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ABSTRACT

Plenary Presentations

The struggle to establish purinergic signalling

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Early experiments in the 1960's will be described leading to the purinergic neurotransmission hypothesis proposed in 1972. The influence of publications by key figures such as Andrew Szent-Györgyi, Pamela Holton, Robert Berne, Mike Rand and Jack Eccles is recognised. Unfortunately, the hypothesis was regarded with scepticism by many for the next 25 years and stories of this resistance will be recounted. The cloning of receptors for purines and pyrimidines in the early 1990's was an important turning point in the acceptance of the hypothesis and the important contributions of many outstanding scientists will be related. Current strong interest in the pathophysiological roles of purinergic signalling and exploration of the therapeutic potential for a number of disease conditions will be discussed.

From chemoreception to eye development -fundamental roles of ATP signalling

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Chemosensory transduction, allows the brain to sense and regulate the levels of blood gasses such as CO2 and important metabolites such as glucose. At least one class of CO2 chemosensory cell at the ventral surface of the medulla releases ATP as a key step in the detection of CO2. We have studied the mechanisms of CO2-dependent ATP release at the ventral medullary surface and have discovered a new principle of CO2 chemosensory transduction. ATP is released via the gating of connexin 26 (Cx26) hemichannels to mediate the adaptive ventilatory response to elevated CO2. The Cx26 channels are located only in the pia mater and sub-pial astrocytes. This very outermost layer of the brain, comprised of non-neuronal cells, thus has a key role in the detection of CO2.
I will review our data and that of others suggesting a role for ATP-signalling in development, comparing and contrasting development of later structures such as the retina, and cortex with very early developmental events -genetic specification of the eye field itself. I will suggest that there are general principles of ATP signalling in these developmental contexts: namely release of ATP through gap junction hemichannels to evoke intracellular Ca2+ waves in neighbouring cells through activation of P2Y receptors. This suggests two important conclusions -that gap junction hemichannels may play a critical role in intercellular signalling in early development; and that their more general involvement in ATP-signalling can act as a unifying mechanistic principle across highly divergent physiological and developmental signalling contexts.

Adenosine –an endogenous distress signal

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Adenosine is known to modulate the function of most organ systems of the body. It is formed from breakdown of intra- or extracellular adenine nucleotides – mostly in response to stressful situations such as low energy supply or cell damage. Adenosine therefore is an endogenous distress signal. Adenosine acts on four G protein coupled receptors - A_1, A_{2A}, A_{2B} and A_3. Adenosine is more potent at A_1, A_{2A}, and A_3 than at A_{2B} receptors. The degree of stimulation by endogenous adenosine is determined, not only by the levels of extracellular adenosine, but also by the density of the receptors. Therefore even the low basal levels of adenosine that are always present in extracellular fluids may be sufficient to activate e.g.

adenosine A_{2A} receptors where they are very abundant. It is known that A_{2A} receptors are particularly abundant on a subset of neurons in the basal ganglia that project i.a.- to globus pallidus. This tonic activation of A_{2A} receptors can be antagonized by the most widely used of all drugs- caffeine. The overview will briefly deal with roles of adenosine in several organ systems and from my own and other laboratories. I will also pay brief homage to a pioneer in the area – Dr John W. Daly who passed away earlier this year.

Microglia listening to neurons through purinergic receptors

Kazuhide Inoue
Japan

Accumulating evidence indicates that nucleotides play an important role in cell-to-cell communication through P2 purinoceptors. Nucleotides are released or leaked from non-excitable cells as well as neurons in physiological and pathophysiological conditions. One of the most exciting cells in non-excitable cells is microglia. Microglia express many types of P2 purinoceptors and are known as resident macrophages in the CNS. ATP and other nucleotides work as ∞gwarning molecules∞h especially through activating microglia in pathophysiological conditions. Microglia play a key role in neuropathic pain through P2X4 receptors (P2X4Rs), and they show chemotaxis and phagocytosis through nucleotide-evoked activation of P2X12 and P2Y6 receptors, respectively. Neuropathic pain is a severely disabling state that affects more than 15 millions of people in the world. This type of pain may be experienced after nerve injury. In the neuropathic pain state, touch stimulation frequently evokes strong pain sensation. We have shown that P2X4Rs, which are upregulated in activated microglia in the spinal cord after nerve injury (Nature, 2003), and the stimulation of P2X4Rs causes release of brain-derived neurotrophic factor (BDNF) (Nature, 2005). BDNF causes a collapse of transmembrane anion gradient of lamina I neurons, resulting in the change of the inhibitory action of GABA to excitatory one. Thus, we postulate that BDNF released by the stimulation of P2X4Rs in microglia is a crucial signalling molecule for neuropathic pain. We also reported that UDP from damaged neurons stimulates microglial phagocytosis (Nature, 2007).
These evidence for extracellular nucleotide signalling places nucleotides in the central stage of microglia-neuron communications.

Purinergic signaling in astrocytes

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Over the past few years, a virtual revolution has occurred in our understanding of the cell biology and physiology of astrocytes, and in our understanding of their interactions with neurons and the vasculature. Astrocytes are electrically non-excitable cells, which communicate predominantly by P2Y receptor mediated calcium signaling. Astrocytes release ATP through connexin-hemichannels, and possible other pathways, highlighting the key role of connexins in non-synaptic signaling in brain.
The complex roles of purine receptors and connexin hemichannels in protoplasmic astrocytes will be discussed focusing on in vivo imaging approaches using 2-photon laser scanning microscopy.

TR(i)Ps from truly remarkable proteins to disease

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The Transient Receptor Potential (TRP) superfamily comprises now 28 mammalian cation channels which are subdivided into six subfamilies: TRPC, TRPV, TRPM, TRPP, TRPML and TRPA. For all these TRP channels, the enormous diversity of gating mechanisms and permeation properties will be reviewed. TRP channels play a unique role as cell sensors, are involved in a plethora of Ca2+-mediated cell functions, and play a role as "gate-keepers" in many homeostatic processes

such as Ca2+ and Mg2+ reabsorption. One possible explanation of the gating diversity of TRP channels is related to their weak voltage dependence. Very often, response of TRP channels to diverse gating stimuli can be explained by a dramatic shift of he voltage-dependent gating. Some of the TRP channels require ATP as a crucial regulator (e.g. TRPM4). Obviously, a close interaction of ATP with some TRP channels is required for regulation of their dramatic modulation by membrane phosphatidylinositols, e.g. PIP2 (e.g. TRPM4, TRPM8). The closely related TRPM5 channel is required to trigger release of ATP from taste bud cells as a response to sweet, umami and bitter stimuli. Responses of TRP channels, which function as temperature sensors in keratinocytes, also trigger ATP release for activation of thermosensory nerve fibers. Some TRP channels are mechanosensors. An especially striking example is TRPV4. We show that trpv4 deficient mice develop an incontinence bladder phenotype. Crucial for this defect is a decreased release of ATP from urothelial cells upon distension of the bladder. TRPV4 is also required for the final differentiation of osteoclasts. Therefore, trpv4 deficient mice develop an increased bone mass and do not develop, when unloaded, osteoporosis as wild type mice. Concerning human diseases, in which TRP channels are widely involved (TRPathies), it will be described how genetic changes in TRPV4 cause an autosomal dominant bone disease, brachyolmia.

CD39/ENTPD1: At the interface between innate and adaptive immunity

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Extracellular nucleotides activate type-2 purinergic/pyrimidinergic (P2Y and P2X) receptors on platelets, endothelium, leukocytes and immune cells. Ectonucleotidases hydrolyze these mediators, ultimately to the respective nucleosides, to terminate P2-signaling and initiate P1 adenosine-mediated responses. The CD39/ENTPD and CD73/ecto-5′-nucleotidase families are responsible for modulated extracellular nucleotide hydrolysis in the vasculature and immune system. Expression of vascular CD39 is rate-limiting in nucleotide phosphohydrolysis and crucial in regulating innate immunity, leukocyte chemotaxis, platelet activation, acute ischemic insults, altered vascular permeability, angiogenesis and tumor growth. Expression of CD39 by dendritic cells has major impacts on antigen presentation and T cellular responses crucial in the evolution of adaptive immunity. Further, CD39 (with CD73) are important surface markers of adaptive immune cells: viz. T regulatory (Treg) and Natural Killer T (NKT) cells. CD39 serves as an integral functional component providing a unique biochemical signature to distinquish these regulatory cells from other immune effector cells. Modulated expression of CD39 on Treg and adenosine receptors on activated effector T cells (Teff) and NK cells generate paracrine, immunosuppressive loops. Adoptive transfers of Cd39 null Treg fail to inhibit allograft rejection and Cd39 null mice develop autoimmunity with deviated Th1 responses. Inhibition of CD39 on Treg also promotes tumor clearance in vivo. In addition to major thromboregulatory roles, CD39 has a major impact upon cellular immunoregulation and the evolution of auto/alloimmune reactions. Pharmacologic modalities to boost CD39 expression may suppress host reactions that are unwanted, as in transplant rejection and autoimmunity. Alternatively, CD39 blockade may be used to augment host innate protective responses, such as in metastatic cancer.

SYMPOSIA 1: PURINERIGIC FRONTIERS IN BIOLOGY

Pharmacological characterisation of a P2X receptor cloned from the central nervous system of Lymnaea stagnalis

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Background
The CNS of the model organism Lymnaea stagnalis is relatively simple consisting of ~20,000 large identifiable neurons. This, together with the fact that the circuitry underlying complex behaviours such as feeding and respiration are well characterized make Lymnaea an attractive model to investigate the role of P2X receptors in CNS function.
Methods
RT-PCR with degenerate oligonucleotides was used to identify a P2X receptor fragment expressed in the Lymnaea CNS. The full length sequence was obtained by RACE-PCR and the cloned receptor was expressed in Xenopus oocytes to facilitate electrophysiological characterisation.
Results
ATP evokes inward currents at LymP2X with slow desensitisation and an EC50 of 6.2 microM. BzATP is a partial agonist with a maximum response ~70% that of ATP and an EC50 of 2.4 microM whereas alpha, beta-methylene ATP is a very weak agonist and ADP and UTP produce no response. Protons inhibit LymP2X with currents reduced by 55% at pH 6.5 compared to pH 7.5 with no change in EC50. Both PPADS and suramin are antagonists (IC50 9.1 and 27.4 microM respectively). The divalent cations Cu2+ and Zn2+ have biphasic effects potentiating currents at concentrations up to 100 microM and inhibiting currents at 1 mM. LymP2X is insensitive to ivermectin.
Conclusion
This work has increased our emerging phylogenetic knowledge of P2 receptors by adding molluscs to the list of organisms that possess functional P2X receptors. Knowledge of the pharmacological properties of LymP2X allows us now to probe the function of this receptor in vivo in the Lymnaea CNS.

Dual regulation of human penile smooth muscle tone by P2 purinoceptors: possible implications in erectile dysfunction

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ATP acts as a potent relaxant agent in rabbit and human corpus cavernosum (HCC). ATP effect can be partially attributed to the metabolic breakdown of ATP into adenosine by ecto-nucleotidases (see Faria et al., 2006), but also due to a direct stimulation of P2 purinoceptors. We showed that stable adenine nucleotides exert a dual role on HCC tone; the P2-mediated responses were attenuated in vasculogenic erectile dysfunction (ED) patients (Faria et al., 2008). We aimed at characterizing the P2 purinoceptors that mediate contraction and relaxation of HCC strips collected from organ donors and from impotent men submitted to surgery. The Ethics Committees of HGSA and ICBAS-UP approved all the procedures. Isometric muscle tension was measured from longitudinal strips of HCC pre-contracted with phenylephrine. Adenosine deaminase was present in the incubation fluid to inactivate endogenous adenosine.The rank potency order for relaxation of HCC strips was (IC50 microM): ADP(10)~ADPbetaS (10)>ATPgamaS(100)~2-MeSATP(100)>ATP(300); UTP was devoid of effect. PPADS and MRS2179 (P2Y1 antagonist) failed to antagonize relaxation of HCC. In contrast to L-NAME, indomethacin shifted to the right the concentration-response curve of ADPbetaS. Alpha,beta-MeATP (P2X1 and P2X3 receptor agonist), transiently increased the HCC tonus without causing relaxation of the tissue. Nucleotide-induced contractions were blocked by PPADS and NF023 (P2X1 antagonist).Data suggest that P2X1 receptors directly cause contraction of HCC strips, while ADP-sensitive P2Y12 and/or P2Y13 receptors might mediate relaxation of HCC indirectly through the release of prostanoids. Further studies are required to investigate whether P2-induced release of prostanoids from the endothelium is impaired in HCC from patients with vasculogenic ED.

P2X receptors of single celled organisms

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Until recently P2X receptors have only been identified in multicellular organisms, where they act as excitatory receptors for ATP in processes such as sensory neuron and immune cell activation in painful and inflammatory responses, respectively. Although ATP itself is an ancient and fundamental biological molecule, the phylogeny of P2X receptors outside multicellular organisms remains unclear. We have identified and cloned functional P2X receptors from several single celled organisms including amoeba, choanoflagellate and green algae, which are providing useful tools for exploring the molecular operation of this class of receptors, and provide an insight to the evolutionary origins of ATP-gated ion channel signalling. The use of genetics to understand the physiological roles of P2X receptors in protists and how this information can be used to inform the intracellular roles that P2X receptors may play in animal cells will be discussed.

(Invited)

Disordered pancreatic inflammatory responses and inhibition of fibrosis in CD39-null mice

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Chronic pancreatitis (CP) is a debilitating disease characterized by severe inflammation with fibrosis culminating in progressive loss of function. Purinergic P2-signaling responses implicated in inflammatory and immune responses are modulated by ectonucleotidases, such as CD39/ENTPD1. We have previously shown upregulation of vascular, stromal and immune cell CD39, P2X7 and P2Y2 expression in CP. As the specific role of CD39 in mediating pancreatic inflammation and fibrosis is poorly understood, we have studied impact of CD39 expression in a model of pancreatic disease.

Pancreatitis was induced by cyclosporine/cerulein injections; mice were sacrificed at days 2, wk 3 and wk 6. Immunohistochemistry and pancreatic morphometry of fibrosis were performed in wild type and CD39-null mice. Effects of CD39 deletion on immune deviation and proliferation of stromal cells were further investigated in vitro. Wild type mice developed morphological features of pancreatitis with the anticipated development of parenchymal atrophy and fibrosis. CD39 and P2-R were overexpressed in vascular and adventitious tissues. In contrast, CD39-null mice had substantive inflammatory reactions but developed only minor pancreatic atrophy and limited fibrosis. Interferon-gamma became significantly increased in tissues and plasma of CD39-null mice in keeping with the nature of Th1-type immune deviation observed in vitro. CD39-null PSC exhibited decreased rates of proliferation and the expression of procollagen-á1 was significantly inhibited in vitro.

We conclude that CD39 expression is altered in pancreatic disease. Gene deletion decreases fibrogenesis in experimental pancreatitis. Our data implicate CD39 and P2R as modulators of immune reactivity, PSC proliferation and collagen production in CP.

Extracellular ATP and ADP mediate Ca2+ influx in Dictyostelium discoideum

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Introduction
The model eukaryote Dictyostelium has a family of five genes (p2xA-E) with homology to vertebrate P2X receptors. dP2XA has an exclusively intracellular localisation where it plays an osmoregulatory role. It therefore remains unclear whether Dictyostelium utilise P2 receptors for extracellular nucleotides in a manner analogous to vertebrates.
Methods
An aequorin-based assay was used to monitor changes in intracellular Ca2+ evoked by extracellular nucleotides in wild type and knockout strains.
Results
ATP and ADP elicit a transient rise in intracellular Ca2+ with EC50 values of 7.5 and 6.1 microM respectively. Responses peak within 2.9±0.04 seconds, require extracellular Ca2+ and are unaffected by knockout of either the heterotrimeric G protein beta subunit gene or iplA, a gene thought to encode the Dictyostelium IP3 receptor. Deletion of p2xA or p2xE also has no effect on the response to extracellular purines showing that these P2X-like genes are also not involved. Cu2+, Zn2+ and Gd3+ ions inhibit the response whereas suramin and PPADS have no effect. 300 μM Zn2+ completely inhibits the large rapid rise in intracellular Ca2+ revealing the presence of an additional smaller and much slower response.
Conclusions
In addition to P2X receptors with intracellular function, Dictyostelium also possess cell surface P2 receptors which respond to extracellular purines, making this organism an attractive model to explore purinergic signalling. The rapid activation of a Ca2+ permeable ion channel is indicative of the involvement of one or more of the P2X-like receptors, with dP2XB, dP2XC and dP2XD the most likely candidates.

Purines and the anticonvulsant/neuroprotective effects of ketogenic diets

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ATP and adenosine are purines critically involved in neuron-glia interactions, neuromodulation and synaptic plasticity. Metabolic perturbations alter the levels of these purines, and abnormal signaling caused by either acute or chronic metabolic dysfunction characterizes a number of neurological disorders. Strategies which enhance brain bioenergetics (ATP) and/or increase adenosine, an endogenous anticonvulsant and neuroprotectant, may offer therapeutic benefits. Separate but possibly parallel clinical metabolic interventions include ketogenic strategies such as fasting or adhering to high fat, low carbohydrate diets; both are known to reduce seizures and offer neuroprotection against a variety of insults. However, despite intense interest, key mechanisms underlying the clinical success of ketogenic strategies remain unknown. Our hypothesis is that ketogenic diets increase brain levels of adenosine and ATP, and that these molecules contribute significantly to the anticonvulsant and neuroprotective effects of ketogenic diets.
At weaning we placed rats on either a ketogenic or a control diet for at least 3 weeks. We used a head-focused high-energy microwave system to snap-inactivate molecules and measured levels of ATP, ADP, AMP, adenosine, phosphocreatine and creatine using HPLC. We found changes in these molecules in discrete brain regions, including increased ATP levels in hippocampus and cortex. Electrophysiological recordings from acute hippocampal slices of ketogenic diet-fed rats demonstrated changes in paired pulse facilitation and pharmacological responses that were consistent with increased endogenous adenosine levels. These data suggest that ATP and adenosine may play a critical role in the clinical success of a ketogenic diet.

QSAR studies on purine and 2,3-Dihydropurine analogues as antitubercular agents

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A quantitative structure-activity relationship (QSAR) model was developed for compounds acting against Mycobacterium tuberculosis, since TB is still one of the main causes of human death and new compounds, capable of overcoming drug resistance, are strongly required.
It has been recently found that several purine and 2,3-dihydropurine derivatives show considerable antitubercular properties. A data mining approach was used to build a predictive model based o classification trees which is able to predict if new purine and 2,3-dihydropurine derivatives belong to an "Active" or "Inactive" class. The model was built by using a small number of descriptors and allowed highlighting structural features that confer antitubercular activity to new derivatives based on the same heterocycles.
In order to be active against M. tuberculosis, they should have appropriate size and not contain highly charged regions. That may reflect the need to have a quite "neutral" character, in order to be able of crossing the cell walls of the microorganism. The main heterocyclic ring should not bear substituents behaving as strong electron-withdrawer, such as NO2, SO2, CF3 groups, whereas Cl atoms, and amino, alkyl or alkylaryl groups are allowed at different positions.
Acknowledgments
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Comparative genomics of the P2Y11 receptor

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Background. The organisation of the P2Y11 receptor gene and the flanking PPAN and EIF3S4 genes has been investigated in a number of evolutionarily-diverse species genomes.
Methods. The basic local alignment search tool (BLAST) was used to identify the genomic location, organisation and corresponding nucleotide and protein sequences of the PPAN, P2Y11 and EIF3S4 genes in several species (Homo sapiens, P.troglodytes, C.familiaris, B.taurus, M.musculus, R.norvegicus, C.porcellus, M.domestica, L.africana, X.tropicalis, T. nigrovididens, D.rerio, D.melanogaster and C.elegans) by using both mRNA and protein sequences of the human and canine genes to query Genbank, Ensembl and Whole-Genome Shotgun (WGS) Trace Archives.
Results. PPAN and EIF3S4 gene sequences were identified in all species studied. P2Y11 sequences were identified between PPAN and EIF3S4 in almost all species, including the rodent-like lagomorph C.porcellus. P2Y11 sequences were not detected in the D.melanogaster and C.elegans genomes, where PPAN and EIF3S4 are ~1 Mb apart or on separate chromosomes, respectively. P2Y11 sequences were not detected in the rodent Rattus norvegicus and Mus musculus genomes, where the genomic interval between the PPAN gene to the 5′ of P2Y11 and the EIF3S4 gene to the 3′ of PPAN was too short to accommodate a full-length GPCR.
Discussion. The P2Y11 receptor was not detected in invertebrate or rodent genomes, but was present in mammalian, amphibian and in teleost fishes. The genomic organisation of the P2Y11 gene in X tropicalis precludes the possibility of intragenic splicing with PPAN, which is observed in both H.sapiens and B.taurus.

Characterization of high-affinity adenine binding sites in Achromobacter xylosoxidans isolated from contaminated Tris buffer

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[3H]Adenine has previously been used to label the newly discovered G protein-coupled adenine receptors. Recent reports have questioned the suitability of [3H]adenine for adenine receptor binding studies because of curious results, e.g., high specific binding even in the absence of mammalian protein. Using radioligand binding studies we could show that specific [3H] adenine binding to various mammalian membrane preparations increases linearly with protein concentration. We found that Tris-buffer solutions typically used for binding studies that have not been freshly prepared but stored at 4°C may contain bacterial contaminations which exhibit high affinity binding for [3H]adenine. Specific binding is abolished by heating of the contaminated buffer or by filtering it through 0.2 μm filters. Three different aerobic, gram-negative bacteria were isolated from

a contaminated buffer solution and identified as Achromobacter xylosoxidans, Achromobacter denitrificans, and Acinetobacter Iwoffii. Achromobacter xylosoxidans, a common bacterium which can cause nosocomial infections, showed a particularly high affinity for [3H]adenine in the low nanomolar range. Structure-activity relationships revealed that hypoxanthine also bound with high affinity to the [3H]adenine-labelled binding sites in Achromobacter xylosoxidans, while other nucleobases and nucleosides did not. The high-affinity binding sites found in A. xylosoxidans have not much in common with the adenine receptors found in mammals. The nature of the labeled sites in bacteria is not known yet, but preliminary results indicate that it may be a high-affinity purine transporter. We conclude that [3H]adenine is a well-suited radioligand for the labeling of G protein-coupled adenine receptors if bacterial contamination of the buffer solutions is avoided.

Hypoxia-Adenosinergic Immunosuppression and Tissue Protection

M. Sitkovsky

The advances in understanding of hypoxia-driven extracellular adenosine accumulation and signaling via A2A and A2B adenosine receptors (A2AR/A2BR) are informed by the structure-function studies of A2AR and A2BR expression on the cell surface to be reported here. In this presentation, the model of A2AR/A2BR functioning will be presented as a tightly coordinated sequence of biochemical processes that culminate in the inhibition, redirection of immune response and tissue protection and/or remodeling.

The understanding of collaborating functions of extracellular adenosine, A2AR and A2BR with intracellular activities of Hypoxia Inducible Factor 1 alpha allows putting together the cogent model of immunosuppression including the inhibition and redirection of immune response by T regulatory cells.

The promising clinical applications of antagonizing the HIF-1 alpha- and A2AR-mediated Hypoxia Adenosinergic immunosuppression and tissue protection include novel protocols of cancer immunotherapy and treatments of drug-resistant bacteria. The novel use of the recruited by A2AR agonist immunosuppression may lower excessive death rates in intensive care units due to applications of supplemental oxygen to patients with acute respiratory disease syndrome.

(Invited)

Extracellular nucleotides in plants

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A number of publications suggested the presence of extracellular ATP (eATP) in plants and implicated eATP in various physiological processes. We were able to visualize eATP in plants using a luciferase-based reporter system. Current data suggest a key role for eATP in modulating plant cell growth. For example, eATP appears essential for pollen tube growth. More recently, results indicate that eATP is specifically released during the touch response (mechanostimulation) in plants (e.g., during root gravitropic growth). This response appears to be mediated through the action of plant heterotrimeric G-proteins. Indeed, plant mutants defective in heterotrimeric G-proteins also show defects in their response to eATP addition. An open question is whether plants possess P2Y or P2X-like purine receptors. Sequence-based searches of the available databases using animal receptor sequences failed to identify such receptors. However, we were able to identify putative P2Y-like receptors based on a Hidden Markov model derived from the animal P2Y receptor sequences. The putative, plant P2Y-like receptors are induced upon the addition of eATP. The protein predicted from their gene sequence has more than seven transmembrane regions. However, antibody directed against one plant P2Y-like receptor showed that the protein is processed in planta, resulting in a predicted seven transmembrane protein within the plasma membrane. Mutants defective in the expression of this putative receptor protein show defects in their response to eATP addition. Plants defective in a single P2Y-like gene show no phenotype. However, a double mutant shows severe defects in pollen growth, resulting in a male sterile phenotype.

(Invited)

SYMPOSIA 2: PLATELET FUNCTION AND PURINERGIC SIGNALLING

Mechanisms of ATP degradation in platelet

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Genetic defects of the megakaryocyte lineage give rise to bleeding syndromes of varying severity. Blood platelets are unable to fulfill their hemostatic function of preventing blood loss on vessel injury. Spontaneous bleeding is mostly mucocutaneous in nature. Most studied are deficiencies of glycoprotein (GP) mediators of adhesion (Bernard-Soulier syndrome) and aggregation (Glanzmann thrombasthenia) which concern the GPIb-IX-V complex and the integrin alphaIIbbeta3, respectively. Defects of primary receptors for stimuli include the P2Y(12)ADP receptor pathologyAgonist-specific deficiencies in the platelet aggregation response and abnormalities of signaling pathways are common and lead to trauma-related bleeding. Inherited defects of secretion from storage organelles, of ATP production, and of the generation of procoagulant activity are also encountered. In some disorders, such as the Chediak-Higashi, Hermansky-Pudlak, Wiskott-Aldrich and Scott syndromes, the molecular lesion extends to other cells

Platelet P2 receptors in atherothrombosis

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Besides their role in thrombosis, platelets also contribute to the development of atherosclerosis, suggesting their three P2 receptors, P2Y1, P2Y12 and P2X1 to represent important partners in this vascular disease. Both the P2Y1 and P2Y12 receptors contribute to platelet P-selectin exposure, interactions of platelets with leukocytes and exposure of tissue factor on activated leukocytes, which should translate into a direct role of these receptors in atherosclerosis. However, only few data have been reported concerning their involvement in this pathology. Controversial negative and positive results have been reported for P2Y12 and no data at all are available about P2X1.
Concerning P2Y1, we have shown that it contributes to atherosclerosis since P2Y1-/-/ApoE-/- mice display reduced atherosclerotic lesion size and reduced macrophage and smooth muscle cell contents as compared to ApoE-/- mice. Bone marrow transplantation was performed to determine the relative contributions to the development of atherosclerosis of the P2Y1 receptors expressed on blood cells and in the rest of the body. Unexpectedly, the results exclude the involvement of platelet and other blood cell P2Y1 receptors and point to a role of P2Y1 receptors of other tissues, most likely the endothelial or smooth muscle cell receptor. In addition, although this was not statistically significant, plasma cholesterol levels were consistently decreased in P2Y1-/- animals, suggesting that a modification of lipid metabolism could partly explain the observed phenotype.
In view of the known involvement of this receptor in platelet physiology and thrombosis, these findings might be of importance for the development of new anti-atherothrombotic strategies.

(Invited)

Inverse sensitivity to P2Y1 and P2Y12 receptor inhibition in ApoE-/- as compared to WT mice in a model of FeCl3-induced thrombosis

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Background: The P2Y1 and P2Y12 receptors are involved in thrombosis and the latter is the target of the antiplatelet drug clopidogrel while P2Y1 is a potential target for new antiplatelet drugs. Our aim was to evaluate the involvement of the P2Y1 and P2Y12 receptors in atherosclerotic ApoE-/- as compared to WT mice in experimental thrombosis.
Methods: Male WT and ApoE-/- mice aged >25 weeks received the selective P2Y1 antagonist MRS2500 (4 or 10 mg/kg) or clopidogrel (50 mg/kg). Carotid artery injury was induced by topical application of 7.5% FeCl3. Thrombosis was recorded by intravital microscopy and quantified by image analysis.
Results: Clopidogrel strongly inhibited platelet aggregation in both mouse strains, with a greater residual P2Y1-dependent aggregation in ApoE-/- mice as compared to the WT. ApoE-/- mice displayed increased thrombosis in response to FeCl3 as compared to the WT, and inhibition of thrombus formation by clopidogrel was weaker (38±9%) as compared to its effects in WT mice (71±13%). In contrast, the dose-response curve of the P2Y1 antagonist MRS2500 toward inhibition of platelet aggregation and thrombosis was shifted to the left in ApoE-/- mice as compared to the WT, indicating higher potency and sensitivity in the atherosclerotic animals.
Conclusion: These results suggest increased and decreased sensitivity to P2Y1 and P2Y12 inhibition respectively in atherosclerotic mice as compared to the WT, further supporting the concept of the P2Y1 receptor as a target for new anti-atherothrombotic drugs.

Regulation of platelet aggregation by ecto-purine metabolizing enzymes

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ADP is a crucial signaling molecule activating the platelet aggregation (PA). Adenosine and ATP participate in a regulation of this process. PA is also controlled by ecto-nucleotidases and adenylate kinases (AK) present in blood. The aim of presented study is to compare an influence of AK and apyrase on the pig PA.
For experiment we used AK from *Bacilus stearotermofilicus*, potato apyrase and platelet-rich plasma (PRP) isolated from the pig blood. The quantitative and qualitative analysis of purines was made by the HPLC method. PA was analyzed by spectrophotometric method at λ=600 nm.
It is well known that potato apyrase inhibited the platelet aggregation. In our study apyrase suppressed PA activated by 20 mM ADP or 7,5 mg collagen. Efficiency of the inhibition depended on time of enzyme injection into the reaction mixture, and with the decrease of the ATP and ADP and the related increase of the adenosine concentration. However, apyrase did not cause the platelet disaggregation.
The adenylate kinase suppressed PA activated by ADP. At the same time, the concentration of ATP and adenosine in sample increased. In case of PA initiated by collagen, addition of AK into reaction mixture caused its complete disaggregation. That effect was independent from the time of dosage. The disaggregation is accompanied by the increase of ATP and adenosine, and decrease of ADP and inosine concentration.
Qualitative and quantitative differences in products of purines degradation during PA suggest that ADP and collagen activate that process by different pathways. Obtained results indicate the importance of ecto-adenylate kinases present in blood vessels for hemostasis.

Regulation of platelet P2Y receptors

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Adenosine 5′-diphosphate (ADP) plays a central role in regulating platelet function and thrombus formation by the activation of G protein-coupled receptors P2Y1 and P2Y12. Although it is well established that aggregation responses of platelets to ADP desensitize, the underlying mechanisms have been unclear. Using a variety of approaches over recent years, including work on primary human platelets and model cell systems, we have established that these two receptors desensitize by different kinase-dependent mechanisms. P2Y12, but not P2Y1, desensitization is mediated by G protein-coupled receptor kinases (GRKs). In contrast, desensitization of P2Y1, but not P2Y12, is largely dependent on protein kinase C (PKC) activity.

We have shown that these receptors also undergo trafficking in platelets and cell line systems, upon activation. This trafficking involves internalisation through clathrin-mediated endocytosis and subsequent sorting to undergo recycling or lysosomal degradation. Interestingly, we observed that sorting of these receptors takes place much earlier than previously thought, by entry of P2Y1 and P2Y12 into distinct populations of clathrin-coated pit. The mechanisms underlying early sorting again involved differential kinase-dependent processes because internalization of P2Y12 was mediated by GRKs and arrestin, whereas P2Y1 internalization is GRK- and arrestin-independent but requires protein kinase C.

Recently we have gone on to show that responses to both P2Y1 and P2Y12 can rapidly resensitize following agonist-dependent desensitization in human platelets. We show that in human platelets or in 1321N1 cells stably expressing receptor constructs, disruption of receptor internalization, dephosphorylation or subsequent receptor recycling is sufficient to block resensitization of purinergic receptor responses. We also show that, in platelets, internalisation of both of these receptors is dependent upon dynamin, and that this process is required for resensitisation of responses. $P2Y_1$ and $P2Y_{12}$ receptor activity is therefore rapidly and reversibly modulated in human platelets and the underlying mechanism requires receptor trafficking as an essential part of this process.

Finally, we have also recently demonstrated that regulation of P2Y receptors does not take place in isolation of other receptor systems in platelets, since activation of platelets by ADP can alter responsiveness of platelets to another important agonist thromboxane A2. We show that pretreatment of platelets with either ADP or thromboxane substantially desensitizes responses to the other agonist. Responses to thromboxane receptor activation are desensitized by preactivation of P2Y1 but not P2Y12 receptors, and this heterologous desensitization is mediated by a protein kinase C-independent mechanism. Reciprocally, responses to ADP are desensitized by pretreatment of platelets with the thromboxane analogue, U46619, and in this direction desensitization is comprised of two components, a true heterologous component that is PKC-independent, and a homologous component that is mediated through stimulated release of dense granule ADP.

These studies reveal that P2Y receptors in platelets are highly regulated by different kinase dependent mechanisms, and that they may also influence responsiveness to other platelet agonists. This allows very fine control of the critical mechanisms of haemostasis and ultimately thrombosis, and has major bearing on novel approaches to regulate the function of platelets in the treatment of thrombotic disease.

(Invited)

A newly identified role for the A2b adenosine receptor in the control of CXCR4 and platelet activation and migration

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Adenosine inhibits platelet aggregation, which was believed to be due to A2a adenosine receptor (AR) activation. We show that mouse megakaryocytes and platelets express low levels of A2bAR mRNA which is highly elevated upon mitogenic stimuli, including via vascular injury in vivo. A recent study (Amisten S. et al 2007, Thrombosis Research) also detected an active A2bAR in human platelets. To examine whether A2bAR expression in platelets affects platelet function, we measured platelet aggregation (light transmittance) using platelet rich plasma prepared from control C57BL-6 mice or matching congenic A2bAR knockout (KO) mice. ADP-induced or collagen-induced aggregation was greater by about 30–40% in A2bAR KO platelets, compared to control. P-selectin positive platelets from A2bAR KO mice were found to be increased as measured by FACS. Adenosine analog, NECA (10uM) inhibited ADP-induced platelet aggregation by 43±7% in wild-type cells, but only by 31±6% in A2bAR KO platelets. We also examined this response under conditions of induced A2bAR expression in platelets, by using the femoral artery injury model. The inhibition of NECA on ADP-induced platelet aggregation increased from 28% before injury to 50% after one-week injury in wild-type cells, but not in A2bAR KO cells. Platelets highly expressing CXCR4 accumulated as a thrombus in the injured vessel of A2bAR KO mice. SDF-1, CXCR4 ligand, increased ADP-induced platelet aggregation with higher efficiency (about 2 fold) in A2bAR KO cells, as compared to control. Future studies will examine whether A2bAR specific pharmacological activation inhibits platelet aggregation in vitro and in vivo.

(Invited)

Altered hydrolysis of ATP and ADP in rat platelets exposed to homocysteine in vitro and ex vivo

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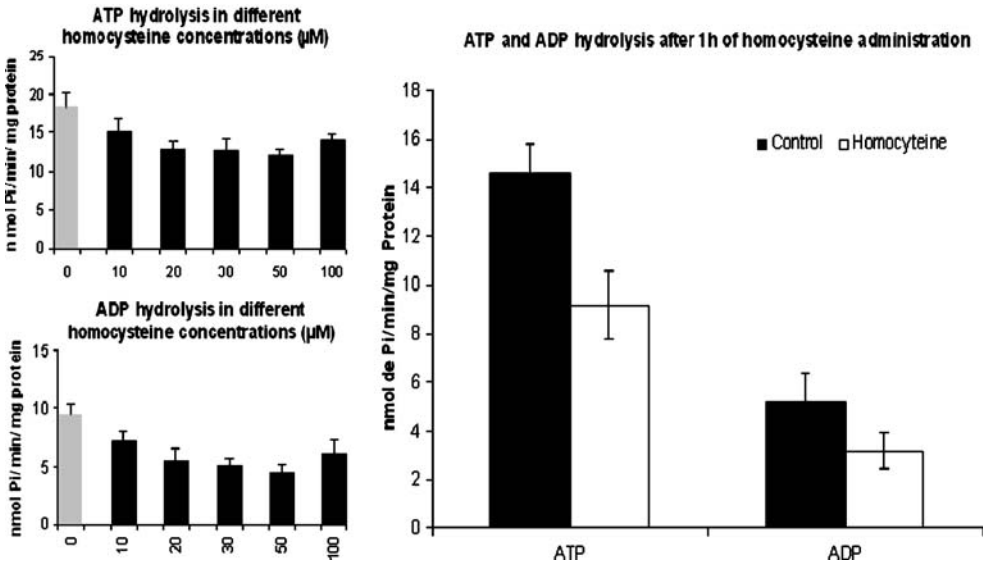
Background: Elevated homocysteine (Hcy) concentration is associated with increased risk of coronary artery disease, stroke, and venous thromboembolism. Platelets develop an important role on the regulating thrombus formation, mainly by the release of active substances such as ADP. ENTPDases enzymes are the main responsible by ATP and ADP hydrolysis. Therefore, considering the importance of adenine nucleotides to the physiological homeostasis, we investigated the homocysteine influence in the ATP and ADP hydrolysis.
Methods: Homocyteine administration was done as described by Streck et al. (2002). Washed platelet-rich plasma was prepared according to the methods of Pilla et al. (1996) and the inorganic phosphate (Pi) released by ATP and ADP hydrolysis was measured by the method of Chan et al. (1986).
Results and Conclusion: The results demonstrated that ATP and ADP hydrolysis of rat platelets exposed to homocysteine in vitro and ex vivo was decreased when compared with the control group. The altered hydrolysis observed on the platelets may contribute to abnormal blood coagulation that is generally related with high homocysteine concentration.



SYMPOSIA 3: NEW PHARMACOLOGICAL TOOLS FOR STUDYING P1 & P2 RECEPTORS

Anthraquinones: combinatorial synthesis and structure-activity relationships as non-nucleotide-derived P2Y2 receptor antagonists

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Background. There is a lack of potent and selective P2Y2 receptors (P2Y2R) antagonists, which are required as pharmacological tools to elucidate the (patho)physiological roles of the receptors.
Methods. A library of anilinoanthraquinone derivatives was synthesized by using a parallel synthesizer. All products were evaluated as antagonists at mouse P2Y2R natively expressed in neuroblastoma glioma hybrid (NG108-15) cells and at human P2Y2R heterologously expressed in 1321N1 astrocytoma cells. Inhibition of UTP-induced calcium mobilization was determined with Fura-2. Selected compounds were incubated with mouse liver microsomes followed by LC/MS analysis in order to investigate their metabolism.
Results. Reactive Blue 2 exhibited an IC50 value of 5.0 μM at mouse and 1.85 μM at human P2Y2R. A much smaller, unsubstituted phenylamino-anthraquinone derivative was nearly as potent (11.1 μM at mouse and 5.16 μM at human P2Y2R). The phenyl residue was essential since the starting compound bromaminic acid was inactive. Chloro-substitution in the meta-position abolished activity, while a number of other mono- and di-substitutions were tolerated. The o-methoxy derivative (PSB-716), which was nearly equipotent at mouse and human P2Y2R, was selected for further preliminary characterization. It was much weaker in inhibiting rat NTPDase1 (Ki>100 μM) and rat ecto-5′-nucleotidase (Ki>100 μM), and in antagonizing the human P2Y4 (IC50 ca. 50 μM) and the rat P2Y6 receptor subtypes (IC50>100 μM) than as P2Y2 antagonist. Several metabolites were identified after incubation with mouse liver microsomes.
Conclusion. 4-Phenylamino-substituted 1-amino-2-sulfoanthraquinones, e.g. 1-amino-4-(2-methoxyphenyl)-2-sulfoanthraquinone (PSB-716), were potent P2Y2 antagonists with IC50 values in the low micromolar range.

Synthesis and structure-activity relationships of new adenine derivatives as ligands for the rat adenine receptor

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A rat orphan G protein-coupled receptor has been found to be activated by the nucleobase adenine (1), and a second subtype has recently been identified in mouse (2). It is highly expressed in the CNS and may enhance nociception and mediate neurotrophic effects (1,3). Potent, selective and stable ligands are required to further investigate the physiological roles of the adenine receptors.
Taking adenine as a lead structure we introduced various 8-substituents, starting from 8-bromoadenine via cross-coupling or nucleophilic substitution. Position 9 was alkylated using various mesylates or halogenides. The exocyclic amino group was derivatized by alkylation or acylation. Receptor affinities were determined in radioligand binding studies at rat brain cortical membranes, and selected compounds were further characterized.
A series of 8-, 9-, and N6-substituted adenine derivatives was obtained. For N6-acylation a novel synthetic pathway was employed. The radioligand binding studies showed, that only minor modifications were tolerated at positions 8 and 9.
In summary, we synthesized a set of 8-, 9-, and N6-substituted adenine derivatives and investigated structure-activity relationships at the rat adenine receptor, revealing N6 as the most promising position for modifications.

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ATP-mimetics derived from 2′(3′)-C-methyladenosine as human P2Y2 agonists

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Background. To investigate the effect of conformational factors of the ribose-modified moiety in ATP mimetics, such as in 2′(3′)-C-methyl-ATP, on the agonist activity at metabotropic P2Y2 receptor.
Methods. The 2′(3′)-C-methyl-ATP were synthesized and assayed for capacity to promote P2Y2 receptor-mediated activation of phospholipase C at recombinant human receptor expressed in astrocytoma cells. Agonist potency of the nucleotides was calculated using a four-parameter logistic equation and the GraphPad software package.
Results. In vitro pharmacological data for 2′(3′)-C-methyl-ATP (compounds 1 and 2, respectively) demonstrated that both ATP derivatives interact with the hP2Y2 receptor and behave as agonists. Nucleotide 2, which has a South conformation, resulted more potent than compound 1, which has the furanose ring in the N-puckered conformation (EC50 values: 1.29 and 11.9 microM, respectively).
Conclusion. The effect of modifications at the ribose moiety of ATP, such as in 2′(3′)-C-methyl-ATP on hP2Y2 receptor was evaluated. The potency of 3′-C-methyl-ATP, which has a South conformation, was higher than that of N-puckered 2′-C-methyl analogue. This is a surprising result because of the Northern conformational preference of hP2Y2 receptor detected by ring-constrained methanocarba analogues of ATP.

P1 receptor ligands: applications on neuroprotection and neurodegenerative diseases

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Adenosine (Ado) affects CNS cells functioning by interacting with GPCRs that can inhibit (A1) or enhance (A2) neuronal communication. Since the involvement of Ado receptors is described under different CNS pathological conditions, the preparation of novel P1 receptor ligands are of utmost importance.
Hence, a series of adenosine A2A receptor (AA2AR) antagonists with adenine structure have been designed, synthesized, and fully characterized in vitro [1,2]. In addition, they have been tested in different in vivo experimental models of Parkinson's disease and proved to reverse locomotor deficits induced by haloperidol and to induce controlateral turning behaviour in treated rats [3]. With aim at explaining differences in potency, the effect of the same compounds on the activity of some metabolic enzymes was investigated [4].Furthermore, a number of polysubstituted Ado derivatives have been prepared and characterized in vitro as partial and full AA3R agonists [5]. This subtype seems to contribute to neuroprotective action of adenosine although the overall modulatory effects of A3 receptors on neurotransmission during cerebral ischaemia are not well defined. Hence, the ability of the new AA3R agonists to reduce field excitatory post-synaptic potential (fepsp) depression in ischemic conditions, in rat hippocampal slices, was investigated [6].

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(Invited)

Novel 4-amido-2-arylpyrazolo[3,4-c]quinolines as highly potent and selective human adenosine A3 receptor antagonists

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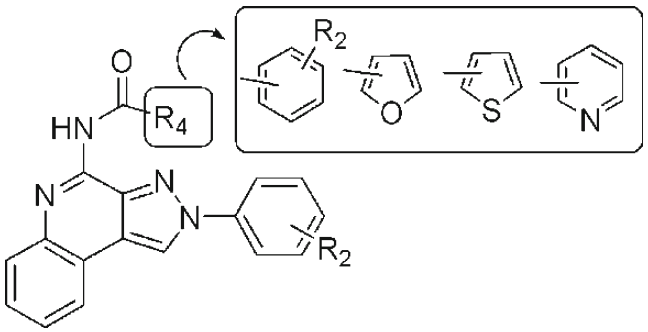
Adenosine A3 receptor (AA3R) antagonists have attracted great attention for their potential therapeutic use in many pathological conditions, such as renal injury, neurodegenerative diseases, and glioblastoma multiforme [1]. In the last few years a series of 2-arylpyrazolo[3,4-c]quinolin-4-amines have been developed in which the presence of acyl residues on the 4-amino group produced profitable effects both for human AA3R affinity and selectivity [2]. On this basis and to further investigate this series of AA3R antagonists, the synthesis of a new set of 2-arylpyrazolo[3,4-c]quinolin-4-amines was undertaken through the introduction of various aroyl/heteroaroyl moieties on the 4-amino group.
The new compounds, tested at human adenosine A1, A2A, A2B, and A3 receptors, showed A3 nanomolar affinities and a complete selectivity versus the other three receptor subtypes. Some derivatives were also tested on human melanoma A375 cells demonstrating to be effective in reducing cell proliferation.
Molecular docking studies have been carried out to rationalize the observed SAR and to depict the hypothetical binding mode of these antagonists at the human AA3R.



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Polysubstituted adenines as human adenosine A2A receptor antagonists

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Adenosine A2A receptor (AA2AR) antagonists have been proposed as an attractive pharmacological tool for the treatment of several diseases, such as motor dysfunctions, because of the interaction between A2A and D2 dopamine receptors in the basal ganglia, and A2A antagonists showed to improve motor dysfunction in in vivo models of Parkinson's disease [1].
In this work our attention has been pointed out toward AA2AR antagonists with adenine structure. Previous studies demonstrated that introducing alkylamine or alkoxy chains in 2 position or a bromine atom in 8 position of 9-ethyladenine

led to compounds with nanomolar affinity for the human adenosine receptors [2,3]. Hence, the synthesis of a new series of 9-ethyladenine derivatives bearing different chains in both 2 and 8 positions was undertaken. Compounds affinities were evaluated at the human recombinant adenosine receptors, stably transfected into CHO cells, utilizing radioligand binding studies (A1, A2A, and A3) or adenylyl cyclase activity assay (A2B). Results showed that the new polysubstituted adenines are endowed with low nanomolar affinity for AA2AR subtype, resulting among the most potent A2A antagonists with adenine structure reported so far. These compounds showed also a slight selectivity for this receptor subtype. Molecular modelling studies have been performed by docking analysis of the compounds in a rhodopsin-based model of the human AA2AR, and the results have been compared with the binding data.

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Discovery and optimization of P2X3 and dual P2X3/P2X2/3 antagonists with therapeutic potential

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Purinergic receptors are a family of ligand gated ion channels whose endogenous ligand is ATP. Homomeric P2X3 and heteromeric P2X2/3 receptors are selectively localized at the peripheral and central terminals of non-myelinated afferent nerve fibers and, along with ATP, have been implicated in the transmission of sensory signals. Blockade of these signals with antagonists offers the potential to treat a broad range of pain conditions. This presentation will describe the lead discovery, optimization and SAR of selective drug-like antagonists; in vivo efficacy in a number of preclinical models outlining the therapeutic potential will be also presented.

(Invited)

Topic: medicinal chemistry and drug development

Synthesis of new uracil nucleotide derivatives and analogs as P2Y receptor agonists

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Background. Potent, selective and metabolically stable ligands for the uracil nucleotide-activated P2Y receptor subtypes (P2Y2,4,6,14) are still lacking.
Methods. In the present study, UDP and UTP were used as lead structures, and novel derivatives and analogs were synthesized essentially according to described procedures.1 Initial screening was performed by measuring inositol phosphate accumulation in 1321N1 astrocytoma cells expressing the human P2Y2, P2Y4, or P2Y6 receptors.[1] Results. A series of compounds of the general structures I and II were prepared, purified by anion exchange chromatography using FPLC followed by reversed phase HPLC and characterized by 1H, 13C and 31P NMR and LC/ESI-MS spectroscopy. A phenylamino moiety was successfully introduced at the Pgamma-atom of 5-bromo-UTP yielding triphosphoric acid amides.
Conclusions. We succeeded in synthesizing a series of base-modified UMP and UDP derivatives I, and UTP analogs II in which the triphosphate group was replaced by enzymatically stable residues. Preliminary results showed that all compounds

were P2Y receptor agonists, some exhibiting high affinity, particularly for the P2Y2 receptor subtype, combined with increased stability in comparison with the parent nucleotides (UTP, UDP).

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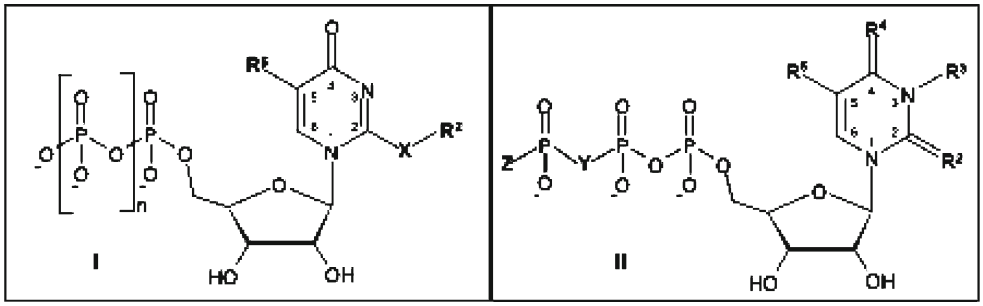

Identification of hydrolytically stable and selective $P2Y_1$ receptor agonists

Shay Elyahu[1], Jean Camden[2], Joanna Lecka[3], Gary A. Weisman[2], Jean Sévigny[3], Sylvie Gélinas[4], and Bilha Fischer[1]

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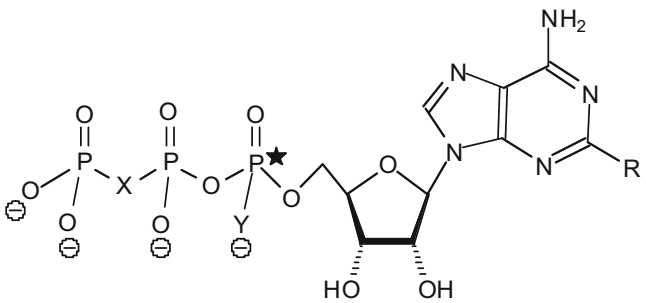
The usage of nucleotides for therapeutic purposes is limited due to their rapid dephosphorylation in extracellular media and their limited chemical stability. Therefore, here, we developed bioisoster-based non-hydrolyzable P2YR agonists to identify potential drug candidates. Specifically, we have synthesized and characterized beta,gama-CH_2-ATP analogues 1-3, evaluated their chemical stability in gastric juice pH, resistance to hydrolysis by alkaline phosphatase, NTPDase1,3,8, and NPP1,3, stability in human blood serum, and activity at $P2Y_{1,2,4,6}$ receptors. The hydrolytic stability ($t_{1/2}$) of analogues 1-3 at conditions simulating gastric juice acidity (pH 1.4/37°C) was 14.5 - 65 h. Analogues 1-3 were found completely resistant to alkaline phosphatase for 30 min at 37°C. Analogues 1-3 in human blood serum were hydrolyzed with $t_{1/2}$ of 12.7, 14.1, 47.1, and 71.9 h respectively. Analogues 1-3 were almost not hydrolyzed by NTPDases (<5% hydrolysis). Likewise, human NPP1 and NPP3 hydrolyzed 1-3 at <10% of the rate of pnp-TMP hydrolysis. Analogues 1 and 3B were agonists of the $P2Y_1R$ with EC_{50} values of 0.08 and 17.2 microM, respectively, as compared to 0.004 microM for 2-MeSADP. These analogues were virtually ineffective agonists of $P2Y_{2,4,6}R$. These features make analogues 1 and 3B attractive and selective therapeutic candidates for health disorders involving the $P2Y_1$-R.



1	X= CH_2	R= SMe	Y= O
2A, **2B**	X= CH_2	R= H	Y= BH_3
3A, **3B**	X= CH_2	R= SMe	Y= BH_3

Hydrolytically stable and selective $P2Y_{1/6}$ receptor agonists

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Most P2YR agonists proposed as drugs consist of a nucleotide scaffold, but their use is limited due to their chemical and enzymatic instability. To overcome the inherent instability of nucleotide drug candidates, yet, to retain potency and selectivity at P2YRs, we developed modified dinucleotides and isoster-based non-hydrolyzable nucleotides. We synthesized several ATP-betagamma-CH_2 analogues. These analogues exhibited $t_{1/2}$ values of 14.5 - 65 h in gastric juice pH, were completely resistant to alkaline phosphatase for 30 min at 37°C and slowly hydrolyzed in human blood serum ($t_{1/2}$ 12.7 - 71.9 h). In comparison to ATP, these analogues were barely hydrolyzed by NTPDase1,2,3,8 (<8% hydrolysis), and NPP1,3 (≤10% hydrolysis). Two of these analogues were selective agonists of the $P2Y_1R$ with EC_{50}'s of 0.08 and 17.2 microM, respectively. In addition, we developed boranophosphate dinucleotides ($Np_n(B)N$). Ap_5(gamma-B)A, was equipotent to 2-MeS-ADP (EC_{50} 0.063 microM), making it one of the most potent and selective $P2Y_1$-R agonists currently known. $Np_n(B)N$ analogues exhibited remarkable and sufficient chemical stability at physiological and gastric juice, respectively. The hydrolysis of Ap_3(beta-B)A by NPP1 and NPP3 was slowed by 40% and 59%, respectively, as compared to Ap_3A. The hydrolysis of Ap_4(beta-B)A and Ap_4(alpha-B)A (EC_{50} at $P2Y_1R$: 0.3 and 0.148 microM) by NPP1 was slowed by 90% and 85%, respectively, as compared to Ap_4A. Up_3(alpha-B)U was stable to NPP1 hydrolysis, equipotent to UDP at $P2Y_6R$ (EC_{50} 0.3 microM) and showed no activity at $P2Y_{1,2,4}Rs$. These features make the specified ATP-betagamma-CH_2 and $Np_n(B)N$ analogues potential therapeutic agents for health disorders involving the $P2Y_1R$ and $P2Y_6R$.

(Invited)

Exploring steric and electronic requirements for molecular recognition of UTP analogues by uridine nucleotide receptors ($P2Y_{2/4/6}$-Rs)

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$P2Y_{2/4/6}$-Rs are involved in mucociliary clearance in the respiratory tract, ocular surface regulation, and cardioprotection against hypoxic damage. Therefore, $P2Y_{2/4/6}$-Rs' agonists are potential drug candidates. To explore the stereo-electronic requirements for recognition of a $P2Y_{2/4/6}$-R agonist, we performed data-mining studies of recognition patterns in uracil-nucleos(t)ide-binding-proteins. Recognition of uracil-nucleos(t)ide involves: H-bonds (N3-H (98% of cases) and O2/4 (80/93%)); π-π (59%) and π-cation (25%) interactions. Based on these findings we designed UTP analogues bearing electron donating groups (EDG) at C5 and C6 positions. These substitutions were expected to improve π-π and π-cation interactions and H-bonding. The new analogues were tested at $P2Y_{2/4/6}$-Rs by stimulation of HEK 293 cells expressing r-$P2Y_{2/4/6}$-GFP and evaluation of $[Ca^{2+}]_i$. 6-Substituted analogues were inactive at $P2Y_{2/4/6}$-Rs. The 5-substituted analogue was slightly less active than UTP and UDP at $P2Y_{2/6}$-Rs (EC_{50}=2 and 0.9 microM, respectively). Using NMR we analyzed the conformation of theses analogues and determined the acid-base equilibria (pK_a) by UV measurements. 6-Substituted analogues showed a preference for the ribose *North* conformation, and *syn* or *high-syn* conformation around the glycosidic bond. The 5-OMe-UTP showed a preference for the *South* and *anti* conformer. The pK_a values of all ligands were only slightly different than that of UTP (~9.5). Therefore, we assume that the 6-substituted analogues are forced into the *syn*

conformation because of steric hindrance. The *syn* conformers are not recognized by the receptors. The 5-substitution permits *anti* conformation which retains the ligand's activity. We also concluded that EDGs at positions 5 and 6 of the uracil ring did not have a significant effect on the electron density of the uracil ring.

N-(9-Benzyl-2-phenyl-9H-8-azapurin-6-yl)-amides: a new class of A1 adenosine receptor ligands

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In the past we have obtained a number of ligands of A1 and A3 adenosine receptors modifying the substituent on the 2, 6 and 9 positions of 8-azadenine nucleus. A phenyl ring in the position 2 and a benzyl group in the position 9 were good substituents to assure binding with A1 and A3 receptors but not for A2A ones. Reguarding the subsutituent in position 6, we have shown that cyclohexyl, alkyl and alkylphenylamino groups, substituted or not with a hydroxyl group, are the best ones to obtain A1 selective ligands (A). [1]. In the same position, a phenyl-ureido function shifts affinity towards A3 receptors (B) [2]. We judged interesting to prepare and assay 2-phenyl-9-benzyl-8-azapurines having an amido group in the position 6 (C) interposed between the purine moiety and a cycloalkyl or a substituted phenyl group, to verify the effect of this function on the affinity of these molecules toward A1, A2A and A3 receptors.
Biological results indicate that these compounds show good or very good affinity towards A1 adenosine receptors, and low or no activity towards A2A and A3 receptors, disclosing a new class of selective A1 adenosine ligands.

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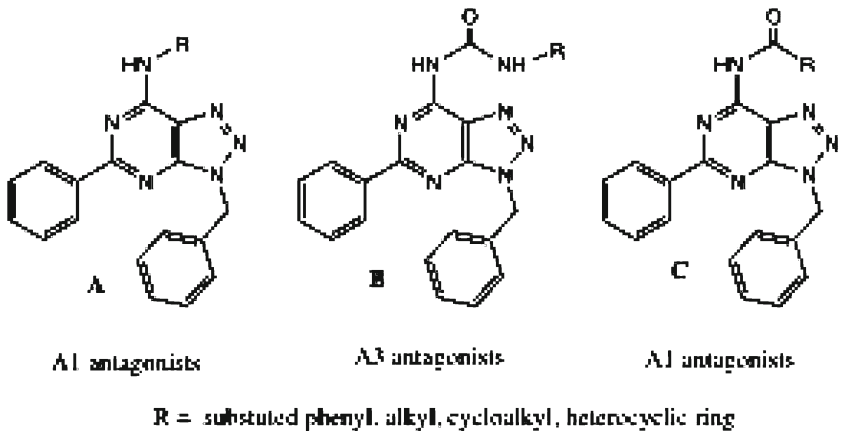

The suramin derivatives NF770 and NF778 are nanomolar potent antagonists of recombinant rat P2X2 receptors

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Background. P2X2 receptors are crucial for sensory transmission and modulation of synaptic function. The classical P2X receptor antagonists suramin, PPADS and TNP-ATP block P2X2 receptors with micromolar potency only (pIC50~4.5–6).

Antagonists with nanomolar potency have been identified for P2X1 and P2X3 receptors, but apparently not for P2X2 receptors. Using suramin as a lead, we have previously shown that the potency for the P2X1 and P2X3 receptor can be increased significantly by structural modifications. Here, we screened 157 additional suramin derivatives in an attempt to identify highly potent P2X2 receptor antagonists.
Methods. TECV electrophysiology was used to assess the inhibitory potency of suramin derivatives at various P2X receptor subtypes expressed in X. laevis oocytes. Results. The most potent inhibitor of rP2X2 channels identified in the screen was the suramin derivative NF770 (7,7′-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1 (4-methyl-phenylene)carbonylimino)) bis(1-methoxy-naphthalene-3,6-disulfonic acid), which blocked rat P2X2 receptors with a Ki of 13±1 nM. Other P2X receptor subtypes were blocked with the following rank order of potency based on Ki values: P2X2=P2X3 >= P2X2+3>P2X1 >= P2X1>P2X3, P2X2+3 >>> P2X4, P2X7).Conclusion. NF770 is a novel P2X2 receptor antagonist with nanomolar potency. As demonstrated previously for P2X1 and P2X3 receptor selective antagonists, the positions of sulfonic acids (or methoxy groups) are also of crucial importance for P2X2 subtype selectivity and potency of suramin derivatives. These data can be helpful in guiding structural modifications aimed at increasing P2X2 receptor selectivity.

Potent antagonistic action of an analogue of reactive blue-2 at the human platelet P2Y12-receptor

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Background: The P2Y12-receptor plays an important role in platelet aggregation. In the present study, we searched for amino acid residues of the human P2Y12-receptor involved in the binding of non-nucleotide antagonists.
Methods: Receptor function was assessed by measuring the cAMP response element (CRE) directed luciferase expression in Chinese Hamster Ovary cells. The cellular cAMP production was accelerated by forskolin and 2-methylthio-ADP was used to activate the recombinant human P2Y12-receptor.
Results: 2-Methylthio-ADP inhibited the CRE-dependent luciferase expression with an IC50 concentration of about 1 nM. The anthraquinone derivative reactive blue-2 shifted the concentration-response-curve to the right with an apparent pKB-value of 7.4. Its analogue PSB-0739 showed a markedly higher antagonistic potency with a pKB value of 9.5. In contrast, suramin and the suramin analogue Bay u9421 (sym-bis(benzoyl-1-naphthylamino-3,6-bisulfonate)chlorotriazine had low antagonistic potencies with pKB values of 5.5 and 5.9, respectively. In cells expressing the R256A-mutant receptor, the potencies of both reactive blue-2 (pKB 5.9) and PSB-0739 (pKB 8.9) were decreased, whereas the potencies of suramin and Bay u9421 were identical to those determined at wild type receptors. pKB values determined for all four antagonists in cells expressing the S101A mutant receptor did not differ from those obtained at wild type receptors.
Conclusions: The results demonstrate that reactive blue-2 and its analogue PSB-0739 are potent antagonists at the human P2Y12-receptor. Both antagonists are likely to interact with the residue Arg256 of the receptor protein. PSB-0739 appears to be the most potent non-nucleotide antagonist at the human P2Y12-receptor described so far.

Recognition of nucleotide agonists and antagonists by amino acid residues of the human platelet P2Y12-receptor

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Background: The P2Y12-receptor plays a crucial role in ADP-induced platelet aggregation. In the present study, we analyzed the role of polar amino acid residues in recognition of nucleotide agonists and antagonists by site-directed mutagenesis. Methods: Human wild-type P2Y12-receptors and mutant receptors were stably expressed in CHO Flp-In cells. Receptor function was determined by measuring the 2-methylthio-ADP induced inhibition of cellular cAMP levels stimulated by forskolin (using a radioaffinity assay or a CRE-directed reporter gene assay).

Results: In cells expressing wild-type receptors, 2-methylthio-ADP caused a concentration-dependent inhibition of the cAMP production with an IC50 concentration of about 1 nM. The same was observed in cells expressing S101A mutant receptors or R256K mutant receptors. However, in cells expressing R256A mutant receptors, there was a clear decrease in agonist potency with an IC50 concentration of about 10 nM. Several mutations caused a very marked or even total loss of receptor function; these include R256D, Y259D, K280A and H253A/R256A constructs. Cangrelor and 2-methylthio-AMP act as antagonists at the wild-type P2Y12-receptor with apparent pKB values of 9.1 and 5.8, respectively. Almost identical pKB values were determined for cangrelor and 2-methylthio-AMP at the R256A mutant receptor.
Conclusions: Arg256 (TM6) plays a crucial role in the recognition of nucleotide agonists, but not in the recognition of the nucleotide antagonists studied. Our results demonstrate in addition, that Tyr259 (TM6) and Lys280 (TM7) are also important for receptor function.

RN-1698: a novel P2X7 antagonist optimized for drug-like properties

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Background:
P2X7 is an ATP-gated ion channel that has generated interest within the pharmaceutical industry based on its function in IL-1 processing/release and possible role in pain as well as inflammation. Recently, AstraZeneca reported positive clinical results with AZD9056, a P2X7 antagonist, in patients with Active Rheumatoid Arthritis on background treatment of methotrexate or sulphasalazine.
Methods:
Property based drug design and a battery of potency, safety and DMPK assays were used in the hit to lead optimization of RN-1698 as a potent, selective and bioavailable P2X7 antagonist.
Results:
RN-1698 binds to hP2X7 with high affinity (Ki<10 nM) and antagonizes channel function based on calcium influx (IC50< 10 nM) and inward current (IC50<10 nM). In addition, RN-1698 inhibits IL-1beta release from THP-1 cells (IC50<10 nM) and retains activity in an ex-vivo human whole blood assay (IC50<150 nM). RN-1698 shows no activity towards other P2X receptors tested. Mechanistic studies indicate that RN-1698 binds to a site separate from the ATP binding site and functions as a negative allosteric modulator.
RN-1698 displays low hERG channel inhibition activity and is negative in the AMES and micronucleus assays. In CYP inhibition assays RN-1698 shows moderate inhibition of CYP 2C9 and 2C19 with no CYP induction. In rats and dogs RN-1698 displays high oral bioavailability with low clearance and volume of distribution.
Conclusion:
RN-1698 displays several characteristics desirable in a lead candidate compound. It has good potency and selectivity, acceptable risk profile, and good pharmacokinetic properties.

Prodrugs of A2B adenosine receptor antagonists

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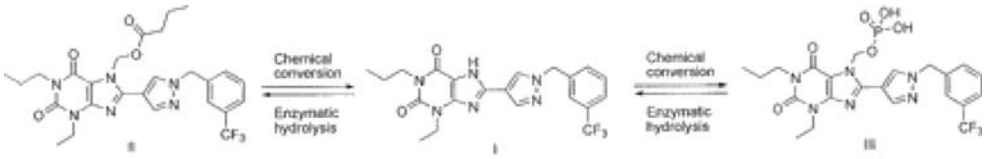
In our recent publications, we have disclosed the synthesis of several high affinity and selective A2B adenosine receptor antagonists. One of these analogs CVT-6883 (I) has successfully completed two Phase I clinical studies as a candidate for chronic airway inflammatory diseases. While in the discovery approach, to further improve the oral absorption and increase the oral bioavailability, we synthesized several prodrugs containing groups like alkyl, oxymtheyl carbamates, oxymethyl

esters (II) and oxymethyl phosphonates (III) at the N-7 position of the xanthine core, as xanthine analogs are generally known to display low oral absorption. The oxymethyl ester (II) and oxymethyl phosphonate (III) groups exhibited substantially high levels of drug substance in the plasma of rats and dogs compared to the parent compound and no prodrug was observed in the plasma.



Macromolecular ligands for G protein-coupled receptors: Poly(amidoamine) dendrimer conjugates of A2A adenosine receptor agonists

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Activation of the A2A adenosine receptor (AR), a G protein-coupled receptor (GPCR), by extracellular adenosine is antiaggregatory in platelets. An approach we have used in GPCR ligand design has been to apply structural modifications at permissive distal sites away from the pharmacophore, which has led to the drug design approach of functionalized congeners. These distal structures may impart additional selectivity and affinity. For instance, the pharmacological profile of ligands for GPCRs may benefit from attachment to a carrier molecule for delivery, and the chemical functional groups of a linker moiety can be varied, as guided by receptor affinity. Dendrimers are treelike macromolecules, which possess characteristics advantageous to their use as drug carriers. These include structural integrity, controllable component functional groups and physical properties by chemical synthesis, conjugation of multiple functional units at the periphery and the interior, and low enzymatic degradation rates. Poly(amidoamine) (PAMAM) dendrimer is composed of aliphatic amino and amido groups, and its biocompatibility has led to many applications in biomedicine. Here, we report the first example of covalent drug conjugates of dendrimers used to induce intracellular signal transduction across GPCRs. Multiple copies of an A2A AR agonist, the nucleoside CGS21680, were coupled covalently to PAMAM dendrimers and characterized spectroscopically. A fluorescent PAMAM-CGS21680 conjugate inhibited the aggregation of washed human platelets and was internalized. Multivalent dendrimer conjugates are envisioned to improve overall pharmacological profiles compared to the monovalent GPCR ligands. The binding of multivalent dendrimer conjugates simultaneously to multiple sites on receptor dimers and higher order structures may prove possible using this approach.

The glucose moiety of uridine 5'–diphosphoglucose is structurally permissive in activation of the human P2Y14 receptor

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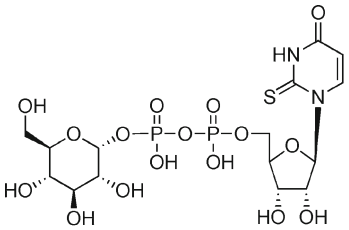
The P2Y14 receptor is a signaling protein in the eight member P2Y nucleotide receptor family that is activated by UDP-glucose (UDPG) and other uridine nucleotides. The P2Y14 receptor plays a role in the neuroimmune system, with expression in T cells, dendritic cells, and hematopoietic stem cells, and also is expressed in other tissues. We have systematically explored the structure

activity relationships of UDPG analogues at this receptor. With the exception of 2- and 4-thio modifications, most modifications of the nucleobase or ribose moieties diminished activity. UDPG also activates the P2Y2 receptor, but its 2-thio analogue (MRS2690) was selective for the P2Y14 receptor and activated this receptor with 7-fold greater potency than UDPG. An analogue in which the glucose moiety was replaced with a simple beta-methyloxy group (MRS2745) retained agonist activity at the P2Y14 receptor. Replacement of the ribose moiety with a rigid methanocarba group, either in the North or South conformation, abolished agonist activity. Many sugar moieties, such as mannose and fructose, may be substituted for the glucose with retention of activity. Thus, the glucose moiety is the most structurally permissive region of the ligand for derivatization.



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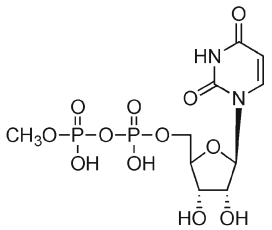

MRS2745

Identification of 2′-fluoro-pyrimidine modified aptamers as high-affinity inhibitors of P2X2 and P2X4 receptors

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Purinergic P2X receptors as ligand-gated ion channels activated by ATP are expressed in pre- and post- synaptic regions, thereby contributing to neurotransmitter release and neurotransmission. Until today, there are no specific antagonists available as subtype-specific inhibitors of these ionotropic receptors. Therefore, we have used the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) technique to develop stable 2′-F-pyrimidine-modified RNA aptamers as specific inhibitors of P2X2 and P2X4 receptors.

Following nine SELEX cycles using 1321N1 astrocytoma cells expressing recombinant rat P2X4 receptors as target, we verified that the selected aptamers bound to and inhibited P2X4 receptor-mediated ion flow in low micromolar concentrations, but had no effect on recombinant P2X2 or P2X7 receptors as determined by radioligand-binding studies and whole-cell current recording. RNA aptamers identified in 11 reiterative in vitro selection cycles of the combinatorial 2′-F-RNA pool against recombinant P2X2 receptors in 1321N1 astrocytoma cells resulted in the isolation of P2X2 receptor-specific aptamers. Sequenced individual aptamers 65 and 69 inhibited whole-cell currents induced by 50 υM with IC50 values of 8.7±3.1 μM and 3.5±2.7 υM, respectively.

These nuclease-resistant aptamers as novel inhibitors of P2X2 and P2X4 receptors are suitable for in vivo use and could turn into therapeutics of diseases in which P2X2 or P2X4 receptors are involved.

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Design, synthesis, and biological activity of new ligands for P2X3 receptors

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The purinergic P2X3 receptor subtype is an ATP-gated ionotropic receptor thought to mediate nociception especially in conditions like neuropathic and chronic pain. Hence, the availability of new selective P2X3 ligands might provide novel analgesics for the treatment of neuropathic pain.
A series of P2X3 ligands consisting of phosphorylated adenine derivatives were designed and synthesized starting from the observation that xanthine and adenine derivatives are purinergic P1 receptor antagonists and that several phosphorylated xanthine derivatives show high affinity at P2X receptors [1,2]. The new compounds were tested to explore their potential agonist or antagonist activity on HEK293 cells expressing rat P2X3 receptors. P2X3 receptor mediated effects were recorded, under voltage clamp, as inward currents evoked by the full agonist alfa,beta-meATP by using the patch clamp technique. Results showed that certain derivatives behave as agonists of P2X3 receptors.
Since several non-nucleotide diammino-pyrimidine derivatives were recently reported to block P2X3 receptors with high potency [3], a second series of P2X3 ligands was designed considering the structure of the above mentioned pyrimidines and modeling data obtained from comparing pyrimidine and purine scaffolds. Hence, purine derivatives bearing different substituents in 9 position were synthesized and evaluated for their potential antagonist activity. Data show that some of these new compounds blocked responses mediated by P2X3 receptor activation.

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High-throughput screening of potential drug candidates for GPR-17 receptor by FAC-MS

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The binding assay is a tool for all stages of drug discovery, including the study of disease mechanisms and the screening for the identification of lead compounds. Bioaffinity screening methods have emerged as an alternative to classical screening strategies. Recently, GPR17, a previously orphan receptor belonging to the "purin receptor cluster" has been identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. GPR17s were found to be highly expressed in organs typically undergoing ischemic damage, i.e., brain, heart and kidney. [1]
In this study, cellular membrane fragments obtained from cell lines expressing the target GPR17 were immobilized on the surface of an immobilized artificial membrane (IAM) liquid chromatography stationary phase following a previously reported procedure [2] and the resulting GPR17-IAM stationary phases used to create different columns for the optimisation of the chromatographic system. The columns were then used in frontal chromatography coupled with mass spectrometry detection (FAC-MS) studies to characterize ligand-GPR17 interactions.
The binding affinities (Kd) for known ligands for GPR17 were determined and correlated with literature values. The results indicate that GPR17 have been successfully immobilized with retention of its binding activity.
The chromatographic system was then used to screen a wide number of new nucleotide derivatives in order to select high affinity GPR17 ligands as lead compounds for the development of novel pharmacological strategies for acute and chronic neurodegenerative diseases.

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Monitoring cell surface expression of purinoceptors and nucleotide-metabolizing ecto-enzymes with antibodies directed against proteins in native conformation (adapincs)

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Extracellular purine nucleotides like ATP and NAD transmit signals either by direct binding to purinoceptors (i.e. the nucleotide-gated P2X ion channels and the G-protein coupled P2Y receptors) or by serving as substrates for various families of ecto-enzymes including, among others, ecto-nucleoside triphosphate diphosphohydrolases (ENTPDs, CD39 family) and ecto-nucleotide pyrophosphatase/phosphodiesterases (ENPPs, CD203 family). These enzymes regulate the availability of extracellular nucleotides and/or generate metabolites that serve signalling functions of their own. Investigation of these receptors and enzymes has been hampered by a lack of suitable antibodies, especially ones that recognize these proteins in their native conformation. This study reports the use of genetic immunization to generate such antibodies against P2X1, P2X4, P2X7, ENTPD1, ENPTD2, ENPTD5, ENPTD6, ENPP2, ENPP3, ENPP4, ENPP5, and ENPP6. Genetic immunization ensures expression of the native protein by the cells of the immunized animal and yields antibodies directed against proteins in native conformation (ADAPINCs). Such antibodies are especially useful for immunofluorescence and immunoprecipitation analyses, while antibodies raised against synthetic peptides usually function well only in Western-Blot analyses. Here we illustrate the utility of the new antibodies to monitor the cell surface expression of and to purify some key players of purinergic signalling.

Mutational analysis for the hetero-oligomerization of the P2Y11 with the P2Y1 receptor to control receptor internalization and P2Y11 receptor ligand selectivity

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Tissue-specific nucleotide signaling requires functional diversity within purinergic receptors. Here, hetero-oligomerization between P2Y1 and P2Y11 receptors is established. They associate physically, co-expressed in HEK293 cells, detected by co-pulldown, immunoprecipitation and FRET. P2Y11-P2Y1 receptor interaction has functional consequences. Interaction promotes agonist-induced P2Y11 receptor internalization.The P2Y11 receptor itself is completely endocytosis-reluctant. Co-internalization of these receptors was also seen in 1321N1 astrocytoma cells, co-expressing P2Y11 and P2Y1 receptors, with ATP or the P2Y1 receptor specific agonist 2-MeS-ADP. In HEK293 cells, the P2Y11 receptor associates functionally with the endogenous P2Y1 receptors. Pharmacological characteristics of the P2Y11 receptor expressed in HEK293 cells, determined by Ca2+ responses, revealed unique ligand-specificity, different from the P2Y11 usual agonist profile. The P2Y11 receptor shows stereospecific ligand recognition of P-alpha-substituted ATP derivatives (ATP-alpha-S isomers), candidates for development of selective P2Y11 receptor agonist immune modulators. We studied molecular determinants of the stereoselective recognition at the P2Y11 receptor. Glu186 in extracellular loop 2 and Arg268 in transmembrane domain-6, part of the nucleotide binding pocket, were selected for mutational analyses. In Arg268/Ala or Gln, stereospecific recognition of the ATP-alpha-S isomers at the P2Y11 receptor was lost. At the Glu186Ala mutant, stereoselective differentiation was increased. The stereospecificity of the P2Y11 receptor for Palpha-substituted ATP derivatives is largely determined by Arg268 in TM6. Thus, hetero-oligomerization of the P2Y1 and P2Y11 receptors allows novel receptor functions of the P2Y11 receptor in response to extracellular nucleotides, and stereospecific anylysis will allow designing receptor-subtype selective ligands.

New trisubstituted adenosine derivatives as potent and selective agonists for the human adenosine A3 receptor

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Adenosine A3 receptor (AA3R) agonists have cardioprotective and cerebroprotective potential, and also may have a role in the treatment of asthma, as anti-inflammatory and immunosuppressive agents, and in cancer therapy as cytostatics and chemoprotective compounds [1]. Since 4′-methylcarboxamidoadenosine (MECA) derivatives bearing in 2 position (ar) alkynyl chains and in N6 position a methoxy group showed good affinity and different degree of selectivity for the human AA3R subtype [2], the synthesis of a new series of N6-substituted 2-alkynyl-MECA derivatives was undertaken.
Compounds affinities were evaluated at the human recombinant adenosine receptors, stably transfected into CHO cells, utilizing radioligand binding studies (A1, A2A, and A3) or adenylyl cyclase activity assay (A2B).
Results demonstrated that these new derivatives are endowed with very high affinity and selectivity for the human AA3R subtype, with Ki hA3 values at sub-nanomolar level and high selectivity versus the other adenosine receptor subtypes. Functional studies, carried out to evaluate the ability of compounds to inhibit forskolin-stimulated cAMP production via human AA3R in comparison with the full A3 agonist Cl-IB-MECA, demonstrated that the new compounds behave as full agonists resulting to be among the most potent and selective ligands at the human AA3R subtype reported so far.
Docking studies in a Rhodopsin-based homology model of human AA3R guided in the analysis and rationalization of structure-activity relationships for this new class of compounds.

[1] Cristalli, G.; Volpini, R. Curr. Top. Med. Chem. 2003, 3, 355.
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Carbon monoxide is a rapid modulator of recombinant and native P2X2 ligand-gated ion channels

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Carbon monoxide (CO) is a potent modulator of a wide variety of physiological processes such as sensory signal transduction. Many afferent sensory pathways are dependent upon purinergic neurotransmission, but direct modulation of P2X receptors by this important, endogenously produced gas has never been investigated. Using conventional, whole-cell patch-clamp of heterologously and natively expressed P2X2, P2X2/3 and P2X3 receptors, we demonstrate that CO is a potent and selective modulator of native and recombinant P2X2 receptors. Brief pre-application of a CO donor resulted in dramatic enhancement of P2X2 currents at sub-EC50 concentrations of ATP and modest inhibition of P2X2 currents at high ATP concentrations. Furthermore, CO evoked a slowing of the apparent ATP off-rate. The CO donor effect had an EC50 of between 1.5 and 2 μM. CO was without effect on recombinant P2X2/3 and P2X3 receptors. Since neither pre-incubation with 8Br-cGMP nor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ - a potent blocker of soluble guanylyl cyclase) ameliorated the ability of CO to enhance the ATP-evoked P2X2 currents, we suggest that CO binds to the receptor itself or to an associated protein partner. These data provide a compelling explanation for how CO might regulate sensory neuronal traffic in physiological reflexes such systemic oxygen sensing but also show that CO can be used as a discriminating pharmacological tool to probe for the involvement of homomeric P2X2 receptors in native physiological systems.

Boranophosphate isosters of dinucleoside polyphosphates are potent and selective P2Y-receptor agonists

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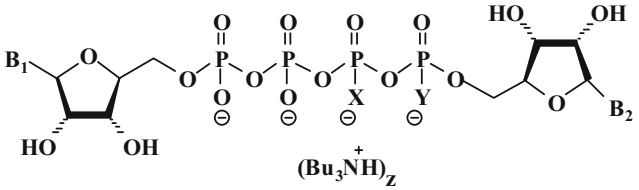
Dinucleoside polyphosphates, NPnN', exert their physiological effects via P2 receptors. NPnN' are attractive drug candidates as they offer better stability and specifity compared to nucleotides, the most common P2-receptor ligands. To further improve the properties of NPnN', which are still pharmacologically unsatisfactory, we developed novel boranophosphate isosters of dinucleoside polyphosphates, denoted as NPn(beta-B)N' and NPn(alpha-B)N. NPn(beta-B)N' analogues were obtained as the exclusive products in unusual multi - component reaction probably facilitated by pre-organization of three reactant molecules by a Mg2+ ion. The diastereoselectivity of the reaction supports this mechanism. NPn(alpha-B)N analogues were obtained in a two-step synthesis involving the reaction of nucleoside phosphorimidazolide with NDP(alpha-B). The potency of these new analogues as P2Y- receptor agonists was evaluated by the agonist – induced Ca2+ release of 1321N1 cells expressing the tP2Y1, hP2Y2, hP2Y4 or rP2Y6-receptors. Compounds 1-8 were found to be selective P2Y receptor agonists. The most potent P2Y1-R agonist was AP3(alpha-B)A, 5 (A Isomer, EC50 0.18 microM). The most potent P2Y6-R agonist was UP3(alpha-B)U, 7 (B Isomer, EC50 0.3 microM). These results indicate that the receptor subtype selectivity can be controlled by the position of the borano moiety on the polyphosphate chain and the nucleobase identity.



$(Bu_3\overset{+}{N}H)_z$

1	$B_1 = B_2 = A$;	n = 1;	$X = BH_3$;	Y = O;	z = 4
2	$B_1 = B_2 = U$;	n = 1;	$X = BH_3$;	Y = O;	z = 4
3	$B_1 = A$; $B_2 = U$;	n = 1;	$X = BH_3$;	Y = O;	z = 4
4	$B_1 = U$; $B_2 = A$;	n = 1;	$X = BH_3$;	Y = O;	z = 4
5	$B_1 = B_2 = A$;	n = 0;	X = O;	$Y = BH_3$;	z = 3
6	$B_1 = B_2 = A$;	n = 1;	X = O;	$Y = BH_3$;	z = 4
7	$B_1 = B_2 = U$;	n = 0;	X = O;	$Y = BH_3$;	z = 3
8	$B_1 = B_2 = U$;	n = 1;	X = O;	$Y = BH_3$;	z = 4

SYMPOSIA 4: PURINERGIC SIGNALLING IN CELL MIGRATION

Involvement of the P2Y4 receptor in inflammation and angiogenesis

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The study of knockout mice recently revealed the role of the P2Y2 and P2Y12 receptors in the chemotaxis of neutrophils and microglia respectively. Beside chemotaxis, leukocytes adhesion to microvascular endothelial cells is a key step in their migration to inflammation sites. It is classically assumed that endothelial cells express P2Y1 and P2Y2 receptors, but this concept needs revision. Indeed UDP induces endothelium-dependent relaxation of the aorta and this effect was abolished in the recently generated P2Y6-/- mice. Concerning microvascular endothelial cells, we have observed the expression of P2Y4 mRNA in endothelial cells isolated from mouse heart but not lung. The effects of ATP and UTP on the migration, proliferation and VCAM-1 expression of cardiac endothelial cells were strongly decreased in P2Y4-deficient mice. This was correlated with a reduction of UTP-induced macrophage adhesion. P2Y4-null mice displayed an angiogenesis defect in the matrigel and air pouch granuloma models. This expands the phenotype of P2Y4-deficient mice, which so far was characterized only by the loss of epithelial chloride secretion induced by ATP and UTP in jejunum and colon.

(Invited)

CD73 is required for efficient entry of lymphocytes into the central nervous system during EAE

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Background: CD73 is an extracellular enzyme of the purine catabolic pathway that catalyzes the breakdown of AMP to adenosine. Due to the strong immunosuppressive and anti-inflammatory properties of adenosine, we investigated the role of CD73 in experimental autoimmune encephalomyelitis (EAE) the animal model for multiple sclerosis (MS). MS is an inflammatory disease of the central nervous system (CNS) mediated by autoagressive immune cells. We predicted that cd73-/- mice would develop severe EAE due to their inability to produce extracellular adenosine to modulate CNS inflammation.
Methods/Results: Surprisingly, cd73-/- mice were resistant to EAE. CD4 T cells from cd73-/- mice were able to proliferate normally, secrete more proinflammatory cytokines than wild type mice and are capable of potentiating EAE when transferred into naïve CD73+/+ recipients. Therefore, the protection from EAE observed in cd73-deficient mice was not due to a deficiency in T cell function. Immunohistochemical analysis showed cd73-/- mice had fewer infiltrating lymphocytes in the CNS compared to wild type mice. Importantly, susceptibility to EAE could be induced in cd73-/- mice following the transfer of wild type derived CD73+/+ CD4+ T cells. Consistent these data, CD73 is expressed on the choroid plexus of wild type mice, which is the site of the blood-CSF barrier. Finally, blockade of adenosine receptor signaling with an antagonist specific for the A2a adenosine receptor inhibits lymphocyte migration into the CNS and protects wild type mice from EAE.
Conclusion: Thus, we conclude that CD73 expression and adenosine receptor signaling are required for the efficient entry of lymphocytes into the CNS during EAE.

E-NTPDase1 and alkaline phosphatase control chemotaxis of human neutrophils by generating adenosine from released ATP

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Polymorphonuclear neutrophils (PMN) release ATP in response to stimulation by chemoattractants, such as the peptide f-met-leu-phe (fMLP). Released ATP and its hydrolytic product adenosine regulate chemotaxis of PMN by activating P2 and P1 receptors, respectively. Here we studied the ecto-enzymes of PMN that mediate the generation of extracellular adenosine. Human PMN efficiently hydrolyze ATP and ADP to AMP, while the conversion of AMP to adenosine is much slower. We have found that ecto-nucleoside-triphosphate-diphosphohydrolases 1 (E-NTPDase1, CD39), which hydrolyses ATP to AMP, and alkaline phosphatase (ALP), which converts AMP to adenosine, are highly expressed in human PMN and that E-NTPDase1 is recruited to the leading edge upon polarization of PMN in a chemotactic gradient. Inhibitors of E-NTPDase1 reduce ATP hydrolysis while inhibitors of ALP block the generation of adenosine. Inhibition of both enzymes significantly reduces the migration speed of PMN towards chemoattractant in a gradient field. Migration speeds of PMN isolated from the bone marrow of CD39 knockout (CD39 KO) mice are significantly slower, compared to wild-type (WT) cells. Similarly, in vivo migration of cells towards a chemotactic peptide injected into the peritoneal cavities of CD39 KO mice are significantly diminished, when compared to WT mice. Together these data identify the concerted actions of CD39 and ALP in the regulation of PMN chemotaxis by generating extracellular adenosine from endogenously released ATP (Funding: Novo Nordisk, NIH, and DoD).

ATP signaling regulates membrane protrusions and chemotaxis in macrophages: role of P2Y2 receptors

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Macrophages express a repertoire of P2Y and P2X receptors but little is known about the function of nucleotide signaling in these cells. We have recently shown that mouse resident peritoneal macrophages strongly express ATP- and UTP-selective P2Y2 receptors, but not P2Y4 receptors. Here, we investigated the effects of ATP on lamellipodia formation, the initial step in cell migration. Resident peritoneal macrophages were isolated from wild-type (WT) or P2Y2 receptor knockout (P2Y2-/-) mice, and challenged with ATP, ATPgammaS, UTP or UTPgammaS. When applied globally, triphosphate nucleotides dramatically induced membrane protrusions (lamellipodia), such that cell 2-dimensional area increased by a factor of 3-4 within 5 min. Combined fluorescence imaging and topographic scanning by atomic force microscopy in aqueous buffer revealed that lamellipodia were extremely thin (~100 nm in height). ATP- and UTP-induced membrane protrusions were absent in P2Y2-/- cells or WT cells treated with the Go/i inhibitor pertussis toxin. RT-PCR analyses revealed that purified macrophages express Gi2 and Gi3, but not Gi1 or Go. Furthermore, in preliminary time-lapse imaging experiments using a novel chemotaxis chamber (Ibidi, Martinsried, Germany) we found that macrophages migrate toward complement component C5a but not in the presence of the ATP scavenging enzyme apyrase. P2Y2-deficient macrophages migrate toward C5a but less effectively compared to WT cells. We conclude that ATP and UTP induces lamellipodia by acting at P2Y2 receptors coupled to Gi2/3. Our findings support a potential role for extracellular ATP in controlling lamellipodia dynamics, leading edge formation and chemotaxis in macrophages.

The endothelial P2Y2 receptor mediates LPS-induced neutrophil transendothelial migration

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Background: Endothelium functions as an interactive barrier between blood and tissue that regulates the extravasation of circulating neutrophils to surrounding tissue. The exposure of endothelium to Gram-negative bacteria lipopolysaccharide (LPS) evokes a rapid neutrophil transendothelial migration (TEM). In this work, we hypothesized that LPS-induced neutrophil TEM was mediated by extracellular nucleotides via P2 receptors.
Methods: Neutrophil migration across LPS-stimulated HUVEC monolayers was investigated using a Boyden chamber system. For the P2Y2 and P2Y4 knockdown, HUVEC were transfected with specific siRNAs.
Results: In agreement with our hypothesis, the general P2 receptor antagonists, suramin and RB-2, completely inhibited the TEM of human neutrophils when added together with LPS to the basolateral (the bottom chamber) side of HUVEC. Interestingly, these antagonists had no effect when added to the upper chamber. HUVEC express P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11. The involvement of P2Y1, P2Y6 and P2Y11 in this system was excluded as the selective antagonists of these receptors, MRS2500, MRS2578 and NF157, did not affect neutrophil migration. The siRNA experiment showed that P2Y2, but not P2Y4, knockdown in HUVEC diminished significantly neutrophil recruitment compared to the control HUVEC. This LPS-induced neutrophil TEM may involve the activation of the Rho kinase-dependent signaling pathway as the pre-incubation of HUVEC monolayers with the Rho kinase inhibitor Y-27632 significantly inhibited (> 50% decrease) neutrophil migration.
Conclusion: These data indicate that the endothelial P2Y2 receptor mediates LPS-induced neutrophil TEM in vitro.

Contribution of ATP-gated P2X1 ion channels to the control of neutrophil chemotaxis

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Background: Extracellular ATP has recently been shown to regulate neutrophil chemotaxis through P2Y2 receptors. Whether neutrophils express ATP-gated P2X ion channels is currently unclear and their role in chemotaxis has never been investigated.
Methods: RT-PCR, Western blotting, immunofluorescence and whole-cell patch clamp experiments were performed to assess expression and functionality of P2X1 ion channels. P2X1-mediated actin cytoskeletal reorganization was analyzed by confocal microscopy. Neutrophil migration was studied in Boyden Chambers and on collagen IV-coated Ibidi chemotaxis microslides.
Results: Both human and mouse neutrophils express functional P2X1 ion channels. Patch clamp recordings showed rapidly desensitizing currents of around 100 pA/pF in human peripheral neutrophils upon application of P2X1-selective agonists, alpha, beta-methylene ATP and beta, gamma-methylene ATP. Beta,gamma-methylene ATP induced 60 pA/pF currents in wild-type mouse peritoneal neutrophils that were absent in P2X1-/- cells. Alpha,beta-methylene ATP elicited rapid reorganization of the actin cytoskeleton in human peripheral and mouse peritoneal neutrophils adhered on glass or type IV collagen. In Boyden chambers, alpha,beta-methylene ATP provoked random cell migration and enhanced fMLP- and W-peptide-induced chemotaxis of human and wild-type mouse neutrophils, respectively. Chemotaxis of P2X1-/- neutrophils was not impaired under these conditions. Time-lapse videomicroscopy revealed that collagen IV-adhered P2X1-/- neutrophils oriented better in a gradient of W-peptide but covered shorter distances than wild-type neutrophils, which correlated with their defective static adhesion on this substrate.
Conclusions: P2X1 ion channels act to control neutrophil adhesion and migration in a chemoattractant gradient.

Molecular mechanisms of ATP-induced microglial chemotaxis

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In response to pathological stimuli microglia rapidly cause morphological change and migrate toward the lesioned site. These microglial responses are crucial for tissue repair of a damaged CNS. Extracellular ATP is known to regulate physiological functions of microglia through various subtypes of P2 receptors. We have previously shown that ATP induces membrane ruffling and chemotactic migration of microglia. The ATP-induced migration is mediated by P2Y12 and P2X4, and requires the activation of phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC). Recently ATP has been reported to induce the extension of microglial processes through P2Y12 in response to neuronal death in a mouse brain. In order to investigate the signal transduction underlying the process extension, we established three-dimensional (3D) culture system of microglia in collagen gels. Microglia in 3D gels extended their processes toward 50 μM ATP. Experiments with agonists and antagonists against P2Y12 or P2X4 suggested that both P2 receptors mediate the process extension. The effects of PI3K and PLC inhibitors indicated that PI3K and PLC signaling pathways are involved in the process extension. We also found that microglia did not migrate into 3D gels within 2 h after ATP stimulation, however, they migrated into 3D gels 4 h after ATP stimulation. These observations suggest that the cell migration of microglia in 3D matrices requires the activation of some other signals in addition to those underlying the process extension.

(Invited)

Regulation of neutrophil chemotaxis by P2Y2 and A3 receptors

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Chemotaxis allows polymorphonuclear neutrophils (PMN) to rapidly reach infected and inflamed sites. However, excessive influx of PMN damages host tissues. Better knowledge of the mechanisms that control PMN chemotaxis may lead to improved treatments of inflammatory diseases. Recent studies have shown that ATP release and autocrine feedback via P2Y2 and A3 receptors play important roles in the control of PMN chemotaxis. We found that PMN release ATP in response to stimulation of formyl peptide receptors (FPR) with chemoattractant peptides. ATP release occurs primarily at the leading edge, resulting in the activation of adjacent P2Y2 receptors that amplify chemotactic signals. Subsequent polarization of PMN within the chemotactic gradient field results in the translocation of A3 adenosine receptors and CD39 to the leading edge. CD39 is involved in the formation of adenosine from released ATP, resulting in a second feedback loop via A3 adenosine receptors that controls the migration speed of PMN chemotaxis. These findings suggest that ATP release, extracellular adenosine, and purinergic receptors are potential therapeutic targets that could be used to modulate PMN chemotaxis in a number of inflammatory and infectious diseases (Supported by NIH, DOD, and Novo Nordisk).

(Invited)

SYMPOSIA 5: NEUROPROTECTION IN THE BRAIN

Pharmacological modulation of P2 receptors in neurodegenerative diseases

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In the immune system, extracellularly-released nucleotides (ATP in particular) act as diffusible danger signals and represent an essential defense mechanism in the initial beneficial inflammatory phase (Ferrari et al., J Immunol. 176:3877–83, 2006). Recent data suggest a similar role in the CNS, where nucleotides are released upon injury and P2 receptors function as amplification device to spread the ATP wave. However, if unappropriately sustained, danger signals become detrimental and eventually contribute to neuronal death. In line with this hypothesis, selected P2X/P2Y antagonists reduced brain damage in various in vitro and in vivo neurodegeneration models (Franke et al., *Pflugers Arch.* 452:622–44, 2006). In this respect, we have recently identified a new purinergic receptor likely playing a prominent role in ischemia-associated damage. This receptor (GPR17) is dually activated by uracil nucleotides and cysteinyl-leucotrienes, a chemically-unrelated, arachidonic acid-derived, family of inflammatory mediators which are also released in the ischemic brain (Ciana et al., EMBOJ, 25:4615–27, 2006). Blockade of GPR17 by a pharmacological or an anti-sense strategy markedly reduced the propagation of acute ischemic damage (see also: Lecca et al., this meeting). We have recently confirmed a potential role for GPR17 in the neurodegeneration associated to spinal cord injury (SCI, Villa et al., this meeting). Of high interest for neuroreparative events, GPR17 was also found on brain neural stem cells (Lecca et al.; Ceruti et al., this meeting), where it may regulate differentiation to mature neurons or glia (ibidem; see also: Ciampi et al., this meeting). Globally, our data suggest that acute over-activation of GPR17 during ischemia or SCI may contribute to damage; conversely, presence of GPR17 on neuroprogenitor-like cells also suggests a role in the long-term reparative changes associated to these conditions.

(Invited)

Adenosine receptors are increased and sensitized in the frontal cortex from Alzheimer's disease cases

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Adenosine is a nucleoside widely distributed in central nervous system. Through specific G-protein coupled receptors binding, adenosine acts as a neuromodulator and neuroprotector because it modulates excitatory neurotransmitter release, mainly glutamate. Adenosine receptors have been classified into A1, A2A, A2B and A3 receptors. A1 and A3 receptors inhibit adenylyl cyclase activity while A2A and A2B receptors stimulate this enzymatic activity. Adenosine A1 and A2A receptors were studied in post-mortem human cortex in Alzheimer's Disease (AD) and age-matched controls by using radioligand binding, Western-blotting and real time PCR assays. Total adenosine A1 receptor number, using [3H]DPCPX as radioligand, was significantly increased in AD cases in early and advanced stages without differences with the progression of the disease. A significant increase in A1R levels was also observed by Western blot in early and advanced stages of AD. In addition, increased number of adenosine A2A receptors by using [3H]ZM 241385 as radioligand and Western blot were also observed in AD samples as comparing with control samples. Increased binding and protein expression levels of adenosine receptors were not associated with increased mRNA levels coding A1 and A2A receptors. On the other hand, A1 and A2A receptor-mediated response determined as adenylyl cyclase activity exhibited in the presence of selective ligands was also increased in AD samples. These results show that adenosine A1 and A2A receptors are increased and sensitized in frontal cortex from AD cases, suggesting the involvement of these receptors in the pathogenesis of AD and opening new therapeutic perspectives against this neurodegenerative disease.

P2Y12 receptor protein expression in the lesioned cerebral cortex of multiple sclerosis patients

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Multiple sclerosis (MS) is a disease of the CNS neuropathologically characterized by myelin, oligodendrocyte and axonal loss. Although it is considered as a white matter disease, grey matter demyelination has also been described, but its nature, pathogenesis and clinical implication has not been well evaluated. Demyelination and oligodendrocyte lesions in grey matter appear to be highly scattered and particularly abundant also in cerebral cortex, where they constitute a significant proportion of the overall MS lesions of the brain. Among the exogenous signals lately emerging as prime actors in the neuron-to-glia interplay responsible for brain malfunctioning, is ATP acting on P2 receptors. Oligodendrocytes express a heterogeneous collection of P2 proteins and, among these, a broad distribution of the metabotropic P2Y12 subunit was previously observed to occur in vivo (Laitinen et al., 2001) and in vitro (Agresti et al., 2005) in progenitors cells. Moreover, our group has recently established the cellular and subcellular localization of the P2Y12 receptor in rat brain in vivo, and particularly in oligodendrocytes and myelin sheets in cerebral cortex, subcortical areas, and periventricular white matter, areas highly affected by MS demyelination (Amadio et al., 2006). In the present study, we thus analyze by immunohistochemistry and confocal microscopy the pattern of P2Y12 protein expression and distribution in coronal sections from MS post-mortem human brain tissue (provided by UK MS Tissue Bank). The aim of our study is to correlate the P2Y12 receptor to the extent of grey matter demyelination and pathologic alterations occurring especially in cortical areas.

The neuroprotection afforded by blockade of adenosine A2A receptors against beta-amyloid peptides involves the control of P38 mapk pathway and mitochondrial function

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The administration of soluble beta-amyloid peptide (1–42, Abeta) causes memory impairment and synaptotoxicity, two cardinal events in Alzheimer's disease (AD), which are prevented by caffeine or A2A receptor (A2AR) antagonists in accordance with the inverse relation between caffeine consumption and the incidence of AD. We now tested the molecular pathways associated with this A2AR-mediated protection upon exposure of rat hippocampal cultured neurons (7DIV) to Abeta (500 nM). This caused an increase of p38 (40.5±15.11%, n=7) and JNK (69±21%, n=6) phosphorylation after 2 hours of incubation. At this time point, A2AR blockade with SCH58261 (50 nM) increased JNK and prevented p38 phosphorylation. In contrast, neuroprotection afforded by A2AR blockade did not involve the cAMP/PKA pathway. To probe the putative involvement of mitochondrial dysfunction, we measured mitochondrial viability (MTT reduction) and mitochondrial potential (using a probe, TMRM) in rat hippocampal synaptosomes, where A2ARs are enriched, upon exposure to Abeta (500 nM) for 2 hours. Aâ decreased MTT reduction (8.3±3.6%, n=4) and mitochondrial potential (15.3±3.7%, n=5), which was counteracted by SCH58261 (50 nM). Hence it is concluded that the ability of A2ARs to control Abeta-induced toxicity likely involves the control of p38 MAPK pathway and mitochondrial dysfunction rather than the cAMP/PKA pathway. (Supported by FCT and Pfizer)

The new P2Y purinoceptor GPR17 is differentially expressed during oligodendrocyte precursor cells development

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We have recently demonstrated that the previously orphan GPR17 receptor is a new dualistic P2Y receptor, responding to both uracil nucleotides and cysteinyl leukotrienes (Ciana et al, 2006. EMBO J 25:4615). Interestingly, GPR17 was also found to be one of the 3 genes exclusively expressed by adult neural stem cells (Maisel et al, 2007. Stem Cells 25:1231), suggesting its possible role in cell fate determination. Since progenitor cells can be also found in brain parenchyma, we aimed at characterizing GPR17 expression in primary cultures from rodent cerebral cortex. We utilized murine astrocytic cultures, kept under different proliferating or differentiating conditions, and rat neuronal cultures. GPR17 expression was evaluated by RT-PCR and immunocytochemical techniques, alone or together with lineage specific markers.
In both experimental settings, we found that GPR17 is uniquely expressed by a subpopulation of oligodendrocyte precursor cells (OPCs); moreover, receptor expression is down-regulated under highly proliferating conditions while increasing along with cell differentiation. Interestingly, a prolonged exposure to bFGF and EGF addresses cells towards a higher receptor expression when shifted to differentiating conditions. GPR17 is mainly expressed by Ng2- and O4-positive immature oligodendrocytes, while CNPase- and MBP-positive mature cells progressively loose receptor expression. GPR17 expression sensitizes cells to adenine nucleotide-induced cytotoxicity, whereas its activation with UDP-glucose promotes cells differentiation towards a more mature phenotype. Our results suggest that GPR17 might play a key role in OPCs differentiation, and might therefore represent a potential target for the development of new therapeutic approaches to demyelinating diseases, such as multiple sclerosis.

Locating the adenosine A2A receptors that regulate neuronal survival

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Depending on cell type and injury trigger, brain damage can be attenuated by inactivation or activation of the A2A receptor (A2AR). This apparent paradox is likely due to the complex actions of A2ARs in different cells, including forebrain neurons and bone marrow derived cells (BMDCs), each with different signalling and actions. Thus, it is critical to distinguish the contribution of A2ARs in different cells to modulation of different brain insults. To address this issue, we have developed forebrain A2AR knockout KO mice (fb-A2AR KO) with selective inactivation of A2ARs in forebrain neurons by Cre-loxP strategy and chimeric mice with selective inactivation of A2ARs in BMDCs by bone marrow transplantation. In middle cerebral arterial occlusion (MCAO) model of stroke, we found that selective inactivation of A2ARs in BMDCs significantly reduced infarct volume. Interestingly, the infarct volume was also significantly reduced in fb-A2AR KO mice compared to their wild-type littermates after MCAO. Thus, inactivation of A2ARs in either BMDCs or forebrain neurons attenuates ischemic brain injury, suggesting that A2AR activities in both BMDCs and forebrain neurons contribute to the modulation of ischemic brain injury through the multiple mechanisms. In MPTP model of PD, selective inactivation of A2ARs in BMDCs or forebrain neurons did not affect MPTP-induced dopaminergic neurotoxicity. However, intracerebral injection of the A2AR antagonist KW6002 into fb-A2AR KO mice produced neuroprotection against MPTP. Furthermore, this neuroprotection was associated with attenuation of MPTP-induced microglial and astrocytic activations by KW6002. Thus, A2ARs in glial cells (but not forebrain neurons nor BMDCs) are important contributor to modulation of MPTP neurotoxicity. These findings highlight that the need to delineate the complex actions of A2ARs in different cells before the safe and effective treatment of A2AR antagonists for neurological disorders can be realized.

(Invited)

GPR17 and vascular endothelial growth factor receptor stimulation in PC12 cells, a model of neuronal differentiation

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Background. Purinergic signalling is present in early stages of embryogenesis and is involved in cell proliferation, migration and differentiation. Recently, it has been pointed out that purinergic receptors are involved in the control of mitogenic signalling activated by growth factor receptors [1]. The aim of this work was to evaluate the functional interaction between the new dualistic receptor GPR17 [2] (which responds to both uracil nucleotides and cysteinyl-leucotrienes) and the vascular endothelial growth factor receptor (VEGFR) in PC12 cells, a model of neuronal differentiation.
Methods. PC12 cells were grown in medium containing the differentiation peptide NGF (100 ng/ml) for various time periods (0–10 days).GPR17 expression was evaluated by RT-PCR experiments and immunocytochemistry. NGF treated PC12 cells were exposed to the GPR17 agonist UDP-glucose and LTD4, alone and in the presence of VEGF (100 ng/ml). Cell proliferation was then measured.
Results and Discussion. Experimental data showed that GPR17 is not expressed in PC12 cells in the early stages of differentiation in serum-containing condition. In serum deprivation experiments, GPR17 expression was demonstrated after 10 days NGF-mediated differentiation. In this condition UDP-glucose, LTD4 and VEGF significantly increased cell proliferation. No improvement of cell viability was found in VEGF treated cells in the presence of GPR17 ligands. These results prompt us to investigate the intracellular signals involved in cell proliferation promoted by GPR17 and VEGF receptor stimulation.

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Enhanced impact of adenosine A2A receptor blockade on the control of long term potentiation in the hippocampus of aged rats

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We have recently provided evidence for a particular role of caffeine and of A2A receptors (A2ARs) antagonists in the prevention of memory dysfunction after different brain insults known also to cause an up-regulation of cortical A2ARs. For instance, ageing enhances the density of hippocampal A2ARs and caffeine or A2AR antagonists prevent the age-induced memory dysfunction. We also reported that A2ARs control hippocampal synaptic plasticity phenomena such as long-term potentiation (LTP), a neurophysiological correlate of learning and memory. However, it is not known if the impact of A2AR in the control of hippocampal LTP also augments on aging.
This was investigated using male Wistar rat hippocampal slices from 3 age groups (adults: 10-15; older adults: 36-38; aged: 70-80 weeks old). Field-excitatory post-synaptic potentials were recorded from the CA1 area. LTP was induced by a of high frequency stimulation train (HFS, 100 Hz, 1 s).
The application of SCH 58261 (50 nM) caused an attenuation of the LTP magnitude that was similar in young adults and old adults, by 12.8±4.6% and 16.0±2.3% (n=4, $P<0.05$), respectively, when compared to control. In aged animals, the blockade of A2A receptors elicited a significantly larger effect, decreasing the magnitude of LTP by 36.8±3.6% (n=4, $P<0.05$) in relation to control.
This shows that the impact of A2ARs on LTP is larger in aged rats in accordance with the age-associate increased A2AR density and with the ability of A2AR to prevent memory dysfunction in aged animals. (Supported by FCT and Pfizer)

Mild hypoxia modulates adenosine receptors in C6 glioma cells through A1 adenosine receptor

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The discovery that neural stem cells (NSC) in the developing and adult brain can generate new neurons has raised new possibilities for repairing the nervous system. The mechanisms governing the neural differentiation of NSCs and in particular their specification into defined transmitter types are poorly understood. Production of dopaminergic neurons from NSCs in vitro may provide a new tool for cell therapeutic approaches to Parkinson's disease. We have previously shown that nucleotides stimulate proliferation and dopaminergic differentiation of immortalized fetal basal midbrain-derived human neural precursor cells (Milosevic et al., 2006). Here we analyse the potential of nucleotides to drive neuronal and dopaminergic differentiation of mouse fetal midbrain derived NSCs.
Cells were harvested from E14 embryos and plated in differentiation media. Nucleotides were added on day 1, 3, and 5 after culturing and the multipotent NSCs were analyzed for differentiation into neurons, oligodendrocytes and astrocytes on day 7. Whereas ADPbetaS drastically reduced the number of neurons, UTP and 2MeSATP increased it by the factor of two. 2MeSATP specifically enhanced catecholaminergic differentiation. RT-PCR analysis indicates expression of a variety of P2X and P2Y receptors as well as of the ecto-nucleotidases NTPDase2 and tissue non-specific alkaline phosphatase. These enzymes have previously been identified also in adult NSCs (Mishra et al., 2006). The expression pattern of enzymes and receptors varied between freshly dissected midbrain samples, undifferentiated and differentiated cells.
Our results suggest that nucleotides influence the neuronal differentiation of basal midbrain-derived NSCs and may provide a tool to enhance dopaminergic phenotype formation.

Purinergic receptors, excitotoxicity and neurodegeneration

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Since stimuli triggering neurodegeneration persistently increase extracellular adenosine and ATP, we explored how they control neurodegeneration. Pharmacological or genetic A2A receptor (A2AR) blockade abrogated kainate-induced hippocampal gliosis and neurotoxicity, which might result from combined control of glutamate release and neuroinflammation. Chronic caffeine and A2AR (but not A1R) blockade also prevented β-amyloid peptides (Aβ)-induced neurotoxicity and memory loss, through direct neuronal action since it was reproduced in cultured neurons and there was no evident Aβ-induced neuroinflammation. Chronic caffeine also abrogated memory deficits and synaptotoxicity caused by diabetes and chronic stress (as A2AR blockade). Whereas A2ARs seem to play a deleterious role as executioners of synaptotoxicity that precedes overt neurodegeneration and amplifiers of neurodegeneration through neuroinflammation control, A1Rs seem to act as an initial hurl to initiate neurodegeneration, as shown by their key role in preconditioning whereby restraint stress prevented subsequent kainate- or ischemia-induced toxicity. ATP also controls neurotoxicity since P2Y1R antagonists (or siRNA) prevented glutamate- or Aβ-induced synaptotoxicity and memory impairment. It is concluded that ATP is a direct danger signal in brain degeneration, which is also under opposite control by adenosine A1 and A2ARs, acting through modification of brain metabolism, synaptic viability and neuroinflammation. (Supported by FCT, Fundação Oriente, Pfizer)

(Invited)

Inflammatory microglial response to amyotrophic lateral sclerosis: expression and functions of extracellular ATP and P2 receptors

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Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by selective loss of motor neurons. The etiology of the disease is still unknown, but 2% of cases are associated with mutations in the gene coding for the ubiquitous anti-oxidant enzyme Cu,Zn superoxide dismutase (SOD1). The pathogenesis of familial ALS (fALS) appears multifactorial, involving an intricate interplay among multiple mechanisms such as oxidative stress, excitotoxicity, protein aggregation and damage to critical cellular processes. Recently, there has been growing interest in the role played by non-neuronal neighbouring cells (in particular microglia), via an inflammatory response which is fundamental in the propagation and acceleration of the disease. As one of the key molecule in microglial activation is ATP, and recent evidences showed the overexpression of several P2 receptors in areas of motor neuron degeneration in fALS (Yiangou et al., 2006; Casanovas et al., 2008) we investigated the expression and possible functions of P2 receptors and extracellular ATP in different microglial models of fALS. As detected by western blotting, biotin pull-down and immunofluorescence methodologies, several P2 receptor proteins are modulated in fALS microglia. Moreover, by measurement of extracellular release of ATP and indirect evaluation of ecto-ATPase activities, the purinergic system appears potentiated in fALS microglia. Finally, extracellular ATP determines increased levels of inflammatory markers such as TNFalpha and COX-2, in fALS microglia. These data suggest a new possible mechanism of fALS propagation involving extracellular ATP and P2 receptors.

Effect of nucleotides on neuronal differentiation and catecholaminergic specification of mouse fetal neural precursor cells

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The discovery that neural stem cells (NSC) in the developing and adult brain can generate new neurons has raised new possibilities for repairing the nervous system. The mechanisms governing the neural differentiation of NSCs and in particular their specification into defined transmitter types are poorly understood. Production of dopaminergic neurons from NSCs in vitro may provide a new tool for cell therapeutic approaches to Parkinson's disease. We have previously shown that nucleotides stimulate proliferation and dopaminergic differentiation of immortalized fetal basal midbrain-derived human neural precursor cells (Milosevic et al., 2006). Here we analyse the potential of nucleotides to drive neuronal and dopaminergic differentiation of mouse fetal midbrain derived NSCs.
Cells were harvested from E14 embryos and plated in differentiation media. Nucleotides were added on day 1, 3, and 5 after culturing and the multipotent NSCs were analyzed for differentiation into neurons, oligodendrocytes and astrocytes on day 7. Whereas ADPbetaS drastically reduced the number of neurons, UTP and 2MeSATP increased it by the factor of two. 2MeSATP specifically enhanced catecholaminergic differentiation. RT-PCR analysis indicates expression of a variety of P2X and P2Y receptors as well as of the ecto-nucleotidases NTPDase2 and tissue non-specific alkaline phosphatase. These enzymes have previously been identified also in adult NSCs (Mishra et al., 2006). The expression pattern of enzymes and receptors varied between freshly dissected midbrain samples, undifferentiated and differentiated cells.
Our results suggest that nucleotides influence the neuronal differentiation of basal midbrain-derived NSCs and may provide a tool to enhance dopaminergic phenotype formation.

Adenosine receptors differentially regulate the expression of regulators of G-Protein Signalling (RGS) 2, 3 and 4 in astrocyte-like cells

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Background: The "regulators of G-protein signalling" (RGS) comprise a large family of proteins that limit by virtue of their GTPase accelerating protein domain the signal transduction of G-protein coupled receptors. Since expression of several RGS proteins such as RGS-2, -3, and -4 appear to be altered in neuropsychiatric diseases that involve adenosinergic mechanisms we hypothesized that adenosine might regulate RGS expression in astrocyte-like cells.
Methods: Expression of RGS-2, -3, and -4 mRNA and cyclophilin B mRNA (as internal reference) was measured in cultured astrocytes and U373 astrocytoma cells by quantitative RealTime-PCR.

Results: The expression of RGS-3 and RGS-4 was down regulated by adenosine agonists while the expression of RGS-2 was up regulated. The order of potency of adenosine agonists was NECA>CPA/IB-MECA>CGS21680. The specific A2B-antagonist MRS1706 but not the specific A1-antagonist DPCPX antagonized the effects of NECA. These results were clear-cut in the case of RGS-2 and -3 and thus indicate that the effects are mediated by adenosine A2B-receptors. However, in the case of RGS-4 the concentration-response curve was much shallower and the order of potency of adenosine agonists and the antagonistic effects of MRS1706 were much less distinct and did not allow an unambiguous classification of the receptors involved, indicating perhaps an involvement of more than 1 type of adenosine receptor.
Conclusion: Expression of RGS mRNA in astrocyte-like cells is regulated by adenosine. Thus, adenosinergic mechanisms should be considered as a potential cause of alterations in RGS expression in the brain under various pathological conditions.

Chronic GMP treatment decreases the immunocontent of glutamatergic system related proteins in cortex of mice

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During the last 10 years, our group has been concerned in investigate the potential neuromodulatory role of the guanine based purines (GBPs) in the CNS. We already demonstrated that GBPs are able to antagonize some brain cellular response to glutamate in vitro (; i.e. GFAP phosphorylation [Neuroreport, 1995, 6:249], ROS [Neurochem Res, 1998, 23: 519], cAMP [Neurochem Res, 2000, 25: 181] and calcium influx [NeuroReport, 2000, 11:2303] evoked by glutamate). In vivo, some studies evidenced that acute and chronic GBPs administration promotes neuropretective effects in events whose glutamate is involved (i.e.anticonvulsant against i.c.v. QA infusion [Brain Res 2000, 2001, 2003, 2004] and against neonatal hypoxic- ischemic insult [Moretto et al., Exp. Neurol. 2005, 195: 400]) in both mice and rats, however the exactly mechanism of GBPs actions is unknown. In the way to investigate the potential glutamatergic system modulation of GBPs, we administrated GMP (1,5 mg/ml) orally for 21 days to mice with the aim to investigate the immunocontent of some glutamatergic receptors (NMDA, AMPA), transporter (EAAC1) and some synaptic proteins (PSD95 and SNAP 25) using Western blotting. Our results demonstrated that the GMP treatment statistically decreases the immunocontent of NMDA, AMPA, EAAC1 and PSD95 (decreases 42, 39, 40 and 25% comparing to control animals, $p< 0,05$) without modulate the SNAP25 in cortex of mice. In conclusion, our results could interesting demonstrated that GBPs has a great modulatory potential upon glutamatergic system and this interaction needs more attention since neuroprotective effects was already observed.

Role of adenosine A2a receptor in regulation of cerebral blood flow (CBF) during induced dynamic hypotension

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Background:
The aim of this study is to evaluate the role for adenosine A2a receptor during induced hypotension in mice.
Methods:
Wild type (WT) mice were anesthetized and ventilated to keep arterial blood gases and pH constant within physiological range. The ipsilateral femoral artery was cannulated for continuous measurement of mean arterial pressure. The contralateral femoral artery was used for withdrawal and returning the arterial blood. MABP was lowered to various degrees by the 50% of the initial MABP within 30seconds. CBF was measured using the laser-Doppler flowmetery and then cerebral vascular resistance (CVR) at the nadir of MABP was calculated. We also studied the effects of specific A2a receptor antagonist, ZM-241385 (1 mg/Kg, 5 mg/Kg, and 10 mg/Kg, ip).

Results:
Values of changing in MABP and CVR were plotted and slopes were gained with linear regression analysis in each group (Fig 1). The slopes of WT mice (n=5) and WT treated with 1 mg/Kg (n=5) and 5 mg/Kg (n=5) were not significantly different. In contrast, the slopes of WT and WT+10 mg/Kg (n=6) were significantly different ($P<0.05$).
Conclusions:
These results suggest that A2a receptor is involved in the regulation of CBF during induced hypotension.
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Design, synthesis, and biological activity of a novel series of adenosine A2A antagonists for the treatment of Parkinson's disease

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Background: Considerable data indicate that adenosine A2A antagonists may provide clinical benefit in the treatment of Parkinson's disease. The identification of adenosine A2A antagonists with enhanced potency, selectivity, and physicochemical properties may lead to improved therapy for these patients.
Methods: A novel series of adenosine A2A antagonists, based upon the 7H-pyrrolo[3,2-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine core, were designed, synthesized, and evaluated in radioligand binding assays with recombinant hA1, hA2A, and hA3 receptors expressed in Chinese hamster ovary (CHO) cells. Functional activities at the adenosine A2B receptor were evaluated in CHO cells expressing the recombinant A2B receptor. In vivo efficacy was assessed in a 6-hydroxydopamine-lesioned rat model of Parkinson's disease.
Results: In radioligand binding studies this series shows affinities ranging from 0.3 nM to greater than 200 nM, with KPRD-516 showing an affinity of 0.5 nM. Selectivities over the hA1 and hA3 receptor subtypes were as high as 3000-fold, dependent upon the compound. In functional assays, KPRD-516 and its analogues show no activity at the recombinant adenosine A2B receptor expressed in CHO cells. The structure-activity relationships developed for this series of compounds will be presented. Salt formation led to modest enhancements in solubility and bioavailability. KPRD-516 shows dose-dependent in vivo efficacy over 3 to 30 mg/Kg in rats.
Conclusion: A novel series of adenosine A2A antagonists with high affinity for the target receptor and excellent selectivity over other adenosine receptors has been identified. This series, typified by KPRD-516, is being developed as a potential new therapy for the treatment of Parkinson's disease.

Expression pattern of GPR17, a dual receptor for uracil nucleotides and cysteinyl-leukotrienes, during brain ischemia in mice

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We have recently demonstrated that GPR17, a previously "orphan" G protein-coupled receptor, can be activated by both uracil nucleotides and cysteinyl-leukotrienes (cysLTs), two chemically and structurally unrelated families of ligands. We also showed

that in vivo inhibition of GPR17 dramatically reduces ischemic damage in a rat focal ischemia model, suggesting GPR17 as a molecular target mediating brain damage under pathological conditions (Ciana et al., 2006. EMBO J, 25:4615–27).
As a first step to the set-up of a mouse model where the pathophysiological roles of GPR17 could be investigated, we cloned the previously unidentified mouse ortholog of GPR17 and confirmed its dual pharmacology with [35S]GTPgammaS binding on 1321N1 transfected cells.
We then analyzed, by immunohistochemistry, the changes of this receptor in ischemic mouse brain after induction of Middle Cerebral Artery occlusion (MCAo). Starting from 24 hours after ischemia induction, GPR17, which is basally expressed by cortical neurons, was found to be up-regulated in several neurons within the infarct area. A significant up-regulation of GPR17 was later visible in the ischemic penumbra: some of these cells, were also positive for HSP70, a marker of sublethal stress. Moreover, 3 days after MCAo, GPR17 was found on microglia/macrophages infiltrating the lesioned area.
In the intact brain, GPR17 was also found to be expressed in subsets of ramified cells with the morphology of neural progenitors. After ischemia induction, the number of these cells was highly increased in both the penumbra area and ipsilateral striatum.
Taken together, these results suggest that GPR17 is not only involved in ischemic neuronal death, but may also have a role in the activation of both inflammatory cells and precursor cells in the ischemic brain.

BzATP is coupled to GSK-3 inhibition and neuroprotection in cerebellar granule neurons

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GSK-3 inhibition is a key convergent point for survival signalling pathways in granule neurons. We investigated if nucleotide receptors, highly expressed in these cells, could be coupled to GSK-3 signalling and neuroprotection.
We found that the P2X7 agonist BzATP induced GSK-3 phosphorylation (in Ser21/9 residues) in granule neurons, which is associated with the inhibition of its catalytic activity. The EC50 for this effect was around 20 microM, and was sensitive to the antagonists BBG and PPADS, suggesting that this effect could be mediated by the activation of a P2X7 receptor. The BzATP-mediated effect was dependent on extracellular calcium and PKC, and independent on the PI3-K/Akt pathway, a survival route mainly used by neurotrophic factors. This could mean some advantage when this survival route is inhibited. Indeed, the pharmacological inhibition of the PI3-K/Akt pathway with LY-294002 induced apoptosis in these cells (measured by MTT assay and TUNEL), being BzATP and 3 mM ATP the only nucleotidic agonists able to rescue granule neurons from cell death. Again, these survival effects were abolished with the antagonists BBG and PPADS. In addition, BzATP prevented the progressive GSK-3 dephosphorylation and its subsequent activation occurring during LY-294002 treatment, this being evaluated by measuring the phosphorylation state of the GSK-3 substrate Tau. The protective effects of BzATP were reproduced by the specific GSK-3 inhibitor, SB-216763. These results support an important role of GSK-3 inhibition in the survival response of BzATP, and that BzATP could provide a relevant survival route alternative to that triggered by classical neurotrophic factors.

Expression levels of adenosine receptors in hippocampus and frontal cortex in argyrophilic grain disease

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Expression of adenosine receptors of the A1, A2A and A2B type has been examined in the post-mortem frontal cortex and hippocampus in argyrophilic grain disease (AGD), a tauopathy affecting the hippocampus but usually not the frontal cortex, in an attempt to learn about the modulation of the adenosine pathway in this disorder. Significant increased levels of A1, but

not of A2A and A2B, have been observed in AGD in the hippocampus but not in the frontal cortex, when compared with age-matched controls. This is accompanied by increased levels of adenylyl cyclase (AC), an effector of A1, and by increased (although not significant) percentage of inhibition of forskolin-stimulated AC by the A1 agonist cyclohexyladenosine in the hippocampus in AGD. These findings indicate sensitization of A1/AC in the hippocampus in AGD, and support a putative activation of the A1/AC pathway that may facilitate protection of this preferentially involved region in AGD.

Caffeine improves recognition memory and alters brain-derived neurotrophic factor and its receptor TrkB immunocontent in the hippocampus of adult and aged-mice

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Caffeine (a non selective adenosine receptor antagonist) presents positive effects on cognitive functions, but studies exploring its effects on cognitive decline with aging are still scarce. Brain-derived neurotrophic factor (BDNF) is a neurotrophin which promotes neuronal maturation and differentiation, being its BDNF signalling through tyrosine kinase B (TrkB) receptors essential for memory processes. In this study, it was investigated if the effects of caffeine on recognition memory in the object recognition task (ORT) could involve changes in the BDNF and TrkB immunocontent in adult and aged-mice. Adult mice received caffeine (1 mg/mL, p.o) or water during 12 months, saline (0.9 g %, i.p) or caffeine (10 mg/kg, i.p) during four days. After treatments, animals performed ORT and their hippocampi were dissected out and immunoblotted for BDNF and TrkB. Aged-mice treated with caffeine presented a performance similar to adults, whereas their aged-matched controls showed impairment in the ORT. Caffeine (10 mg/kg, i.p, 4 days) enhanced mice performance in the ORT in all intervals between training and test session. Hippocampus from aged-mice presented an increase in the BDNF and TrkB immunocontent compared to adults and caffeine administered during 12 months prevented it. Caffeine treatment during four days caused an increase in the BDNF and TrkB immunocontent compared to controls. Thus, caffeine prevented aged-cognitive decline with decrease in the BDNF and TrkB immunocontent, while in adult mice caffeine improved the performance with concomitant increase in both proteins. Since adenosine has been described to modulate BDNF-mediated effects on synaptic transmission, our results showed that BDNF may play a role on the effects mediated by pharmacological manipulation of adenosine receptors. Finally, caffeine-mediated effects on cognition suggest participation of one of the neurotrophins involved in learning and memory processes.

Towards the development of an adenosine A1 receptor agonist tracer for positron emission tomography (PET)

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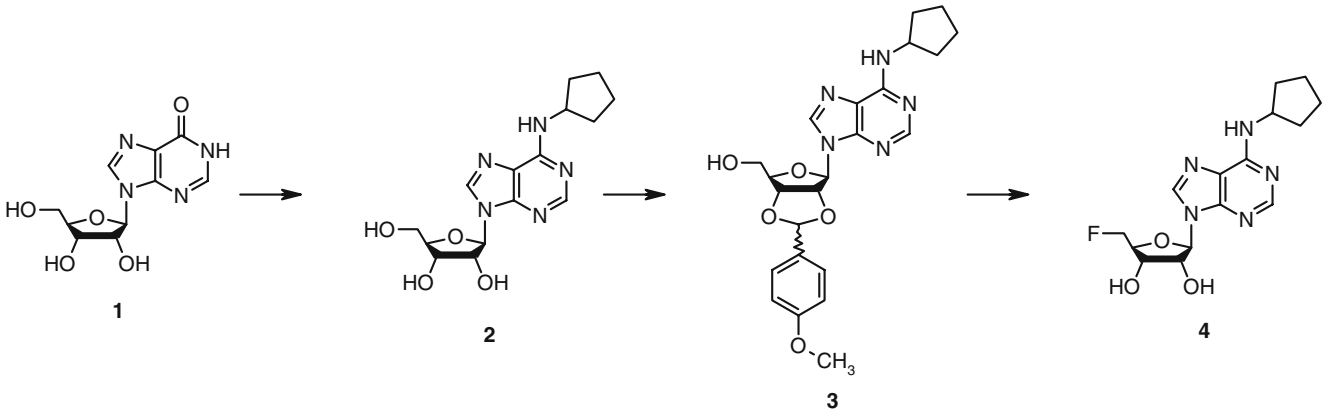
Background. Positron emission tomography (PET) is a promising new method for the early diagnosis of neurodegenerative diseases. A1 antagonist PET ligands, such as [18F]DPFPX, have been applied, however, selective A1 agonist PET ligands are not available so far. Such diagnostics could give valuable insights since they preferentially label active receptor conformations. We recently identified N6-cyclopentyl-5′-deoxy-5′-fluoroadenosine as a nanomolar full agonist for rat and human adenosine A1 receptors. The present study was aimed at providing a suitable precursor for radioactive labelling with fluorine-18, a positron emitter with a favourable half-life of 110 min.
Methods. Inosine (1) was used as a starting compound.
Results. Acetylation of the ribose hydroxyl groups, chlorination at C6 and subsequent treatment with cyclopentylamine and concomitant deacetylation yielded 2. Protection with p methoxybenzaldehye afforded 3. The 5′-hydroxyl group was converted to the mesylate, but for radiolabelling, the 5′-mesylate was unsuitable due to its thermal lability. Therefore, the tosylate was used. The non-radioactive product 4 was obtained by reaction with tetrabutylammonium fluoride upon microwave irradiation, followed by removal of the protecting group.

Conclusion. A precursor has been prepared which should be suitable for radiolabelling to yield [18F]N6-cyclopentyl-5′-deoxy-5′-fluoroadenosine as an agonist PET tracer for A1 receptors.



Adenosine A_{2A} receptors and chronic stress in the hippocampus of the rat

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Selective blockade of the adenosine A_{2A} receptors constitutes a novel therapy for neurodegenerative diseases. The aggressiveness of A_{2A} receptor knockout mice and augmented levels of these receptors upon acute stress implies their involvement in stress, as well. We now evaluated the levels and responses of A_{2A} receptors in synaptic plasticity in the rat hippocampus, in a model of chronic stress by maternal separation.
Male Wistar rats were assigned to control (CTR) or maternal separated (MS) group. The MS were separated from their mothers for 3 h/day from 2–14 postnatal days (PND) while CTR were undisturbed. At 40–80 PND, after testing behaviour, their hippocampi were dissected and blood collected. Long-term potentiation (LTP) in hippocampal slices (High frequency stimulation, 100 Hz, 1 s) was evaluated. A_{2A} receptor levels were assessed by Western blot.
MS animals displayed higher anxiety-related behaviour in elevated-plus maze and their corticosterone levels in plasma were increased (181.5±5.8 ng/ml) compared to CTR animals (62.8±6.5 ng/ml, $n=6$; $P<0.05$). MS displayed a lower magnitude of LTP (27.9±0.9%) than CTR animals (44.7±1.0%, $n=5$; $P<0.05$) which was restored by blockade of A_{2A} receptors (SCH58261;50 nM.). The A_{2A} receptor immunoreactivity in hippocampus was higher in MS than in CTR group.
The neonatal stress induces sustained changes in both behaviour and plasma corticosterone levels in adulthood. The lower LTP reported in MS animals indicates that learning and memory might be impaired but rescued by blockade of A_{2A} receptors. The concomitant increase in A_{2A} receptors suggests that they might play a role in chronic stress in the hippocampus. (Supported by FCT)

Adenosine A2A receptor antagonism increases neuronal nitric oxide syntase expression in the striatum of hungtington transgenic mice

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Huntington disease (HD) is an autosomal, dominantly inherited neurodegenerative disorder characterized by progressive motor and cognitive disturbances caused by an expansion in CAG repeats in the IT15 gene which encodes the huntingtin

protein. Medium spiny GABAergic projection neurons are progressively lost in HD, whereas there is preferential sparing of the few interneurons co-expressing NPY, somatostatin and neuronal nitric oxide synthase (nNOS).
We investigated the effect of the selective adenosine A2A receptor antagonist SCH58261 (0.01 mg/kg, i.p., acutely and chronically administered) on nNOS striatal expression and motor impairment in R6/2 transgenic mice in clearly symptomatic phase (10–11 weeks old mice).
SCH58261, acutely administered (90 min before sacrifice), increased the number of nNOS immunoreactive neurons (nNOS-IR) (+22%) in the striatum of HD mice at 11 weeks of age (n=4) in comparison to wild-type mice (n=8). SCH58261, chronically administered (twice a day for two weeks), significantly increased the number of nNOS-IR (+35%)in the striatum of R6/2 mice at 11 weeks of age (n=6) in comparison to wild-type mice (n=8). At this age, no glial activation was detected in the striatum or cortex. SCH58261 also improved walking in the inclined plane test but not motor capability evaluated by the rotarod test.
These findings demonstrate for the first time a role of adenosine A2A receptors in regulating nNOS expression in the striatum. We suggest that the protective effect of A2A antagonism in HD is related to the increase in striatal nNOS-IR neurons.
(The work was supported by a grant of Fondazione Monte dei Paschi di Siena, Italy)

Desensitisation of adenosine A1-receptors during hypoxia: involvement of endogenous adenosine

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Adenosin acts protective mainly due to the A1-receptors (A1Rs), one of its G-protein-coupled receptor subtypes. Desensitisation of the A1R yields to loss of neuroprotection. We used CHO cells stably transfected with A1R, to investigate the effects of oxygen-glucose-deprivation (OGD) on the release of adenosine and the A1R functionality. Extracellular adenosine was detected by HPLC. Cell viability was determined using LDH assay and fluorescence dye intercalation. Desensitisation of A1R was investigated by measurement of adenylate cyclase inhibition using ALPHA-Screen-technology. OGD reduced significantly the cell viability after 36 h. The decreased viability was correlated with an increase of LDH-activity and fluorescence dye intercalation. The involvement of adenosine seems to be involved because ODG increased the adenosine concentration in cell culture supernatants. The inhibition of the adenylate cyclase activity by activation of A1R decreases time dependently during OGD. Since CPA preincubation was able to mimic the effects evoked by OGD, we propose the desensitisation of the A1R. This desensitisation occurs after 12 to 24 hours of OGD as well as after CPA preincubation. Additionally, we found an increase of the basal forskolin-stimulated cAMP-production after desensitisation suggesting a switch in G-Protein-coupling of the A1R. In conclusion we have shown that OGD induces the release of adenosine and leads to a time dependent desensitisation of the A1R mediated adenylate cyclase inhibition. The reduced function can not exclusively explained with an increased cell death, since the desensitisation is accompanied with a stimulation of the basal cAMP production.

Blocking adenosine A2A receptors during oxygen-glucose deprivation delays anoxic depolarization and ameliorates neuronal survival in the rat CA1 hippocampus

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The role of adenosine A2A receptor activation during oxygen and glucose deprivation (OGD) was investigated using extracellular recordings of field excitatory post-synaptic potentials (fEPSPs) from the CA1 region of hippocampal slices, acutely prepared from 2-month-old rats. The cell-death marker propidium iodide (PI) uptake was used to evaluate cell viability before and after OGD application.

Seven and 30-min OGD elicit an irreversible loss of fEPSP and were invariably followed by the appearance of anoxic depolarization (AD), an unambiguous sign of neuronal damage. The application of the selective adenosine A2A receptor antagonist 4–2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-yl-amino]ethyl phenol (ZM 241385, 100–500 nM., n=32) did not modify the fEPSP outcome during OGD, but significantly prevented or delayed AD appearance (during 30 min OGD: from 6.8±0.3 min, n=11 in the absence to 9.0±0.4 min, n=11 in the presence of A2A antagonist) and permits a significant (P<0.001) recovery of neurotransmission after 7-min OGD (94.4±5.0%, n=22, in comparison to 5.4± 2.8%, n=24 found in untreated OGD slices). In related experiments, slices were subjected to 7 min OGD followed, after 2 hours from the end of OGD, by 1 hour staining with the PI (5 μg/ml). Substantial CA1 pyramidal neuronal damage occurred in OGD untreated slices that was significantly decreased by 100 nM. ZM 241385.
Data indicate that the selective block of adenosine A2A receptors provides neuroprotection after severe OGD in the CA1 region of rat hippocampus.

Function of adenine receptors and interactions with adenosine a1 receptors in the rat cingulate cortex

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The aim of the present study was to evaluate the effect of adenine on the synaptic transmission on pyramidal cells of the rat cingulate cortex and to investigate an interaction with adenosine A1 receptors (A1Rs). Therefore, mRNA expression was investigated in tissue probes of the rat cingulate cortex. Functional investigations were done electrophysiologically by intracellular recordings on pyramidal cells in layer V of the cingulate cortex in rat brain slices. Postsynaptic potentials (PSPs) were evoked by electrical stimulation with a bipolar electrode in layer I. Adenine (1 mM) depressed reversibly the PSPs but had no influence on membrane potential and input resistance. Both the NMDA and non-NMDA component of the PSPs were inhibited suggesting an influence on presynaptic glutamate release. In further experiments the interaction with A1Rs was tested. Adenine and the selective A1R agonist N6-cyclopentyladenosine CPA were superfused successively without washout, each in a concentration which induced maximum inhibition in individual experiments. After preincubation of adenine (1 mM) the influence of CPA (10 μM) was significantly reduced compared with the CPA effect when given alone. In contrast, after preincubation of 100 mM adenine a low concentration of CPA (1 nM) induced an increase of the PSPs. In further experiments the selective A1R antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was superfused 5 minutes before application of adenine. DPCPX (0.1 μM) did not influence the adenine induced PSP inhibition. The current results confirm the functional expression of adenine receptors in the rat cingulate cortex and suggest that preincubation of adenine alters A1R activation by agonists.

Fine tuning of cannabinoid-mediated effects by striatal adenosine A2A receptors

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Adenosine A2A receptors (A2ARs) regulate cannabinoid CB1R-mediated effects in the striatum. A2ARs exert also a permissive role on the metabotropic glutamate receptors 5 (mGlu5Rs), which, in turn, trigger endocannabinoid release. The aim of the present study was to further investigate, by striatal electrohysiology, the functional interaction among A2A, mGlu5 and CB1 Rs.
Corticostriatal slices (300 μm thick) were taken from Wistar rats, and extracellular field potentials (FPs) were recorded in the striatum. FP amplitude was measured and expressed as % of baseline.
The CB receptor agonist WIN 55,212–2 (2 ìM) depressed FP amplitude (55.39+2.8% of basal; N=7 P<0.05, Mann-Whitney U-test), an effect which was completely blocked by the CB1 antagonist AM251 (N=3 P<0.01). As expected, the A2AR antagonist ZM 241385 (100 nM) significantly reduced WIN-mediated effects, confirming that a basal level of A2A activation is requested to allow CB1 effects (81.91+4.65%; N=8; P<0.01). However, WIN-mediated effects were reduced as well by the A2AR agonist CGS 21680 (100 nM) (84.24+6.4%; N=5; P<0.01 vs WIN alone). The effects of CGS21680

were reproduced by forskolin and prevented by the PKA inhibitor KT 5720, indicating the involvement of the cAMP/PKA pathway. Finally, the mGlu5R agonist CHPG markedly potentiated the effects of a concentration of WIN (1 μM) which induced a mild decrease of FP amplitude (WIN alone: 90+3.98%; N=5; P<0.05; CHPG + WIN: 65.59+9%; N=5; P<0.05 vs WIN alone). Interestingly, ZM 241385 (100nM) completely prevented the potentiating effects of CHPG towards WIN (N=3; P<0.05).

These results show that, while an endogenous state of activation of A2ARs is required to allow CB1 effects, a stronger activation of the same receptors significantly inhibits cannabinoid effects. These findings reveal a complex "fine tuning" exerted by A2ARs (either directly and through other Rs) in the regulation of cannabinoid functions in the striatum.

GPR17 expression in healthy and injured spinal cord

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Acute spinal cord injury (SCI) results in immediate tissue damage, followed by long-lasting secondary injury associated to the release of inflammatory molecules, including nucleotides, which may contribute to damage development by activating specific membrane receptors. In this respect, the pathological over-activation of the new purinergic receptor GPR17 has been recently proposed to participate to brain ischemia (Ciana et al., 2006. EMBO J 25:4615). In this study we aimed at evaluating GPR17 expression in the spinal cord and its possible role in SCI-associated damage.

Adult mice were subjected to extradural SCI with an aneurysm clip after T6-T9 laminectomy and injected with bromodeoxyuridine to evaluate cell proliferation. GPR17 expression was evaluated by immunohistochemistry 24h, 72h and 1 week after injury.

In sham-operated mice, GPR17 was expressed by many BetaIII-tubulin-positive neurons and CC1-positive oligodendrocytes, but not by GFAP-positive astrocytes. Ependymal cells and the 29.8±2.1% of cells expressing the neuroprogenitor marker NG2 also expressed GPR17. After SCI, cell proliferation strongly increased in parallel with the total number of GPR17-expressing cells. In fact, despite the massive death of GPR17-positive neurons and oligodendrocytes, a proliferating population of GPR17- and IB4-positive microglia/macrophages emerged. GPR17 was never found in Nestin- and GFAP double-positive reactive astrocytes.

Taken together, in a similar way to brain ischemia, our data show an increase in GPR17 expression that may contribute to acute SCI damage. On the other hand, presence of GPR17 on neuroprogenitor-like cells also suggests a potential role of this receptor in the long-term reparative changes associated to SCI.

Purinergic regulation of adult neural stem cells: new perspectives for brain repair

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New neurons continue to be formed in the adult mammalian nervous system in specific neurogenic niches predominant in the subventricular zone (SVZ) and the hippocampus. The molecular cues initiating the cell cycle of multipotent stem cells, driving proliferation of intermediate precursors, directing the migration of immature neurons or finally inducing neuronal differentiation, functional integration and survival are poorly understood. Small signaling molecules including nucleotides play a key integrative role in controlling neurogenesis.

We have previously demonstrated the presence of ectonucleotidases at progenitor cells in the neurogenic niches and demonstrated a functional role of nucleotides and adenosine in SVZ-derived stem cell proliferation in vitro. Using

immunocytochemistry and quantitative Western blotting we have now investigated in more detail the intracellular signaling pathways induced in cultured neural stem cells by agonists of the P2Y1 and P2Y2 receptor as well as their relation to epidermal growth factor-induced signaling. Furthermore, using a retroviral approach, we analyzed the outcome of knockdown of ecto-nucleotidases as well as of the overexpression of P2 receptors on stem cell proliferation and induced in vitro differentiation of stem cells into astrocytes, oligodendrocytes and neurons.
Our data suggest that nucleotides contribute to the control of adult and most likely also of embryonic and postnatal neurogenesis and that small molecule approaches targeting the nucleotide signaling pathway may contribute to promote self repair processes in the diseased or damaged brain.

(Invited)

HIF-1 alpha is an essential effector for purine nucleoside-mediated neuroprotection against hypoxia in PC12 cells and primary cerebellar granule neurons

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Background: Hypoxia-inducible factor-1 alpha (HIF-1 alpha) and purine nucleosides are critical mediators of physiological responses to acute and chronic hypoxia. The specific aim of this study was to evaluate the potential role of HIF-1 alpha in purine-mediated neuroprotection.
Methods: Cell death was quantified in Hoechst 33342/ propidium iodide-stained cells. Fluorescence microscopy and western blotting analyzed intracellular HIF-1 alpha. To knockdown HIF-1 alpha, cells were transiently transfected with synthetic siRNA.
Results: We showed that adenosine and inosine efficiently rescued PC12 cells as well as primary cerebellar granule neurons from hypoxic insult, and that HIF-1 alpha is critical for purine-mediated neuroprotection. As a possible result of increased protein stabilization or synthesis an up to 2.5 fold induction of HIF-1 alpha accumulation was detected in PC12 cells. In cerebellar granule neurons, purine nucleosides induced an up to 3.1 fold HIF-1 alpha accumulation in cell lysates. Concomitant with these results, siRNA-mediated reduction of HIF-1 alpha completely abolished adenosine- and inosine-mediated protection in PC12 cells and severely hampered purine nucleoside-mediated protection in primary neurons.
Conclusion: Data presented in this study thus clearly demonstrate that HIF-1 alpha is a key regulator of purine nucleoside-mediated rescue of hypoxic neuronal cells. Funded by the Austrian FWF Project P19578-B05

SYMPOSIA 6: CONTROL OF INSULIN SECRETION AND GLUCOSE HOMEOSTASIS

ADP mediates inhibition of insulin secretion by activation of P2Y13 receptors

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The aim of this study was to determine the effect of ADP mediated signaling on insulin and glucagon secretion.
Apyrase was found to increase both insulin (+122%, $p<0.001$) and glucagon (+45%, $p<0.001$) secretion at low (1 mM) glucose, whereas at high glucose (20 mM), insulin secretion was inhibited (-45%, $p=0.003$) and glucagon secretion was promoted (+118%, $p<0.001$). qPCR identified P2Y1 (expression normalized to 100%) and P2Y13 (19% of P2Y1 expression in islets, 45% of P2Y1 expression in beta-cells) in mouse islets and beta-cells, but only trace amounts of P2Y12. Co-incubation of the ADP analogue 2-MeSADP (10 uM) with the selective P2Y1 antagonist MRS2179 (10 uM) at 8.3 mM glucose resulted in a marked decrease in insulin secretion (-70%, $p<0.001$).
2-MeSADP in combination with the selective P2Y13 receptor antagonist MRS2211 strongly increased insulin secretion (+112%, $p<0.001$). Blocking of P2Y13 by MRS2211 alone resulted in elevated secretion of both insulin (8.3 mM glucose: +92%, $p<0.001$; 20 mM glucose: +38%, $p=0.005$) and glucagon (8.3 mM glucose: +76%, $p<0.05$; 20 mM glucose: +69%, $p=0.005$). Finally, islets from P2Y13 null mice displayed an enhanced insulin secretion upon in vitro glucose challenge compared to wild type control islets (10 mM glucose: +44.3%, $p<0.05$; 20 mM: +50.7%, $p<0.005$; 25 mM: +52.9%, $p<0.005$).
We propose that P2Y13 plays an important role in regulating islet hormone release and that inhibition of the P2Y13 receptor might serve as a novel diabetes therapy aimed at increasing insulin secretion from pancreatic islets.

Ischemia-reperfusion stress produced inosine-mediated hyperglycemia through A3 adenosine receptor

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The molecular mechanism underlying stress-induced hyperglycemia has not been comprehensively clarified. Recently, we demonstrated in ischemia-reperfusion (I-R) stress-subjected liver that inosine and adenosine are mainly responsible for the hyperglycemia observed. OBJECTIVE: to advance in the knowledge of the role of inosine and adenosine as mediators of hepatic-mediated hyperglycemia detected after I-R in lower limbs. METHOD: Ischemia was performed in anesthetized rats by occluding abdominal aorta and cava vein; then, reperfusion was allowed. Blood samples from hepatic or abdominal cava veins were taken throughout the experiments to measure glucose, inosine, and adenosine. Antagonists to adenosine (AdoR) and adrenaline receptors (AdrR) were administered during I-R to analyze their effect on hepatic glucose release. RESULTS: Ischemia up to 60 min produced minor increases of glucose and nucleotides blood values, but 5 min of ischemia followed by (2–10) min reperfusion increased glucose 23%, and those of inosine or adenosine by 100%. After 60 min of ischemia and 10 min of reperfusion, glycemia rises 2-fold and blood inosine and adenosine, 3.3- and 2.7-fold, respectively. A linear positive correlation, $r2=0.839$ between glucose and both nucleosides was calculated. The hyperglycemia response to I-R decreased by 0, 25, 33, 45, and 100% after selective inhibition of A2B AdoR, A2A AdoR, alpha1B AdrR, A1 AdoR, and A3 AdoR, respectively. CONCLUSIONS: inosine-adenosine couple through its hepatic A3 AdoR is the main signal for releasing glucose from liver glycogen and for promoting hyperglycemia following experimental injury of I-R from lower limbs.
Grants: DGAPA IN201208, UNAM

Hepatic changes in levels of adenosine 3′-monophosphate, an intracellular P-site inhibitor of adenylate cyclase, and its forming enzyme activity in streptozotocin-induced diabetic mice

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To elucidate the pathophysiological roles of adenosine 3′-monophosphate (3′-AMP) forming enzyme in mice, the effect of streptozotocin (STZ) on the enzyme activities and 3′-AMP levels in the ICR mice liver was examined. After 2 weeks, treatment with a single dosage of STZ (100, 150 or 200 mg/kg i.p.) induced a dose-dependent hyperglycemia and hypoinsulinemia but had no effect on serum alanine aminotransferase activity, indicating that STZ generated type 1 diabetes without hepatitis. In the diabetic liver, the superoxide dismutase (SOD) activity, and ATP levels decreased, and the microsomal CYP2E1 activity increased. The activities of 3′-AMP forming enzyme, one of ribonucleases, in hepatic homogenates were not changed, however, in the STZ 200 mg/kg group, the cytosolic forming enzyme activities were increased, and inversely, the mitochondrial activity was reduced significantly, indicating that the decrease in the mitochondrial activity may be accelerated by development of diabetes due to the reduction of the antioxidant defense system. With the decrease in the 3′-AMP forming enzyme activity, mitochondrial 3′-AMP levels were significantly reduced. These results obtained suggested that change in the mitochondrial 3′-AMP forming enzyme activity might reflect the pathophysiological change of mitochondrial function with the development of diabetes. Our results also suggested that change in cytosolic 3′-AMP forming enzyme activity might serve as a new biomarker of oxidative stress because significant negative correlation between the activities of cytosolic 3∞f-AMP forming enzyme and SOD was found in the early stage of diabetes.

Physiological role of extracellular ATP in insulin release pulsatility

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Background. Pancreatic islets are coupled oscillators generating 3–6 min cycles of circulating insulin.
Methods. Cytoplasmic Ca2+ was measured with ratiometric fura-2 technique in islets and beta-cells isolated from mice, rats and humans. Insulin, glucagon and somatostatin were measured during perfusion of pancreas or superfusion of islets.
Results. Each beta-cell is an oscillator responding to glucose stimulation with 2–10 min insulin pulses evoked by rhythmic depolarisation with entry of Ca2+. Intermittent stimulation of P2Y1 receptors aids to entrain beta-cells into a common rhythm by generation of Ca2+ transients and closure of KATP channels. Within the islets, external ATP is complementary to gap junctions for co-ordinating the beta-cells. Regenerative release of ATP propagates Ca2+ signals both among the beta-cells and between these cells and capillary endothelial cells. Moreover, ATP is the principal candidate for a neurotransmitter locking the different islets in the pancreas into the same oscillatory phase. Studies of hormone release from rat pancreas indicated that 4–5 min pulses of insulin coincide with those of somatostatin but are antisynchronous to glucagon. Inhibition of P2Y1 receptors (1 μM MRS 2179) removed the pulses of glucagon and somatostatin with maintenance of insulin pulses. ATP released from the beta-cells is hydrolyzed by ecto-nucleotidases to adenosine. Recent studies indicate that knock out of the A1 receptor amplifies the insulin pulses and prolongs those of glucagon and somatostatin.
Conclusions. Extracellular ATP is important for pulsatile release of islet hormones. Purinoceptors participate in the islet control of the liver production of glucose by regulating amplitude and duration of antisynchronous pulses of insulin and glucagon.

(Invited)

Endogenously released ATP serves as a positive autocrine feedback loop for the human pancreatic beta cell

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ATP is present in insulin containing granules and is co-released with insulin from the pancreatic beta cell during glucose-induced exocytosis. There are conflicting reports with regard to ATP effects in the islet, and a comprehensive study in human islets is not available. Our goal was to define the role of released ATP in human beta cell insulin secretion. We investigated human islet P2 receptor expression with immunohistochemistry, Western Blotting, and RT-PCR. Cytoplasmic free Ca2+ concentration, [Ca2+]i, was measured to study P2 receptor cellular localization and signaling mechanisms. Dynamic hormone release was measured to evaluate the functional role of these receptors.
We show for the first time the expression of P2X receptors in human islets. P2X3 receptors were present in beta cells and P2X4 receptors were expressed in alpha cells. In the absence of extracellular Ca2+, [Ca2+]i and insulin responses to ATP were strongly diminished. By contrast, thapsigargin had only a small effect on [Ca2+]i and insulin responses to ATP. Glucose-induced insulin release was decreased by apyrase, suramin and isoPPADS (20%, 40% and 30%, respectively). Reactive blue 2 had no effect. This positive autocrine feedback loop of extracellular ATP on beta cell function was present in primate but not in rodent islets.We have demonstrated the presence of a positive autocrine ATP signaling loop that modulates insulin secretion in the human islet. In contrast to rodent models, P2X receptors predominate in the ATP signaling pathway in primate pancreatic islets, amplifying insulin secretion in response to increases in glucose concentration.

Expression and pharmacology of P2 purinergic receptors in the pancreatic beta-cell

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Pancreatic beta-cells express P2 ionotropic (P2X) and metabotropic (P2Y) purinergic receptors which, upon binding of ATP or structural analogs, induce or modulate insulin release.
Different molecular subtypes of P2X receptors (P2X1 and P2X4) have been observed in Langerhans islets. ATP signaling via P2X receptors leads to a transient insulin release that may be mediated by KATP-channel closure. ATP was consistently shown to increase cytoplasmic free calcium concentration, notably in human pancreatic islets and beta-cells.
Activation of purinergic P2Y receptors by selective agonists results in amplification of insulin release in a glucose-dependent way. Coupling mechanisms involve phosphoinositide hydrolysis, intracellular calcium mobilization, or activation of the cAMP/PKA pathway. Importantly, the response to P2Y receptor activation was shown to be independent of KATP channels and to require glucose metabolism. Pancreatic beta-cells express different molecular subtypes: P2Y1, P2Y2, P2Y4, P2Y6 and P2Y12, in the INS-1 rat insulinoma cell line. This expression profile may explain the complex interaction of P2Y ligands on high- and low-affinity binding sites for ATP-alpha-S. Additionally, the expression of P2Y11 and P2Y12 receptor subtypes, known to be coupled to adenylate cyclase, was reported in human pancreatic islets.
Finally, consistent data suggest the potential of P2Y receptor agonists as drug candidate for the treatment of type 2 diabetes. Indeed, selective P2Y receptor ligands trigger amplification pathways of glucose-stimulated insulin release. They also increase insulin response in pancreas from diabetic animal models and, interestingly, improve glucose tolerance after oral administration.

The future challenge will be to better understand the roles of the different receptor subtypes not only in islet cells but also in relevant insulin-sensitive tissues, and to design compounds with high receptor-subtype selectivity and efficient bioavailability.

(Invited)

Regulation of insulin secretion by NTPDase3

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Background: Nucleoside triphosphate diphosphohydrolases (NTPDases) are plasma membrane bound enzymes that hydrolyse nucleotides at the cell surface. By their biochemical activities, NTPDases have the ability to regulate the biological actions exerted by extracellular nucleotides on P2 receptors. In pancreatic Langerhans islets, extracellular nucleotides modulate insulin secretion by beta cells. The presence of an ATPase activity at the surface of Langerhans islet cells suggests the implication of this enzyme in the modulation of insulin secretion. The aim of this work was to identify this ectonucleotidase and to determine its potential function.
Methods: The involved NTPDase was identified and localized by enzyme histochemistry, using ATP and ADP as substrate and immunofluorescence, using specific antibodies developed in the laboratory and cell markers of the endocrine pancreas. Insulin secreted by beta cell lines upon glucose stimulation, in the presence or absence of NTPDase inhibitors, was quantified by ELISA.
Results: Enzyme histochemistry, immunofluorescence and flow cytometry experiments demonstrated that NTPDase3 was the major ectonucleotidase at the surface of Langerhans islet cells, including mouse, rat and human beta cells as well as the beta cell lines beta-TC3 and Ins-1(832/13). A significant increase in insulin secretion mediated by glucose stimulation was measured in the presence of NTPDase3 inhibitors on the beta cell lines.
Conclusion: These results suggest a modulatory role of NTPDase3 activity in insulin secretion mediated by nucleotide release upon glucose stimulation. These results open a new avenue of research for the potential treatment of diabetes mellitus.

A role for P2Y6 receptor in regulation of islet hormone release

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We examined the presence of uracil nucleotide metabotropic P2Y receptors such as P2Y2, P2Y4 and P2Y6 in the isolated mice pancreatic beta-cells and whether activation of these purinoceptors can be functionally linked to insulin and glucagon release. Real-time PCR were used to analyse the presence of mRNA for P2Y2, P2Y4, P2Y6 and compared with P2Y1 in the isolated islets and in beta-cells purified by counterflow elutriation and gradient centrifugation. Static incubation experiments on isolated islets were used to examine the secretory pattern of insulin- and glucagon release upon activation of P2Y2, P2Y4 and P2Y6 in isolated islets. A marked expression of mRNA for P2Y1 and P2Y6 but not of P2Y2 and P2Y4 was found in both purified b-cells and whole islets. In vitro experiments revealed that P2Y6 activation by a specific agonist, UDP-betaS, dose-dependently enhanced insulin and glucagon release during a short-term incubation while P2Y6 activation during a longer period (24 h) resulted only in a marked increase of insulin release. These effects were more pronounced in the presence of a high glucose levels. MRS2578 a selective antagonist of P2Y6 suppressed the effects of UDP-betaS indicating that the P2Y6 purinoceptor is functional. The negative RT-PCR results and the lack of response to UTPgammaS a selective P2Y(2/4) agonist further rule out the involvement of P2Y(2/4) receptors in the islet hormone release.Taken together these results suggest a role for P2Y6 signalling in the regulation of insulin and glucagon release from mouse pancreatic islets.

Role of agonists and antagonists of adenosine receptors including diadenosine polyphosphates on glucose homeostasis

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Role of agonists and antagonists of adenosine receptors including diadenosine polyphosphates on glucose homeostasis
E.J. Verspohl, Münster, Germany
Purinergic receptors:
The solution for the controversy on ATP effects may be that ATP has a dual effect in increasing insulin release (EC50~0.0032 μM) and inhibiting insulin release (EC50~0.32 μM). According to using ADPßS (rather specific for P2Y1 receptors), 2-Methylthio-ATP (P2X and P2Y1 agonist), UTP (P2x and P2Y agonist), PPADS (P2X and P2y1,4,6 antagonist) and adenosine desaminase it is concluded that ATP increases insulin at low concentration via P2Y receptors and decreases insulin release at high concentrations via its metabolite adenosine.
Diadenosine polyphosphates (ApxA) receptors:
The compounds inhibited insulin release in vitro which was overcome by Ip5/6I (inosine derivatives; antagonists of AP4A effects). In vivo Ap4A (0.75 mg/kg) increased blood glucose dramatically without a parallel increase in plasma insulin. AP4A increased glucose release from perfused rat liver and glucose uptake was reduced in 3T3 adipocytes (decrease in translocated GLUT-4). Ap4A induces a diabetic situation.
Adenosine receptors:
A1 receptor: An inhibitory effect mediated by A1 receptor agonism was detected at a low NECA concentration (10 nM) and was confirmed by adding the A1 receptor antagonist PSB-36 (1-butyl-8-(3-noradamantyl)-3-(3-hydroxy-propyl)xanthine). The selective A1 receptor agonist CHA (N6-cyclohexyladenosine) inhibited insulin release which was reversed by DPCPX (A1 receptor antagonist).
A2B receptor: The A2B receptor antagonists PSB-53 and PSB-1115 counteracted the inhibitory effect of NECA. The same results were obtained in vivo and in diabetic GotoKakizaki rats. Thus A2B-receptor antagonists show an anti-diabetic potential mainly by increasing insulin levels.

(Invited)

SYMPOSIA 7: EXOCRINE SECRETION AND EPITHELIAL TRANSPORT

Adenosine receptors regulate secretion in rabbit lacrimal gland

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Background
The wide-spread disorder Dry Eye is often connected to inflammation in the lacrimal gland, leading to reduced tear secretion (1).To investigate a possible stimulatory role of adenosine on secretion, adenosine receptor expression and function in rabbit lacrimal acini were studied.
Methods
Reverse Transcriptase PCR was performed from snap-frozen lacrimal glands. For secretion studies, lacrimal gland acinar cells were isolated and cultured for 2 days according to our standard procedure (2). Cultured cells were treated with adenosine receptor agonists/antagonists with or without the cholinergic agonist carbachol for 30 min, after which levels of the secretion marker beta-hexosaminidase were measured. Immunohistochemistry from tissue was performed using antibodies towards adenosine receptors.
Results
Presence of A1 (2), A2a and A2b receptors was confirmed through RT-PCR and sequencing of the product. Immunohistochemistry also indicated presence of the mentioned receptors. Secretion studies revealed that adenosine receptor agonists generate an approximate 2-fold increase of secretion. A synergistic effect with carbachol for both A1 and A2a/b agonists was observed. Adenosine receptor antagonists significantly decreased the synergistic effect.
Conclusion
Adenosine receptors A1, A2a and A2b are present in the rabbit lacrimal gland. Activation of the receptors leads to increased secretion and induces synergy with the cholinergic agonist carbachol. Adenosine may therefore be a useful tool in the treatment of Dry Eye, due to its anti-inflammatory effects along with the stimulatory role presented here.

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2. M. C. Edman et al., Exp Eye Res 86, 110–7 (2008).

Purinergic Signaling in the Pathogenesis of Biliary Cirrhosis

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Biliary cirrhosis is the cause of 20% of liver failure in adults and the majority of liver failure in children. Biliary cirrhosis is distinct in several ways: (1) it is invariably characterized by disordered bile ductular proliferation, (2) the progression of fibrosis is rapid, and (3) the cells that mediate biliary cirrhosis are distinct from those that mediate other forms of cirrhosis. The two cell types that are pivotal in the pathogenesis of biliary cirrhosis are bile duct epithelia (BDE) and portal fibroblasts (PF). We report that purinergic signals are key in the cross-talk signaling between BDE and PF. First, PF regulate BDE proliferation via expression of NTPDase2. We reduced expression of NTPDase2 in PF; doing so blocked the ability of PF to regulate BDE proliferation, which is P2Y-mediated. Second, PF lose expression of NTPDase2 in biliary cirrhosis. Biliary cirrhosis, but not non-biliary cirrhosis, transcriptionally downregulates NTPDase2. Loss of NTPDase2 expression in this setting blocks the ability of PF to regulate BDE proliferation. Third, secretion of the cytokines CCL2 and IL-6 by BDE induces downregulation of NTPDase2 in PF. IL-6 arrests NTPDase2 transcription via distinct IL-6 response elements in the NTPDase2 promoter. Fourth, extracellular ATP regulates BDE IL-6 release. The receptor that regulates this phenomenon is functionally similar to the cloned P2Y11 receptor, as it is activated by ATPγS > ATP and mediated by cAMP and intracellular Ca2+ and a downstream cAMP-response element in the IL-6 promoter. In summary, BDE and PF regulate each other via purinergic signals, and this inter-regulation is disturbed in biliary cirrhosis. PF regulate BDE via expression of NTPDase2 function. In turn, BDE regulate PF NTPDase2 expression via cytokine release, which is itself regulated by a P2Y11-like receptor. We propose that this elegant cross-talk signaling system is a prototype model for functionally relevant ecto-nucleotidase/P2 receptor interactions.

(Invited)

Purinergic signaling in cholangiocyte secretion and bile formation

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ATP in bile is a potent secretogogue, stimulating biliary epithelial cell (cholangiocyte) Cl- and fluid secretion via binding to membrane P2 receptors, and represents a dominant pathway in bile formation based on the following observations: First, while P2 receptors can be detected in both the apical and basolateral domains, ATP has potent secretory effects when applied to the apical surface facing the duct lumen. Second, ATP released into the canalicular space between hepatocytes has access to apical P2 receptors located "downstream" on cholangiocytes, constituting a mechanism for hepatobiliary coupling, or coordination of the separate hepatocyte and cholangiocyte contributions to bile formation. Third, ATP stimulates cholangiocyte secretion through activation of "non-CFTR" Cl- channels, and thereby represents a strategy for overcoming the secretory defect associated with cystic fibrosis. Lastly, we have recently identified fluid flow/shear stress as an important stimulus for cholangiocyte ATP release, providing a new "mechanosensitive" paradigm for bile formation. In human and rat biliary epithelial models, exposure to flow increased relative ATP release which was proportional to the shear force. In parallel studies, flow/shear was associated with an increase in [Ca2+]i and membrane Cl- permeability which were both dependent on extracellular ATP and P2 receptor stimulation. The finding that fluid-flow can regulate membrane transport suggests that mechanosensitive ATP release may be a key regulator of biliary secretion and an important target to modulate bile flow. Understanding the plethora of purinergic processes along the apical axis of biliary epithelium offers great promise for new strategies to modify the volume and composition of bile in the treatment of cholestatic liver disorders.

(Invited)

Purinergic receptors stimulate Ca(2+) fluxes in pancreatic ducts: role of Na(+)/Ca(2+) exchanger and other Ca(2+) binding and transporting proteins

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Background: Disturbances in Ca(2+) homeostasis in exocrine pancreas may be associated with pathological conditions, but only little is known about the mechanisms of Ca(2+) transport and regulation in this tissue. Since purinergic signalling has been shown in this tissue, we aimed to investigate whether pancreatic duct cells from human and rat pancreas express Ca(2+) transporting and Ca(2+) binding proteins and whether purinergic signalling might regulate Ca(2+) transport processes.
Methods: Molecular biology and immunochemical approaches were used on human pancreatic duct cell lines and isolated rat pancreatic ducts to identify proteins dealing with pancreatic Ca(2+) homeostasis. Fura-2 measurements and imaging were used to test purinergic regulation.
Results: It was shown that activation of purinergic receptors can modulate Na(+)/Ca(2+) exchange in rat and human pancreatic ducts. Expression analysis showed that pancreatic ducts of rat and human express the NCX1 splice variant NCX1.3 and NCX1.7, Ca(2+)-sensing receptor (CaR) and the cytoplasmic calbindin (CBD9K).
Conclusion: Pancreatic duct cells express proteins suitable for transepithelial Ca(2+) transport. Since, Na(+)/Ca(2+) exchange was stimulated by purinergic receptor stimulation, we propose that P2 receptors and Ca(2+) transporting/binding proteins might be involved in pancreatic Ca(2+) homeostasis and maintenance of low Ca(2+) concentrations in pancreatic juice.

Purinergic receptors and calcium signalling in human pancreatic duct cells lines

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Background: Purinergic signalling may regulate ion transport and exocrine secretion in human pancreas, but little is known about which purinergic receptors are expressed in this tissue. Thus, we aimed to identify P2 receptor subtypes in human exocrine duct cell lines that are commonly used in ion transport studies.
Methods: In PANC-1 and CFPAC-1 cell lines Fura-2 and single-cell imaging was used to test the effect of various nucleotide analogues on intracellular calcium signals and obtain a pharmacological profile. Additionally, P2 receptor expression was examined with RT-PCR on PANC-1, CFPAC-1 and Capan-1 cell lines.
Results: PANC-1 and CFPAC-1 responded to all nucleotides tested with the following efficacy: UTP ≥ ATP = ATP[gamma]S > BzATP. The functional experiments revealed differences in responses regarding the shape of the calcium signals and the percentage of responsive cells. ATP, UTP and ATP[gamma]S elicited oscillatory signals. BzATP, commonly used to stimulate P2X7 receptors, elicited non-oscillatory and transient calcium signals. Ivermectin, a P2X4 potentiator, enhanced signals evoked by ATP. All cell lines expressed RNA transcripts for the following receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-14 and P2X1, P2X2, P2X4, P2X5, P2X6, and P2X7.
Conclusion: Human ducts express a number of P2 receptors similar to ducts in other species. The single cell calcium measurements indicate functional expression of P2Y2 and other P2Y receptors, and notably expression of P2X4 and P2X7 receptors.

Na^+-absorptive function and purinergic receptor in the epithelial cells of Reissner's membrane during development

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Background. The epithelial cells of Reissner's membrane (RM) are known to be capable of transporting Na^+ out of cochlear endolymph via epithelial Na^+ channels (ENaC) (Lee and Marcus, 2003). However, little is known in developing age.
Methods. Rats were used at the age of postnatal day (PND) 1, 3, 5, 7, 14, and 21. After the cochlear lateral wall was dissected from the apical turn of the cochlea, the stria vascularis was removed. The attached portion of RM was folded over the suprastrial portion of the spiral ligament and perfused at 37°C. The vibrating probe technique was chosen to measure transepithelial currents under short circuit conditions due to the small extent of the RM epithelial domain.
Results. Results showed that the short-circuit current (*Isc*) toward apical to basolateral direction in the physiologic saline gradually increased during early neonatal development. The amiloride-sensitive ENaC current also increased from PND 1 to PND 7, when the endolymphatic Na^+ concentration is known to reach the lowest level like in adult. The *Isc* decreased by application of UTP at early neonatal ages. The response to UTP was not inhibited by 100 μM suramin or PPADS. The present results point to the P2Y purinergic receptor as the $P2Y_4$. However, the cellular localization of P2Y4 and its functional role should be determined further.
Conclusion. Our results support that the RM is responsible for the maintenance of the endolymphatic low Na^+ concentration. $P2Y_4$ receptor is expressed in neonatal RM, but its role is uncertain as yet.

Colonic ion transport regulated by extracellular nucleotides

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Renal distal tubular and distal colonic ion transport is crucial for body salt and water homeostasis. At both transport sites systemic and local hormonal control define the final composition of the excreted fluids.
Extracellular nucleotides are general modualtors of epithelial ion transport. They do so by activation either luminal and basolateral purinergic receptors. The colonic epithelium is a bi-functional epithelium with absorptive surface cells and secretory

crypt cells. The current state of knowledge will be reviewed in this presentation. Three major ion transport processes are regulated. 1. ENaC-mediated electrogenic Na+ absorption is inhibited via luminal P2Y2 receptor. 2. Crypt derived anion secretion is activated by luminal and basolateral nucleotides. 3. Electrogenic K+ secretion is stimulated via luminal P2Y2 and P2Y4 receptors. We have established that colonic K+ secretion occurs via the KCa1.1 (BK) potassium channel, a phenomenon previously not fully appreciated. The comprised functional consequences of puringeric stimulation, is net secretion of fluids and electrolytes into the luminal compartment. Nucleotide-dependent modulation of colonic ion transport is believed to be present under normal resting conditions and provides a modulating "puringeric tonus" of colonic ion transport.

(Invited)

Role of ATP and adenosine in exocrine pancreas

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Pancreatic acini secrete digestive enzymes and in addition ATP and ATP-handling enzymes. Secretion is led through a series of pancreatic ducts, which contribute with fluid rich in bicarbonate and poor in calcium. Our studies aim to understand whether purinergic singalling contributes to short- and long-term communication between acini and ducts. Special focus is on ductal tissue, which is physiologically important for maintenance of pancreatic secretion. Malfunction of this epithelium can be a factor in cystic fibrosis, pancreatitis and pancreatic cancer.
Molecular biology, immunochemical approaches, electrophysiological recordings and bioimaging of intracellular calcium and chloride concentrations were used on isolated rat pancreatic tissues, human duct cell lines (PANC-1, CFPAC-1 and Capan-1) and oocyte expression system.
Both rat and human duct cells express various P2 receptors, including P2Y2, P2X4 and P2X7 receptors. P2Y2 receptors inhibit maxi-K channels and thus would down-regulate secretion. P2 receptors also stimulate Na/Ca exchange, and since both rat and human ducts also express calcium sensing and binding proteins, it is likely that purinergic receptors contribute to pancreatic calcium homeostasis. Our recent study shows pancreatic ducts also express adenosine receptors, most notably the A2A receptors, which opens CFTR chloride channels, and thus would support secretion. Taken together, various components of the purinergic signalling pathway regulate short-term exocrine function of pancreas. Long-term effects and exocrine to endocrine cell interaction is yet to be elucidated.

(Invited)

Differential regulation of anion secretion by apical vs. basolateral P2Y6 receptors in human bronchial epithelia

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Apical and/or basolateral membranes of polarized epithelia express P2Y receptors which regulate the transport of fluids and electrolytes. In the airway, P2Y receptors modulate Cl- secretion and Na+ reabsorption through the phospholipase C and calcium signaling pathways. Recent evidence suggests that P2Y6 receptors are expressed in bronchial epithelium and coupled to the cAMP/PKA pathways. We examined P2Y receptor subtype expression, including P2Y6, and the effect of extracellular nucleotides on basal short-circuit current (ISC) and intracellular calcium concentration ([Ca2+]i) in a human bronchial epithelial cell line (16HBE14o-). Real-time PCR demonstrated P2Y1, P2Y2, P2Y4 and P2Y6 receptor expression and confirmed that transcript levels were not altered when cells were grown under varied conditions. It was determined that P2Y agonists (ATP, UTP, UDP) stimulated a concomitant increase in ISC and [Ca2+]i. Apical nucleotides stimulated increased [Ca2+]i more efficiently than basolateral nucleotides, however, P2Y agonistic effects on ISC were greater when applied

basolaterally. Since the P2Y6 receptor differentially regulate apical and basolateral UDP-induced ISC and [Ca2+]i, we investigated membrane-resident P2Y6 receptor functions using Cl- or K+ channels blockers. Apical and basolateral UDP-activation of ISC was inhibited by applying DIDS apically or TRAM-34 and clotrimazole basolaterally. Although both apical and basolateral UDP increased PKA activity, only apical UDP-induced ISC was sensitive to a CFTR inhibitor. These data demonstrate that apical and basolateral P2Y agonists stimulate Ca2+-dependent Cl- secretion across P2Y receptor expressing human bronchial epithelia and that the cAMP/PKA pathway regulates apical but not basolateral P2Y6 receptor-coupled ion transport in human bronchial epithelia.

SYMPOSIA 8: ADENOSINE, INFLAMMATION AND IMMUNITY

Over-expression of the A_3 adenosine receptor in auto-immune inflammatory diseases: molecular mechanism involved

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Background: A_3 adenosine receptor (A_3AR), a Gi protein associated cell surface receptor, is highly expressed in the inflammatory cells and in peripheral blood mononuclear cells (PBMCs) of adjuvant induced arthritis (AIA) rats. The aim of this study was to investigate A_3AR expression levels in inflammed tissues and peripheral blood mononuclear cells (PBMCs) from Rheumatoid Arthritis (RA), Crohn's disease (CD) and Psoriasis (PS) patients. The molecular mechanism involved with receptor over-expression is presented.
Methods: A_3AR expression levels was examined by immuno-histochemistry, RT-PCR and Western blot analysis. Bioinformatic analysis was performed to explore transcription factors (TFs) present in the A_3AR gene promoter.
Results: A_3AR over-expression was found in inflamed synovial, colon and skin tissues from patients with RA, CD and PS respectively, in comparison to adjacent normal tissues. Receptor over-expression was also found in the patients' PBMCs and was directly correlated to disease severity. Mechanistic studies to explain A_3AR over-expression in the autoimmune inflammatory diseases revealed that TNF-α present in the inflammatory microenvironment, induced up-regulation of NF-κB and CREB TFs. Bioinformatic analysis revealed the presence of DNA binding sites of these TF in the A_3AR gene promoter, supporting their involvement in mediation A_3AR over-expression.
Conclusion: A_3AR over-expression in PBMCs from patients with auto-immune inflammatory diseases reflects receptor status in the inflammed organ and is directly correlated to disease severity. Receptor over-expression correlates with up-regulation of the TFs NF-κB and CREB. These findings and our former data suggest A_3AR as a possible therapeutic target and biological marker in autoimmune conditions.

Modulation of murine dendritic cells function by adenine nucleotides and adenosine: involvement of the A2B receptor

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Adenosine triphosphate (ATP) has been shown previously to induce a semi-maturation state of human monocyte-derived dendritic cells characterized by the up-regulation of co-stimulatory molecules, the inhibition of IL-12 production and the up-regulation of some genes involved in immune tolerance, such as thrombospondin-1 (TSP-1) and indoleamine-2,3-dioxygenase: these actions are mediated by the P2Y11 receptor. Since there is no functional P2Y11 gene in the murine genome, we investigated the action of adenine nucleotides on murine dendritic cells (DCs). ATP*ƒ*'S and adenosine inhibited the production of IL-12p70 by bone marrow-derived DCs (BMDC). These inhibitions were relieved by 8-p-sulfophenyltheophylline, an adenosine receptors antagonist. The use of subtype-selective ligands suggested the involvement of the A2B receptor. This was confirmed by experiments on A2B-/- BMDC. A microarray experiment, confirmed by qPCR, showed that, in presence of LPS, NECA (the most potent A2B receptor

agonist) regulated the expression of several genes: arginase I and II, TSP-1, and VEGF were up-regulated whereas CCL2 and CCL12 were down-regulated. We further showed that NECA, in combination with LPS, increased the arginase I enzymatic activity. In conclusion the described actions of adenine nucleotides on BMDC are mediated by their degradation product, adenosine, acting on the A2B receptor and will possibly lead to an impairment of Th1 response or tolerance.

Gene expression profilling defines ATP as a key regulator of human dendritic cells functions

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Several papers described the role of extracellular nucleotides such as ATP in the regulation of the immune response. To identify new promising target proteins, we have extensively compared gene expression profiles of ATPgammaS and PGE2 (prostaglandin E2) in human monocyte-derived dendritic cells (MoDCs). At 6 h of stimulation, ATPgammaS initiated an impressive expression profile (1125 regulated genes) compared to that of PGE2 (133 regulated genes). Many target genes involved in inflammation have been identified and validated by quantitative RT-PCR experiments. We have then focused on novel ATP target genes in MoDCs such as colony-stimulating factor-1 (CSF-1), MCP-4/CCL13 chemokine, neuropilin-1 (NRP-1) and vascular endothelial growth factor (VEGF-A). ATPgammaS and PGE2 inhibited CSF-1 secretion involved in monocyte and DC differentiation. Additionally, ATPgammaS inhibited the secretion of several chemokines involved in monocyte and DC migration including CCL2/MCP-1, CCL3/MIP-1alpha, CCL4/MIP-1beta, CCL8/MCP-2 and CCL13/MCP-4. Furthermore, flow cytometry experiments have demonstrated that ATPgammaS, ATP and PGE2 down-regulate NRP-1, a receptor involved in the interaction between T cells and DCs.
Finally, we have demonstrated that VEGF-A and other angiogenic factors were secreted by MoDCs in response to ATPgammaS, ATP or PGE2 - alone or in synergy with LPS - and could promote endothelial cell proliferation.

Diabetes affects iNOS expression control by adenosine in rat vascular smooth muscle cells

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Background: Induction of nitric oxide synthase (iNOS) by inflammatory cytokines is involved in diabetic vascular dysfunction. Considering the cytoprotective properties of adenosine, its influence on iNOS expression was investigated in vascular smooth muscle cells (VSMCs) from diabetic in comparison with normoglycaemic rats.
Methods: Diabetes was induced in Sprague-Dawley rats by intravenous injection of streptozotocin 4 weeks before sacrifice. VSMCs obtained from normal and diabetic rat aortas were incubated for 24 hours in the presence of LPS (1 microgram/ml), IL-1beta (10 nanograms/ml), TNF-alpha (25 nanograms/ml) and INF-gamma (10 nanograms/ml). iNOS protein was detected by Western blotting. Adenosine, AMP and their metabolites in the incubation medium were determined by HPLC.
Results: In VSMCs, diabetes have not altered the ability of adenosine deaminase (ADA, 0.01–1 U/ml) to increase cytokine-induced iNOS expression. Exogenous adenosine (1 millimolar) was ineffective in control cells, but potentiated the response to cytokines in diabetes. This effect was prevented by the ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (1 micromolar). Similarly, the adenosine precursor AMP (1 millimolar) enhanced iNOS induction in diabetic but not in control VSMCs. At the end of incubation with adenosine or AMP, inosine and hypoxantine were the sole metabolites detected, accounting for 100% and 60–70% of the added compounds, respectively. The inosine to hypoxanthine ratio was increased in diabetes.
Conclusion: Diabetes makes VSMCs sensitive to the potential proinflammatory effect of high adenosine concentrations, without altering the protective action of the endogenous nucleoside. The observed changes in purine metabolism may be partly involved in this effect.

Caffeine blocks MPTP-induced increases in blood-brain barrier leakage in mouse striatum

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Caffeine blocks MPTP-induced increases in blood-brain barrier leakage in mouse striatum
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Background: The blood-brain barrier (BBB) is important physiologically, and pathologically BBB disruption has been implicated in a wide spectrum of neurological disorders including Parkinson's disease. Recent studies indicate that caffeine is protective against Parkinson's disease, but by poorly understood mechanisms. Using a MPTP neurotoxin model of Parkinson's disease, we tested the hypothesis that caffeine protects against MPTP-induced BBB leakage.
Methods: FVB mice were pretreated with caffeine (10 mg/kg, i.p.) or saline for 7 days and 10 min before each dose of MPTP (20 mg/kg, i.p.) for another 7 days. Striatum (and for some studies hippocampus and cerebral cortex as well) were evaluated for BBB leakage, tight junction protein expression, integrity of dopaminergic neurons, and neuroinflammation by immunostaining, immunoblotting and real-time PCR techniques.
Results: We found that caffeine blocked MPTP-induced decreases in TH positive dopaminergic neuron, increases in leakage of Evan's blue dye and FITC-albumin specifically in striatum but not in cerebral cortex or hippocampus, decreases in levels of the tight junction proteins occludin and ZO-1, and increases in reactive astrocytosis.
Conclusion: Our results suggest that caffeine might protect against Parkinson's disease, in part, by stabilizing the BBB.
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Adenosine A2A receptor activation inhibits Th1 and Th2 cell development and effector function

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Adenosine is an immunosuppressive nucleoside, and adenosine A2A receptors inhibit T cell activation in response to TCR stimulation. We, therefore, investigated the role of A2A receptors in regulating Th1 and Th2 cell development and effector function. We show that A2A receptor stimulation suppresses the development of TCR-stimulated naïve T cells into both Th1 and Th2 cells, as indicated by decreased IFN-gamma production by cells developed under Th1-skewing conditions and decreased IL-4, IL-5, and IL-10 production by cells developed under Th2-skewing conditions. Using A2A receptor-deficient mice we demonstrate that A2A receptor activation inhibits Th1 and Th2 cell development by decreasing the proliferation of naïve T cells irrespective of whether the cells are expanded under Th1- or Th2-skewing environment. This decreased proliferation of T cells caused by A2A receptor activation was linked to suppression of IL-2 production by both developing Th1 and Th2 cells. Using in vivo established Th1 and Th2 cells, we further demonstrate the non-selective nature of A2A receptor-mediated immunosuppressive effects, because A2A receptor activation decreased IFN-gamma and IL-4 secretion by TCR-stimulated effector Th1 and Th2 cells, respectively. The decreased production of IFN gamma and IL-4 was associated with decreased levels of mRNA for both cytokines. A2A receptor mRNA expression in Th2 effector cells increased following TCR stimulation, indicating the existence of an autoregulatory immunosuppressive feedback loop. In summary, these data demonstrate that A2A receptor activation has strong inhibitory actions during early developmental as well as late effector stages of Th1 and Th2 cell responses.

Altered IL-1ß secretion in a patient affected by Schnitzler's syndrome

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Background: Schnitzler's syndrome is a rare and often underdiagnosed disorder defined by two constant features, chronic urticarial rash and monoclonal IgM gammopathy. Successful treatment with anakinra of patients affected by Schnitzler's syndrome supports the hypothesis that IL-1 plays an important role in the pathogenesis. Aim of the study was to investigate the IL-1ß processing machinery in peripheral blood mononuclear cells (PBMCs) from a patient affected by a variant-type Schnitzler's syndrome.
Methods: PBMCs were examined for ability to secrete IL-1ß and IL-18, expression and function of the P2X7 receptor, and expression of inflammasone components,ASC and NALP3, before and after therapy with high prednisone doses. Expression of inflammasome components was measured by Real Time PCR and Western blotting.
Results: Schnitzler PBMCs showed a high spontaneous and LPS-stimulated IL-1ß release, but were poorly responsive to stimulation with the P2X7 agonist benzoyl ATP. P2X7 expression was about seven fold increased, while ASC expression was dramatically decreased compared to PBMCs from healthy controls. NALP3 expression was unchanged. Prednisone treatment caused remission of clinical symptoms and normalized IL-1ß secretion and P2X7 and ASC expression.
Conclusion: Schnitzler's PBMCs have already undergone an inflammatory priming that allows substantial IL-1ß release in the absence of the "second hit" usually required to trigger efficient release, showing an altered pathway of IL-1ß processing and release. Therapies aimed at normalizing inflammasome function might help correct the unbalanced IL-1ß secretion typical of this syndrome.

The anti-inflammatory effect of A3 adenosine receptor agonists: a novel targeted therapy for rheumatoid arthritis

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Targeting the A3 adenosine receptor (A3AR) to combat inflammation is a new concept based on two findings: a) A3AR is highly expressed in inflammatory cells, whereas low expression is found in normal tissues; and b) A3AR activation with a specific agonist induces de-regulation of the NF-kappa B signaling pathway in inflammatory cells. The A3AR agonist CF101 (known generically as IB-MECA) induces anti-inflammatory effects in experimental animal models of collagen- and adjuvant-induced arthritis (CIA and AIA, respectively). Combined therapy with CF101 and Methotrexate (MTX) in AIA rats yielded additive anti-inflammatory effect. MTX induced up-regulation of A3AR, rendering the inflammatory cells more susceptible to CF101.
In Phase I and in Phase IIa human studies CF101 was safe and well tolerated and showed strong signal of an anti-inflammatory effect in Rheumatoid Arthritis patients. In PBMC withdrawn from the patients at base line, statistically significant correlation between A3AR expression level and response to the drug was noted. It is suggested that A3AR may serve as a biological marker to predict patient response to the drug.
Taken together, this information suggests that A3AR agonists may be a new family of orally bioavailable drugs to be developed as potent inhibitors of autoimmune inflammatory diseases

(Invited)

A2B receptor activation decreases bacterial invasion, inflammation, and immune cell apoptosis in sepsis

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Background. Sepsis, a disease caused by a dysregulated immune system following infection, is a leading cause of multiple organ failure and death in hospitalized patients. Endogenous adenosine has been shown to regulate the immune pathophysiology of sepsis via adenosine receptor activation; however, the role of A2B receptors is unknown. Methods: We studied the role of A2B receptors by subjecting A2B receptor KO and WT mice to cecal ligation and puncture (CLP). Immune system dysfunction following CLP was evaluated by measuring bacterial load, assessing inflammatory cytokine concentrations, detecting immune cell apoptosis using immunoblotting of cleaved caspase-3 and cleaved PARP, and analyzing immune cell subset abundance using flow cytometry. Signaling pathways in the spleen were examined using immunoblotting for IkappaB, p-AKT, p-PTEN, and p-p42/44. Results. We found markedly increased numbers of bacteria in the peritoneal fluid of A2B KO mice. Levels of inflammatory cytokines (IL-6, IL-1beta, TNFalpha, IL-12p40, MIP-2, and MCP-1) were increased both in the blood and in the peritoneum of A2B KO mice. Spleen cell apoptosis was augmented in A2B KO mice. Analysis of white blood cell subsets indicated a decrease in neutrophil and B cell numbers in both spleen and blood of A2B KO mice. We found a decreased level of IkBalpha, diminished Akt and PTEN phosphorylation, and enhanced p42/44 phosphorylation in A2B KO mice. Conclusion. A2B receptor activation decreases bacterial load and inflammation in sepsis.

Discovery of novel non-nucleoside adenosine kinase inhibitors

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Background. Adenosine kinase (AK) inhibitors are of considerable interest as novel site- and event-specific indirect adenosine receptor agonists. They exhibit potent antinociceptive, anticonvulsive and antiinflammatory activity without the limiting cardiovascular side effects exhibited by direct adenosine receptor agonists. The aim of the present study was to establish a fast and convenient high-throughput 96 well-plate assay and to identify lead compounds for the development of structurally novel adenosine kinase inhibitors.
Methods. A radioactive screening assay in a 96-well-plate format was established using [3H]adenosine as a substrate and separating the formed [3H]AMP from [3H]adenosine by precipitation with lanthanum chloride, which was subsequently filtered off using a harvester. Computational virtual screening analysis was performed using 2D similarity search of ZINC and MDDR databases with known AK inhibitors as reference molecules, structurally diverse compounds were selected and analyzed in docking using UCSF DOCK and FlexX.
Results. Selected compounds from our library initially developed for interaction with purinergic receptors were tested for AK inhibition. In addition, structures proposed by the virtual screening approach were investigated. From a series of 59 compounds four novel AK inhibitors belonging to three different structural classes were identified. The most potent compound showed a Ki value of 109±4 nM.
Conclusion. The identified hits will provide valuable lead structures for the development of structurally novel AK inhibitors.

The A2A receptor and T cell regulation of gastrointestinal inflammation

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It has been appreciated for a long time that intestinal T cells prevent excessive responses to luminal dietary or microbial antigens by the induction of oral tolerance. More recently, it appears that different Th cell subsets with “regulatory” function also exist. One factor that mediates anti-inflammatory activity is adenosine. Adenosine accumulates in inflamed or hypoxic

tissues largely due to CD39 mediating the dephosphorylation of ATP to ADP then to 5′-AMP while CD73 catalyzes the terminal reaction to convert 5′-AMP to adenosine. Magnetic bead-purified CD4+ Th cells from murine spleen or human biopsy specimens were stimulated with anti-CD3/CD28 and assayed for the expression CD39/CD73, the 4 adenosine receptor subtypes and cytokine expression by flow cytometry, ELISA or RT-PCR. Th cells expressed both CD39 and CD73 which was up-regulated after activation. The human CD25+ Foxp3 regulatory T cells expressed higher levels of these enzymes suggesting that the formation of adenosine by Treg contributes to the pool of mediators of suppression. A2AAR mRNA was the predominant subtype expressed in Th cells or gastric T cells. A2AAR activation reduced production of pro-inflammatory cytokines (including IL-2, IFN-gamma, TNF-alpha) while IL-10 and TGF-beta remained increased. Moreover, using the CD45RB adoptive transfer, we showed that Th cells from mice lacking A2AAR had impaired Treg activity. In addition to the direct effects of adenosine on T cells, we also show that the immunogenicity of antigen presenting cells is impaired due to adenosine. Thus, the expression of CD39 and CD73 by T cells along with the effects of adenosine suggest that T cells as well as antigen presenting cells contribute and/or respond to local adenosine accumulation. Moreover, adenosine appears to contribute as a mediator of suppression within the regulatory T cell network in the gastrointestinal tract.

(invited)

Adenosine receptors on human T lymphocytes and Jurkat T cells

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Background. T cells play a central role in cellular immune reactions. Activation of adenosine A2A receptors on T cells has been reported to cause immunosuppressive effects. The current project was aimed at investigating the expression and function of adenosine receptor subtypes on phytohemagglutinin(PHA)-stimulated and unstimulated human T lymphocytes and Jurkat T cells.
Methods. Real-time RT-PCR and radioligand binding studies were performed to study the mRNA transcription and protein expression levels of the four adenosine receptor subtypes in native human lymphocytes as well as in Jurkat T cells, a permanent human T cell line. A [3H]thymidine incorporation assay was used to study effects of selective adenosine receptor ligands on T cell proliferation, and fluorimetric [Ca2+]i measurements were used to investigate functional responses.
Results. By real-time RT-PCR we detected mRNA for all four adenosine receptor subtypes in unstimulated lymphocytes. Highest levels were found for A2A receptors, which were further increased upon PHA-stimulation. The increase in A2A receptor expression by PHA-stimulation could be confirmed on the protein level. In Jurkat T cells, whose proliferation could not be further stimulated by PHA, mRNA levels for A1, A2A and A2B receptors were high. Both, A2A and A2B were coupled to effects on intracellular calcium concentration. Subtype-selective agonists (A1, A2B and A3) showed concentration-dependent antiproliferative effects in native T lymphocytes. Selective antagonists (A1, A2A and A3) also inhibited proliferation possibly due to an upregulation of the receptors.
Conclusion. All four adenosine receptor subtypes appear to have effects on lymphocyte proliferation.

A P2X7-dependent process mediates the formation of multinucleated macrophages by GM-CSF

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Activation of the purinergic P2X7 receptor in heterologous cell expression systems and in macrophages has been linked to the formation of multinucleated giant cells (MGC), a hallmark of chronic inflammatory reactions. In the present study we investigated the relevance of P2X7 in the generation of MGC driven by the inflammatory cytokine GM-CSF, an important stimulus for MGC formation. To this aim, various macrophage preparations were used including rat alveolar macrophages,

murine J774 macrophage clones selected for either high (P2X7 hyper) or low (P2X7 hypo) expression of the receptor and peritoneal macrophages obtained from wild-type and P2X7 knockout mice. Macrophages were stimulated with GM-CSF (10 ng/ml) for 48–72 h, and their ability to form MGC determined by digital imaging microscopy and capture image analysis. Oxidized ATP, an irreversible inhibitor of P2X7 function was found to block GM-CSF promotion of MGC in primary cultures of rat alveolar and mouse peritoneal macrophages. In addition, P2X7 hyper macrophages formed increased number of MGC in response to GM-CSF (5–20 ng/ml) whereas P2X7 hypo macrophages harboring defective P2X7 function did not respond to GM-CSF. Moreover, the GM-CSF promoting effect was lost in peritoneal macrophages obtained from mice lacking the P2X7 receptor. These observations clearly demonstrate that the P2X7 receptor mediates MGC promotion by GM-CSF and suggest that P2X7 activation may represent an efficient amplification pathway for the formation of multinucleated macrophages under inflammatory conditions. (Supported by NSERC)

Induction of inhibitory adenosine A2A receptors on NKT and NK cells limits ischemia-reperfusion injury

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Background: Activation of NKT or NK cells results in rapid cytokine release followed by long term anergy. In this study we sought to determine if NKT and NK cell activation is accompanied by induction of inhibitory adenosine A2A receptors (A2AR). Methods: CD1d-restructed iNKT cells were activated in vivo by injecting C57BL/6 mice with alpha-galactosylceramide a-GalCer). Liver injury was assessed enzynmatically and histologically. Cytokine production was measured used fluorescent antibodies and FACS. Induction of A2AR transcription and protein in NKT and particularly in NK cells following a-GalCer was demonstrated using an A2AR promoter-eGFP reporter mouse, and by anti-A2AR immunoreactivity, respectively. Results: FACS analysis of dispersed liver cells revealed sequential production of proinflammatory cytokines in NKT cells and transactivated NK and T cells that peaked in 2, 4, and >24 hours, respectively after a-GalCer injection. These cytokine responses and liver necrosis were elevated in A2AR -/- mice. In A2aR +/+ mice inflammation and injury were suppressed by the selective A2AR agonist, 4-{3-[6-Amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-piperidine-1-carboxylic acid methyl ester (ATL313). Over 85% of NK cells were positive the for A2AR transcript reporter and for receptor protein 20 hours after a-GalCer injection. The activation of A2ARs on NK cells with ATL313 in vitro inhibited the release of IFN-$f^{\cdot}$ half maximally at a concentration of 0.7 nM. Conclusions: These results demonstrate that inhibitory A2ARs are induced following activation of NKT and NK cells and may contribute to anergy that limits persistent activation of these cells.

(Invited)

Adenosine A2A receptor occupancy promotes dermal fibrosis by modulating Il-13 and Fli1 expression

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We have previously reported that adenosine enhances dermal matrix production. Adenosine A2A receptor(A2AR)-deficient mice are resistant to bleomycin-induced dermal fibrosis. To further clarify how adenosine induces dermal matrix accumulation, we explored the effects of A2A receptor occupancy on key fibrogenic mediators in the dermis.
IL-13 was significantly increased in mice with elevated dermal levels of adenosine (ADA-deficient mice) (1.47±0.06 vs. 2.54±0.21 pg/mg protein, ADA WT vs. ADA KO, ELISA, $p<0.001$), and reversed by A2AR antagonist, ZM241385

(10 μM, $p<0.01$). IL-13Ralpha1 mRNA expression was upregulated by A2AR agonist CGS21680 (10 μM) in human dermal fibroblasts (DF) (real-time PCR), and IL-13Ralpha1 protein expression was increased by CGS21680 in membrane preparations from DF (Western blot, 24 hrs). In vivo, treatment of ADA KO mice with ZM241385 decreased IL-13Ralpha1 mRNA ($p<0.01$, n=4) while that for the decoy receptor, IL-13Ralpha2, was unchanged.
Fli1 is a known transcriptional repressor of fibrillar collagen genes and connective tissue growth factor (CTGF/CCN2) in DF. A2AR stimulation with CGS21680 (10 μM) suppressed Fli1 mRNA by 47.0±18.2% (vs. control) in DF nuclear extracts (4 hrs, real-time PCR, n=4, $p<0.05$). Fli1 protein was reduced by CGS21680 (24 hrs, 31.9±12.8% reduction, Western, n=5, $p<0.05$). Furthermore, IL-13 induces a 38±7% ($p<0.05$, n=4) reduction in Fli1 protein in the nucleus. CGS21680 also increased CCN2 secretion by DF (2.6-fold).
Conclusion: A2AR occupancy promotes dermal matrix production by (1) inducing IL-13, (2) suppressing Fli1, and (3) augmenting CCN2 secretion by DF, suggesting that modulation of A2AR function may be a novel therapeutic approach to limit dermal fibrosis as seen in scleroderma.

Adenosine A2A agonist and A2B antagonist mediate an inhibition of inflammation-induced contractile disturbance of a rat gastrointestinal preparation

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Adenosine can show anti-inflammatory as well as pro-inflammatory activities, and the contribution of the specific adenosine receptor subtypes in various cells, tissues and organs is complex. In this study, we examined the effect of the adenosine A2A receptor agonist CGS 21680 and the A2B antagonist PSB-1115 on acute inflammation induced by TNBS on rat ileum/jejunum preparations. Preincubation of the ileum/jejunum segment with TNBS for 30 min resulted in a concentration-dependent inhibition of ACh-induced contractions.
Pharmacological activation of the A2A receptor with CGS 21680 (0.1–10 μM) preincubated simultaneously with TNBS (0.01 M) restored concentration-dependently the TNBS-induced inhibition of the ACh-contractions. Stimulation of A2B receptors with the selective agonist BAY 60–6583 (10 μM) did neither result in an increase nor in a further decrease of ACh-induced contractions compared to the TNBS-induced inhibition. The simultaneous preincubation of the ileum/jejunum segments with TNBS (0.01 M) and the selective A2B antagonist PSB-1115 (100 μM) inhibited the contraction-decreasing effect of TNBS. A significant amelioration of the TNBS-diminished contractility was found by the combination of the A2AR agonist CGS 21680 and the A2B antagonist PSB-1115 at subthreshold concentrations of both agents, which was in the same range as the effect induced by 1 μM methotrexate. Our results demonstrate that the activation of A2A or the blockade of A2B receptors can decrease the inflammation-induced disturbance of the ACh-induced contraction in TNBS pretreated small intestinal preparations. The combination of both may be useful for the treatment on inflammation bowel diseases.

HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak

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Background: Extracellular adenosine has been implicated in vascular adaptation to hypoxia. Based on the observation that increases in intracellular adenosine can effectively elevate extracellular adenosine, we studied the contribution of adenosine kinase (AK, intracellular conversion of adenosine to AMP) to vascular adenosine responses.

Methods and Results: Initial in vitro studies of ambient hypoxia revealed prominent repression of endothelial AK transcript (85±2% reduction), protein and function. Transcription factor binding assays and hypoxia inducible factor (HIF)-1alpha loss and gain of function studies suggested a role for HIF-1alpha in transcriptional repression of AK. Moreover, repression of AK by ambient hypoxia was abolished in conditional HIF-1alpha mutant mice in vivo. Studies of endothelial barrier function revealed that inhibition or siRNA repression of AK is associated with enhanced adenosine-dependent barrier responses in vitro. Moreover, in vivo studies of vascular barrier function demonstrated that AK inhibition with 5′-iodotubericidin (1 mg/kg prior to hypoxia) significantly attenuated hypoxia-induced vascular leakage in multiple organs and reduced hypoxia-associated increases in lung water.
Conclusions: Taken together, our data reveal a critical role of AK in modulating vascular adenosine responses and suggest pharmacological inhibitors of AK in the treatment of conditions associated with hypoxia-induced vascular leakage (e.g. sepsis or acute lung injury).

Anti-inflammatory preconditioning by agonists of adenosine A1 receptor

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Background: We previously reported that in a model of peritonitis the peak A1R expression during leukocyte recruitment, is followed by a peak in A2AR during inflammation resolution. In this study, we examined whether A1R activation sequentially induces A2AR expression and reverses inflammation.
Methods: Peritonitis was induced in CD1 mice by intraperitoneal injection of E. coli. Protein and mRNA were extracted from the peritoneum or from peritoneal mesothelial cells (PMC) or peritoneal macrophages (PM) and adenosine receptor or chemokine levels were analyzed by western blot and real time PCR analysis. TNF-alpha and IL-6 levels were determined in peritoneal fluid or sera by ELISA.
Results: A1R agonists, CHA or CCPA upregulated A2AR expression in cultured PM and PMC. Induction of A2AR was inhibited by Pertussis toxin and partly dependent on A2AR stimulation. CHA administration to healthy mice reduced A1R expression and induced A2AR production in PMC. Preconditioning with CHA (0.02 mg/kg) or CCPA (0.1 mg/kg) 24 hours before E. coli-inoculation reduced leukocyte recruitment (to 66% from vehicle) and exhibited decreased TNF-alpha and IL-6 sera levels (to 25% and 38% from vehicle, respectively) and reduced MIP-2 and MCP-1 mRNA level in PMC (to 25% and 38% from vehicle, respectively).Preconditioning was blocked by pretreatment with A1R antagonist (DPCPX, 1 mg/kg) or by treatment with A2AR antagonist (ZM241385, 1 mg/kg) before inoculation and was absent in A2AR-/- mice.
Conclusion: A1R-agonist preconditioning promotes inflammation resolution by inducing the production of A2AR. Future implications may include pretreatment before anticipated inflammatory events, such as surgical procedures.

Adenosine A2A receptor-deficient mice are resistant to induction of peritoneal fibrosis

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Background: Peritoneal dialysis (PD) has emerged an important treatment for patients with end stage renal disease. During PD, the peritoneum is exposed to bioincompatible dialysis fluids, which are associated with fibrosis and loss of peritoneal

membrane function.Adenosine through its A2A receptor (A2AR) was shown to promote tissue repair and extracellular matrix production. In this study we examine the role of adenosine and it's A2AR in peritoneal fibrosis development.
Methods: Peritoneal fibrosis was induced by i.p. injection of either chlorhexidine gluconate (CG) for two weeks or 4.25% glucose peritoneal dialysis fluid (PDF) for one month. WT (A2AR+/+) mice were compared to A2AR deficient mice (A2AR-/-) or to WT mice treated with caffeine, an A2AR antagonist, in drinking water (0.1%). In addition, collagen deposition was evaluated in primary peritoneal fibroblasts (PF) following treatment with A2AR agonist and antagonist.
Results: In both in vivo models of fibrosis, A2AR-/- mice or caffeine treated-WT mice had a reduction in submesothelial thickness in histological preparations stained with Masson's trichrome (-30%) and a reduction in collagen deposition measured by Sircol assay (-30%). In addition, those mice exhibit reduced levels of fibroblast specific protein-1 mRNA (-60%) and transforming growth factor-beta (TGF-beta) in the lavage fluid. Consistent with these results, adenosine or the A2AR agonist CGS21680 stimulated collagen production in PF that was blocked by A2AR antagonists (ZM241385, 10nM or caffeine, 100microM).
Conclusion: Our data suggest that adenosine through it's A2AR promotes peritoneal fibrosis and therefore should be considered as a target for pharmacological intervention in PD-treated patients.

CF502 targets the A_3 adenosine receptor to induce anti-inflammatory effect via down-regulation of the NF-κB signaling pathway

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Background. The A_3 adenosine receptor (A_3AR) has recently been shown to be up-regulated in inflammatory cells and was suggested as a target to combat inflammation. In this study we looked at the anti-inflammatory effect of CF502, a highly selective agonist to the human A_3AR.
Methods. Affinity of CF502 to the human A_3AR was tested by competition assays on CHO cell membranes. The capability of CF502 to activate A_3AR was tested by cAMP accumulation assay. Effect of CF502 on fibroblast like synoviocytes (FLS) obtained from patients with active Rheumatoid arthritis was evaluated by MTT assay. The effect of CF502, administered orally, on the development of Adjuvant Induced Arthritis (AIA) was examined. Western blot and immuneo-histochemistry analysis were used to look at protein profile.
Results. CF502 was highly selective at the A_3AR compared with other adenosine receptor subtypes, demonstrating binding affinity of 0.29±0.04 nM.. The drug inhibited forskolin-stimulated adenylyl cyclase (EC_{50}=0.38±0.12 nM.). CF502 exerted a dose-dependent anti-proliferative effect of RA FLS. A_3AR expression level was down-regulated shortly after treatment, demonstrating receptor response to the drug. This was followed by a decrease in the expression level of PI3K, PKB/Akt, IKK, IκB, NF-κB resulting in inhibition of TNF-α. CF502, ameliorated the arthritic clinical and pathological manifestations, via the same mechanism of action demonstrated in vitro.
Conclusion. The data provide evidence that the selective A_3AR agonist CF502 reduces the inflammatory manifestations of arthritis and support the concept that A_3AR agonists may be considered as drug candidates for Rheumatoid Arthritis treatment.

Anti-inflammatory effect of adenosine in rat vascular smooth muscle cells

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Background: The potential vasoprotective effect of adenosine was evaluated by studying its influence on nitric oxide (NO) release and inducible NO synthase (iNOS) expression in vascular smooth muscle cells (VSMCs) under inflammatory conditions.
Methods: VSMCs obtained from intimal-medial layers of rat aorta were incubated for 24 hours with LPS (1 microgram/ml) combined with IL-1 beta (10 nanograms/ml), TNF-alpha (25 nanograms/ml) and INF-gamma (10 nanograms/ml) to mimic the environment of some vascular inflammatory events in vivo. The activity of iNOS was determined by the Griess' reaction in culture medium samples and iNOS protein was detected by Western blotting.
Results: In stimulated VSMCs, adenosine deaminase (ADA, 1 U/ml) doubled cytokine-driven NO release. This effect was reduced by the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1 micromolar) and enhanced by nitrobenzylthioinosine (10 micromolar) and dipyridamole (10 micromolar), two adenosine transport blockers. By contrast, inhibition of ecto-5′nucleotidase with alpha,beta-methyleneADP (200 micromolar) did not affect NO accumulation. Exogenous adenosine (0.1–1 millimolar) hindered the stimulated NO production. This effect was maintained in the presence of EHNA, but was abolished by the adenosine receptor antagonist, 8-phenyltheophylline (10 micromolar), which mimicked ADA action. Changes in NO release were linearly related to iNOS protein levels.
Conclusion: The overproduction of NO by VSMCs under inflammatory conditions is limited by adenosine that is released from the cells and activates P1 receptors. This protective effect primarily involves adenosine-induced changes in iNOS expression.

Diagnostic potential of sputum adenosine deaminase activity for chronic obstructive pulmonary disease and asthma

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Background: Airway hyperresponsiveness to AMP displays higher specificity than methacholine to distinguish asthmatic from COPD patients, but worsens eosinophilia. Since AMP is metabolized into adenosine (ADO), we hypothesized that markers of ADO regulation, measured in induced sputum, may provide non-invasive alternatives to differentiate the two diseases. Methods: Induced sputum from healthy volunteers, mild atopic asthmatics and mild-to-moderate COPD patients were tested for differential cell counts, cytokines, ADO concentration and ADO deaminase (ADA1 and ADA2) activities. Results: The asthma samples exhibited eosinophilia, macrophage dominance (>65%), high GM-CSF and low IL-6 levels, compared to normal secretions. The COPD samples were neutrophil dominant (>60%) with low lymphocyte counts, elevated IL-6 and IL-8 concentrations. The diseases were clearly distinguished based on ADO concentrations, which were 3-fold higher in asthma, and 2-fold lower in COPD, than in normal sputum. We identified ADA1 as the isoform regulating airway ADO elimination (COPD > normal > asthma). In COPD, ADA1 activity correlated positively with IL-8 and neutrophils. In contrast, ADA2 activity correlated positively with GM-CSF and macrophages in all groups. Lung functions (FEV1 and FEV1/FVC) correlated positively with ADO concentration, and negatively with ADA1 activity, in COPD patients. Finally, sputum ADA1/total ADA activity distinguished normal (6.1), COPD (9.0) and asthmatic (1.9) subjects with 85% specificity. Conclusion: This study demonstrates that COPD and asthma exhibit opposite deregulations of airway ADO related to the type of inflammation, and supports sputum ADA1/total ADA activity as a new non-invasive tool to diagnose the two diseases.

The adenosine A2A receptor triggers key 'stop signals' in inflammatory human neutrophils: a genomics approach

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-Background: Activation of the adenosine A2A receptor (A2AR), elevations in intracellular cAMP concentrations, or PGE2, each elicit potent anti-inflammatory events in human neutrophils, by inhibiting phagocytosis, superoxide production,

adhesion and cytokine release. However, the intracellular pathways that mediate these events remain poorly understood. In the present study, we investigated the impact of anti-inflammatory signals, including A2AR activation, on the gene expression profile of stimulated neutrophils.
-Methods: Blood neutrophils were obtained from healthy donors, purified, and stimulated with a cocktail of inflammatory agonists, in combination with one of the following anti-inflammatory factors: the adenosine A2AR agonist, CGS21680; prostaglandin (PG) E2; or a cAMP-elevating mixture. Total RNA was isolated and processed for the Affimetrix genechip technology in order to identify differentially-expressed genes. Real-time PCR was utilized to validate data obtained with genechips.
-Results: The mRNA expression of a group of approximately 20 genes was significantly altered, either positively or negatively, by anti-inflammatory agents. Activation of the A2AR, PGE2, and the cAMP-elevating mixture all affected the gene expression profile in a similar fashion, supporting a central role for these genes in limiting cell activation. These genes include COX-2, TNF-alpha, MIP-1, DUSPs, NOR, and several others.
-Conclusions: A set of genes, for which the expression is specifically altered by anti-inflammatory factors, was identified. This set of genes is likely to include some of the most important "stop-signals" that are under A2AR control and responsible for limiting neutrophil activation.

Adenosine A2B receptors on host immune cells promote carcinoma growth

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Recent studies suggest that tumor-infiltrating immune cells can benefit the tumor by producing factors that promote angiogenesis and suppress immunity. Because the tumor microenvironment is characterized by high adenosine levels, we hypothesized that the low-affinity A2B adenosine receptor located on host immune cells may participate in these effects. In the current study we tested this hypothesis in a Lewis lung carcinoma (LLC) isograft model using A2B receptor knockout (A2BKO) mice. These mice exhibited significantly attenuated tumor growth and longer survival times after inoculation with LLC cells compared to wild type (WT) controls. LLC tumors in A2BKO mice contained significantly lower levels of VEGF compared to tumors growing in WT animals. This difference was due to VEGF production by host cells, which comprised 30% of total tumor cell population. Flow cytometry analysis of CD45+ cells showed that the proportion of CD11b+/Gr-1+ myeloid suppressor cells was 3-fold lower in tumors grown in A2BKO mice compared to WT control. In contrast, the proportion of cells expressing CD3epsilon chains of T cell receptor was 3-fold higher in tumor-infiltrating CD45+ cells isolated from A2BKO mice compared to WT control. Stimulation of adenosine receptors on WT tumor-infiltrating CD45+ immune cells increased VEGF production five-fold, an effect not seen in tumor-associated CD45+ immune cells lacking A2B receptors. In contrast, we found no significant difference in VEGF production between CD45- tumor cells isolated from WT and A2BKO mice. Thus, our data suggest that tumor cells promote their growth by exploiting A2B adenosine receptors in host immune cells.

Adenosine receptor expression and regulatory effects of A2AR activation on IL-6 and IL-8 secretion in human kidney epithelial cells

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Background
Adenosine acting via A2A receptors is known to down-regulate inflammation in many tissues but it is not yet known whether A2A receptor activation regulates the host response and inflammation in urinary tract infection (UTI).
Methods
Human kidney epithelial cells (A498) were stimulated with proinflammatory cytokines (IL-1beta, TNF-alpha and IFN-gamma) or uropathogenic Escherichia coli (UPEC). The expression of adenosine receptors was investigated using semi-quantitative RT-PCR, Western blot analysis and immunocytochemistry. Secretion of IL-6 and IL-8 was determined by ELISA.

Results
The A1, A2A and A2B, but not the A3, receptor subtypes were detected in A498 cells and the expression of the A2A receptor increased when exposed to proinflammatory cytokines and UPEC. UPEC-induced production of IL-6 and IL-8 increased from 27±7.1 to 223±50 pg/ml/1000 cells and from 26±7.9 to 493±145 pg/ml/1000 cells in A498 cells, respectively. UPEC-evoked secretion of IL-6 and IL-8 decreased significantly, by 34±6% and 31±3% ($p<0.01$) respectively, when exposed to the A2A receptor agonist CGS 21680 (1 μM). SCH 58261 (10 nM), an A2A receptor antagonist, reversed the CGS 21680-induced inhibitory effect.
Conclusions
Expression of A2A receptors in kidney epithelial cells was up-regulated in UTI-like conditions and activation of the A2A receptor reduced UPEC-induced IL-6 and IL-8 secretion. These findings suggest that adenosine, via A2A receptor activation, may be a previously unrecognized regulator of the mucosal response in UTI.

Allergen induced airway inflammation is attenuated in ecto-5′-nucleotidase (CD73) deficient mice

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Extracellular purines are involved in allergen induced airway inflammation and regulation of Cl- secretion by the airway epithelium. To study the role of extracellular purines in inflamed airways, mice were sensitised and challenged with ovalbumin (OVA). 24 and 72 h after OVA challenge of BALB/c mice infiltration of eosinophils and increased airway responsiveness to methacholine was found. This was not observed in ecto-5′-nucleotidase (CD73) deficient mice (CD73 -/-). In tracheas of BALBc mice purine induced Cl- secretion was enhanced after OVA challenge. In addition increased expression of mRNA was found for the purinergic receptors P2Y6 and A2B and the Cl- channel CFTR. AMP induced Cl- secretion was not detectible in trachea of CD73 -/-, indicating that formation of extracellular adenosine in the trachea is due to degradation of AMP through ecto-5′-nucleotidase. Enhanced sensitivity to purines on Cl- secretion was not observed in CD73 -/- mice. Application of ATP (10 μM) facilitated mucociliary clearance in BALB/c and CD73, but was strongly induced in OVA challenged mice. In conclusion, release of ATP and increased expression of purinergic receptors during airway inflammation enforce mucociliary clearance. In addition ATP and adenosine formation by ecto-5′-nucleotidase cause acute airway inflammation. This may reflect a novel target for therapeutic intervention.
This study is supported by Else Kröner- Fresenius- Stiftung and SFB 699 A7.

Effect of adenosine on the production of tissue inhibitor of metalloproteinases-1 (TIMP-1) in interleukin (IL)-4-activated macrophages.

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Background: Macrophages activated by IL-4 or IL-13, also known as alternatively activated macrophages, can produce increased levels of factors involved in tissue remodeling such as TIMP-1. Adenosine has been shown to contribute to tissue remodeling. In this study we investigated the effect of adenosine on the production of TIMP-1 by macrophages activated by IL-4. Methods: Thioglycollate-elicited peritoneal macrophages were plated into 96-well plates at 2x105 cells/well, and treated with adenosine (100 mikroM) or adenosine agonists (CCPA for A1, CGS-21680 for A2A, IB-MECA for A3, as well as NECA, a non-selective agonist) (1–100 nM) 30 min prior to administration of IL-4 (20 ng/ml), followed by incubating the cells for 24 hours. The level of TIMP-1 was determined from cell supernatants taken at the end of the incubation period using enzyme-linked immunosorbent assay. In some experiments adenosine receptor specific antagonists (1–100 nM) such as DPCPX for A1, ZM241385 for A2A, MRS-1754 for A2B, and MRS-1523 for A3 were added 30 min prior to adenosine treatment. Results: Adenosine significantly increased the IL-4-induced production of TIMP-1. CGS-21680 and NECA increased IL-4-evoked TIMP-1 production with 22.09 nM and 36.65 nM EC50 values, respectively, while CCPA and IB-MECA had no significant effect at the studied concentrations (1–100 nM). ZM241385 blocked the stimulatory effect of

adenosine on IL-4-induced TIMP-1 production with 20.75 nM IC50 value, while other antagonists had no significant effect on it. Conclusion: The effect of adenosine on the production of TIMP-1 in IL-4-activated macrophages is mediated via adenosine A2A receptors.

Sulphate-reducing bacteria infection modulates ATP-induced cell death of human intestinal epithelial cells

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Background: Sulphate-reducing bacteria (SRB) are anaerobic organisms found colonizing the human gut. They were associated with the development of ulcerative colitis, a chronic inflammatory bowel disease (IBD). We investigated whether the interaction of SRB with human intestinal epithelial HCT8 cells can interfere on ATP-induced apoptosis.
Methods: HCT8 cells were infected or not with SRB strain Desulfovibrio indonensiensis. After 24 h of infection, cells were treated or not with ATP for 24 to 48 h. Lactate dehydrogenase content was measured by colorimetric assay on cells supernatant. Cells were processed for hypo-diploid DNA content measurements and transmission electron microscopy (TEM) analysis.
Results: ATP induced apoptosis in dose-response manner (EC50=1 mM), generating 7±0.5% of apoptotic cells with 2 mM ATP. SRB infection induced specific apoptosis on 11±1% of cells. SRB infection followed by ATP treatment showed stimulatory effect on apoptosis, achieving 23±1% of cells. Bacterial supernatant alone did not induce apoptosis, but after ATP treatment was capable to increase it, generating 9.5±0.8% of apoptotic cells. While ATP induced only apoptosis, SRB induced both necrosis and apoptosis. TEM analysis showed that both ATP and SRB induced chromatin condensation and changes related to autophagy such as swollen mitochondria and large intracellular vacuoles.
Conclusion: SRB infection can interfere with P2 receptor signaling on HCT8 cells. It is possible that SRB and ATP released from infected cells can together be involved with immune cells recruitment and establishment of IBD.
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The role of CD39 in Treg-mediated immune-suppression

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The ecto-nucleoside triphosphate diphosphohydrolase NTPDase 1 (CD39) is expressed on the surface of regulatory T cells (Treg). It hydrolyzes pro-inflammatory, extracellular ATP to AMP, thereby exhibiting anti-inflammatory activity. An effect further amplified by the conversion of AMP to the immune-suppressive adenosine by CD73, another ectonucleotidase on Treg cells.
In mice, CD39 is expressed constitutively by all Treg cells. Retroviral transfection revealed that it is driven by Foxp3, the key-transcription factor of Treg cells. Notably, the catalytic activity of CD39 was found to depend on the activation state of the Treg cell. “Naïve” Treg cells showed only little ATP hydrolysis whereas Treg cells activated by TCR ligation show high hydrolytic activity. As a consequence only activated Treg cells were able to prevent ATP-induced DC maturation in vitro. The dramatic increase in CD39 activity is not due to the expression level, but rather depends on posttranslational modifications. This is in contrast to CD73, whose activity increased only 4fold and correlated with enhanced surface

expression after activation. Treg activation resulted not only in increased degradation of ATP, but also in an efficient generation of adenosine, a process largely regulated by CD39 activity.
Thus, on Treg cells CD39 activity may represent one important element to mediate immune suppression. In mice the significance of CD39 is supported by the observation that CD39-/- mice are more susceptible to experimental autoimmune encephalomyelitis (EAE). Even more remarkable, in humans reduced numbers of CD39+ Treg cells have been observed in the blood of Multiple Sclerosis.

Activation of the A3 adenosine receptor inhibits fMLP-induced rac activation in mouse bone marrow neutrophils

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We have previously shown that activation of the A3 adenosine receptor (AR) inhibits superoxide production and chemotaxis of mouse bone marrow neutrophils in response to various activating agents. The goal of this study was to determine the signaling mechanisms by which the A3AR inhibits neutrophil functions, focusing on the monomeric GTPase Rac. Mouse bone marrow neutrophils were isolated by Percoll density gradient centrifugation. Isolated cells were pretreated with vehicle, the selective A3AR agonist CP-532,903, or the selective A2AAR agonist CGS 21680 for 30 min at 37 C prior to stimulation with the tripeptide fMLP. Total activated Rac (isoforms 1, 2, & 3) in cell lysates was quantified using a commercially available ELISA kit (G-LISA kit, Cytoskeleton, Inc) that utilizes 96-well plates coated with a Rac-GTP binding domain-containing effector protein and a non-specific anti-Rac antibody. Treatment with fMLP (1 microM) produced a rapid increase in Rac activation that peaked at 5 sec and returned to baseline levels after 5 min. Pretreatment with CP-532,903 (100 nM), but not with CGS 21680 (100 nM) markedly inhibited Rac activation. Subsequent studies showed that treatment with CP-532,903 reduced fMLP-stimulated f-actin formation (assessed by flow cytometry after staining with FITC-conjugated phalloidin), a downstream action of Rac, but did not increase cAMP production or inhibit calcium transients induced by either fMLP or ATP. Our findings support the theory that the A3AR signals to suppress neutrophil functions by inhibiting activation of the monomeric GTPase Rac.

Coupling of adenosine a2b receptors to the transcription factor NFkappaB in human mast cells

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Background: The activation of adenosine A2A-receptors has been shown to promote anti-inflammatory effects in human mast cells, whereas the activation of A2B-receptors mediates pro-inflammatory responses including the production and secretion of cytokines such as IL-4 and IL-8. In the present study, we searched for changes in transcription factors due to activation of A2A- and A2B-receptors in cells of a human mast cell (HMC1) line.
Methods: Receptor-mediated changes in transcription factors were assessed by a reporter gene (luciferase) assay in cultured HMC1 cells transiently transfected with either the pAP1 (activator protein-1)-luc, the pCRE (cAMP response element)-luc, the pNFAT (nuclear factor of activated t cells)-luc or the pNFkappaB (nuclear factor kappa B)-luc vector.
Results: Adenosine (10 to 30 mikroM) and its analogue NECA (N-ethylcarboxamido-adenosine; 0.3 to 10 mikroM) increased the AP1-, CRE-, NFAT and NFkappaB-controlled luciferase expression about 10-fold, 50-fold, 5-fold and 2-fold, respectively. The A2B-receptor antagonist MRS 1754 (1 mikroM) markedly reduced (CRE, NFAT) or almost abolished (AP1, NFkappaB) the responses to NECA. The A2A-receptor agonist CGS21680 (0.3 to 3 mikroM) increased the CRE-directed luciferase expression about 30-fold and the NFAT-directed luciferase expression about 2-fold, but had no effects on the AP1- and NFkappaB-directed luciferase expression.

Conclusions: The results indicate that adenosine mediates stimulatory effects on the transcription factors NFkappaB and AP1 in human mast cells by activation of A2B-receptors, but not by A2A-receptors. The data suggest a role of NFkappaB and AP1 in pro-inflammatory responses to adenosine.

Control of major histocompatibility class II (MHCII) transactivator (CIITA) and MHC II expression by the A2b adenosine receptor

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Some of the hallmarks of inflammation include upregulation of histocompatibility class II (MHC II) through its master regulator Class II Transactivator (CIITA). We have previously reported that IFN-gamma is a major inducer of CIITA and hence, of MHC II in smooth muscle cells (SMCs). Here, we demonstrate that CIITA and MHC II expression in response to IFN-gamma is elevated at gene promoter, mRNA and protein levels in SMCs from A2b adenosine receptor (A2bAR-/-) knockout mice, as compared to cells derived from matched control mice. An A2-type adenosine receptor agonist suppresses CIITA expression and inhibits IFN-gamma-mediated MHC II upregulation in wild type cells, which can be blocked by an A2bAR specific antagonist. We further identify that increased cellular cAMP level is responsible for the this effect as evidenced by the following data: 1) direct activation of adenylyl cyclase activity is both necessary and sufficient to suppress the IFN-gamma response; 2) inhibition of phosphodiesterase activity attenuates the above IFN-gamma-induced transcription events; and 3) direct treatment with cAMP analog abrogates CIITA activation and MHC elevation. Therefore, our data establish a possible crosstalk between the adenosine signaling through cAMP and IFN-gamma during regulation of CIITA expression. To test this phenomenon in vivo, we subjected A2bAR-/- and control mice to femoral artery injury, which is accompanied by elevated IFN-gamma. There was a clear increase in the level of CIITA in the vasculature of the knockout mice at base line, and more so after injury. This is the first study to link A2bAR expression to the control of the regulator of inflammation, CIITA.

Role of purinergic signaling in the regulation of endothelial permeability

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Extracellular nucleotides and adenosine are important signaling molecules controlling immune responses and endothelial permeability under various inflammatory conditions. The magnitude and duration of purinergic signalling in the vasculature is governed by a network of purine-converting ectoenzymes. Endothelial and lymphoid cells are generally characterized by counteracting ATP-inactivating and ATP-regenerating/ adenosine-eliminating phenotypes, respectively. By using cultured human umbilical endothelial cells and normal or leukemic lymphocytes, we have identified a link between the adhesion cascade and extracellular purine turnover. Upon adhesion, the lymphocytes could facilitate their transendothelial migration via (a) inhibition of endothelial ecto-5'-nucleotidase/CD73-mediated AMP hydrolysis, (b) rapid deamination of the remaining adenosine, and (c) maintenance of external levels of proinflammatory ATP at relatively high levels. The important role of ecto-5'-nucleotidase/CD73 and adenosine signaling in the control of endothelial permeability was further confirmed in studies with human pulmonary and umbilical vein endothelial cells. In these cells, induction of CD73 level by immunomodulatory cytokines interferon-beta and interferon-gamma was accompanied by marked decrease in endothelial barrier function, as assessed by flux of FITC-labeled dextran. Together, these studies suggest an important role of ecto-5'-nucleotidase as a "master switch" between two counteracting, nucleotide-inactivating and ATP-regenerating, pathways. In this scenario, selective inhibition or up-regulation of this endothelial ectoenzyme could directionally shift purinergic signaling cascade towards the pro- or anti-inflammatory phenotype respectively, and in this way, regulate endothelial barrier function and leukocyte trafficking between the blood and tissues.

SYMPOSIA 9: CLINICAL USE OF PURINERGIC ANTIPLATELET DRUGS

Clopidogrel resistance, and other potential disadvantages of first-generation thienopyridines

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Ticlopidine and clopidogrel are structurally related compounds, belonging to the thienopyridine family of ADP receptor antagonists; they are pro-drugs that are inactive *in vitro* and need to be metabolized *in vivo* by the hepatic cytochrome P-450 1A enzymatic pathway to active metabolites, which have very short half-lives. They irreversibly and specifically inhibit the function of the platelet $P2Y_{12}$ receptor. The use of ticlopidine and clopidogrel in the clinical setting, despite their proven antithrombotic activity, has some drawbacks. 1) The need for their metabolism to active metabolites accounts for their delayed antiplatelet effects: a maximum plateau of inhibition of ADP-induced platelet aggregation is observed 4–5 days after daily oral administration of 500 mg ticlopidine or 75 mg clopidogrel. It should be noted, however, that the delayed onset of action of clopidogrel can be reduced to about 2 - 5 h by a loading dose of 300 - 600 mg. 2) As a consequence of the irreversible inhibition of $P2Y_{12}$ function, the inhibitory effect of thienopyridines on circulating platelets lasts for approximately 10 days, which corresponds to the lifespan of a circulating platelet. Although the ability of thienopyridines to inhibit irreversibly $P2Y_{12}$ with their short-lived metabolites has theoretical advantages, it may represent a problem for patients who need to undergo coronary bypass surgery, because treatment with clopidogrel within 4 - 5 days of the procedure is associated with increased blood loss, reoperation for bleeding, increased transfusion requirements and prolonged intensive care unit and hospital length of stay. 3) Finally, there is a substantial inter-individual variability in platelet inhibition by ticlopidine and clopidogrel, which is mostly due to inter-individual differences in the extent of metabolism of the pro-drug to the active metabolite. Preliminary, small-sized studies demonstrated an association between insufficient platelet function inhibition by clopidogrel and heightened incidence of vascular events. Increasing the dose of clopidogrel might reduce the number of poor responders. However, safety issues should caution against this policy, as severe toxic effects of the drug such as bone marrow aplasia and microangiopathic thrombocytopenia, albeit less frequent than ticlopidine, have been described, which might be dose-dependent. The above limitations of ticlopidine and clopidogrel have fostered the search for new $P2Y_{12}$ antagonists.

(Invited)

The reversible oral P2Y12 antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature

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Aims The platelet P2Y12 receptor has long been used as a target for antithrombotic drugs of the thienopyridine family such as clopidogrel and ticlopidine. These compounds are prodrugs and their bioactive metabolites bind irreversibly to the P2Y12 receptor on the platelet. The half-lives of these compounds are short, and systemic concentrations are low. AZD6140, the first reversible oral P2Y12 antagonist, is currently in a Phase 3 clinical trial (PLATO) for acute coronary syndromes (ACS). It has been shown that P2Y12 receptors are distributed on vascular smooth muscle cells and mediate a contractile function after stimulation by ADP. This could contribute to local vasospasm seen in patients with ACS. This study investigates whether AZD6140, in contrast to clopidogrel, can inhibit ADP-mediated arterial contractions.
Methods Mice were treated with clopidogrel, 50 mg/kg, the day before and also 2 h before the experiment was started. Thoracic parts of aorta were used from both clopidogrel-treated and untreated mice. Vessels were dissected free from connective tissue and denuded. Ring segments were mounted into temperature-controlled tissue baths. Segments were

precontracted with a submaximal dose of norepinephrine and AZD6140 10 μM was added 20 min before the stable analogue 2-MeSADP. Contractions of human left internal mammary arteries and omental resistance arteries were studied in an identical manner.
Results Clopidogrel treatment per os did not inhibit 2-MeSADP-induced contractions. However, AZD6140 inhibited ADP contraction both in the systemic clopidogrel-treated group and in the untreated group. Contractions in response to 2-MeSADP in both human left internal mammary arteries and omental resistance arteries were inhibited by AZD6140.
Conclusions These data demonstrate in both murine and human tissue that the antiplatelet drug AZD6140 has an effect on large and small arteries, where it blocks the contractile effect of ADP via P2Y12 receptors.

Addressing clopidogrel's variable antiplatelet effect with Prasugrel, a novel P2Y12 antagonist

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Activation of platelet P2Y12 ADP receptors at sites of atherosclerotic plaque rupture promotes the formation of platelet aggregates and occlusive thrombus formation. P2Y12 is the target of the thienopyridine class of oral antiplatelet agents with clopidogrel being the most commonly used. Despite providing important clinical benefit, clopidogrel's platelet inhibitory profile has several limitations; namely its onset of action is relatively slow and the extent and consistency of inhibition are highly variable. Prasugrel is a 3rd generation thienopyridine P2Y12 antagonist which, like clopidogrel, is a prodrug requiring metabolism in vivo to an active metabolite. Prasugrel's distinct chemical structure permits rapid and efficient conversion to this active metabolite which has been shown to inhibit a wide range of ADP-mediated platelet activities. Clinical data indicated greater platelet inhibition and consistency with prasugrel compared with clopidogrel. Pharmacokinetic studies have shown that the amount of each active metabolite generated in vivo is quite different, with prasugrel generating substantially more of its active metabolite compared with clopidogrel despite being used at a lower dose. These data provide a mechanistic basis for the distinct profile of more consistent platelet inhibition observed with prasugrel. The recently completed phase 3 trial (TRITON TIMI-38) demonstrated that compared to standard clopidogrel dosing prasugrel resulted in a 19% relative reduction in the risk of ischemic events in patients with acute coronary syndrome undergoing percutaneous coronary intervention. Notably there was a 52% relative reduction in the risk of stent thrombosis in the prasugrel treated patients compared to those on clopidogrel. In the overall TRITON population, prasugrel was associated with 32% increase in the relative risk of major bleeding compared to patients treated with clopidogrel.

(Invited)

AZD6140, a reversible oral P2Y12 antagonist: possibilities for clinical benefit beyond platelet inhibition?

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AZD6140 is the first reversible oral P2Y12 antagonist in development for prevention of arterial thrombotic events in patients with acute coronary syndromes (ACS). Chemically distinct from thienopyridines, AZD6140 is a cyclopentyl-triazolo-pyrimidine (CPTP) that binds directly to the P2Y12 receptor. Preclinical studies indicate that AZD6140 potently and selectively inhibits ADP-induced platelet aggregation. Animal studies have shown that AZD6140 effectively prevents arterial thrombosis but demonstrates shorter bleeding time increases than the thienopyridine dose required to achieve similar antithrombotic effect.
Data from the phase I program and phase IIa DISPERSE study have shown that AZD6140 produced greater and more consistent levels of ex vivo inhibition of platelet aggregation (IPA) vs clopidogrel with a more rapid onset of antiplatelet activity. The phase IIb DISPERSE2 study found that higher platelet inhibition with AZD6140 vs clopidogrel did not lead to significant differences in major/minor bleeding rates, confirming earlier preclinical observations.

AZD6140 may also have beneficial effects beyond IPA, including effects on vascular smooth muscle cells. In an ex vivo mouse study, AZD6140 was found to block ADP-induced vasoconstriction in denuded aortic rings, regardless of whether mice received in vivo pretreatment with clopidogrel. Dog studies have also shown that AZD6140 can augment local adenosine-mediated perfusion increases. These data suggest that AZD6140 may modulate vasoreactivity by locally elevating concentrations of ADP or adenosine in vivo, potentially enhancing coronary blood flow.
AZD6140 is currently being investigated in PLATO - a large head-to-head outcomes study vs clopidogrel in ACS patients.

(Invited)

SYMPOSIA 10: NUCLEOTIDE RECEPTOR MEDIATED INTRACELLULAR SIGNALING

P2X7R shields cells from death trough ER Ca2+ uptake and NFATc1 activation

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P2X7 receptor is involved apoptosis, necrosis, cell-cell fusion, cytokine secretion but also cell proliferation and survival. Most of these phenotypes are linked to P2X7 potential of regulating intracellular calcium content.
Here we show how the endoplasmic reticulum (ER) calcium content can influence both the proliferative and pro-apoptotic effects of P2X7 expression.
Intracellular, mitochondrial and reticular calcium were all increased in hP2X7 HEK transfectants (HEKhP2X7) versus mock cells and the rate of calcium uptake in the ER resulted faster. Treatment with the P2X7 inhibitor oATP decreased the reticular calcium content. The same compound had also a negative effect on P2X7 induced cell survival. Moreover, we observed a decreased toxicity on HEKhP2X7 of different apoptotic stimuli such as C2-ceramide, TPEN and staurosporin trough mechanisms as different as: cell blebbing, cytosolic histone-DNA association and trypan blue permeability. The link among the increased survival of HEKhP2X7 and the augmented levels of calcium resulted to be NFATc1. This transcription factor is activated by the calcium/calcineurin pathway and is nuclear-traslocated functioning moiety is doubled in P2X7 transfectants. Treatment with VIVIT, that specifically blocks the interaction among calcineurin and NFAT, is able to obliterate P2X7 dependent survival.
The activation of P2X7R by its agonist BzATP causes apoptosis accompanied by ER structure fragmentation. This phenotype is dependent on calcium entry from the extracellular milieu as is obliterated by EGTA treatment. In summary here we describe a mechanism by which P2X7R mediates resistance to apoptosis via increased store Ca2+ and, when maximally activated, apoptosis via massive extracellular calcium entry.

The contribution of lipid rafts to the regulation of P2X and P2Y receptors

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An increase in intracellular Ca^{2+} represents a key signalling event coupled to both P2X and P2Y receptors. Previous reports have shown that disruption of cholesterol-rich membrane lipid rafts reduced $P2X_1$ receptor-mediated Ca^{2+} increases while the $P2Y_1$ receptor-dependent Ca^{2+} response was unaffected. We have extended this work to look at the cholesterol sensitivity of $P2X_{2,3,\&4}$ receptors expressed in HEK293 cells and $P2Y_{1,\ 2,\ 4\ \&\ 11}$ receptors expressed in 1321N1 cells. $P2X_1$ receptor currents were inhibited by 90%±3% or 93%±2% following cholesterol depletion with methyl beta cyclodextrin (MβCD) or filipin treatment respectively. In contrast ATP evoked $P2X_{2,3\ \&4}$ currents were unaffected. $P2Y_{2,\ 4\ \&\ 11}$ receptor mediated Ca^{2+} rises were reduced by MβCD treatment to 74%±14%, 75%±6% and 58%±10% of control respectively. However, $P2Y_1$ responses were unchanged. To determine whether the sensitivity of P2X receptors results from differential raft localisation we used sucrose density centrifugation following low pH solubilisation to isolate lipid rafts. $P2X_{1\text{-}4}$ receptors were detected in flotillin rich lipid raft fractions. Furthermore $P2X_4$ receptors showed predominant expression within lysosomal compartments as indicated by LAMP-1. The use of high concentration triton X-100 (1%) resulted in detection of $P2X_{2\text{-}4}$ receptors outside of the lipid raft microdomains, indicating a subtype of raft sensitive to high detergent concentration. In conclusion subtype P2X and P2Y receptors are differentially regulated by cholesterol depletion. In the case of P2X receptors this does not correlate to their presence within rafts

Role of Purinergics in Cyclooxygenase-2 (COX-2) and Prostaglandin E2 (PGE2) production in activated rat microglia

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Purinergic receptors are expressed in both the central and peripheral nervous system, where they mediate various forms of intercellular communication and modulate several biological functions. Large amounts of ATP can be rapidly released from different cell types following hypoxia, stress, and tissue damage. In this study, we investigated the effects of stable ATP analogue 3′-O-(4-benzoylbenzoyl)-ATP (BzATP) on Cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production in primary microglial cells prepared from rat pups. We characterized the expression of different P2 receptors on rat microglia. In rat microglia, exogenously added BzATP dose dependently induced COX-2 Protein and PGE2 release after 24 hours. To know responsible receptor/s for this effect some specific and non-specific agonist and antagonist were used. Suramin which is non-specific P2 receptor antagonist was able to block this effect both at COX-2 and PGE2 level. We also used specific antagonists of P2X7 and P2X1 but there was no significant inhibition was found. To know if this effect is through metabotropic receptor/s, P2Y agonist was also used. In addition, we found mitogen-activated protein kinases (MAPK) and protein kinase C (PKC*f*w) to be involved in bzATP-induced PGE2 synthesis. bzATP mediated Prostaglandins were blocked with MAPK and in parts with PKC inhibitors. At this stage, the exact receptor/s explaining these findings are unknown, a different P2 receptors or subtypes might be involved. These results could have important implications for future studies investigating the effects of purinergics on activated microglia, which could be relevant to neuroinflammatory processes occurring in neurodegenerative disorders.

Functional characterisation of A1:P2Y1 heterodimeric receptors

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Background. We have investigated the functional properties of heterodimeric A1: P2Y1 receptors expressed at a density corresponding to physiological levels in the CHO.K1 cell line.
Methods.
Clonal cell lines expressing wild-type or mutant A1 adenosine receptors in CHO.K1 cells, which express endogenous P2Y1 and P2Y2 receptors, were studied. Receptor activation of the Gi- and Gq/11-coupled signal transduction pathways was assayed by inhibition of forskolin (FSK)-stimulated de novo cAMP production, and by quantification of intracellular calcium. Results are expressed as mean+SEM (n=3).
Results. 10microM FSK increased cAMP to 5911+190 dpm from control levels (141+5 dpm). At 100 microM, the P2Y1 receptor ligand ADPbetaS inhibited FSK-stimulated cAMP by 36.2% (3770+283 dpm) in the mutant CHO.G14T cell line, but had no effect on CHO.K1, wild-type CHO.A1 or mutant CHO.T277A cells. The effect of ADPbetaS was blocked by 100microM MRS2179 (5914+87 dpm) and 10microM DPCPX (6547+518 dpm). The EC50 values of ADPbetaS in the calcium mobilisation assay were: CHO.K1, 5.45+0.10microM; CHO.A1, 6.05+2.42microM; CHO.T277A, 9.45+0.84microM. The CHO.A1 data were best fit to a sigmoidal concentration-response curve with variable slope (nH=0.73+0.02). The EC50 for CHO.G14T, 46.57+8.68microM, was significantly different from CHO.K1 ($P<0.001$), CHO.A1 ($P<0.01$) or CHO.T277A ($P<0.01$) (ANOVA, Tukey's posthoc test).
Discussion. Detection of cAMP-dependent ADPbetaS responses required co-activation of the A1 receptor achieved, in these experiments, through use of the constitutively-active G14T mutant. The reduced ADPbetaS potency in the CHO.G14T

calcium assay may be due to decreased Gq-coupled P2Y1 receptor density as a result of increased A1:P2Y1 receptor formation.

Inflammatory mediator inhibition and modulation of early growth response transcription factors in microglia by Purinergic P2X7 receptors

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Whereas the beneficial versus neurotoxic roles of microglia in CNS injury and disease remain controversial, we hypothesize that P2 nucleotide receptors have the ability to shift this balance to constrain microglial activities. We find that activation of microglial P2X7 receptors by BzATP reduces their expression/release of tumor necrosis factor-alpha (TNF) and inducible nitric oxide synthase (iNOS)/ nitric oxide following stimulation with lipopolysaccharide. Microarray analyses of BzATP-treated microglia revealed up-regulation of several target genes of the early growth response (Egr) transcription factor family, effects consistent with our observations of Egr gene induction within as little as one hour of treatment. Egr factors regulate the expression of many inflammatory genes in immune cells and we have identified putative Egr binding sites in both the TNF and iNOS gene promoters, supporting the idea that BzATP-mediated repression of pro-inflammatory gene expression may involve regulation by Egr factors in microglia. Egr gene induction by BzATP is prevented by inhibitors of the MEK-ERK and p38 MAP kinase pathways, but not by inhibitors of calmodulin kinase II. Additionally, the P2X7 receptor antagonists Brilliant Blue G, KN-62 and oxidized ATP block BzATP-induced Egr factor induction, implying that P2X7 receptor-dependent MAP kinase pathway activation is necessary for these Egr effects. Because RNAi to microglial P2X7 receptors prevents the BzATP-mediated decreases in both TNF and iNOS, a role for P2X7 receptors in these responses is suggested. Studies are currently underway to assess the role of Egr factors in the regulation of TNF and iNOS inflammatory gene expression in BzATP-treated microglia.

Translocation of arrestin induced by human A3 adenosine receptor ligands in an engineered cell line: comparison with G Protein-dependent pathways

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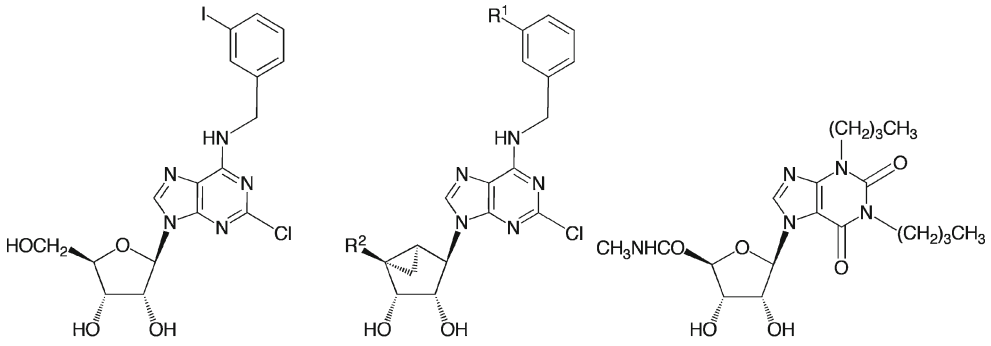
Structurally diverse ligands were studied in an A3 adenosine receptor (AR)-mediated beta-arrestin translocation assay using a PathHunterTM engineered CHO cell line. The agonist potency and efficacy were similar, although not identical, to their G protein signaling. However, differences have also been found. MRS542, MRS1760, and other adenosine derivatives, A3AR antagonists in cyclic AMP assays, were partial agonists in beta-arrestin translocation, indicating possible biased agonism. The xanthine 7-riboside DBXRM, a full agonist, was only partially efficacious in beta-arrestin translocation. DBXRM was shown to induce a lesser extent of desensitization compared with IB-MECA. In kinetic studies, MRS3558, a potent and selective A3AR agonist, induced ≤-arrestin translocation significantly faster than IB-MECA and Cl-IB-MECA. Non-nucleoside antagonists showed similar inhibitory potencies as previously reported. PTX pretreatment completely abolished ERK1/2 activation, but not arrestin translocation. Thus, lead candidates for biased agonists at the A3AR have been identified with this arrestin-translocation assay, which promises to be an effective tool for ligand screening.



Regulation of $P2Y_1$ receptors

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We have investigated the molecular mechanisms underlying agonist-induced internalization of the $P2Y_1$ receptor in MDCK epithelial cells. Receptor internalization was quantified by either intact cell radioligand binding assays with [^{32}P]MRS2500 or by intact cell radioimmunoassays. The rate and extent of agonist-promoted internalization of exogenously expressed $P2Y_1$ receptors were identical to those of endogenously expressed canine $P2Y_1$ receptors, indicating that the level of expression afforded by retroviral expression of the receptors does not influence internalization kinetics. Deletion of residues 340-374 in the C-terminal tail abolished internalization, and within this region we identified three residues (Ser-352, Ser-354, and Thr-358) that play a major role in agonist-promoted internalization, presumably by serving as sites of phosphorylation. Surprisingly, $P2Y_1$ receptors in which all Ser and Thr residues in the C terminus were mutated to Ala ($P2Y_1$-0P) internalized in response to agonist with the same kinetics as wild type receptors, and mutational analysis revealed that internalization of these receptors was due to mutation of Ser-336. $P2Y_1$ receptors in which Ser-336 was mutated to Ala markedly increased both the rate and extent of agonist-promoted internalization. Moreover, $P2Y_1$ receptors in which Ser-336 was mutated to Asp to mimic phosphorylation did not internalize in response to agonist, whereas receptors with a Ser-336 to Asn mutation internalized with kinetics similar to those with a Ser-336 to Ala mutation. These data suggest that Ser-336 is a key residue in agonist-promoted internalization of $P2Y_1$ receptors.

Purinergic signaling in adult neural stem cells

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The subventricular zone of the lateral ventricles is one of the major neurogenic zones of the adult mammalian brain. The molecular cues controlling neural precursor proliferation, migration, differentiation and survival are poorly understood. We have previously shown that cultured neural precursors express functional P2Y1 and P2Y1 receptors. Here we analyzed intracellular signaling pathways initiated by agonists (ADPbetaS, UTP) of these two receptors and compared it to that of signaling via EGF. According to RT-PCR, neural precursors express a variety of P2X and P2Y receptors. Immunocytochemistry demonstrates that ADPbetaS, UTP and EGF induce ERK1/2 and CREB phosphorylation in undifferentiated nestin-positive precursors. Following induced differentiation of precursors into astrocytes, neurons and oligodendrocytes, only astrocytes retained ERK1/2 and CREB phosphorylation, suggesting that neurons and oligodendrocytes have down regulated P2Y1 and P2Y1 receptors.

As revealed by quantitative Western blotting, ADPbetaS, UTP and EGF but not UDP induced time and concentration-dependent ERK1/2 and CREB phosphorylation. ADPbetaS and UTP had little effect on Akt and Src phosphorylation. Further experiments using a variety of inhibitors of intracellular signaling pathways suggest that ADPbetaS and UTP pathways for ERK1/2 phosphorylation converge on MEK but differ in the upstream signaling pathways. In particular, transactivation of the EGF receptor does not contribute to UTP-induced ERK1/2 or CREB phosphorylation. The signaling pathways leading to CREB phosphorylation are similar for ADPbetaS- and UTP-induced P2 receptor activation. Our data suggest that nucleotides can contribute to the regulation of neurogenesis, synergistically interacting with growth factors.

Adenosine receptors modulate the activity of Nox 2 hepatic-cell plasma membrane

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NADPH oxidase (Nox) in non-phagocytes cells is probably the main cellular system responsible for H2O2 controlled synthesis devoted to signalization. In previous studies it has been shown that adrenaline promotes, in a concentration-dependent manner via the activation of alpha 1B receptor. Activation of this adrenoceptors, increase Nox activity (identified as Nox 2) present in plasma membranes of highly enriched parenchyma cells isolated from rat liver (Diaz-Cruz et al, 2007 FRR 41,663)
The aim of this work was to investigate a potential role of activated adenosine receptors in liver to modulate Nox activity and thus participate in ROS generation. Plasma membranes of hepatic parenchyma cells were prepared for this purpose. Nox activity was measured in the presence of NADPH after activation with specific adenosine receptor agonists. Nox modulation by adenosine receptor agonist is dependent of GTP and Ca2+. Addition of specific A2A and A2B receptor agonist enhance Nox activity, whereas addition of specific A3 receptor agonist resulted in an inhibition of Nox activity. Specific antibodies again Nox 2 produced a complete inhibition of specific adenosine receptor agonists tested; whereas antibodies against Nox 1 and Nox 4 have no effect. Grants CONACYT 45003-M and DGAPA IN201208, UNAM.

Autocrine-paracrine signaling by ATP and P2 receptors

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Although investigators who study extracellular nucleotides and nucleotide receptors recognize that cellular release of ATP and other nucleotides can have autocrine and paracrine functions, this concept is not fully appreciated in the broader scientific community. This is particularly surprising since one could argue that release of nucleotides and the subsequent activation of P2, and at least in some cases adenosine, receptors accompanies virtually every experiment conducted in cell culture or with perfused tissues. A number of mechanisms have been proposed (and in some cases documented) by which cells release and respond to nucleotides. Work by my colleagues and me has involved studies of nucleotide release and response of cells associated with inflammation (including leukocytes and fibroblasts) and in the kidney, in particular in renal epithelial cells. In this talk I will review some of these findings and will identify some key, unanswered questions regarding whether nature has “chosen” nucleotides as a general means to detect and respond to altered homeostasis through release, metabolism and receptor activation.

(Invited)

Characterisation of the effects of the phopholipase C inhibitor U73122 on P2Y receptor-mediated contractions of the rat isolated pulmonary artery

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P2Y receptor agonists induce vasodilation via endothelial P2Y receptors and vasoconstriction via smooth muscle P2Y receptors (Chootip et al., 2002). However, the intracellular signalling pathways by which vasoconstriction is induced are not well characterised. In this study we determined the role of phospholipase C (PLC) in nucleotide-evoked vasoconstriction of rat small intrapulmonary artery (SPA) using the PI-PLC inhibitor U73122.
5mm rings of rat SPA were mounted under isometric conditions at 37°C and 0.5g resting tension. Contractions were elicited by addition of the P2Y receptor agonists UTP and UDP (300 microM), the FP receptor agonist PGF_{2alpha} (10 microM) or KCl (40 milliM) to the bath.
UTP, UDP and PGF_{2alpha} each evoked slowly developing contractions, which reached a peak within 5min. Pre-incubation with U73122 (1-3 microM) for 15min had no effect on the resting tone of the arteries nor the amplitude of contractions evoked by UTP, UDP, PGF_{2alpha} or KCl (n=5 each). Increasing the concentration of U73122 to 10 microM and the preincubation period to 30min, also left the responses to UTP, UDP and KCl unchanged (n=6). In contrast, the contractions to PGF_{2alpha} were inhibited significantly under these conditions ($77 \pm 6\%$ of control, n=6, $P < 0.05$).
These results show that the PI-PLC inhibitor U73122 had no effect on P2Y receptor-mediated contractions of rat pulmonary artery under conditions that inhibited the responses induced by PGF_{2alpha}. This calls into question the involvement of PLC in the P2Y receptor-mediated contraction of rat pulmonary artery and further studies are ongoing to determine the involvement of other intracellular signalling components.

Chootip, K, Ness, K.F., Wang, Y., Gurney, A.M. & Kennedy, C. (2002). Br. J. Pharmacol., 137, 637-646

Interaction partners of the A2a-adenosine receptor binding to its carboxy terminus alter the receptors properties

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Regulation of the A2A-adenosine receptor is mediated by accessory proteins that bind to the extended receptor carboxyl terminus (c-tail). We have identified three putative interaction partners from a human brain cDNA library. ARNO, the nucleotide exchange factor for the small GTPase ARF6, is required for signalling to the mitogen-activated protein kinase (MAPK) and for shutting off Gs-mediated cAMP formation when the agonist has dissociated from the receptor. Agonist occupancy recruits ARNO to the receptor where it binds to the proximal part of the c-tail. By contrast, SAP102 (synapse associated protein) binds to a five amino acid motif (DVELL) in a more distal segment. In primary neurons over-expression of SAP102 attenuates the lateral mobility of the receptor. The ubiquitin splitting protease USP4 controls receptor export from the endoplasmic reticulum by removing ubiquitin and thus rescuing the receptor from ER-associated degradation. The DUSP (present in ubiquitin specific proteases) domain of USP4 binds to a 15 amino acid segment of the peripheral c-tail. The interaction of the receptor with USP4 seems to be highly specific as it cannot be substituted for by its closest homologue USP15. Further analysis of these interacting domains and their influence on the receptors properties in heterologous expression systems will allow the generation of transgenic animals to demonstrate the importance of the c-tails interaction partners in the in vivo background.

Membrane rafts and P2Y1 receptor signaling

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The micro-regionalization of the nucleotide $P2Y_1$ receptor ($P2Y_1R$) in the cell plasma membrane has not been systematically investigated notwithstanding its wide expression in both the endothelial and smooth muscle cells of the blood vessel wall. We used sodium carbonate and OptiPrep sucrose density gradients, western blot analysis, reduction of tissue cholesterol content and vasomotor assays to demonstrate that in the smooth muscle cells of human placental superficial blood vessels, the $P2Y_1R$ is associated to membrane rafts as evidenced by its co-localization with flotillin-1 and caveolin-3. Cell extracts with Triton X-100 indicated the non-caveolae nature of the raft. Methyl-beta-cyclodextrin reduced tissue cholesterol and shifted the receptor from raft domains, obliterating the $P2Y_1R$-mediated contractions but not the vasomotor response elicited with serotonin or KCl. Perfusion of chorionic artery segments with 100 nM. 2-MeSADP, or 10 nM. MRS 2365, displaced within 4-min the $P2Y_1R$ localization out of membrane rafts. MRS 2179, did not cause a similar displacement, but blocked the agonist-induced exit from rafts. Structural analogs such as adenosine, uridine triphosphate, inactive $P2Y_1R$ agonists, did not displace the $P2Y_1R$ from the membrane raft, further evidencing the pharmacodynamics of the ligand-receptor interaction. Vascular reactivity assays showed desensitization of the vasomotor response, a finding that correlated with the $P2Y_1R$ exit from raft domains. These results demonstrate for the first time the functional dependence of the $P2Y_1R$ to non-caveolae membrane rafts highlighting the role of raft domains for $P2Y_1R$ agonist/antagonist interactions (Funded by FONDAP 13980001 and MIFAB P04–071-F grants).

CF102 induces growth suppression and apoptosis in an orthotopic model of hepatocellular carcinoma

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Background: The A3 adenosine receptor (A3AR), a Gi protein associated cell surface receptor was suggested as a target for cancer treatment. In this study the expression and functionality of the A3AR in Hepatocellular carcinoma (HCC) was examined.
Methods: RT-PCR, western blot analysis and Immuno-histochemistry were used to evaluate the level of A3AR and cell growth regulatory proteins in tissues and peripheral blood mononuclear cells (PBMCs). A rat orthotopic model of N1S1 HCC tumor cells was used.
Results: A3AR was found to be highly expressed in tumor tissues derived from patients and rats with HCC. A3AR over-expression was also found in the PBMCs, reflecting receptor status in the remote organ. The high expression level of the receptor was directly correlated to over expression of NF-kappaB, known as a transcription factor of A3AR. CF102, a synthetic highly selective agonist at the A3AR, induced growth suppression of rat HCC in an orthotropic model when given orally. Analysis of tumor lesions excised from CF102-treated animals demonstrated: a) de-regulation of the NF-kappaB and the Wnt signal transduction pathways; b) up-regulation of BAD, BAX and Caspase-3 resulting in apoptosis.
Conclusion: A3AR is highly expressed in tumors and PBMCs of HCC patients and tumor bearing rats. Oral administration of CF102 induced tumor growth suppression and apoptosis of HCC. These data suggest A3AR as a novel targeted therapy to treat HCC.

Adenosine and its receptors play a central role in ethanol-induced fatty liver

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Background: Metabolism of ethanol requires ATP breakdown with extracellular purine release. We have recently demonstrated that high levels of adenosine are released by livers from mice fed ethanol, and CD73 mediated-adenosine

production and adenosine A2A receptors play a critical role in hepatic fibrosis. Alcoholic fatty liver is not well understood. Because ethanol causes adenosine release we determined whether adenosine and its receptors play a role in fatty liver.
Results: Mice lacking adenosine A1 and A2B receptors, but not those lacking A2A receptors were significantly protected against development of fatty liver, as reflected by hepatic triglyceride levels and steatosis scores. Similarly, both DCPX and the enprofylline provided similar protection against development of fatty liver. mRNA expression of ACL, FAS, SREBP1 and PPARã was significantly decreased in A1KO mice and DCPX treated-mice compared with WT mice after ethanol treatment, whereas ACC, CPT and PPARá mRNA was greatly decreased in WT mice compared with A2BKO or enprofylline treated-mice after ethanol treatment. The triglyceride levels, ACL and FAS protein levels and nuclear SREBP1 and PPARã protein levels in AML-12 hepatocytes were significantly increased after A1 agonist treatment and DCPX almost completely blocked the increase. The A2B agonist also increased triglyceride levels of hepatocytes and decreased nuclear PPARá protein expression, whereas it had no significant effects on ACL and FAS protein levels or nuclear SREBP1 and PPARã protein in hepatocytes, and the selective A2B antagonist MRS1706 blocked NECA increased-triglyceride levels and decreased nuclear PPARá levels in AML-12 cells.
Conclusion: These results indicate that adenosine, acting at adenosine A1 and A2B receptors plays a central role in alcoholic fatty liver by different and complementary mechanisms: A1 receptors increase lipid synthesis whereas A2B receptors diminish hepatocyte lipid oxidation and utilization.

Evaluation of nucleotides, nucleotide-sugars, and lipid mediators as agonists of GPR17 and GPR87

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A recent study (Ciana et al. 2006 EMBO J 25:4615–4627) reported that UDP, UDP-sugars, and cysteinyl-leukotrienes are agonists at the orphan receptor GPR17, while a different study identified lysophosphatic acid (LPA) as an agonist of the presumptive P2Y receptor, GPR87 (Tabata et al. 2007 BBRC 363, 861–866). To further investigate the ability of these compounds to activate these receptors, HA-GPR17 and HA-GPR87 were transiently expressed in HEK293 and COS-7 cells and stably expressed in 1321N1 and CHO cells. A cell surface radioimmunoassay confirmed expression of the receptors in all four cell types. In 1321N1 or CHO cells stably expressing GPR17, UDP, UDP-sugars, and LTC4 all failed to promote accumulation of inositol phosphates, mobilization of intracellular calcium, or inhibition of cAMP accumulation. Likewise, none of these compounds promoted accumulation of inositol phosphates in COS-7 or HEK293 cells transiently transfected with GPR17 alone or co-transfected with GPR17 and Gqi5, which links Gi-coupled receptors to the Gq-regulated phospholipase C signaling pathway. In contrast, each of these ligands promoted robust accumulation of inositol phosphates in cells expressing their cognate receptors. Moreover, none of these compounds promoted internalization of GPR17 in 1321N1-GPR17 cells. These data argue against the inclusion of GPR17 as a member of the P2Y receptor family. Similarly, adenine and uracil nucleotides, nucleotide-sugars, and LPA were inactive in cells expressing GPR87 (with or without Gqi5), and none of these compounds induced internalization of the receptor. Although sequence identity (~54%) with other Gi-coupled P2Y receptors suggests that GPR87 is likely to be a member of the P2Y receptor family, its endogenous agonist remains unknown.

(Invited)

Influence of microglia on nucleotide-induced astroglia proliferation

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In injured brain, ATP released by damaged cells can trigger reactive astrogliosis upon P2-receptor-activation (Franke et al, 2006, Eur. J. Physiol. 452). Microglia can sense astrocyte activity and modulate astrogliosis (Rohl et al, 2007, Brain Res. 1129, 43).
2. The present study aimed to investigate the influence of microglia on the astrocyte proliferative response to nucleotides. Primary cultures of astrocytes, co-cultures of astrocytes and microglia were prepared from brain cortex of newborn rats (P0-P2) and incubated with nucleotides for 48 h. The effect of nucleotides on cell proliferation was measured by methyl-[3H]-thymidine incorporation. Metabolites formed during incubation with nucleotides were quantified by HPLC-UV. In astrocyte cultures, ATP, ATPgamaS and ADPbetaS (0.01–1 mM), increased cell proliferation up to 382±33% (n=5, P<0.05). UTP (up to 1 mM) had no effect and 2-MeSADP (0.1–1 mM) inhibited cell proliferation up to 40±5% (n=4, P<0.05).
In co-cultures, the proliferative effect of ATP, ATPgamaS and ADPbetaS was attenuated to 180±7% (n=5, P<0.05) whereas UTP and 2-MeSADP caused an inhibition of cell proliferation of the same magnitude (26±3%; n=4, P<0.05).
There were no measurable differences in metabolism of nucleotides between cultures. Nucleotides mediate opposite effects on astroglial proliferation. Attenuation of the proliferative effects observed in the presence of microglia cannot be explained by differences in the metabolism of nucleotides.
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P2 receptor-subtypes in the modulation of reactive gliosis

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P2-receptors induce glial cell proliferation and the earliest response of microglia after injury (Bianco et al., 2005, Brain Res Rev 48, 144) but the subtypes involved have not been identified. Using a pharmacological approach we investigated the subtypes involved in these responses.
Primary cultures of astrocytes and co-cultures of astrocytes and microglia where prepared from brain cortex of newborn rats and incubated with P2-receptor agonists for 48 h. Antagonists were added to the medium 1 h before agonists. The effect of drugs on cell proliferation was measured by methyl-[3H]-thymidine incorporation.
In astrocyte cultures, ADPâS (100 microM) increased cell proliferation up to 219±12% (n=23, P<0.05), an effect attenuated by MRS 2179 (30 microM; 140±10%; n=4, P<0.05), by MRS 2395 (10 microM; 129±10%; n=4, P<0.05), by inhibition of PLC with U-73122 (1 microM; 150±8%; n=5, P<0.05), PKC with Ro 32–0432 (1 microM; 142±9%; n=5, P<0.05) and Mek1/2 with U0126 (10 microM; 156±12%; n=4, P<0.05). 2-MeSADP (100 microM) inhibited cell proliferation by 42±3% (n=22, P<0.05). The metabolic derivatives of 2-MeSADP; 2-MeSAMP (100 microM) and 2-MeSADO (30 microM) caused an inhibition of same magnitude.
In co-cultures, UTP (100 microM) inhibited cell proliferation (44±3%; n=16, P<0.05), an effect antagonised by MRS 2578 (1 microM; 10±3%; n=6, P<0.05) and reversed by ARL 67156 (100 microM; 149±10%; n=5, P<0.05). The proliferative effect of UTP observed in the presence of ARL 67156 was abolished by suramin (100 microM).
P2Y1 and P2Y12 receptors induce proliferation of astroglial cells by activation of PLC-PKC pathway and ERK1/2. In co-cultures P2Y2 receptors also stimulate whereas the P2Y6 subtype inhibits glial proliferation.
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P2Y receptor-subtypes in the modulation of noradrenaline release in rat brain cortex

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In the rat brain, neuron-glia signalling can be mediated by ATP released from astrocytes in response to noradrenaline (Gordon et al, 2005, Nat. Neurosci. 8, 1078), which by activation of P2 receptors modulates neuronal noradrenaline release

(von Kügelgen et al,1994, Br. J. Pharmacol. 113, 815). Our aim was to identify the P2 receptor-subtypes involved in this signalling.
Cortical occipito-parietal slices from male Wistar rats were incubated with 0.1 microM of [3H]-noradrenaline, superfused with a physiological solution and stimulated with trains of 100 pulses at 5 Hz. The effects of P2 agonists and antagonists on evoked noradrenaline released were estimated as [3H]-overflow.
P2-receptor agonists inhibited tritium overflow up to 57±7% (n=5; P<0.05) with the following potency order: ADP>ATP>ADP≈ÂS>UDP=UTP. The inhibitory effect of ADP (300 microM; 44±3%, n=7; P<0.05) was attenuated by adenosine deaminase (ADA; 2U/mL; to 29±6%; n=5; P<0.05), by DPCPX (0.1 microM; to 17±3%; n=10; P<0.05) and by RB2 (30 microM; to 28±1%; n=5; P<0.05). ADPbetaS (300 microM) caused an inhibition of 33±4% (n=6; P<0.05) that was not changed by ADA (2U /mL; 42±6%; n=4) but was antagonised by DPCPX (0.1 microM; 12±4%; n=6; P< 0.05) and MRS 2179 (100 microM; 12±2%; n=4; P<0.05) whereas the inhibitory effect of UDP (100 microM; 19±3%, n= 5; P<0.05) was abolished by MRS 2578 (1 microM). DPCPX and MRS 2179 did not change noradrenaline release but RB2 increased tritium overflow by 34±2% (n=6; P<0.05). P2-agonits inhibited noradrenaline release by activation of: i) P2Y1 receptors; ii) P2Y6 receptors and iii) receptors with pharmacology similar to that of A1/P2Y1 heterodimers (Nakata et al, 2005, J. Mol. Neurosci. 26, 233).
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Desensitization of the dilatation response mediated by endothelial P2Y receptors in pressurized rat small mesenteric artery

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Background: Endothelial P2Y1 and P2Y2 receptors are activated by extracellular nucleotides, leading to EDHF-type vasodilatation in resistance arteries. The signalling initiated by these G-protein coupled receptors desensitizes upon prolonged stimulation with the agonists. Our aim was to evaluate the mechanisms underlying P2Y1 and P2Y2 receptor desensitization in pressurized rat small mesenteric arteries.
Methods and Results: Luminal perfusion with the non-hydrolyzable selective P2Y1 agonist, ADP-beta-S (3μM) evoked vasodilatation that desensitized rapidly over time, and at this time, co-perfusion with either ADP (1μM, P2Y1 receptor agonist) or UTP (3μM, P2Y2 receptor agonist) did not evoke further dilatation. Evaluation of the homologous desensitization of P2Y1 receptors showed that luminal incubation with the PKC inhibitor BIS-I (1μM) tended to augment dose-response dilatation to ADP (10nM-1μM) and ADP-beta-S (0.1-3μM). Pretreatment with Go6976 (1μM), a calcium-dependent PKC isoform inhibitor only caused partial attenuation of P2Y1 receptor desensitization to various dose of ADP or ADP-beta-S. In separate experiments, P2Y1 and P2Y2 receptor activation by luminal infusion of the agonists increased endothelial cell [Ca2+]i in pressurized arteries, which also desensitized. This desensitization was also significantly reduced by PKC inhibition.
Conclusion: These data demonstrate that desensitization of P2Y1 receptors in pressurized rat mesenteric arteries heterologously regulates P2Y2 receptors and this event is mediated by conventional PKC isoforms. A role for other PKC isozymes or other protein kinases cannot be discarded. These findings reinforce the importance of purine nucleotides as vasoactive agonists by acting on endothelial P2Y receptors.

Different transducing systems operated by adenosine A2A receptors in HEK-293 cells

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Adenosine A2A receptors (A2ARs) are classically classified as coupling to the cAMP/ PKA pathway. Recruitment of this pathway is known to afford neuroprotection. However, it is A2AR blockade (rather than activation) that affords

neuroprotection in adults, implying the involvement of alternative transducing systems. We now transfected HEK293 cells with human A2ARs to explore their efficiency in recruiting cAMP and MAP kinases (MAPK) that are known effectors of neurodegeneration. CGS21680 (A2AR agonist) caused a larger cAMP-induced levels ($P<0.05$, $n=3$ at 90 min) with 300nM (460±47%) than with 10nM (283±44%), and was prevented by SCH58261 (50nM, A2AR antagonist). CGS21680 also triggered ERK1/2 activation maximally at 5 min with equi-effective effects ($P>0.05$, $n=5–6$) at 10nM (181±28%) and 300nM (194±26%) in a SCH58261-sensitive manner. The same was observed for p38 and for JNK phosphorylation (both increased similarly up to 90 min with 10nM and 300nM in a SCH58261-sensitive manner, $n=6$) and these effects were unaffected by the PKA inhibitor H89 (1ìM). CGS26180 also decreased Akt activation, with an effect larger ($P<0.05$, $n=5$, after 15 min) for 300 nM (40±7%) than 10 nM (18±3%) that was sensitive to SCH58261 (50nM) and reverted by H89 (1ìM). These results show that A2ARs are more efficacious to activate MAPK pathways than the cAMP/PKA pathway. Furthermore, the ability of A2ARs to activate JNK and p38 and decrease Akt activation, which are hallmarks of greater susceptibility to damage, provide a rationale to understand the neuroprotection afforded by A2AR blockade. (Supported by FCT)

Blockade of P2Y1 receptors prevent glutamate-induced toxicity in cultured hippocampal neurons

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Over-activation of glutamate receptors can trigger excitotoxicity, which is involved in the demise of most neurodegenerative diseases. ATP is considered a general danger signal, but its role in neurodegeneration is still unclear. We now tested if P2 receptors (P2Rs) modulate the neurodegeneration caused by exposure of rat hippocampal neurons (7 DIV) to glutamate (100 μM during 25 min). This lead to a sustained increase of extracellular ATP levels up to 24 h and caused an initial synaptotoxicity (MAP-2 and synaptophysin labeling at 12 h) followed by a subsequent overt neurodegeneration (Hoescht 33348 labeling at 24 h), both prevented by a P2YR (PPADS, 20 μM) and P2Y1R antagonists (MRS2179, 10 μM). Accordingly, P2Y1R silencing (80nM of 3 different siRNAs decreasing P2Y1R mRNA levels by 51% after 72 h) also prevented glutamate toxicity. Glutamate induced toxicity was also abrogated by apyrase (20U/ml, hydrolyses ATP) and reinstated by ADPâS (1 μM, P2Y1R agonist). We then explored the ability of P2Y1Rs to modulate Ca2+ influx and recruitment of stress-activated kinases, which mediate glutamate-induced neurotoxicity. We found that MRS2179 (10 μM) decreased by nearly 1 μM the glutamate-induced 45Ca2+ influx and prevented the glutamate-induced phosphorylation of JNK and p38 kinases. Thus, glutamate excitotoxicity requires the release of ATP and the activation of P2Y1Rs that triggers a Ca2+- and JNK-/p38-dependent early synaptotoxicity, which later evolves into an overt neuronal death. This shows that increased extracellular ATP levels contribute to neuronal toxicity and suggests that P2Y1R antagonists may be novel neuroprotective drugs. (Supported by FCT)

Extracellular nucleotides induce activation of AMP-activated protein kinase in hepatocytes

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Introduction: AMP-activated protein kinase (AMPK) is the key energy regulator and a major pharmacological target for the treatment of metabolic disorders, including diabetes. Activation of AMPK stimulates glucose transporter translocation, increases glucose and fatty acids oxidation in skeletal muscle and inhibits glucose production in liver. The aim of this study

was to identify a purinergic signaling pathway of AMPK phosphorylation in hepatocytes and investigate possible outcomes of the AMPK activation.
Methods: AMPK phosphorylation was analyzed by Western blot. Identification of mRNA for purinergic receptors was done by RT-PCR. In vivo study was performed in C57/BL6 mice injected with ATP or saline.
Results: We have detected mRNAs for P2Y1, P2Y2, P2Y4, P2Y6, P2Y13 and P2Y14, and most of the P2X receptors in both human hepatoma cell line HepG2 and mouse primary hepatocytes. ATP, ADP and UTP, but not UDP, induced phosphorylation of AMPK in time and dose-dependent manners. Pharmacological and genetic evaluations indicate that AMPK activation is mediated by P2Y1, P2Y2 and/or P2Y4 receptors. Moreover, we identified calcium/calmodulin-dependent kinase kinase (CaMKK), and excluded LKB1, as the upstream activator of AMPK in the signaling pathway initiated by nucleotides. Importantly, phosphorylation of AMPK was confirmed in mouse liver following ATP administration. The effects of AMPK activation, including glucose output in hepatocytes, are under investigation.
Conclusion: Extracellular nucleotides induce AMPK phosphorylation in HepG2 cells, as well as in primary hepatocytes and mouse liver via CaMKK pathway. We hypothesize that P2 receptors could be a new target for the regulation of glucose levels and thus management of diabetes.

Mechanism of purinergic activation of endothelial nitric oxide synthase in endothelial cells

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Background
Decreased endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production are critical contributors to endothelial dysfunction and vascular complications observed in many diseases, including diabetes mellitus. Extracellular nucleotides activate eNOS and increase NO generation, however the mechanism of this observation is not fully clarified.
Methods
To elucidate the signaling pathway(s) leading to nucleotide-mediated eNOS phosphorylation at Ser-1177, human umbilical vein endothelial cells (EC) were treated with ATP, UTP, and ADP in the presence or absence of selective inhibitors. eNOS phosphorylation and activity were analyzed by Western blot and cGMP measurement.
Results
We identified P2Y1, P2Y2 and possibly P2Y4 as the purinergic receptors involved in eNOS phosphorylation, and demonstrated that this process was adenosine-independent. Nucleotide-induced eNOS phosphorylation and activity were inhibited by BAPTA-AM (an intracellular free calcium chelator), rottlerin (a protein kinase C (PKC) delta inhibitor) and siRNA specific to PKC delta. In contrast, blockade of AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent kinase (CaMK) II, CaMK kinase (CaMKK), serine/threonine protein kinase B (Akt), protein kinase A (PKA), extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) did not affect nucleotide-mediated eNOS phosphorylation. Importantly, activation of eNOS by extracellular nucleotides was maintained in EC exposed to high glucose. This contrasts with glucose-mediated impairment of other eNOS activating pathways, including phosphoinositol 3-kinase (PI3K)/Akt, ERK and p38 MAPK.
Conclusions
Extracellular nucleotide-mediated eNOS phosphorylation is calcium and PKC delta dependent. This newly identified signaling pathway is resistant to high glucose. Accordingly nucleotide-based therapies could be highly beneficial for the management of EC dysfunction in diabetes.

Blockade of adenosine A2A or P2Y1 receptors prevents interleukin-1Beta-mediated JNK phosphorylation in hippocampal neurons

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We have previously shown that P2Y1 or A2A receptor (A2AR) blockade affords neuroprotection. This might involve a control of neuroinflammation, since A2AR blockade prevents both neuroinflammation-induced depression of long-term potentiation and increases of interleukin-1beta (IL1B) levels. However, it is unclear if A2ARs only control IL1B levels or also its effects and it is not known if IL1B receptor-I (IL1R1) is located where P2Y1 and A2AR are most abundant, i.e. synaptically. Western blot analysis revealed that IL1RI is more abundant in whole hippocampal membranes but is also present in synaptosomal membranes of adult rats, where it is evenly located pre- (41.7±3.9%) and post-synaptically (43.3± 6.3%, n=4). This indicates that IL1R1 is mostly located extra-synaptically but is also present at synapses. We then tested if P2Y1 or A2ARs controlled IL1B-mediated effect through quantification of IL1B-induced phosphorylation of JNK and p38 in rat hippocampal cultured neurons. Exposure to IL1B (100 ng/ml, 15 min) enhanced the phosphorylation of JNK (123.6± 3.9%, n=12) and p38 (152.2±17.3%, n=5), which was prevented both by the A2AR antagonist SCH58261 (50 nM) or the P2Y1 receptor antagonist MRS2179 (10 μM). These results indicate that both P2Y1 and A2ARs control the neuronal actions of IL1B. This further supports the hypothesis that the neuroprotective effects afforded by antagonists of these receptors also involve the direct neuronal control of IL1B actions, a pro-inflammatory cytokine playing a pivotal role in neuroinflammation. (Supported by FCT)

Airway P2X ion channels mediate IL-8 expression through CaMKII-dependent NF-kappaB activation

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Background: In airway epithelia, extracellular nucleotides regulate the expression of pro-inflammatory cytokines by acting at G-protein coupled P2Y receptors. P2Y1, P2Y2 and P2Y6 receptors that are activated by ADP, ATP/UTP and UDP, respectively, have been identified. ATP-gated P2X ion channels mediating sustained Ca2+ entry are also expressed. However, the identity of these channels and their role in airway inflammation remain unknown.
Methods: The human tracheal and pulmonary epithelial cell lines, NT-1 and A549, and polarized human primary nasal epithelial cells were treated with ATP, activating both P2Y and P2X receptors, or with its stable analogs, alpha,beta-meATP and BzATP, mainly acting at P2X subtypes. NF-kappaB activation was analyzed by electromobility shift assays, reporter gene and kinase assays as well as by chromatin immunoprecipitation experiments. The expression of interleukin-8 was studied by real-time RT-PCR and ELISA.
Results: ATP and its analogs activated NF-kappaB through the IkappaB kinase complex, a process requiring extracellular Ca2+, calmodulin (CaM) and Ca2+/CaM-dependent kinase II (CaMKII). Both alpha,beta-meATP and BzATP induced Ca2+-dependent binding of phosphorylated p65 (S536) NF-kappaB subunit to the endogenous IL-8 gene promoter. While these agonists did not induce significant IL-8 gene expression by NT-1 cells, they markedly enhanced TNF-alpha-induced NF-kappaB activation, resulting in synergic stimulation of IL-8 expression and release. In contrast, application of alpha, beta-meATP or BzATP at the apical side of polarized human primary nasal epithelial cells sufficed to cause CaMKII-dependent IL-8 release.
Conclusions: ATP induces Ca2+ entry through P2X ion channels that activates NF-kappaB via CaMKII, enhancing IL-8 gene transcription and protein release.

Modulation of P2Y11 by compounds with activity at guinea-pig taenia coli

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Background. The activity of the methylene phosphonate isosteres of ATP, (including alpha,beta-methylene-ATP (abmA) and beta,gamma-methylene-ATP (bgmA)) shown to cause relaxation of guinea-pig taenia coli, have been investigated at the recombinant human P2Y11 receptor.
Methods. The human P2Y11 receptor cDNA was expressed in astrocytoma 1321N1 cells and P2Y11 receptor activation of the Gs- and Gq/11-coupled signal transduction pathways was assayed by quantification of de novo cAMP production and increases in intracellular calcium, respectively.
Results. Human P2Y11 receptors were activated in a concentration dependent manner by abmA and bgmA. The following EC50 values (mean±SEM, for n=3) were calculated for cAMP production and calcium mobilisation, respectively (indicated in parentheses): abmA (20.5+3.3 μM and 34.1+6.3 μM), bgmA (61.3+16.5 μM and 25.4+6.9 μM). Reactive Red and A317491 inhibited P2Y11-mediated calcium responses in a concentration-dependent manner with IC50 values (mean +SEM, for n=3) of 45.7+15.3 μM and 16.9+4.7 μM, respectively. Acid blue, phenol red and trinitrophenyl-ATP had no effect at concentrations up to 100 μM, whereas 100 μM of either PPADS or MRS2179 increased P2Y11-mediated calcium responses (Student t-test; p=0.027 and p=0.021, respectively).
Discussion. The methylene phosphonate compounds abmA and bgmA, which cause profound relaxation in guinea-pig taenia coli, are P2Y11 receptor agonists, causing activation of both the Gs and Gq/11 pathways. Reactive Red and A317491 have been identified as novel antagonists of the P2Y11 receptor, while high micromolar concentrations of PPADS and MRS2179 have been shown to potentiate P2Y11 receptor responses.

P2Y2 receptor signaling pathways

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Previous studies demonstrate that the Gq-coupled P2Y2 receptor (P2Y2R) contains motifs that interact with integrins, growth factor receptors and their signaling pathways. An Arg-Gly-Asp (RGD) sequence in the P2Y2R promotes its association with alphav integrins whereby ATP or UTP can activate integrin-associated pathways involving G12 and Go proteins. UTP-induced activation of G12 and G12-mediated events, including RhoA activation, cofilin and myosin light chain-2 phosphorylation, stress fiber formation and chemotaxis were inhibited by mutation of the RGD domain to RGE in the human P2Y2R expressed in 1312N1 cells that are devoid of endogenous P2 receptors. Similarly, UTP-induced activation of Go and Go-mediated events, including activation of Rac and vav2, a guanine nucleotide exchange factor for Rac, as well as chemotaxis towards UTP, were inhibited by the RGD to RGE mutation in the P2Y2R. Furthermore, the P2Y2R contains a Src homology-3 (SH3) binding motif in its intracellular C-terminus that enables the activated receptor to recruit Src and to stimulate growth factor receptors thereby increasing the expression of VCAM-1 that promotes binding of leukocytes/lymphocytes. The SH3 binding motif was found to be important for UTP-induced transient association of the P2Y2R with vascular endothelial growth factor receptor-2 (VEGFR-2) and VE-cadherin, a transmembrane component of endothelial adherens junctions. Recent results indicate that the C-terminal tail of the P2Y2R (amino acids 322 to 333 including the SH3-binding domains) is linked to cytoskeleton through interaction with the actin-binding protein filamin A (FLNa), which cross-links actin polymers and acts as a scaffold for various signaling molecules. This interaction was required for FLNa phosphorylation induced by nucleotides and caused spreading and migration of smooth muscle cells. This diversity of signaling molecule interactions with the P2Y2R is unique among G protein-coupled receptors.

(Invited)

SYMPOSIA 11: REAL TIME MEASUREMENTS OF PURINE RELEASE

Simultaneous measurement of adenosine and dopamine concentration in rat caudate-putamen

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Title: Simultaneous measurement of adenosine and dopamine concentrations in rat caudate-putamen.
Background: The purpose of the study was to observe the effects of receptor activation or inactivation on transient changes in dopamine and adenosine concentrations.
Methods: Fast-scan cyclic voltammetry at the carbon-fiber microelectrode was used to detect adenosine and dopamine simultaneously with subsecond temporal resolution. Electrical stimulation trains were applied to the substantia nigra every 3 minutes and the chemical changes were measured in the caudate-putamen of anesthetized rats. Drugs were dissolved in 1 mL of saline and injected i.p. Effects 45 minutes after administration were measured for the following drugs: alpha-methyl-DL-tyrosine methyl ester (AMPT, 250 mg/kg); SCH-23390 (0.1 mg/kg); MSX-3 (3 mg/kg); and N6-cyclopentyladenosine (CPA, 5 mg/kg).
Results: First we tested the effects of dopamine drugs on adenosine concentrations. The dopamine synthesis inhibitor AMPT decreased dopamine release by 50%, while adenosine concentration decreased only slightly, by 20%. SCH-23390, a D1R antagonist increased adenosine concentrations by 40%, but did not affect the dopamine release. Therefore, the amount of adenosine released is not directly correlated to dopamine release. Then we tested the effects of adenosine receptor agents on dopamine and adenosine release. MSX-3, an A2A antagonist, decreased dopamine release by 20% and adenosine concentrations by 40%. CPA, an A1 agonist, decreased the dopamine release by 50%, and the adenosine peak completely disappeared after drug injection.
Conclusion: A1 receptors mediate both electrically-stimulated adenosine and dopamine release in the rat caudate-putamen.

Simultaneous measurement of ATP and adenosine in intact tissues by biosensors

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A fundamental prerequisite for the myriad actions of the purines is their release from cellular elements into the extracellular space where they can interact with their many G-protein coupled receptors and ligand-gated ion channels. This release can occur in a basal, constitutive, “housekeeping” mode to provide a background tone of purines capable of influencing cellular and network activity via higher affinity receptors, but also via the stimulus-evoked, and often activity-dependent, release of purines. The release, presence and actions of purines have classically been inferred through the use of selective receptor antagonists. Here we run into the first problem: the requirement for selective antagonists. When such antagonists exist they seldom inform as to the kinetics of purine release and the spatio-temporal profile of extracellular purine accumulation and dissipation. Hence the need to measure, directly, in real time and with high spatio-temporal resolution, purine release from anatomically-defined regions during patho/physiologically-relevant conditions.
Enzyme-based microelectrode biosensors for the purines (adenosine, inosine, xanthine, ATP) solve many of these problems. Our lab has been using them for a decade to probe various aspects of purine release, primarily in the context of in vitro models of cerebral ischemia and seizure activity. Our observations and those of others using the biosensor have shed new light on purine release, and some unexpected surprises. This presentation will focus on the principles behind the purine sensors, the applications in which they can be used, and some of the questions direct measurement of purine release has raised.

(Invited)

Three different types of ATP-release in mammary epithelial cells revealed by ATP imaging

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Mammary epithelial cells are highly sensitive to stimuli such as mechanical stretch or touch, and release ATP. The ATP acts on secretory and myo- epithelial cells in mammary alveoli as autocrine and paracrine mediators, and regulates or modulates milk ejection. To investigate the mechanism of ATP release from the cells, we have developed a real-time ATP imaging system under microscope. Luciferin-Luciferase bioluminescence of released ATP is detected by a high sensitive cooled-CCD camera with an image intensifier equipped on an upright microscope. The system also enables simultaneous imaging of a Nomarski DIC-IR image with ATP luminescence image. Using this, we observed (1) mechanical stimulations (touch and stretch) induced ATP release; (2) spontaneous ATP release in Calcium-free solution (CFS); (3) oxytocin and UTP induced ATP release, in cultured mammary epithelial cells. We found that the stretch stimulation induces intense ATP release from a limited number of cells, and that the number increases with increase of stretch length. Kinetic analysis of ATP release shows that the time course of spontaneous ATP release is ten times slower than by stretch or touch induced one. Adding pharmacological results, we conclude that three different types of ATP release, i.e., mechanically induced, ligand binding induced, and spontaneous ATP releases, are included in a mammary cell.

Characterization of K^+ depolarization evoked ATP and adenosine release in rat hippocampus: a microelectrode biosensor study

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It is well known that depolarization evokes stimulation-dependent purine release in various parts of the brain, including the hippocampus. The release mechanism of ATP and adenosine efflux have been characterized in details in previous studies. However, the recently developed microelectrode technique allows the detection of the efflux of endogenous neurotransmitters with better temporal resolution than conventional neurochemical methods. Therefore we aimed to characterize K^+ depolarization-evoked ATP and adenosine efflux in the *in vitro* rat hippocampus.
ATP, Null and ADO/INO (sarissaprobe®) biosensors were each inserted through the 400 μm hippocampal slice in a way that most of the sensing part of the sensor was intimate contact with the tissue. ATP and adenosine sensor exhibited a rapidly increasing current during 25 mM K^+ depolarization, which reached its peak at 1.21±0.08 μmol /L (ATP) and 11.15 ±0.63 μmol/L (ADO/INO) concentration. Removal of extracellular Ca^{2+} from the medium abolished both ATP and adenosine release in response to K^+ depolarization (0.22±0.1 μmol/L). In contrast, Na^+-channel blocker TTX (1 μmol/L) had no effect on either ATP or adenosine release. The gap junction hemichannel blocker carbenoxolone (100 μmol/L) significantly decreased 25 mM K^+ evoked ATP and adenosine efflux (0.17±0.08 μmol/L).
We have used successfully this fast-responding sensor to monitor ATP and adenosine release upon neuronal activity in the rat hippocampus. Our data suggest that both ATP and adenosine release during K^+ depolarization require extracellular Ca^{2+} and this release is directly or indirectly mediated by connexin hemichannels.

FRET-based assays of local ATP release

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It would be informative to non invasively measure ATP release and activation of defined transmitter-gated cation channels in living organisms, with high resolution in defined cell populations. We present such an approach and validate it for ATP-

gated P2X receptors. The method we report exploits the fact that most transmitter-gated cation channels, including P2X receptors, have appreciable calcium fluxes. We genetically engineered P2X receptors to carry sensors for calcium ions attached in the vicinity of the inner aspect of the channel pore, and thus within a mandatory nano domain to sense calcium ions. We rigorously tested the method for P2X2 receptors expressed in human embryonic kidney cells and brain hippocampal neurons in vitro. Within a given neuron this method allows one to image the location and activation of P2X receptors by imaging. The sensitivity of the method is equivalent to that of whole-cell patch clamp recording, with the major advantages that the approach is non invasive and provides micrometer scale spatial information. Our experimental data suggest the approach will work for any cation channel with fractional calcium currents greater than ~1%. The approach thus represents a general, sensitive, genetically encoded way to image activation of defined transmitter-gated cation channels. The method should help shed light on when, where and how different receptors are activated during physiological processes in vitro and in vivo, as well as provide measures of ATP release.

(Invited)

Real time detection of extracellular ATP in tumor microenvironment

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Background: The tumor microenvironment plays a critical role in tumor initiation, progression and invasion. Nucleotides, such as ATP, have a surprisingly wide range of modulatory effects on tumor cells, however techniques for measuring ATP in the extracellular environment are still rudimentary. Here we show an in vivo method for measuring extracellular ATP release in tumor microenvironment.
Methods: We stably expressed pmeLUC, a chimeric luciferase targeted to the extracellular side of the plasma membrane, in HEK293 cell lines. A stable cell clone (HEK293-pmeLUC) was then injected anesthetized nude/nude mice bearing or not a tumor. Bioluminescence was then monitored by IVIS imaging system (Xenogen).
Results: Endovenous, intraperitoneal and subcutaneous inoculation of HEK293-pmeLUC cells in healthy nude mice caused a modest, barely detectable, increase in luminescence. On the contrary, bioluminescence observed after injection of the probe into mice bearing the OVCAR-3 human ovarian carcinoma or the MZ2-MEL human melanoma increased strongly. We then validated HEK293-pmeLUC cells as ATP reporters using the ATP-hydrolyzing enzyme, potato apyrase. Apyrase injection cause a significative drop in luminescence. We then performed an in vitro calibration which revealed that ATP in the tumor interstitium is in the hundrend micromolar range, while it is basically undetectable in healthy tissues. Our approach offers a new tool for the investigation of the biochemical composition of tumor milieu and for development of novel therapies based on the modulation of extracellular purine-based signalling.

Direct electrochemical detection of adenosine in vivo

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Adenosine modulates cerebral blood flow and neurotransmission and is neuroprotective during pathological conditions such as stroke. Adenosine receptors have a wide range of affinities and effects, therefore, the concentration of adenosine available to bind to and activate these receptors controls the physiological response to adenosine. Microelectrodes can detect electrochemically-active neurochemicals and are advantageous because of their small size and ability for fast measurements. We have developed a microelectrode sensor to measure adenosine changes with sub-second temporal resolution. Fast-scan cyclic voltammetry of adenosine at carbon-fiber microelectrodes was used. The expected oxidation potential of adenosine is

around +1.3 V, so the electrode potential was scanned from -0.4 to 1.5 V and back at 400 V/s with a 10 Hz repetition rate. Two oxidation peaks and a small reduction peak were detected for adenosine. By varying the scan rate, we have shown that detection of adenosine is adsorption controlled. Adsorption of adenosine was maximized to obtain detection limits of less than 15 nM, less than basal levels estimated to be 50–200 nM. The electrode is 5 times more sensitive for adenosine detection than ATP detection because the negative holding potential of the potential waveform. The electrode was used for in vivo detection of adenosine after electrical stimulations. Pharmacological data confirmed the identity of the measured species as adenosine. This sensor will be ideal for measuring adenosine release in brain slices or in vivo models of ischemia.

(Invited)

SYMPOSIA 12: CARDIOPROTECTION

Opposite effects of uracil and adenine nucleotides on the survival of murine cardiomyocytes

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Based on evidence suggesting that, both in humans and rodents, plasma levels of ATP and UTP markedly increase during myocardial infarction, we decided to study the effects of adenine and uracil nucleotides on murine HL-1 cardiomyocytes. RT-PCR analysis showed that HL-1 cardiomyocytes express all known P2X receptors (except for P2X2), as well as the P2Y2,4,6,14 subtypes. A 24-hr exposure of HL-1 cardiomyocytes to ATP induced apoptosis and necrosis, as shown by flow cytometric analysis of PI-stained nuclei and Annexin V-stained cell outer-membrane. Cell death was exacerbated by tumour necrosis factor (TNF-alpha), a cytokine implicated in chronic heart failure progression. To shed light on the specific receptor subtypes involved, HL-1 cells were exposed to ATP and TNF-alpha in the presence of various P2 antagonists. Suramin was the most potent antagonist in inhibiting apoptotic death induced by ATP and TNF-alpha; a partial, but significant inhibition was observed with PPADS and RB2. Based on the expression profile of P2 receptors in HL-1 cells, we suggest that P2Y2/P2Y4 are the most likely candidates for induction of cell death. Interestingly, uracil nucleotides (UTP, UDP and UDPglucose) fully counteracted the deleterious effects induced by ATP and TNF-alpha. Cardiomyocytes do not need to be "primed" by uracil nucleotides to become insensitive to adenine nucleotides-induced death, suggesting the existence of a possible "therapeutic" window for uracil nucleotides-mediated protection. Thus, release of UTP during cardiac ischaemia and in chronic heart failure may protect against myocardial damage, setting the basis for developing novel cardioprotective agents that specifically target uracil-preferring P2Y receptors.

Coordination of extracellular adenosine generation and signaling during ischemic preconditioning of the heart

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BACKGROUND: Ischemic preconditioning provides strong cardioprotection from ischemia, but its molecular mechanisms remain largely unknown. Convincing evidence confirms a central role of hypoxia-inducible factor (HIF)-1 in mammalian oxygen homeostasis. Thus, we pursued HIF-1 as central component to cardioprotection by IP.
METHODS AND RESULTS: Murine studies of in situ preconditioning revealed a robust activation of cardiac HIF-1alpha. Moreover, in vivo siRNA repression of cardiac HIF-1alpha resulted in abolished cardioprotection by IP. In contrast, pretreatment with HIF-activator dimethyloxalylglycine was associated with similar cardioprotection as ischemic preconditioning itself. Finally, selective siRNA-repression of prolylhydroxylase PHD2 resulted in significant activation of HIF-1alpha and attenuated myocardial infarct sizes (0.44±0.09-fold). As endpoint of HIF-dependent cardioprotection, we defined the role of A2B adenosine receptor (A2BAR) signaling. While the cardiac A2BAR was induced with HIF activation, HIF-dependent cardioprotection was abolished in A2BAR-/-mice.
CONCLUSIONS: Taken together, these studies provide evidence for a critical role of HIF-1 in ischemic preconditioning via enhancing purinergic signaling pathways.

(Invited)

A1 receptor stimulation induces changes in calcium homeostasis

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Adenosine is well known to contribute to delayed cardioprotection but the mechanisms of action of its effect are poor understood. We investigated the expression of sarcolemmal L-type Ca(2+) channel, Na(+)/Ca(2+) exchanger, sarcoplasmic reticulum (SR) Ca(2+)-ATPase, phospholamban, and ryanodine receptor upon adenosine stimulation. Since adenosine release is proportional to energy demand, the effects of adenosine were compared to the effects of changes in myocardial work load. We measured gene expression, SR Ca(2+) uptake, and irreversible tissue injury in an acute model, isolated perfused working rat hearts treated with R-phenylisopropyladenosine (R-PIA), an A(1) receptor agonist; in a subacute model, rats treated with R-PIA e.v. and sacrificed after 24 h; and in a transgenic model, mice overexpressing A(1) adenosine receptors. Gene expression was also determined after perfusion at high atferload (180 cm vs 100 cm) or with a cardioplegic solution. A(1) stimulation induced a significant up-regulation in phospholamban gene expression in each model. SR Ca(2+) uptake and irreversible tissue injury from ischemia were significantly reduced in subacute and transgenic models. On the other hand, significant decrease in the expression of the SR Ca(2+)-ATPase gene was observed in the high afterload group while no change in gene expression was observed in the cardioplegia group. In conclusion, A(1) adenosine receptor stimulation increases phospholamban gene expression, which reduces the rate of SR Ca(2+) uptake within 24 h. This effect is not reproduced by changes in myocardial work, which however modulates SR Ca(2+)-ATPase expression. A1 receptor stimulation may contribute to the delayed cardioprotective effect by inducing changes in Ca(2+) homeostasis.

The anti-inflammatory and cardioprotective properties of A2A adenosine receptor agonists

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Background: Reperfusion injury after MI is associated with intense inflammation lasting 24–48 hours that contributes to ongoing myocyte injury. We previously showed that infusion of ATL-146e, a selective adenosine A2A receptor agonist, at the time of reperfusion, decreased inflammation and infarct size in dogs examined 2 hours post-MI without increasing coronary blood flow. We next sought to determine if the reduction in inflammation and infarct size persists beyond the acute stage, and if there is any further reduction in infarct size if the treatment is extended for 24 hours. Methods: Twenty five adult dogs underwent 90 minutes of LAD and visible collateral vessel occlusion. Thirty minutes before reperfusion, a low dose infusion of ATL-146e (0.01 micrograms/kg/min) (n=13) or vehicle (n=12) was started and continued for 2.5 hours (Group 1) or 24 hours (Group 2). Hearts were removed and assessed for risk area and infarct size by TTC staining and neutrophil numbers in the reperfused zone were determined by microscopy. Results: Heart rate and mean arterial blood pressure was comparable between ATL-146e and vehicle-treated animals at all time points. Importantly no fall in blood pressure was observed consequent to ATL-146e infusion. TTC Infarct size was smaller in ATL-146e treated vs. control dogs (16.7±3.7% vs. 33.3±6.2% risk area, p=0.027; Group 1). ATL-146e also significantly reduced neutrophil accumulation in the infarct region. ATL-146e infusion for 24 hours did not confer significant additional infarct size reduction. Conclusions: A relatively brief 2.5 hour infusion of ATL-146e beginning at reperfusion resulted in a marked and persistent reduction in inflammation and infarct size, with no untoward hemodynamic effects, and shows promise as a potential therapy in the setting of reperfused acute myocardial infarction.

(Invited)

Regulation of adenosine A1 receptor from rat neonatal cardiomyocytes after caffeine exposure

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Adenosine is a nucleoside widely distributed in both central and peripheral nervous system. Actions of adenosine are mediated through G-protein coupled receptors which can be blocked by caffeine, stimulatory substance presents in coffee, tea and cola beverages. The aim of the present work was to identify adenosine A1 receptor (A1R) in neonatal rat cardiomyocytes and to determine the possible effects of caffeine treatment on A1 receptor numbers and functionality expressed in these cells. Cardiomyocytes were isolated from neonatal Wistar rat heart and identified by immunofluorescence. An expression profile of Adenosine A1 receptors was determined at different DIV by binding assays using 8-Cyclopentyl-1,3-dipropylxanthine ([3H]DPCPX) as radioligand. Cell cultures at 7 DIV were subjected to 100 μM caffeine exposure at different times (2, 24 and 48 hours). Caffeine treatment caused an increase in total adenosine A1R number at 2 h. However, a significant decrease in A1 receptors was observed at 24 and 48 h of treatment. On the other hand, mRNA level coding adenosine A1 receptor was also analyzed by real time PCR and no significant differences were detected after caffeine treatment suggesting a posttranslational regulation. Therefore, we can conclude that caffeine affects adenosine A1 receptors endogenously expressed in rat cardiomyocytes in a biphasic manner depending on the time of exposure, causing an up-regulation of these receptors at short and a down-regulation at long-time of drug exposure.

Ischemic preconditioning of isolated mouse hearts is independent of the A2B adenosine receptor

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Background: The A2B adenosine receptor (A2BAR) has been proposed to contribute to myocardial ischemic preconditioning (IPC) based on studies using an in vivo mouse model of infarction. We examined this hypothesis using an isolated mouse heart model of global ischemia/reperfusion injury with A2BAR gene "knock-out"/reporter gene "knock-in" (A2BKO) mice in which the A2BAR gene is disrupted by the gene encoding for beta-galactosidase.
Methods: Langendorff-perfused hearts from C57BL/6J wild-type (WT) and congenic C57BL/6J A2BKO mice were subjected to 20 min of global ischemia followed by 45 min of reperfusion. IPC was induced by two cycles of 3-min ischemia/3-min reperfusion. Left ventricular pressure was assessed throughout the experiments and necrotic cell injury was measured by release of cardiac troponin I (cTnI) into the coronary effluent. Results: IPC improved recovery of contractile function following 20 min of global ischemia and 45 min of reperfusion to a similar extent (~30–40%) in both WT and A2BKO hearts. Interestingly, the release of cTnI was less in A2BKO hearts compared to WT hearts in the absence of IPC, but was markedly reduced by IPC in both genotypes. Staining for beta-galactosidase in A2BKO hearts was not detected in cardiomyocytes, although it was prominent in the smooth muscle layer of coronary vessels. Moreover, A2BAR mRNA expression in isolated cardiac myocytes determined by quantitative RT-PCR was extremely low (199 versus 20,361 copies/50 ng total RNA detected in cardiac fibroblasts that routinely contaminate cardiomyocyte preparations). Conclusion: IPC of isolated mouse hearts is not dependent on the A2BAR.

Caffeine-mediated embryonic programming of body composition and cardiac function in adulthood

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A1 adenosine receptor (A1AR) activation has been recently shown by us to play a major role in protecting the embryo against hypoxia (Proc Natl Acad Sci U S A. 2007 5;104(23):9697–702). Adenosine action is antagonized by caffeine, which is a non-selective adenosine antagonist. Although caffeine is consumed by large numbers of women during pregnancy, our understanding of the acute and long-term affects of prenatal caffeine exposure are minimal. To address this issue, pregnant mice were exposed to hypoxia or room air from embryonic days (E) 8.5–10.5, and treated with caffeine or vehicle. Hypoxic exposure from E8.5–10.5 acutely reduced embryo growth. Exposure to a single dose of caffeine in hypoxia or room air did not affect embryo growth; however, caffeine treatment inhibited cardiac ventricular development. Caffeine exposure resulted in marked inhibition of hypoxia-induced HIF1-alpha protein expression in embryos, as well. When offspring from dams treated with a single dose of caffeine were studied in adulthood, we observed that caffeine treatment alone caused resulted in decreased in cardiac function as assessed by echocardiography. We also observed increased body fat content in male mice exposed to caffeine. These results suggest that exposure to a single dose of caffeine during early embryogenesis can result in long term effects on body composition and cardiac function in adulthood.

The role of equilibrative nucleoside transporters in modulating flux of purine nucleosides across cardiomyocyte membranes

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Nucleoside transporters are integral membrane proteins that are responsible for the flux of non-lipophilic nucleosides and analogs across cellular membranes. The equilibrative nucleoside transporters (ENTs) comprise the SLC29 family of transporters and four isoforms (ENT1–4) are known. ENTs are widely distributed in the body and are routes of uptake for nucleoside analog drugs used in anti-cancer and anti-viral therapies and as targets for the drug dipyridamole (DIPY), a coronary vasodilator.
To investigate the role of ENT1 and ENT2 in the heart we used the mENT1 knockout model which has not previously been used for cardiovascular studies. ENT expression profile and function were characterized in adult cardiomyocytes, isolated from mENT1-/- (KO) and mENT1+/+ (WT) littermates, using qRT-PCR, NBTI-binding and purine nucleoside uptake assays. Concentrations of extracellular and intracellular nucleosides, nucleotides and nucleobases in WT and KO cardiomyocytes were measured, in the presence and absence of DIPY and hypoxic challenge, by HPLC.
Cardiomyocytes from KO mice show identical expression of mENT2–4 to WT but no mENT1 expression or NBTI binding. Chloroadenosine and inosine uptake were significantly affected in KO cardiomyocytes supporting a major, but not exclusive, role for ENT1 in purine nucleoside transport. Analyses of prevailing concentrations of nucleotides, nucleosides and nucleobases under the various treatment conditions suggests a complex relationship between purine nucleoside/tide pools, and the bi-directional ENTs, which control purine nucleoside flux. These studies support the use of the mENT1-/- model as a tool in the study of the role of the ENTs in purine nucleoside dependent physiology of the heart.

Uridine-5′-triphosphate (UTP) reduces infarct size and maintains cardiac mitochondrial function following hypoxic stress

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Massive amounts of nucleotides are released during ischemia in the cardiovascular system. Whereas the effect of purine nucleotides (ATP) in myocardial infarction was intensively studied, the cardioprotecting role of pyrimidine nucleotides (UTP) under hypoxic condition has not been demonstrated.
We found that UTP significantly reduced cardiomyocyte death caused by hypoxia. Even incubation (1 hour) with UTP, 24 hours before exposing the cells to hypoxic conditions, protected the cells. The cardioprotective effect of UTP was

reduced in the presence of the non-selective P2 receptor antagonist – suramin. To further define the role of UTP on ischemic heart in vivo, heart function was tested 24 h post left anterior descending (LAD) ligation in UTP (0.44 μg/kg) treated rats. UTP's beneficial effect in LAD ligated hearts was expressed by high ATP levels, improved mitochondrial activity (Complex I, II and IV) and reduced infarct size. A modest reduction (12%) in the mitochondrial membrane potential was demonstrated when the cultured cardiomyocytes were subjected to UTP. This reduction was abolished by the P2Y receptor antagonist, reactive blue 2, but not with 5 hydroxydecanoate, a mitochondrial K_{ATP} channel inhibitor, or by BAPTA-AM, an intracellular calcium chelator. Using Rhod-2 loaded cardiomyocytes, we found that UTP reduced mitochondrial calcium elevation following hypoxia. In conclusion, early or late UTP preconditioning is effective, demonstrating reduced infarct size and superior myocardial function. The reduction in mitochondrial calcium overload can partially explain the beneficial effect of UTP in cardiac protection following ischemic injury.

(Invited)

SYMPOSIA 13: PURINERGIC SIGNALLING IN THE KIDNEY

Purinergic Signaling in the Juxtaglomerular Apparatus

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In the juxtaglomerular apparatus (JGA) of the kidney, the loop of Henle contacts its parent glomerulus. This close vicinity between the tubular system and the vasculature of the afferent arteriole enables specialized tubular cells (macula densa cells, MD) to control both pre-glomerular resistance (and by that single nephron glomerular filtration rate), and renin secretion from granular cells of the afferent arteriole.
The tubular control of preglomerular resistance (tubuloglomerular feedback, TGF) is initiated by the detection of the tubular NaCl concentration by MD cells. An increase in tubular NaCl concentration triggers a signaling cascade which eventually leads to a vasoconstriction of the afferent arteriole. The signaling cascade between MD cells and the vascular effector cells of the afferent arteriole involves regulated release of ATP from MD cells, extracellular dephosphorylation of ATP by ecto-NTPDases and ecto-5′-nucleotidase, and activation of adenosine receptors (A1AR) of the afferent arteriole. Thus, pharmacological blockade or genetic ablation of proteins responsible for each signaling step results in compromised or absent TGF responsiveness. Conversely, enhanced TGF responses were observed in a transgenic mouse model with vascular over-expression of A1AR. Similar to TGF, also the MD control of renin secretion depends on purinergic signaling. Thus, in A1AR-deficient mice, the suppression of renin secretion in response to an increase in NaCl at the MD is absent. In summary, the formation of adenosine in the confines of the JGA appears to be an indispensable prerequisite for an intact TGF function and for a functional MD control of renin secretion.

(Invited)

P2Y12 receptor blockade or anti-inflammatory treatment preserve renal microvascular function in hypertension

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Hypertension is associated with renal inflammation and impaired autoregulatory control. The autoregulatory impairment observed in hypertension reflects a marked reduction in afferent arteriolar (AA) reactivity to P2X receptor activation. We postulated that anti-inflammatory treatment or P2Y12 receptor inhibition would preserve renal microvascular function in hypertension. Autoregulatory behavior and AA reactivity to P2X receptor activation were assessed in normotensive and Ang II-infused (60 ng/min; 14 days) hypertensive rats. Selected rats received the P2Y12 receptor antagonist, clopidogrel (10 mg/kg/day) or the anti-inflammatory agent, pentosan polysulphate (PPS). Systolic arterial pressure in Ang II infused rats reached 200±6 mmHg after 13 days of infusion. Tail cuff blood pressure was not affected by clopidogrel or PPS. Reducing renal perfusion pressure from 140 to 90 mmHg reduced renal blood flow by 23±2% in Ang-II infused animals indicating impaired autoregulatory efficiency compared to controls (4±5%). Conversely, autoregulation was well-preserved (5±7%) in hypertensive animals receiving clopidogrel. AA autoregulatory responses and P2X receptor-mediated vasoconstriction were impaired in hypertension. Increasing perfusion pressure from 100 to 160 mmHg, reduced afferent diameter to 73±4% of baseline in kidneys from PPS treated hypertensive rats compared to 97±6% of baseline in untreated hypertensive rats. Improved autoregulatory behavior coincided with normalized P2X receptor-mediated vasoconstriction in clopidogrel or PPS treated rats. These data establish that P2Y12 receptor inhibition with clopidogrel or anti-inflammatory treatment with PPS preserves normal renal microvascular autoregulatory function during Ang II-induced hypertension. Preservation of autoregulatory behavior is supported by preservation of afferent arteriolar reactivity to P2X receptor activation.

Renal purinergic system - a potential drug target for the treatment of lithium-induced diabetes insipidus

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Lithium (Li), an established treatment for bipolar disorders, is emerging as a potential neuroprotective therapy for acute and chronic neurodegenerative diseases. However, a significant side effect of Li therapy is nephrogenic diabetes insipidus (NDI), which is due to increased production of PGE2 by the medullary collecting duct (mCD). Current therapies aimed at direct inhibition of PGE2 biosynthesis (e.g. indomethacin or COX-2 inhibitors) are associated with serious side effects, including Li intoxication. Replacement of these side effect-prone drugs with a new class of agents based on improved understanding of the molecular pathophysiology of Li-induced NDI should result in improved efficacy and fewer side effects in the clinic. In this context we discovered that increased production of PGE2 by mCD in Li-induced NDI may be related to the hypersensitivity of P2Y2 receptor, the predominant purinergic receptor in the mCD. Preliminary studies conducted in rat model showed that administration of potato apyrase (a soluble ecto-nucleotidase) causes significant reduction in Li-induced polyuria, associated with normalization of augmented release of P2Y2 receptor-stimulated PGE2 release by the mCD. We also found that genetic deletion of P2Y2 receptor results in marked resistance to the development of Li-induced polyruia. Thus our studies suggest that hypersensitivity and enhanced signaling through P2Y2 receptor may a play a significant role in vivo in the development of NDI by markedly increasing the production of PGE2 by mCD. Hence renal purinergic system may be a potential target for the development of a new class of drugs for the treatment of Li-induced NDI.

Purinergic regulation of renal water transport
(Invited Lecture)

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Water homeostasis is achieved by the inherent capacity of the kidneys to conserve or eliminate water as per the needs of the body. The renal collecting duct (CD) system is the site of regulated “free water” transport that fine tunes body water homeostasis. Defective transport of water by the CD results in various water balance disorders. Arginine vasopressin (AVP), a circulating hormone acts on the CD and increases its capacity to conserve water. However, during the past decade, locally produced autocrine and/or paracrine factors have emerged as potent modulators of handling of water by the CD. One such local factor is extracellular nucleotides (ATP/UTP), which act through P2Y2 receptor, the predominant purinergic receptor expressed in the CD. Studies conducted by us and other investigators are unraveling the potent interactions among AVP, prostanoid and purinergic systems in the CD, and the perturbations of these interactions in water balance disorders. Specifically, we identified perturbations in the purinergic-prostanoid interactions in hydrated, dehydrated and AVP excess states. In addition, we uncovered enhanced purinergic-prostanoid interactions in acquired nephrogenic diabetes inspidus (NDI), which may be one of the key factors for AVP-resistant polyuria. Furthermore, it appears that the renal purinergic system may be a potential target for the development of a new class of drugs to treat water balance disorders in the clinic. Such disorders can be either water-losing conditions such as acquired NDI that may cause hypernatremia, as well as water-retaining conditions associated with hyponatremia, such as congestive heart failure or cirrhosis of liver.

(Invited)

Spontaneous and stimulated renal tubular ATP secretion

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Background
Extracellular ATP is, via P2 receptors a crucial regulator of ion and water transport along the renal tubule. Epithelial cells release ATP, the mechanism and regulation, however, is unknown.
Methods
Here we have established an ATP bio-sensing method, where ATP indicator cells (132–1N1 cells stably transfected with hP2Y2 receptors) were positioned in the luminal out-flow of isolated perfused mouse nephron segments. [Ca2+]i was measured in parallel using fluo-4 in indicator cell and the perfused tubulus.
Results
We find that freshly isolated medullary thick ascending limbs and cortical collecting ducts spontaneous secrete ATP in amounts sufficient to stimulate bio-sensor cells. Intriguingly, arginine-Vasopressin (10nM) stimulated additional renal tubular ATP secretion.
Conclusion
This study provides novel insight into the physiological understanding of spontaneous and hormone-induced ATP signalling in the mouse nephron.

Visualization of the release and actions of ATP in the intact kidney

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ATP is an important autocrine/paracrine regulator of kidney function. ATP release from macula densa (MD) cells into the interstitium of the juxtaglomerular apparatus (JGA) is an integral component of tubuloglomerular feedback (TGF) that controls glomerular filtration rate. Recently, confocal fluorescence calcium imaging of cultured glomerular endothelial cells, the in vitro microperfused isolated JGA-glomerulus complex and the intact kidney was performed to visualize extracellular ATP-dependent calcium waves including that during TGF. Gap junction uncoupling or Cx40 siRNA treatment, an ATP scavenger enzyme cocktail and pharmacological inhibition of P2 purinergic receptors, but not adenosine A1 receptor blockade, abolished the changes in [Ca2+]i and cell-to-cell propagation of the calcium waves. These studies provided evidence that both gap junctional communication and ATP release through connexin hemichannels are integral components of the TGF and endothelial calcium waves.
Other studies tested if apical membrane Cx30 hemichannels in the distal nephron release ATP and regulate renal electrolyte and water transport. Using a biosensor approach, significant ATP release was detected from intact intercalated but not principal cells. Importantly, ATP release was completely absent in Cx30-/- mice. Mice were also surgically instrumented for pressure natriuresis measurements. In response to step increases in MAP, urine flow increased 4.2-fold in Cx30+/+ compared to only a 2.6-fold increase in Cx30-/- animals. Also, fractional urinary Na+ excretion increased 5.1-fold in Cx30+/+ compared to only a 2.8-fold increase in Cx30 -/- animals. These studies suggest that distal tubular Cx30 hemichannels release ATP which inhibits renal salt and water reabsorption.

(Invited)

DOCA unmasks salt-sensitivity of blood pressure in mice lacking P2Y2 receptors

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Mice that lack P2Y2 receptors (ko) have salt-resistant hypertension, suppressed plasma aldosterone concentrations, and require smaller increases in plasma aldo to adapt renal Na+ excretion to restricted intake, suggesting greater aldosterone sensitivity

(FASEB J 21:3717–26, 2007). Here we performed cell-attached patches on apical plasma membranes of principal cells in cortical collecting ducts and found that the open-probability of the aldosterone-regulated epithelial sodium channel, ENaC, is greater in ko compared with wild-type (WT) mice (0.51±0.05 (n=18, N=6) vs. 0.28±0.05 (n=12, N=3); P<0.05) and significantly lowered by extracellular ATP only in WT. To clamp the mineralocorticoid system at high levels, we administered deoxycorticosterone acetate (DOCA, 2.4 mg/day s.c. pellet/21 day release) plus access to water and 1%NaCl in drinking water. DOCA-treated WT and ko mice initially (on day 1) preferred 1%NaCl vs. tap water when given the choice, consistent with DOCA-induced salt appetite. WT mice responded similarly on subsequent days but ko mice preferred water. In spite of lower salt intake, ko mice had a greater blood pressure (BP) increase than did WT (Ä BP vs. basal: 14±3 vs. 5±2 mmHg; N=5–6, P<0.05). If only able to drink 1%NaCl, WT and ko mice had similar salt intake but ko mice had a greater increase in BP than did WT (ko: Ä BP vs. basal: 23±3 vs. WT: 6±3 mmHg, p<0.05). These data show that activation of P2Y2 receptors lowers ENaC open-probability and that suppression of the mineralocorticoid system masks salt-sensitivity of BP in mice that lack P2Y2 receptors.

(Invited)

Luminal factors affecting apical P2 receptor mediated regulation of renal EnaC

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Epithelial Na+ channels (ENaC) control Na+ reabsorption along the collecting duct (CD). We have previously demonstrated that ENaC activity is inhibited by activation of apical P2Y2 and/or 4 and P2X4 and/or 4/6 receptors when extracellular Na+ concentrations are high (145 mM), and that apical P2X4 and/or 4/6 receptors switch to being potentiators of ENaC activity when extracellular Na+ is lowered (to 50 mM); the latter being a more physiological concentration of luminal Na+ (not previously used in vitro). Here we address the effects of altering extracellular pH on P2 receptor-mediated regulation of ENaC in CD.
Adult Sprague Dawley rats were maintained on a low (0.01%) sodium diet to increase ENaC expression in CD. Kidneys were removed, micro-dissected and CD segments split-open to expose the apical membrane of individual cells for whole-cell perforated-patch clamp experiments.
ATP- and UTP-evoked currents reduced, and 2meSATP- and ATPgammaS-evoked currents increased, the amplitude of subsequent ENaC-mediated currents. Lowering extracellular pH from 7.4 to 6.5 did not significantly alter these effects. Further acidification of the extracellular medium to pH 5.5 increased the degree of inhibition by UTP and abolished the modulatory effects of 2meSATP or ATPgammaS-evoked currents.
These data suggest the ENaC activity is further inhibited by apical P2Y2 and/or 4 receptors when extracellular pH is lowered (to pH 5.5), and that apical P2X4 and/or 4/6 receptors cease to have an effect. The relationship between apical P2R and ENaC activity in rat CD is complex and dependent on both luminal Na+ and tubular fluid pH.

(Invited)

SYMPOSIA 14: ECTONUCLOTIDASES: PHYSIOLOGICAL IMPLICATIONS

The role of ecto-5′-nucleotidase/CD73 in glioma cell line proliferation

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Malignant gliomas are the most common and devastating primary tumors in the brain and, despite treatment, patients with these tumors have a poor prognosis. The participation of ecto-5′-NT/CD73 per se as a proliferative factor, being involved in the control of cell growth, maturation, differentiation, invasion, migrations and metastasis processes has been previously proposed. In the present study, we evaluated the activity and functions of ecto-5′-NT/CD73 during the proliferation process of rat C6 and human U138-MG glioma cell lines. Increasing confluences and culture times led to an increase in ecto-5′-NT/CD73 activity in both C6 and U138-MG glioma cells. RT-PCR analysis and flow cytometry showed a significant increase in ecto-5′-NT/CD73 mRNA and protein levels, respectively, comparing confluent with sub-confluent cultures in human U138-MG glioma cells. Ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73) may regulate the extracellular AMP and adenosine levels, which have been described as a proliferative factor. Treatment with 1 mM APCP, a competitive ecto-5′-NT/CD73 inhibitor, caused a significant reduction of 20% in U138-MG glioma cell proliferation. In addition, 100 mM adenosine increases cell proliferation by 25%. AMP (1 mM and 3 mM) decreased U138-MG glioma cell proliferation by 29% and 42%, respectively. Taken together, these results suggest the participation of ecto-5′-NT/CD73 in cell proliferation and that this process is dependent upon the enzyme's production of adenosine, a proliferative factor, and removal of AMP, a toxic molecule for gliomas.

Functional distribution of ecto-enzymes involved in extracellular adenosine generation in rat intestinal smooth muscle cells

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Background. Adenosine participates to the physiological regulation of gut motor functions. CD73/ecto-5'nucleotidase and ecto-adenosine deaminase (ecto-ADA) directly control extracellular adenosine levels. The purpose of this study was to assess CD73 and ecto-ADA expression and their functional role in adenosine metabolism in intestinal smooth muscle cells (ISMCs). Methods. ISMCs were prepared by primary explant technique from freshly isolated longitudinal muscle of rat ileum. The formation of AMP metabolites in ISMCs incubated with exogenous AMP (50 micromol/L) was monitored by HPLC. CD73 and ADA were detected by immunoprecipitation and/or immunofluorescence. Results. In ISMCs, exogenous AMP metabolism was rapid (t1/2=4.1min) and associated with a marked increase in adenosine, without affecting inosine levels. Increasing AMP concentration up to 100 micromol/L did not alter CD73 kinetics. Pretreatment with alpha,beta-methylene-ADP (200 micromol/L) or concanavalin A (0.1 milligram/mL) reduced AMP clearance to a similar extent (t1/2= 9.9 and 9.7min, respectively), in parallel with adenosine formation. Moreover, levamisole (10 millimol/L) did not modify AMP levels in the incubation medium. Immunofluorescence showed CD73 surface expression, partially colocalized with vinculin and ZO-1 at the level of intercellular junctions. In contrast, ADA staining appeared mainly in the cytosolic compartment. Immunoblotting revealed CD73 presence also in freshly isolated enterocytes and longitudinal smooth muscle. Conclusion. CD73 appears to be responsible for generation of extracellular adenosine from exogenous AMP and an important way for cell-cell and cell-environment interactions. Ecto-ADA may represent a catabolic pathway to prevent long-

lasting increases of extracellular adenosine. Thus, CD73 and ecto-ADA are critical points for extracellular adenosine regulation in ISMCs.

Synthesis and characterization of nucleotide mimetics as inhibitors of ecto-5′-nucleotidase (CD73) and NTPDase1 (CD39)

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Background. A major source of extracellular, P1 receptor-activating adenosine is the hydrolysis of extracellular ATP by ectonucleotidases. NTPDases1, 2, 3, and 8 or NPP1-3 are dominant ectonucleotidases that hydrolyze the P2 receptor agonists ATP and/or ADP leading to final production of AMP. AMP can then be further dephosphorylated by ecto-5′-nucleotidase (ecto-5′-NT, CD73) yielding adenosine. By regulating the levels of the ligands for P1 and P2 receptors, these enzymes may regulate a variety of (pathological) functions such as cancer progression.
Methods. A series of pyrimidine and purine nucleotide mimetics was synthesized. The phosphate chain was replaced by dipeptides, consisting of an acidic C-terminal amino acid and an omega-amino acid. The benzyl- or ethyl-protected dipeptides were coupled to the nucleoside-5′-position, that had been oxidized to a carboxylic acid function, via an amide linkage. Hydrogenolysis of the amino acid benzyl esters also led to a hydrogenation of the uracil 5,6-double bond yielding 5,6-dihydrouracil nucleotide mimetics. Alkaline hydrolysis of the ethyl esters yielded uracil derivatives. The new compounds were investigated for their potency to inhibit rat ecto-5′-NT and human NTPDase1 using a recently developed capillary electrophoresis-based enzyme assay.
Results. Among the 5,6-dihydrouracil nucleotide mimetics selective inhibitors of ecto-5′-NT with submicromolar Ki values were identified, while one of the compounds was a dual inhibitor of ecto-5′-NT and NTPDase1. Nucleotide mimetics containing the natural, non-hydrogenated uracil nucleobase were generally less potent enzyme inhibitors. These inhibitors showed no affinity for P2Y2, 4, 6, 12 receptors.
Conclusion. We identified N-[4-((2S,3R,4S,5R)-5-(2,4-dioxo-3,4,5,6-tetrahydropyrimid-1(2H)-yl)-3,4-dihydroxytetrahydrofuryl-2-carboxamido)butyryl]-(S)-aspartic acid (PSB-08193) as a new, potent, selective, and metabolically stable inhibitor of rat ecto-5′-NT.

Cholinergic nerve hyperactivity in obstructed human bladders: on the role of sustained P2x purinoceptors activation

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Contractions of normal human bladder are exclusively mediated by acetylcholine (ACh) via muscarinic M_3 receptors. Nerve-evoked ATP release acting via P2X receptors may also regulate bladder contractility, particularly under pathological conditions such as bladder outlet obstruction. This prompted for a new (purinergic) target for pharmacological manipulation of bladder overactivity. There is, however, a gap of information concerning the mechanisms of extracellular ATP inactivation and whether purines control the release of ACh release from cholinergic nerve terminals in obstructed bladders. Human bladder samples were collected from patients with outflow obstruction submitted to surgery and from cadaveric organ donors. Ethics Committees of HGSA and ICBAS-UP approved all the procedures. Isometric contractions and [^{3}H]-ACh release were measured from stimulated (10 Hz, 200 pulses) detrusor strips. The kinetics of ATP (30 μM) hydrolysis and metabolites formation was evaluated by HPLC.

PPADS (10 μM)-sensitive contractions induced by ATP (0.01–3 mM) were more powerful in obstructed bladder strips (1.13± 0.24 mN/g, n=28) than in controls (0.16±0.05 mN/g, n=6), while the opposite was observed when comparing the effect ACh (0.1–100 μM). The total amount of [^{3}H]-ACh released increased from 11.1±0.2x10^3 DPM/g to 26.7±2.7x10^3 DPM/g in obstructed bladder samples. Desensitization of P2X receptors with α,β-methylene ATP (30 μM) reduced [^{3}H]-ACh release by 32±1% (n=4) and 58±8% (n=4) respectively in control and obstructed samples. The extracellular hydrolysis of ATP was slower in obstructed bladders (t 1/2=41±7 min, n=4) than in control samples (t 1/2=30±6 min, n=3).
Data indicate that cholinergic nerve hyperactivity may be correlated with decreased hydrolysis of extracellular ATP leading to sustained activation of prejunctional P2X receptors. This mechanism might contribute to bladder instability in obstructed patients.

Co-expression of ecto-5′-nucleotidase/CD73 with NTPDases in the liver distinctly regulates adenosine formation

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Background. Extracellular nucleos(t)ides modulate various liver functions upon activation of specific P1 and P2 receptors. The concentrations of hepatic extracellular nucleos(t)ides are controlled mainly by nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39), NTPDase2/CD39L1, NTPDase8 and ecto-5′-nucleotidase/CD73. NTPDases hydrolyze ATP and ADP to AMP which is converted into adenosine by CD73. The purpose of this study was to characterize the impact of the cellular distribution of these hepatic ectonucleotidases on the levels of neighbouring P1 and P2 receptor agonists.
Methods. NTPDase1, 2, 8 and CD73 immunolocalization was performed by confocal microscopy. ATPase activity assays were performed with recombinant enzymes (rat or human) from transfected COS-7 cell lysates and aliquots were collected at different time points and nucleotide contents evaluated by HPLC.
Results. CD73 was co-expressed with each NTPDase, not necessarily in the same cells but in the same structures: with NTPDase1 at the vascular wall level, with NTPDase2 in the periductular/portal area and with NTPDase8 in bile canaliculi. The incubation of ATP with both NTPDase1 and CD73 completely converted ATP into adenosine, whereas NTPDase2 and CD73 combination hardly produced this nucleoside, in agreement with the fact that ATP and ADP, the latter accumulating in the medium, are natural inhibitors of CD73. The ATP hydrolysis in presence of NTPDase8 and CD73 generated a transient accumulation of ADP that led to a delay in adenosine production.
Conclusion. The co-expression of CD73 with NTPDases in specific liver cellular compartments may lead to differential activation of P1 and P2 receptors that may be critical in some physiological and pathological conditions.

Role of purine-converting ecto-enzymes in hypoxia-induced angiogenic phenotype of pulmonary artery adventitial vasa vasorum endothelial cells

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We recently reported that extensive neovascularization of the vasa vasorum network occurs in the pulmonary artery adventitia of chronically hypoxic calves. Given that isolated vasa vasorum endothelial cells (VVEC) exhibit a unique pro-angiogenic phenotype with an augmented sensitivity to extracellular ATP and ADP, these studies were undertaken to test the hypothesis that nucleotide-converting ecto-enzymes might contribute to angiogenic phenotype of VVEC via regulation on local nucleotide concentrations. By using TLC assay, we found that cultured VVEC hydrolyze various 3H-labelled nucleotides with relative ATPase/ADPase/AMPase ratios of ~4.4/9.3/1 and in addition, display high ecto-adenylate kinase activity. Measurement of purine-converting activities in VVEC isolated from hypoxic vessels revealed significant decreases in ADPase and ecto-5′-nucleotidase activities by 30 and 45% respectively, whereas ATPase and adenylate kinase remained at the same level as in control cells. Noteworthy, decreased ecto-ADPase activity in hypoxic VVEC is consistent with potent

pro-angiogenic effects of extracellular ADP in these cells. Furthermore, in VVEC isolated from both control and hypoxic pulmonary arteries, ATP- and ADP-hydrolyzing activities are much lower than in pulmonary artery endothelial cells and lung microvascular endothelial cells, while adenylate kinase in VVEC is much higher. These findings are consistent with more potent mitogenic effect of exogenous nucleotides in VVEC. Together, these results suggest that endothelial ATP/ADP-inactivating ecto-enzyme nucleoside triphosphate diphosphohydrolase (NTPDase), in concert with ecto-5′-nucleotidase/CD73 and adenylate kinase, may contribute to pro-angiogenic phenotype of VVEC and that nucleotide-dependent angiogenic activation of VVEC observed under hypoxic conditions may be due to down-regulation of ecto-ADPase/NTPDase and ecto-5′-nucleotidase activities.

Novel and specific inhibitors of ectonucleotidases

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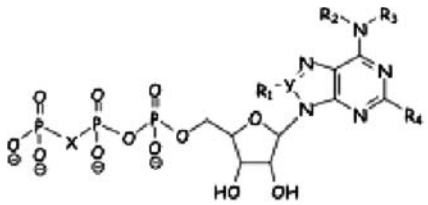
NTPDase inhibitors may be valuable to potentiate the effect of P2 agonists, by preventing the hydrolysis and subsequent inactivation of nucleotides. To study the structure-activity relationship of ATP analogues at NTPDase and to identify more potent and enzyme-sub-type selective inhibitors we synthesized and evaluated a series of novel ATP-derivatives, analogues 1-8 modified both at the phosphate chain and the base. To evaluate the importance of N6-substition we synthesized analogues 1-4. 8-BuS-ATP is the most potent inhibitor found thus far. Therefore, we evaluated two 8-BuS-ATP analogues 5-6 where the beta,gamma-bridging oxygen is replaced by NH2 or CH2 groups. The hydrolysis rate of 2-BuS-ATP by NTPDase1 is 50%. Therefore, here we synthesized 2-substituted ATP analogues with beta,gamma-CH2 bridge 7-8. All N6-substituted ATP derivatives (1-4) were hydrolyzed up to 90% of the rate of ATP hydrolysis and were poor inhibitors of NTPDases1,2,3,8. 8-BuS-beta,gamma-imido-ATP (5) was not hydrolyzed by NTPDases and inhibited NTPDase1 by about 60%. 8-BuS-beta,gamma-CH2-ATP (6) was a poorer inhibitor, inhibiting NTPDase1 by ca. 40%. 2-HexS-beta,gamma-CH2-ATP (8) was not hydrolyzed by NTPDases either, and inhibited selectively the rate of ATP hydrolysis NTPDase2 by 70%. 8-Aza-2-HexS-ATP (7) gave similar results. None of the analogues inhibited NTPDase8. In conclusion, we found that the most selective inhibitors of NTPDase1 and NTPDase2 are 8-BuS-beta,gamma-imido-ATP (6), 2-HexS-beta,gamma-CH2-ATP, respectively (8).



(1) R_1 = H; R_2 = Me; R_3 = H; R_4 = H; X = O; Y = C
(2) R_1 = H; R_2 = Me; R_3 = Me; R_4 = H; X = O; Y = C
(3) R_1 = H; R_2 = Et; R_3 = Et; R_4 = H; X = O; Y = C
(4) R_1 = H; R_2 = Bz; R_3 = Bz; R_4 = H; X = O; Y = C
(5) R_1 = BuS; R_2 = H; R_3 = H; R_4 = H; X = NH; Y = C
(6) R_1 = BuS; R_2 = H; R_3 = H; R_4 = H; X = CH_2; Y = C
(7) R_1 = --; R_2 = H; R_3 = H; R_4 = HexS; X = CH_2; Y = N
(8) R_1 = H; R_2 = H; R_3 = H; R_4 = HexS; X = CH_2; Y = C

Anthraquinones: structure-activity relationships as inhibitors of ecto-nucleoside triphosphate diphospho-hydrolases (E-NTPDases)

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Background. The ecto-nucleoside triphosphate diphosphohydrolases (EC 3.6.1.5) represent a major and ubiquitous family of ecto-nucleotidases. Inhibitors of ecto-nucleotidases could thus represent valuable tools for amplifying the biological effects induced by extracellularly released nucleotides. Reactive Blue 2 (RB-2) had been characterized as a relatively potent E-NTPDase inhibitor with some selectivity for NTPDase3.

Methods. In search for the pharmacophore and to analyze structure-activity relationships we synthesized and investigated a series of truncated derivatives and analogs of RB-2, including 1-amino-2-sulfo-4-ar(alk)ylaminoanthraquinones, 1-amino-2-methyl-4-arylamino-anthra-quinones, 1-amino-4-bromoanthraquinone 2-sulfonic acid esters and sulfonamides, and bis-(1-amino-4-bromoanthraquinone) sulfonamides, at preparations of rat NTPDase1, 2 and 3 using a capillary electrophoresis assay. Several 1-amino-2-sulfo-4-ar(alk)ylaminoanthra-quinone dervatives inhibited E-NTPDases in a concentration-dependent manner.
Results. The 2-sulfonate group was found to be required for inhibitory activity, since 2-methyl-substituted derivatives were inactive. 1-Amino-2-sulfo-p-chloro-anilino-anthra-quinone was identified as a non-selective competitive blocker of rat NTPDases1, 2 and 3 (PSB-069, Ki 16–18 μM), while 1-amino-2-sulfo-4-(2-naphthyl-amino)-anthraquinone was a potent inhibitor with preference for NTPDase1 (Ki 0.328 μM) and NTPDase3 (Ki 2.22 μM). Its isomer, 1-amino-2-sulfo-4-(1-naphthylamino)anthraquinone (PSB-06126), was a potent and selective inhibitor of rat NTPDase3 (Ki 1.5 μM).
Conclusion. We have investigated 25 anthraquinone derivatives related to RB-2 for their potency at inhibiting rat NTPDases1, 2 and 3 in order to obtain initial information about their structure-activity relationships. Certain 1-amino-2-sulfo-4-arylaminoanthraquinones were identified as potent NTPDase inhibitors.

Long-term facilitation of spontaneous astrocytic calcium oscillations with endogenous adenosine in the hippocampus of the rats

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Astrocytes express various purinergic receptors, including adenosine and ATP receptors, in the hippocampus. Astrocytes exhibit spontaneous calcium oscillations and calcium wave propagations. The role of purinergic receptors in the spontaneous activity of astrocytes, however, has not been yet identified. To analyze the physiological role of purinoceptors in spontaneous astrocytic calcium oscillations in situ, fluorometric measurements using Fluo-4 with a fluorescent microscope were made from hippocampal organotypic slice cultures obtained from postnatal rat pups.
Application of ATP (100 μM) for one minute induced an increase in the frequency of spontaneous calcium oscillations in astrocytes for over 20 minutes. A long-term and significant increase in the frequency of calcium oscillations was mimicked by a one-minute application of adenosine (100 μM) or the non-metabolizable adenosine analogue (NECA; 100 μM), and inhibited by the non-selective adenosine A(1)/A(2) receptor antagonist (CGS15943; 30 μM) and a selective adenosine A (2B) receptor antagonist (MRS1754; 10 μM). The NECA-induced long-term facilitation of the frequency of calcium oscillations was reduced by adenosine deaminase, the ecto-ATPase inhibitor (ARL67156; 100 μM) and the ecto-5′-nucleotidase inhibitor (alpha, beta methyleneADP; 100 μM).
These results suggest that activation of adenosine A(2B)-like receptors modulates the spontaneous astrocytic calcium oscillations with endogenous adenosine produced via extracellular breakdown from ATP in the hippocampus of the rats.

Thienopyridines as specific inhibitors of NTPDase1

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Background:
The thienopyridine drugs ticlopidine (Tyklid) and clopidogrel (Plavix) are antiplatelet compounds that exert their effect by irreversibly inhibiting platelet P2Y12 receptors but only once they have been converted to the active forms by the liver. Recently we showed that these pro-drugs inhibited NTPDase1 activity. Here we tested the specificity of these pro-drugs towards human and murine NTPDases and ecto-5′-nucleotidases.

Methods:
Activity assays, with and without thienopyridines, were carried out by Pi determination using both intact cells and protein extracts from transfected COS-7 cells as enzymes sources. Intact human umbilical vein endothelial cells (HUVEC) and human liver and pancreas tissues in situ were also tested.
Results:
Both pro-drugs inhibited NTPDase1 but not NTPDase2, 3 or 8. The concentration that produces 50% of the maximal inhibition (IC50app) was 40 microM for ticlopidine and 76 microM for clopidogrel. The activity of the native NTPDase1 at the surface of HUVEC was inhibited at about 75% by 60 microM ticlopidine and 81% by 20 microM clopidogrel. These results were supported by activity assays with intact cells transfected with human NTPDase1 where ADPase activity was totaly abolished by 100 microM ticlopidine or 60 microM clopidogrel.
At 1 mM they also affected the activity of rat, but not human, ecto-5′-nucleotidase. The inhibition was also confirmed in situ where NTPDase1 activity could be discriminated from the other ectonucleotidases.
Conclusion:
Both thienopyridines are potent and specific NTPDase1 inhibitors. Ticlopidine, due to better solubility and stability at neutral pH than clopidogrel, appears as a good inhibitor of NTPDase1.

Specific NTPDases' inhibitors

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Background:
Ectonucleotidases regulate the concentration of extracellular nucleotides and, in extension, their effects on P2 receptors. These enzymes differ in substrates' affinity and avidity. Potent and specific inhibitors to study these enzymes, as well as nucleotides' signalling, are still lacking. Here, we present the preliminary characterization of novel adenosine derivatives and various other molecules, commonly used as ectonucleotidases' inhibitors or P2 receptor antagonists, as NTPDases' inhibitors.
Methods:
Enzymatic and kinetic assays were performed with the Malachite Green method using recombinant proteins of NTPDase1, 2, 3, 8 or ecto-5′-nucleotidase incubated with 100 microM substrates. Qualitative analyses were also performed by histochemical techniques with sections from human livers and pancreases analysed by light microscopy.
Results:
At the concentration of 100 microM 8BuS derivatives of AMP and ADP were most specific inhibitors of NTPDase1 with inhibition ranging from 70 to 85%. Other NTPDases were not affected by these molecules and ecto-5′-nucleotidase activity was inhibited at about 50%. The analogue 2HxATP with a methylene group between the beta and gamma phosphates was a selective inhibitor of NTPDase2 (70% inhibition). IC50app of these respective inhibitors were 35 microM, 28 microM and 39 microM respectively. In addition, a monoclonal antibody that we have generated against human NTPDase3 specifically inhibited this enzyme.
Conclusion:
Novel molecules have been identified as specific inhibitors of NTPDase1, 2 and 3. Further assays on P2 receptors will tell if these molecules can be considered as biochemical and pharmaceutical tools to study both extracellular nucleotides' signaling and the functions of these ectonucleotidases.

NTPDase1 modulates $P2X_7$ responses in murine peritoneal macrophages

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Background:
Nucleoside triphosphate diphosphohydrolase-1 (NTPDase1), also known as CD39, is the dominant ectonucleotidase responsible for the clearance of extracellular nucleotides at the surface of murine peritoneal macrophages; it accounts for over 85% of their total ATPase activity. Here we investigated the potential function(s) of this enzyme in mouse macrophages.
Methods:
Macrophages were collected 4 days after intraperitoneal injection of 3% thioglycollate medium. Expression of NTPDase1 and P2 receptors was determined by RT-PCR, flow cytometry and immunoblotting. IL-1beta release was quantified by ELISA. ATP-induced death was evaluated by trypan blue exclusion. The mouse "air pouch" model was used to confirm some in vitro experiments.
Results:
Entpd1$^{-/-}$ macrophages were more susceptible to 2 mM ATP-induced death as 77±7% compared to 12±8% for *entpd1*$^{+/+}$ macrophages died. This difference vanished at concentration ≥5 mM of ATP. P2 receptor expression was similar in both *entpd1*$^{-/-}$ and *entpd1*$^{+/+}$ macrophages. IL-1beta release, from macrophages pre-stimulated with various PAMPs (LPS, Pam_3CSK_4, flagellin) followed by ATP (≥1 mM), was also increased in *entpd1*$^{-/-}$ macrophages. $P2X_7$ receptor antagonists or apyrase protected macrophages from ATP-induced death and blocked IL-1beta release, while ivermectin ($P2X_4$ potentiator) had no effect. In the air pouch model, 4 hours after injection of 100 ng/mL LPS, higher level of IL-1beta were detected in *entpd1*$^{-/-}$ (46±4 pg) compared to *entpd1*$^{+/+}$ (20±2 pg) macrophages ($p \leq 0.001$).
Conclusion:
These results show that NTPDase1 controls ATP-induced death and IL-1beta release in murine peritoneal macrophages by modulating $P2X_7$ activation. This suggests a role of NTPDase1 in early inflammatory responses and pathologies associated with IL-1beta.

Ecto- and exo-enzymes involved in purine metabolism during in vitro incubation of rat ileum

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Background. Considering the role of adenosine (Ado) and its precursors in the pathophysiology of gastro-intestinal tract, we investigated the activity of ecto-enzymes involved in purine metabolism, and their release during in vitro incubation of rat ileum.
Methods. Longitudinally cut strips of distal ileum from male Wistar rats were incubated at 37 °C in 2 ml of aerated (95% O2, 5% CO2) Tyrode solution. Conditioned medium was obtained by removing the tissue after a 30 minutes incubation. ATP, cAMP, AMP or Ado (50 micromolar) were added in the absence or in the presence of enzyme inhibitors. Purine nucleotides and their metabolites were quantified by HPLC.
Results. Exogenous Ado, AMP and ATP (50 micromolar) are fully metabolized within 60 minutes by rat ileum as well as by conditioned medium. By contrast, cAMP concentration is reduced by 50% in the presence of ileum strips but not in conditioned medium. No spontaneous degradation occurs during incubation in fresh medium. The adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (1 micromolar) and the ecto-5′-nucleotidase (ecto-5′-Nu) inhibitor, alpha,beta-methylene-ADP (200 micromolar) allow total recovery of Ado and AMP after 1 and 5 minutes of incubation, respectively. Suramin, a partial inhibitor of ATPases (100 micromolar), does not affect ATP metabolism.
Conclusions. In rat ileum both ADA and 5′-Nu exist as ecto- and exo-enzymes. This also holds true for ATPases, which are not sensitive to inhibition by suramin. cAMP phosphodiesterase (cAMP-PDE) exists as ecto-enzyme but is not released by the tissue.

Localization of the ecto-NTPDases in the murine reproductive tract

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BACKGROUND: Extracellular nucleotides act as autocrine and/or paracrine signalling mediators in most tissues, including those from the reproductive system. Such purinergic signalling is influenced by certain nucleoside triphosphate diphosphohydrolases (NTPDases) expressed on the plasma membrane of various cell types, namely NTPDase1, 2, 3 and 8. The purpose of this study was to identify and localize the expression of these NTPDases in the mouse reproductive system.
METHODS: The general tissue distribution of NTPDases was evaluated by Western blot (for the presence of the proteins) and by the malachite green technique (active enzymes). The precise cellular localization of NTPDases was assessed by immunohistochemistry (proteins) and by histochemistry (active enzymes).
RESULTS: Typical isoforms of NTPDase1 and 2 are highly expressed in the female and male reproductive tract. Aside from the expected expression on blood vessels and smooth muscle cells, NTPDase1 was detected in interstitial cells in the testis and in granulosa cells in select ovarian follicles. NTPDase2 expression was localized to connective tissue and, interestingly, in the seminiferous epithelium in association with some spermatogenic cells. NTPDase3 was expressed by secretory epithelial cells in the male reproductive system as well as by oviductal epithelium. NTPDase8 was not detected in any tissues studied here.
CONCLUSION: Differential localization of the plasma membrane bound NTPDases in the reproductive system has been shown. Our studies suggest close regulation of extracellular nucleotide levels in the genital tracts by NTPDases, that in turn, may affect specific biological functions in this system.

Generation and characterization of a monoclonal antibody as the first specific inhibitor of human NTPDase3

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Background: The study and potential therapeutic modulation of purinergic signaling is hindered due to the lack of specific inhibitors for nucleoside triphosphate diphosphohydrolases (NTPDases), the terminating enzymes for these processes. In addition, little is known of the NTPDase protein structural elements that affect enzymatic activity and could be used as targets for inhibitors. Here we report the generation of the first inhibitory monoclonal antibodies specific for human NTPDase3.
Methods: Antibody specificity was assessed by Western blot, flow cytometry and immuno(cyto/histo)chemistry. Inhibition assays were performed using recombinant NTPDases from protein extracts and intact transfected cells, and human pancreas sections. Epitope determination was performed using mutants of human NTPDase3 and chemical cleavage by cysteine substitution mutagenesis.
Results: The monoclonal antibodies inhibit exclusively human NTPDase3 by about 75%. Antibody recognition is attenuated by denaturation with SDS, and abolished by reduction with DTT, indicating the significance of the native conformation and the disulfide bonds in NTPDase3 for epitope recognition. The SDS-resistant parts of the epitope are located in two fragments, Leu220-Cys347 and Cys347-Pro485, both required for antibody binding. In the first fragment, Ser297 is likely to directly interact with antibody. In the second fragment, the principal part of the epitope is likely located near the disulfide bond Cys399-Cys422.
Conclusion: We identified the first inhibitory antibodies of an NTPDase and partially defined their epitope. These monoclonal antibodies are likely to be a valuable tool for both biochemical studies and for modulation of purinergic processes controlled by NTPDase3, including insulin secretion by pancreatic islet cells.

Distinct Biochemical Microenvironments for Airway Adenosine in Cystic Fibrosis and Asthma

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Background. Adenosine (ADO) is a signaling molecule mediating key aspects of airway defenses, including inflammatory responses and mucus clearance. A critical role is emerging for chronically-elevated ADO in lung diseases, but the underlying mechanisms are poorly understood. This study compares the regulation of airway ADO by sputum and epithelial surfaces from healthy subjects and patients with cystic fibrosis (CF) or asthma. Methods. Sputum samples were analyzed for inflammatory cells, IL-8 and nucleotide composition, and ADO deaminase activity (ADA_1: ADO → inosine). Bronchial epithelial surfaces were assayed for nucleotide composition, ecto 5′-nucleotidase (ecto 5′-NT: AMP → ADO) and ADA1 activity. Results. While both diseases raise sputum ATP and AMP levels by > 4-fold, ADO was also elevated in asthma, but reduced 6-fold in CF. On epithelial surfaces, there was no significant difference in ADO concentrations among groups, despite the elevated ecto 5′-NT/ADA1 activity ratios in both CF and asthma. However, stress-induced ATP release established chronically-elevated ADO in both diseases. The discrepancy between sputum and epithelial AMP/ADO ratios, in CF, may result from neutrophil dominance (>90%), compared to macrophage dominance (>65%) in asthma. Incidentally, neutrophil and IL-8 correlated positively with AMP, but negatively with ADO in all groups. Since neutrophils lack the ability to convert extracellular AMP into ADO, CF sputum may constitute a distinct biochemical microenvironment for airway ADO. Conclusion. This study suggests that chronic obstructive diseases, dominated by neutrophil infiltrations, would exhibit regional differences in airway ADO concentrations along the airways, and between sputum and epithelial surfaces.

Cerebrospinal fluid nucleotide pyrophosphatase/phosphodiesterse (NPP) activity: A preliminary report

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Brain expresses several members of ecto-nucleotide pyrophosphatase/ phosphodiesterase (E-NPP) family. NPP1 ectoenzyme is located in plasma and synaptic membranes while NPP2 is secreted into cerebrospinal fluid (CSF). NPP1 hydrolyzes ATP and diadenosine polyphosphates (ApnA), hence regulating nucleotide signalling. NPP2 is identical to the tumour cell motility-stimulating protein autotaxin (ATX) which also displays lysophospholipase D activity. However, properties and functions of CSF NPP2/ATX remain poorly known. Thus we undertook characterization of native CSF NPP activity.
Photometric assays detecting p-nitrophenol enzymatically released from the substrates TMPpnp, pNppp and bis-pNpp, and fluorimetric assays to detect cleavage of the fluorogenic ApnA-derivatives, di-ethenoadenosine polyphosphates were used. CSF hydrolyzed the substrates TMPpnp, pNppp and bis-pNpp (1 mM) at alkaline pH. Enzymatic hydrolysis was strongly inhibited by the potent NPP2/ATX inhibitors lysophosphatidic acid (IC50 0.03 microM) and sphingosine 1-phosphate (0.6 microM), but unaffected by ApnA. P2 receptor antagonists suramin and the related compound NF279 displayed diverse behaviours depending on the substrate. Suramin (IC50 5 microM) and NF279 (IC50 0.5 microM) were potent inhibitors of TMPpnp hydrolysis. However, suramin weakly inhibited pNppp hydrolysis (IC50>50 microM) while NF279 (0.5–2 microM) activated it. Against bis-pNpp, suramin was an inhibitor (IC50 10 microM) but not NF279. NF449 behaved as a weak inhibitor (IC50>50 microM) against all substrates while NF023 did not display noticeable effects. Di-ethenodenosine polyphosphates, useful NPP1 substrates, were not cleaved by CSF.
Results indicate that NPP2/ATX accounts for most of CSF NPP activity and suggest that suramin and NF279 effects on NPP2/ATX could be exploited to investigate enzyme-substrate interactions.

Regions of growth are regions of highest release of ATP and highest expression of ectonucleotidases AtAPY1 and AtAPY2 in Arabidopsis

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Among the seven members of the apyrase (NTPDase) family in Arabidopsis thaliana, two of them, AtAPY1 and AtAPY2, share 87% sequence identity and play a major role in growth control. Double-knock out mutants atapy1atapy2 are lethal because pollen from these mutants cannot germinate or grow. Complementation of these mutants with wild-type apyrase genes under control of a pollen-specific promoter allows pollen to germinate, leading to normal fertilization, seed formation and germination, but plants from these seeds are extreme dwarfs and infertile. Expression of AtAPY1 and 2 is highest in rapidly growing tissue and tissue exposed to high levels of extracellular ATP (eATP), e.g., at wound sites. Because plant growth requires the delivery of secretory vesicles to growth sites, and secretory vesicles typically have a high [ATP], these data suggest that apyrase expression is highest where the rate of ATP release into the ECM is highest. To test this hypothesis, we generated transgenic plants expressing an extracellular luciferase and used these to assay relative levels of eATP in growing tissues. This assay revealed that regions of most rapid growth and/or secretion in roots corresponded to regions of highest release of ATP and highest expression of AtAPY1 and 2. Pollen germinates and grows rapidly in culture, and after pollen germination, suppression of AtAPY1 and 2 activity by polyclonal antibodies results in a transient increase in [eATP] and subsequent suppression of pollen tube growth. These data suggest there is an eATP-induced signaling pathway leading to growth control in plants. We are using an inducible RNAi construct to suppress the expression of AtAPY1 and 2 and assess the gene changes and other signaling steps that connect this suppression to changes in growth. Supported by NSF grant to SR.

A retroviral approach to address the role of nucleotide signaling in the control of proliferation in adult neural stem cell cultures

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During adult neurogenesis cells pass through a series of stages leading from multipotent neural stem cells (NSC) to proliferating progenitors and finally to mature neurons and glial cells. These transitions are controlled by signalling cues from the stem cell niche. Recent work implicates signals acting on nucleotide receptors in this process.
To further investigate the role of nucleotide signalling pathways we applied a culture model of adult NSCs using adherent conditions. To interfere with protein expression, we used the transgenic mouse line Tg(GFAP-TVA)5Hev/J which harbours a copy of the quail tva-receptor-gene driven by a 5 kbp-fragment of the human GFAP-promoter. Tva-positive cells are highly susceptible to infection with the avian RCAN(BP)A-virus. This approach enables us to target more than 50% of cells in proliferating cultures for either knockdown or overexpression of proteins.
To address the functional role of ectonucleoside triphosphate diphosphohydrolase 2 and tissue non-specific alkaline phosphatase on the proliferation or differentiation of NSC-cultures we introduced shRNA-expression cassettes into the viral vector, thereby abrogating the associated protein function. In addition we induced forced expression of nucleotide receptors (P2Y1, P2Y2) to study their effects on precursor proliferation and differentiation.
Our experiments demonstrate the advantage of viral infection over prevalent transfection protocols and open the prospect of reliably establishing stable gain-of-function and loss-of-function conditions in highly proliferative NSC-cultures. Furthermore, they imply a functional role of nucleotides in the control of adult neurogenesis.

Crystal structure and catalytic function of NTPDase2

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In addition to the receptors involved in purinergic signalling, the ecto-nucleotidases are promising drug targets as well as protein drugs themselves. To facilitate the rational development of specific inhibitors of NTPDases for pharmaceutical and biological studies, as well as for a better characterization of the catalytic mechanism, we have determined the crystal structure of an NTPDase ectodomain. Following E. coli expression, refolding, purification and characterization of the ectodomains of rat NTPDases 1–3, NTPDase2 could be crystalized [1]. In agreement with previous modelling studies [2], the active site is located at the interface between the two domains of the actin/hsp70/sugar kinase superfamily fold [3]. Co-crystal structures with products and substrate analogs suggest a mechanism, in which a water molecule deprotonated by Glu-165 attacks the nucleotide′s terminal phosphate group, which is positioned by coordination to a divalent metal ion. The specificity for ATP and ADP is achieved by an alternative binding mode of the alpha-phosphate group. An analysis of sequence diversity among different NTPDases in the active site region suggest that the development of type-specific inhibitors might be a feasible task.

Overexpression of airway CD39 in transgenic mice enhances lipopolysaccharide-induced inflammation

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Background: Several in vitro studies indicate that activation of airway P2 receptors by extracellular ATP may modulate innate immunity and pathogen-induced inflammatory responses. Clearance of ATP by ectonucleotidases likely plays a central role in the control of inflammation as accelerated ATP metabolism occurs in chronic infectious lung diseases. Our study aimed at investigating the role of mucosal ATP in innate defense against bacterial infection.
Methods: We have generated transgenic mice overexpressing the human ectonucleoside triphosphate diphosphohydrolase CD39 (NTPDase 1) under the control of the airway-specific Clara cell 10-kDa protein gene promoter.
Results: Immunohistochemistry verified lung-specific overexpression of the transgene. Luciferase assays and reversed-phase HPLC performed on bronchoalveolar lavage (BAL) fluids of transgenic mice indicated increased lung ectonucleotidase activity as compared to wild-type samples. These mice did not show any spontaneous lung inflammation. However, intratracheal instillation of E. coli lipopolysaccharide (LPS) revealed accelerated recruitment of neutrophils in the airways of transgenic mice whereas macrophage counts remained identical.
Conclusions: The mCC10-hCD39 transgenic mice develop enhanced airway inflammatory response upon bacterial challenge. They constitute new tools to further investigate the role of mucosal ATP in airway innate immunity as well as to evaluate nucleotide therapeutic potentials in chronic lung infectious diseases.

CD73-generated adenosine regulates thrombomodulin expression

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Background: Thrombomodulin (TM), an endothelial cell surface protein, serves as a thrombin modulator. When bound to TM, thrombin's substrate specificity changes dramatically, and it preferentially cleaves protein C, rather than triggering clot formation. In addition to inhibiting coagulation, TM also decreases inflammation and vascular permeability. As TM expression is regulated by cAMP, we asked whether adenosine generated by CD73, an ecto-enzyme that dephosphorylates AMP, can regulate TM expression via adenosine receptor signaling.
Methods/Results: Mouse endothelial cells were cultured with the general adenosine receptor agonist NECA and TM expression was measured. Protein levels increased 2-fold in response to NECA; mRNA levels increased 4-fold. Protein and mRNA levels also increased after AMP treatment and these increases were abrogated by a 5′-nucleotidase inhibitor. Treatment of mice with NECA resulted in 4- to 6-fold increases in TM mRNA in lung, heart, and liver. NECA-treated mice

also showed a two-fold increase in the generation of activated protein C when injected with thrombin, demonstrating that the NECA-induced increase in TM expression translated into increased TM function.
TM expression is known to be down regulated during inflammation. To determine whether CD73-generated adenosine can modulate this down regulation, wild type and $CD73^{-/-}$ mice were treated with LPS and TM expression was measured in lung tissue. TM expression was substantially more down-regulated in the lungs of $CD73^{-/-}$ mice.
Conclusions: CD73-generated adenosine can modulate TM expression both in vitro and in vivo. The anti-inflammatory action of CD73 may be mediated in part by maintenance of TM expression.

(Invited)

Regulatory mechanisms of purinergic signaling in the vasculature in normal and pathological states

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Extracellular nucleotides and adenosine are important signaling molecules known to be implicated in an array of cell-specific responses both in an autocrine and paracrine fashions. Current models of nucleotide turnover include (i) release of ATP, ADP and other agonists; (ii) triggering of signalling events via a series of ligand-gated (P2X) and metabotropic (P2Y) receptors; (iii) nucleotide inactivation by membrane-bound and soluble enzymes; (iv) interaction of the resulting adenosine with own nucleoside-selective receptors; and finally, (v) adenosine deamination and/or uptake by the cells. Despite the significant progress in our understanding of the purinergic machinery as a multistep cascade, current knowledge on the whole sequence of "release-signalling-inactivation" is rather limited. General schemes of nucleotide and nucleoside inactivation in the cardiovascular system have included a role for ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ecto-nucleotide pyrophosphatase/ phosphodiesterase (E-NPP) family, ecto-5′-nucleotidase/CD73 and adenosine deaminase. In contrast to traditional paradigms that focus on inactivating mechanisms, it has now become clear that "classical" intracellular ATP-regenerating enzymes, adenylate kinase and nucleoside diphosphate (NDP) kinase, can be co-expressed on the cell surface and also freely circulate in the bloodstream. Here, I summarize recent advances in this field, with particular emphasis on major nucleotide-converting pathways in the cardiovascular system and on the role of intravascular nucleotides in the immunomodulatory and thromboregulatory responses.

(Invited)

SYMPOSIA 15: ADENOSINE IN THE REGULATION OF SLEEP

Adenosine A1 receptor control of the homeostatic sleep response: functional implications

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During sleep, the mammalian CNS undergoes widespread, synchronized slow wave activity (SWA) that directly varies with prior waking duration(Borbely, 1982;Dijk et al., 1990). When sleep is restricted, an enhanced SWA response follows in the next sleep period. Enhanced SWA has been associated with improved cognitive performance(Huber et al., 2004), but it is not clear whether a selective reduction of SWA is associated with decreased performance. Here we show a conditional, CNS knockout of the adenosine receptor, AdoA1R gene, selectively attenuates the enhanced SWA response to restricted sleep, but not sleep duration. During sleep restriction, wild phenotype animals, express a large enhancement of SWA and pre-sleep restriction performance of working memory is maintained. However, the knockout animals show a reduced capacity for both the enhancement SWA and the maintenance of cognitive function during waking, suggesting a role for enhanced synchronized SWA that includes maintenance of cognitive performance.

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Astrocytic adenosine controls mammalian sleep homeostasis

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Astrocytes are known to modulate synaptic transmission and neuronal activity by the release of chemical transmitters in a process termed gliotransmission. However, the roles of gliotransmission in the control of mammalian behavior are not known. Since SNARE-dependent gliotransmission is known to regulate extracellular adenosine and adenosine exerts sleep promoting effects, we used a conditional genetic system to inhibit gliotransmission to determine if astrocytes play an unsuspected role in sleep regulation. Following sleep deprivation, there is a compensatory increase in total sleep time and sleep continuity that we demonstrate are prevented by the inhibition of gliotransmission. Inhibition of gliotransmission attenuated A1 receptor-dependent inhibition of cortical synaptic transmission and selectively blocked the sleep-suppressing effects of the A1 receptor antagonist CPT. Chronic in vivo administration of CPT to wildtype mice mimicked the transgenic phenotype by preventing the homeostatic response to sleep deprivation. This study provides the first evidence that astrocytes and gliotransmission play an essential role in a complex mammalian behavior, and we identify glial-dependent A1 receptor activation as a critical pathway underlying the effects of sleep deprivation.

(Invited)

Sleep homeostasis: A role for adenosine in humans?

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Sleep is an active state, which is finely regulated. The homeostatic facet of sleep-wake regulation is keeping track of changes in 'sleep propensity' (or 'sleep need'), which increases during wakefulness and decreases during sleep. Increased sleep propensity following sleep deprivation is counteracted by prolonged sleep duration, but also by enhanced sleep intensity as measured by EEG slow-wave activity (SWA, power within ~ 1–4 Hz) in non-rapid-eye-movement (nonREM) sleep. The neurochemical mechanisms of nonREM sleep homeostasis are unknown. Our studies in humans provide compelling and convergent evidence that adenosinergic neurotransmission plays a role in nonREM sleep homeostasis. Thus, a functional polymorphism in the adenosine metabolizing enzyme, adenosine deaminase, contributes to the high inter-individual variability in slow wave sleep duration and intensity. Moreover, the adenosine receptor antagonist, caffeine, attenuates the EEG markers of nonREM sleep homeostasis during sleep, as well as during wakefulness. Finally, adenosinergic mechanisms modulate individual vulnerability to the detrimental effects of sleep deprivation on neurobehavioral performance, and EEG indices of disturbed sleep after caffeine consumption. These convergent findings support an important contribution of adenosine and adenosine receptors to nonREM sleep homeostasis in humans.

(Invited)

The role of the basal forebrain cholinergic cells in regulation of recovery sleep

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Basal forebrain (BF) is one of the sleep-wake rhythm regulating areas in the brain. Cholinergic, GABAergic and glutamatergic cells from this area send projections to cortex to control for vigilance. Previous results from our laboratory have shown that during prolonged wakefulness extracellular concentrations of adenosine and nitric oxide (NO) increase in this area. Further, both these compounds proved to be important in the induction of sleep increase (recovery sleep) after prolonged wakefulness. We hypothesized that the cholinergic cells of the BF would be important in the regulation of recovery sleep. We used IgG-saporin, which specifically destroys cholinergic cells. Baseline sleep and sleep after sleep deprivation, as well as BF extracellular concentrations of adenosine and NO, were measured before the lesion and 2 weeks after the lesion in the same animals. While the untreated animals had normal increase in sleep after sleep deprivation, this increase was absent in the lesioned animals. Also the increases in adenosine and NO were absent in the lesioned animals. We conclude that the BF cholinergic cells are central in the production of recovery sleep and that this response is mediated through adenosine and NO.

(Invited)

SYMPOSIA 16: NUCLEOTIDE REGULATION OF MICROCIRCULATION

Purinergic regulation of cerebral arterioles and hypoxia/reoxygenation injury

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Adenosine Tri-phosphate (ATP) is a potent regulator of cerebro-vascular blood flow. Ischemia, the prolonged cessation of blood flow, followed by the reinstatement of blood flow or reperfusion has long been recognized to lead to tissue damage aptly named Ischemia/Reperfusion injury. Here especially the availability of oxygen during the initial phase of reperfusion is considered a main event in the development of subsequent tissue injury. While numerous studies have demonstrated that after cerebral ischemia blood vessel responses are diminished, it has not been shown if cerebral penetrating arterioles are directly impaired by the Ischemia/Reperfusion (I/R) or if the observed vascular impairment is due to parenchymal factors. If microvessels are a directly impaired due I/R, microvessel may be a target for direct treatment to improve blood flow after I/R injury. The goal of this study was to test if vasomotor responses of isolated and cannulated rat penetrating arterioles are impaired after a hypoxic insult and to elucidate if such an impairment can be alleviated. To simulate I/R we subjected cerebral arterioles to Hypoxia/Reoxygenation (H/RO) injury and observed changes in vessel tone and responses to adenosine tri-phosphate before and after H/RO ex vivo. Possible mechanisms involved in vascular regulation such as NO, oxygen radicals or potassium channel activity were studied. We find that I/R causes vessel constriction and impairs dilation to ATP in penetrating arterioles. Scavenging of reactive oxygen species and sub-threshold calcium-sensitive potassium channel activation can restore dilatory response to ATP. This opens the possibility to restore vascular reactivity after I/R.
Supported by NIH NS30555 and NS32636.

(Invited)

Investigating microvascular regulation of oxygen supply in vivo

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Background: This study explores the role of the red blood cell in regulating oxygen (O_2) supply to tissue to match the tissues O_2 demands.
Methods: Our approach involves applying a systems biology approach in which in vivo experiments provide information that can be used in mathematical simulations to aid in understanding the experimental results and in predicting new factors or mechanisms that need to be tested experimentally. An experimental protocol was developed that tests the local O_2 regulatory system without triggering regulatory mechanisms associated with metabolism. A gas exchange chamber was built in the stage of an inverted intravital video microscope to alter the O_2 environment at the muscle surface (rat extensor digitorum longus) using computer controlled gas flowmeters. The microvascular O_2 regulatory system responded to changes in the percent O_2 in the chamber to compensate for the amount of O_2 supplied to or extracted from the tissue by the chamber. Quantitative experimental data for the computational model was obtained using our functional imaging system which measured the changes in RBC O_2 saturation in surface capillaries and in capillary RBC velocity, hematocrit and supply rate.
Results: We found a time delay between the change in chamber O_2 and the regulatory response that likely corresponds to signal conduction velocity along vascular endothelium from capillaries to upstream arterioles.
Conclusion: Our systems biology approach is enabling us to explore the integration of information needed to regulate tissue oxygenation.
Supported by NIH grant (HL-089125) and a CIHR operating grant to Ellis & Goldman

(Invited)

Erythrocyte-derived ATP and the regulation of skeletal muscle circulation: purinergic receptors and metabolites involved

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ATP release from erythrocytes is thought to play an important role in the regulation of muscle blood flow by matching O2 delivery to the local metabolic demand. In vitro and in vivo observations support this hypothesis by demonstrating that i) erythrocytes release ATP in association with the offloading of O2 from the hemoglobin molecule, ii) plasma ATP is tightly correlated to alterations in O2Hb with exposure to hypoxia, hyperoxia and CO, and iii) ATP, when infused intraarterially, causes both marked muscle vasodilatation and complete inhibition of alpha-adrenergic vasocontriction. ATP exerts its vasodilatory effect directly and not via degradation compounds, as comparison of the relative vasoactive potencies of ATP, and other nucleotides (ADP, AMP), adenosine and UTP, reveals the following rank order of vasoactive potency: ATP (100) = UTP (100) >ADP (2.7) >(1.7). Further, both ATP and UTP, but not ADP, AMP or adenosine infusions, fully inhibit alpha-adrenergic vasoconstriction in a manner similar to exercise and hypoxia. Human skeletal muscle expresses mainly P2Y2 purinergic receptors located in the vascular endothelium. The P2Y4, the only common ATP/UTP receptor, cannot be detected at the mRNA level in skeletal muscle. Therefore, these findings support that erythrocyte-derived ATP acts as an important mediator of O2 sensing transduction between the erythrocyte and the muscle vascular endothelium causing both vasodilatation and sympatholysis by binding to endothelial ATP/UTP selective P2Y2 purinergic receptors and stimulating the vascular endothelium to release vasodilator-sympatholytic substances, including NO, prostaglandins and EDHFs.

(Invited)

Role of P2Y4 nucleotide receptor in angiogenesis and inflammation

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Human P2Y4 receptor is a G-protein coupled nucleotide receptor responsive to UTP. Murine P2Y4 has been described as a receptor responsive to UTP and ATP. We detected P2Y4 mRNAs in primary endothelial cells isolated from mouse heart but not in endothelial cells isolated from mouse lung. Loss of P2Y4 expression in cardiac endothelial cells inhibited their migration as well as their growth in response to UTP and ATP. We also observed by flow cytometry experiments that the increase of VCAM-1 expression on endothelial cells treated by UTP, is strongly inhibited in P2Y40/- primary endothelial cells isolated from heart. This inhibition of VCAM-1 expression was correlated with a strong reduction of monocyte adhesion on P2Y40/- endothelial cells. Moreover, P2Y4-null mice display angiogenesis defects in two in vivo models involving microvascular endothelial cell proliferation after subcutaneous injection of matrigel or gelatine/carmine red mixture. P2Y4 receptor was effectively detected in endothelial cells isolated from matrigel plugs. In conclusion, our study highlights P2Y4 receptor as an important regulator of angiogenesis and inflammation.

Excess adenosine contributes to murine prolonged penile erection (priapism) via A2B adenosine receptor

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BACKGROUND: Priapism is defined as prolonged penile erection occurring unassociated with sexual interest. 40% of male sickle cell disease patients display priapism. The disorder is dangerous given its association with erectile dysfunction. Current treatments are poor due to lack understanding of the pathophysiology of priapism.
METHODS AND RESULTS: We found that male adenosine-deaminase (ADA)-deficient mice, characterized by high circulating levels of adenosine, display features of priapism seen in humans, including spontaneous prolonged penile erection, increased corpus cavernosal strip relaxation following neurostimulation with subsequent penile vascular damage and fibrosis. Reducing the accumulation of adenosine by ADA enzyme therapy relieved priapic activity both in vivo and in vitro. Moreover, the analysis of four adenosine receptor deficient mice revealed that the A2BR is essential for adenosine-dependent penile vascular smooth muscle relaxation and erection. Using primary corpus cavernosal smooth muscle cells, we found that adenosine, as a potent vasodilator, stimulates both cAMP and cGMP production via A2BR activation. Finally we showed that priapic activity in sickle cell disease (SCD) transgenic mice, a well accepted animal model of priapism, is also due to elevated adenosine with activation of the A2BR.

Contribution of annexin ii and plasminogen inhibitor-1 (PAI-1) in the proangiogenic effect of adenosine A2A receptor activation

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Background: The fibrinolytic system is an essential regulator of angiogenesis and wound healing. Because adenosine and its A2A receptor promote wound healing, we examined the effect of the selective adenosine A2A receptor agonist CGS-21680 on production of tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1) and Annexin II, a plasma membrane tPA and plasminogen co-receptor, in human microvascular endothelial cells (HMVEC). Methods: Subconfluent HMVEC were incubated in the presence or absence of 10–6 M CGS-21680 in 0.1% BSA supplemented EBM-2 media for 24 h (37oC, 5% CO2). Conditioned media were analyzed by ELISA and immunoblotting. Whole cell lysates were analyzed by Western Blot or annexin II was immunoprecipitated and the pellets subjected to immunoblotting for tPA. mRNA levels were measured by real time PCR. Results: 10–6 M CGS-21680 significantly diminished PAI-1 secretion by HMVEC supernatants (79,28±12,09 ng/ml, versus control 171,2±9,25 ng/ml, n=7, $p<0.01$), but had no effect on tPA (1,13±0,13 ng/ml, versus control 1,44± 0,11 ng/ml, n=13, p=NS), and uPA (0,75±0,11 ng/ml, versus control 0,79±0,08 ng/ml, n=6, p=NS) levels. In contrast, Western Blot analysis of whole cell lysates revealed that CGS-21680 failed to induce any change in uPA, but increased tPA and annexin II production by HMVEC. CGS-21680 markedly increased tPA levels coimmunoprecipitated with annexin II. Conclusion: These results are consistent with the hypothesis that adenosine A2A receptors regulate the fibrinolytic system and further suggest the novel hypothesis that A2A receptor-mediated regulation of the fibrinolytic system plays a role in the proangiogenic effect of adenosine A2A receptor activation.

TGF-ß1 inhibits adenosine transport in human fetal endothelium

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Background: TGF-ß1 increases the synthesis of nitric oxide (NO), a gas that inhibits adenosine transport via human equilibrative nucleoside transporter 1 (hENT1) in umbilical vein endothelium (HUVEC). We studied whether TGF-ß1 alters hENT1 expression and/or activity in HUVEC. Methods: Cells expressing a truncated receptor type II for TGF-ß (Ad-tTßRII) were exposed to TGF-ß1 (2 ng/ml, 0–6 h) in absence or presence of N^G-nitro-L-arginine methylester (L-NAME, 100 μM) or S-nitro-*N*-acetylpenicillamine (SNAP, 100 μM). [^{3}H]Adenosine transport (4 μCi/ml, 20 s, 22°C) was measured in absence or presence of nitrobenzylmercaptopurine ribose (NBMPR, 1 μM). hENT1 mRNA and protein were detected by RT-PCR and western blot. TGF-ß1 effect on *SLC29A1* promoter activity (-2154 and -1114 bp plasmid contructs from ATG) was also analyzed. Results: TGF-ß1 reduced (~55%) the V_{max} for hENT1-mediated adenosine transport, mRNA expression (~50%), protein abundance (~60%), and promoter transcriptional activity. TGF-ß1 effects were blocked by L-NAME, unaltered by SNAP, and absent in cells transduced with Ad-tTßRII. Conclusion: Activation of receptor type II for TGF-ß1 inhibits hENT1-mediated adenosine transport, likely due to NO-dependent reduced *SLC29A1* expression in HUVEC.

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Extracellular ATP is a pro-angiogenic factor for systemic microvascular endothelial cells

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Angiogenic expansion of the vasa vasorum contributes to the progression of both systemic and pulmonary vascular diseases. We recently reported that neovascularization of the vasa vasorum network occurs in the pulmonary artery (PA) adventitia of chronically hypoxic calves. Because extracellular adenine nucleotides are important regulators of vascular functions and can be released from vascular and blood cells in response to hypoxia, we hypothesized that extracellular ATP may have a direct angiogenic effect on vasa vasorum endothelial cells (VVEC). We found that ATP dramatically increases DNA synthesis, migration, and tube formation on Matrigel in isolated VVEC. The PI3K/mTOR and ERK1/2 play a critical role in ATP-induced mitogenic and migratory responses. However, only ERK1/2 contribute in part, to ATP-induced tube formation on Matrigel. In contrast to VVEC, mitogenic responses to extracellular ATP were not observed in aortic and PA endothelial cells, indicating that VVEC, isolated from the sites of active angiogenesis, exhibit a unique pro-angiogenic phenotype with an augmented sensitivity to extracellular ATP. Interestingly, VEGF had only modest effect on VVEC mitogenesis suggesting that VEGF is important, but not exclusive for VVEC growth. Further, using an in vivo Matrigel plug assay, we found that subcutaneous injection of Matrigel containing MeSATP, ATP [gamma]S, MeSADP, and ADP[beta]S resulted in plug neovessel formation (n=3). However, adenine nucleotides diminished the effect of total platelet lysates (n=11) on plug neovascularization (n=3). Together, our studies demonstrated that a fundamental molecule, ATP could be recognized as a potent regulator and/or modulator of angiogenesis in systemic microvascular endothelial cells.

SYMPOSIA 17: PURINERGIC REGULATION OF PAIN SENSATION

Effects of P2 receptor ligands in animal models of neuropathic and thermal pain

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Recent studies show that many subtypes of the purinergic P2X and P2Y receptors are gateways of pain signalling in animal models of neuropathic pain, and these receptors are promising pharmaceutical targets for the treatments of neuropathic disorders. Male Wistar rats were submitted to partial ligation of the sciatic nerve (Seltzer et al). 7–14 days after surgery, the effects of PPADS (P2X1/P2X3/P2X7 receptor antagonist), UDP (P2Y2/P2Y4 receptor agonist), Brilliant Blue G (BBG, selective P2X7 receptor antagonist) and MRS 2365 (selective P2Y1 agonist) were investigated on the injury-induced mechanical allodynia, using a dynamic plantar aesthesiometer with an automated von Frey type filament.We also investigated the effects of these compounds on thermal nociception using an increasing temperature hot plate system. Treatments with PPADS, BBG and UDP dose-dependently attenuated the mechanical allodynia after surgery. MRS 2365 attenuated mechanical allodynia at a dose of 0.03 mg/kg. The minimal effective dose of PPADS was 12.5 mg/kg, while UDP was effective at 25 mg/kg. The minimal effective dose of BBG was 10 mg/kg. In the hot plate test, PPADS and UDP caused a dose-dependent thermal hypoalgesia, while BBG decreased nociceptive threshold at a dose of 10 mg/kg. These results suggest that several purinergic receptor subtypes are involved in the pathophysiology of neuropathic and thermal pain. Compared with current literature, the role P2X7 and P2Y1 receptors are highlighted, and drugs which act on these receptor subtypes may provide a novel approach in the treatment of neuropathic and other pain disorders.

Purinergic receptor antagonists: novel treatments for pain and overactive bladder

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ATP activation of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors is believed to play a crucial role in nociception and sensory modulation of the micturition reflex. Blockade of these receptors with novel antagonists offers the potential to treat a variety of pain conditions and visceral sensory dysfunctions. Preclinically, this is supported by the unique localization of $P2X_3$ and $P2X_{2/3}$ receptors on the peripheral and central terminals of unmyelinated C-fiber and thinly myelinated Aδ sensory afferent neurons that innervate somatosensory and visceral sensory organs. Studies using P2X gene knockout and gene knockdown mice and the $P2X_3$, $P2X_{2/3}$ antagonists TNP-ATP and A-317491 have also demonstrated a role for peripheral and central $P2X_3$ and $P2X_{2/3}$ receptors in pain transmission and bladder sensory function. Over the past several years, we have made considerable advancements in developing novel, small molecule $P2X_3$, $P2X_{2/3}$ antagonists that serve as ideal probes to further explore the role of peripheral and central $P2X_3$ and $P2X_{2/3}$ receptors in pain and micturition. This presentation will describe our evaluation of dual $P2X_3$, $P2X_{2/3}$ antagonists in rat models of persistent inflammatory, neuropathic and bone cancer pain, as well as in the anesthetized rat Refill model of bladder sensory function. Collectively, our data demonstrate a role for $P2X_3$ and $P2X_{2/3}$ receptors in chronic pain. Our data also support a role for both peripheral and spinal $P2X_3$ and $P2X_{2/3}$ receptors in sensory modulation of the micturition reflex.

(Invited)

Adenosine and ATP in the management of postoperative pain: a randomised, double-blind, placebo-controlled trial

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Background: In patients undergoing major surgical procedures, the incidence of moderate to severe pain has been reported to be as high as 25%–50%. Inadequate management of postoperative pain can worsen patient outcome and result in prolonged hospitalisation. In the multimodal treatment approach of postoperative pain, adenosine/ATP seem promising treatment options because of their low cost, their simple mode of administration (i.v. during surgery), and their long-lasting analgesic effects (up to several days post-surgery).
Study questions:
1. What is the postoperative analgesic effect of peroperative adenosine and ATP infusion in patients undergoing major surgery, as compared to placebo?
2. Can intravenous adenosine and ATP reduce the need for opioids during the first 48 hours after major surgery, as compared to placebo?
Study population: 75 subjects scheduled for major abdominal or urological elective surgery undergoing total anaesthaesia.
Design and methods: Double-blind placebo-controlled randomised clinical trial with three parallel arms. Randomisation is stratified according to study centre, gender and type of surgery. Patients receive adenosine, ATP or placebo infusions during surgery. Outcome measures (baseline, 48 h, 7 d and 30 d post-surgery) include regular pain measurements (pain diary, VAS), use of analgesics, appetite, fatigue and quality of life. Blood will be collected to measure inflammatory response, stress hormones and genetic polymorphisms.
Results: So far, 55 patients have been included. Infusions during surgery are tolerated well. Effects on pain management are expected in 2009.

Effect of acid pH on purinergic P2X3 receptors depends on the histidine-206 residue

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Whole-cell patch-clamp investigations were carried out to investigate the pH sensitivity of native and recombinant P2X3 receptors. In HEK293 cells permanently transfected with human P2X3 receptors, acidic pH shifted the concentration-response curve for alpha,beta-methylene ATP (alpha,beta-meATP) to the right and increased its maximum. Alkalic pH did not alter the effect of alpha,beta-meATP. Low pH value increased the activation time constant of the alpha,beta-meATP current; the fast and slow time constants of desensitization were at the same time also increased. Acidification accelerated the recovery of P2X3 receptors from the desensitized state. Replacement of histidine-206, but not histidine-45 by alanine abolished the pH-induced effects on hP2X3 receptors transiently expressed in HEK293 cells. Changes in the intracellular pH had no effect on the amplitude or time-course of the alpha,beta-meATP currents. The voltage-sensitivity and reversal potential of the currents activated by alpha,beta-meATP were unaffected by extracellular acidification. Similar effects were observed in a subpopulation of rat dorsal root ganglion neurons expressing homomeric P2X3 receptor-channels. It is suggested that acidification may have a dual effect on P2X3 channels, by decreasing the current amplitude at low agonist concentrations (due to a decrease in the rate of activation) and increasing it at high concentrations (due to a decrease in the rate of desensitization). Thereby, a differential regulation of pain sensation during e.g. inflammation may occur at the C fibre terminals of small DRG neurons in peripheral tissues.

Spinal administration of an Adenosine-1 receptor partial agonist in a model of acute postoperative pain: effects on mechanical and thermal withdrawal thresholds

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Background: Adenosine-1 receptors modulate nociception in the spinal cord. Little is known about partial agonists of the adenosine-1 receptor in the setting of surgical pain.
Methods: 12 Groups of rats were randomly assigned to receive 10, 30, 100 nmol of a partial adenosine-1 receptor agonist (Drug supplied by CV Therapeutics) or vehicle (DMSO) in a 10μL saline spinal injection, followed by hind-paw incision. Mechanical testing (von Frey filaments) was performed at the site of incision, at distance of the incision and on the opposite paw. Thermal testing (radiant heat) was done on both paws. These tests were performed before incision and after at various time points (1 h, 2 h, 4 h, 6 h), then daily until day 11.
Results: Effect of drug versus vehicle peaked one and two hours after incision. Values returned progressively to base-line around day nine.
TABLE:

Mechanical testing - withdrawal treshold (Mn) ± SEM						Thermal testing - withdrawal threshold (sec) ± SEM					
Drug	Test site	Vehicle Base-line	Drug Base-line	Vehicle 2h post-op.	Drug 2h post-op.	Drug	Test site	Vehicle Base-line	Drug Base-line	Vehicle 2h post-op.	Drug 2h post-op.
10 nmol	At incision	483±46 (n=10)	494±27 (n=10)	112±9 (n=9)	182±56 (n=10)	10 nmol					
	At distance	359±39 (n=10)	355±29 (n=10)	185±41 (n=9)	276±80 (n=10)		Incised paw	13.5±0.93 (n=5)	14.4±0.48 (n=10)	5.3±0.77 (n=5)	8.1±0.33 (n=10)
	Opposite	502±50 (n=10)	532±32 (n=10)	585±73 (n=9)	614±70 (n=10)		Opposite	14.03±0.87 (n=10)	13±0.34 (n=10)	12.96±0.31 (n=5)	13.4±0.5 (n=10)
30 nmol	At incision	491±32 (n=17)	514±56 (n=10)	141±15 (n=17)	199±35 (n=10)	30 nmol *					
	At distance	3.66±31 (n=17)	3.45±51 (n=10)	202±22 (n=17)	252±42 (n=10)		Incised paw	17.3±1.13 (n=12)	19.8.±0.91 (n=9)	6.3±0.67 (n=12)	10.07± 0.85 (n=9)
	Opposite	5.32±32 (n=17)	5.52±49 (n=10)	590±40 (n=17)	684±76 (n=10)		Opposite	17.32±1.01 (n=12)	19.4±0.77 (n=9)	15.57±1.03 (n=12)	16.86±1.63 (n=9)
100 nmol **	At incision	571±35 (n=8)	613±70 (n=9)	341±66 (n=8)	259±48 (n=9)	100 nmol					
	At distance	515±67 (n=8)	425±56 (n=9)	581±63 (n=8)	392±70 (n=9)		Incised paw	17.50±0.57 (n=9)	19.26±0.84 (n=9)	5.98±0.76 (n=9)	6.16±1.99 (n=9)
	Opposite	633±40 (n=8)	676±48 (n=9)	810±70 (n=8)	642±38 (n=9)		Opposite	18.47±0.84 (n=9)	18.08±0.66 (n=9)	18.81±1.48 (n=9)	16.51±1.56 (n=9)

Three factor ANOVA with repeated measures showed no differences at individual time-points. *Overall effect in favor of the drug (p=0.017). **Overall in favor of vehicle (p=0.025)
Conclusions: Intrathecal partial adenosine-1 receptor agonist revealed thermal anti-hypersensitive properties at 30 nmol. A tendency towards effect, increasing from 10–30 nmol should be confirmed. The opposite effect at 100 nmol cannot be explained by partial agonist properties and needs further investigation.

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Peroperative adenosine versus remifentanil infusion during breast surgery: short and long term analgesic effect a randomized double - blind study

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BACKGROUND:
In a randomized double-blind study during breast surgery procedures, we compared the perioperative analgesic effects of intravenous adenosine (ADO) versus remifentanil (RF). Their possible consequence on delayed neuropathic pain was also evaluated.

METHODS:
Thirty-one women, ASA I and II, were randomly assigned to one of the two drug treatment group. After intubation and before skin incision, a basal rate infusion of either ADO (75 μg/kg/min) or RF (0,225 μg/kg/min) was started. Anesthesia was maintained with sevoflurane 2%, and air 50%, in oxygen. During surgery, and in order to maintain stable hemodynamics, we allowed for a ±25% modulation of the basal infusion rate. Postoperative evaluations of the analgesic effect, include morphine consumption at 2 (MH2) and during the first 24 hours (MH24) post-procedure and persistent pain at one year post surgery (PPY1).
RESULTS:
There was no statistical difference between the two groups for morphine consumption at MH2, MH24 or in the PPY1.(see table 1&2)
CONCLUSION:
In the present perioperative setting we conclude that ADO is as efficient as RF as an analgesic The long term trend for fewer cases with persistent pain deserves further study including more patients.

table 1:

group(n)	H2 mg(mean± SEM)	H24 mg(mean± SEM)
ADO (13)	7± 4,8	15,4±9,0
RF(15)	8,9±6,9	18,0±14,9

table 2:

PPY1	ADO(n)	RF(n)
Yes	0	2
No	12	12

Identification of novel, orally active antagonists of P2X3 and P2X2/3 purinergic receptors

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Background: P2X3 and P2X2/3 receptors are ATP-gated ion channels currently being investigated for their therapeutic potential in treating pain and urinary incontinence. Previous studies showed that P2X3 and P2X2/3 knockout animals have reduced responses to formalin-induced pain. In addition, antagonist, antisense, or siRNA mediated knockdown of receptor activity is analgesic in models of inflammatory and neuropathic pain. Taken together, these studies demonstrate an essential role for P2X3 and P2X2/3 signaling across a broad spectrum of pain indications.
Methods: Selected battery of in vitro potency, DMPK, and in vivo pharmacology assays
Results:
We have developed proprietary potent and selective P2X3 and P2X2/3 antagonists with good drug-like properties. One compound, RN-1838 has been selected as a biological proof of concept molecule. In vitro, RN-1838 blocks alpha, beta-methylene ATP-induced P2X3 or P2X2/3-mediated calcium influx with IC50 < 30nM, and <200nM respectively. Electrophysiological studies demonstrate potent (<30nM) block of agonist-induced current in cells heterologously expressing P2X3 or P2X2/3, or in rat nodose ganglia neurons. RN-1838 also shows excellent selectivity against other P2X family members.
Pharmacokinetic profiling shows that RN-1838 is a good candidate for proof of concept biological studies. The compound demonstrates good oral bioavailability (%F=43), moderate clearance (Cl=0.84 L/Kg/hr), and acceptable oral exposure (AUC=23294 min*ng/mL). Preliminary results show that RN-1838 is efficacious in the formalin model of pain.
Conclusions:
We have developed novel dual P2X3 and P2X2/3 antagonists with excellent potency and pharmacokinetic properties. Preliminary studies demonstrate that RN-1838 is efficacious in animal pain models, confirming a role for P2X3 and P2X2/3 in nociception.

Regulatory domains of P2X3 receptors; agonist binding, pH sensitivity and dessensitization properties

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Whole-cell patch-clamp recordings were performed on human embryonic kidney (HEK) 293 cells permanently or transiently transfected with the human (h) wild-type P2X3 receptor or its various mutants. In order to determine the role of certain conserved, positively charged amino acids in the nucleotide binding domains of hP2X3 receptors, the lysine (K)-63, -65, -176, -299 as well as the arginine (R)-281 and -295 residues were substituted by the neutral amino acid alanine. We observed no effect of the selective P2X3 receptor agonist alpha, beta-methylene ATP (a,b-meATP) at the K63A, K176A, R295A, and K299A mutants and a marked decrease of agonist potency at the K65A and R281A mutants. Acidic pH shifted the concentration-response curve to a,b-meATP to the right and increased its maximum. Alkalic pH did not alter the effect of a,b-meATP. Replacement of histidine 206, but not histidine 45 by alanine abolished the pH-induced effects on hP2X3 receptors. High-affinity desensitization (HAD) by nanomolar agonists was described to shape the ability of P2X3 receptors for mediating pain sensation. These receptors are activated by micromolar ATP, but nanomolar ATP is sufficient to effectively desensitize them. HAD of P2X3 receptors could occur without a preceding desensitization in the resting state. In conclusion, conserved, positively charged amino acid residues in the nucleotide binding domains 1-4 (NBD1-4) serve as binding sites for the negatively charged phosphate moiety of ATP. Acidification may have a dual effect on P2X3 receptor-channels, by decreasing the current amplitude at low agonist concentrations (because of a decrease in the rate of activation) and increasing it at high concentrations (because of a decrease in the rate of desensitization). A simple allosteric kinetic model in which only triliganded receptors could open the channel fails to reproduce receptor behaviour during HAD; introduction of an additional diliganded open state is necessary.

(Invited)

Purinergic modulation of consolidated synaptic potentiation in the cerebral amygdala of the neuropathic pain models

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Presynaptic inhibition is a major mechanism for effective control of the synaptic efficacy in the brain. For example, adenosine A1 receptor-mediated inhibition of transmitter release is a prevailing form of presynaptic inhibition ubiquitously found in most of the central excitatory synapses. Furthermore, in most of these synapses, ATP can be their powerful endogenous activator after extracellular conversion to adenosine (e.g., Kato and Shigetomi, J Physiol 2001; Kawamura, Kato et al., J Neurosci 2004), suggesting a “purine-signaling complex” composed of ecto-nucleotidase, A1 receptors and release machinery located at such synapses. We have recently demonstrated, in the rat models of the neuropathic pain, that the excitatory transmission from the lateral nucleus of the parabrachialis (NPB) to the laterocapsular part of the central amygdala (CeLC) becomes potentiated in a manner dependent on pain behaviors (Ikeda et al., Pain, 2007). As CeLC plays a major role in the link between nociception and pain-related emotion (Gauriau & Bernard, 2002), this synaptic potentiation indicates that the pain-evoked emotion is enhanced in these animals with neuropathic pain. In the brain slices prepared from young rats in which an unilateral spinal nerve ligation was made, adenosine as well as ATP significantly reduced the amplitude of excitatory postsynaptic current (EPSC) by ~20% in a manner sensitive to adenosine A1 receptor antagonist. Surprisingly, these potentiated synapses in the contralateral CeLC were significantly more prone to the inhibition by

adenosine and ATP (reduction by ~50%) than in the ipsilateral CeLC and in the sham groups. Our interpretation of these results is that, in the central amygdala, the synapses with the purine-signaling complex predominantly show plastic changes in response to enhanced nociception-related inputs. Supported by Brain Science Foundation, Novartis Foundation and MEXT, Japan 20107024.

(Invited)

Effects of the selective adenosine A1 receptor agonist 5′-chloro-5′-deoxy-N6-(2-endo-norbornyl)adenosine on nociception in mice

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Background. To investigate the analgesic effect of 5′-chloro-5′-deoxy-N6-(2-endo-norbornyl)-adenosine (5′Cl5′d-(±)-ENBA), a very selective agonist with subnanomolar affinity for the human A1 adenosine receptor (A1AR), the formalin test was applied in freely moving mice.
Methods. The Formalin test-induced pain is a widely used animal model of persistent pain. Nociceptive responses are divided into an early, short lasting first phase (0–7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15–60 min) of tonic pain. The total time of the nociceptive response was measured every 5 min and expressed as the total time of the responses in min (mean ± SEM). Recording of nociceptive behaviour commenced immediately after the formalin injection and was continued for 60 min.
Results. Systemic administration of 5′Cl5′d-(±)-ENBA (1–2 mg/Kg, i.p.), 10 min before formalin reduced the late nocifensive behaviour induced by formalin in a dose-dipendent manner. The higher dose 5′Cl5′d-(±)-ENBA used reduced both the early and the late phases of the formalin test, and this was prevented by the DPCPX (3 mg/Kg, i.p.), a selective A1 receptor antagonist.
Conclusion. These data further evidence that the adenosine A1 receptor stimulation results effective in reverting formalin-induced nocifensive behaviour in mice and suggest that the presence of adenosine receptors within the endogenous antinociceptive pathway may play a critical role in pain modulation. Moreover, it was demonstrated that the substitution of 5′-OH group with a chlorine in adenosine derivatives is not detrimental for the antinociceptive effect mediated by A1AR.

Interferon-γ and its receptor are required for spinal microglia activation and neuropathic pain after peripheral nerve injury

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Neuropathic pain is a result of peripheral nerve injury in diabetes or cancer. A hallmark of this pain is abnormal sensory perception of pain such as allodynia (hypersensitivity to light stimuli). We have previously shown that spinal microglia exhibiting activated form have a crucial role in producing allodynia after nerve injury. However, the mechanisms of microglia activation remain unknown. In the present study, we examined the role of interferon-γ (IFN-γ), reported to be upregulated in the spinal cord of animal models of neuropathic pain, in microglial activation and neuropathic allodynia. We found that intrathecal administration of IFN-γ produced long-lasting allodynia. The expression of IFN-γ receptor mRNA in the spinal cord of naïve rats was observed only in microglia. In the dorsal horn of IFN-γ-treated rats, microglia dramatically

changed their morphology into hypertrophy and also proliferated, both of which are prominent features of activated microglia. We next examined the role of IFN-γ in tactile allodynia and microglia activation after peripheral nerve injury using mice lacking IFN-γ receptor (*ifngr*$^{-/-}$). We found that a strong allodynia and microglia activation in spinal dorsal horn were markedly attenuated in *ifngr*$^{-/-}$ mice compared with wild-type (WT) mice. We also found that $P2X_4$ receptor-deficient mice reduced the IFN-γ-induced allodynic behavior as compared with WT mice. Together, our present findings suggest that the IFN-γ/IFN-γ receptor system contributes to nerve injury-induced tactile allodynia through microglia activation and $P2X_4$ receptor.

The algesic action of NGF involves plastic upregulation of P2X3 receptors of trigeminal sensory neurons

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Because the neurotrophin NGF is known to induce algogenic effects which may be a component of the severe headache of migraine, blocking NGF is potentially a novel analgesic approach to this disease. It is unclear how nociceptors, in particular ATP-gated P2X3 receptors, of trigeminal ganglion neurons that signal headache may be modulated by manipulating NGF levels. Using cultured mouse trigeminal sensory neurons, we studied changes in P2X3 receptors natively co-expressed with NGF receptors.
NGF antibody (24 h) treatment decreased P2X3 receptor mediated currents (recorded under patch clamp) and Ca2+ transients (monitored with cell imaging), without affecting TRPV1 channels or GABA receptors. Furthermore, P2X3 receptors remained longer refractory to activation. Exogenously-applied NGF had the opposite effect. NGF neutralization was associated with decreased threonine phosphorylation of P2X3 subunits, presumably accounting for their reduced responses and slower recovery. Anti-NGF treatment also increased the residual current typical of heteromeric P2X2/3 receptors in accordance with the observation of enhanced membrane location of P2X2 subunits, including the P2X2e splicing variant which confers reduced function. These data suggest that NGF controlled plasticity of P2X3 subunits and their co-assembly with P2X2 subunits. Using an in vivo model of mouse trigeminal pain, we confirmed that anti-NGF pretreatment blocked responses evoked by P2X3 receptor activation. The potent algogenic peptide CGRP enhanced P2X3 receptor activity, an effect partly mediated by the neurotrophin BDNF and independent from NGF. These results indicate distinct neurotrophin-dependent pathways to regulate sensory nociceptors and suggest an important contribution by neurotrophin signalling to nociception of trigeminal sensory neurons. Supported by the Telethon Foundation and the Italian Institute of Technology.

(Invited)

P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury

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Background: Extracellular nucleotides have been implicated as signaling molecules used by microglia to sense adverse physiological conditions through purinoceptors, especially P2Y12 receptor (P2Y12R). In this study we purposed to assess the role of microglial P2Y12Rs in neuropathic pain in which activated spinal microglia are key cellular intermediaries.
Methods: Male Wistar rats, and 10-week-old wild-type and P2ry12 knockout mice were used for neuropathic pain models in which the left L5 spinal nerve was injured. The left hindpaw withdrawal threshold was measured using von Frey filaments to evaluate tactile allodynia. The L5 spinal cord was subjected to quantitative RT-PCR, in situ hybridization and immunohistochemistry.

Results: We found that the level of P2Y12R mRNA expression was markedly increased in the spinal cord ipsilateral to the nerve injury, and that this expression was highly restricted to Iba1-positive microglia. Blocking spinal P2Y12R by the intrathecal administration of its antagonist prevented the development of tactile allodynia. Mice lacking P2ry12 displayed impaired tactile allodynia after nerve injury without any change in basal mechanical sensitivity. Moreover, a single intrathecal or an oral administration of P2Y12R blockers, to nerve-injured rats, produced a striking alleviation of existing tactile allodynia.
Conclusion: Together, our findings indicate that activation of P2Y12Rs in spinal microglia may be a critical event in the pathogenesis of neuropathic pain, and suggest that blocking microglial P2Y12R might be a viable therapeutic strategy for treating neuropathic pain.

Role of P2Y receptors in trigeminal pain signalling

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Migraine is a chronic neurovascular disorder associated to activation and sensitization of the trigeminovascular system. Since little information is available on purinergic transmission in the generation of migraine pain, we aimed at studying the expression and function of P2 receptors in trigeminal ganglia (TG), with particular focus on P2Y receptors. We chose an in vitro model of mixed primary TG cultures from P11 C57 black/6 mice. To study P2 receptor expression, RT-PCR and immunocytochemistry analysis were utilized, while receptor function was evaluated by single cell calcium imaging.
Comparison with intact ganglion showed that cultured neurons retain, at least in part, their physical relationships with satellite glia. RT-PCR indicated expression of P2X2/P2X3 subtypes and of all cloned P2Y-receptors. Single cell calcium imaging revealed the presence of functional neuronal P2X3, as well as of ADP-sensitive P2Y1,12,13 and UTP-activated P2Y2/P2Y4 receptors on both neurons and glia. Glial cells also responded to UDP, suggesting the presence of functional P2Y6 receptors. Exposure to the pro-algogenic agent bradykinin (BK) modified neuronal P2X3 receptor function in a biphasic way, with sensitization after an acute exposure followed by a reduction of P2X3-mediated calcium responses upon chronic application. Interestingly, the function of P2Y receptors in SGCs was significantly enhanced by chronic exposure to BK, suggesting that a complex crosstalk between the two systems may exist.
Thus, TG cultures represent a reliable model to study purinergic receptors signaling after acute or chronic algogens application, and P2Y receptors might represent new interesting pharmacological target for migraine pain therapy.Sponsored by the Italian Telethon Grant #GGP07032A.

SYMPOSIA 18: ENTERIC NERVOUS SYSTEM

Extracellular cyclic amp-adenosine pathway: functional role in rat intestinal motility

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Background. Recently, we have shown that extracellular cAMP is not only a source of adenosine in rat ileum but also affects intestinal motility. The aim of the present study is to gain better understanding of the extracellular cAMP-induced changes on ileum tension. Methods. Muscular function was assessed in vitro by pharmacological stimulation of rat ileum segments. The formation of cAMP metabolites in ileum strips (ISs) and longitudinal muscle strips (LMs) incubated with exogenous [3H]cAMP was monitored by radio-HPLC. Results. Exogenous cAMP (0.1–1 millimol/L) and AMP (0.1–3 millimol/L) concentration-dependently increased ileum muscle tension. Pretreatment with 8-phenyltheophylline (10 micromol/L) or with adenosine deaminase (0.5 U/mL) attenuated cAMP (0.5 millimol/L)-induced contractions by 42% and 27%, respectively, and also AMP (0.1 millimol/L)-evoked contractions by 76% and 60%, respectively. Forskolin-stimulated endogenous cAMP induced concentration-dependent ileum relaxations. Forskolin-induced relaxations were abolished by H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-iso-quinolinesulphonamide; 10 micromol/L), an inhibitor of protein kinase A, while cAMP-induced contractions were not. In ISs and LMs, the generation of [3H]cAMP-derived AMP, adenosine, and inosine was time-dependent in a manner consistent with exogenous cAMP being processed through the extracellular cAMP-adenosine pathway. Accordingly, [3H]cAMP uptake in ISs and LMs was negligible. Conclusion. Extracellular cAMP triggers unique functional responses in the ileum, both directly, by binding to specific smooth muscle cell membrane sites and indirectly through its degradation products, AMP and adenosine. Its effects on muscular tension are opposite to those induced by an increase in intracellular cAMP levels, suggesting the involvement of other intracellular signalling pathways.

Symposium ‘Purinergic signalling in the enteric nervous system’ Purinergic mechanosensory transduction: peristalsis and pain

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Intrinsic sensory neurones in the submucous plexus of the gut, as well as extrinsic sensory nerves, show positive immunoreactivity for $P2X_3$ and $P2X_{2/3}$ receptors. It has been proposed by Burnstock (2001) that during distension, ATP is released from mucosal epithelial cells to act on $P2X_3$ and $P2X_{2/3}$ receptors on subepithelial sensory nerve endings. Further, that action potentials in low threshold intrinsic sensory nerves in response to moderate distension modulate peristaltic reflexes, while during excessive (colic) distension, activity in high-threshold extrinsic sensory nerve fibres leads to initiation of nociceptive impulses that pass messages through the dorsal root ganglion (DRG) to pain centres in the central nervous system. Activity recorded in extrinsic sensory nerve fibres supplying the rat colorectum during distension is mimicked by ATP and reduced by ATP antagonists (Wynn et al., 2003). Peristalsis is impaired in $P2X_3$ receptor knock-out mice (Bian et al., 2003). Purinergic mechanosensory transduction has also been implicated in reflex control of intestinal secretion, whereby ATP released from mucosal epithelial cells acts on $P2Y_1$ receptors on enterochromaffin cells to release 5-hydroxytryptamine, which leads to regulation of secretion either directly or via intrinsic reflex activity (Cooke et al., 2003). $P2X_3$ purinergic signalling enhancement in an animal model of colonic inflammation has been described, due, at least in part, to the appearance of $P2X_3$ receptor expression in a greater number of calcitonin gene-related peptide-labelled small nociceptive neurons in the DRG (Wynn et al., 2004). $P2X_3$ receptor expression is increased in the enteric plexuses in human irritable bowel disease suggesting a potential role in dysmotility and pain (Yangiou et al., 2001).

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(Invited)

Tachykinergic control of acetylcholine release from myenteric neurons requires high levels of endogenous adenosine activating A2A receptors

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The underlying mechanism, by which capsaicin-sensitive primary afferent neurons control gastrointestinal motility, is not fully understood. Recently, we showed that in addition to inhibitory adenosine A1 receptors expressed on both cholinergic and tachykinergic myenteric neurons, activation of A2A receptors facilitate ACh release from myenteric neurons (Duarte-Araújo et al., 2004).
Here, we investigated the influence of endogenous adenosine on tachykinin-mediated facilitation of [3H]-ACh release from stimulated (5 Hz, 200 pulses) longitudinal muscle-myenteric plexus (LM-MP) preparations of the ileum of control rats (C) and of rats treated with capsaicin in the neonatal period (CAP).
TheNK1 receptor agonist, Sar9,Met(O2)11-Substance P (s,m-SP, 100–300 nM), failed to modify evoked [3H]-ACh release from both C and CAP rats. S,m-SP (300 nM) facilitated the release of [3H]-ACh when A2A receptors were activated with CGS21680C (3 nM, 48±8%, n=4), but not when the A1 receptor agonist, R-PIA (300 nM, 1±10%, n=3), was used. Increasing endogenous adenosine accumulation, by inhibiting adenosine deaminase with EHNA (50 μM, 27±6%, n=5), mimicked facilitation of s,m-SP (300 nM) by CGS21680C (3 nM) in both C and CAP rats. A similar effect was observed with the adenylate cyclase activator, forskolin (3 μM, 31±6%, n=4).
Data indicates that facilitation of [3H]-ACh release by NK1 receptor activation requires high levels of endogenous adenosine activating A2A receptors and that synergism between these two receptors requires stimulation of the adenylate cyclase/cyclic AMP pathway.

ATP signaling in the subepithelial fibroblasts of intestinal villi

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Subepithelial fibroblasts of intestinal villi locate under the basement membrane of the epithelium, and form contractile cellular networks via gap junctions. They sheathe lamina propria and are in close contact with neural and capillary networks, smooth muscles, and immune cells. They have unique characteristics such as: cAMP-dependent rapid reversal cell-shape changes from flat to stellate, cell-shape dependent mechanosensitivity that induces ATP release as autocrine and paracrine mediator, contractile ability, and expression of various receptors for endothelins (ETA receptors, ETB receptors), ATP (P2Y1 receptors), substance-P, bradykinin, serotonin etc. Mechanical stimulations (touch and stretch) evoke Calcium-increase and ATP-release from the subepithelial fibroblasts, which elicit Ca2+-wave propagation through the cellular network via P2Y1 activation. In a co-culture of

neuronal cells (NG108-15) and subepithelial fibroblasts, mechanically induced Calcium-waves propagated from subepithelial fibroblasts to neural cells. Uptake of food, water and nutrients may cause mechanical stress on subepithelial fibroblasts in the villi. The ATP released by mechanical stimulation elicits Calium-wave propagation through the subepithelial cellular network and also may activate P2X on terminals of mucosal sensory neurons and also P2Y/P2X on the capillary network to regulate villous movement, absorption, and peristaltic motility. In the intestinal villi, subepithelial fibroblasts work as a mechano-sensor and signal transduction machinery, which are regulated locally and dynamically by rapid cell-shape conversion.

Purinergic synaptic transmission and modulation in the enteric nervous system

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There are more than thirty potential neurotransmitters found in neurons of the enteric nervous system (ENS). While the central importance of acetylcholine as an enteric neurotransmitter is established, the role of most of the other potential neurotransmitters is not clear. However, there is growing evidence that ATP and other purines contribute to synaptic excitation or synaptic modulation in the ENS. The myenteric plexus is the division of the ENS that controls of gut motor function. ATP acting at P2X2 and/or P2X3 receptors is a fast synaptic excitatory transmitter in the myenteric plexus and the nerve fibers that release ATP have an oral-anal projection. Functional studies showed that P2X receptors mediate synaptic excitation of inhibitory motor neurons supplying the muscle layers. ATP is also a transmitter released by the inhibitory motorneurons. ATP acts at P2Y1 receptors to relax GI muscle. Therefore, purinergic synaptic transmission contributes to peristalsis. The submucosal plexus is the division of the ENS that controls secretory function. ATP, acting at P2X receptors, is a fast excitatory synaptic transmitter in the submucosal plexus. ATP also acts at P2Y1 receptors to cause slow synaptic excitation of submucosal neurons. Adenosine may also be an excitatory neurotransmitter in the myenteric and submucosal plexuses and adenosine can act as a presynaptic modulator synaptic transmission in both plexuses. Purinergic neurotransmission is important for normal motor and secretory function in the gut. Future work may identify pathophysiological changes in purinergic neurotransmission that contribute to motility or secretory disorders.

(invited)

Alpha, beta-methylene-ATP: a P2Y11 agonist and smooth muscle relaxant

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Background. alpha,beta-methylene-ATP (abmA) is known to inhibit gastrointestinal smooth muscle. The involvement of the P2Y11 receptor in this inhibitory effect was investigated in the guinea-pig taenia coli, where P2Y receptor pharmacology was first established.
Methods. Human P2Y11 receptors were expressed in astrocytoma 1321N1 cells. The P2Y11 receptor couples to Gq and Gs signalling proteins when activated; therefore, calcium mobilisation was monitored by FLIPR assays and adenylate cyclase activity by de novo cAMP production. Also, nucleotide evoked relaxant responses of carbachol precontracted smooth muscle strips of guinea-pig taenia were studied in organ bath experiments.
Results. Human P2Y11 receptors were activated in a concentration dependent manner by abmA, with pEC50 values (mean ±SEM, for n=4) of 4.23±0.09 (calcium assay) and 5.23±0.10 (cAMP assay). Reactive Red was a competitive antagonist here, with a pKB value of 4.76±0.04 (n=4). Guinea-pig taenia was potently relaxed in by abmA, with a pEC50 value of 5.70±0.03 (n=4), which was due to activation of P2X3 receptors and release of ATP to activate P2Y1 receptors. After blocking these P2 subtypes, abmA evoked prolonged relaxations with a pEC50 value of 4.09±0.09 (n=4). Prolonged relaxations were antagonised competitively by Reactive Red, with a pKB of value 4.86±0.14 (n=4).
Discussion. An inhibitory P2 receptor in guinea-pig taenia coli resembles the human P2Y11 receptor. Subsequently, a partial P2Y11 cDNA was isolated from guinea-pig taenia and P2Y11 immunoreactivity was identified in dispersed smooth muscle cells. It is concluded that P2Y11 is inhibitory in gastrointestinal smooth muscle.

Benzoyl-benzoyl-ATP: a smooth muscle relaxant and P2Y11 agonist

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Background. Benzoyl-benzoyl-ATP (BzATP) can activate guinea-pig myenteric neurons but, surprisingly, it is inactive at the guinea-pig isoform of the P2X7 receptor. The inhibitory effect of BzATP in the guinea-pig taenia coli was investigated to identify the nature of P2 receptors involved.
Methods. Nucleotide evoked relaxant responses of carbachol precontracted smooth muscle strips of guinea-pig taenia were studied using organ experiments. Also, BzATP activity at human P2Y11 receptors expressed in astrocytoma 1321 N1 cells was detected in FLIPR assays for intracellular calcium mobilisation and enzyme assays for de novo cAMP production.
Results. Guinea-pig taenia was potently relaxed by BzATP, with a pEC50 value of 5.56±0.04 (mean±SEM; n=4). Concentration-response curves were right-shifted by the P2Y1 antagonist, MRS2179 (100 microM), and shifted again by the further addition of the P2X3 antagonist, A317491 (100 microM). New pEC50 values were 4.11±0.09 (n=4) and 3.50± 0.17 (n=4), respectively. Relaxant responses were antagonised by Reactive Red, with a pKB value of 4.86±0.14 (n=4). However, relaxations were unaffected by either tetrodotoxin (1 microM) or omega-conotoxin (0.1 microM). BzATP activated human P2Y11 receptors, with pEC50 values (n=4) of 5.08±0.06 (calcium assay) and 6.06±0.08 (cAMP assay); Reactive Red was a competitive antagonist, with a pKB value of 4.76±0.04 (n=4).
Discussion. BzATP directly activated neuronal P2X3 receptors to release ATP which then activated P2Y1 receptors on smooth muscle cells. After blocking P2X3 and P2Y1 receptors, BzATP directly activated P2Y11 receptors on smooth muscle cells. The localisation of these P2 receptors was confirmed by immunohistochemistry.

Purinergic signalling in gut pathology

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There is overwhelming evidence that purinergic substances play an important role in several physiological activities in the mammalian gastrointestinal tract under normal and pathological conditions. Variable expression levels of both P2X and P2Y receptors has been reported in several neuronal and non-neuronal cell types,including immune cells, during inflammation of the gastrointestinal tract. ATP and adenosines have been associated with the development of gastric ulcers and colon carcinomas. Significant changes in the distribution pattern of distinct purinergic receptor subtypes have been demonstrated in diabetes, motility-related disorders, Hirschsprung's disease, Chagas' disease, intestinal schistosomiasis, etc..
The pathophysiological roles of ATP and related compounds in gut signalling and their possible validity and potential in therapeutic strategies will be discussed.

(Invited)

A2A receptors mediate a positive feedback mechanism that controls extracellular adenosine levels at the myenteric plexus of the rat ileum

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Myenteric neurons endowed with facilitatory A2A receptors are the main source of adenosine released per se in response to electrical stimulation (Correia-de-Sá et al., 2006). However, it remains to be elucidated whether adenosine plays a role regulating its own extracellular levels.

We used longitudinal muscle-myenteric plexus (LM-MP) preparations of the rat ileum, which were stimulated (10 Hz, 3000 pulses) in the presence of test drugs. Samples were collected before and after stimulus application and retained for analysis of nucleosides content by HPLC.
Stimulation of the LM-MP caused the outflow of adenosine (ADO, 74±6%, n=6) above baseline. Inhibition of adenosine kinase by 5′-iodotubericidin (ITU, 10 microM) facilitated stimulation-induced ADO outflow by 51±6%, (n=8). The nucleoside transport inhibitor, dipyridamole (DIPY, 0.5 microM), was more effective in inhibiting (-59±9%, n=4) extracellular ADO accumulation as compared with the ecto-5′-nucleotidase inhibitor, concanavalin A (Con A, 0.1 mg/ml, -27±1%, n=5). Activation of A2A receptors with CGS 21680C (3 nM) increased stimulation-induced ADO outflow by 129±10% (n=5). The facilitatory effect of CGS 21680C (3 nM) was prevented by the selective A2A antagonist, ZM 241385 (50 nM) and by DIPY (0.5 microM), while Con A (0.1 mg/ml) was ineffective. The adenylate cyclase activator, forskolin (FSK, 3 microM), mimicked (158±14%, n=4) the facilitatory effect of CGS 21680C (3 nM); the effect of FSK (3 microM) was also attenuated by DIPY (0.5 microM).
These results indicate that adenosine regulates its own transport outside myenteric neurons via the activation of A2A receptors coupled to the adenylate cyclase/cyclic AMP pathway.

“Plurichemical and purinergic transmission” in the human enteric nervous system

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Background. Our goal was to determine the contribution of purinergic transmission in the human enteric nervous system (huENS,AJP,294:G554-G566,2008). Methods: A Zeiss LSM/REN-410 laser-scanning confocal system was used to monitor fast (msec,ROI=8×8microm2) or slow (sec,frame imaging) synaptic Ca2+ transients in submucous plexus from human jejunum loaded with Fluo-4/AM Ca2+ indicator. TTX-sensitive synaptic Ca2+ responses were elicited by focal-electrical stimulation of fiber tracts (FTS). Results: Analysis was done on 359 neurons from 51 experiments and 12 Roux-en-Y specimens. FTS elicited frequency-dependent synaptic Ca2+ transients (0.1–100 Hz/3 s) with single or multiple fast (msec) and slow (sec) components. Adenosine (1nM-1 mM) reduced peak 25 Hz/FTS Ca2+ responses by 58±7% (n=152;p=6.9e-5);the IC50=25microM. Adenosine deaminase (AdoDase,0.5–2 U/ml) augmented FTS responses by 58.5±7.73% (p=2.5e-10;n=27/72). Apyrase (1.5–8 U/ml) suppressed (68.5±6.8%;23/28 neurons;p=4.0e-6) whereas ARL 67156 (5microM) augmented 25 Hz/FTS responses (13.7±3.9%;n=7,p=0.0177). The P2X2/3 antagonist 2′3′-O-trinitrophenyl-ATP (20 nM TNP-ATP) reduced FTS responses in 15/64 neurons by 49.2±9.2% (p=0.005); 5microM TNP-ATP reduced responses in different neurons by 54.1±4.4% (31/56 neurons;p=5e-10); 9/101 neurons responded to 20 nM and 5microM TNP-ATP. ATP, UTP or ADP-beta-S (P2Y agonist) elevated Ca2+ levels. ADP-beta-S (5microM) inhibited FTS responses by 66.4±5.4% (n=41,p<0.0001);in 8/25 neurons, FTS responses were also sensitive to 5microM TNP-ATP. Some neurons sensitive to TNP-ATP responded to AdoDase. The NK3 antagonist SB218795 (100 nM) reduced FTS responses by 68.7±13.0% (n=7/25;p=0.0062) in AdoDase-insensitive neurons; some neurons responded to apyrase and SB218795. Conclusion: Purines play a role in synaptic transmission via putative adenosine, P2X2, P2X3 and P2Y receptors. An emerging concept is ‘plurichemical and purinergic transmission’ in huENS (NIHDK44179-13;NCRR-1S10RR11434).

Compromised intestinal motility and adenosinergic function in herpes simplex virus-1 (HSV-1) infected rats

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Background Neurotropic viruses are implicated in digestive neuropathies but the mechanisms through which such viruses affect the enteric nervous system (ENS) are unknown. This study aims to assess the effects of HSV-1 infection on intestinal motility and adenosinergic function in the gut. Methods Rats were inoculated intranasally (10^3pfu) and 4 weeks (W) later again intragastrically (i.g., 10^8pfu) with HSV-1. Infected or mock infected rats were sacrificed 1 and 6 W after the i.g. inoculation. The presence of HSV-1 infection was determined by PCR amplification of HSV-1-tk gene, RT-PCR for HSV-1 latency associated transcripts (LATs) and early gene ICP-4. Neuromuscular function was assessed in vitro by pharmacological and electrical field stimulation (EFS) of ileum segments. Adenosine receptors (ARs) expression was detected on ileum sections by immunofluorescence. Results HSV-1 infected rats did not show clinical and histological abnormalities in the ileum. In the brain and ENS HSV-1 established a latent infection demonstrated by the presence of viral tk-DNA and LAT mRNA. Emax values for carbachol (0.01–100 microM)-evoked contractions of ileum segments were increased by 45% and 72% at 1 and 6 W postinfection (PI), respectively. Responses to EFS were significantly augmented at 1 W. Contractile responses to *R*(-)-N^6-(2-phenylisopropyl)adenosine (0.1–15 microM) were increased at 1 W whereas adenosine (0.1–1.25 milliM)-induced contractions were decreased at 1 and 6 W PI. A1Rs and A2aRs immunoreactivity was increased on smooth muscle at 1 and 6 W and at 1 W PI, respectively. Conclusions Following i.g. delivery, HSV-1 establishes a latent infection in the ENS that significantly affects intestinal contractility, enteric neurotrasmission and adenosinergic function.

SYMPOSIA 19: GENETIC P2 RECEPTOR VARIANTS AND ALTERED FUNCTION

P2-polymorphisms and cardiovascular disease

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Cardiovascular disease is a complex multifactorial and polygenic disorder. Family history is an independent predictor of acute myocardial infarction (AMI). The underlying pathologic spectrum causing cardiovascular disease is broad, ranging from cholesterol regulation, endothelial and vascular smooth muscle cell function to inflammation and platelet aggregation. Purinergic receptors are known to be involved in the regulation of several of these mechanisms and genetic differences in P2-receptors could be important the risk of developing cardiovascular disease.
We examined all P2 receptors in silico for non-synonymous and relatively prevalent single nucleotide polymorphisms (SNPs) and examined their contribution to myocardial infarction and cardiovascular risk factors (diabetes, cholesterol, blood pressure and inflammation) in a large clinical material consisting of more than 10 000 individuals.
The P2Y2 SNP leu46Pro was not associated with AMI or riskfactors. The P2Y11 Ala87Thr SNP was associated with an increased risk of both AMI, odds ratio (OR)1.21 and inflammation (increased CRP). The risk was more pronounced in patients with family history (OR1.32) and homozygots (OR 1.94). We have now examined the effects on receptor function of the SNP and found decreased efficacy in ATP stimulated generation of cAMP. The synonymous P2Y12 H2 haplotype that has been suggested to be involved in platelet function and cardiovascular disease was not associated with AMI or risk factors. The P2X4 Gly242Ser SNP was associated with increased inflammation indicating a role in immunomodulation. The loss of function P2X7 Glu496Ala SNP which has been associated with decreased interleukin release and impaired ATP-mediated killing of mycobacteria was associated with AMI in smokers. Another loss of function P2X7 SNP (Ser357Thr) was not associated with AMI or risk factors.

(Invited)

The haplotype block structure of P2RX7 in a Caucasian population

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Background
Genome-wide association studies using non-synonymous SNPs have found a potential role for P2RX7 as a susceptibility gene in bipolar affective disorder and major depressive disorder. The presence of a number of non-synonymous SNPs in P2RX7 that can interact and affect receptor function makes it important to investigate linkage disequilibrium (LD) between SNPs and define P2RX7 haplotypes.
Methods
We recruited 1755 Caucasian subjects for direct sequencing of P2RX7. For LD between 10 non-synonymous SNPs we calculated D' and r2 measures and defined haplotype blocks using HAPLOVIEW (http://www.broad.mit.edu/mpg/haploview/index.php). ResultsA haplotype block was found to include 1068G>A, 1096C>G, 1405A>G and 1513A>C (figure 1). For these 4 SNPs 99.9% of subjects had 1 of 5 haplotypes: GCAA, ACAA, GCAC, ACGA and GGAA.
Conclusion
In this study we report linkage disequilibrium between 4 non-synonymous P2RX7 SNPs that form a haplotype block in the P2XR7 gene. In Caucasians 99.9% of subjects are represented by 5 distinct haplotype blocks. Interaction of SNPs within these blocks and LD to other SNPs within P2RX7 must be considered when analysing clinical association studies and functional studies of the P2X7 receptor.

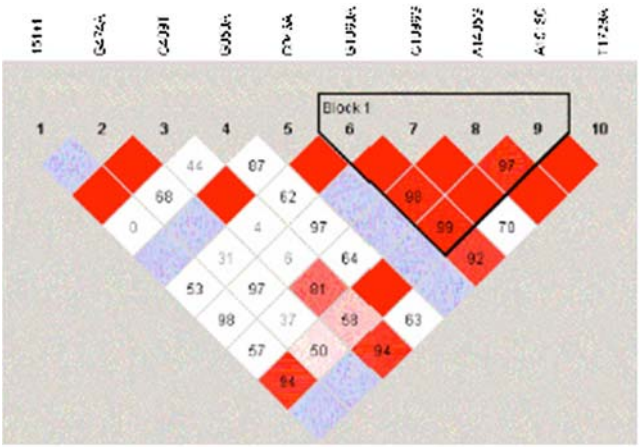

Figure 1: *P2RX7* haplotype block structure in Caucasian subjects (n=1755). Shading represents D'/LOD with D' value shown

Identification of the promoter regions of the human P2RX7 and P2RX4 genes

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The genes for human P2X7 and P2X4 receptors lie immediately adjacent on chromosome 12q24.31, separated by 27 kb of intron. The relative expression of P2X7 in cells of monocyte/macrophage origin is upregulated by interferon-gamma, but the transcriptional control of P2RX4 is unknown. To delineate the promoter region of P2RX7 and P2RX4 genes, we constructed 22 fragments (size 100~6000 bp) covering the 6 Kb upstream of these two genes. We used a dual-colour luciferase report vector system, to measure the promoter activities in both transfected HEK-293 cells and COS-7 cells for each fragment extracted from 5~9 randomly picked colonies. The transcription initiation sites were also defined by using a novel sequencing based primer extension method with 5-FAM tagged primers. The regions -344 to -158 upstream of start codon was found to have high promoter activity for P2RX7, while the promoter region for P2RX4 was -134 to 0 upstream of ATG codon. The transcription initiation sites were -149/-148 and -72 for P2RX7 and P2RX4, respectively. The related transcription factors in these two defined regions were further characterized using Strepavidin coated Dynabeads and biotin-labeled dsDNA from mononuclear cell crude nucleic extracts. These promoter regions are important in regulating expression of P2RX7 and P2RX4 in cells of haemopoietic, endothelial and neural origin.

P2 receptor single nucleotide polymorphisms and bone metabolism

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Single nucleotide polymorphisms (SNP) have been identified in a number of genes coding for P2 receptor subtypes. Receptor activation is involved in bone turnover and regulates both bone formation and bone resorption. Thus, defects in receptor function could potentially lead to altered bone mass and quality leading to metabolic bone diseases.
Most of the studies examining the association of P2 receptor SNP and bone status have been performed in a cohort study of app. 2,000 Danish postmenopausal women. Ohlendorff et al demonstrated an association between both the Glu496Ala and the Ile568Asn and risk of vertebral fractures 10 years after menopause. In vitro investigations on osteoclasts showed clear associations between genotypes and apoptosis in the cells. In a later study, other SNP were examined. Interestingly, a significant association was found between the Arg307Gln SNP and the rate of bone loss in the hip five and ten years after menopause, and between the Ala348Thr SNP and fracture incidence ten years after menopause.Next, the association

between SNP in the gene coding for the P2Y2 receptor and bone status was examined. Though the P2Y2 receptor is involved in regulation of bone turnover, no significant associations could be demonstrated.
In conclusion, genetic variations in the genes coding for the P2 receptors are clearly associated with altered bone turnover and thus loss of bone and bone fragility fractures. Further studies are warranted to consolidate the associations, and to elucidate the potential of genotyping of individuals to be used as a marker of fracture risk.

(Invited)

Pharmacological characterization of the P2Y11 Thr-87 variant

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The Thr-87 polymorphism (SNP) of the P2Y11 receptor has previously been associated with an increased risc of developing acute myocardial infarction (AMI). As the Ala-87-Thr SNP is located in TM II the increased incidence of AMI might be related to an altered receptor function. In order to address this issue the molecular pharmacology of the P2Y11 Thr-82 variant was explored and compared to the wt (Ala-87).
Concentration-response curves for ATPγS mediated cAMP accumulation were compared at maximal and sub-maximal tissue response. The data was fitted to the operational model and K_a and τ were estimated. While the results revealed no difference in affinity they indicated a tendency to a lower efficacy for the Thr-82 variant (table 1). To test the validity of these results the efficacy was further evaluated at different receptor densities and at a constant ATPγS concentration (1 μM). The results confirmed that the Thr-82 variant has a reduced efficacy (≈ 20%, P<0,0001) compared to the wild type.

Table 1

Parameter	wt	Thr-87
pK_a	4,762	4,948
τ (20 ng)	8,21	6,46
τ (0.2 ng)	0,31	0,113

Cysteine substitution mutagenesis at human P2X2 and P2X4 receptors indicates a common mode of ATP action at P2X receptors

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Conserved motifs commonly found in ATP binding proteins, e.g. the Walker motif, are absent from the P2X receptors and suggest that they have a distinct mechanism for ATP recognition. Mutagenesis studies of conserved amino acids in the mammalian P2X receptor family using the P2X1 receptor as a model have established the paradigm that three lysine residues, as well as FT and NFR motifs play an important role in mediating ATP action. We have determined the effects of equivalent cysteine mutants in P2X2 and P2X4 receptors on ATP potency and regulation by methanethiosulfonate (MTS) compounds. All the mutants (except the P2X2 K69C and K71C that were expressed, but non-functional) showed a significant decrease in ATP potency, with >300 fold decreases for mutants of the conserved asparagine, arginine and lysine residues. MTS reagents had no effect at the phenylalanine of the FT motif, in contrast cysteine mutation of the threonine was sensitive to MTS reagents and suggested a role of this residue in ATP action. The lysine substituted receptors were sensitive to the charge of the MTS reagent consistent with the importance of positive charge at this position for co-ordination of the negatively charged phosphate of ATP. At the NFR motif the asparagine and arginine residues were sensitive to MTS reagents whilst the phenylalanine was either unaffected or showed only a small decrease. These results support a common site of ATP action at P2X receptors and suggest that non-conserved residues also play a regulatory role in agonist action.

Altered depression-like behavior, dysregulated monoaminergic transmission and HPA hypoactivity in P2X7 receptor knockout mice

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Recent linkage studies have associated a polymorphism in P2X7 receptor gene with bipolar affective disorder and major depression, but experimental evidence was not available on the role of P2X7 receptors in the mood related behavior until know. In this study, we tested the role of P2X7 receptors in the development of depression- and anxiety-like behavior using a mouse line genetically deficient in P2X7 receptors (P2X7R -/-). P2X7R -/- animals did not develop "behavioral despair", a depression-like phenomenon in the Porsolt's forced swim test, and no change was observed in the elevated plus-maze test and in open field activity. In parallel, elevated levels of norepinephrine, serotonin and dopamine were found in the amygdalae of P2X7R -/- mice, norepinephrine levels were reduced in the hippocampus and monoamine levels were unchanged in the prefrontal cortex. The resting- and electrically-induced release of newly incorporated [3H]5-HT and [3H] NE were unaffected by the genetic deletion of P2X7 receptors, with the exception of electrical stimulation-evoked efflux of [3H]NE, which was decreased in the hippocampus. In contrast, P2X7R -/- mice exhibited diminished plasma ACTH and corticosterone response upon restraint stress and attenuated elevation of ACTH secretion in response to CRH in isolated pituitary cells. Our findings show that P2X7 receptor gene disruption results in likely interrelated changes in monoamine bioavailability, stress response and depression-like behavior, suggesting that this receptor plays a role in the regulation of emotional states.

Functional effects of the 1405A>G (Q460R) polymorphism in the human P2RX7 gene

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Background: The P2X7 receptor is known to mediate many of the pro-inflammatory effects of extracellular ATP. Over 10 non-synonymous single nucleotide polymorphisms (SNPs) have been discovered in the human P2X7 gene. The majority of these are loss-of-function mutations with the exception of bp 489 C>T (H155Y) which has been shown to confer a gain-of-function effect on P2X7 responses. Recently a SNP at bp 1405A>G (Q460R) has been linked with major depressive and bipolar disorder in two independent genetic association studies.
Aim: To examine the functional effect of the 1405A>G SNP in the C-terminus and to investigate its genetic linkage to other SNPs in P2RX7.
Methods: ATP-induced ethidium uptake was measured in transfected HEK293 cells and in native human monocytes, lymphocytes and erythrocytes. Site directed mutagenesis was used to introduce mutations into a human P2X7 plasmid in isolation and in combinations to recreate the observed haplotypes.
Results: We have genotyped 1700 Caucasian subjects at 10 SNPs in P2RX7 and Haploview analysis shows that 4 SNPs in exon 13 (bp 1068, 1096, 1405 and 1513) are inherited as a haplotype block with only 5 variants. The haplotype containing the 1405G minor allele shows strong linkage disequilibrium with the gain-of-function 489 T allele. We show that in isolation the 1405G mutation reduces P2X7 mediated ethidium uptake but the combination of 489 T with 1405G rescues this loss-of-function effect.
Conclusion: Genetic association of the 1405G minor allele with mood disorders and inflammatory disease should be re-analysed to determine which particular haplotype variant of P2X7 is associated.

Major differences between modulation of human and rat P2X2 receptors by zinc

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Background: Zinc has a biphasic effect on mouse and rat P2X2 receptors (rP2X2). At concentrations of 1–100 μM zinc potentiates responses to low concentrations of ATP, while at higher concentrations (200–1,000 μM) zinc substantially attenuates responses to the same low concentration of ATP. Two histidine residues of rP2X2 (H120 and H213) are known to be required for zinc potentiation and likely directly coordinate the zinc, while the molecular nature of the lower potency inhibitory site remains unknown. Human P2X2 (hP2X2) has a histidine at the position analogous to rat H120, but it has an arginine at the position analogous to H213, which suggested that this receptor might be unable to respond to zinc in the low μM range.
Methods: Receptors were expressed in Xenopus oocytes voltage clamped at -50 mV.
Results: There was no detectable zinc potentiation of hP2X2, but zinc was a highly potent inhibitor of ATP responses, with an IC50 of approximately 8 μM when 1/10 maximal ATP was applied. In contrast it required over 100 μM zinc to reach the IC50 for rP2X2. Acidic pH and negative membrane potential are also powerful positive allosteric modulators of rP2X2. Both of these modulators were far less effective at enhancing ATP evoked currents of hP2X2.
Conclusion: As the potency of zinc potentiation in rP2X2 and of zinc inhibition in hP2X2 are very similar, our results suggest that zinc released from synaptic terminals onto cells bearing P2X2 receptors would have very different effects on the neuronal circuits of rats and humans.

The role of the P2X1 receptor amino terminus in regulation by phorbol ester and GPCRs

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P2X1 receptors currents can be potentiated by phorbol ester and GPCR stimulation, however the molecular mechanisms of this regulation are unclear. To determine whether the intracellular amino terminal contributes to regulation we generated an amino terminal P2X1 receptor minigene. Overexpression of the mini-gene reduced both PMA and mGlur1alpha receptor stimulated potentiation of P2X1 receptor currents. To address further the role of the amino terminal of the P2X1 receptor cysteine point mutants of residues Y16-G30 were expressed in Xenopus oocytes. The mutants T18C, Y16C and R20C had normal levels of surface channel expression however ATP evoked currents were reduced in amplitude. The majority of mutants showed changes in the time-course of desensitization of ATP evoked currents indicating the important role of this region in regulation of channel properties. PMA and mGluR1alpha receptor potentiation was abolished for the mutants Y16C, T18C, R20C, K27C and G30C. At D17C and V29C mutants PMA potentiation was abolished but the response to mGluR1alpha*f*nstimulation was unaffected. Cysteine reactive MTSEA inhibited ATP evoked currents at Y16C, P19C and G30C mutants indicating that these residues contribute to ionic permeation. These results demonstrate that the conserved amino terminal YXTXK/R sequence incorporating a consensus PKC site and a second region with a conserved glycine residue close to the first transmembrane segment contribute to PMA and GPCR regulation of P2X1 receptors.

Polymorphic variations in the P2x7 receptor and their disease associations

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Many evolutionary pressures shape the emergence and persistence of human mutations and the association of certain polymorphic variants of the P2X7 gene with human disease suggest this molecule plays an important role in the nervous system, in bone strength and in the innate immune response to certain pathogens. We and others have found over 10 polymorphic variants in the P2RX7 gene which decrease function (and in one case increase function) of this receptor. The 1513A—C (Glu-496 to Ala) single nucleotide polymorphism (SNP) has been associated with failure of macrophages to accomplish ATP-induced killing of Mycobacterium tuberculosis in
vitro while in epidemiological studies we and others have shown an association of 1513C allele with reactivation of latent (primary) tuberculosis. The same 1513C SNP has also been implicated in
infection with Psittacosis trachomatis. The observation of reduced bone density in the P2X7 gene-deleted mouse has prompted a search for P2X7 polymorphisms in genetic studies of osteoporosis.Thus a large Danish study has found an increased prevalence of the 1513C loss-of-function SNP in patients with fractures of the lumbar spine. A different SNP, 1405G (Gln-460 to Arg),has been implicated in mood disorders. The 1405G allele has been associated with major depression and bipolar disorder in two independent studies. The functional significance of 1405G and its linkage to other SNPs has been studied by our group. Ten SNPs with functional significance were genotyped in the P2X7 gene from 1755 Caucasian subjects and analysed by Haploview. The 1405 and 489 positions were in linkage disequilibrium with a D' of 0.81 with the1405G allele occurring predominantly on a gain-of-function 489 T background. In experiments with transfected HEK-293 cells we showed that mutated P2RX7 containing 1405G has reduced function but in combination with 489 T the function is restored. Our data show the need for haplotype analysis in genetic studies of P2RX7 in human disease.

(Invited)

SYMPOSIA 20: P2X7 RECEPTOR AND PORE FORMATION

Characterization of P2X7 isoform B sheds light on the role of pore versus channel activity in P2X7 mediated life and death

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P2X7 isoform B (Gene bank AY847299, P2X7B) is a naturally occurring splice variant of P2X7 full-length receptor (Gene Bank accession n NT-009775, P2X7A). P2X7B mRNA retains introns between exons 10 and 11 thus causing the loss of the C terminal tail and incorporation of 18 amminoacids. If compared to P2X7A, P2X7B variant shows a reduced activity as ion channel and a complete loss of the pore forming ability.
We engineered and stably expressed P2X7B in HEK293 cells were we could confirm that its activity is restricted to that of small anion channel. P2X7B also loose P2X7A ability of ATP release in the extracellular milieu.
Nevertheless, P2X7B expression confers to HEK293 cells a longer survival in the absence of serum. P2X7B transfectants also gain an increased competence in growth and migration in serum free soft agar, suggesting a possible role of the receptor in facilitating tissue infiltration.
This increased resistance to unfavorable colture conditions is dependent on extracellular ATP, as treatment with apyrase significantly reduces it. P2X7B transfectants show an higher content of intracellular ATP if compared to mock cells, suggesting an increased metabolic activity. This hypothesis is also supported by a greater content of reticular calcium in P2X7B cells and by an increased nuclear translocation of the calcineurin activated nuclear factor NFATc1. Moreover, in the same cells there is and loss of Bz-ATP evoked cell blebbing.
It is tempting to speculate that while P2X7B expression will represent a proliferative advantage for the cell its counterpart, P2X7A, will also expose it to death. These different phenotypes will be dependent on channel versus pore forming activity.

The carboxyl tail of the P2X7 receptor is a regulatory module of its ion channel activity - Electrophysiological evidence

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A distinctive feature of the P2X7 receptor subtype is the large C-terminal domain which is over 100 amino acids longer than of the other P2X family members. It is believed to interact with several intracellular proteins and to be involved in the induction of large pores permeable to organic cations after prolonged application of high ATP concentrations. The role for the ion channel function of the C-terminal domain was investigated by heterologous expression of C-terminal truncated receptors in Xenopus oocytes and measurement of hP2X7 receptor-dependent ion currents by means of the two microelectrode voltage clamp and single channel patch clamp recording. Truncation of hP2X7 receptors reduced the ATP4- induced membrane currents, altered its kinetics and abolishes detectable hP2X7 receptor-dependent single channel currents. This could be explained by an inhibition of the effect of the low-affinity ATP4- binding site on hP2X7 ion channel kinetics. Coexpression of the C-terminal end fully restored ion channel current and kinetics. Quantitative coexpression data support a model where all three hP2X7 receptor subunits must have a C-terminal domain to constitute a functional receptor. We conclude that the C-terminal domain 435-595 is necessary for the normal ion channel function of the hP2X7 receptor. The carboxyl tail may be considered as gating regulatory module. This work was supported by the Deutsche Forschungsgemeinschaft (Ma 1581/12-1 to FM; Schm536/6-3 to GS).

Purinergic modulation of LPS-induced IL-1β production in the hippocampus: an in vivo study

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$P2X_7$ receptors ($P2X_7R$) have important role in the posttranslational processing of the biologically active proinflammatory cytokine IL-1β in the periphery. However, it is not known, whether the cytokine response of the hippocampus to bacterial lipopolysaccharide (LPS) in vivo is also subject to regulation by $P2X_7R$. The levels of IL-1β production were quantified in the hippocampi of rat, wild-type and $P2X_7R^{-/-}$ mice, using an ELISA Kit.
Intraperitoneal injection of LPS elevated IL-1β production in rat hippocampus in a concentration-dependent (300–500 μg kg^{-1}) and time-dependent manner (2–6 h). LPS (300 μg kg^{-1})-induced response was attenuated by PPADS (25 mg kg^{-1}) and by the selective $P2X_7$ receptor antagonists BBG (100 mg kg^{-1}) and oxidised ATP (oATP; 100 μM kg^{-1}). LPS induced IL-1b production was also diminished by ATP (1 mM kg^{-1}) and the P2X receptor agonist BzATP (300 μM kg^{-1}). The inhibitory effect of ATP (1 mM kg^{-1}) was reversed by the A1 adenosine receptor-selective antagonist DPCPX (10 mg kg^{-1}).
LPS (250 μg kg^{-1}) also induced IL-1β production in the hippocampus of wild-type mice, which was significantly less in the hippocampus of $P2X_7R^{-/-}$ mice. Systemic endotoxin induced IL-1b response was strongly inhibited by BBG (100 mg kg^{-1}) and oATP (300 μM kg^{-1}) and their inhibitory effect was significantly attenuated in the absence of $P2X_7$ receptor.
In conclusion our data reveal that endogenous activation of $P2X_7R$ promotes LPS-induced IL-1β production in the hippocampus, whereas the activation of A1 adenosine receptors has an inhibitory role.

Dual signalling mechanism of P2X7-like receptors in rat cerebellar astrocytes

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P2X7 receptor is a member of the P2X family, the ATP-gated ion channels. The cellular localization and functionality of P2X7 receptors in the central nervous system remains controversial. In a preliminary work we detected the presence of functional P2X7 subunits in cerebellar astrocytes, which after stimulation with BzATP, far from inducing cellular lysis, evoked morphological changes, which were not reproduced by any other nucleotide. In order to corroborate the presence of P2X7 in this cellular model we conducted the characterization of BzATP-induced calcium responses. We found that BzATP calcium responses were biphasic in the majority of the tested cells and exhibited two components. The first one was transient and metabotropic in nature, independent of extracellular calcium and sensitive to PLC inhibitors and the second one was ionotropic, sustained and slowly rising during the stimulation period. The ionotropic feature of the second component was also assessed by monitoring Mn2+ quenching of fura-2 fluorescence (F360) and by measuring non-selective cationic currents (Erev+5 mV) in whole cell configuration and in the perforated-patch configuration of the patch-clamp. The two components differed in their sensitivity to P2X7 antagonists. The first one was only sensitive to PPADS, whereas the ionotropic component was sensitive to the two antagonists of P2X7, PPADS and BBG. Furthermore, the ionotropic component was also modulated by extracellular divalent cations, Mg2+ and Zn2+, a distinctive feature of P2X7 receptor. These results reveal that P2X7-like receptor present in rat cerebellar astrocytes represents another example of ionotropic receptors displaying metabotropic activity.

Antibiotics and natural peptides as modulators of P2X7 functions

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Among natural antibiotics, polymyxin B (PMB) and hCAP-18/LL37 have recently been shown to modulate responses induced by stimulation of the P2X7 receptor subtype.
PMB, a cationic cyclic antibiotic produced by Bacillus polymyxa, potentiates responses elicited by ATP or BzATP. Expression of P2X7 is mandatory for up-regulation of Ca2+ influx, plasma membrane permeabilization, IL-1beta secretion and cytotoxicity. The N-terminal fatty amino acid 6-methylheptanoic/octanoic-diaminobenzoic residue of PMB is also required for up-modulation of ATP- and BzATP-induced responses.
Cathelicidin peptides bind to bacterial surface thus leading to microbial killing. hCAP-18/LL-37 is the only cathelicidin of humans. It is stored in granules of neutrophils, in lymphocytes, macrophages and epithelial cells from skin and mucose membranes.
LL-37 stimulates the growth of murine fibroblasts through activation of P2X7. This effect does not depend on helix sense (the all-D analog is active) but requires a strong helix-forming propensity in aqueous solution (a scrambled analog and primate orthologs devoid of this property are inactive). In P2X7-expressing cells, LL-37 raises intracellular Ca2+, synergizes with BzATP to increase intracellular Ca2+ and allows cellular internalization of ethidium bromide. The activity of hCAP-18/LL-37 has an absolute requirement for P2X7 expression, as it is fully blocked by the potent P2X7 inhibitor KN-62 and is absent in cells lacking P2X7. Peculiarly, hCAP-18/LL-37 restores the pore-forming activity in cells expressing a truncated P2X7 receptor.
Our results show that besides a direct effect on microbes, PMB and hCAP-18/LL-37 interact with a receptor expressed by eukariotic cells, thus adding complexity to the picture of the antibiotic-mediated effects.

(Invited)

$P2X_7$-mediated apoptosis inhibits DMBA/TPA-induced skin papilloma formation in mice

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Background: In epithelia the main function of the $P2X_7$ receptor is regulation of cell growth through mediation of apoptosis. ATP is the naturally occurring ligand of the receptor and it is present in the extracellular space at concentrations that suffice to activate the receptor. Protein and mRNA levels of $P2X_7$ are lower in cancer epithelial tissues than in the corresponding normal tissues. Since defective apoptosis may lead to cancer, we hypothesized that the decreased cellular expression of $P2X_7$ could be causally related to the development of cancer. The objective of the present study was to understand the pro-apoptotic role of $P2X_7$ in the development of skin papillomas, precursors of skin cancer in mice.
Methods: Adult female FVB mice (15 in each group) were treated with DMBA (carcinogen) followed by biweekly TPA (cancer promoter), applied directly on their shaved dorsal skin. Test animals were co-treated with the $P2X_7$ agonist BzATP, applied on the dorsal skin. Control animals were treated with BzATP or with DMBA/TPA.

Results: (1) Skin papillomas appeared 3–4 weeks after the DMBA/TPA treatment; (2) Co-treatment with BzATP decreased formation of papillomas at week 12 (Table); (3) Papillomas were not formed in the BzATP-only group; (4) Treatment with BzATP augmented apoptosis of epidermal keratinocytes; (5) Treatment with BzATP alone had no toxic effects.

	BzATP	DMBA/TPA	BzATP+DMBA/TPA
Percent animals with papillomas	0	87	54 ($p=0.055$)
Number of papillomas per animal	0	2.27	1.23 ($p<0.04$)
Papilloma size (mm) per animal	0	5.86	3.46 ($p<0.01$)

Summary and Conclusion: Local treatment aimed at activation of the $P2X_7$ receptor inhibited skin papilloma formation in mice. Activation of $P2X_7$-mediated apoptosis may prevent skin cancer development. (Support: NIH AG15955).

3Live cell imaging of caspase activity induced by P2x7 receptor stimulation in mouse macrophages

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P2X7 receptors are expressed in macrophages, and activation of these ATP-gated ion channels is implicated in caspase-dependent cytokine processing and cell death. It is controversial whether apoptotic signaling is an early or late event in relation to cytokine processing. Here, we investigated the effects of P2X7 receptor activation on cell morphology, cytosolic [Ca2+], and intracellular caspase activity in single mouse resident peritoneal macrophages. Millimolar ATP (required to activate P2X7) elicited a large cytosolic Ca2+ spike, followed by a sustained elevation of [Ca2+]. The sustained Ca2+ component was absent in cells isolated from P2X7-deficient (P2X7-/-) mice. Time-lapse contrast and fluorescence imaging, combined with topographic scanning by atomic force microscopy, revealed the hallmarks of early apoptosis, membrane phosphatidylserine translocation and extensive blebbing, within 60 s of ATP application. Intracellular caspase 3 activity, measured by live cell fluorescence imaging, appeared within 30 min of ATP application and coincided with massive bleb formation (cell destruction). Apoptotic signaling was absent in P2X7-/- macrophages. We now plan to measure intracellular caspase-1 activity using a novel probe, and thereby dissect the kinetics of P2X7-mediated “inflammatory” versus “apoptotic” caspase signaling in single cells.

Expression of P2X7 transcripts in neurons of the rat brain

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The ATP-gated P2X7 receptor is present in immune cells as well as epithelial and endothelial tissues. It is an appealing drug target because of its function in neuropathic and inflammatory pain, interleukin secretion, and bone formation. Recent genetic study also suggests an involvement in familiar depressive disorders. However, reports regarding cellular distribution in the central nervous system are controversial. In this study, we performed in situ hybridization using DIG-labeled cRNA probes to investigate the distribution of P2X7 transcripts in the rat brain.
We detected clear cytosolic signals in all regions of rat brain sagittal sections compared to control probe staining. P2X7 mRNA is widely expressed in many types of neurons in the adult rat brain. P2X7 mRNA was strongly expressed in

excitatory principal cells and inhibitory interneurons in both, the neocortex and the hippocampus, whereas in the cerebelulm only purkinje cells and inhibitory interneurons exhibiedt a strong expression. Cerebellar granule cells showed rarely a weak signal. Glial cells in the hippocampus and cerebellum showed generally a rather weak signal, but in the neocortex at least some glial cells also expressed P2X7 mRNA at high levels. Sporadic positive signals were also observed in nerve fibers among the corpus callosum and the fimbria of hippocampus.
Our findings with a clear cellular resolution are in agreement with a recent report from Shimada and co-workers (Brain Res. 2008) and show that the P2X7 receptor is expressed predominantly in neurons rather than glia cells in the rat central nervous system.

Inhibitory effects of ATP on pannexin-1 currents

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Background. Pannexin-1 (panx1) has been identified as the 'large pore' activated by the ATP-gated P2X7 receptor. There is currently little known about panx1 as an ion channel. We have asked whether ATP has direct effects on panx1 currents in the absence of P2X7 receptors.
Methods. Whole-cell patch clamp recordings were performed on HEK cells transiently expressing human or mouse pannexin-1.
Results. Panx1 currents were distinguished by their characteristic current-voltage (IV) relationship: they showed depolarization-evoked outward rectification and were rapidly and reversibly blocked by 30 microM carbenoxolone (CBX). The current was not affected by lanthanum (2 mM), which blocks endogenous Trp-like currents in HEK cells. Extracellular ATP inhibited the outward panx1 currents following in a dose-dependent manner with an IC50 of 630 microM. Similar inhibitory effects on panx1 currents were also observed with UTP and GTP, the IC50s of which were 1200 microM and 1122 microM, respectively. Pyrophosphate (PPi) did not inhibit panx1 currents at either 0.1 or 5 mM while ADP and AMP only minimally inhibited panx1 currents.
Conclusions. Nucleoside triphosphates, but not their phosphate groups, directly inhibit panx1 currents when panx1 is expressed in cells lacking the P2X7 receptor.
Topic: Cellular and molecular biology of receptors

ATP elicited intracellular reactive oxygen species generation via NADPH oxidase in murine macrophages can induce cell death

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Extracellular ATP has been observed to elicit the generation of reactive oxygen species (ROS) in macrophages. We are currently investigating the nature of these ROS; mechanisms involved, location of generation and their role in cell death. Murine J774.2 macrophages were loaded with 10 micromolar 2′ 7′-dichlorodihydrofluorescin-diacetate to measure ROS generation. Membrane necrosis was detected by the release of cellular lactate dehyrogenase (LDH) into cell supernatants. Stimulation of lipopolysaccharide-primed J774.2 cells with ATP triggered a sustained generation of ROS. The ATP concentration-response curve was observed to be bell shaped peaking at ~ 2 mM with an up-phase EC50=3.24±0.04 (n=10). ATP (1 mM) induced oxidation was inhibited with the flavoprotein oxidoreductase inhibitor diphenyleneiodonium (100 micromolar DPI) by 58+/-2% (n=3) and the NADPH-oxidase inhibitor Apocynin (100 micromolar) by 25+/-6% (n=4). Inhibitors of Xanthine oxidase (allopurinol), Mitochondrial complex I and III (rotenone, myxothiazole) and nitric oxide (L-NAME) did not inhibit ROS formation (n=3, P>0.05).
Addition of extracellular superoxide dismutase also failed to modulate ATP elicited ROS generation (n=3). Stimulation of cells with 1 mM ATP led to an increase in LDH release (n=3) within 30 min. Pre-incubating cells with 100 micromolar DPI inhibited LDH release by 49+/-5% (n=3).
We conclude that ATP induced cell death occurs via the generation of intracellular ROS by NADPH Oxidase.

Functional characterization of a P2X7 splice variant with an alternative transmembrane domain 1

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We have identified a P2X7 splice variant (P2X7(k)) in which the intracellular N-terminus and 80% of the proposed first transmembrane domain are replaced by a highly lipophilic sequence derived from a yet unidentified exon in the rat P2X7 gene. Here we describe the tissue distribution and functional properties of this variant and compare it with the originally cloned P2X7(a) variant.
Duplex RT-PCR with primer pairs selective for the P2X7(a) and P2X7(k) isoforms revealed the presence of the P2X7(k) transcript in a wide variety of tissues with a dominant expression in the liver. Two-electrode voltage-clamp analysis of the variants in Xenopus oocytes showed a rapid activation followed by a slow current increase upon application of saturating ATP concentrations. This current was reversible in case of the P2X7(a) isoform but appeared irreversible or only slowly reversible in case of the P2X7(k) receptor, thus complicating further analysis. Analysis of reversal potentials in extracellular NMDG solution showed a different relative permeability increase for NMDG of the two isoforms. Unlike the P2X7(a) receptor, the splice variant exhibited already a high permeability ratio (PNMDG/PNa) at the beginning of the ATP application suggesting that the channel is constitutively dilated upon opening. Patch clamp analysis and Ca2+ imaging experiments of HEK cell-expressed isoforms showed that the P2X7(k) receptor has a higher Bz-ATP sensitivity and much longer inactivation time. We conclude that residues critically involved in channel activation and pore dilation are located within the TM1 domain.

Homotrimeric complexes are the dominant assembly state of P2X7 subunits

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P2X4 and P2X7 subunits show an abundant and overlapping expression in a variety of tissues and both subunits appear to be functionally involved in processes such as bone formation, inflammatory pain and neuropathic pain. Biochemical and functional evidence for the existence of heteromeric P2X4/7 receptors was recently shown.
In this study, we validate the use of subtype specific antibodies in combination with BN-PAGE and SDS-PAGE analysis and subsequent western blotting as an approach to decipher P2X receptor complexes solubilized from membrane extracts of native tissues and from cRNA-injected Xenopus oocytes. Our results show that specific P2X7 and P2X4 receptor staining was abundant in brain, lymph nodes, bone marrow and salivary gland. The native and recombinant P2X7 complexes showed identical size that clearly differed from the size of the P2X4 complexes. Native and recombinant P2X4 and P2X7 complexes were composed of three subunits. No complexes corresponding to more than three subunits could be detected suggesting that possible interactions with other proteins are only transient or did not resist the process of solubilization or BN-PAGE analysis. No complexes of intermediate size between P2X4 and P2X7 complexes were detected. Also, no complexes reactive to both P2X4 antibodies and P2X7 antibodies were detected. We conclude that heteromerization

between P2X4 and P2X7 subunits occurs either not in form of stable trimeric complexes or that P2X4/7 heteromers do not represent a dominant subtype in the investigated tissues.

Pannexin-1 as a key component for the large pore formation by the ATP-gated P2X7 receptor

Pablo Pelegrin
UK

The P2X7 receptor (P2X7R) is unique among ion channels in that its activation not only opens a "typical" ion channel selective for small cations including calcium but also leads to the gradual opening over seconds to minutes of a larger pore that allows passage of molecules up to 900 dalton, also in macrophages is one of the main physiological signals that leads to the processing and release of the pro-inflammatory cytokine, interleukin-1beta (IL-1b). It has not been known what this large pore is, or whether it is involved in downstream signalling to IL-1b release. We discover a novel hemichannel protein, pannexin-1 (panx1), that is highly expressed in human and mouse macrophage, is upregulated under inflammatory conditions, and co-immunoprecipitates with the P2X7R protein. Selective inhibition of panx1 by siRNA targeting panx1 and by a panx1-mimetic inhibitory peptide inhibited P2X7R-mediated dye uptake without altering the associated membrane current or calcium influx, and without altering P2X7R protein expression. Over-expression of panx1 increased the rate of P2X7R-mediated dye uptake and induced constitutive dye-uptake in the absence of P2X7Rs. Also, inhibition of panx1 blocked caspase-1 cleavage as well as IL-1b processing and release from a repertoire of LPS-primed mouse and human macrophage in response to P2X7R activation. Thus, we have identified panx1 as the large pore pathway activated by P2X7Rs and show that signalling through panx1 is required for processing and release of mature IL-1b induced by this receptor.

(Invited)

ATP-induced P2X7-associated uptake of large molecules involves distinct mechanismsfor cations and anions in macrophages

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Background: P2X7 receptor is an ATP-gated cation-selective channel that has also been associated with the opening of a non-selective pore that allows the passage of large organic ions. In macrophages, cationic dyes such as ethidium and YO-PRO-1 and anionic dyes such carboxyfluorescein and Lucifer Yellow are taken up upon ATP stimulation. It has been assumed that cations and anions enter the cells through the same pathway. However, only the uptake of cationic dyes have been reported in HEK-293 and other cells transfected with P2X7 receptors.
Methods: We used patch-clamp recordings, intracellular Ca2+ measurements and fluorescent dye uptake assays to compare P2X7-associated transport phenomena of macrophages and HEK-293 cells transfected with P2X7 receptors (HEK-P2X7 cells).
Results: Macrophages and HEK-P2X7 cells presented inward currents, increase of free intracellular Ca2+ and uptake of cationic dyes upon exposure to ATP, as described. However, contrary to macrophages, HEK-P2X7 cells did not uptake anionic dyes and did not display the 440 pS channels (Z pores) under cell-attached patch-clamping configuration. Moreover, the transport mechanism of anionic dyes can also support dye efflux and, once activated at 37°C, it remains active at 4°C, while cationic dyes follow a temperature-dependent unidirectional uptake-only pathway.
Conclusions: Our results indicate that the mechanism of ATP-induced dye uptake, usually called "permeabilization phenomenon" can be ascribed to at least two distinct mechanisms in macrophages: a diffusional pathway, possibly

associated with the Z pores, and a cation uptake mechanism that is not diffusional and should be ascribed to a yet non-identified transport mechanism.

ADP-ribosylation at R125 gates the P2X7 ion channel by presenting a covalent ligand to its nucleotide binding site

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The cytolytic P2X7 purinoceptor plays an important role in inflammation because of its capacity to sense extracellular ATP and NAD+ released from damaged cells and to induce calcium signalling and cell death. The P2X7 ion channel can be gated by its soluble ligand ATP or by ADP-ribosylation mediated via the arginine-specific ADP-ribosyltransferases (ARTs) that transfer the ADP-ribose moiety from NAD+ onto specific target proteins. The aim of this study was to identify the target arginine(s) of the toxin-related ecto-ADP-ribosyltransferase ART2.2 that is responsible for receptor gating. We examined the responses of arginine mutants of mouse P2X7 in transfected human embryo kidney (HEK) cells to soluble and covalently bound ligands by live cell calcium imaging. Cell surface expression levels were assessed by flow cytometry using three different antibodies that recognize P2X7 in native conformation. We show that Lysine substitutions at R206, R276 and R277 result in gain of function mutants with enhanced ATP and NAD+ responses, while lysine substitution at R294 results in loss of channel function. Perfusion of HEK cells co-transfected with ART2.2 and the gain of function mutants with low concentrations of ecto-NAD+ induced gating of P2X7 following ADP-ribosylation at R125. Arginine 125 lies on the prominent cysteine-rich finger at the interface of adjacent receptor subunits. ADP-ribose shares an adenine-ribonucleotide moiety with ATP. We propose that ADP-ribosylation of R125 positions this common chemical framework to fit into the nucleotide-binding site of P2X7 and thereby gates the channel.

Are cathelin-derived peptides natural agonists of P2X7 receptors?

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Recent data suggest that LL-37, the only human cathelicidin, might be the natural agonist for P2X7 receptors since at low concentrations it can activate the P2X7 receptors expressed by human macrophages. But we observed that in mouse peritoneal macrophages LL-37 blocked the non-selective cation channel coupled to P2X7 receptors (Pochet et al., in preparation). The purpose of our work was to test for a possible species specificity (man versus mouse) of this family of peptides by measuring the response of murine macrophages to CRAMP, the mouse orthologue for LL-37. One millimolar ATP provoked a biphasic increase of the [Ca2+]i: the initial peak was followed by a sustained increase. In cells from P2X7-KO mice only the initial peak was observed. ATP also increased the catabolism of phospholipids and the secretion of IL-1beta. Longer incubations with ATP provoked the release of LDH and cell death (measured by the MTT test). One μM CRAMP was without effect by itself but in combination with ATP the peptide blocked the sustained increase of the [Ca2+]i without affecting the initial peak and inhibited the release of oleic acid. It had no effect on the release of IL-1beta provoked by ATP
In conclusion CRAMP is not an agonist of murine P2X7 receptor but rather an inhibitor of some responses mediated by this receptor. It is concluded that the ATP- like properties of LL-37 on human P2X7 receptors are specific to this peptide and that the cathelin-derived peptides cannot be considered as natural agonists of all P2X7 receptors.

Haemolysis induced by alpha-haemolysin from Escherichia coli requires P2X receptor activation

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Escherichia coli is the dominant facultative bacterial strain in the normal intestinal flora. E.coli is, however, also responsible for serious extraintestinal infections. Invasive E.coli-strains frequently produce virulence-factors as alpha-haemolysin (HlyA), which causes haemolysis by creating pores in the erythrocyte membrane. The present study reveals that this pore-formation per se is not sufficient to produce haemolysis. HlyA demands intact purinergic signalling to mediate its haemolytic action.
Methods
HlyA was collected from the supernatant of E.coli strain ARD6. Haemolysis was documented by optical density (OD540) measurements of the erythrocyte supernatant or by time laps microscopy.
Results
The non-selective P2-receptor antagonist (suramin) and ATP scavengers (apyrase and hexokinase) concentration-dependently decreased HlyA-induced lysis of equine, murine, and human erythrocytes. The pattern of responsiveness to more selective P2-antagonists indicate that the relevant P2-receptor is the P2X7-receptor in human -, and a combination of P2X7 and P2X1 in murine and equine erythrocytes. These findings were verified in erythrocytes from P2X7-/- mice, which showed lower levels of haemolysis compared to controls. The pore-forming protein pannexin1 also seems to be implicated in HlyA-induced haemolysis, as non-selective inhibitors of this channel attenuated haemolysis in all three species. The principal is not restricted to HlyA-induced haemolysis as similar results were found with á-toxin from Staphylococcus aureus. In conclusion, activation of P2X-receptors is a requirement for hemolysis induced by HlyA. These findings have clear clinical perspectives as P2-antagonists may ameliorate symptoms during sepsis with haemolytic bacteria and offer a time window for antibiotic treatment to become effective.

Comparison of the P2X7 Receptor in Canine and Human Erythrocytes and Mononuclear Leukocytes

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We have previously demonstrated that canine erythrocytes express P2X7 and that this receptor has greatly increased function compared with human erythrocytes. Using cation flux measurements we further compared P2X7 in erythrocytes and mononuclear leukocytes from these species. ATP and BzATP stimulated rubidium efflux from canine erythrocytes in a NaCl medium with EC50 values of ~519 and ~17 microM respectively. These values were reduced to ~271 and ~12 microM respectively in KCl (sodium-free) medium. EC50 values for ATP and BzATP for human erythrocytes were ~150 and ~15 microM (NaCl) ~84 and ~5 microM (KCl) respectively. KN-62 and magnesium inhibited ATP-induced rubidium efflux from canine erythrocytes with IC50 values of ~7 nanoM and ~124 microM respectively, which was similar to human erythrocytes (~25 nanoM and ~79 microM respectively). KN-62 and magnesium also inhibited ATP-induced choline uptake by >90% in erythocytes from both species. ATP and BzATP but not ADP and NAD induced ethidium uptake into canine T and B lymphocytes and monocytes. KN-62 reduced the ATP-induced ethidium uptake into these cells by >90%. In contrast to human leukocytes, where monocytes had six-fold greater P2X7 function than lymphocytes, P2X7 function was greatest in canine T lymphocytes, with values twice that of canine B lymphocytes and monocytes. P2X7 function into canine lymphocytes was five times greater than that of the human lymphocytes, while P2X7 function in canine monocytes was half that of human monocytes. In conclusion, the relative P2X7 function differs greatly between equivalent blood cell sub-populations from dogs and humans.

Biophysics and transcriptomics of hemichannels and their genes

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Cells respond to a variety of stimuli with the opening of a permeability pathway through which moderately large molecules can enter or leave the cell. From the standpoint of purinergic signaling such a pathway is important, both because the permeability pathway is activated by prolonged exposure to high concentrations of ATP and because ATP is permeable to this pathway. Several channel proteins have been proposed to mediate this permeability pathway, with gap junction "hemichannels" or connexons, specifically formed of the gap junction protein connexin43 (Cx43), being favored by many investigators. However, the new gap junction protein pannexin1 (Panx1) readily forms highly permeable nonjunctional channels, is activated by P2 receptor stimulation, displays sensitivity to pharmacological agents known to block gap junction channels, and in brain displays coordinated expression with a similar set of genes that are coordinately expressed with Cx43. These finding suggest the possibility that Panx1 might mediate ATP release and dye uptake that have been attributed to Cx43 "hemichannels". This talk will summarize what one channel type can do that the other cannot, emphasizing similarities and differences between biophysical properties and pharmacodynamics of connexons and pannexons as well as their transcriptomic linkages to other genes

(Invited)

GL261 mouse glioma cell line is sensitive to ATP cytotoxicity

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Background: Gliomas are the most common and aggressive CNS tumor and their prognosis remains dismal. Previously we showed that interference with the purinergic signaling could reduce glioma growth. The objective of this study was investigating high ATP doses in glioma survival. Methods: Citotoxicity was measured by LDH release, propidium iodide incorporation and MTT assay. RT-PCR and Western Blot protocols were performed to analyze P2X7 mRNA and protein presence. Results: Here we show that ATP is cytotoxic in the mouse glioma cell line GL261, contrary to several other glioma cell lines tested. ATP 5 mM induced increased LDH release, PI incorporation and reduced cellular viability in GL261 cultures. ATP was toxic only at concentrations above 2 mM whereas other purinergic receptors agonists such as adenosine, ADP and UTP were not toxic at 5 mM. BzATP, also at mM concentrations, stimulated cell death. The P2 purinergic receptors antagonists suramin, RB2, BBG, TNP-ATP did not reversed ATP effects while PPADS and oATP significantly reversed ATP-induced toxicity. P2X7 expression, at mRNA and protein levels, in GL261, was confirmed. Conclusion: These results suggest the involvement of P2X7 receptor in ATP-induced death, which were corroborated by the expression of the mRNA and protein of this receptor in GL261. In contrast, the rat glioma C6 and the human gliomas U87, U138 and U343 showed a high resistance to ATP-induced cytotoxicity even at 5 mM. Interestingly, the P2X7 mRNA and protein was found some of these gliomas, suggesting that P2X7 is important but not sufficient for ATP-induced cell death in gliomas.

Over-expression of P2X7 receptor enhances tumor growth in vivo, but not in vitro

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The ATP-gated P2X7 receptor is a plasma membrane receptor belonging to the family of P2X purinoceptors. Activation of P2X7 receptor leads to multiple downstream events including influx of ions, pore formation to allow the passage of larger molecular weight species and cell death by apoptosis and/or necrosis. Until now, it has not yet been determined a role of P2X7 receptor in tumor cells.
To investigate the effect of over-expression of P2X7 receptor in tumor cells, mouse P2X7 receptor cDNA were transfected into B16 melanoma. High P2X7-expressed clones (C1, C6) and low-P2X7 expressed clone (C3) were selected. Apoptotic activity of P2X7 receptors of these clones was examined by ATP-induced LDH release and apoptotic cell shrinkage. C1 and C6 clones were more sensitively died by treatment with 1 mM ATP than C3 and wild type (WT). Moreover, number of apoptotic cell shrinkage was significantly higher in C1 and C6 cells. These data suggested that C1 and C6 clones have a higher activity of P2X7 receptor than WT and C3 cells. These clones were transplantated into mouse footpad. After transplantation, tumor volume was measured for a month. Tumor volumes of C1 and C6 cells significantly became larger than WT and C3 cells, indicating high expression of P2X7 receptor would cause tumor growth in vivo. However, cell proliferation rates of C1 and C6 cells were not higher than C3 and WT.
These results suggested that tumor growth was significantly enhanced in vivo, but not in vitro, by over-expression of P2X7 receptors.

A non-covalent association between P2x7 receptor core and the carboxyl tail strongly modulates P2x7 channel gating

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The P2X7 receptor has a carboxyl tail extension that is 120-200 amino acids longer than that of other P2X family members. Here, we used the split protein approach in X. laevis oocytes to assess the role of the tail extension in P2X7 receptor function. Carboxyl tail truncated receptor cores (residues 1-408, 1-436 or 1-505) formed homotrimers that appeared abundantly at the cell surface yet mediated only small currents. Co-expression of the tail extension (residues 434-595) as an individual protein greatly enlarged ATP-gated current amplitudes of 1-436 and 1-505 hP2X7 receptor cores, but not of the 1-408 core (as shown in detail in the accompanying poster of Becker et al.). Chemical cross-linking disclosed a direct interaction of unity stoichiometry between receptor fragments 1-436 and 434-595 or 465-595, but not between fragments 1-408 and 434-595 or 1-436 and 495-595. The full-length hP2X7 receptor could not be cross-linked with the co-expressed 434-595 tail domain. This suggests that the tail binding site is unoccupied and hence accessible in the truncated 1-436 receptor core, but blocked within the non-split full-length hP2X7 receptor by stable intrachain interactions with contiguous carboxyl tails. Altogether, these data demonstrate a functionally important and stable non-covalent association between hP2X7 residues 408-436 and 465-495, and identify the tail extension as a regulatory gating module.

Methylation-sensitive 549 bp region downstream the P2X7 promoter gene inhibits P2X7 gene transcription

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Background: The P2X7 receptor regulates cell growth through mediation of apoptosis. P2X7 levels are lower in cancer epithelial cells than in normal cells. The objective of the study was to understand the role of DNA methylation in P2X7 gene transcription. Methods: Experimental study using human cervical tissues and cultured cervical epithelial cells. DNA methylation assayes utilized the Combined Bisulfite Restriction Analysis (COBRA) method.
Results: (1) A 549 bp region of the human P2X7 gene (nt -66 to +483, from the putative translation start), including the distal 5'UTR, Exon-1, and the proximal Intron-1, contains 20 CpG sites. (2) Cytosines within CpG sites 102-103 (and/or

120-121), 238-239, and 370-371 (and/or 372-373) in that region were found to be hypermethylated in cancer cells/tissues compared to normal cells/tissues. (3) Treatment with the de-methylation agent 5-aza-2′-deoxycytidine (Aza-dC) decreased methylation. Aza-dC also increased P2X7 mRNA and protein expression, and augmented baseline apoptosis. (4) Promoter location assays revealed maximal P2X7 transcriptional control within a region of approximately 250 bp upstream the CpG-rich 549 bp region. (5) Heterologous expression of the putative promoter ligated with the 549 bp region in HEK293 cells revealed inhibition of transcription.
Summary and Conclusion: The identified 549 bp region downstream to the promoter inhibits P2X7 gene transcription. Hypermethylation of CpG sites within this region, e.g. in cancer epithelial cells augments silencing, while de-methylation inhibits the inactivation. (Support: NIH AG15955).

Micro-RNAs miR-186 and miR-150 regulate P2X7 mRNA stability

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Background: The P2X7 receptor regulates cell growth through mediation of apoptosis. P2X7 levels are lower in cancer epithelial cells than in normal cells. The objective of the study was to understand regulation of P2X7 mRNA stability.
xMethods: Experimental study using human cultured cells, and transfections with luciferase 3′UTR-P2X7 reporter.
Results: (1) Overexpression of a full-length 3′UTR-P2X7-reporter, or reporters containing parts of the 3′UTR-P2X7 were associated with increased stability of the construct in normal cells and decreased stability in cancer cells. (2) Overexpression of a reporter containing the middle part of the 3′UTR-P2X7 was associated with decreased stability in normal and cancer cells. (3) Sequences within the 3′UTR-P2X7, which are putative target sites for miR-186 (middle segment) and miR-150 (distal segment), conferred instability to the P2X7 transcript. (4) Overexpression in cancer cells of mutated miR-186 and miR-150 target sites was associated with attenuated degradation of the reporter genes. (5) In normal cells overexpression of the mutated miR-186 target site was associated with marked stability, but overexpression of the miR-150 target-site reporters, wild-type and mutant, did not change over time. (6) Levels of miR-186 and miR-150 were more abundant in cancer than in normal cells, and treatment with miR-186 and miR-150 inhibitors increased P2X7 mRNA. (7) In HEK293 cells transfected with full-length 3′UTR-P2X7 luciferase reporter, miR-186 and miR-150 inhibitors increased, while miR-186 and miR-150 mimics decreased luciferase activity. Summary and Conclusion: miR-186 and miR-150 stimulate degradation of P2X7 mRNA by activation of miR-186 and miR-150 targets located at the 3′UTR-P2X7. (Support: NIH AG15955).

SYMPOSIA 21: LUNG PHYSIOLOGOY

Purinergic stimulation of ciliary beat frequency in adenosine receptor knockout mouse tracheal rings is dependent on A_{2B} adenosine receptor activation

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Background: Ciliary Beat Frequency (CBF) is a key regulator of innate airway defense and adenosine (ADO) is recognized as an important modulator of many physiological and pathological processes including ciliary motility. However, the contribution of specific adenosine receptors to key ciliary motility processes are unclear. The aim of this study was to evaluate adenosine regulation of CBF in mouse tracheal rings from adenosine receptor knockout mice (A_1, A_{2A}, A_{2B} or A_3, respectively) using an *ex vivo* tissue model.
Methods: Mouse tracheal rings (MTR) were excised and stimulated with a known activator of CBF, procaterol (10 μM), in the presence or absence of NECA (10 μM), a non-selective adenosine receptor agonist. We measured CBF digitally using the Sisson-Ammons Video Analysis (SAVA) system.
Results: Procaterol stimulated CBF in tracheal rings from both wild type and adenosine knockout mice by ~3 Hz over control media-treated rings. Tracheal rings from A_1, A_{2A} and A_3 knockout mice stimulated with NECA also demonstrated an increase in CBF. However, NECA failed to stimulate CBF in tracheal rings from A_{2B} knockout mice.
Conclusion: Mouse tracheal rings of adenosine receptor knockout mice confirmed the concept drawn from previous pharmacological data in our lab that stimulation of A_{2B} receptor(s) potentiate epithelial ciliary motility. Collectively, the data indicate purinergic stimulation of CBF is reliant on A_{2B} adenosine receptor(s) activation.
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Role of adenosine signaling in lung inflammation and remodeling, inferences for asthma, COPD and pulmonary fibrosis

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Asthma, COPD and pulmonary fibrosis are distinguishable pulmonary disorders with clear diagnostic differences. However, these chronic lung diseases share features such as persistent inflammation and airway remodeling and/or destruction. Little is known about the factors that regulate the chronic nature of these disorders. Adenosine is produced in response to injury and has clear anti-inflammatory properties and can orchestrate processes such as matrix production and angiogenesis that aid in the protection or repair of tissues following injury. However, in situations of chronic and unremitting pathogenesis adenosine may contribute to overactive wound healing responses such as fibrosis and thus contribute to the progression of certain disorders. Adenosine levels are elevated in individuals with chronic lung disease where it has been proposed to regulate features of chronic lung disease. My laboratory has been using adenosine deaminase (ADA)-deficient mice that develop adenosine-dependent pulmonary inflammation and damage to study the contribution of adenosine signaling to features of chronic lung disease. We utilize adenosine receptor knockout mice as well as pharmacological approaches to examine specific cellular mechanisms involved in these processes. My presentation will summarize our findings to date concerning the role of specific adenosine receptors in this model and draw references to their impact on human disease. Attention will be given to the ability of elevated adenosine to activate the A2BR to regulate macrophage activation, angiogenesis and fibrosis in the lung.

(Invited)

Stimulation of Nucleotide Release by Changes in Airway Mucus Viscoelasticity

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Background: In the airways, extracellular ATP and adenosine represent major regulators of the "hydration" of the airway surface, critical for maintaining proper mucus clearance. The goal of this work was to test whether airway epithelia sense and respond to increases in the viscoelastic properties of the overlying mucus by stimulating ATP release, as a consequence of stresses generated by cilia beating.
Methods: For these studies, we used agarose as a "mucus simulant", as this high-molecular weight polymer exhibits similar rheological properties found in native airway mucus at low concentrations (0.1–0.5%). Human airway cultures were exposed to agarose over a range of viscoelasticities for 30 min prior to quantitation of apical [ATP] by luciferase bioluminescence.
Results: Our data demonstrate that steady-state [ATP] increased as a function of [agarose]. Interestingly, significant changes in [ATP] were only observed at viscoelasticities greater than that of "normal" mucus. We also observed that the yield stress of the agarose showed a high correlation with [ATP], suggesting that this property could be sensed by the cilia. In contrast, [ATP] in airway cultures from patients with immotile cilia was not significantly altered when exposed to high viscoelastic agarose, suggesting that beating cilia is involved in sensing/transducing the viscoelastic properties of the mucus.
Conclusions: Our data shows that as the viscoelasticity of the agarose increases, the cilia experience an increased resistance to beating, leading to stimulation of ATP release. As a result, we predict that this provides an "autoregulatory" feedback mechanism to rehydrate the mucus layer, optimizing mucociliary clearance.

Nucleotide-mediated inhibition of alveolar fluid clearance in influenza virus-infected BALB/c mice

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Pulmonary infections can impair alveolar fluid clearance (AFC), contributing to formation of lung edema and hypoxemia. However, effects of influenza virus on AFC are unknown.
BALB/c mice were infected intranasally with influenza A/WSN/33 (10,000 FFU/mouse). AFC was measured in anesthetized, ventilated mice by instilling 5% BSA into the dependent lung. Infection with influenza A resulted in a steady decline in body weight and arterial oxygen saturation, together with increased lung water content. AFC was significantly inhibited from 1 hour after infection, and remained suppressed over days 1 to 6. AFC inhibition at early timepoints (1–4 hours after infection) did not require viral replication, while AFC inhibition later in infection was replication-dependent. Impaired AFC at day 2 resulted primarily from reduced amiloride-sensitive transport, which could be reversed by addition to the AFC instillate of agents that block the pyrimidine-purinergic receptor axis: a de novo pyrimidine synthesis inhibitor (A77-1726), inhibitors of volume-regulated anion channels (fluoxetine or ROCK inhibitor), enzymes that degrade UTP (apyrase, UDP-glucose pyrophosphorylase) but not ATP (hexokinase), and P2Y purinergic receptor antagonists (suramin or XAMR-0721).
However, an additional component of AFC impairment derived from activation of adenosine receptors leading to cAMP stimulation of increased CFTR-mediated anion secretion.
Influenza infection also increased bronchoalveolar lavage fluid nucleotide levels. These data indicate that nucleotide-mediated AFC inhibition may be an important feature of the pathogenesis of influenza virus infection. Its blockade may reduce the severity of pulmonary edema and hypoxemia associated with influenza pneumonia.

Role of purinergic signalling on allergen-induced lung inflammation

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Allergic asthma is one of the most common chronic diseases in western society, characterized by mucus hypersecretion by goblet cells, structural remodelling of the airway wall, variable airway obstruction, bronchial hyperresponsiveness (BHR) to non-specific stimuli and infiltration of the airway wall with T-helper type 2 (Th2) cells, eosinophils and mast cells. Increasing evidences shows that lung dendritic cells (DC) play an important role in the induction and maintenance of asthmatic airway inflammation. In addition airway and bronchial epithelial cells are also involved in regulating airway inflammation and airway remodeling

In the past others and we could show that all structural and inflammatory cells involved in the pathogenesis of asthma do express functional P2R and that activation of these receptors could modulate the function of the different cells in a "pro-asthmatic manner". Recently we provided evidence that ATP serves as an important mediator in the pathogenesis of asthma. 1) ATP levels are elevated in allergen- but not in saline-challenged airways of OVA-sensitized and -challenged asthmatic mice. 2) Decreasing extracellular ATP levels in the airways or blocking airway P2-receptors potently inhibits features of asthma in the mouse model. 3.) Administering ATP enhances Th2 immunity in the lung by causing mDC recruitment and activation. ATP enhances the capacity of mDCs to prime for Th2 responses directly. Together these data show that extracellular ATP might be a key mediator of allergic inflammation in asthma, therefore targeting P2R might be a novel and power strategy for the treatment of allergic asthma.

(invited)

The respiratory phenotype of the adult and neonatal adenosine A1 receptor knock out mouse

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Background: The nucleoside adenosine has been implicated in the regulation of respiration especially during hypoxia. However, it is still a matter of debate which of the subtype of adenosine receptors is mediating the respiratory depression during hypoxia. This study aimed at investigating the role of adenosine A1 receptors during hypoxia in vivo. We used adult and neonatal adenosine A1 receptor knockout mice (A1R-/-) as tools to explore the importance of the A1 receptor in respiration.
Material and Methods: Respiration of unrestrained A1R-/- and wildtype mice (A1R+/+) was measured using a plethysmographic device.
Results: Under normoxia and mild hypoxia, no difference in respiration patterns was observed between adult A1R+/+ and A1R-/-. However, at more severe hypoxia A1R+/+ exhibited a transient increase of respiration followed by decrease of the minute volume (MV). In sharp contrast to this, the MV of A1R-/- was strongly and constantly increased under these severe hypoxic conditions. No differences in respiration of A1R-/- and A1R+/+ were observed during hypercapnia (3-10% CO2). Interestingly, under hyperoxia (100% O2) only the A1R-/- mice showed a transient respiratory depression. Neonatal A1R+/+ and A1R-/- did not show obvious differences in MV during hypoxia. However, neonatal displayed an increased rate of breathing arrests during hypoxia.
Conclusion: This is the first in vivo study demonstrating that the adenosine A1 receptor is a critical determinant of the hypoxia-induced down-regulation of respiration in adult mice. In neonatal A1R-/-, however, the stability of the respiratory control appears to be diminished as indicated by the increased risk of hypoxia-induced apnea.

Mucin secretion-dependent and -independent release of ATP from airway epithelium

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We recently illustrated that ATP is secreted from goblet airway epithelial cells during Ca2+-regulated mucin exocytosis, suggesting that ATP is a releasable cargo-molecule within the mucin granule (Kreda et al, J Physiol 584:245). Here, we describe that Calu-3 cell mucin granules can be loaded with MANT-ATP. Moreover, mucin granules isolated from Calu-3 cells are enriched in adenyl-nucleotides. Thus, ATP is likely released from mucin granules to signal neighbouring (ciliated) cells to promote ion/water transport for hydration of secreting mucins in the airway lumen.

In addition to Ca2+-promoted mucin secretion-associated ATP release, Calu-3 cells display robust ATP release in response to hypotonic shock. Hypotonicity-triggered ATP release is partially inhibited by BAPTA and bafilomycin-A, suggesting that an exocytotic mechanism is in part responsible. However, hypotonic shock does not stimulate mucin secretion from Calu-3 cells. Confocal microscopy studies indicate that in hypotonically-challenged Calu-3 cells, FM1-43-labelled granules moved rapidly close to the apical membrane without being secreted. We hypothesize that hypotonicity-mediated ATP release does not involve "complete" exocytosis. Rather, a kiss-and-run mechanism (as described in neuronal and endocrine cells) may account for the rapid release of ATP from granules, while the large mucin molecules remain unsecreted. Additionally, hypotonicity could elicit the insertion and/or activation of a plasma membrane channel that allows efflux of cytosolic ATP. This possibility is supported by data illustrating that hypotonically-released ATP is partially abrogated by connexin hemichannel inhibitors. In sum, our data suggest that airway cells release ATP by different mechanisms that likely reflect the histological and physiological complexity of the airway epithelium.
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Involvement of the P2Y1 receptor in asthmatic airway inflammation

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BACKGROUND Extracellular nucleotides have been shown to exacerbate the severity of allergic asthma in vivo by activating dendritic cells (DCs). However, the mechanisms and the subtypes of P2 receptors involved are still unknown. The aim of the present study was to investigate the role of the P2Y1 receptor in this pathology. METHODS The functionality of the P2Y1 receptor in human and mice DCs was tested by measuring intracellular calcium mobilization ([Ca2+]i). The consequences of this activation were examined by dosing cytokine secretion in response to ADP. To determine the role of the P2Y1 receptor in asthma, WT and P2Y1 deficient mice have been sensitized and challenged to ovalbumin. Features of allergic airway inflammation were evaluated in bronchoalveolar lavage and in plasma. RESULTS ADP induced a [Ca2+]i rise in human and in mice DCs which was inhibited by selective P2Y1 antagonist indicating that the P2Y1 is functional in DCs. This activation increased the production of IL-10 in DCs, showing that the P2Y1 receptor could influence the orientation of the immune response. In vivo, the increase of IgE was of 143% in WT mice after sensitization and challenge to ovalbumin, it was only of 43% in P2Y1 deficient mice. Finally, analysis of bronchoalveolar lavage showed that the recruitment of eosinophils was severely affected in mice deficient for the P2Y1 receptor (39%) as compared to control group (64%). CONCLUSION Altogether, these results provide evidence that the P2Y1 receptor is involved in allergic airway inflammation which could be at least partly explained by its role in the regulation of DCs maturation.

Morphofunctional attenuation of acute lung injury in $P2X_7$ knockout mice

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Background. Immune cells secrete nucleotides (ATP) at inflammatory foci as a result of cell injury. $P2X_7R$ activation could modulate the secretion of proinflammatory cytokines. We have investigated the roles for $P2X_7R$ activation in mice model of acute lung injury (ALI).
Methods. C57BL/6 WT and $P2X_7R$ knockout mice were intratracheally instillated with LPS (n=6) (E. coli-60ug/50uL-LPS-KO e LPS-WT) or saline (n=5) (50 uL, C-KO e C-WT). Lung histology (amount of elastic, collagen fibers and mast cells in the alveolar septa), the expression of $P2X_7R$, F4/80, BSL-1 lectin and TGF-beta, mechanical parameters, and biochemical analysis of lactate dehydrogenase (LDH) and nitric oxide (NO) in bronchoalveolar lavage fluid (BALF) were analyzed after 24 hours.
Results. Histomorphometric analysis of lung parenchyma demonstrated diminished polymorphonuclear and mononuclear cells in LPS-KO group ($p<0.05$), absence of mast cells (alcian-blue pH2.5) and decrease in alveolar collapse ($p<0.05$). The

LPS-KO and C-KO animals did not change the amount of collagen and elastic fibers, as well as secretion of LDH and NO in BALF ($p>0.05$). The expression of $P2X_7R$ was increased in LPS-WT group when compared with WT animals ($p<0.05$). Macrophages ($F4/80^+$) did not change after LPS treatment in LPS (WT and KO) groups ($p>0.05$), however the macrophage activation ($BSL\text{-}1^+$ lectin) was abolished in LPS-KO group ($p<0.05$). The increase of TGF-beta expression was smaller ($p<0.001$) on LPS-KO group. The animals LPS-KO did not have altered pulmonary functions ($p>0.05$).
Conclusion. The $P2X_7R$ participates in ALI on the recruitment of neutrophils and mast cells, in macrophage activation and in extracellular matrix remodeling.
Financial support: PRONEX, FAPERJ, CNPq.

A3 adenosine receptor signaling regulates inflammatory cell recruitment, eosinophil degranulation and extracellular matrix remodeling in bleomycin-induced lung injury

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Rationale: Adenosine has been implicated in the regulation of many pulmonary disorders by selectively engaging various adenosine receptors. The focus of the current study is to examine the involvement of A3 adenosine receptor (A3R) signaling in the bleomycin model of pulmonary inflammation and fibrosis.
Methods: Saline alone or 3.5 U/kg bleomycin was administered intratracheally to A3R-deficient (A3R-/-) and wild type (C57BL6, 8–10 weeks old) female mice. Inflammatory and fibrotic responses were examined fourteen days later.
Results: Bleomycin-treated A3R-/- mice exhibited enhanced inflammation that included a marked increase in pulmonary eosinophils. Expression of inflammatory chemokines and growth factors was upregulated. Despite the increase in eosinophil numbers, eosinophil peroxidase activity was diminished in the lavage fluid of bleomycin-treated A3R-/-mice, suggesting that the A3R is necessary for eosinophil activation in this model. Although inflammation was increased in A3R-/- mice exposed to bleomycin there was no difference in the degree of pulmonary fibrosis. Transforming growth factor (TGF)-*fÒ*1 RNA expression was highly elevated, but there was no concomitant increase in active TGF-*fÒ*1 protein levels. Furthermore, fibronectin expression was diminished in the lungs of A3R-/- mice exposed to bleomycin while the expression levels of matrix metabolizing enzymes were altered.
Conclusions: Together these results suggest that the A3R serves anti-inflammatory functions in the bleomycin model, and is also involved in regulating aspects of fibrosis and matrix metabolism.
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Impact of inflammation on airway epithelial ATP release

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Purinergic signaling regulates lung defense mechanisms. Airway inflammation has been linked to increased ATP concentrations in sampled respiratory fluid from cystic fibrosis (CF) patients (Esther, Eur Resp J 2008). We, therefore, investigated the contribution of airway epithelia to the raised ATP concentrations during inflammation. Well-differentiated primary human bronchial epithelial (HBE) cell cultures were challenged with secretion from CF airways or respiratory syncytial virus (RSV), to model bacterial and viral infection-induced airway inflammation, respectively.
In both models, basal ATP release did not differ between acutely inflamed vs. control HBEs. However, basal ATP release was increased in goblet cell metaplastic HBEs 6 weeks after RSV infection, and correlated with increased basal UDP-glucose and mucin release. IL-13-treated HBEs, a sterile model of goblet cell metaplasia, also exhibited increased basal ATP release, confirming the correlation between basal ATP release and goblet cell secretion.A different pattern was observed for

stimulated ATP release. HBEs just after exposure to CF airways secretion or RSV infection released 2–3 times more ATP following hypotonic challenge than controls. The increase in peak ATP concentrations correlated with the degree of inflammation (as indexed by IL-8) and the increase in ATP-triggered intracellular calcium mobilization. The inflammation-induced increase in ATP release was sensitive to inhibition of exocytosis by BAPTA, brefeldin A, monensin or N-ethylmaleimide. These data suggest a positive feedback loop between increased ATP release by exocytosis and increase in ATP-triggered intracellular calcium mobilization in inflamed HBE cells.
In conclusion, we propose that airway epithelia exhibit multiple mechanisms of increasing ATP release into the lumen in response to inflammation.

Modeling purinergic signaling reveals new therapies for obstructive airway diseases

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Background. In the airways, extracellular nucleotides support epithelial functions essential for host defenses. Released by the epithelium into the airway surface liquid (ASL), they stimulate mucus clearance through P2 (ATP) receptors, and following surface metabolism, through P1 (ADO) receptors. The complexity of the biochemical network regulating ATP and ADO concentrations has considerably hindered our progress in the identification of the key proteins regulating ATP- and ADO-mediated mucus clearance. Methods. A mathematical model was developed to integrate all known components of ASL nucleotide regulation: nucleotide release, the enzymes supporting the linear dephosphorylation of ATP into ADP, AMP and ADO, ADO deamination into inosine, and nucleoside uptake. The model also includes the non-linear ecto-adenylate kinase activity and feed forward inhibition of ADO production by ATP and ADP. Results. The model parameters were optimized using experimental data of the steady-state and transient concentration profiles generated by adding ATP to primary cultures of human bronchial epithelial cells. The model captures major aspects of ATP and ADO regulation, including their > 4-fold increase in concentration during mechanical stress mimicking normal breathing. An interactive approach between simulations and assays also reveals that feed forward inhibition is mediated by selective inhibition of ecto 5′-nucleotidase. Furthermore, the model identifies NTPDase 1 and ecto-adenylate kinase as key regulators of ATP, and proposes novel strategies for the treatment of airway diseases characterized by impaired nucleotide-mediated mucus clearance. Conclusion. These new insights into the biochemical processes supporting ASL nucleotide regulation illustrate the potential of this mathematical model for fundamental and clinical research.

Effect of A2B adenosine receptor gene ablation on pro-inflammatory adenosine signaling in mast cells

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Pharmacological studies suggest that A2B adenosine receptors mediate pro-inflammatory effects of adenosine in human mast cells in part by upregulating production of Th2 cytokines and angiogenic factors. This concept has been recently challenged by the finding that mast cells cultured from bone marrow (BMMCs) of A2B knockout (A2BKO) mice display an enhanced degranulation in response to FcepsilonRI stimulation. This was interpreted as evidence of anti-inflammatory functions of A2B receptors and it was suggested that antagonists with inverse agonist activity could promote activation of mast cells. In this report, we demonstrate that genetic ablation of the A2B receptor protein has two distinct effects on BMMCs, one is the previously reported enhancement of antigen-induced degranulation which is unrelated to adenosine signaling; another is the loss of adenosine signaling via this receptor subtype that upregulates IL-13 and vascular endothelial

growth factor (VEGF) secretion. Genetic ablation of A2B receptors had no effect on A3 adenosine receptor-dependent potentiation of antigen-induced degranulation in mouse BMMCs, but abrogated A2B adenosine receptor-dependent stimulation of IL-13 and VEGF secretion. Adenosine receptor antagonists MRS1706 and DPCPX with known inverse agonist activity at the A2B subtype inhibited IL-13 secretion induced by the adenosine analog NECA, but did not mimic the enhanced antigen-induced degranulation observed in A2BKO BMMCs. Thus, our study confirmed the pro-inflammatory role of adenosine signaling via A2B receptors and the anti-inflammatory actions of A2B antagonists in mouse BMMCs.

ATP released from pulmonary neuroepithelial bodies activates Clara-like cells in the NEB microenvironment via P2Y2 receptors

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Pulmonary neuroepithelial bodies (NEBs) reside in the airway epithelium as densely innervated groups of neuroendocrine cells that are shielded from the airway lumen by Clara-like cells. In ex vivo mouse lung slices, NEB cells respond with a [Ca2+]i rise upon membrane depolarisation with high K+, followed by a delayed [Ca2+]i increase in the neighbouring Clara-like cells, suggestive of an indirect activation. Aim of the present study was to unravel the mechanism of this potential interaction between NEBs and Clara-like cells. Because accumulation of ATP is known for NEB cells, we focused on a possible purinergic signalling pathway. Using 'sniffer-patching' to detect ATP release, stimulation with high K+ was shown to elicit quantal ATP release from NEBs. Use of enhanced ATP hydrolysis and the P2 receptor blocker suramin confirmed that ATP was responsible for activating Clara-like cells following application of high K+. A combined pharmacological and Ca2+ imaging approach revealed the involvement of functional P2Y2 receptors, and immunohistochemistry verified the expression of P2Y2 receptors on Clara-like cells. It may be concluded that stimulation of NEBs in ex vivo lung slices evokes exocytosis of ATP and subsequent selective purinergic activation of surrounding Clara-like cells in the NEB microenvironment. which recently has been assigned a potential pulmonary stem cell niche.
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Expression of P2X receptors in LAD-2 cells and Human Lung Mast Cells

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Mast cells play an important role in the pathophysiology of many allergic diseases including asthma. This is via the release of mediators into surrounding tissues following a rise in intracellular calcium. The molecular identity of the calcium channels supporting this influx have not yet been fully elucidated. P2X receptors are non-selective cation channels which may contribute to the influx of extracellular calcium in mast cells. The purpose of this study was to determine if P2X purinoceptors are functionally present in the LAD-2 mast cell line and primary human lung mast cells (HLMC's).
RT-PCR revealed P2X1, 4 and 7 transcripts in both cell types; mRNA for P2X6 was also detected in LAD-2 cells. Under whole-cell patch clamp recording conditions, ATP (1 s application) consistently evoked a P2X1-like current in both cell types. This current could also be elicited by alphabetamethyleneATP (10 microM, 93.55% cells, n=31) and was antagonised in LAD-2 cells by NF 449 (1 microM) and Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (10 microM). A P2X7 -like non-desensitising current in response to high concentrations of ATP (1–5 mM) was also seen in both cell types (96% LAD-2 cells, n=24; 54% HLMC's, n=24) and could be evoked in LAD-2 cells by the agonist 2′(3′)-0-(4-benzoylbenzoyl)ATP (300microM). A P2X4 like current was seen in response to 100microM ATP (80% LAD-2 cells, n=10; 21% HLMC's, n=29), the amplitude and duration of which was potentiated by Ivermectin. In summary, the electrophysiological recordings support the molecular studies and confirm the presence of functional P2X1, 4 and 7 receptors in LAD-2 and HLMC's.

SYMPOSIA 23: Mechanisms of ATP release

A non-junctional role for a "gap junction protein"

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Pannexins, a second family of gap junction proteins in vertebrates, have sequence homology with the invertebrate innexins. However, it appears that in contrast to their invertebrate counterparts, pannexins have lost the ability to form gap junction channels. Instead, they provide a pathway across the non-junctional plasma membrane for the exchange of molecules in the size range of second messengers between cytoplasm and the extracellular space. The ability to form non-junctional membrane channels is also realized in (some) innexins, in addition to their well-characterized gap junction function in invertebrates. Thus, in vertebrates with the acquisition of connexins for gap junction function, pannexins were retained solely for the non-junctional role. Pannexin1 forms a large channel with a unitary conductance of 475 pS. The channel is highly permeable for ATP and can be activated at the resting membrane potential by either mechanical stress or by cytoplasmic calcium. When co-expressed with purinergic receptors, the pannexin channel also can be activated by extracellular ATP. Based on these properties and on its expression profile the pannexin channel has to be considered a prime candidate for the elusive ATP release channel, which is a crucial component in the initiation and propagation of intercellular calcium waves. Another non-junctional function of pannexin is the formation of the large pore in the P2X7 receptor complex, which is involved in inflammation processes.

(Invited)

Evidence for P2X7 receptor-mediated release of ATP in HTC hepatoma cells

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Liver epithelial cells secrete ATP into extracellular space. Once outside the cell, ATP acts as a potent autocrine signal that regulates a variety of important liver cell functions. The cellular mechanisms responsible for release of ATP are not known. Recent studies in astrocytes indicate that the P2X7 receptors may mediate release of ATP. We have recently demonstrated that hepatocytes express multiple P2X receptors, and the P2X4 and the P2X7 being most abundant. Thus, we assessed the role of P2X7 receptors in ATP release in a model liver cell line. Immunohistochemistry showed that similar to primary hepatocytes, HTC cells also express the P2X7 receptors. The measurements of ATP in the media using luminometry showed that exposures to hypotonic solution (30%) increased the amount of ATP released from HTC cells by ~5–10 fold. Addition of P2X7 inhibitors Zn2+ (300 microM), Cu2+ (100 microM) or Brilliant Blue G (0.1 microM) to the extracellular solution rapidly inhibited hypotonic-induced ATP release. To further assess for the presence of P2X7 in the plasma membrane, intracellular [Ca2+] ([Ca2+]i) was measured in response to BzATP. Exposures to BzATP (100 microM) evoked a rapid increase in [Ca2+]i that slowly decayed over ~10 minutes to a steady level above the basal level. BzATP-dependent increases in [Ca2+]i were potently inhibited by a pretreatment with Zn2+, Cu2+ or Brilliant Blue G. These results provide support for the hypothesis that hepatic P2X7 receptors may be permeable to ATP, and contribute to cellular secretion of ATP.

Intracellular acidification causes adenosine release during states of hyperexcitability in the hippocampus

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ABSTRACT
Adenosine is a known anticonvulsant and neuroprotective molecule, and recent studies lend insight into its regulation in a number of different brain regions. In particular, studies in hippocampus, spinal cord, and medulla demonstrate that a decrease in pH can increase endogenous extracellular adenosine. However, these studies differed in their conclusions regarding whether the critical factor for increasing adenosine is a decrease in intracellular and/or extracellular pH. Previously we showed that under physiological conditions, a decrease in extracellular pH is critical and a decrease in intracellular pH alone is not sufficient to alter extracellular adenosine in a hippocampal slice preparation. Here we demonstrate that when synaptic excitability is increased by either GABAA receptor blockade in CA1 or induction of persistent bursting in CA3, lowering intracellular pH without changing extracellular pH is now sufficient to: 1) elevate extracellular adenosine concentration, 2) activate adenosine A1 receptors 3) decrease synaptic transmission (CA1) and 4) decrease burst frequency in an in vitro seizure model (CA3). Taken together, these data strengthen and add significantly to the evidence outlining a change in pH as a stimulus influencing extracellular adenosine. We identify specifically the role of intracellular pH in regulating extracellular adenosine concentration, and highlight the clinically-relevant increased sensitivity of this regulation during conditions of enhanced neuronal excitability.

Regulation and mechanisms of ATP release from osteoblasts

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Bone cells have been shown to express multiple P2 receptor subtypes. Extracellular ATP, working in concert with local and systemic hormones, regulates osteoblastic and osteoclastic activity and probably plays a major role in mechanotransduction in bone. In order to understand how ATP appears in the bone microenvironment, we investigated the release of ATP from primary human osteoblasts and three osteoblastic cell lines, SaOS-2, MG63 and Te85. ATP was detected by a combination of end-point and real-time assays, utilising luciferin/luciferase bioluminescence. Real-time assays revealed that ATP is constitutively released by osteoblasts and that release is exquisitely sensitive to changes in fluid flow. In response to increased flow, there was a rapid rise in ATP concentration in medium, followed by a slower decline to resting levels. At steady state, the concentration of ATP in the medium of osteosarcoma cells lines was usually less than 5nM ATP. In contrast, the ATP concentration in the medium of primary osteoblasts was up to six fold higher, depending on the donor. End-point assays revealed that medium displacement led to a rise in ATP. After six displacements there was an approximate 10-fold increase in control cultures and 16-fold in cultures treated with ionomycin. Treatment with N-ethylmaleimide inhibited constitutive and fluid displacement-stimulated release, probably by blocking N-ethylmaleimide sensitive factor, a protein involved in exocytosis. Addition of a monoclonal antibody which blocks the action of the P2X7 receptor was ineffective in blocking ATP release. These results suggest that there are multiple mechanisms of ATP release from osteoblasts and that release can be stimulated by fluid flow.

P2X7-Pannexin1 complex mediates astrocyte intercelular calcium waves

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Purinergic P2 receptors and gap junctions are two groups of proteins involved in the transmission of intercellular Ca2+ waves (ICWs) between astrocytes. The extent to which ICWs spread among also depends on the amount of ATP released, which can occur through ion channels. Our previous studies showed that P2X7R-Pannexin1 complex contributes to ICW spread between astrocytes (Suadicani et al., 2006 J Neurosci; Scemes et al., 2008 Neuron Glia Biol). Using electrophysiological, biochemical and fluorescence imaging techniques, we provide further support for the participation of P2X7R-Pannexin1 in ICW spread and indicate that a tyrosine kinase mediates Panx1 activation through the P2X7R. Whole cell voltage clamp recordings in astrocytes revealed voltage activated Panx1 channels sensitive to carbenoxolone (CBX: 50 μM) and mefloquine (MFQ: 10nM). Panx1 currents sensitive to CBX and MFQ were also recorded at resting membrane potential following P2X7R activation with 50ìM BzATP. Dye uptake studies as well as measurements of the extent of ICW spread performed on astrocytes untreated and treated with CBX, MFQ and Panx1 siRNA indicated that

Panx1 provides sites for diffusion of large molecules, such as ATP. The src tyrosine kinase inhibitor PP2 (10iM) attenuated BzATP-induced Panx1 currents, dye uptake, and prevented the amplification of ICW. A membrane permeant peptide mimicking the src homology 3 (SH3) binding site of the P2X7R carboxyl terminal domain also prevented BzATP-induced dye uptake and amplification of ICW. Based on these results, we suggest that a src tyrosine kinase is involved in the signal transduction pathway of the P2X7R-Pannexin1 complex.

Are astrocytes the cerebral source of adenosine?

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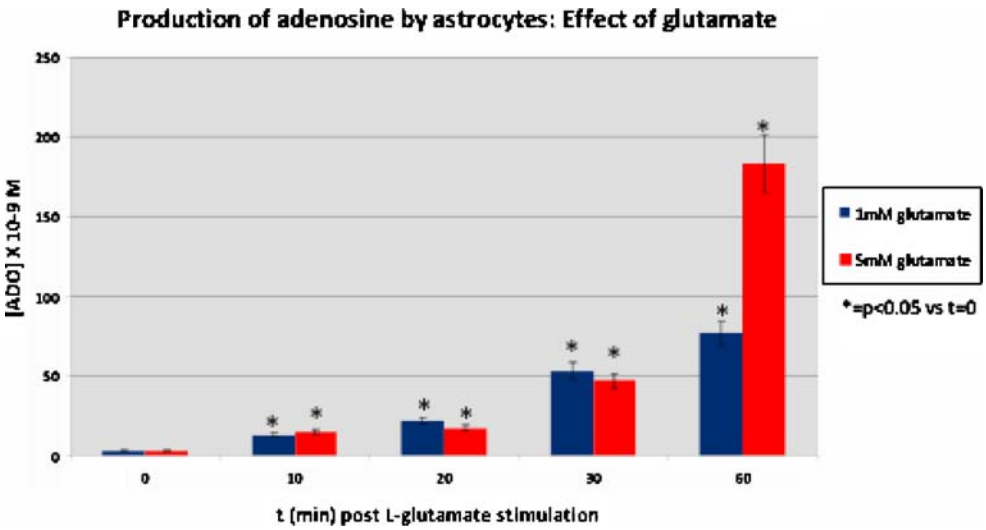
To investigate the source of adenosine (Ado) in the brain, we examined the role of astrocytes pertaining to the release of Ado during glutamatergic stimulation. We established a highly (>90%) pure culture of astrocytes, as verified by immunolabeling. Astrocytes were grown on microcarrier beads suspended in spinner flasks under controlled conditions.

To analyze and quantify the concentration of Ado and its breakdown products, we developed a HPLC ion-exchange gradient method. Compounds of interest were identified by retention time and wavelength spectrum, and confirmed by enzymatic peak-shift technique and spiking of samples.

During our studies we found Ado baseline levels in the low nanomolar range. We established a temporal profile of [Ado] in response to 1 mM and 5 mM L-glutamate stimulation. At every time the concentration was significantly higher than in the unstimulated control group ($p<0.05$). Within 60 min [Ado] rose to 77.1 nM±5.9 and 183.0 nm±11.9 in response to 1 mM and 5 mM L-glutamate stimulation respectively (refer Fig.1).

Conclusion: The increase in [Ado] in response to stimulation with L-glutamate is caused by a release of Ado from astrocytes.



G protein regulation of ATP release from lung epithelial cells. Release of UDP-sugars from the secretory pathway

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Nucleotides and nucleotide-sugars promote cellular responses by interacting with widely distributed purinergic receptors, but the mechanisms by which nucleotide species are released from cells are poorly understood. Our initial approach was to manipulate the expression level of well-characterized ER/Golgi-resident UDP-N-acetyl glucosamine (UDP-GlcNAc) transporters to ask whether entry of UDP-GlcNAc into the secretory pathway precedes the cellular release of this UDP-sugar. We developed an assay that quantifies UDP-GlcNAc with nanomolar sensitivity. Robust constitutive release of UDP-GlcNAc was observed in several cell types, including human airway epithelial cells and yeast. Airway epithelial cells overexpressing the human Golgi UDP-GlcNAc transporter HFRC1 displayed enhanced release of UDP-GlcNAc. Yeast mutants lacking YEA4 (the ER/Golgi UDP-GlcNAc transporter endogenously expressed in yeast) exhibited reduced UDP-sugar release. Thus, the ER/Golgi lumen is an important source of extracellular UDP-sugars and, likely, other nucleotides. In a second approach, we sought to identify regulatory elements upstream of ATP release. The protease-activated receptor (PAR) agonist thrombin promoted robust release of ATP from lung epithelial A549 cells. Thrombin-promoted ATP release was inhibited by BAPTA-AM and thapsigargin. Surprisingly, UTP, which induced robust Ca2+ mobilization and inositol phosphate formation responses in A549 cells, elicited negligible ATP release from these cells. Thus, Ca2+ was involved in receptor-promoted ATP release, but additional signaling was needed. PAR-elicited ATP release was impaired by Rho-kinase inhibitors, or by transfecting cells with either the dominant negative RhoA mutant RhoAT19N or GEF-deficient p115-RhoGEF RGS. Our results suggest that Gq/PLC/Ca2+ and G12/13/RhoGEF/RhoA activation are required for receptor-elicited ATP release in lung epithelial cells.
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(Invited)

ATP release from peripheral nerves and cultured schwann cells

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Connexins are the proteins responsible of forming gap junctions. Six subunits are necessary to form a hemichannel or connexon anchored in the plasma membrane. Two hemichannels of adjacent cells form a gap junction. At present, there are, at least, 20 isoforms of connexins described. Particularly, mutations in Connexin 32 (Cx32) have been associated to the X-linked form of Charcot-Marie-Tooth disease, a neurodegenerative illness affecting the peripheral nervous system. The Cx32 is expressed in Schwann cells, on paranodal zones. We detected ATP release including luciferin-luciferase reaction in the bath medium of isolated nerves or in cultured cells. Imaging of ATP release was performed capturing the luminescence with an Orca II camera (Hamamatsu). Electric stimulation of isolated rat sciatic nerves resulted in a release of ATP. Albino mice sciatic nerve fragments released ATP under mechanical stress, using hypotonic solutions (150 mOsm). Teased and isolated single nerve fibres released ATP, with preference from paranodal zones of Schwann cells. Using of cultured Schwann cells from adult mice, we found that they released ATP under hypotonic conditions. Using a microplate reader photomultiplier, we found that the release was transient reaching the maximal value after 4 s of stimulation. We found Cx32 in cultured SC using immunoflourescence techniques. These results open the possibility that ATP may be released through Cx32 hemichannels. This work is supported by SAF2005/736 of Spanish Government, Generalitat de Catalunya and by La Marató de TV3.

Vesicular release of ATP in the central synapses

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Background:
The role of ATP as a fast excitatory neurotransmitter has been firmly established over the last decade. However, the biophysical analysis of ATP-mediated synaptic events and mechanism of ATP release in central synapses is far from complete.

Methods:
We recorded ATP-mediated miniature postsynaptic currents (mEPSCs) in the hippocampal and cortical neurons in situ and compared them to the synaptic currents mediated by glutamate and GABA.
Results:
The mEPSCs recorded from pyramidal CA1 and cortical neurons exhibited two independent populations of synaptic events: larger glutamatergic currents and smaller currents mediated by P2X purinoreceptors. The relative frequency of spontaneous purinergic synaptic currents was 20–40% of control. Stimulation of single axon elicited unitary ATP-mediated synaptic current in the approximately the half of pyramidal neurons tested. The evoked P2X receptor-mediated current was always accompanied by glutamatergic synaptic current; there was no difference in their latency time. Purinergic mEPSCs were asynchronous with those mediated by glutamate or GABA; as was shown by analysis of their kinetics and voltage dependence. The P2X receptor-mediated synaptic currents originated from vesicular release of ATP as was evidenced by effects of bafylomicin and latrotoxin.
Conclusions:
These data suggest that ATP is released from a specific set of vesicles which are present in glutamate-containing terminals but this component of transmission occurs with lower probability only at a subset of synapses. Yet, the amplitude of ATP-mediated synaptic events is comparable (with ratio of 0.4–0.8) to the amplitude of glutamate-mediated unitary synaptic currents. The ATP receptors can therefore mediate a considerable fraction of excitatory input in sub-populations of synapses. The purinergic component of synaptic transmission may have specific physiological role, in particular due to high calcium permeability of the P2X receptors.

Role of autocrine signaling in mechanosensitive ATP release

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Extracellular nucleotides regulate surfactant secretion in alveoli and mucociliary clearance in airway epithelia, but the mechanism(s) of their release and their regulatory pathways remain incompletely understood. Different forms of mechanical stress, including shear, cell stretch, cell deformation and hypotonic swelling cause Ca2+-dependent ATP release. In particular, we showed that hypotonic swelling of A549 epithelial cells induces Ca2+-dependent secretion of several adenosine and uridine nucleotides, implicating regulated exocytosis. In this study, we examined sources of intracellular Ca2+ ([Ca2+]i) elevation evoked by acute 50% hypotonic stress and the role of autocrine purinergic signalling in Ca2+-dependent ATP release. We employed Fura-2 fluorescence imaging for [Ca2+]i measurements. ATP release from confluent A549 cell monolayers was investigated in a custom-made flow-through chamber, and ATP content in the samples was measured by lucifersase-luciferin assay. We found that ATP release depends entirely on Ca2+ mobilization from intracellular stores. Both thapsigargin-sensitive and thapsigargin-insensitive stores are involved, contributing approximately 70% and 30% respectively to total ATP released by hypotonic shock. Importantly, we also found that despite rapid perfusion of the experimental chamber, autocrine stimulation of P2Y6/P2Y2 receptors, by co-released uridine and adenosine nucleotides, plays a major role in amplifying the initial hypotonic stress-induced [Ca2+]i response and further promoting ATP release. Our study reveals a novel paradigm in which ATP release is amplified by the synergistic autocrine/paracrine action of co-released uridine and adenosine nucleotides. We suggest that a similar mechanism of purinergic signal propagation operates in other cell types. (Supported by CIHR and CCFF).

Different types of mechanical loading induce differentitial ATP release from osteoblasts grown on 3-dimensional scaffolds

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The maxi-anion channel is widely expressed and well characterized biophysically while its physiological functions remain poorly understood. We found that this channel is closed in the resting state and opens in response to various ATP-releasing stimuli, such as osmotic, ischemic and hypoxic stresses. The cells tested included mammary C127 cells (Sabirov et al., J. Gen. Physiol. 2001, 118: 251–266), kidney macula densa cells (Bell et al., Proc. Natl. Acad. Sci. U S A 2003, 100: 4322–4327), cultured neonatal (Dutta et al., J. Physiol. 2004, 559: 799–812) and acutely isolated adult cardiomyocytes (Dutta et al., Biophys. J. 2008, 94: 1646–1655) and primary cultured astrocytes (Liu et al., Glia 2006, 54: 343–357). A weak ATP-binding site with Kd of 12–13 mM located in the middle of the pore together with a wide pore with a radius of ~1.3 nm (Sabirov and Okada, Biophys. J. 2004, 87: 1672–1685) provides suitable environment to accommodate and pass ATP4- and MgATP2- (~0.6–0.7 nm) with PATP/PCl and PMgATP/PCl of around 0.1. Thus the maxi-anion channel perfectly fits its physiological function as an ATP-conductive channel. This inference is supported by the pharmacological profile of the maxi-anion channel, which resembled that of the stimulated ATP release.

(Invited)

Maxi-anion channel-mediated ATP release

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The maxi-anion channel is widely expressed and well characterized biophysically while its physiological functions remain poorly understood. We found that this channel is closed in the resting state and opens in response to various ATP-releasing stimuli, such as osmotic, ischemic and hypoxic stresses. The cells tested included mammary C127 cells (Sabirov et al., J. Gen. Physiol. 2001, 118: 251–266), kidney macula densa cells (Bell et al., Proc. Natl. Acad. Sci. U S A 2003, 100: 4322–4327), cultured neonatal (Dutta et al., J. Physiol. 2004, 559: 799–812) and acutely isolated adult cardiomyocytes (Dutta et al., Biophys. J. 2008, 94: 1646–1655) and primary cultured astrocytes (Liu et al., Glia 2006, 54: 343–357). A weak ATP-binding site with Kd of 12–13 mM located in the middle of the pore together with a wide pore with a radius of ~1.3 nm (Sabirov and Okada, Biophys. J. 2004, 87: 1672–1685) provides suitable environment to accommodate and pass ATP4- and MgATP2- (~0.6–0.7 nm) with PATP/PCl and PMgATP/PCl of around 0.1. Thus the maxi-anion channel perfectly fits its physiological function as an ATP-conductive channel. This inference is supported by the pharmacological profile of the maxi-anion channel, which resembled that of the stimulated ATP release.

(Invited)

ATP release and x-linked charcot-marie-tooth disease

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X-linked Charcot Marie Tooth (CMTX) is an inherited neurodegenerative disease affecting both motor and sensory peripheral nerves. CMTX is due to mutations in connexin 32 (Cx32) and more than 200 mutations have been so far described. Cx32. Schwann cells express Cx32 in the Schmidt-Lanterman incisures and in the paranodes in which may form hemichannels. In this communication, we have explored if Cx32 hemichannels support the release of ATP. Human Cx32 was expressed in Xenopus oocytes and ionic currents and ATP release were simultaneously recorded under voltage-clamp configuration. ATP release was measured as light emission by luciferine-luciferase reaction. Oocytes expressing hCx32, showed an outward current of 1041+/-

81 nA (n=135) when depolarized from -40 mV to +80 mV during 30 s. When returning to -40 mV, it appeared an inward transient current (tail) with an amplitude of -387+/-46 nA which was coincident with a peak of ATP release of 274+/-88 fmole, (n=16). Outward and tail currents were sensitive to stretch. There was a good correlation between the tail current charge and the amount of ATP released. Deconvolution of the light signal was coincident with the time course of the tail current. We exclude the vesicular pathway of ATP release because no inhibition was found after application of Brefeldin A and no increase of membrane capacitance was recorded after the depolarization pulse. In oocytes expressing P87A, R220ST, DEL111–116 mutations of hCx32, found in CMTX, ATP release was severely inhibited. Acknowledgment, SAF 2005/736 of the Spanish Government.

SYMPOSIA 24: NUCLEOTIDES IN THE EYE

The P2Y2 receptor is critical for corneal epithelial injury response and cell migration

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Background. Our goal was to determine the role of the P2Y2R in corneal epithelial injury and repair by examining Ca2+ waves, ERK signaling, and cell migration.
Methods. The P2Y2R was downregulated by RNAi. Cells were incubated in Fluo-3AM and monitored on a Zeiss LSM510. Real time PCR confirmed receptor downregulation. Live-cell imaging in a Zeiss environmental chamber was used to monitor wound healing.
Results. Human corneal limbal epithelial cells were stimulated with various nucleotides, and the Ca2+ wave was imaged. Cells transfected with P2Y2 siRNA and stimulated with UTP showed a significant reduction in the Ca2+ increase (73% less than nontargeting control siRNA). ATP showed a smaller but not significant reduction (22% less than control) while cells stimulated with ADP showed no reduction. UDP showed a significant reduction (43% less than control). Transfected cells were wounded and the Ca2+ wave imaged. In nontargeting control cells, a typical Ca2+ wave propagated from the site of injury. The wave is reduced by 90% in cells where P2Y2R is downregulated. Similar results were shown in scratch wound assays and ERK phosphorylation. Cells transfected with nontargeting control siRNA show an increase in migration upon UTP stimulation while P2Y2 siRNA transfected cells showed no increase above unstimulated. In addition, ERK phosphorylation is reduced.
Conclusion. Downregulating P2Y2R leads to reduction in the UTP and injury induced Ca2+ responses. These cells display impaired UTP induced wound closure. P2Y2R is essential for the injury induced Ca2+ wave, ERK activation, and migration of corneal epithelial cells.

P2X7 Purinergic Receptors Mediate Protein Secretion and ERK Activation in Rat Lacrimal Gland

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The lacrimal gland is responsible for secretion of the aqueous portion of the tear film to protect the ocular surface. To determine if purinergic receptors play a role in secretion, we determined the presence and location of P2X purinergic receptors and the effect of P2X7 purinergic receptors on protein secretion and ERK activation. Lacrimal glands from male Sprague-Dawley rats were subjected to western blot analysis to determine the presence of P2X receptors. Acini were isolated by collagenase digestion and intracellular [Ca2+] ([Ca2+]i) was measured after stimulation with UTP, which is more specific for P2Y receptors, ATP, a nonspecific P2X purinergic receptor agonist, or the P2X7 agonist benzyl-ATP (BzATP). Peroxidase, a marker of protein secretion, was measured after stimulation. Activation of ERK was measured 10 min after addition of BzATP (10–4 M) and western blot analysis was performed using antibodies to activated and total ERK. Western blot analysis indicated that all P2X receptors but P2X5 were present in lacrimal gland. UTP (10–4 M) did not increase the [Ca2+]i, while ATP increased [Ca 2+]i by 49.7 nM above basal at 10–3 M ATP. BzATP (10–4 M) caused a maximum increase of 150 nM above basal. BzATP increased secretion 1.24±0.05 fold and ERK activation 2.36±1.35 fold. A 15 minute preincubation with the P2X7 specific inhibitor brilliant blue G (10–5 M) completely inhibited protein secretion and ERK activation. We conclude that the lacrimal gland contains functional P2X7 receptors that stimulate an increase in [Ca2+]i, protein secretion, and ERK activation.

The role of purines in retinal degeneration

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Background: Extracellular ATP activation of P2X7 receptors (P2X7R) is thought to modulate photoreceptor function and a possible role in retinal degeneration has been suggested. The aim of this study was to investigate whether excessive stimulation of P2X7R in photoreceptors leads to apoptotic cell death, and whether agents that block P2X receptors slow retinal degeneration in animal models.
Methods: Photoreceptor expression of P2X7R in rodents was confirmed immunocytochemically. To investigate the possible role of overactivation of P2X7 receptors, eyes of Sprague Dawley rats were then injected intravitreally with either a sham injection (PBS) or 1 mM–45 mM ATP, ATPgammaS, Adenosine, ATP+PPADS. Following 2 hrs–7 days retinae were processed for histological analysis, apoptosis (TUNEL), retinal function (ERG and agmatine), activation of microglia and glia (IB4, GFAP).
Results: Photoreceptor expression of P2X7R was confirmed. Injection of ATP or analogues induced photoreceptor death in a dose dependent and irreversible manner. A combined injection of ATP and PPADS reduced apoptosis. Complete loss of retinal function was observed by 7 days. Reactive gliosis and increased numbers of microglia were observed after significant levels of photoreceptor apoptosis (18 hours). Treatment of rd/rd mice with either the P2X antagonists, PPADS or TNP-ATP, in one eye at P14 slowed photoreceptor degeneration.
Conclusions: These data suggest that excessive stimulation of purinergic receptors leads to selective apoptotic cell death of photoreceptors. Moreover, application of an agent that blocks the action of P2X receptors slowed photoreceptor death in an animal model of retinal degeneration. These findings suggest a novel means for preventing retinal degeneration.

Purinergic regulation of aqueous humor outflow

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Background: Nucleotides and dinucleotides are natural compounds found in the aqueous humor (AH). In the rabbit eye, corneal application of dinucelotides, modify intraocular pressure (IOP). In addition, increased ATP levels have been found in the AH of glaucomatous patients. Here we studied the purinergic signaling in the trabecular meshwork (TM) and its involvement in AH outflow regulation. Methods: Western blott and immunocytochemistry were used to detect the presence of P2Y receptors in bovine TM cells in culture. Intracellular calcium concentration ([Ca2+]i) was measured in TM cells loaded with Fura-2. Outflow facility studies were performed in bovine anterior segments perfused at constant pressure. Determination of dinucleotides in the AH of control or glaucomatous patients was performed by HPLC. Results: TM cells express P2Y1, P2Y2 and P2Y4 receptors. Stretching of TM cells induces ATP release that is rapidly cleaved by nucleotidases. After inhibition of nucleotidases, released ATP increases [Ca2+]i and modulates BKCa channels. Smaller [Ca2+]i increments were seen when stimulating TM cells with Ap3A, Ap4A, Ap5A while Up4U produced the most important [Ca2+]i mobilization. Perfusion of Ap3A or Ap4A increased AH outflow whereas perfusion with Up4U or Ap5A did not change it significantly. Interestingly, Ap4A was increased in glaucomatous patients (296±32 nM) compared to controls (19±9 nM), while no differences were found for Ap5A. No association between elevated levels of Ap4A and other systemic diseases such as diabetes mellitus or hypertension were found. Conclusions: Purinergic compounds in the AH target TM cells to modify trabecular outflow and IOP. Hypotensive effects of Ap4A appear mediated by an increase in AH outflow, likely activating P2Y1 receptors. Elevated concentrations of Ap4A in the AH of glaucomatous patients may reflect a release of nucleotides and/or dinucelotides due to elevated IOP or an homeostatic mechanism to compensate for elevated IOP.

$P2X_7$ knock out mice exhibit decreased corneal wound healing and altered stromal collagen

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Background: The purpose of this study is to examine the role of $P2X_7$ in the healing of corneal epithelial wounds and the structure of stromal collagen, where alignment of fibrils is critical for vision.
Methods: Central superficial corneal wounds were performed on $P2X_7^{-/-}$ and WT mice. The wound area was measured after 16 hours and corneas were sectioned and analyzed. Analysis included immunohistochemistry, light microscopy, and electron microscopy. Cuprolinic blue staining was performed to examine glycosaminoglycans in the stroma.
Results: Corneal re-epithelialization was delayed in $P2X_7^{-/-}$ mice, which had a mean wound area 23% larger than WT. Immunohistochemistry revealed differential staining of pannexin-1, $P2Y_4$, and p-paxillin between WT and $P2X_7^{-/-}$. The average central corneal thickness was reduced by 25.33 μm and stromal ultrastructure was altered in wounded and unwounded stromas of $P2X_7^{-/-}$ mice. There were fewer lamellae in the $P2X_7^{-/-}$ stroma and the average lamellae thickness was 489 nm greater than in WT. A larger interfibrillar distance was accompanied by 30% fewer sulfated glycosaminoglycans along collagen fibrils in the $P2X_7^{-/-}$ mice. Additionally, the diameter of the collagen fibrils in the $P2X_7^{-/-}$ stroma was 19.5% smaller than in WT.
Conclusion: $P2X_7$ is required for proper healing of corneal epithelial abrasion wounds and organization of collagen in the corneal stroma. Lack of the receptor leads to changes in expression of epithelial proteins and delayed wound healing. The absence of $P2X_7$ and the resulting change in fibril size and sulfation of GAGs may account for the stromal disorganization observed.

P2X7 receptor kills retinal ganglion cells with glutamate release and excitotoxic NMDA receptor activation

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Background: Retinal ganglion cells (RGCs) carry the visual message from the retina to the brain through their axons in the optic nerve. Death of RGCs leads to the loss of vision in glaucoma. RGCs have been shown to express P2X7 receptors on a molecular, immunohistochemical, and functional level, with agonist BzATP acting exclusively on P2X7 receptors to raise calcium and kill the neurons. The mechanisms linking P2X7 receptor activation and cell death are unclear, but do not involve large pores. This study asked whether the NMDA receptor and excitotoxic death were involved in the loss of RGCs accompanying P2X7 receptor activation. Methods: Fura-2 was used to examine intracellular calcium levels, NADPH flourescence to detect extracellular glutamate and retrograde labeling to quantify cell loss. All experiments were performed on fresh isolated rat RGCs. Results: The calcium rise in response to BzATP was partially attenuated by the three distinct NMDA antagonists MK-801, AP-5 and memantine. These three antagonists also gave near-complete protection from BzATP-mediated death. BzATP triggered a rapid release of glutamate from isolated RGCs, providing an agonist for the NMDA receptor. Removal of extracellular glutamate with glutamate pyruvate transaminase greatly reduced the response to BzATP, further supporting a role for released glutamate. Conclusions: Stimulation of P2X7 receptors on RGCs leads to the release of glutamate and autostimulation of NMDA receptors. This suggests that excitotoxic processes are responsible for at least some of the cell death following P2X7 receptor activation in RGCs.

(Invited)

Dinucleotides and mononucleotides in the aqueous humour and the modulation of intraocular pressure

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Diadenosine polyphosphates are a family of dinucleotides with emerging biochemical, physiological, pharmacological and therapeutic properties in the eye and other tissues. These compounds are formed by two adenosine moieties linked by their ribose 5-ends to a variable number of phosphates. Diadenosine polyphosphates are present in ocular secretions such as tears and aqueous humour and can activate P2 purinergic receptors present on the ocular structures. Ap4A and Ap5A have been detected in the aqueous humour by high-performance liquid chromatography. Levels found were 0.34 and 0.08 mM for Ap4A and Ap5A, respectively. When diadenosine polyphosphates are applied topically to the cornea of New Zealand white rabbits, intraocular pressure changes as expected. Among all the tested dinucleotides, Ap2A, Ap3A and Ap5A significantly increase IOP in a concentration-dependent manner. On the contrary, Ap4A behaves as an hypotensive dinucleotide, reducing IOP in a dose-dependent manner (IC50 0.13 nmol). Characterisation of the receptors activated by Ap4A, performed by cross-desensitization and antagonist analysis, shows a receptor profile belonging to the P2X subtype since beta, gamma-me ATP completely inhibits the hypotensive effect of Ap4A and it is also antagonized by suramin and PPADS. This receptor seems to be located in the cholinergic terminals present at the ciliary body but also, it has been demonstrated that Ap4A can activate P2Y1 receptors located in the trabecular meshwork. In summary di- and mononucleotides are active substances in the aqueous humour that control the dynamics of this ocular fluid being relevant as therapeutic agents for the treatment of ocular pathologies such as glaucoma.

(Invited)

P2Y2 Agonists for the Treatment of Dry Eye

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Background: Diquafosol tetrasodium is a uridine-containing dinucleotide that is a potent and selective P2Y2 agonist. P2Y2 receptors are located on ocular surface tissues including cornea, conjunctiva and meibomian glands. Dry eye disease has multiple etiologies but involves disruption of any of the three components of tear film: aqueous, mucin, or lipid. Methods: Diquafosol has been evaluated in several Phase 3 clinical studies that included measurement of objective and subjective endpoints. Endpoints studied included corneal fluorescein staining, conjunctival lissamine green staining, Schirmer wetting test, tear break-up time and subject reported symptoms. Total clearing of staining from the ocular surface has been evaluated with emphasis on clearing of fluorescein staining from the central region of the cornea.
Results: In studies 105 and 109, subjects receiving 2% diquafosol demonstrated lower (p less than 0.001) mean corneal and conjunctival staining compared to subjects receiving placebo following 6 weeks of QID dosing. In addition, subjects treated with diquafosol were (p less than 0.05) more likely to have zero staining in the central region of the cornea following diquafosol treatment compared to placebo. Positive changes in central region of the cornea may be associated with improvements in ocular function.
Conclusions: Diquafosol has demonstrated significant differences from placebo in several objective and subjective measures, suggesting that diquafosol may have positive effects on the ocular surface health in subjects with dry eye disease.

(invited)

SYMPOSIA 25: PURINERGIC SIGNALLING IN BONE AND SKELETAL MUSCLE

Purinergic signalling in human osteoporosis: design of a genetic epidemiological study on associations between P2 receptor polymorphisms and osteoporosis risk

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Background: Single nucleotide polymorphisms (SNPs) in the $P2X_7$ receptor gene were recently found to be associated with fracture risk.[1]
Objectives: Within WP6 of a EU Seventh Framework project, we will investigate associations between P2 receptor SNPs and osteoporosis risk in humans. Genetic data from a fracture cohort in the Netherlands with high prevalence of osteoporosis will be analyzed. Furthermore, effects of aberrant P2 receptor signalling on bone turnover markers will be assessed ex vivo. As a second step, data will be pooled with two ongoing Danish cohorts.
Subjects and methods: The Dutch cohort will include app. 1,000 patients ≥50 yrs with a clinical fracture, who will be recruited at the University Hospital Maastricht. Standard medical follow-up includes assessment of bone mineral density (BMD) by Dual-Energy X-ray Absorptiometry (DXA).[2] Prior to medication for osteoporosis, blood samples will be collected from fracture patients to be genotyped for P2 receptor SNPs, and analyzed for biochemical markers of bone turnover. Systemic correlates of osteoporosis will be compared between osteoporotic subjects (low BMD) and non-osteoporotic controls (normal-high BMD). Subsequently, whole blood assays in patient subgroups (n=20 per subgroup), based on BMD and P2 SNPs, will be performed to evaluate ex vivo effects of ATP and related nucleotides on bone markers. Within the EU project, the data will be pooled with data from the Danish Osteoporosis Prevention Study (n=2,000 postmenopausal women)[3] and the Glostrup Cohort Study (n=5,000 men and women aged 18–69 yrs).
Results: Patient recruitment is currently ongoing and will be completed in 2009.
References:
[1]Ohlendorff et al. Pharmacogenet Genomics 2007;17(7):555–67
[2]van Helden et al. J Bone Joint Surg 2008;90(2):241–8
[3]Mosekilde et al. Maturitas 1999;31:207–19

Exogenous ATP regulates excitation-induced force in isolated muscle through the P2Y receptors

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In skeletal muscles, contractions elicit a release of ATP into the extracellular space. Extracellular ATP has been recognized as an autocrine and paracrine signaling molecule acting on two purinergic receptor families; the metabotrophic P2Y receptors and the ionotropic P2X receptors. In this study we investigate the role of P2 receptors on the excitability and contractility of isolated skeletal muscle.
Tetanic force production and M-wave amplitude and area were investigated in isolated rat soleus muscles incubated in Krebs-Ringer buffer. Exogenously added P2-receptor agonist (ATP) and phospholipase C (PLC) inhibitor (U73122) was used to investigate P2-receptor mediated effects on muscle function under conditions with compromised muscle excitability.

When the excitability of muscles was depressed by elevating [K+]o to 10 mM, tetanic force was reduced to 24±2% of initial force at 4 mM K+. However, upon addition of 1 mM ATP, force was recovered to 65±8% of initial force at 4 mM K+ ($P<0.001$, $n=5$). The force recovery was accompanied by a recovery of M-wave area and amplitude from 20±5 to 81±11% ($P<0.05$) and 8±1 to 47±11% ($P<0.05$) of initial M-wave area and amplitude at 4 mM K+, respectively ($n=4$). ATP induced force recovery could be eliminated by 50 μM PLC inhibitor indicating that the force recovery was mediated by the P2Y receptor family. Danish Medical Research Council (j. nr. 271-05-0304)

Extracellular ATP synergises with glucose-dependent insulinotropic polypeptide (GIP) to induce c-fos in osteoblastic cells

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We previously demonstrated that activation of osteoblastic P2Y1 and/or P2Y2 receptors leads to a moderate induction of c-fos, a key transcription in bone remodelling, whereas dual activation of P2 receptors and parathyroid hormone receptors leads, through multiple levels of interaction in downstream signalling, to a massive synergy in c-fos induction. This synergy provides a mechanism whereby locally released extracellular nucleotides can sensitize cells to systemic PTH and thereby activate bone remodelling at discrete sites. In recent studies, we have demonstrated the expression of receptors for gut hormones, including glucose-dependent insulinotropic polypeptide (GIP) in primary human osteoblasts and in the osteoblast-like osteoscarcoma cell line, SaOS-2. We now report that GIP induces c-fos expression in osteoblastic cells and furthermore that there is a synergistic activation when cells are co-stimulated with GIP and extracellular ATP. Using a c-fos-driven reporter gene in stably transfected SaOS-2 cells, we observed a small but consistent induction of c-fos expression up to 40% above control values when cells were exposed to ATP or GIP alone ($P<0.01$), whereas when both ligands were added there was a robust induction up to 4 fold ($P<0.001$). These results indicate that osteoblast function might be modulated in the postprandial and post-absorptive states when gut hormones are released in response to changes in dietary intake. They also provide another example of how locally released nucleotides interact with systemic hormones in bone. The findings add further weight to the theory that synergy between extracellular ATP and other endocrines is a ubiquitous phenomenon in homeostasis.

A polymorphism in the P2X7 receptor gene is associated with loss of lumbar spine bone mineral density in postmenopausal women

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The gene for the P2X7 receptor (P2RX7) is highly polymorphic; four SNPs (c.474G>A, c.1096C>G, c.1513A>C, and c.1729T>A) are known to cause amino acid changes that result in reduced function by as much as 70% in the heterozygous state. We have previously shown that both osteoblasts and osteoclasts express the P2X7R, and a recent report has detailed the association of two P2RX7 polymorphisms with an increased 10-year fracture risk in elderly postmenopausal Danish women. The aim of this study was to survey a cohort of younger women for an association between functional P2RX7 polymorphisms and a key determinant of fracture risk bone mineral density (BMD). We have genotyped 508 perimenopausal women from the Aberdeen Prospective Osteoporosis Screening Study (APOSS) for known P2RX7 polymorphisms. Females were recruited randomly from among the general population in the North East of Scotland from 1990–94 when aged 45–54 y. Lumbar spine (LS) and femoral neck (FN) BMD was measured at baseline and at 6–7 year follow up. P2RX7 genotyping was performed by homogeneous mass extension. Genotype frequencies for all P2RX7 variants were in Hardy-Weinberg equilibrium (p-values 0.2–1.0). We found association of c.946A with LS-BMD at both baseline ($p=0.006$, beta=-0.12) and follow-up ($p=0.004$, beta=-0.13). Although the c.946 G>A polymorphism is of low

frequency (1–2%) in the Caucasian population, it has a major effect on LS BMD in younger postmenopausal women, thus confirming P2X7R as possible bone anabolic therapeutic target, and early diagnostic tool for prevention of bone loss.

Adenosine and osteoporosis: Adenosine A1 receptor blockade reverses bone loss in ovariectomized mice and deletion of adenosine A2A receptors leads to diminished bone density

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Adenosine regulates a wide variety of physiological processes via interaction with one or more of four G protein-coupled receptors. Because we have previously reported that adenosine A1 receptors promote fusion of stimulated human monocytes to form giant cells whereas A2A receptor activation inhibits in vitro giant cell formation we determined whether there was a similar requirement for A1 & A2A receptor occupancy in osteoclast formation. Electron microscopy of bone sections of bones from A1, A2A knockout and wild type mice was carried out. A1KO and A2A KO mice were analyzed by DEXA (PIXImus) to measure the BMD and by MicroCT. Four week old C57BL/6 mice underwent ovariectomy (OVX) or SHAM surgery; those mice were treated with DPCPX (50 mg/kg/day) or vehicle (VEH) for 5 weeks. The A1R antagonist DPCPX inhibits osteoclast formation in a dose-dependent fashion (P=0.0012). Splenocytes from A1R KO mice formed few osteoclasts. MicroCT and PIXImus examination from A1R KO mice shows osteopetrosis, where A2A KO mice shows Osteoporosis. Electron microscopy from A1KO femur shows an absence of ruffled borders of osteoclasts and resorption of bone. Where A2A mice shows that the osteoclasts are pretty actively in resorbing bone, MicroCT and PIXImus scan measurements indicated that by 5 weeks of DPCPX treatment trabecular bone density (TbBD) in the proximal femur increased in both OVX and SHAM compared to VEH. Ex vivo analysis of whole mice by DEXA (PIXImus) scan confirmed increased BMD in DPCPX treated mice. These results indicate that endogenously-released adenosine plays a critical role in regulating bone turnover via interaction with adenosine A1 & A2A receptors on osteoclasts and their precursor cells. Moreover the A1 & A2A receptors may be useful targets in treating diseases characterized by excessive bone turnover such as osteoporosis.

P2 receptors are functionally expressed in MLO-Y4 osteocytes

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Background: P2 purinergic receptors are involved in mechanically induced intercellular calcium signalling among osteoblasts and between osteoblasts and osteoclasts. The signalling is mediated by both P2Y and P2X receptors and plays an important role in the bone remodelling process, as extracellular ATP affects both bone resorption and formation. Osteocytes are situated in the mineralized bone matrix, and are the mechanosensors of bone. Thus, they are believed to regulate local bone remodelling. The aim of this study was therefore to investigate the functional expression of P2 receptors in the osteocyte-like cell line MLO-Y4 and their response to mechanical load.

Methods: The expression of P2 receptor mRNA was investigated by the RT-PCR. ATP release was investigated using Luciferin-Luciferase reaction during mechanical stimulation with an instant fluid flow stimulus. The expression of

functional P2 receptors was estimated by monitoring intracellular calcium responses to different P2 receptor agonists using Fura-2 loaded cells. Experiments investigating the propagation of calcium waves were also conducted in the calcium imaging system with micropipette-induced mechanical stimulations.
Results: RT-PCR and pharmacological analysis showed that P2Y2 and P2X7 are functionally expressed in MLO-Y4. Stimulation of cells with a short fluid flow stimulus induced ATP release, and ATP release was proportional to injection speed indicating that ATP release is induced by mechanical load. Finally MLO-Y4 were able to propagate mechanically induced calcium waves.
Conclusion: In conclusion results demonstrate the expression and function of P2 receptors in MLO-Y4 osteocytes, as well as the ability of the cells to release ATP upon mechanical stimulation.

The role of adenosine and related substances in exercise hyperaemia

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It has long been recognised that adenosine contributes to vasodilatation when oxygen delivery does not meet oxygen demand. Accordingly, earlier studies showed release of adenosine into the venous efflux of exercising muscle under restricted flow conditions. However, it is now known that ATP, ADT, AMP, adenosine and inorganic phosphate (Pi) accumulate in the interstitium of exercising muscle under free flow conditions. It seems probable they originate directly or indirectly from skeletal muscle fibres. However, ATP is also released as a co-transmitter from sympathetic nerve fibres, which are activated in exercise. Further, ATP and/or adenosine are released from endothelial cells by increased shear stress and hypoxia, while ATP is released from red blood cells during haemoglobin de-oxygenation, conditions that can be expected in exercising muscle. ATP acting on P2X receptors on vascular smooth muscle causes vasoconstriction. However, adenosine has the potential to induce vasodilatation by stimulating A1 or A2A receptors on vascular smooth muscle, while ATP and adenosine can cause vasodilatation by acting respectively on P2Y receptors and on A1 or A2A receptors on endothelium and releasing NO. On the basis of current evidence it can be argued that Pi and adenosine originating from muscle fibres make a major contribution to the hyperaemia of dynamic, as well as static exercise, and that the adenosine acts on A2A, not A1 receptors on the vascular smooth muscle. By contrast, ATP and adenosine released into the blood stream and acting on endothelial P2Y, A1 or A2A receptors are unlikely to be important, particularly because NO makes little active contribution to exercise hyperaemia.

(Invited)

NO and prostaglandins are important mediators of adenosine induced vasodialtion

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Arterial infusion of adenosine can induce vasodilation in human skeletal muscle, but the contribution of adenosine in blood flow regulation and the mechanisms by which adenosine induces vasodilation remain unclear. We measured leg haemodynamics before and during intra-arterial adenosine infusion (83±2 μg min-1 kg leg mass-1) and one-legged knee-extensor exercise (24±3 W; 30% of peak power; mean ± S.E.M.) in nine healthy male subjects (age 24±1 years, weight 80±3 kg, height 183±2 cm and VO2max 47±2 ml O2 min-1 kg-1) with and without inhibition of NO (L-NMMA) and prostaglandin (indomethacin (INDO)) synthesis and/or adenosine receptors (theophylline; TEO). During adenosine infusion LBF increased from a basal value of 0.36±0.04 l min-1 to 2.4±0.2 l min-1. When adenosine was co-infused with L-NMMA, INDO, or L-NMMA + INDO LBF was reduced ($P<0.05$) by 39±6%, 49±5% 72±1%, respectively, compared to adenosine alone. During exercise, LBF was 2.0±0.13 l min-1 in control. LBF during exercise was lower during infusion

with TEO (15±4%), L-NMMA+INDO (31±6%) or all three inhibitors (28±4%) ($P<0.05$). These results show that part of the vasodilatory effect of intraluminal adenosine is mediated by formation of NO and prostaglandins, which indicates a close interaction between these three substances. These findings may explain why no additional reduction in exercise hyperemia was observed when adenosine receptor blockade was added to the inhibition of prostaglandin and NO synthesis.

Presynaptic A_1 Inhibitory/A_{2A} facilitatory adenosine receptor activation balance is impaired in toxin-induced *Myasthenia gravis*

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While adenosine acts predominantly as an inhibitory signal (via A_1 receptors) under resting conditions, during high frequency nerve stimulation amplification of neuromuscular transmission depends on tonic activation of A_{2A} receptors (Correia-de-Sá *et al.*, 1996, *J. Neurophysiol.*, 79, 3910–19). This may offer the potential for regulating neurotransmitter exocytosis and, hence, minimization of transmission deficits observed in myasthenic patients.
Wistar rats (70–100 g) were chronically injected once every 48 h with saline (controls) or α-bungarotoxin (α-BTX, an irreversible muscle-type α1 nicotinic receptor antagonist) (toxin-induced *Myasthenia gravis* model, TIMG). [^{3}H]-ACh release was evoked by phrenic nerve stimulation with either 5 Hz-trains (750 pulses) or 50 Hz-bursts (5 bursts of 150 pulses, 20-s interburst interval).
In contrast with control animals, inactivation of endogenous adenosine with adenosine deaminase (ADA, 2.5 U/ml) and blockade of A_1 receptors with 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX, 10 nM.) failed to increase [^{3}H]-ACh release evoked by 5 Hz-trains in TIMG animals. ADA (2.5 U/ml)-induced decreases of [^{3}H]-ACh release caused by 50 Hz-bursts was smaller in TIMG animals (-18±6%, $n=6$) than in control rats (-54±8%, $n=5$). Likewise, the inhibitory effect of the selective adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM.), was smaller in TIMG animals (-19±9%, $n=4$) as compared with controls (-46±7%, $n=6$). Muscle paralysis due to selective blockade of muscle action potentials with μ-conotoxin GIIIB (0.6 microM), reduced stimulation-induced adenosine release measured by HPLC.
Data suggest that impairment of contractile activity in myasthenic rats reduces retrograde signalling mediated by adenosine outflow from the muscle, which causes profound changes in the control of neuromuscular transmission by the nucleoside. These changes are particularly evident during high frequency nerve stimulation.

Purines, bisphosphonates and bone mineralisation

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Our recent work has shown that ATP and UTP act potently on primary osteoblasts to increase intracellular calcium and inhibit bone mineralisation. We also found that expression of the ATP/UTP-selective P2Y2 receptor is upregulated strongly during terminal differentiation of osteoblasts into the bone-forming phenotype. These findings suggested that the P2Y2 receptor could function as a negative regulator of bone formation/mineralisation in vivo. In support of this notion we observed increases of up to 40% in the volume or density of lower limb bones of 8 week old P2Y2-deficient mice, measured by computed tomography and X-ray absorptiometry. However, we also obtained strong evidence that ATP and other NTPs can block bone mineralisation via a non-receptor-mediated mechanism. NTP hydrolysis by members of the ENPP family yields pyrophosphate (PPi), long-recognised as a key physicochemical inhibitor of tissue mineralisation. We found that PPi, at physiologically relevant concentrations (10uM), blocks mineralisation of bone matrix deposited by osteoblasts; bisphosphonates, which are stable analogues of PPi, exert similar effects. We are now studying in detail the expression of ENTPDases (which hydrolyse NTPs to yield phosphate, a promoter of bone mineralisation) and ENPPs by osteoblasts. We

find that mRNAs for ENTPDases 1–3 are upregulated markedly in mature, bone-forming osteoblasts (compared with immature, proliferating cells). Expression of mRNAs for ENPPs 1–3, on the other hand, does not alter greatly during osteoblast differentiation. Thus, evidence to date indicates that ATP and other NTPs play complex and potentially important roles in the regulation of mineralisation in bone.

MicroCT analysis of P2Y1 and P2Y2 receptor knockout mice demonstrates significant changes in bone phenotype

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Extracellular nucleotides, signalling through P2 receptors, play a significant role in bone biology modulating both osteoblast and osteoclast function. In vitro, P2Y1 receptor activation by ADP/ATP stimulates osteoclast formation and activity; whilst P2Y2 receptor activation by ATP/UTP inhibits bone mineralisation. This study investigated the skeletal effects of P2Y1 and P2Y2 receptor deletion using 2-month-old knockout mice. DEXA analysis (Lunar PIXImus) demonstrated 5–14% decreases in total, hind-limb and spinal bone mineral density (BMD) and bone mineral content (BMC) of P2Y1R-/- mice; although total BMC and BMD were unchanged in P2Y2R-/- mice, a 9–17% increase in hind-limb BMC was observed. More detailed analyses using MicroCT (SkyScan 1172) showed decreases in the trabecular bone volume (BV/TV) of the P2Y1R-/- tibia (23%, $p<0.05$) and femur (35%, $p<0.01$). Trabecular number was reduced by 25% ($p<0.01$) and 32% ($p<0.0001$) in the tibia and femur, respectively. In P2Y2R-/- mice, BV/TV was increased by 43% ($p<0.01$) and 21% in the femur and tibia, respectively. Increases in femoral trabecular thickness (17%, $p<0.01$) and trabecular number (33%, $p<0.05$) were also observed. The differences in the trabecular architecture of the P2Y1R-/- mice were unexpected because P2Y1 receptor activation stimulates bone resorption in vitro; consequently, receptor deletion may predominantly affect osteoblast function. The increased trabecular bone parameters in P2Y2R-/- mice are consistent with the in vitro effects on mineralisation; thus, P2Y2 receptor deletion could potentially limit the negative actions of extracellular nucleotides on bone. These data provide further evidence for the important role of purinergic receptors in modulating bone remodelling in vivo.

On adenosine excitatory effects at the rat neuromuscular junction

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Adenosine (ADO) modulates acetylcholine release from the rat phrenic nerve via inhibitory (A1) and excitatory (A2A) ADO receptors. There is a predominance of adenosine A2A in relation to A1 receptors on ADO effects in young rats. In the present work we investigated if this occurs in ageing animals.

Evoked (EPPs) and miniature end plate potentials (MEPPs) were recorded from single fibres of innervated strips of the rat (male, Wistar, 3–4; 12–16; 36–38 weeks old) diaphragm at room temperature. The phrenic nerve was stimulated supramaximally with rectangular pulses (20 μs, every 2 s). Muscle twitches in response to stimulation were prevented with high $Mg2+$ (17–20 mM). The quantal content was calculated as the mean EPP/mean mEPP amplitudes recorded simultaneously.

In young (3–4), adult (12–16) and aged (36–38 weeks old) rats the stable ADO analogue, 2-chloroadenosine (CADO, 30nM), induced an increase in the mean amplitude of EPPs (31±8.0%, n=4; 11±1.0%, n=3 and 18±4.3% n=5; respectively). These excitatory effects of CADO observed in all ages, were prevented by the A2A receptor antagonist, ZM 341385 and were presynaptic, since CADO caused an increase in MEPPs frequency without affecting their mean amplitude and increased the quantal content of EPPs.

The results suggest that ADO effects on neuromuscular transmission change with age. The activation of A2A receptors predominates over A1 receptors in young, adult and aged rats, being these A2A effects more marked in young and aged, than in adult animals.

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Is the adenosine that contributes to exercise hyperaemia released from skeletal muscle fibres or endothelium?

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Adenosine is implicated in vasodilatation in situations in which oxygen supply is diminished (hypoxia) or oxygen demand is increased (exercise). We showed that vasodilatation evoked by systemic hypoxia in hindlimb muscle of rats is mediated by adenosine, released from the endothelium, acting via endothelial A1-receptors to release nitric oxide (NO) even though exogenous adenosine can also induce vasodilatation via A2A-receptors. We have now investigated the role of adenosine in exercise hyperaemia.

In three groups of anaesthetised rats, we recorded arterial blood pressure (ABP), femoral blood flow (FBF) and tension in extensor digitorum longus (EDL). Isometric twitch contractions were evoked by sciatic nerve stimulation at 4 Hz. Integral femoral vascular conductance (IntFVC) was calculated off-line. Group 1 (n=7) was the time control for Group 2 (n=10), which received A2A-receptor antagonist ZM241385 before the third, and 8-SPT (non-selective adenosine receptor antagonist) before the fourth contraction. Group 3 (n=10) received L-NAME (NOS inhibitor) before the third and ZM241385 before the fourth contraction.

Time controls showed consistent tension and hyperaemic responses. ZM241385 tended to reduce contraction-evoked hyperaemia (IntFVC; +10.88±1.12 vs +9.39±1.15 CU, p=0.07), 8-SPT had no further effect. L-NAME substantially reduced contraction-evoked hyperaemia (+13.91±1.31 vs +9.52±1.09 CU, p=0.0002) and this was further attenuated by ZM241385 (to 5.46±1.12 CU; p=0.0004).

In contrast to systemic hypoxia, adenosine acts via A2A-receptors independently of NO to mediate exercise hyperaemia in rat hindlimb. It is therefore likely that adenosine originates from muscle fibres during contraction and acts directly on vascular smooth muscle.

Adenosine regulates differentiation of human osteoblast cells

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Extracellular purines are important local modulators of bone cell function. Surprisingly, there are a few reports of cell function regulation by adenosine in the human bone. Recently we provided evidence that ATP is metabolised sequentially into adenosine, which accumulates extracellularly in human osteoblast cell cultures. We know, aimed at investigating the role of the nucleoside in the proliferation and differentiation in non-modified human osteoblasts.

Human bone marrow was collected from patients submitted to orthopaedic surgery (procedure approved by the Ethics Committee). Bone marrow was cultured in supplemented α-Minimal Essential Medium for up to 28 days in the absence and in the presence of stable adenosine analogues. Cultures were characterized for morphology, cell viability/proliferation (MTT assay), total protein content (method of Lowry), and alkaline phosphatase (ALP) activity.

The results obtained after a 14-day period of incubation of osteoblast cell cultures with the stable adenosine analogues are shown in the following table:

Drugs	MTT	ALP/total protein content
Control	0.626±0.112 (9)	1.150±0.154 (5)
CPA (30 nM.)	0.687±0.088 (9)	0.640±0.141 (5) *
CGS21680C (10 nM.)	0.651±0.086 (11)	0.600±0.074 (5) *
NECA (0.3 μM)	0.535±0.075 (10)	0.590±0.038 (5) *
2-Cl-IB-MECA (10 nM.)	0.626±0.094 (9)	0.480±0,086 (5) *

* $P<0.05$.

Stable adenosine analogues are devoid of effect on cell viability/proliferation (MTT assay), but they consistently affected differentiation of human osteoblasts in culture as demonstrated by the reduction of ALP activity. Characterization of adenosine receptor subtypes involved requires further investigations.

Role of P2X7 nucleotide receptors in bone remodeling

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ATP and other nucleotides are released from cells in response to mechanical stimuli. Studies by us and others have shown that extracellular nucleotides interact with osteoblasts and osteoclasts through multiple subtypes of P2 cell-surface nucleotide receptors. The P2X receptor family are ligand-gated cation channels, whereas the P2Y family are G protein-coupled receptors. The P2X7 receptor is bifunctional, under some conditions behaving as an ion channel and under others causing the formation of nonselective membrane pores. The P2X7 receptor knockout mouse exhibits a unique skeletal phenotype – diminished periosteal bone formation, excessive trabecular resorption, and impaired response to mechanical loading. Our current studies focus on the signaling pathways and roles of P2X7 receptors in bone. These investigations have led to the discovery of a novel signaling axis that couples P2X7 receptors on osteoblasts through phospholipases to production of lysophosphatidic acid, which in turn stimulates osteogenesis. In contrast, activation of P2X7 receptors on osteoclasts leads to cell death through acute cytolysis or apoptosis. Thus, nucleotides released in response to mechanical stimulation may serve as autocrine/paracrine regulators of bone cell function, providing a biological basis for mechanotransduction in the skeleton. Moreover, the P2X7 receptor is a potential target for the development of drugs with combined anabolic and antiresorptive effects. These studies are supported by the Canadian Institutes of Health Research.

(Invited)

SYMPOSIA 26: P2 RECEPTORS IN IMMUNITY

Knock-out mice reveal a role for the P2Y6 receptor in macrophages, endothelial cells and vascular smooth muscle cells

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The P2Y6 receptor is a G-protein coupled receptor selectively activated by UDP. In order to unambiguously identify its physiological roles in vivo, we have generated a P2Y6 gene targeted null mouse strain. ES cells with LoxP sites flanqued allele of the P2Y6 gene were aggregated with CD1 morulae to produce chimeric mice. These were bred with CD1 females to obtain P2Y6 +/LoxP mice. The inactivation of the P2Y6 gene was obtained by breeding these mice to mice expressing the ubiquitous Cre recombinase. The P2Y6 knock-out mice are viable and are not distinguishable from the wild type mice in terms of growth or fertility. In thioglycollate elicited macrophages, the production of inositol phosphates in response to UDP stimulation was lost, indicating that P2Y6 is the unique UDP-responsive receptor expressed by mouse macrophages. Furthermore, the amount of interleukin-6 and macrophage-inflammatory protein-2, but not tumor necrosis factor-alpha, released in response to lipopolysaccharide stimulation was significantly enhanced in the presence of UDP and this effect was lost in the P2Y6 KO macrophages. The endothelium-dependent relaxation of the aorta by UDP was abolished in P2Y6-/- mice. The contractile effect of UDP on the aorta, observed when endothelial nitric oxide synthase is blocked, was also abolished in P2Y6-null mice. In conclusion, we have generated P2Y6-deficient mice, and shown that these mice have a defective response to UDP in macrophages, endothelial cells and vascular smooth muscle cells. These observations might be relevant to several physiopathological conditions like atherosclerosis or hypertension.

The antigen receptor-induced Ca2+ response in human B lymphocytes is influenced by activation of the P2X7 receptor

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Cells release ATP during inflammatory processes or tissue damage. Activation of the P2X7 receptor raises a depolarizing nonspecific cationic ion current which carries sodium, calcium and potassium. B cell receptor (BCR) ligation induces an increase in the global intracellular calcium concentration which is dependent on the degree of the depolarisation of the cell membrane. In our study we investigated the interaction of P2X7 receptors with the calcium signalling of B lymphocytes induced by B cell receptor (BCR) ligation. B lymphocytes were isolated from human tonsils and purified by depletion of T lymphocytes by sheep red blood cells. The cells were loaded with the Ca2+-sensitive dye FLUO-4. The fluorescence signals were collected by single cell imaging. The cells were stimulated by the ligation of the BCR by human F(ab')2 anti-μ antibodies in combination with rising concentrations of the P2X7 receptor agonists BzBzATP or ATP. We found a concentration-dependent effect of the agonists on the BCR-dependent Ca2+ response in P2X7 receptor-expressing cells which was inhibiting at low concentrations and additive at higher concentrations of the agonists. The depolarization and hyperpolarization of the membrane was mimicked by gramicidin and valinomycin, which diminished or augmented the BCR-dependent Ca2+ response, respectively. The interaction of the calcium response induced by P2X7 receptors and BCR stimulation might be significant for the modulation of the immune response.

Regulation of expression and trafficking of P2X4 and P2X7 receptors in immune cells

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P2X4 and P2X7 are the predominant purinergic receptor subtypes expressed in macrophages, microglia and epithelial cells, and they are potentially important therapeutic targets for treatment of pain and inflammation. For both subtypes, there is evidence that plasma membrane expression is tightly regulated. P2X4 receptors are prominently localized to lysosomes and resist degradation by virtue of N-linked glycans decorating the intra-luminal loop of the receptor. P2X7 receptors are reported to be predominantly intracellular in monocytes and are upregulated at the plasma membrane upon differentiation of monocytes to macrophages. We have previously shown an interaction between P2X4 and P2X7 receptors, suggesting that they might form an association. The mechanisms that regulate their plasma membrane expression are not well understood, and we have used biochemical methods to look at the size and distribution of the native complexes in a variety of cell types in which they are co-expressed. We have compared the proportion of receptors expressed at the cell surface in cultured microglia and macrophages following exposure to modulators of microglial/macrophage activation. Surface expression was analysed by biotinylation of exposed proteins and by cross-linking proteins with membrane impermeant cross-linkers, followed by SDS-PAGE and western blotting. The modulators included lipopolysaccharide (LPS), ATP and phorbol esters. Cross-linking of surface receptors also provides a means of analysing the subunit composition of the complexes at the plasma membrane, based upon the size difference of P2X4 and P2X7 subunits. These results are compared with those obtained using blue native (BN)-PAGE analysis of the total P2X receptor population.

ATP infusion therapy in patients with rheumatoid arthritis: design of a multicenter double-blind placebo-controlled RCT

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Background: Based on previous findings with ATP infusions in non-small-cell lung cancer patients[1,2] and the growing recognition that ATP plays a role in immunity and inflammation, we have initiated a multicenter double-blind placebo-controlled RCT to evaluate effects of ATP infusions in patients with rheumatoid arthritis.
Objectives: To investigate effects of ATP infusions on blood markers of chronic inflammation, disease activity, fatigue and quality of life (QoL).
Subjects and methods: Patients with rheumatoid arthritis are included, who meet criteria for active disease (CRP≥20 mg/l and/or BSE≥28 mm/h, in combination with ≥4 swollen/painful joints), have previous treatment failure of at least two anti-rheumatic drugs and are currently on stable medication. Patients are recruited at hospitals in the southeastern region of the Netherlands. Patients are randomly assigned to receive weekly ATP or placebo infusions for 10 weeks. The first infusion is given under medical supervision at the day care center of the University Hospital Maastricht, subsequent infusions are given at the patient's home by a trained nurse of the infusion team of regional community care organizations. Outcome parameters include serum levels of cytokines and acute phase proteins, disease activity score (DAS) and disease-specific and generic questionnaires of functional status and QoL, such as SF-36. Outcome parameters are assessed on a regular basis (every 1–3 weeks), until 20 weeks (i.e. 10-week follow-up after finishing ATP/placebo infusions).
Current status: Patient enrollment is currently ongoing. To date, 14 patients have been included. First results are expected in 2009.

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Role of purinergic signalling in cytokine production by human enterocyte-like Caco-2 cells

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Background: It is well-recognized that extracellular purines modulate leukocyte function. We hypothesized that ATP and adenosine, in addition to affecting leukocyte function, might affect the immune function of human enterocyte-like Caco-2 cells during an inflammatory reaction.
Methods: Effects of ATP and adenosine on basal and IFN-gamma/IL-1-beta-stimulated production of cytokines was explored in Caco-2 cells. Cytokines were assessed by ELISA. Moreover, extracellular ATP and adenosine metabolism was determined by HPLC, and the presence of P2 and P1 receptor mRNA was determined by RT-PCR.
Results: Caco-2 cells metabolized both ATP and adenosine in a time-dependent fashion with sequential formation of their metabolites (ADP, AMP, inosine, hypoxanthine). Caco-2 cells expressed mRNA for P2Y1,2,11,12 and A2A,2B,3 receptor subtypes. Basal levels of IL-8 in the supernatant of Caco-2 cells were increased by both ATP and adenosine. The production of IL-8 as well as TNF-alpha in response to stimulation of Caco-2 cells by IFN-gamma and IL-1-beta was enhanced by ATP. In contrast, ATP inhibited the IFN/IL-1-induced production of IL-6. The P2 receptor agonist ATP-gamma-S had similar effects on IL-8 and IL-6, but did not affect TNF-alpha production. Adenosine increased the IFN/IL-1-induced production of IL-8 and decreased IFN/IL-1-induced production of IL-6 by Caco-2 cells.
Conclusions: Our results show that (1) Caco-2 cells exhibit significant ecto-enzyme activity, (2) P2 and P1 receptor mRNA is co-expressed by Caco-2 cells, and (3) ATP and adenosine affect the generation of cytokines by Caco-2 cells. Our findings warrant further investigation on the role of purinergic signalling in an enterocyte-driven inflammatory reaction.

P2X7 receptors activation modulates toxoplasma gondii clearance in infected macrophages

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BACKGROUND: The P2X7 receptor has recently emerged as an important component of the innate immune response against intracellular pathogens. Toxoplasmosis is a parasitic disease caused by the protozoan Toxoplasma gondii, which actively invade host cells avoiding the host lysosomal pathway and proliferates inside its parasitophorous vacuole.
METHODS: Permeabilization assay was performed by differential uptake of ethidium bromide (EB). J774 macrophages cells line were treated with or without ATP and EB at 37°C for 10 min. In order to determine the parasite load, macrophages were incubate or not with ATP for 30 min after 2 h of infection. Following 16 h of treatment the cells were stained with Panotico and infection percentage was evaluated counting parasite by light microscopy. Intracellular calcium measurements were monitored continuously in J774 cells loaded with 2.5 mM Fura-2-AM by the use of a fluorescence photometer. T. gondii-infected and control macrophages were processed for transmission electron microscopy (TEM) analysis.
RESULTS: We have observed that ATP-treatment decreased the parasite load on infected macrophages. In addition, the infected cells are more sensitive to ATP-induced EB uptake and calcium changes. TEM analyses showed ultra-structural changes on T. gondii after ATP treatment, especially swollen of parasite endoplasmic reticulum profiles and mitochondria with loss of mitochondrial cristae morphology.

CONCLUSION: The P2X7 receptors seems to be positively modulated during T. gondii infection and its activation might be involved with the clearance of this parasite inside host macrophages.
Acknowledgments: FAPERJ, CNPq and CAPES. Thanks to Erica Martins and Leandro Lemgruber for experimental assistance.

Intestinal inflammation up-regulates the expression of the G protein-coupled receptor P2Y2 at the transcriptional level

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Background: Intestinal inflammation increases the expression of the $P2Y_2$ receptor ($P2Y_2R$) in the colon of both DSS treated mice and in patients having IBDs. However, the exact molecular mechanisms regulating the expression of the $P2Y_2R$ are not known. Methods: The transcription starting point (+1) was determined by 5′RLM-RACE. The proximal region of the human $P2Y_2R$ promoter was cloned into the pGL4.10-Luc vector ($pGL4/P2Y_2$). Caco-2/15 cells were co-transfected with the $pGL4/P2Y_2$ construction or with deletion mutants generated by restriction enzyme digestions and with the transcription factors C/EBPs or NFκB/p65. The interactions were determined by luciferase assays and identification of C/EBPs and NFκB/p65 potential binding sites determined by ChIP assays, EMSA. Colitis was induced in mice with 5%DSS in water for 7 days. The colonic tissues were extracted and PCR assays were performed to evaluate the presence of $P2Y_2R$. Tissue samples were also parafilm-embedded and immunofluorescence was performed for the detection of $P2Y_2R$, C/EBPs and NFkB expression pattern. Results: We demonstrated that $P2Y_2R$ expression was up regulated in colitic mice. We established that the transcription factors C/EBPs and NFκB/p65 can bind to the promoter region of $P2Y_2$ and transactivated the luciferase gene. We also demonstrated, *in vivo* by ChIP assays, that these transcription factors can bind to specific region of the $P2Y_2R$ proximal promoter. Conclusion: These results showed that inflammation up-regulates the expression of the $P2Y_2R$ and that the expression of this gene is regulated at the transcriptional level by C/EBPs and NFkB.

Modulation of immune response by extracellular ATP and P2 receptors

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Extracellular ATP is an ubiquitous messenger endowed with many different actions (1). Until recently its role was thought to be relevant mainly in neurotransmission, however there is now compelling evidence that ATP has a crucial role as immunomodulator (2). This evidence is supported by the finding that many pro-inflammatory factors, whether of bacterial or chemical origin, or of endogenous origin (e.g. cytokines) trigger non-lytic ATP release from immune and non-immune cells. Furthermore, ATP accumulates in high concentrations at inflammatory sites as indicated by *in vivo* experiments exploiting cell-associated chimeric luciferase. In addition, recent reports show that ATP accumulates in the bronchoalveolar lavage fluid of sensitized mice exposed to antigen challenge (3). *In vivo* evidence rests on solid *ex vivo* and in vitro data showing that ATP may act as an immunodepressive or immunostimulating agent depending on the dose and conditions of stimulation (4), (5). As an immunodepressive agent ATP is an inducer of Th2 shift as well as a IL-10 secretion, thus inducing a tolerogenic phenotype. As an immunostimulant ATP cohordinates a complex pro-inflammatory response in mononuclear phagocytes involving stimulation of nitrogen- and oxygen-reactive species, release of lysosomal enzymes, stimulation of processing and release of cytokines of the IL-1 family, formation of multinucleated giant cells, killing of intracellular parasites (6). Availability of P2 receptor selective agonist and antagonists and of genetically modified mice, as

well as preliminary data from *in vivo* experiments lead to identification of P2 receptor and intracellular pathways involved. A picture is emerging in which extracellular ATP should be included in the family of so-called "DAMPs" (Damage Associated Molecular Patterns), i.e. those molecules which are released from tissue in response to an infection, an immuno-mediated aggression or a chemical or physical injury, and which act in association with "PAMPs" (Pathogen Associated Molecular Patterns) to trigger and/or modulate the innate immune response. The role of extracellular ATP as a DAMP is relevant not only for our understanding of host reaction to exogenous or endogenous noxious agents, but also for the development of novel anti-inflammatory therapies, innovative vaccines included. In this presentation I will summarize novel in vitro and in vivo data supporting the notion that ATP should be now considered a *bona fide* inflammatory mediator.

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(Invited)

P2X7 receptor activation triggers two pathways for extracellular release of antigen-presenting MHCII membranes: linking localized innate immune response to remote activation of the adaptive immune response

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The P2X7 receptor regulates a remarkably diverse array of membrane trafficking responses in inflammatory effector cells. We now report that P2X7 receptor activation in macrophages and dendritic cells (DC) triggers the rapid extracellular release of two pools of membranes containing the antigen-binding and presenting MHCII protein. In macrophages primed with both interferon-gamma and LPS or DC primed with LPS, extracellular ATP stimulated the export of ~15% of the total cellular MHCII pool within 90 min. The released MHCII was present within two morphologically and biochemically distinct populations of membrane vesicles: 1) plasma membrane-derived microvesicles (100–500 nm diameter) that sediment at 10,000xg and also contain P2X7 receptor protein, actin, and the LAMP1 lysosomal membrane protein; and 2) multivesicular body-derived exosomes (30–80 nm diameter) that sediment at 100,000xg and lack the P2X7, actin, and LAMP1 markers. P2X7-stimulated export of the exosomal MHCII pool, but not the microvesicle pool, was inhibited in macrophages that lack ASC or Nalp3/cyropyrin which are critical adapter proteins for assembly of caspase-1 inflammasome signaling complexes. Conversely, release of microvesicles, but not exosomes, was dependent on P2X7-gated Ca2+ influx. Significantly, both pools of released, purified MHCII membranes were capable of binding antigenic peptide and activating T cell receptor-dependent IL-2 production in antigen-specific T cell hybridoma cells. These observations link the well-characterized actions of the P2X7 receptor on innate immune responses within highly localized regions of microbial infection to a possible non-juxtacrine intercellular communication pathway for delivery of microbial antigen to T cells with consequent long-range engagement of the adaptive immune response.

(Invited)

Intestinal inflammation increases P2Y6 receptor expression on epithelial cells and the release of CXCL8 by UDP

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Background: Epithelial cells participate to the immune response of the intestinal mucosa. Extracellular nucleotides have been recognized as inflammatory molecules. We investigated the role of extracellular nucleotides and their associated P2Y receptors in the secretion of cytokines by epithelial cells. Methods: The effect of intestinal inflammation on P2Y6 receptor expression was determined by PCR in the mouse, rat and human. Localization of the P2Y6 was determined by immunofluorescence microscopy in the colon of normal and DSS-treated mice. The effect of P2Y6 activation by UDP on cytokine expression and release by epithelial cells was determined using a combination of western blots, luciferase assays, RT-PCR, cytokine antibody arrays and ELISA assays. Results: Inflammation up-regulates P2Y6 receptor expression in the mucosa of the colon of colitic mice. In vitro, we demonstrated that UDP could be released by Caco-2/15 cells. We have confirmed the increased expression of P2Y6 by challenging IEC-6 and Caco-2/15 cells with TNF-alpha and IFN-gamma and showing that stimulation of epithelial cells by UDP results in an increased expression and release of CXCL8 by an ERK1/2-dependent mechanism. The increase in CXCL8 expression was associated with a transcriptional activation by the P2Y6 receptor. Conclusion: This study is the first report demonstrating the implication of P2Y nucleotide receptors in the inflammatory response of intestinal epithelial cells. We show for the first time that P2Y6, as well as P2Y2, expression is increased by the stress associated with intestinal inflammation. These results demonstrate the emergence of extracellular nucleotide signaling in the orchestration of intestinal inflammation.

Induction of cell death by ATP and NAD through P2X7 receptors in murine T lymphocytes

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Background. The P2X7 receptor is responsible for ATP or NAD-dependent cell death of murine T lymphocytes through the formation of membrane pores permeable to large molecules. However, there is room for further investigation into the difference between ATP and NAD in T cell functions. In this study, we investigated the characteristics of P2X7 receptor signalling by ATP and NAD in murine T lymphocytes.
Methods. Pore formation and cell death were assayed by ethidium and propidium uptake into cells, respectively. Exposure of phosphatidylserine on the outside surface of the cell membrane was detected by annexin V binding. Cell size was determined by the change in forward light scattering property on a flow cytometer.
Results. ATP and NAD induced cell death in murine splenocytes and the responding cells were T cells. Much higher ATP than NAD concentrations were required to induce pore formation and phosphatidylserine exposure. ATP also induced cell shrinkage and decreased extracellular Cl- suppressed ATP-induced cell shrinkage and cell death, but not pore formation and phosphatidylserine exposure. Moreover, NAD did not induce cell shrinkage even in CD4+CD25+ T cells, which are more sensitive to death induction by P2X7 receptor signalling than conventional T cells.
Conclusion. These observations indicate that the signalling pathways by ATP and NAD to lead T cell death are not the same. The balance between ATP and NAD could play an important role in P2X7 receptor-mediated T cell functions.

An important role for redox signalling in P2X7 receptor mediated interleukin 1beta processing in human monocytes

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The inflammatory protease, caspase-1, is responsible for the generation of pro-inflammatory interleukin 1beta. ATP-gated P2X7 receptors (P2X7R) couple to the rapid formation of mature interleukin 1beta in endotoxin-primed human monocytes. This study investigated the role of cellular oxidation in coupling P2X7 receptors to rapid interleukin 1beta processing.
A human monocyte cell line (THP-1) and primary human monocytes were primed with lipopolysaccharide prior to stimulation with ATP. Cellular oxidation was monitored with a fluorescent detector of reactive oxygen species, H2DCF-DA. Purinergic receptor stimulation, including P2X7Rs, triggered rapid cellular oxidation that was blocked by diphenylene iodonium (DPI), the antioxidant N-acetylcysteine (NAC) or extracellular superoxide dismutase (SOD). In THP-1 cells, ATP-mediated interleukin 1beta processing was also blocked when NADPH oxidase was inhibited (100 micromolar DPI), in the presence of 10–20 millimolar NAC or a peroxynitrite decomposer (FeTPPS). Conversely, SOD augmented ATP-mediated interleukin 1beta processing. In THP-1 cells, nigericin (potassium proton antiporter) also triggered the generation of superoxide anions detected using dihydroethidium. DPI (100 micromolar) blocked nigericin-mediated oxidation, caspase-1 activation and interleukin beta processing. In primary human monocytes, ATP mediated interleukin 1beta processing was attenuated by the addition of 100 micromolar DPI or FeTPPS.
These data demonstrate that signalling via NADPH oxidase is an important upstream step for the generation of mature interleukin-1beta by P2X7R stimulation.

Involvement of NFAT in chemokine release from microglia induced by ATP

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Microglia is a potential prominent source of inflammatory factors in damaged central nerve system. ATP leaked from damaged cells has been revealed to be a potent activator of glial cells including microglia and been reported to release cytokines from microglia. However, little is known about the role of ATP in release of chemokines from microglia. Here we show that ATP evoked rapid production and release of MIP-1α (a CC chemokine) from MG-5, a microglial cell line. The increase of MIP-1α was also induced by Bz-ATP, an agonist of $P2X_7$ receptor and prevented by brilliant blue G, an antagonist of $P2X_7$ receptor. Activation of $P2X_7$ receptors induced de-phosphorylation and nuclear translocation of nuclear factor activated T cell (NFAT) in a calcineurin-dependent manner. Furthermore, the ATP-induced MIP-1α production and release were prevented by a NFAT inhibitor. Our results suggest that activation of $P2X_7$ receptors by ATP stimulates rapid production and release of MIP-1α from microglia via NFAT activation.

P2 receptor(s) mediate IL-8 synthesis and release by human umbilical vein endothelial cells (HUVEC) stimulated with TLR3 and TLR4 agonists

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Background: The pathogen-associated molecular patterns (PAMPs) via Toll-like receptors (TLRs) are powerful stimuli of IL-8 release by various cell types. Recent studies have suggested that the release of inflammatory cytokines/chemokines by

PAMPs can be mediated by extracellular nucleotides via P2 receptors. In this work, we have investigated if P2 receptors are involved in PAMP-induced IL-8 secretion by HUVEC.
Methods: HUVEC TLRs were stimulated with Pam3CSK4 (TLR1/2), poly(I:C) (TLR3), LPS (TLR4) and flagellin (TLR5), and IL-8 secretion was quantified by ELISA.
Results: In the presence of poly(I:C) and LPS HUVEC secreted IL-8. Pam3CSK4 and flagellin had no effect. IL-8 release by poly(I:C)- and LPS-stimulated HUVEC was fully inhibited by the general P2 receptor antagonists, suramin and RB-2, suggesting that P2 receptors were involved in these responses. The selective P2 antagonists MRS2500 and NF157 did not affect IL-8 release by HUVEC excluding the involvement of P2Y1R and P2Y11R, respectively. IL-8 was also secreted by HUVEC stimulated with ATP, ATPgS and UTP but not with UDP and beta-NAD. We are currently identifying the P2R subtype responsible for IL-8 release by HUVEC using gene silencing techniques.
Conclusion: This novel function of P2 receptors in IL-8 release by endothelial cells may play an important role in inflammatory neutrophil recruitment.

Caspase-1 independent processing of IL-1beta in fish

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IL-1beta is a potent pro-inflammatory cytokine which is translated as a leaderless 33 kDa precursor protein (proIL-1beta) that is processed into its 17 kDa active form by the enzyme caspase-1, which activation can be triggered by different stimuli including the engagement of P2X7 receptors by extracellular ATP or infection with invasive bacteria. While the recognition site for caspase-1 is well conserved in mammals, most of the non-mammalian IL-1beta genes cloned so far lack this conserved site. In addition, we have previously shown that extracellular ATP fails to induce the processing and release of this cytokine by P2X7 activation in the bony fish gilthead seabream. In this study, we report that the stimulation of seabream leukocytes with flagellin or bacterial DNA leads to the processing of proIL-1beta into a 20 kDa form, although it largely remains intracellular. However, the infection of leukocytes with salmonella results in the efficient and specific release of the mature form. Interestingly, the processing of IL-1beta in this species is not prevented by a specific inhibitor of caspase-1 or a pan-caspase inhibitor. In addition, we have detected a calcium dependent processing of the recombinant seabream proIL-1beta after incubation with seabream leukocytes lysates. Whatever the case, our results suggest that the processing and release of IL-1beta in early vertebrates could be mediated by cell death upon bacterial infection by a caspase-1 independent mechanism, while a more sophisticated mechanism operated by activation of ATP-gated P2X7 receptors appeared later in evolution. However, the enzyme responsible for such processing has not been identified yet.

Modulation of phagocytosis and clearance of dead cells by macrophages via P2 receptor activation

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Background: The macrophage system of defense includes internalization of different particles through endocytosis. The internalization of apoptotic cells leads to immunossupression, while the uptake of necrotic cells leads to pro-inflammatory cytokines production by macrophages and up-regulated expression of assessory molecules, such as CD11b. Methods: Peritoneal and J774 macrophages were plated at different times. The drugs ATP, alpha, beta-methyl ATP, ADP, benzoyl benzoyl ATP (BzATP) and apyrase were added for 30 minutes before exposure to particles. The antagonists, suramin and pyridoxalphosphate-6-azonphenyl-2′,4′-disulphonic (PPADS) were added 30 minutes before addition of nucleotides. The expression of the macrophage marker, CD11b, was assessed by flow cytometry analysis. Calcium measurements were

observed on FURA-2 loaded cells. Adhesion assay was carried out by exposing macrophages to apoptotic lymphocytes (APO-1) for 3 hours and subsequent counting using an optical microscope. Results: We have shown that alpha, beta-methyl ATP, BzATP, ATP and ADP increased endocytosis/macropinocytosis of dextran and phagocytosis of latex beads in macrophages and that this is inhibited by pre-treatment with the P2 receptor antagonists PPADS and suramin. The P2X1 and P2X3 receptor agonist, alpha, beta-methyl ATP, induced intracellular calcium changes when the cells were pre-treated with apyrase. Immunofluorescence showed that alpha, beta-methyl ATP, BzATP, ATP and ADP induced an up regulation of CD11b/CD18 (Mac-1) after 24 hours in culture. In addition, ATP and alpha, beta-methyl ATP induced a 2–5 times increase in adhesion of apoptotic cells on macrophages. Conclusion: We propose that engagement of P2 receptors by ATP released from dying cells results in an increased ability of macrophages to bind apoptotic bodies, whose antigens would be internalized and thus be processed more effectively by the stimulated macrophages.

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NAD and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells

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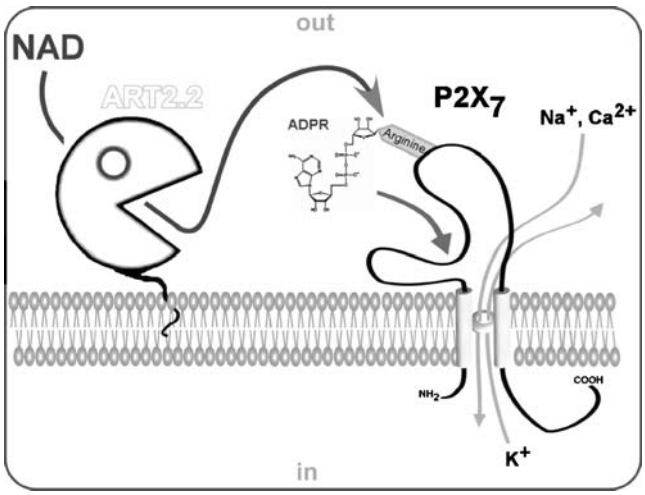
Extracellular NAD and ATP trigger the P2X7-dependent shedding of CD62L and the externalization of phosphatidylserine (PS) on murine T cells. While ATP activates P2X7 as a soluble ligand, gating by NAD requires ecto-ADP-ribosyltransferase ART2.2-catalyzed transfer of the ADP-ribose moiety from NAD onto Arg125 of P2X7. The goal of this study was to identify potential endogenous sources of these nucleotides. We report that hemolysis (rupture of erythrocytes) releases sufficient levels of NAD and ATP to activate P2X7. Dilution and incubation at 37°C reveals that signaling via ATP fades more rapidly than that by NAD. Further, the routine preparation of primary cells from spleen or lymph nodes releases sufficient NAD for ART2.2 to activate P2X7 via ADP-ribosylation, even at 4°C. Gating of P2X7 occurs when cells are returned to 37°C, inducing CD62L-shedding and PS-externalization. This "spontaneous" activation of P2X7 during preparation of primary T cells can be prevented by inhibiting ART2.2 in vivo, i.e. by intravenous injection of ART2.2-blocking single domain antibodies before sacrificing. We conclude that injured cells release NAD and ATP in sufficient concentration to activate P2X7. On cells co-expressing ART2.2, P2X7 is far more sensitive to activation by NAD than by ATP.

(Invited)



The secretion of IL-1beta in response to extracellular ATP: do only $P2X_7$ receptors matter?

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The purpose of this work was to test the contribution of P2X receptors other than $P2X_7$ to the activation of macrophages. To this end we compared the response to ATP in the absence or in the presence of ivermectin which potentiates the activation of the $P2X_4$ receptors by ATP. Macrophages were isolated from the peritoneal cavity of normal (WT) or $P2X_7$-knock-out (KO) mice. Concentrations of ivermectin lower than 100 μM had no effect on cell viability (measured by the MTT test). In the 0.1 to 30 μM concentration range, ivermectin potentiated the rapid increase of the $[Ca^{2+}]_i$ in response to ATP both in WT and $P2X_7$-KO mice. These results suggested that murine peritoneal macrophages expressed $P2X_4$ receptors. This was confirmed by a Western analysis. Ivermectin increased the release of IL-1beta in response to 10 μM–1 mM extracellular ATP both in WT and KO mice. Ivermectin partially inhibited the release of oleic acid elicited by ATP in WT mice.
These results suggest that the activation of $P2X_4$ receptors contributes to the secretion of IL-1beta from murine peritoneal macrophages. This response is not regulated by phospholipid metabolism since the activation of $P2X_4$ receptors inhibits rather than stimulates the release of oleic acid.

This work was supported by grant n° 3.4.528.07 from the Fonds de la Recherche Scientifique Médicale and by grant n° BFU2007–62728 from MEC.

Murine epidermal langerhans cells and keratinocytes express functional P2X7 receptors

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We have previously demonstrated that human epidermal and monocyte-derived Langerhans cells (LC) express functional P2X7 receptors (J Invest Dermatol, 125: 482, 2005), while others have found P2X7 receptors associated with terminally differentiated keratinocytes in rat and human stratum corneum (Exp Dermatol, 12: 860, 2003; Arch Dermatol Res 298: 301, 2006). Using flow cytometric measurements of ethidium uptake we examined whether P2X7 is present in LC, as well as in keratinocytes (KC) and dendritic epidermal T cells (DETC) from C57Bl/6 mice. ATP induced ethidium uptake into LC and KC, with EC50 values of 516 and 447 microM respectively, but not into DETC. Other nucleotides (BzATP > ATP > ATPgammaS > ADP) also induced ethidium uptake into LC and KC, while UTP and ADP were ineffective. NAD was also unable to induce ethidium uptake into LC and KC despite inducing ethidium uptake into murine lymphoid T cells. ATP-induced ethidium uptake in LC and KC was abolished in the presence of magnesium, as well as in LC and KC from P2X7 knockout mice. These results demonstrate that murine LC and KC express functional P2X7 receptors and thus mice may provide suitable models to study the role of this receptor in the skin immune system.

A role for purinergic P2Y2 receptor in MCP-1 chemokine secretion from macrophages

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Background: Macrophages play a key role in inflammation through synthesis and release of pro-inflammatory cytokines and chemokines. Extracellular nucleotides released at sites of tissue damage may be an early danger signal for immune cells, and ATP-gated P2X7 receptors are well known to mediate rapid release of pro-inflammatory IL-18 and IL-1beta. Aim: To identify whether other purine receptor subtypes are involved in the release of cytokines or chemokines from macrophages. Methods: A protein antibody array was used initially to address whether extracellular ATP could release inflammatory cytokines and chemokines from rat NR8383 alveolar macrophages, which lack the P2X7 receptor. ELISA was used to detect rat MCP-1/CCL2 in supernatants and semi-quantitative RT-PCR was used to detect MCP-1 transcripts in NR8383 cells. Results: UTP, ATPgammaS, and ATP increased the release of MCP-1 (CCL2) a pro-inflammatory chemokine. Pharmacological profiling with selective agonists and antagonists for purinergic receptor subtypes identified the receptor responsible as the G-protein coupled P2Y2 receptor. Activation of P2Y2 receptors increased MCP-1 release above basal levels after 60 minutes and MCP-1 mRNA levels were increased 3–4 fold within 30 minutes of receptor activation. Investigations into downstream signalling pathways using various inhibitors revealed that PLC, PI3K and MAPK pathways were not involved in P2Y2 receptor-induced synthesis of MCP-1 although PLC blockade did affect the release of MCP-1. NF-kappaB transcription factor is known to regulate MCP-1 gene transcription however we found no evidence of nuclear translocation of p56 NF-kappaB following P2Y2 receptor activation suggesting this pathway is not involved in P2Y2-induced transcription of MCP-1.

Microglial P2X4 receptor upregulation by fibronectin is regulated via two distinct signaling pathways

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Upregulation of P2X4 receptors (P2X4Rs) in spinal microglia is implicated as a crucial event in neuropathic pain. We have previously found that fibronectin (FN) increases microglial P2X4R protein through activation of PI3K- and MEK/ERK-mediated signaling pathways. However, the roles of the two distinct pathways in controlling P2X4R expression remain unknown. In the present study, we demonstrated that an increase in P2X4R mRNA by FN in microglia was suppressed by LY294002, an inhibitor of PI3K, but not by U0126, an inhibitor of MEK. FN increased the level of phosphorylated Akt, which was prevented by LY294002. Interestingly, FN failed to increase the level of P2X4R protein in MG-5, microglial cell line lacking p53, a transcription factor.. To determine the signaling pathway to the nucleus and the role of p53, we treated microglial cells with pifthrin, an inhibitor of p53, and found that pifthrin-treated microglia resulted in increasing gene expression of P2X4R, indicating that p53 may function as a transcriptional repressor. FN decreased the level of p53 protein, an effect that was prevented by LY294002 and an Akt inhibitor, but not by U0126. On the other hand, FN markedly increased the level of phosphorylated-eIF4E (eukaryotic initiation factor 4E), a translation initiation factor, via ERK. We also found that the eIF4E inhibitor suppressed P2X4R upregulation. Together, these results suggest that the PI3K/Akt pathway regulates P2X4R expression at the transcriptional level through p53 and the MEK/ERK pathway regulates P2X4R expression at the translational level.

Extracellular nucleotides mediate TLR2-induced neutrophil migration and IL-8 secretion

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Background:
Monocytes express several P2Y receptors that may play important roles in inflammation. Here, we investigated a role of P2 receptors in Toll-like receptor 2 (TLR2)-induced interleukin 8 (IL-8) release from monocytes and neutrophil migration.
Methods:
Human primary monocytes and monocytic cell lines were incubated with the specific TLR2 agonist, Pam3CSK4, a synthetic mimetic of gram-positive bacteria lipoproteins. These assays were performed either in the presence or absence of apyrase, a nucleotide scavenger, or P2 receptor antagonists. IL-8 release of stimulated monocytes was measured by ELISA. The role of P2 receptors in TLR2-induced neutrophil migration was investigated with a modified Boyden chamber assay and a murine air pouch model.
Results:
Pam3CSK4 stimulated secretion of IL-8 from human monocytes and monocytic cell lines (THP-1 and U937) that was decreased significantly by apyrase (30% and 75%, respectively) and the non-specific P2 receptor antagonists reactive blue 2, PPADS and suramin. MRS2578, a P2Y6 specific antagonist, decreased IL-8 release by ¡Ö80% suggesting the implication of this receptor in IL-8 secretion by monocytes.
The supernatants from Pam3SCK4-treated monocytes were chemoattractant for neutrophils due to IL-8 secretion as seen in the Boyden chamber assay. The supernatants of the cells stimulated in the presence of apyrase recruited significantly less neutrophils. In keeping with the role of P2 receptors in neutrophil migration in vitro, apyrase significantly inhibited neutrophil migration to the murine air pouches injected with Pam3SCK4.
Conclusion:
P2 receptors mediate TLR2-activated IL-8 secretion from monocytes and neutrophil migration.

Autocrine regulation of T cell activation by ATP release and P2X7 receptors

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T cells play a central role in the adaptive immune response. Activation of T cells triggers a rapid rise in cytosolic Ca2+, which is required for the formation of the immunological synapse, activation of Nuclear Factor of Activated T Cells (NFAT), and synthesis of cytokines such as interleukin-2 (IL-2). The sustained influx of Ca2+ is mediated by Calcium Release-Activated Ca2+ (CRAC) channels on the plasma membrane of T cells. Here, we examined the role of P2X7 receptors in the regulation of T cell activation. We found that T cell stimulation triggers the release of ATP from Jurkat T cells and that ATP release is required for calcium influx, NFAT activation, and the expression of IL-2. Additional data indicate that these effects are caused by feedback through P2X7 receptors, as removal of extracellular ATP, inhibition of ATP release, or silencing of P2X7 receptors blocked calcium entry and inhibited downstream T cell activation events. Moreover, we found that T cell activation up-regulates P2X7 receptor gene expression and that P2X7 receptors co-localize with STIM1 and Orai1, two recently identified proteins that associated with CRAC channels. These findings suggest a novel autocrine feedback loop that controls T cell activation by ATP release, up-regulation of P2X7 receptor expression, and control of Ca2+ signaling through the stimulation of P2X7 receptors. (Support: NIGMS GM51477, GM60475, DOD PR043034, W.G.J.; and GCRC, UCSD).

SYMPOSIA: 27 ATP IN SENSORY TRANSDUCTION

ATP and afferent control of breathing

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There has been increasing interest in the role of purines in sensory transduction. Results obtained in our laboratory indicate that ATP-mediated purinergic signalling plays a key role in the afferent mechanisms controlling breathing. Using transgenic animal models we have demonstrated that ATP acting via ionotropic P2X2 receptors is essential for the development of normal ventilatory response to hypoxia. Chemosensitive glomus cells of the carotid body (main peripheral chemosensitive organ in mammals) upon stimulation release ATP to activate afferent fibres of the carotid sinus nerve, which transmit information about arterial levels of O2, CO2 and pH to the CNS respiratory centres. It was also found that ATP mediates central respiratory CO2 chemosensory transduction in the medulla oblongata. Recently we obtained evidence suggesting that ATP is also involved in central afferent processing - ATP is released with glutamate from the central terminals of slowly adapting lung stretch receptors to activate second-order relay neurones. Thus, it emerges that ATP acts as a common mediator of peripheral and central chemosensory transduction and also contributes to neurotransmission at a first central synapse of the afferent arm in the Breuer-Hering reflex pathway. These data reveal the key role of purinergic signalling in the sensory mechanisms contributing to the control of breathing (Supported by the Wellcome Trust).

(Invited)

ATP release from sympathetic nerve endings is modulated differentially by CGRP or substance P

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To assess whether sensory neurons modulate sympathetic co-transmission, we determined the co-release of extracellular ATP/total purines, noradrenaline (NA), and neuropeptide tyrosine (NPY) and ascertained its putative modulation by the sensory neuropeptides calcitonin gene related peptide (CGRP) or substance P (SP). Electrical field stimulation (16 Hz, 60 V, 1 ms for 1-min) elicited an extracellular outflow of ATP/metabolites, NA and NPY from the prostatic end of rat vas deferens nerve endings maintained in Tyrode buffer (37 Celsius, 95% O2/5% CO2) added with desipramine. Extracellular neurochemicals were analytically determined by fluorescence, voltametry and RIA. Upon transmural electrical depolarization, we detected a burst of extracellular of ATP/metabolites, NA and NPY, which was reduced by 10 nM CGRP. In contrast, 1–10 nM SP reduced the outflow of NA and NPY, but increased extracellular ATP/metabolites. Following co-application of both peptides, the overflow of ATP/purines was not additive. We conclude that sensory neuropeptides have a dual and differential action on the overflow of sympathetic co-transmitters; the cellular mechanisms involved are actively pursued. The present results highlight that sensory neurons in addition to their role in sensory input to the CNS, have a novel role as modulators of sympathetic nerve endings. Funded by FONDAP and MIFAB grants.

	Control (6)	10 nM CGRP (4)	10 nM SP (4)
ATP (pmol)	56±9	11.5±4**	110±7**
Total Purines (pmol)	674 ±110	261±70*	827±88
NA (pmol)	35±4	17±2*	17±3*
NPY (fmol)	22±1	15±2*	11±3*

Results refer to mean ± S.E.M. *P<0.05, **P<0.01 (Dunnett's tables).

ATP and taste buds: a novel method of transmitter secretion in peripheral chemosensory sensory organs

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Taste buds are the peripheral sensory organs of gustation. Until recently, neuroscientists believed that taste buds were merely passive interfaces between the external chemical environment and afferent sensory axons. The role of taste buds was simply to transduce chemical stimuli into electrical signals. Recent evidence, however, indicates that there are synaptic connections between cells within taste buds and that a certain degree of signal processing takes place in these peripheral sensory organs. ATP and serotonin have been identified as taste neurotransmitters in taste buds and novel mechanisms for transmitter secretion involving pannexin hemichannels have been described.
This presentation reviews the evidence for signal processing in taste buds, focusing on the role of ATP and the unusual mechanism by which ATP is secreted during taste stimulation.

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(Invited)

Differential expression of P2X receptors at suprachiasmatic and supraoptic nuclei

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Hypothalamus is a prominent region of P2X-type ATP receptor expression. Previous studies have shown the presence of several P2X receptor subtypes in the supraoptic nucleus (SON), P2X2 immunoreactivity has been described at both pre-and postsynaptic sites and ATP is considered to be a dominant messenger for neuron-glia and glia-glia communication. In this study we examined the distribution of P2X subunits in the suprachiasmatic nuclei (SCN) that control circadian changes in a variety of physiological and behavioral functions, including metabolism, body temperature, hormone secretion, feeding and sleep and activity cycles. In this study we compared the expression of P2X receptor mRNAs and proteins in the SCN with that in the SON. In-situ hybridization, immunohistochemistry combined with confocal microscopy and calcium imaging were used. Experiments were performed on brain slices from adult or 21-day-old rats. In-situ hybridization showed the presence of P2X2, P2X3 and P2X4 receptor transcripts both in the SON and the SCN. Immunohistochemistry revealed the presence of P2X2, P2X3, P2X4 and P2X5 receptor proteins in the SON but not in the SCN. In contrast to the SON, there was also a lower percentage of extracellular ATP-induced calcium responses in the SCN of Fura2AM-loaded acutely isolated hypothalamic slices. These results demonstrate differential distribution of P2X receptors within hypothalamus and support functional interaction of extracellular ATP with the SON but not with the SCN. Further studies are required to elucidate whether low functional expression of P2X receptors in the SCN is related to its specific function as endogenous clock pacemaker and/or is due to low number of glia cells.

Interaction of P2X3 receptors with other macromolecules relevant for pain regulation

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Small to medium-sized neurons in the dorsal root ganglion (DRG) convey nociceptive information to the spinal cord and are endowed with homomeric P2X3 and heteromeric P2X2/3 receptors. Additionally, the co-expression of TRPV1 receptors with P2X3 receptors has been demonstrated in many DRG neurons. Although the signalling mechanism of the cationic P2X3 receptor-channel is relatively simple, various macromolecules may modify its function. We recorded by the whole-cell patch-clamp technique macroscopic currents through native P2X3 receptors in DRG neurons or their recombinant counterparts expressed in HEK293 cells. In the following, three different mechanisms, which may play a role in the modulation of P2X3 receptor-function, will be described. (1) An extracellularly localized protein kinase C (ecto-PKC) is able to increase the conductance of P2X3 receptor-channels by phosphorylating consensus PKC sites at the extracellular loop of this receptor. (2) However, the increase of P2X3 receptor-currents by PKC-activation was observed only, when a negative interaction between native P2Y receptors and native or recombinant P2X3 receptors was inhibited by the intracellular application of guanosine 5′-O-(3-thiodiphosphate (GDPbetaS). Such an inhibitory P2Y/P2X3 receptor-interaction has been shown to be Gq protein-dependent. (3) We observed a bidirectional cross-talk between both native and recombinant TRPV1 and P2X3 receptors in DRG neurons and HEK293 cells, respectively. This cross-talk was probably due to a close physical association established by means of a structural motif located at the C-terminal end of the P2X3 receptor distal to Glu362. In conclusion, we believe that a better understanding of the interaction between P2X3 receptors and a number of macromolecules in the cell membrane will allow the precisely tuned modulation of pain transmission in sensory neurons.

(Invited)

OTHER TOPICS

Characterization of a novel mouse adenine receptor by radioligand binding and functional experiments

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Background. Adenine was recently identified as the endogenous ligand of an orphan rat G protein-coupled receptor by a "reverse pharmacology" approach.1 Even though no gene sequence could be identified by bioinformatic methods there is evidence that human adenine receptors may exist.2 A mouse ortholog (m MrgA10) of the adenine receptor was identified by sequence analysis and comparison.1 In the present study, we report on the pharmacological characterization of an additional Gi-coupled receptor for adenine (Genbank accession code DQ386867).
Methods. mRNA for this receptor was obtained from mouse brain and the mouse neuroblastoma x rat glioma hybrid cell line NG108-15. The functional coupling of the mouse adenine receptors was studied. Adenine and 2-fluoroadenine inhibited the adenylate cyclase activity of the recombinant mouse adenine receptor expressed in human 1321 N1 astrocytoma cells. In addition, competition assays of selected ligands were performed in membranes from Sf21 insect expressing the new receptor, as well as in mouse neuroblastoma x rat glioma NG108-15 cell membranes, and compared to data obtained from rat brain cortical membranes.
Results. Adenine and 2-fluoroadenine inhibited the isoproterenol-induced cAMP formation with IC50 concentrations of 8 and 15 nM, respectively. In competition assays, the rank order of potency of selected ligands was adenine > 2-fluoroadenine > 7-methyladenine > 1-methyladenine > N6-dimethyladenine.
Conclusion. Our data show that a second mammalian DNA sequence encodes for a Gi-coupled GPCR activated by low, nanomolar concentrations of adenine.

1 Bender et al., Proc. Natl. Acad. Sci. USA 2002, 99, 8573–8578.
2 Gorzalka et al., Mol. Pharmacol. 2005, 67, 955–964.

Effect of ATP on survival, tumour response, nutritional status and quality of life in lung cancer patients: a multicentre, double-blind randomized trial

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Background: In a previous randomized clinical trial, we found that regular infusions of adenosine 5′-triphosphate (ATP) stabilized weight, muscle strength, appetite, fatigue and quality of life in non-small-cell lung cancer (NSCLC) patients. In weight-losing patients, ATP also enhanced median survival from 3.5 to 9.3 months (1). Several in vitro and animal studies suggest that ATP reduces radiation induced damage, probably by the down-regulation of proinflammatory cytokines (2). We here present the design of an ongoing study to assess the effects of concurrent ATP and radiotherapy treatment on outcome in NSCLC patients.
Methods: Eighty NSCLC patients selected for radiotherapy will be recruited by 12 participating clinical centres in the Netherlands and Belgium. Patients are randomized to ATP/placebo (proportions: 2/1). Regular ATP/placebo infusions are administered to the patients starting the day before the first radiotherapy session. Weekly infusions during radiotherapy are followed by bi-monthly and monthly infusions, totalling 16 infusions in 32 weeks. The primary outcome measure is survival. Secondary outcome measures include tumour response (CT-scan, X-ray), somatic side effects of radiotherapy, pulmonary function, nutritional status (weight, body composition), muscle strength, dietary intake (food record), fatigue and

quality-of-life (questionnaires). Blood samples are stored for future assessment of biomarkers for oxidative stress, inflammation, etc. Patients are followed-up until one year after randomization. Data will be analyzed according to intention-to-treat, with survival analysis or repeated-measures ANCOVA after adjusting for confounders.
Results: First results are expected in 2010.

1. Agteresch HJ, Dagnelie PC, van Der Gaast A, Stijnen T, Wilson JH (2000) J Natl Cancer Inst 92: 321–328.
2. Swennen EL, Dagnelie PC, Van den Beucken T, Bast A (2008) Biochem Biophys Res Commun 367: 383–387.

Intravenous ATP infusions can be safely administered in the home setting: A study in pre-terminal cancer patients

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Background: To investigate the safety of adenosine 5′-triphosphate (ATP) administration at home in pre-terminal cancer patients.
Methods: Included were patients with cancer for whom medical treatment options were restricted to supportive care, who had a life expectancy of less than 6 months, a WHO performance status 1 or 2, and suffered from at least one of the following complaints: fatigue, anorexia or weight loss >5% over the previous 6 months. Side effects were registered systematically on a standard form according to the National Cancer Institute (NCI) Common Toxity Criteria.
Results: Fifty-one patients received a total of 266 intravenous ATP infusions. Of these, 11 infusions (4%) were given at the lowest dose of 20 mcg/kg.min, 85 infusions (32%) at 25–40 mcg/kg.min, and 170 (64%) at the highest dose of 45–50 mcg/kg.min ATP. The majority of ATP infusions (63%) were without side effects. Dyspnea was the most common side effect (14% of infusions), followed by chest discomfort (12%) and the urge to take a deep breath (11%). No symptoms of cardiac ischemia occurred in any of the infusions. All side effects were transient and resolved within minutes after lowering the ATP infusion rate. Side effects were most frequent in patients with cardiac disorders.
Conclusion: ATP at a maximum dose of 50 mcg/kg.min can be safely administered in the home setting in cancer patients, even in the pre-terminal stage.

A2B adenosine receptors and MAP-kinase activity in the breast cancer cell line MDA-MB-231

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The estrogen-receptor negative breast cancer cell line MDA-MB-231 expresses high levels of A2B adenosine receptors as the sole adenosine receptor subtype. In addition to a stimulation of adenylyl cyclase the A2B receptor mediates a Ca2+ signal in these cells, most likely via activation of Gq. Attempts to detect a potential A2B-mediated MAP-kinase activation revealed a very high basal MAPK activity essentially identical to maximal activity as no further stimulation was achieved with FCS or EGF. This high basal activity in MDA-MB-231 cells was dependent on src and her2 as inhibitors of both proteins abolished the high basal ERK1/2 phosphorylation almost completely. Interestingly, we observed that stimulation of A2B adenosine receptors with NECA caused a time-dependent inhibition of ERK1/2 phosphorylation. A similar inhibition was seen after forskolin stimulation suggesting the involvement of cAMP in this effect. However, cAMP seems to act independently of the PKA pathway as shown by the lack of an effect of PKA inhibiton. The presence of U73221 abolished the NECA effect suggesting a

role for PLC for the A2B receptor-mediated inhibition of ERK1/2 phosphorylation. Similarly, Ca2+ was shown to be required as chelation of intracellular Ca2+ with BAPTA abrogated the inhibition of ERK1/2 phosphorylation. The investigation of cell proliferation showed that stimulation of A2B adenosine receptors caused a transient inhibition of cell growth. The A2B receptor may therefore be a target in the therapy of breast cancer independent of the expression of estrogen receptors.

A comparison of the membrane diffusion of Adenosine-A1, -A2A and -A3 receptor homo-oligomers in living cells

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Bimolecular fluorescence complementation (BiFC, Hu, Mol. Cell, 9, 789, 2002) allows the specific detection of interacting proteins which are fused to the non-fluorescent N- or C-terminal fragments of yellow fluorescent protein (N-YFP or C-YFP), by allowing reconstitution of full length fluorescent YFP (wtYFP). We have specifically monitored the diffusion of homo-oligomers of the A1-, A2A- and A3-adenosine receptors in microdomains of living cells using BiFC and fluorescence correlation spectroscopy (FCS).
FCS measurements were carried out as previously described (Briddon, PNAS, 101, 4673, 2004) on the upper cell membrane of CHO-K1 cells transiently transfected with the constructs shown below, and the diffusion co-efficients of the receptor species determined.
Homo-oligomeric versions of the A1- and A2A-ARs (Table 1) showed the same diffusion characteristics as the total receptor population. In comparison, the homo-oligomeric A3-AR appeared to represent a faster diffusing fraction of the total receptor population. These data show that a combination of BiFC and FCS can be used to study, in isolation, the diffusional characteristics of homo-oligomeric forms of ARs.

Table 1 Diffusion co-efficients for the indicated ARs as determined by FCS

	Diffusion Co-efficent ($\times 10^{-9}$ cm^2/s)	
	AR-wtYFP (Total Pop.)	AR-N-YFP+AR-C-YFP (Oligomers)
A_1 AR	4.0±0.3 (27)	4.9±0.6 (33)
A_{2A} AR	5.1±0.3 (29)	4.8±0.4 (34)
A_3 AR	4.3±0.3 (28)	6.0±0.5* (33)

Data are shown as mean±s.e. mean for (n) different cells. *$P<0.01$ vs. wtYFP construct. (one way ANOVA, post-hoc Newman-Keuls test).

A randomized, double-blind, placebo-controlled crossover study on oral ATP administration

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Background: ATP-infusions exert beneficial effects on survival, nutritional status, and quality of life in cancer-patients. Drawbacks of I.V.-administrations (patient burden, medical supervision, logistics, costs) prompted us to compare two oral ATP-administration modes.

Methods: Healthy men (n=2) and women (n=6) received five single dosages of 5000 mg ATP or placebo in 100 ml water by naso-duodenal tube or as enteric-coated pH-dependent pellets (Eudragit® L30D-55 for duodenal, FS30D for ileac release). Wash-out period was one week. Whole blood, collected until 7 h post-administration, was analyzed by RP-HPLC for ATP and metabolites. Li2CO3 (ET-AAS measurement in plasma) was added to the pellets to evaluate their content-release properties.
Results: No changes in blood ATP-concentrations were found. However, plasma urate started to rise at 30 (tube) and 75 min (L30D-55-pellets) post-administration, reaching 1.5˙ baseline values (198 microM) at 105 and 195 min. The FS30D-pellets caused only an 8% rise starting >5 h post-administration. Lithium preceded urate by ~15 min.
Conclusion: Single-dose oral ATP-administration does not lead to an increase in blood ATP-concentrations, indicating either intraluminal ecto-nucleotidase activity or a strong first-pass effect. However, both duodenal L30D-55-coated pellets and administration by tube gave similar increases in plasma urate. Terminal ileac release (FS30D-coating) was ineffective. Lithium proved useful in evaluating release-profiles.

ATP infusions improve disease-related symptoms and physical functioning in patients with advanced non-small-cell lung cancer

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Background: We earlier reported that infusions with adenosine 5′-triphosphate (ATP) had favourable effects on weight loss, muscle strength, and quality of life in patients with advanced non-small-cell lung cancer (NSCLC). The aim of the present randomised clinical trial was to investigate the effect of ATP on disease related symptoms and physical functioning in NSCLC patients in the palliative treatment stage.
Methods: Fifty-eight patients with NSCLC in stage IIIB and IV were randomized to receive either 10 ATP infusions of 30 hours every 2–4 weeks, or no ATP infusions. Outcome measurements were obtained every four weeks using the EORTC QLQ-LC13 and the RSCL questionnaires, for a period of 28 weeks. Between-group differences were tested for statistical significance by repeated measures analysis of covariance.
Results: Twenty-eight patients were assigned to the ATP group and 30 to the control group. Dyspnoea deteriorated significantly in the control group, whereas a stabilization of dyspnoea was found in the ATP group. A significant favourable effect of ATP was also found for pain in the arm and sore muscles. The results for coughing showed no significant between group differences. For tiredness and difficulty sleeping a favourable effect of ATP was found. Most items of the physical functioning deteriorated in the control group, whereas all items remained stable in the ATP group. A significant favourable effect of ATP was found on seven out of eight physical functioning items.
Discussion: Regular ATP infusions have significant favourable effects on dyspnoea, tiredness and physical functioning. Combining these data with previous findings, we conclude that ATP infusions appear to have palliative potential in NSCLC.

Do ATP infusions enhance survival in pre-terminal cancer patients? - a randomized clinical trial

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Background: To investigate the effect of intravenous infusions of adenosine 5′-triphosphate (ATP) on nutritional status and survival in pre-terminal cancer patients.
Methods: Ninety-nine pre-terminal cancer patients (estimated life expectancy 1–6 mo) with mixed tumor types were randomly allocated to receive either ATP i.v. weekly (8 h/wk, max. 50 ìg/kg.min) for 8 weeks, or no ATP (control group). Nutritional status parameters were assessed until 8 weeks, and analyzed by repeated-measures analysis of covariance. The effect of ATP on short term (0–8 weeks) and long term (0–6 months) survival was appraised by Cox proportional hazards model.
Results: Fifty-one patients were randomized to ATP and 48 to the control group. Results showed a significant difference in short-term survival (0–8 weeks) in favor of the ATP group (HR: 0.40, p=0.037), and a significant favorable effect of ATP on triceps skin fold thickness (between-group difference per 8 weeks: 1.76 mm, 95% CI: 0.48 to 3.12 mm, p=0.009). In weight-stable as well as in lung cancer patients, long term survival (0–6 months) was also significantly better in ATP-treated patients (weight stable patients: HR: 0.40, p=0.014; patients with lung cancer: HR: 0.35, p=0.025).
Conclusion: In this population of pre-terminal cancer patients, ATP infusions, at the dose and schedule studied, had a favorable effect on triceps skin fold thickness and survival, especially in weight-stable patients and patients with lung cancer.

Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist-administration

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The precise mechanism by which mutant-huntingtin elicits its toxicity remains unknown. However, synaptic alterations and increased susceptibility to neuronal death are known contributors to Huntington's disease (HD) symptomatology. While decreased metabolism has long been associated with HD, recent findings have surprisingly demonstrated decreased neuronal apoptosis in C. elegans and Drosophila models of HD by drugs that diminish ATP production. Furthermore, extracellular ATP has been recently reported to elicit neuronal death through stimulation of P2X7 receptors. These are ATP-gated cation channels known to modulate neurotransmitter release from presynaptic terminals. We hypothesized that alteration in P2X7 mediated calcium permeability may contribute to HD synaptic dysfunction and increased apoptosis. Using mouse and cellular models of HD, we demonstrate increased levels of this receptor and altered P2X7 mediated calcium permeability in somata and terminals of HD neurons. Furthermore, cultured neurons expressing mutant-huntingtin showed increased susceptibility to apoptosis triggered by P2X7 receptor stimulation. Finally, in vivo administration of a P2X7 antagonist to HD mice prevented neuronal apoptosis and attenuated body-weight loss and motor-coordination deficits. These in vivo data strongly suggest that altered P2X7 receptor level and function contribute to HD pathogenesis and highlight the therapeutic potential of P2X7 receptor antagonists.

P-glycoprotein overexpression confers resistance to A3 adenosine receptor agonists 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA) in human leukemia cells

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BACKGROUND: 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA) the A3 adenosine receptor (A3AR) agonist has been implicated in modulation of cell proliferation and cell death. Effects of Cl-IB-MECA strongly depend on the concentration used. Thus, nanomolar concentrations of Cl-IB-MECA stimulate cell proliferation and protect

cells from death in some experimental models. On the other hand, micromolar concentrations of Cl-IB-MECA are cytotoxic and induce apoptosis itself. Interestingly, antagonistic effects of Cl-IB-MECA were attributed to the same signalling pathway, which is triggered upon ligation of A3AR. We studied cytotoxic effects of Cl-IB-MECA in K562/Dox cell line, which exhibits multidrug resistance phenotype.
METHODS: K562/Dox cells were derived from K562 cell line, by long-term cultivation in the presence of increasing concentration of doxorubicin. K562/Dox is resistant to doxorubicin and also to other drugs due to P-glycoprotein (ABCB1, MDR1) overexperssion. Gene expression was confirmed by western blot analysis. P-glycoprotein function was confirmed using rhodamine 123 efflux assay monitored by flow-cytometry.
RESULTS: We found that K562/Dox cell line was significantly more resistant to Cl-IB-MECA than the maternal cell line K562, which did not express P-glycoprotein. Interestingly, inhibitors of P-glycoprotein function, including cyclosporin A restored sensitivity of K562/Dox cell line to Cl-IB-MECA.
CONCLUSION: The cytotoxic effect of Cl-IB MECA might be at least in par due to its intracellular accumulation.
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Presence of functional P2Y1 and P2Y1 receptors in human SK-N-MC neuroblastoma cells

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We have analyzed the presence of functional P2 receptors in the human neuroblastoma SK-N-MC cell line. If such receptors were present in these cells, SK-N-MC could serve as a useful model to analyze different aspects of the signalling mediated by nucleotides in neural tissues. Western blotting and immunocytochemical analysis indicated the presence of P2Y1, P2Y6, P2Y11, P2X1, P2X4, P2X5, P2X6 and P2X7 proteins in the SK-N-MC cells. Functional analyses were performed by single-cell imaging after loading cells with the calcium dye Fura-2. SK-N-MC cells showed transient [Ca2+]i increases upon application of ADP, 2MeSADP or ADPbetaS. Responses to these agonists are likely mediated by a P2Y1 receptor, as demonstrated by the almost complete blockade exerted by the P2Y1 receptor antagonist MRS2179. ATP was also able to induce [Ca2+]i increases in the SK-N-MC cells. Responses to ATP were partially blocked by both MRS2179 and the P2X antagonist TNP-ATP, this suggesting that ATP could interact with two different P2 receptors in the SK-N-MC cells: a P2Y1 and a P2X receptor. To further characterize the P2X receptor that is responsible for the MRS2179-resistant component of the ATP response we analyze the effect of several P2X agonists. No cells showed responses to either alpha-beta-meATP or Bz-ATP, although calcium transients could be observed when cells were challenged with CTP. Moreover, both the response to CTP and the MRS2179-resistant component of the ATP response were potentiated in the presence of ivermectin, such pharmacology profile suggesting the presence of a functional P2X4 receptor in the SK-N-MC cell line.

A role of conserved tyrosine in the first transmembrane domain in purinergic P2X receptor function

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Among the TM residues of P2X subunits, only four are highly conserved: glycine and tyrosine of TM1 and glycine and aspartic acid of TM2. Here we examined the effect of alanine substitution of tyrosine residue on receptor function using rat P2X1, P2X2, P2X3, P2X4 and P2X7 subunits. We also examined effects of replacement of the P2X4 Tyr-42 with residues

of different hydrophobicity, volume, or charge. Wild type (WT) and mutant receptors were expressed in HEK293 cells and examined using whole-cell patch clamp recording. The P2X1[Y43A] mutant was expressed in the plasma membrane but was non-functional. The P2X2[Y43A] and P2X4[Y42A] mutants were functional, exhibited 10-15 fold enhanced sensitivity for ATP accompanied with prolonged deactivation, and developed sensitivity to alpha-beta-meATP, with the EC50 values of 10 μM and 0.7 μM, respectively. P2X3[Y37A] and P2X7[Y40A] mutants showed 2-fold leftward shifts in the EC50 values for ATP and 2′,3′-O-(benzoyl-4-benzoyl)-adenosine triphosphate, respectively, accompanied with longer deactivation times. The P2X4-Tyr-42 substitution with residues of different properties enhanced the sensitivity of receptor for ATP and prolonged deactivation time in order: Gly > Ile > Ala > Cys > Trp > Phe > Tyr; logarithmic plot showed a strong inverse relationship between EC50 values and deactivation time. Effects of Y42A substitution on receptor activation and deactivation were lost in the Y42A+W46A mutant, whereas the receptor function of the W46A mutant was not affected. These results indicate that TM1 aromatic residues play important roles in three-dimensional structure of P2X receptors required for agonist binding and/or gating.

Is there a role for P2 receptors other than the P2X1 subtype in parasympathetic cotransmission in the urinary bladder?

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Parasympathetic nerves innervate the detrusor muscle of the urinary bladder, but a non-cholinergic component of neurotransmission was recognised over 100 years ago (Langley & Anderson, 1895). Subsequently, ATP was identified as the cotransmitter with acetylcholine (Kasakov & Burnstock, 1983). Here we have determined the effects of P2X receptor antagonists on the non-cholinergic component of neurotransmission.
Strips of guinea-pig urinary bladder were mounted under isometric conditions in 2 ml baths at 35 C. Contractions were elicited by 4 Hz EFS, ATP (300 microM) & alpha,beta-meATP (1 microM). Atropine (1 microM) & prazosin (100 nanoM) were present throughout.
PPADS (0.1–100 microM) & suramin (1–300 microM) inhibited atropine-resistant contractions to 4 Hz EFS with IC50s (95% cl) of 6.9 microM (0.7–78.9 microM, n=5) & 13.8 microM (7.9–24.1 microM, n=6), but 30–40% of the response was resistant. TTX (1 microM) abolished all responses. PPADS & suramin also inhibited contractions elicited by ATP & alpha, beta-meATP, with potencies similar to those against 4 Hz EFS (n=4–8). Whilst responses to alpha,beta-meATP were abolished, 30–40% of the peak response to ATP was not. The atropine-resistant neurogenic contractions were also only partially inhibited by 100 microM NF279 (by 31.9±3.7%, n=7), MRS2159 (by 57.0±4.4%, n=5) and RB2 (by 32.8±5.2%, n=6), all of which abolished responses to alpha,beta-meATP. In contrast, desensitisation of P2X1 receptors abolished responses to 4 Hz EFS (n=12), alpha,beta-meATP (n=4) and ATP (n=5).
Thus, whilst the non-cholinergic component of neurotransmission is abolished by desensitisation of the P2X1 receptor by alpha,beta-meATP, it is only partially inhibited by P2X1 antagonists. The mechanism underlying the atropine- and P2X1 receptor antagonist-resistant component of neurotransmission is unclear.
Langley, KN & Anderson, HK (1895). J. Physiol., 19, 71–139.Kasakov, L & Burnstock, G (1983). Eur. J. Pharmacol., 86, 291–294.

The role of Ca2+ sensitisation in P2X receptor-induced contractions of rat small pulmonary artery

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P2X receptors are Ca2+-permeable, ligand-gated cation channels, which are activated by ATP. In arteries, such as the rat small pulmonary artery (SPA), they elicit vasoconstriction (Chootip et al., 2002), which is dependent upon extracellular Ca2+. An increase in the sensitivity of the contractile proteins to cytoplasmic Ca2+ via Rho kinase (RhoK) and protein kinase C (PKC) can also play a role in vasoconstriction. Thus, the aim of this study was to determine the involvement of Ca2+ sensitisation in P2X receptor-mediated contractions of rat SPA, using the RhoK inhibitor Y27632 and PKC inhibitor GF109203X.

5 mm rings of rat SPA were mounted under isometric conditions in 1 ml organ baths at 37°C and a resting tension of 0.5 g. Contractions were elicited by addition of the P2X receptor agonist alpha,beta-meATP (10 microM) or KCl (40 milliM) to the bath. Data are expressed as mean±sem and were compared by Student's t-test or one-way ANOVA as appropriate.
alpha,beta-meATP-induced contractions were significantly decreased by preincubation with Y27632 (10 microM, 70.5±2.4% of control, n=4) or GF109203X (10 microM, 62.8±3.4% of control, n=4) and were further depressed when the inhibitors were co-applied (37.8±6.6% of control, n=5, P<0.01). KCl-induced contractions were also significantly decreased by Y27632 (10 microM) (77.4±3.4% of control, n=5, P<0.05) and GF109203X (10 microM) (44.8±4.7% of control, n=4, P<0.001). Co-application of the two inhibitors (10 microM each) had no greater effect than GF109203X alone (48.7±7.0% of control, n=10, P<0.001).
These data show that RhoK and PKC play a role in P2X-evoked contraction of SPA. This is surprising, as the activation of these enzymes is thought to be dependent upon activation of G proteins.
Chootip, K, Ness, K.F., Wang, Y., Gurney, A.M. & Kennedy, C. (2002). Br. J. Pharmacol., 137, 637–646

Ca2+-signalling pathways involved in P2X receptor-induced contractions of rat small pulmonary artery

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P2X receptors are Ca2+-permeable, ligand-gated cation channels, which are activated by ATP. In arteries, including the rat small pulmonary artery (SPA), they elicit vasoconstriction (Chootip et al., 2002), which is dependent upon extracellular Ca2+. The aim of this study was to determine the relative contributions of Ca2+ influx via the P2X receptor pore and voltage-dependent CaV1.2 (L-type) Ca2+ channels, and the role of Ca2+-induced Ca2+ release (CICR) in P2X receptor-mediated contractions of rat SPA.
5 mm rings of rat SPA were mounted under isometric conditions in 1 ml organ baths at 37°C and a resting tension of 0.5 g. Contractions were elicited by addition of the P2X receptor agonist alpha,beta-meATP (10 microM) or KCl (40 milliM) to the bath. Data are expressed as mean ± sem and were compared by Student's t-test or one-way ANOVA as appropriate. Contractions evoked by alpha,beta-meATP were abolished when tissues were bathed in Ca2+-free buffer (n=5) and inhibited by 56±6% by nifedipine (1 microM) (n=5, P<0.05), 47±9% by CdCl2, (100 microM) (n=5, P<0.05) and 56±9% by nifedipine (1 microM) plus CdCl2, (100 microM) (n=6, P<0.01). These treatments each abolished contractions evoked by KCl (n=4–5). To study the role of CICR, the sarcoplasmic Ca2+ stores were depleted by pretreatment with ryanodine (10 microM) and caffeine (10 milliM). Under these conditions the contractions to alpha,beta-meATP were unchanged (99.9±7.5% of control, n=7), whereas the response to KCl was significantly reduced (54.4±3.6% of control, n=5, P<0.05).
These data show that P2X-evoked contraction of SPA predominantly depends equally upon influx of extracellular Ca2+ through the P2X receptor pore and CaV1.2 voltage-operated Ca2+ channels. However, CICR from sarcoplasmic reticulum Ca2+ stores does not appear to play a role.
Chootip, K, Ness, K.F., Wang, Y., Gurney, A.M. & Kennedy, C. (2002). Br. J. Pharmacol., 137, 637–646.

Down-regulation and desensitization of adenosinw A1 receptor/adenylyl cyclase pathway after r-pia intake during pregnancy

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Little is known about G-protein coupled receptors (GPCRs) desensitization process during pregnancy. Wistar pregnant rats were treated with (R)-N6-(1-Methyl-2-phenylethyl)adenosine (R-PIA), adenosine A1 receptor agonist, in their drinking water during pregnancy and the effect on A1R/adenyly cyclase system was studied in both maternal and fetal brain. In mothers' brain, binding assays revealed a significant decrease in total receptor numbers in plasma membranes (27%), with no significant changes on receptor affinity. The effect of R-PIA on plasma membranes from fetal brains was more marked, being detected approximately 42% of the total receptors detected in control fetuses. No variations on mRNA coding A1 receptor were found in any case,

suggesting post-transcriptional modulation as responsible for the lost of receptors detected previously. Immunoblotting and RT-PCR analysis did not revealed any significant changes in either protein or mRNA levels for alphaGi1,2 and alphaGi3 subunits, respectively, neither in mothers nor fetuses. On the other hand, forskolin- and forskolin plus GTPgammaS (guanosine-5′-O (3-thiotriphosphate) tetralithium salt)-stimulated adenylyl cyclase activity was decreased only in fetal brain. Nevertheless, adenylyl cyclase inhibition elicited by N6-cyclohexyladenosine (CHA), selective adenosine A1 receptor agonist, was significantly decreased in both mothers and fetuses, suggesting a desensitization of adenosine A1 receptor/adenylyl cyclase pathway. Therefore, this results suggest that R-PIA intake during pregnancy causes desensitization of A1R-mediated inhibitory transduction pathway in both mother and fetus brain, probably due to the down-regulation of adenosine A1 receptors at the membrane surface.

Identification of extracellular residues of P2X7 receptor involved in functional inhibition by acidic pH

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Background: We investigated the role of extracellular histidine, lysine, aspartic acid, and glutamic acid residues in the potent inhibition of P2X7 receptor function by acidic pH.Methods: We introduced point mutations by site-directed mutagenesis, expressed wildtype (WT) and mutant P2X7 receptors in human embryonic kidney 293 cells and, used patch clamp recording to characterize effects of the acidic pH on 2′-3′-O-(4-benzoylbenzoyl)ATP evoked receptor currents.
Results: Increase in extracellular acidity or proton concentrations inhibited BzATP-evoked currents in cells expressing WT P2X7 receptor with half-maximal inhibition concentration (IC50) for proton of 0.2 microM. The major effect of acidification was suppression of the maximal current response without altering the agonist sensitivity. Alanine mutation of five positions (His85, Lys110, Lys137, Asp197 and His219) altered the inhibition by acidic pH; compared to WT, the H85A, H219A, K137A mutants became 2–3 fold more sensitive, whereas the K110A and D197A mutants were 2.5 and 9 fold less sensitive. Double alanine substitution of Lys110 and Asp179 resulted in 23-fold decrease in the sensitivity to inhibition by acidic pH. Furthermore, charge neutralization (K110M and D197N), but not charge conserving mutation (K110R and D197E) attenuated the inhibition by acidic pH.
Conclusion: The inhibition of P2X7 receptor by acidic pH may involve extracellular His85, Lys110, Lys137, Asp197 and His219 residues, among which Asp197 is critical.

A comparison of binding kinetics of fluorescent agonist at adenosione -A1 and -A3 receptors in single living cells

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This study has used the fluorescent adenosine agonists ABEA-BY630 and ABA-BY630 to directly measure agonist dissociation kinetics at the adenosine-A1 and -A3 receptors. CHO cells stably expressing either the adenosine-A1 or -A3 receptor were exposed to 100 nM fluorescent agonist for 3 minutes. Single confocal slices were obtained at 2 second intervals for 130 seconds with the addition of 10 μM xanthine amine congener (XAC) at 10 seconds. Agonist dissociation was biphasic with the rate of both components being considerably slower at the adenosine-A1 receptor (ABA-BY630: k-1= 3.77±0.7 min-1, k-2=0.08±0.03 min-1; ABEA-BY630: k-1=3.95±1.6 min-1, k-2=0.04±0.03 min-1) as compared to the -A3 receptor (ABA-BY630: k-1=19.3±2.9 min-1, k-2=1.3±0.9 min-1; ABEA-BY630: k-1=8.3±2.3 min-1, k-2=1.1±1.0 min-1). In addition, at the adenosine-A1 receptor the proportion of agonist showing slow dissociation was more then double that at the adenosine-A3 receptor. This study is the first to directly measure adenosine agonist dissociation in whole cells and has shown that agonist dissociation appears significantly slower at the adenosine-A1 receptor as compared to the -A3 receptor.

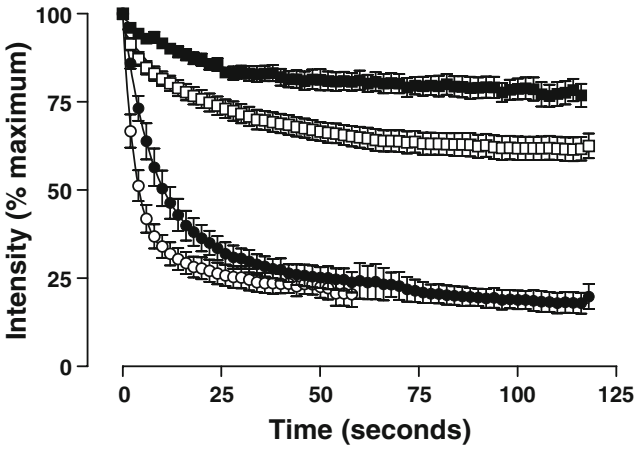

Figure 1. Dissociation of ABEA-BY630 (closed symbols) and ABA-BY630 (open symbols) from the adenosine-A_1 (squares) and -A_3 (circles) receptors stably expressed in CHO cells. Data points represent mean ± S.E.M. of 30-130 cells from 3-13 separate experiments.

Induction of apoptosis by adenosine A3 receptor agonists N6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamide (IB-MECA) in human leukemia cells: a possible involvement of intracellular mechanism

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BACKGROUND: The A3 adenosine receptor (A3AR) has been implicated in modulation of several physiological and pathological processes, including cell proliferation and cell death. IB-MECA the A3AR agonist exhibits dual activity on mammalian cells depending on the concentration used. While micromolar concentrations induce apoptosis, nanomaolar concentrations may even protect cells from death. Both effect were attributed to the signalling pathway, which is triggered upon ligation of A3AR by IB-MECA. In our laboratory, we addressed the question whether there exists also other mechanism in addition to A3AR mediated that may offer alternative explanation for proapototic effects of IB-MECA.
METHODS: HL-60 cells were cultivated in growth medium containing micromolar concentrations of IB-MECA. Cell extracts were analysed using reversed phase HPLC/MS.
RESULTS: We observed that IB-MECA was transported into cells, where it was further metabolised. Both events, transport and metabolism, were time and concentration dependent. We found that IB-MECA interfered with biochemical pathways that are utilised for metabolism of adenosine. We speculate that IB-MECA might exert its pro-apoptotic effects at least partly through biochemical pathway functioning independently of A3AR.
CONCLUSION: IB-MECA is transported into cells where it is metabolised. Intracellular IB-MECA can enter biochemical pathways important for metabolism of adenosine.
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P2X agonist BzATP interferes with amplex-red-coupled fluorescence assays

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The amplex red (AR) reagent (10-acetyl-3,7-dihydroxyphenoxazine) is a fluorigenic substrate for horseradish peroxidase (HRP) used in many oxidations that produce hydrogen peroxide. In the presence of hydrogen peroxide, AR is converted to the fluorescent compound, resorufin, which has an emission maximum of 590 nm.
The interference of the P2X7 receptor agonist BzATP in AR-coupled-assays was first evidenced when the AR glutamic acid/glutamate oxidase assay was used in experiments of glutamate release regulation by BzATP. In the absence of added hydrogen peroxide, the sole addition of BzATP to a working solution of L-glutamate, resulted in a significant increase in the maximum of the fluorescence emission at 590 nm. Thus, HRP in the presence of BzATP was able to oxidize the nonfluorescent AR to fluorescent resorufin. This was reproduced by 4-benzoilbenzoic acid, indicating that the benzophenone group of BzATP was responsible for such effect. In addition, it was necessary the presence of oxygen, as this effect was decreased under anaerobic conditions. The proposed mechanism involves the benzophenone (BHP) abstracting one electron from the previously formed AR radical, leading to a ketyl radical and resorufin. This BHP radical reacts with oxygen to produce benzophenone and superoxide radical, which is transformed into hydrogen peroxide. The hydrogen peroxide generated is then used by HRP in a subsequent reaction to oxidize AR. This explains how BzATP enhances the HRP-mediated AR oxidation in the absence of added hydrogen peroxide. Based on these results, caution might be needed when using BzATP in AR-coupled assays, suggesting that other ways of P2X7 receptor activation could be undertaken.

Expression of A2A and A2B adenosine receptors in human breast tumoes

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Background: After identification of the expression profile, signal transduction, molecular function and cell growth modulation of adenosine receptor subtypes in the human breast cancer cell lines, we decided to investigate the possible roles of adenosine receptors in the human breast tissues. In this study, we used RT-PCR to assess A2A and A2B gene expression in normal and tumor breast tissues.
Methods: Breast tumors and non-neoplastic mammary tissues (n=15) were collected immediately after mastectomy and stored at -80°C until use. All tumors were histologically confirmed to be breast cancer. Total RNA was extracted and reverse transcribed to cDNA. PCR primers were synthesized from human adenosine receptor cDNA sequences. PCR was performed under optimized condition for each receptor subtype. Amplification of beta-actin mRNA served as control for RT-PCR. The PCR products were separated on 1.5% agarose gels.
Results: To elucidate the expression of A2A and A2B mRNA in breast carcinoma and normal tissues, we compared the level of A2A and A2B mRNA expression by RT-PCR analysis. All breast tumor tissue specimens (n=11) expressed A2A and A2B adenosine receptor transcripts. In contrast, only one of the four breast specimens from patients without carcinoma expressed no A2A mRNA. Moreover, there were no observable differences between normal and tumor tissues, when normalized against that of beta-actin.
Conclusion:
In conclusion, these results indicate for the first time, to our knowledge, the expression profile of A2A and A2B adenosine receptors in the human breast carcinoma. However, further studies based on the Real-time quantitative RT-PCR are needed to identify gene expression levels.

Behavioral responses to caffeine and ethanol are altered in human ENT1 transgenic mice

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Background Mice expressing human equilibrative nucleoside transporter subtype 1 (hENT1) under the control of a neuron specific enolase promoter were developed and characterized in biochemical and behaviour experiments.

Methods Experiments to verify transgene expression included tritiated adenosine uptake assays, tritiated nitrobenzylthioinosine binding assays and reverse transcriptase polymerase chain reactions (RT-PCR) with hENT1 specific primers. For behaviour experiments, mice were injected (i.p.) with ethanol (3.6 g/kg) or caffeine (25 mg/kg) and monitored for loss of righting reflex (LORR) or locomotor activity, respectively.
Results Tritiated adenosine uptake into synaptosomes was increased 2–3 fold and tritiated nitrobenzylthioinosine binding to cortical membranes was increased up to 20-fold in samples from hENT1 transgenic mice, relative to wild type littermates. hENT1 expression was detected, by RT-PCR, in cortex, striatum, hippocampus and cerebellum but not in liver, heart, lung, kidney or skeletal muscle. Ethanol induced LORR was increased 58% but caffeine-stimulated locomotor activity was reduced in hENT1 transgenic mice, relative to wild type littermates.
Conclusion Mice with increased ENT1 activity in neurons have altered behavioural responses to ethanol and caffeine, drugs that increase and decrease, respectively, adenosine receptor signalling.

Forced unbinding of GPR17 ligands form wild-type and R255I mutant receptor models

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GPR17 is a "hybrid" GPCR that responds to two unrelated ligand families, extracellular nucleotides and cysteinyl-LTs. Its in vivo blockade reduces progression of cerebral ischemic damage, highlighting GPR17 as a novel therapeutic target for ischemia (Ciana et al, 2006. EMBO J 25:4615).
To explore the binding mode of the "purinergic" and "leukotrienic" components of the receptor, we studied the binding and the forced unbinding of two GPR17 ligands (the endogenous purinergic agonist UDP and the leukotriene receptor antagonist pranlukast) from both wild-type and a mutant (R255I) model of GPR17, by means of docking and molecular dynamics simulations techniques (MD).
MD suggests that GPR17 nucleotide binding pocket is enclosed between the helical bundle and EL2. The driving interaction involves R255 and the UDP phosphate moiety. This hypothesis was also supported by steered MD experiments, showing that the energy required to unbind UDP is higher for the wild-type receptor than for R255I.
Three potential binding sites for pranlukast where instead found. In one of its preferential docking conformations, pranlukast tetrazole group is close to R255 and phenyl rings are placed into a subpocket highly conserved among GPCRs. Pulling forces developed to break polar and aromatic interactions of pranlukast were comparable. No significant differences between the wild-type and the mutant receptor were found for the unbinding of pranlukast from GPR17.
MD simulations thus suggest a crucial role for R255 in binding of nucleotides to GPR17. Aromatic interactions are instead likely to play a predominant role in the recognition of pranlukast, suggesting that two different binding sites are present on GPR17.

The effect of ATP, alone and in combination with EGF, on breast cancer cell survival

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There is increasing evidence that ATP and other extracellular nucleotides play an important role in the growth and survival of various human cancer cell types in vitro. We have previously demonstrated that ATP enhances epidermal

growth factor (EGF) activation of c-fos in Hs578T and T47D breast cancer cell lines. c-fos is an immediate early gene and proto-oncogene that plays an important part in cell proliferation, apoptosis and differentiation. To determine the downstream physiological effects of EGF and ATP-induced activation of c-fos we have investigated the effect of EGF and ATP on breast cancer cell survival. Hs578T and T47D cells were treated with either EGF or ATP alone or in combination and the effect on cell proliferation determined. Whilst EGF had different effects on the proliferation of both cells lines due to their estrogen status, ATP had an anti-proliferative effect. We also measured the levels of apoptosis in treated cells and found that EGF and ATP, both alone and in combination, had strong effects on apoptosis of these cells. Using a transwell migration assay we found that migration was unaffected in either cell line by any treatment. Microscopic examination of Hs578T cells revealed an altered morphology from their typical flattened, stellate cell morphology to a more spindle-like morphology with combined EGF and ATP treatment. These results have highlighted the role of ATP in the survival of Hs578T and T47D breast cancer cells and may represent novel targets for therapeutic intervention in breast cancer.

Ectodomain residues Asp331 and Met336 near the transmembrane domain 2 of P2X4 receptors are important for the action of ethanol

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Purinergic P2X receptors (P2XRs) are ligand-gated ion channels that are gated by synaptically released extracellular ATP. Although investigations are in the early stages, P2XRs have been linked to a growing list of behaviors and physiological functions affected by ethanol. At the present time, sites of ethanol action on P2XRs are poorly understood. Recent findings from our laboratory using P2X2R/P2X3R chimeras combined with site-directed mutagenesis, suggest that ectodomain regions near the TM domains of these P2XR play a role in determining ethanol action. The present work extends this investigation to P2X4Rs which are widely expressed in the CNS, and in vitro are inhibited by ethanol. We performed alanine scanning mutagenesis of the region (residues 321–337) at the ectodomain-TM2 domain interface and expressed the mutated receptors in Xenopus oocytes. Changes in ethanol sensitivity (100 mM) were determined using two-electrode voltage clamp (-70 mV). Among all mutants, mutations of residues Asp331 and Met336 significantly reduced ethanol inhibition compared to WT P2X4Rs. Moreover, simultaneous mutation of positions 331 and 336 to Ala further reduced ethanol inhibition compared to those of individual mutant P2X4Rs. Overall, our results suggest that D331 and Met336 are important targets for ethanol action in P2X4Rs. These data support our previous findings on the important role of the residues at the ectodomain-TM domain interface in ethanol action in P2X2 and P2X3 receptor subtypes. Taken together, these results represent an important step forward in identifying sites and mechanisms for ethanol action in P2XRs. Support: NIAAA/NIH F31 AA017029–01, AA013922, AA03972 and USC School of Pharmacy.

Single receptor/channel imaging of P2X4 receptor

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ATP is released or leaked from cells when they are stimulated or damaged and participates in various events in both physiological and pathological conditions. P2X4 receptor (P2X4R) is crucial as regards intractable chronic pain. It is therefore receiving a lot of attention as an important therapeutic target. Biochemical/electrophysiological studies have

revealed that P2XRs are ATP-gated cation channels that are thought to consist of three subunits and that gate the membrane-spanning pore. However, there is no direct evidence for this structural hypothesis. Here, we show the trimer structure, real-time conformational changes and pore formation of P2X4R, which we determined with fast-scanning atomic force microscopy (AFM). The ATP-induced conformational change in P2X4Rs had two components: (i) an increase in the gaps between subunits, and (ii) flip-flop changes in extracellular domains (ECDs), which were associated with the cavity formation by ECDs. A deeper pore surrounded by ECDs was observed in the cavity, which was considered to be the mouth of the transmembrane pore. Additionally, real-time imaging at a scan rate of 2 frames per second showed that the ATP-induced conformational change occurred within 0.5 sec, and that the two types of P2X4R were different conformations of P2X4R before and after the application of ATP. Together, these results suggest that P2X4Rs are composed of three subunits, they change their conformation with an increasing gap between subunits, and cause flip-flop changes in ECDs that accompany pore opening in response to ATP stimulation.

Increased cytotoxicity of cisplatin in a human large cell lung carcinoma cell line by ATP

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Background. Lung cancer, a highly aggressive type of cancer, is one of the leading causes of cancer-related death throughout the world. Cisplatin is a widely used anti-cancer agent for the treatment of lung cancer, however, the development of resistance to cisplatin is a major obstacle in cancer therapy. Several in vitro studies indicate that multi-drug resistance is associated with increased intracellular glutathione (GSH)-levels. The aim of the study was 1. to investigate the effect of ATP on the cytotoxicity of cisplatin in a large cell lung carcinoma cell line, and 2. to examine the involvement of GSH depletion by ATP in this effect.
Methods. H460 cells were harvested and seeded (5% CO2; 37 C). Subsequently, cells were incubated with medium, ATP (0.03, 0.3 and 3 mM) or BSO (a known GSH depleter), followed by an incubation with cisplatin. Cytotoxicity screening was analyzed by the sulforhodamine B (SRB) colorimetric assay and total GSH levels were analyzed by the recycling method.
Results. 72 h incubation with 0.3 and 3 mM ATP strongly decreased the IC50 value of cisplatin (67% for 300 μM ATP and 83% for 3 mM ATP). BSO also decreased IC50 (68%) and reduced GSH levels as expected. However, ATP, despite lowering the IC50 of cisplatin, did not modulate GSH levels in H460 cells.
Conclusion. 1. ATP increases the cytotoxicity of cisplatin in a human large cell lung carcinoma cell line. 2. This effect of ATP is not mediated via GSH depletion, refuting previous reports [1; 2].

[1] J.M. Estrela et al. Nat Med 1 (1995) 84–8.
[2] T. Palomares et al. Melanoma Res 9 (1999) 233–42.

Radioprotective effects of ATP in human blood ex vivo

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Background. Damage to healthy tissue is a major limitation of radiotherapy treatment of cancer patients, presumably partly due to radiation-induced release of pro-inflammatory cytokines. The aim of the present study was to investigate the

protective effects of extracellular ATP on markers of oxidative stress, radiation-induced inflammation and DNA damage in irradiated blood ex vivo.
Methods. Blood from eight healthy volunteers was incubated with medium or 300 μM of ATP. Following a 30 min incubation period at 37 C, each blood sample was divided into two parts: one was exposed to 16 Gy (irradiated sample, at t=0); the other was not exposed to irradiation. Immediately (t=5 min), 3 h and 6 h after irradiation, samples were analyzed for oxidative stress markers, cytokines (ELISA) and DNA damage (Comet assay).
Results. ATP inhibited radiation-induced TNF-alpha release and increased IL-10 release. The inhibitory effect of ATP on TNF-alpha release was completely reversed by adenosine 5′-O-thiomonophosphate, indicating a P2Y11 mediated effect. Furthermore, ATP attenuated radiation-induced DNA damage immediately after irradiation, as well as 3 h and 6 h after irradiation.
Conclusion. Our study indicates that ATP administration alleviates radiation toxicity to blood cells by inhibiting radiation-induced inflammation and DNA damage.

P2X purinergic receptor expression and activity during neural progenitor differentiation: a possible cross-talk with the kallikrein-kinin system

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BACKGROUND: Neurotransmitters contribute to the generation of a functional nervous system. We have used neural progenitor cells to study expression and activity of P2X1–7 purinergic receptors along differentiation.
METHODS: Neural rat progenitor cells isolated on day 14 of embryonic development proliferated as neurospheres in the presence of EGF and FGF-2. The absence of these growth factors induced neurosphere differentiation into neural phenotypes. The progress of on-going differentiation was studied by immunofluorescence and flow cytometry analysis; while P2X receptor expression was verified by RT-PCR. Receptor functionality in differentiating neurospheres was observed by calcium-imaging.
RESULTS: Increasing expression of proteins, specific for oligodendrocytes, astrocytes and neurons, and a decrease in the number of nestin-positive cells were noted following induction of neurogenesis. While P2X1 and P2X5 subunits were not present at any stage of differentiation, expression levels of P2X7 and P2X4 subunits remained constant, and P2X2 subunit expression decreased along differentiation. The overall [Ca2+]i response to ATP increased during the course of differentiation, while no such variation was observed in the presence of Bz-ATP. Functional purinergic receptor responses in differentiated neurospheres were partially inhibited when cells had been differentiated in the presence of HOE-140, a specific inhibitor of kinin-B2 receptors.
CONCLUSION: The inhibition of purinergic receptor responses in neurospheres pre-treated with HOE-140 suggests that bradykinin and its receptor participate in neural differentiation as already shown for P19 embryonal carcinoma cells (Martins et al. J. Biol. Chem. 280, 19576, 2005). This is the first evidence for interactions between kinin and purinergic receptors in differentiating neurospheres.
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Role of adenosine receptors on the regulation of gastric ghrelin release

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Ghrelin is a potent orexigenic hormone predominantly released from the stomach. It plays an important role in inducing food intake and regulating blood glucose levels and energy expenditure. Abnormal ghrelin levels are associated with various eating disorders, obesity and diabetes. However, the mechanisms that regulate the release of ghrelin remain uncertain. Our laboratory has demonstrated that adenosine A1 and A2A receptors are involved in modulating the release of gastric regulatory peptides such as somatostatin and gastrin. Therefore, the objectives of the present study were to examine the

effect of adenosine on the release of ghrelin in the isolated vascularly perfused mouse stomach and to identify the localization and distribution of adenosine receptors in relationship to ghrelin-containing cells. Immunohistochemical results show that adenosine A1 receptors are present on ghrelin-containing cells in the mouse. Co-localization of adenosine A1 and A2A receptors and ghrelin were also observed in nerve fibres of the gastric muscle layers and the myenteric plexus. In the perfused mouse stomach, exogenous administration of adenosine (10 micromolar) stimulated ghrelin release, which was blocked by the A2A receptor antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM 241385; 1 micromolar). The selective adenosine A2A receptor agonist, 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680; 0.001 to 10 micromolar) concentration-dependently enhanced ghrelin release. In addition, exogenous administration of adenosine had no significant effect on ghrelin release in adenosine A2A receptor knockout mice (kindly provided by Drs. Chen, J-F & Schwarzschild, M). These results suggest that a novel adenosine-dependent mechanism is involved in the regulation of ghrelin release.

Knock down of P2X2 and P2X7 receptors expression in P19 murine embryonal carcinoma cells and NH15-CA2 neuroblastoma x glioma cells by RNA interference

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Background. P2X receptors are a family of ligand-gated cation channels activated by extracellular ATP and comprise seven subtypes (P2X1-7) involved in neurotransmission. Purinergic signaling has also been implicated in neuroprotection and early neuronal development. The aim of this study was to target P2X2 and P2X7 receptor expression in P19 embryonal carcinoma and NH15-CA2 neuroblastoma x glioma double hybrid cells by small-interfering (si)-RNAs, respectively.
Methods. Real Time-PCR and immunocytochemical staining was used to examine P2X2 and P2X7 receptor gene and protein expression in the absence and presence of siRNA constructs.
Results. Following 24 h of induction of RNA interference (RNAi) in the presence of siRNA-9 or siRNA-34, P2X2 receptor gene-expression was reduced by 64% or 42%, respectively. In the case of the P2X7 receptor (siRNA-2), the reduction of gene-expression was 88% in NH15-CA2 cells after 48 h. Immunocytochemical staining revealed decreases of 70% or 55% in receptor protein-expression in the presence of siRNAs-9 or -34, and 90% in the presence of siRNA-2. Expression profiling of P2X2 and P2X7 receptors was performed during in vitro neuronal differentiation of P19 cells. P2X2 receptor mRNA transcription increased during initial induction of the differentiation followed by a reduction of gene-expression until P19 cells became functional neurons. P2X7 receptors were only present in late differentiation when glial cells dominated the cell culture.
Conclusion. Using RNAi for inhibiting gene expression of P2X2 and P2X7 receptors during differentiation of P19 cells, we will be able to study the participation of these receptors in a model of early and late neuronal development.

P2X receptor-mediated contractile responses of isolated human uterus are increased with progression of the pregnancy

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The aim of the study was to test the hypothesis that in the human uterus, the effectiveness of P2 receptor-mediated contractile responses is up-regulated during pregnancy. Experiments were performed on myometrial samples obtained from women undergoing caesarean section at 28–30 weeks of pregnancy (3 women, Group 1), 32–34 weeks of pregnancy (6 women, Group 2) and 38–41 weeks of pregnancy (16 women, Group 3). Concentration-response relationships for a non-selective P2 receptor agonist, adenosine 5′-triphosphate (ATP), a selective P2X receptor agonist, alpha,beta-methylene-ATP (a,b-meATP), and a frequency-response relationship for non-adrenergic non-cholinergic (NANC) electrical field stimulation (EFS) were obtained using routine pharmacological organ bath technique. Effects of pyridoxalphosphate azophenyl-disulphonic acid (PPADS, 30

microM), a P2 receptor antagonist, were also evaluated. Parametric Student's t-test, non-parametric Wilcoxon T-test, Mann-Whitney U-test, two-way analysis of variance (ANOVA) and Krushkal-Wallis tests were used for statistical analysis. ATP (1–300 microM), a,b-meATP (0.1–30 microM) and EFS (2–32 Hz) evoked contractions of isolated pregnant uterus in all three groups. Uterus responses to ATP were not correlated with the term of pregnancy while the amplitude of uterine contractions to a,bmeATP and EFS was higher in full term pregnancy than in earlier pregnancy. PPADS antagonized uterus responses to a,b-meATP and EFS, but not to ATP, in all three groups. P2X receptor-mediated contractions of human pregnant uterus to a,b-meATP and EFS, but not to ATP, are increased with the progression of pregnancy.