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EXPRESSION OF PHOSPHODIESTERASE ISOENZYMES IN INFLAMMATORY CELLS IN ALLERGIC AIRWAY DISEASE

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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Phosphodiesterase (PDE) enzymes regulate levels of intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by hydrolysis to inactive monophosphates. This regulation is important, because cAMP and cGMP are involved in signalling cascades in all cells. When levels of cAMP, but not cGMP, are elevated in tissues a net anti-inflammatory effect is observed, and inhibitors of cAMP hydrolysing PDE enzymes types 4, 7 and 8 are, therefore, currently considered a therapeutic strategy in asthma. This thesis examines the expression of PDE families in inflammatory cells in patients with allergic asthma, compared with atopic non-asthmatic and normal subjects. The functional effects of PDE inhibitors and cAMP elevating agents on the production and release of tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1), key regulators of proteolytic cascades and fibrinolysis, were investigated in primary human bronchial epithelial cells.

This thesis has investigated the mRNA expression of the PDE4, PDE7 and PDE8 isoforms using RT-PCR, and their contribution to cAMP hydrolysing activity by the scintillation proximity assay (SPA). Western blotting and immunohistochemistry were used to investigate protein expression of PDE4 isoforms, and their specific localisation within cells. PAI-1 and tPA levels in unstimulated normal bronchial epithelial cell culture supernatants were measured by enzyme linked immunosorbent assay (ELISA).

The results showed mRNA for the PDE4 isoenzymes to be differently expressed between the inflammatory cells, with individual cell types having differing profiles. The presence of PDE7 mRNA in eosinophils and PDE8 mRNA in PBMC samples are novel findings. Immunohistochemical analysis revealed PDE4A, PDE4B and PDE4D staining was predominantly in the epithelium of bronchial biopsies from the airways, and these isoforms were demonstrated to have unique intracellular distribution within primary bronchial epithelial cells. The cAMP hydrolysing PDE activity, and its protein expression, were uniquely distributed and expressed within different members of the inflammatory cells. This suggests that different inflammatory cells regulate specific responses, by tailoring expression and distribution of the PDE enzymes. A PDE4 inhibitor, rolipram, and a β agonist, salbutamol, significantly altered the molar ratio of tPA and PAI-1 produced by primary epithelial cells, suggesting that therapeutic strategies that modify intracellular cAMP may be important in containing the tissue remodelling response in asthma.

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
APRT	Adenine phopshoribosyltransferase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AEC	Aminoethylcarbazole
AKAP	A kinase anchoring proteins
ATP	Adenosine triphosphate
BEGM	Basal epithelial growth medium
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CBP	CREB binding protein
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CPM	Counts per minute
COOH	Carboxyl
COX	Cyclooxygenase
CNS	Central nervous system
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
CREM	Cyclic AMP responsive element modulator

DAB	Diaminobenzidine
db cAMP	Dibutyryladenosine 3` 5` cyclic monophosphate
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphates
DTT	Diothiothreitol
EAR	Early asthmatic response
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EGF-R	Epidermal growth factor receptor
EPO	Eosinophil peroxidase
EDTA	Etheylene diaminetetraacetic acid
EGF	Epidermal growth factor
EHNA	Erythro-9-[2-hydroxyl-3-nonyl] adenine
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
FCS	Foetal calf serum
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FMLP	Formyl-methionyl-leucyl-phenylalanine
GDP	Guanosine diphosphate
G3PDH	Glyceraldehyde 3 phosphate dehydrogenase
GMA	Glycol methacrylate
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Guanosine monophosphate

GTP	Guanosine triphosphate
HARBS	High affinity rolipram binding site
15 HETE	15-Hydroxyeicosatetraenoic acid
HBSS	Hank`s balanced salt solution
HEPES	N-[hydroxyethyl]piperazine-N[2-ethanesulphonic acid]
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
IBMX	Isobutylmethylxanthine
IC_{50}	Inhibitory concentration 50%
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IGF	Insulin like growth factor
Km	Michaelis` constant
IL	Interleukin
INF γ	Interferon gamma
LAR	Late asthmatic response
LT	Leukotriene
MBP	Major basic protein
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NH_2	Amino

NO	Nitric oxide
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC_{20}	Provocative concentration of drug, giving a 20% drop in FEV_1
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PGE	Prostaglandin E
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
RACK	Receptor for activated C kinase
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SPA	Scintillation proximity assay
TBS	Tris buffered saline
TCR	T cell receptor
TES	N-tris[hydroxymethyl]methyl 2 aminoethansulfonic acid
TGF β	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF α	Tumour necrosis factor alpha

tPA	Tissue plasminogen activator
TRITC	Tetramethylrhodamine
tRNA	Transfer ribonucleic acid
TSH	Thyroid stimulating hormone
UCR	Upstream conserved region
uPA	Urokinase like plasminogen activator
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Asthma

One in seven children and one in twenty five adults in Britain today are reported to suffer from asthma. This disease affects 3.5 million of the British population (National Asthma Campaign 2000). In spite of advances in treatment and increases in prescribing of various therapies, a recent report has shown that the incidence of allergic asthma in an adult Scottish population has doubled over the last two decades (Upton et al. 2000). It is likely that this study is indicative of a general increase in allergic asthma in the population of Britain.

Asthma is characterised by a combination of the symptoms of chest tightness, wheezing, and dyspnoea. These are the result of reversible airway narrowing and bronchial hyperresponsiveness to an array of different and unrelated stimuli such as allergens, cold air or sulphur dioxide (reviewed by Coward et al. 1998). Asthma is not considered a single disease entity as many variants occur. These variants include atopic asthma, brittle asthma, intrinsic asthma, aspirin intolerant asthma, occupational non-IgE dependent asthma and cough variant asthma (reviewed by Holgate, 1999).

Asthma was initially thought to be due to bronchoconstriction solely; however, it is now appreciated that a more insidious bronchial inflammation underlies this disease (reviewed by Kay, 1991). The inflammatory process in asthmatics has devastating effects on the airways, and clearly contributes to asthma deaths. Post mortems reveal macroscopic evidence of mucus plugs blocking the asthmatic airways and massive oedema of the airway walls. Microscopically there is evidence of large areas of bronchial epithelial desquamation, eosinophil and neutrophil infiltration of both the bronchial submucosa and mucosa, mucous gland hyperplasia and thickening of the subepithelial lamina reticularis (reviewed by Holgate, 1993). These observations, to a lesser extent, have all also been observed in

existing asthmatics by analysis of bronchial tissue obtained at broncoscopy (Djukanovic et al. 1990).

A sub-group of patients with allergic asthma experience two distinct phases of airway narrowing in their exacerbations following exposure to a specific allergen. An early asthmatic response (EAR) is observed, which consists of smooth muscle contraction leading to bronchoconstriction (Holgate, 1993). The release of histamine from activated mast cells is responsible for this rapid narrowing of the airways, which occurs 5-10 minutes after allergen exposure. The hypersensitivity observed in asthmatics is thought to relate to the sensitivity of subepithelial nerve endings which are exposed as a result of bronchial epithelial shedding (Torphy et al. 1994). This epithelial shedding, itself, is the result of destructive mediator release from activated eosinophils. The late asthmatic response (LAR) occurs 4-6 hours later and is characterised by airway oedema which until recently was attributed to inflammatory mediator release from eosinophils and basophils. However, recent studies (Leckie et al 2000) have questioned the dominance of the eosinophil in this response, as discussed in section 1.2.1.3. Leukotriene C_4 (LTC_4), released from activated eosinophils in the submucosa, is both a smooth muscle spasmogen and a stimulus for mucus hypersecretion and plasma exudation (Holgate, 1993). All of these responses potentially contribute to the narrowing of the airways observed in the LAR.

Whilst the airway narrowing observed in the EAR and LAR is largely reversible, many asthmatics exhibit an underlying thickening of the airway wall. This thickening is the result of subepithelial fibrosis with increased deposition of fibronectin and collagen types 3 and 5 in the lamina reticularis together with smooth muscle cells hyperplasia (reviewed by Calhoun and Liu, 1995; Bousquet et al. 2000).

Current asthma therapy aims to relieve bronchoconstriction and inhibit the inflammatory response. Bronchodilators include the β_2 adrenoceptor agonists, such as salbutamol and salmeterol, which are widely used in asthma treatment. The bronchodilatory activity of β_2 agonists is mediated through β_2 adrenoceptors on smooth muscle cells, which initiate the cyclic adenosine monophosphate (cAMP) cascade and electrophysiological changes which lead to smooth muscle relaxation (reviewed by Barnes, 1995a). β_2 agonists have little anti-inflammatory efficacy and are often used in conjunction with corticosteroids, which have a broad spectrum of anti-inflammatory actions. Increased cyclic adenosine monophosphate (cAMP) levels within inflammatory cells have been demonstrated *in vitro* to inhibit T cell chemotaxis, eosinophil degranulation and histamine release from basophils and mast cells (Hidi et al. 2000; Momose et al. 1998; Weston et al. 1997). These anti inflammatory actions of cAMP clearly make it a target for modulation in asthma therapy.

1.2 Role of inflammatory cells in asthma

1.2.1 Eosinophils

These cells were first described over 100 years ago by Erhlich who remarked upon their ability to bind acidic dyes such as eosin. Eosinophils possess a strong negative charge as a consequence of many of their proteins being composed largely of basic amino acids, especially arginine. This therefore gives this cell a strong affinity for acidic dyes such as eosin (reviewed by Wardlaw et al. 1997). In humans, eosinophils constitute between 2-10% of the peripheral leukocytes and have a half life in the blood of around 18 hours (reviewed by Giembycz and Lindsay, 1999).

1.2.1.1 Morphological features

The morphological characteristics of eosinophils are a bi-lobed nucleus filled with partially condensed chromatin and many electron dense granules (Giembycz and Lindsay, 1999). Eosinophils display heterogeneity and there are three distinct populations. The first is normodense resting, unprimed eosinophils and these are normally the largest population. Secondly, hypodense eosinophils which are of relatively light buoyant density, and their numbers are increased in asthma (Fukada et al. 1995). The third category are referred to as primed eosinophils that respond to normally inactive or very weak stimuli (Giembycz and Lindsay, 1999).

Within these eosinophils there are four different types of granules, which differ in their number and composition. Specific granules have an obvious crystalloid core composed of major basic protein (MBP). This core is surrounded by a matrix containing eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO) and cathepsin. Cytokines are also resident within this matrix and they include tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and RANTES (reviewed by Gleich and Loegering, 1984; Giembycz and Lindsay, 1999). Small granules which contain arylsulphase B, elastase, acid phosphatase and possibly catalase are found in tissue eosinophils. Primary granules present in eosinophils house the Charcot-Leyden protein which acts as a lysophospholipase (reviewed by Kroegel et al. 1994a). The fourth type of eosinophil granule are lipid bodies that contain a core of arachidonic acid together with the enzymes cyclooxygenase (COX) and leukotriene C_4 synthase (Giembycz and Lindsay, 1999). The number of lipid bodies is low in unprimed eosinophils and increased numbers have been recorded in eosinophils from asthmatics (Giembycz and Lindsay, 1999).

Eosinophils are known to possess a whole host of receptors upon their cell surface. These include β_2 adrenoceptors, histamine H_1, H_2 and H_3 receptors, adenosine A_2, bradykinin and endothelin receptors. Receptors are also present for PAF, LTB_4, formyl-methionyl-leucyl-phenylalanine (FMLP), IL-8, RANTES, eotaxin, substance P and complement fragments (reviewed by Kroegel et al. 1994a; Giembycz and Lindsay, 1999).

1.2.1.2 Biological properties

The properties of proteins found in eosinophil granules elucidate why these cells are such potent destroyers of bacteria and helminths, which is their primary biological role. MBP is a potent helminthotoxin that damages cells through charge interactions at their membranes, thus increasing their permeability. This protein is also able to cause the degranulation of basophils. ECP, like MBP, is also a helminthotoxin which exerts its effects by creating pores in target cells. ECP promotes mast cell degranulation and is also a weak ribonuclease. EPO in conjunction with peroxidase and halide ions can form the hugely destructive hypohalous acids and highly reactive oxygen singlet. Finally, EDN is a potent ribonuclease (Giembycz and Lindsay, 1999).

Eosinophils are capable of releasing many destructive free oxygen radicals, generated by the actions of a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme catalyses the production of superoxide ions from oxygen, and these superoxide ions can then be converted into hydrogen peroxide by superoxide dismutase. As discussed above, EPO together with this hydrogen peroxide and bromide is able to form the highly destructive hypobromous acid. Finally hydrogen peroxide in combination with superoxide and ferrous ions is able to produce hydroxyl radicals. Numerous lipid mediators and cytokines are also synthesised or released from eosinophils. These include PAF, LTC_4,

PGE_2, IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, TNF-α, GM-CSF, RANTES, eotaxin and transforming growth factor beta (TGF-β) (reviewed by Wardlaw et al. 1997).

1.2.1.3 Role in asthma

The granular proteins, MBP, EPO and ECP, are thought to be responsible for much of the epithelial damage and shedding characteristic of asthma. In fact, the eosinophil is hypothesised to have a fundamental role in the pathology of asthma, and asthmatic patients exhibit increased numbers of eosinophils in their blood, sputum, bronchoalveolar lavage (BAL) and bronchial biopsies (Djukanovic et al. 1992). There is also a strong correlation between markers of eosinophil activation, for example ECP release, and asthma severity (reviewed by Wardlaw and Kay, 1987). However there is some conflicting evidence to question the importance of eosinophils in the asthmatic response. Leckie et al. (2000) found that whilst a single dose of a monoclonal antibody against IL-5 was able to suppress circulating eosinophil numbers in allergic asthmatic patients. There was no corresponding protection from an allergen induced late asthmatic response or bronchial hyperresponsiveness (Leckie et al. 2000). These investigators suggested that the lack of response may be due to the involvement of several cell types in the late asthmatic response and a certain degree of redundancy within this.

Eosinophils are recruited into the airways by mediators produced by the epithelium, T cells and macrophages. For example, RANTES, eotaxin, C3a, LTB_4 and PAF may play a role in eosinophil recruitment (Shahabuddin et al. 2000). Additionally eosinophil numbers in the submucosa are increased by cytokines such as IL-3, IL-5 and GM-CSF, which inhibit eosinophil apoptosis and prolong their survival (Wardlaw 1993). Activation of eosinophils present in the airways by stimuli, such as IgA, C5a and PAF, induces release of the granule

associated basic proteins and also reactive oxygen metabolites such as superoxide anions, with the subsequent formation of hypohalous acids. These are all highly toxic to the epithelium and cause significant damage and epithelial shedding. Once the epithelium has been shed, airway nerves are exposed to stimulation through agents such as bradykinin, resulting in the characteristic bronchial hyperresponsiveness observed in asthmatics (reviewed by Kroegel et al. 1994b; Gleich, 1990).

1.2.2 Neutrophils

Neutrophils constitute half of the circulating white cell population and have a half life in the blood of only 4-6 hours (reviewed by Hellewell and Henson, 1993).

1.2.2.1 Morphological features

Neutrophils are characterised by their polymorphic nucleus and cytoplasmic granules (Hellewell and Henson, 1993). They contain an armoury of proteases including elastase, gelatinase, collagenase and cathepsin G, and the enzymes lysozyme, myeloperoxidase and β glucoronidase (reviewed by Haslett and Chilvers, 1997). Neutrophils are end stage cells and, as a consequence, their ability to synthesise mediators is limited; however, the mediators that they do generate are IL-1, TNF-α, IL-8, IL-6, IL-12, GM-CSF, LTB_4 and PAF. Neutrophils are also able to produce a respiratory burst that leads to superoxide and hydrogen peroxide generation. These in turn can produce hypohalous acids if combined with halide ions in the presence of myeloperoxidase (reviewed by Church, 1999). Receptors for potent neutrophil chemoattractants such as C5a, LTB_4, PAF, FMLP and IL-8 are expressed on the cell surface of neutrophils (Hellewell and Henson, 1993).

1.2.2.2 Biological function

Neutrophils are the primary defence against invading bacteria and other pathogens, which they phagocytose, and their impressive array of proteases detailed above allows them to fulfil this role (reviewed by Wardlaw, 1993). Neutrophils must be able to rapidly gain access to the site of infection and their range of receptors for chemoattractants allows them to migrate easily through the endothelium and into various tissues.

1.2.2.3 Role in asthma

Although the role of neutrophils in asthma is a contentious subject, there is considerable evidence to support a role for these cells in severe asthma (Fahy et al. 1995). Studies have shown that neutrophils comprise a major proportion of the inflammatory cells observed in sputum from asthmatics in acute exacerbation. This neutrophil accumulation may be attributed to respiratory tract infections, which are often a precipitant of acute episodes of asthma (Fahy et al. 1995). This study also proposed that mucus hypersecretion, characteristic of acute asthma, may be due in part to increased levels of neutrophil derived mediators. These cells are recruited in response to high levels of IL-8, and the subsequent release of neutrophil elastase stimulates submucosal and goblet cell mucus secretion. There are no reports of increased numbers of neutrophils in bronchial biopsies from asthmatic patients in comparison to normals. Therefore the role they play in asthma is still unclear.

1.2.3 T Lymphocytes

T lymphocytes are cells that directly recognise and respond to processed antigen. These cells can be classified into two distinct groups, CD4+ and CD8+ cells. A simplistic definition of these groups is that CD4+ (or helper cells) assist B cells in antibody production and produce cytokines, whereas, CD8+ cells (or suppressor cells) are protective against

viral infections and suppress antibody production (reviewed by Wardlaw, 1993). CD4+ and CD8+ can be further subdivided into Th1 and Th2 clones. Whilst Th1 cells produce IFN-γ and TNF-β and are thought to confer protection against intracellular parasites, Th2 cells release IL-4, IL-5 and IL-10 and confer protection against extracellular parasites as well as participating in inflammatory responses (reviewed by Essayan, 1997c). The division of T cells into these two Th1 and Th2 types is again somewhat simplistic in humans, as evidence has shown that both Th1 and Th2 cells are capable of secreting IL-2, IL-3, IL-10, IL-13 and GM-CSF (reviewed by Borish and Rosenwasser, 1997). However, cells do show Th1 or Th2 like responses so this definition continues.

T cells are thought to orchestrate many processes observed in the asthma phenotype. Firstly the generation of the Th2 cytokine, IL-5, serves to promote terminal differentiation of eosinophils. IL-5 also enhances eosinophil activation and survival time (reviewed by Kay, 1998). As mentioned in section 1.2.1 eosinophils, through the release of their mediators, cause much of the tissue damage observed in the lungs. The release of IL-4 from T cells is also able to promote class switching to the production of IgE by B cells (reviewed by Wardlaw, 1993). High levels of IgE are characteristic of atopy and asthma and, when bound to mast cells, allow degranulation upon an antigen trigger. Although there are no reports of increased numbers of T cells in the airways of asthmatics, there are reports of increased numbers of CD25+ (activated) T cells in the bronchial mucosa of asthmatics (Azzawi et al. 1990).

1.2.4 Monocytes

Monocytes constitute between 1-6% of the white blood cell count and have a half life in the circulation of 3 days. They circulate in the blood and migrate into tissues, where many

undergo differentiation into macrophages. Monocytes function effectively as antigen presenting cells. They are able to rapidly internalise particulate and soluble ligands and express antigens of the major histocompatibility complex (MHC II). The cross linkage of the low affinity receptor for IgE (FcεRII) on their cell surface, by IgE immune complexes, triggers the release of numerous cytokines, including PAF, leukotrienes, TNF-α, GM-CSF, IL-2 and IL-8. Monocytes, like eosinophils and neutrophils, are also able to generate superoxide ions, hydrogen peroxide and hydroxyl radicals (reviewed by Church, 1999).

1.2.5 Bronchial epithelial cells

1.2.5.1 Morphological features

The bronchial epithelium provides an important barrier between the underlying bronchial tissue and the external environment. This epithelium consists of columnar and basal cells that rest upon an underlying `true' basement membrane, which is composed of the lamina rara and the lamina densa, (reviewed by Calhoun and Liu, 1995), composed of collagen type 4, laminin, entactin and proteoglycans. Underlying this is the lamina reticularis and lamina propria, which also contain collagens, type 3 and 4, elastin, fibronectin and many proteoglycans.

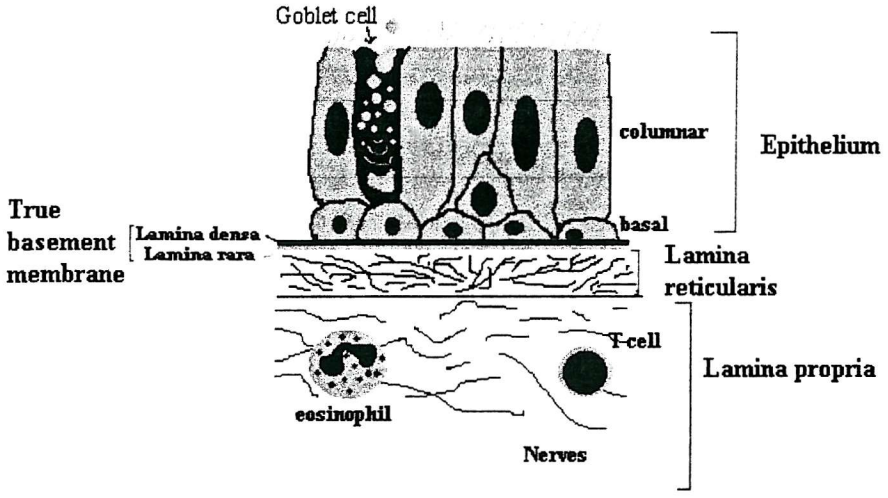


Figure 1.1 Pictorial representation of bronchial epithelium.

Interactions of the basement membrane and lamina reticularis with the epithelial cells regulate numerous cellular processes. The basement membrane is able to influence the gene expression, morphology, motility, cell cycle status and epithelial responses to external stimuli such as growth factors. In turn, the epithelium is able to regulate the status of the extracellular matrix (ECM) through secreting fibronectin and collagen or matrix metalloproteinases (MMPs) that alter its synthesis or degradation respectively (reviewed by Ashkenas et al. 1996). Interactions between the epithelial cells and ECM are thought to be mediated largely through receptors on the basal surface of the epithelium that form contacts with elements of the basement membrane. The most widely known of these receptors are the integrins. These molecules are expressed on the basal surface of epithelial cells and interact intracellularly with cytoskeletal structures. Integrins, for example $\alpha_2\beta_1$, also interact extracellularly with components of the ECM, for example laminin or collagen (reviewed by Ashkenas et al. 1996). There is huge diversity in the integrin family and, through this, the ECM is able to control specific epithelial processes such as motility. The ECM can alter cell shape through integrins, such as $\alpha_3\beta_1$and $\alpha_2\beta_1$, that bind to intracellular structural proteins, such as α actinin or talin. The extracellular matrix can also alter nuclear events through the ability of integrins to assemble cytoskeletal frameworks, which allow the components of the intracellular signalling machinery to interact (reviewed by Damsky and Werb, 1992).

The interactions of epithelial cells with mediators are also controlled via the ECM. For example, growth factors, such as fibroblast growth factor (FGF-2), can be sequestered in the ECM, by binding to heparan sulphate side chains of proteoglycans where they are unable to interact with receptors on the epithelial cells. Integral proteins of the ECM, such as fibronectin, can also regulate functions of the epithelium. The size of fibronectin, whether

intact or present in fragments, also alters its effects on epithelial functions. The glycoproteins and proteoglycans that make up the ECM create a strong meshwork that supports the epithelium, and also acts as a sieve for infiltrating inflammatory cells (reviewed by Damsky and Werb, 1992). The structural state of the ECM, therefore, will profoundly influence the epithelium through alterations in any of the above mentioned mutual control mechanisms.

1.2.5.2 Biological properties

Epithelial cells form the primary barrier between the environment and the underlying bronchial tissue (reviewed by Cohn et al. 1997). They have numerous functions, some of which serve to protect against, and others contribute to, inflammation. The epithelium acts as a defence for underlying cells in a number of ways. The ciliated apical surface of the columnar cells, in combination with mucous produced by the goblet cells, form a protective layer which is swept upwards removing potentially harmful substances, and preventing their contact with underlying cells. The epithelium, by virtue of tight junctions, limits the infiltration of viruses and bacteria and molecules from the environment to the underlying tissues. The epithelium also metabolises arachidonic acid to produce prostaglandin E_2 (PGE_2), which is a potent bronchodilator (reviewed by Jacoby, 1997). Several enzymes found in the epithelium have immunoprotective properties. These include neutral endopeptidase and histamine-N-methyl transferase, both of which degrade potent bronchoconstricting agents. Whilst endopeptidase can degrade tachykinins, bradykinin and enkephalins, histamine-N-methyl transferase can degrade histamine (Jacoby, 1997). Protease inhibitors are also produced by the epithelium and these include elafin, cystatin C, α1-antiprotease, α1-antichymotrypsin and secretory leukocyte protease inhibitors. These

protease inhibitors can inhibit tissue damage associated with the actions of elastase, cathepsin B and G and mast cell chymase (reviewed by Polito and Proud, 1998).

The epithelium can also contribute to and enhance inflammation within the bronchial tissue. It can produce endothelin and $PGF_{2\alpha}$, both of which are bronchoconstrictors (Jacoby, 1997). Epithelial cells convert arachidonic acid via the 15 lipoxygenase pathway to 15-hydroxyeicosatetraenoic acid (15-HETE), which is a proinflammatory molecule acting on mast cells (Polito and Proud, 1998). Potent chemoattractants IL-8, GROα (growth-related oncogene), GROγ, eotaxin, monocyte chemoattractant protein 1 and RANTES are also produced by the epithelium and cause increased inflammatory cell infiltration. Granulocyte-macrophage colony stimulating factor (GM-CSF) is produced by the epithelium and enhances activation of eosinophils and neutrophils, as well as prolonging their survival (Jacoby, 1997). Several proinflammatory cytokines, including IL-6, IL-11 and IL-16, are produced by the epithelium (Jacoby, 1997; Cohn et al. 1997). Finally, transforming growth factor β (TGF-β) is released from epithelial cells, which stimulates mucin secretion, endothelin secretion and epithelial migration (Cohn et al. 1997).

Nitric oxide (NO), produced by the epithelium, can be argued to have proinflammatory and anti-inflammatory actions. It potentiates inflammation, by increasing plasma leakage, and interacts with superoxide anion to form peroxynitrite, a tissue damaging radical. However, nitric oxide can act as a bronchodilator, causing bronchial smooth muscle relaxation via cGMP. Nitric oxide is also speculated to have antiviral actions and it is able to nitrosylate viral molecules, for example reverse transcriptase and nucleocapsid proteins, thus effecting the virus lifecycle (Polito and Proud, 1998, Colasanti et al. 1999).

1.2.5.3 Role in asthma

In bronchial biopsies from asthmatics there is evidence of thickening of the lamina reticularis, resulting from excess fibronectin and collagen type 3 and 5 deposition in the ECM. Subepithelial fibrosis is thought to be the result of a dysregulated repair system that fails to respond normally to the chronic inflammation seen in asthmatics. Asthmatics exhibit increased sensitivity to repeated insults to the epithelium from allergens and the environment compared to normal subjects. The process of inflammation and excessive repair is referred to as airway remodelling (reviewed by Kips and Pauwels, 1999).

Injury to the bronchial epithelium and underlying tissue in asthma is often manifested as epithelial denudation (Jacoby, 1997). This initial injury is mediated by air pollutants, viruses, allergen derived proteases or inflammatory cell derived mediators (Holgate et al. 1999). The injured epithelium is also capable of proinflammatory actions. It can produce inflammatory cell chemokines, such as IL-8, eotaxin, IL-16 and RANTES, which attract T cells, eosinophils and neutrophils (Holgate et al. 1999). Epithelial cells can also produce 15HETE, which activates the lipoxygenase pathway in mast cells, PAF which increases vascular permeability and endothelin which stimulates mucus secretion (Holgate et al. 1999). Initial tissue injury results in an inflammatory response, including increased microvascular permeability and oedema, which aids in the migration of inflammatory cells to the site of injury (Roman, 1996). The ECM has an important role in inflammatory cell migration; it can act as a map guiding cells towards the site of injury. This is mediated by the association of chemoattractants, for example the chemokines, with proteoglycans within the ECM in an increasing concentration gradient (Kips and Pauwels, 1999). The controlled proteolysis of components of the ECM by inflammatory cell proteases, such as MMP-9, is believed to have a permissive role in passage of cells through the tissue. Molecules within

the ECM can also aid in priming and activating these inflammatory cells as they migrate into the tissue. For example GM-CSF, a non fibrogenic growth factor, is activated upon its proteolytic release from the ECM, and can stimulate mediator release from eosinophils, neutrophils and macrophages (Polito and Proud, 1998; Kips and Pauwels, 1999). Structural elements of the ECM can also participate in cell activation, for example fibronectin is able to initiate eosinophil degranulation and mediator release from basophils (Kips and Pauwels, 1999). The ECM, therefore, through numerous functions, has an important role in inflammation. Inflammatory cells contain an armoury of proteases and tissue damaging agents, which include tryptase and chymase from mast cells, elastase from neutrophils, ECP, MBP, EPO and matrix metalloproteinase 9 (MMP 9) from eosinophils. The generation of oxygen free radicals, including superoxide ions, hydrogen peroxide, hypobromous acid and hypochlorous acid from activated eosinophils, neutrophils and macrophages, all contribute to further tissue damage (Woessner, 1991; Holgate et al. 1999).

This tissue injury, and resulting inflammation, is swiftly followed by repair processes. The first stage of this repair is the formation of an essential fibrin meshwork that allows cell accumulation at the site of injury (Idell et al. 1994). Cells that infiltrate this fibrin meshwork include fibroblasts, monocytes and epithelial cells. Once inside the meshwork, fibroblasts and epithelial cells are stimulated to proliferate by agents, such as histamine, tryptase and the growth factors, FGF-2, platelet derived growth factor (PDGF), insulin like growth factor (IGF) and epidermal growth factor (EGF) (Holgate et al. 1999; Idell et al. 1994; Kips and Pauwels, 1999). A major function of these fibroblasts is their production of the ECM components collagen and fibronectin, and this is stimulated by transforming growth factor β (TGF β) histamine and tryptase (Holgate et al. 1999). Monocytes also infiltrate the wound site where they differentiate to become activated macrophages, which

produce TGF-β, tumour necrosis factor alpha (TNF-α) and the growth factors PDGF and vascular endothelial growth factor (VEGF) (Singer and Clark, 1999). In the process of repair, epithelial cells migrate towards the site of injury, where they are stimulated to proliferate by histamine, tryptase, IGF and EGF (Holgate et al. 1999; Kips and Pauwels, 1999). Epithelial cells are stimulated by TGF-β to produce ECM components; for example fibronectin and collagens type 1 and 3 (Kips and Pauwels, 1999). The ECM proteins tenascin and entactin, together with the growth factors EGF, TGF-β and basic fibroblast growth factor (bFGF) or FGF-2, are also produced by the epithelial cells (Polito and Proud, 1998; Holgate et al. 1999). The proliferation of fibroblasts and their secretion of ECM components, serves to repair the tissue injury by the deposition of a new ECM and the proliferation of epithelial cells completes the repair.

The initial fibrin meshwork necessary for the early stages of repair must be degraded, and this is mediated by the protease plasmin. Plasmin is produced by the proteolytic cleavage of its inactive precursor plasminogen, which is produced in the liver, and freely circulates in the plasma, passing into the tissue due to increased microvascular permeability as a result of injury. The cleavage of plasminogen to form active plasmin is mediated by tissue plasminogen activator (tPA), that is produced in the endothelium and urokinase like plasminogen activator (uPA), which is produced by fibroblasts and macrophages as well as inflammatory cells (Elhasade et al. 1998). The provisional ECM formed at the injury site also requires remodelling for normal tissue repair, and this is partly achieved by the ability of plasmin to degrade fibronectin, laminin and thrombospondin, but largely by the actions of the matrix metalloproteinases (MMPs) (Sitrin et al. 1992). The MMPs are a family of substrate specific endopeptidases whose members can degrade most components of the ECM (Kips and Pauwels, 1999). They are produced by macrophages, fibroblasts, epithelial

cells and endothelial cells as inactive precursors that often associate with components of the ECM (Singer and Clark, 1999; Woessner, 1991). Activation of these MMPs, and their release from the ECM is achieved by trypsin, plasmin or hypochlorous acid, produced by neutrophils (Woessner, 1991).

Remodelling mediated by fibrinolysis and proteolysis must be held in tight check, as excess lytic activity may result in further tissue damage or destruction. This is achieved by numerous specific protease inhibitors. The plasmin forming enzymes tPA and uPA, are regulated by the specific inhibitor plasminogen activator inhibitor 1 (PAI-1), which is also produced by the endothelium (Elhasade et al. 1998). Plasmin is itself inhibited by $\alpha 2$ antiplasmin and α_2 macroglobulin both found in the plasma (Elhasade et al. 1998). Finally, the MMP family is inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs), α_1-antitrypsin and α_2 macroglobulin (Kips and Pauwels, 1999). These inhibitors bind MMPs stoichiometrically in a non-covalent fashion (Woessner, 1991).

1.2.6 Mast cells and basophils

Mast cells are recognised by having a cytoplasm filled with large granules. These granules contain heparin and chondroitin sulphate. Mast cells contain an array of mediators, including histamine, tryptase, chymase, chondroitin sulphate, LTB_4, 5-HETE, PGD_2, LTC_4, TNF-α, IL-4 and IL-5 (reviewed by Wardlaw, 1993). Mast cells can be divided into two classes: MC_T mast cells, that contain only tryptase and are localised to mucosal surfaces, and MC_{TC} cells that contain tryptase and chymase and are localised to the submucosa and connective tissue (Irani et al. 1989). MC_T mast cells are increased in numbers in allergic disease, unlike MC_{TC} cells. Mast cells play an important role in the early response observed in asthma via release of histamine, PGD_2 and LTC_4. These agents increase vascular

permeability, leading to mucosal oedema, and also elicit smooth muscle contraction and increased mucus secretion (reviewed by Wassermann, 1985). Tryptase, the major mast cell protease, is also implicated in the pathogenesis of asthma (Church et al. 1997). Reports indicate that tryptase sensitises bronchial smooth muscle to contractile agents and also cleaves matrix components for example fibronectin and gelatinase. Tryptase increases the release of IL-8 from epithelium and is a mitogen for fibroblasts. All of the above actions of tryptase are pro-inflammatory and contribute to the inflammation and subsequent airway damage seen in asthma. Mast cell mediators for example histamine and tryptase, are released when the cells degranulate. This degranulation is triggered by the cross linkage of high affinity IgE receptors which occurs when antigens bind to them (reviewed by Church, 1999).

Basophils are mainly found in low numbers in the blood, and constitute less than 1% of circulating leukocytes. They have many similar properties to mast cells, including stores of histamine and proteoglycans. Basophils are distinguished from mast cells by the absence of tryptase and their lack of the low affinity IgE receptor. These cells do, however, express the high affinity IgE receptor, together with receptors for IL-3, IL-4 and GM-CSF. Basophils also generate tryptase, leukotrienes, IL-4 and IL-13, but do not synthesise PGD_2. Basophils exert many of the same effects as mast cells in asthma, however they are associated predominantly with the late allergic response and are responsible for the majority of histamine release at this time (reviewed by Church, 1999).

1.2.7 Macrophages

These cells result from the differentiation of blood monocytes, and have many similar properties. Macrophages are able to release several inflammatory mediators, including

LTB_4, TNF-α, C5a, platelet derived growth factor (PDGF) and PAF (reviewed by Lee and Lane, 1992).

Macrophages have an important role in asthma, and have been reported to be present in increased numbers in the bronchial mucosa from asthmatic subjects in comparison to normals (Chanez et al. 1991). The role of macrophages in asthma is two fold; firstly they act as antigen presenting cells, ingesting and then processing antigens that they then present to T cells. The second role of macrophages in asthma is the generation of large quantities of pro-inflammatory mediators, including PAF, TNF-α, leukotrienes, GM-CSF, IL-1, IL-6, IL-8 and NO. These mediators allow recruitment, prolonged survival, or activation of granulocytes and lymphocytes at the site of inflammation (Church, 1999)

1.2.8 Endothelial cells

Endothelial cells act as the barrier between blood and tissue and, as such, they are able to control the passage of cells and fluids into the tissues. These cells, like epithelial cells, have the capacity to generate both pro and anti-inflammatory mediators (Tonnel et al. 1997). Endothelial cells can release nitric oxide, which has bronchodilatory actions, and prostacyclin. However, they also have the capacity to generate proinflammatory mediators, including PAF, IL-8, RANTES, LTC_4 and LTD_4. Endothelial cells tightly control the migration of inflammatory cells into the tissue, and this is regulated through the expression of cellular adhesion molecules on their surface. These cell surface markers interact with adhesion molecules expressed on circulating inflammatory cells. The integrins ICAM-1 and 2, VCAM-1 and selectins, E-selectin and P-selectin, are expressed on endothelial cells, and bind to adhesion molecules expressed on inflammatory cells, to allow rolling, adhesion and finally migration into the inflamed tissue (reviewed by Tonnel et al. 1997).

Endothelial cells have an important role to play in asthma pathogenesis. Through increased permeability of the endothelium, inflammatory cells migration into the submucosa is increased. This increased permeability also enhances plasma leakage, which contributes to oedema (reviewed by Raeburn et al. 1993).

1.3 Genetic linkage

Although susceptibility to asthma is not solely defined by a genetic trait, there is clear evidence, through family and twin studies, to suggest a genetic component in this disease (Sandford et al. 1996). Much research has gone into investigating possible links between candidate genes that are known to code for traits or characteristics associated with asthma or atopy.

Chromosome 5q

This chromosome houses the coding regions for the β_2 adrenergic receptor and leukotriene C_4 synthase, a key enzyme in regulation of leukotriene C_4, D_4 and E_4 levels. Chromosome 5q also has coding regions for numerous of the proinflammatory cytokines linked with asthma and allergy. These include IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF which are involved in processes such as immunoglobulin class switching in B cells, basophil, eosinophil and mast cell maturation (reviewed by Bleecker et al. 1997)

Chromosome 6

Coding for tumour necrosis factor alpha (TNF-α), a potent proinflammatory cytokine, is found on this chromosome, together with coding for the human leukocyte antigen region (HLA) (reviewed in Bleecker et al. 1997)

Chromosome 11q

Genes coding for the β subunit of the high affinity IgE receptor (FcεRI), present on the cell surface of basophils and mast cells, are found on this chromosome (Sandford et al. 1993). This receptor is intimately involved in the allergen triggered release of histamine from these cells.

Chromosome 12q

This chromosome houses coding regions for interferon gamma (IFNγ), nitric oxide synthase and leukotriene A_4 hydrolase (reviewed by Bleecker et al. 1997).

Chromosome 14q

The T cell antigen receptor (TCR) and NFκB-1, an important signalling molecule, are found on this chromosome (reviewed by Bleecker et al. 1997).

Several studies have found evidence for links between polymorphisms of the above genes and the asthma/atopy phenotype. At present there is no evidence of a disease related gene for asthma, and it is unlikely to be attributed to a single gene. Work is ongoing, screening large patient populations, in an attempt to find further candidate loci.

Genetic location of the PDE4 genes.

The PDE4 family members (see below) are coded by genes found at distinct chromosomal locations. PDE4A is found at 19p13.2, 4B is coded by a region at 1p31, 4C at 19p13.1 and 4D at 5q12 (Houslay and Milligan, 1997). At present these genetic locations have not been implicated in any hypothesised linkage associated with the asthma phenotype (reviewed by Holgate, 1999).

1.4 Cyclic nucleotides

Cyclic AMP and cyclic guanosine monophosphate (GMP) are important intracellular second messengers that are involved in signalling cascades in nearly all cells of the body. Cyclic AMP is formed by the action of adenylyl cyclase on adenosine tri-phosphate (ATP), and is hydrolysed by the cAMP phosphodiesterases (PDE).

When levels of intracellular cAMP are elevated, there is thought to be a net anti-inflammatory effect in many cells. For this reason, agents that control levels of cAMP in cells and tissue are of interest when considering therapeutic strategies in asthma (Torphy, 1998; Houslay and Milligan, 1997). There is little evidence to suggest that cGMP is an important mediator in control of inflammation, however it has peripheral roles such as contributing to airway smooth muscle relaxation, mechanism of action described in section 1.9, (Diamond 1993). It is the cAMP modulating phosphodiesterases that are found predominantly in inflammatory cells and are therefore examined in this thesis (Torphy, 1998; Berridge, 1985).

There are up to nine different mammalian forms of adenylyl cyclase, and their activity is regulated by many G protein linked receptors for hormones, neurotransmitters and growth factors (reviewed by Houslay and Milligan, 1997). For example, the activation of adenylyl cyclase is mediated through the A_2 adenosine receptors, β_1,β_2, β_3 adrenoceptors, EP_1 and EP_2 prostanoid receptors and histamine H_2 receptors (Dent et al. 1994b). Once activated, adenylyl cyclase converts ATP to cAMP, leading to an increase in intracellular cAMP. This increase in cAMP causes activation of protein kinase A (PKA), an enzyme which phosphorylates a wide range of intracellular proteins, leading to a whole host of biological responses (reviewed by Berridge, 1985). A schematic representation of this cascade is

shown in figure 1.2. Through this cAMP signalling pathway, control is imposed on gluconeogenesis, glycogenolysis, lipogenesis, muscle contraction, apoptosis and ion channel conductance to name but a few cellular responses (Houslay and Milligan, 1997).

Protein kinase A is composed of four subunits, two catalytic (C) and two regulatory (R) units. Following binding of cAMP to the R units these dissociate from the C units. The C units subsequently become active and phosphorylate threonine or serine residues on target proteins. There are two forms of PKA; PKA I, which is mainly cytosolic in cells, and PKA II which is mainly particulate. The subcellular localisation of these PKA forms requires them to be present in the same subcellular location as newly generated cAMP, and may serve to limit their ability to be activated by cAMP (Lewis et al. 1996).

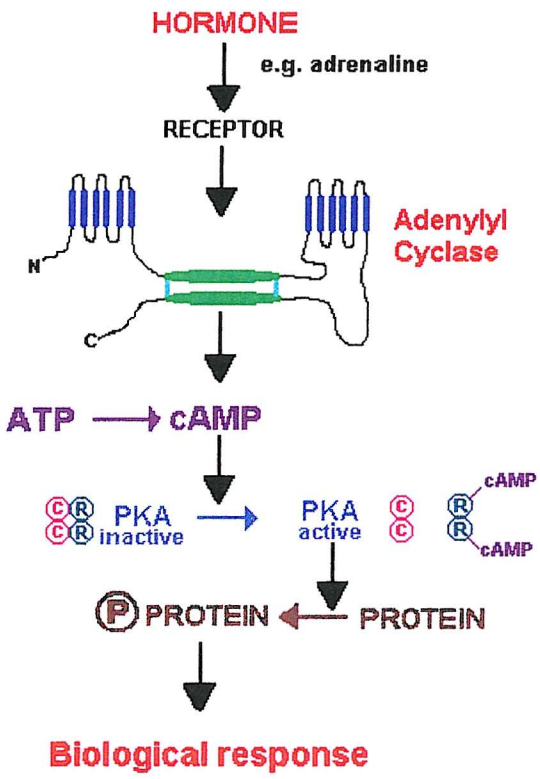


Figure 1.2 Schematic representation of the cAMP signalling cascade.
A hormone, e.g. adrenaline, or a drug, e.g. salbutamol, interacts with a specific β_2 adrenoreceptor, thus activating adenylyl cyclase. Activation of adenylyl cyclase initiates cAMP production, which in turn activates protein kinase A (PKA). PKA can then phosphorylate a host of proteins (adapted from Houslay and Milligan, 1997).

1.5 The phosphodiesterase enzymes

Sutherland and Rall first reported the presence of cyclic nucleotide hydrolysing enzymes in tissue extracts in the late 1950s (Sutherland and Rall, 1957). Phosphodiesterase enzymes cleave cyclic nucleotides by hydrolysis of phosphodiester bonds (Torphy, 1998). There are, at present, eleven known members of the phosphodiesterase family that have differing affinities for their substrates, which are either adenosine 3',5' cyclic monophosphate (cAMP) or guanosine 3',5' cyclic monophosphate (cGMP). The hydrolysis of these substrates is illustrated in figure 1.3

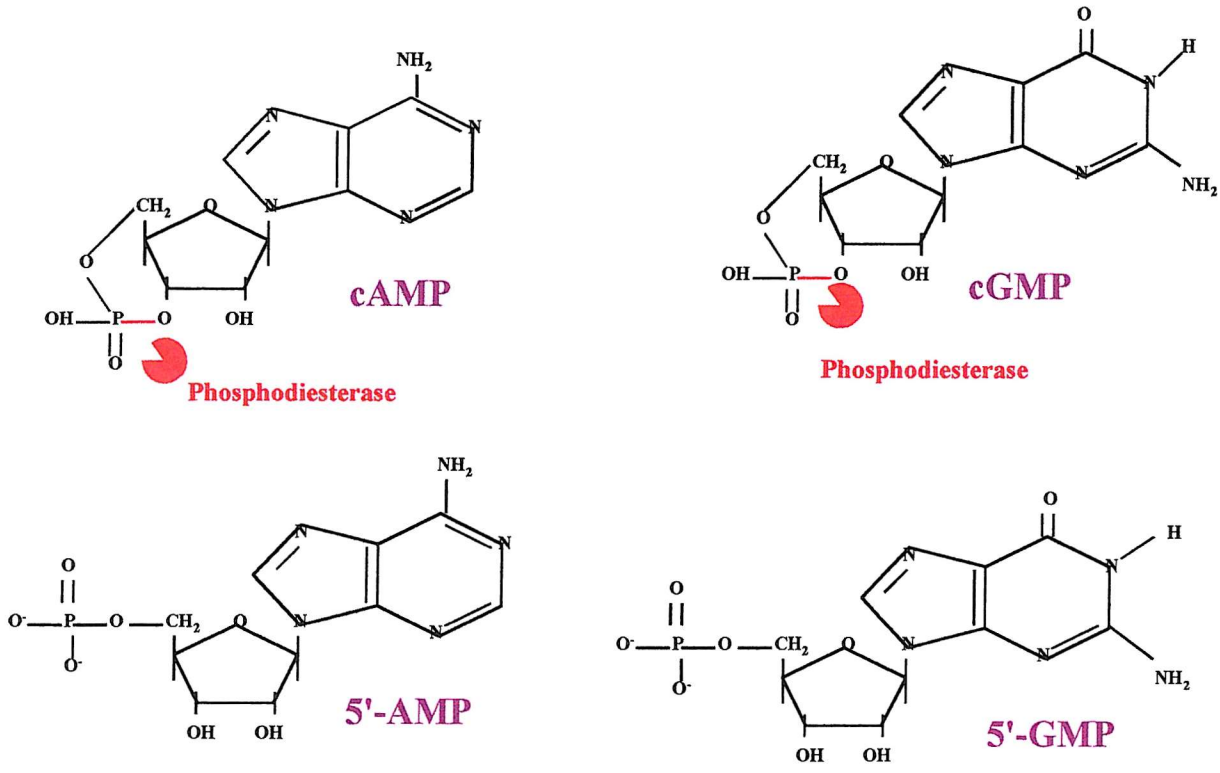


Figure 1.3 Hydrolysis of phosphodiester links in cAMP and cGMP by phosphodiesterases

The presence of at least one member of the phosphodiesterase family in most human cells suggests that there is a fundamental biological role for these enzymes. That biological role is the regulation of cyclic nucleotide levels, both intracellularly and on a larger scale, in organs and systems of the body. All members of the phosphodiesterase family share between 20-25% sequence homology with each other and have three common features, illustrated in figure 1.4 below.

Figure 1.4 The common structure of the PDE enzymes.

The regulatory domain contains regions responsible for enzyme modulation by calmodulin, cGMP or phosphorylation. This region also houses targeting domains and inhibitory domains. The catalytic region shows 50% homology in its amino acid sequence between different family members, and this region houses sites for substrate and inhibitor binding. The role of the variable region is less clear, but is thought to house a dimerisation domain (reviewed by Torphy, 1998).

1.6. PDE family members

1.6.1 PDE1

PDE1 enzymes are characterised by their ability to be activated by a calmodulin/calcium complex. Members of this PDE family are encoded by three distinct genes, all of which can give rise to separate splice variants. The calmodulin/calcium complex binds to a sequence on PDE1 near the amino (NH_2) terminus, and increases the activity of the enzyme several fold. This increase in activity is thought to result from the displacement of an inhibitory domain that holds the enzyme in an inactive state (Reviewed by Sonnenburg et al. 1998). The three members of this PDE1 family differ in their substrate preference. PDE1A and 1B have much greater affinity for cGMP, whilst PDE1C hydrolyses cAMP and cGMP equally. PDE1A and 1B are reported to be phosphorylated by PKA or calmodulin kinase II respectively. However, there is no evidence of any phosphorylation of the 1C isoform. This phosphorylation is thought to decrease the sensitivity of these enzymes to calmodulin/calcium activation (reviewed by Juilfs et al. 1999). In-situ hybridization has localised PDE1 to distinct regions of the brain (Sonnenburg et al. 1998), and expression has also been reported in alveolar macrophages (Tenor et al. 1995a) and epithelial cells (Rousseau et al. 1994). KS505a is the only reported specific PDE1 inhibitor to date.

1.6.2 PDE2

Members of this family are characterised by their ability to be stimulated by cGMP. There are at present three documented PDE2 isoforms, all of which have equal substrate affinity for cAMP and cGMP (reviewed by Giembycz et al. 1997). PDE2 enzymes possess a non catalytic binding site for cGMP that, when occupied, is thought to change the catalytic domain of the enzyme, from a low affinity form to a high affinity form, that hydrolyses cAMP. High concentrations of cGMP, however, inhibit catalysis of cAMP by PDE2, probably through competitive inhibition at the catalytic site. The ability of PDE2 to be stimulated by cGMP has implicated this enzyme in playing a role in mediating the cross talk between cAMP and cGMP regulated pathways. The three individual PDE2 forms are also expressed in different cell locations. PDE2A1 has been termed 'soluble', as it was isolated in the supernatant fractions of bovine heart extracts, and PDE2A2 and 2A3 are termed 'particulate' as this was their location in bovine brain extracts (reviewed by Juilfs et al. 1999). Members of the PDE2 family have been largely reported to be present in distinct areas of the brain with low levels in the lungs, airway smooth muscle, platelets, epithelial and endothelial cells (Giembycz et al. 1997). Erythro-9-[2-hydroxyl-3-nonyl]-adenine (EHNA) is a selective inhibitor of PDE2 enzymes.

1.6.3 PDE3

These enzymes are characterised by their ability to be inhibited by cGMP. The substrate specificity of PDE3 enzymes is the same for both cGMP and cAMP, demonstrated in similar Km values (see table 1.1). Cyclic GMP acts as a competitive inhibitor of cAMP hydrolysis, and is thought to inhibit hydrolysis of cAMP by competition at the catalytic site (Juilfs et al. 1999). This inhibition may allow cGMP to potentiate the effect of cAMP in cells by decreasing its hydrolysis. There are two forms of PDE3; 3A and 3B, which show

distinct cellular expression. Whilst PDE3A is expressed in platelets and cardiac tissue, PDE3B is expressed largely in adipocytes (reviewed by Conti and Jin, 2000). Both forms of these PDE3 enzymes can be phosphorylated by PKA, and this is reported to inhibit activity. Expression of PDE3 has also been reported in T cells (Sheth et al. 1997), basophils (Peachell et al. 1992) and epithelial cells (Rousseau et al 1994). Inhibitors for PDE3 were developed for treatment of congestive heart failure, and they include milrinone, siguazodan, motapizone and vesnarinone.

1.6.4 PDE4

This family hydrolyses cAMP almost exclusively, and members are present in all inflammatory cells with the exception of platelets. This thesis will therefore focus on investigating the PDE4 family, expression, localisation and activity in cells and tissue in asthma.

1.6.5 PDE5

PDE5 enzymes are cGMP specific. They are composed of two identical subunits, each of which contains a non catalytic binding domain for cGMP. Two isoforms of PDE5 have been identified so far, and both are susceptible to phosphorylation by protein kinase G (PKG) and PKA. The effect of this phosphorylation is still unclear, as it does not appear to change their activity (reviewed by Juilfs et al. 1999). Zinc binding domains are also present on PDE5 enzymes and it is thought that association with this ion is necessary for catalytic activity (reviewed by Beavo et al. 1994). Expression of PDE5 has been noted in epithelial cells (Rousseau et al. 1994), and traces in alveolar macrophages (Tenor et al. 1995a). Specific PDE5 inhibitors, however, do not suppress functional responses in these cells (reviewed by Giembycz et al. 1997). More recently a specific PDE5 inhibitor, Sildenafil,

has proved very effective in treatment of penile erectile dysfunction (Glossmann et al. 1999).

1.6.6 PDE6

This group, like PDE5, is also cGMP specific, and these enzymes are present in the rods and cones of the eye. PDE6 is activated by the retinal G protein transducin on light stimulation and this PDE activation leads to a decrease in cGMP and, therefore, closure of the cGMP gated cationic channels of the photoreceptor plasma membrane (Beavo et al. 1994).

1.6.7 PDE7

Enzymes of this subgroup are most like those of PDE4, in that they have a high affinity for cAMP. However, they are unaffected by PDE4 inhibitors such as rolipram (Giembycz et al. 1997). It is of particular interest that the mRNA for PDE7 appears to be relatively abundantly expressed in tissues, but there appears to be tight control over protein expression, with very little protein detected in cells (Li et al. 1999). At present there are two documented isoforms of PDE7; 7A, which has splice variants 7A1 and 7A2, and the recently cloned 7B (Gardner et al. 2000). The expression of PDE7 mRNA has been reported in T cells (Giembycz et al. 1996) and epithelial cells (Fuhrmann et al. 1999). PDE activity possibly attributable to PDE7 has been noted in T cells (Tenor et al 1995b) and B cells (Gantner et al. 1998), however, the absence of any commercially available PDE7 specific inhibitor has made identification of PDE7 difficult.

1.6.8 PDE8

Members of this group hydrolyse, almost exclusively, cAMP with an affinity forty times higher than PDE4 enzymes. For this reason it is speculated that PDE8 may be involved in the regulation of cAMP at low basal levels of this second messenger, whereas PDE4 enzymes may hydrolyse peaks of cAMP occurring during signal transduction. There are two isoforms of this enzyme, PDE8A and PDE8B. Messenger RNA for PDE8A is reported to be present in the human colon, small intestine, ovary and testis (Fisher et al. 1998a), and mRNA for PDE8B was present in the thyroid (Hayashi et al. 1998). Whilst this enzyme is unaffected by many PDE inhibitors, such as IBMX and rolipram, it can be inhibited by dipyridimole (Fisher et al. 1998a).

1.6.9 PDE9

PDE9 is highly cGMP specific, with one of the highest affinities for its substrate of all the PDEs. It is most similar to PDE8, and its high affinity for cGMP may suggest it is active in cells with lower cGMP concentrations than other PDEs (Soderling et al. 1998). High levels of the mRNA for this enzyme have been found in the kidney, spleen and small intestine (Fisher et al. 1998b; Soderling et al. 1998). PDE9 is inhibited only by the PDE1/5 inhibitor SCH51866.

1.6.10 PDE10

This enzyme has dual specificity for its substrates cAMP and cGMP. PDE10 has a sequence that codes for a possible cGMP allosteric binding site (Soderling et al. 1999). Messenger RNA expression of PDE10 has been detected in testis and brain, but was not present in

lungs or peripheral leukocytes (Fujishige et al. 1999). Dipyridamole and SCH51866 are capable of inhibiting this enzyme.

1.6.11 PDE11

PDE11, like PDE10, can hydrolyse cAMP and cGMP and also has the sequence for a potential cGMP allosteric binding site. There may be three isoforms of this enzyme, as demonstrated by three separate molecular weight bands evident through Western blotting. Skeletal muscle, liver, kidney, salivary gland and testis all have mRNA for this PDE. PDE11 can be inhibited by IBMX, zaprinast or dipyridamole (Fawcett et al. 2000).

Table 1.1 summarises the characteristics of the PDE isoenzyme family and catalogues the number of isoforms within these subtypes

Sub family	Characteristic feature	Km (μM) cAMP	Km (μM) cGMP	Isoforms
1	calcium/calmodulin dependent	1-30	3	3
2	cGMP stimulated	50	50	3
3	cGMP inhibited	0.2	0.3	2
4	cAMP specific	4	>3,000	4
5	cGMP specific	150	1	2
6	retinal cGMP specific	2000	60	4
7	high affinity for cAMP	0.2	>1,000	2
8	high affinity for cAMP	0.055	124	2
9	high affinity for cGMP	230	0.17	1
10	dual specificity for cAMP and cGMP	0.26	7.2	1
11	dual specificity for cAMP and cGMP	1.04	0.52	3?

Table 1.1 Characteristics of the multi-gene family of phosphodiesterase isoenzymes

1.7 Sub-families and splice variants of PDE4

Members of the PDE4 family are encoded by four distinct genes, and their transcription results in four isoforms 4A, 4B, 4C and 4D. Within these isoforms further diversity is introduced through alternative splicing, resulting in unique splice variants. Whilst these PDE4 isoforms are unique, in certain areas of their sequence they show large homology with each other. For example, all isoforms have greater than 80% homology in amino acid sequence within their catalytic domain, however, sequences coding for their amino (NH_2) and carboxyl (COOH) terminals show more variability (McLaughlin et al. 1993). All PDE4 isoforms also contain two upstream conserved regions (UCR), UCR1 and UCR2. These regions are distinct from each other, but show individual conservation between PDE4 isoforms suggesting they participate in a fundamental function of these enzymes (Beard et al. 2000). Figures 1.5-1.8 illustrate the four individual PDE4 isoforms, and show where in their sequence splicing occurs.

Figure 1.5 Schematic representation of PDE4A.
Length of isoform in terms of amino acids is represented in numerals shown at the NH_2 end of the sequence (Jacobitz et al. 1996).

Figure 1.6 Schematic representation of PDE4B
Splice variants are illustrated and labelled in red, whilst their amino acid length is shown in numerals at their NH_2 terminals (Huston et al. 1997).

Figure 1.7 Schematic representation of PDE4C
Length of isoform is represented in numerals at the NH_2 terminal (Engels et al. 1995).

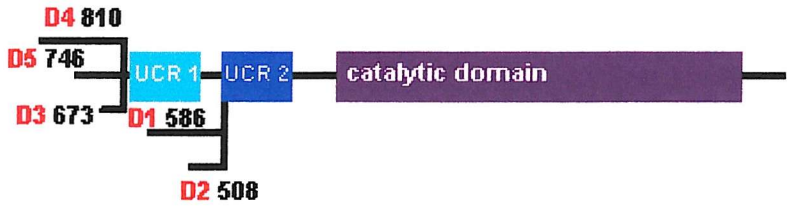


Figure 1.8 Schematic representation of PDE4D
Splice variants are illustrated and labelled in red, and their amino acid length is shown in numerals at their NH_2 terminals (Bolger et al. 1997).

Variations in the NH_2 terminal of PDE4 proteins brought about by alternative splicing are thought to serve several purposes. Firstly, they can target individual isoforms to specific cellular locations. For example engineered RD1, a rat PDE4A version, when stably transfected into human follicular thyroid carcinoma cells, shows distinct localisation with the Golgi complex (Pooley et al. 1997). The role of the NH_2 terminal in intracellular localisation of PDE4s was demonstrated by the expression of another engineered PDE4A, RPDE6, in monkey derived COS 7 cells. Whilst this RPDE6 protein was found to associate with SH3 domains of SRC or SRC related kinases, RPDE39, a version of RPDE6 lacking in a proline rich N terminal region had no association with these SH3 sequences (O`Connell et al. 1996). Splice variants of the PDE4B and 4D isoforms when expressed in transfected cells, not only show differing localisations, but also differ in their affinity of attachment to various structures. This variability in attachment between splice variants is evident through the different conditions needed to break these attachments and solubilise them (Jin et al. 1998; Bolger et al. 1997; Huston et al. 1997). More recently, reports have emerged that this

specific localisation may also target these splice variants to signalling complexes, as is the case with PDE4D5, which binds the protein receptor for activated C kinase (RACK1) in several cell lines (Yarwood et al. 1999). Targeting of splice variants to different intracellular locations may also serve to regulate their activity. Huston et al. (1997), demonstrated that 4B1 and 4B2 splice variants of PDE4B have 40% less catalytic activity, when located in the particulate fraction of COS 7 cells, than when found in the soluble fraction (Huston et al. 1997). Studies of splice variant localisation in non transfected human cells are still limited, but a report by Baroja et al. (1999) in human T cells has demonstrated that the B2 splice variant of PDE4, which lacks a UCR1 region, is associated with the CD3ε chain of the T cell receptor. This association is not observed for the longer B1 version of this isoform (Baroja et al. 1999).

Individual sequences, held within the NH_2 terminals of the PDE4 splice variants, are also known to alter the catalytic activity of these enzymes. The UCR2 region, of both PDE4D1 and PDE4A, is hypothesised to contain an inhibitory region which, if removed by deletion, increases the catalytic activity of these enzymes (Jacobitz et al. 1996; Kovala et al. 1997). Phosphorylation of a sequence within the UCR1 region of the long variant, 4D3, can also significantly increase catalytic activity (Lim et al. 1999; Alvarez et al. 1995). Recently it has been hypothesised that UCR1 and UCR2 regions in long splice variants are held together by electrostatic interactions. This interaction holds the enzyme in a conformation with low catalytic activity. Phosphorylation by PKA of a site within the UCR1 releases its interaction with UCR2, thus transforming the enzyme into a more active catalytic conformation (Beard et al. 2000). Differences in the presence of this UCR1 region between long and short splice variants of PDE4 are exploited by cells to change activity of individual splice variants. Whilst ERK2 phosphorylation of 4D long variants amplifies the inhibitory

effect of UCR1 and UCR2 on the catalytic domain, ERK2 phosphorylation of short 4D variants, which have only a UCR2 region, causes activation of the enzyme (MacKenzie et al. 2000). The ability of cells to increase catalytic activity by changes in conformational states induced by phosphorylation is observed in many cells. This increase in activity by phosphorylation can occur in a short time, allowing cells to quickly regulate stimulated increases in cAMP by rapid induction of a long form PDE4 that can degrade it. Cells regulate cAMP levels in the long term by induction of protein synthesis for PDE4s. This temporal regulation of cAMP levels by cells was illustrated in the FRTL-5 thyroid cell line. Thyroid stimulating hormone (TSH) causes a quick increase in PDE4 activity within 15 minutes by the phosphorylation of 4D3. Long term incubation with TSH results in increased 4D2 expression through cAMP dependent activation of its promoter (Jin et al. 1998).

Finally, differences between the individual PDE4 splice variants lead to variability in their inhibition by PDE4 inhibitors. This was illustrated for recombinant 4A, whilst the long version has a high affinity for rolipram with an IC_{50} of 204nm, truncated versions of this isoform show low affinity with an IC_{50} of 1022nm (Owens et al. 1997). The intracellular localisation of the splice variants also affects their IC_{50} for rolipram. 4B2 shows a ten fold increase in sensitivity to rolipram inhibition when located in the particulate fraction of COS7 cells (Huston et al. 1997). RACK1 association with 4D5, however, decreases its sensitivity for rolipram inhibition (Yarwood et al. 2000). Phosphorylation also changes affinity for inhibitors. For example the phosphorylated version of 4D3, when expressed in U937 cells, shows a 100 fold increase in sensitivity to RS25344 (a PDE4 inhibitor) (Alvarez et al. 1995).

The amazing diversity of PDE4 expression through different isoforms and splice variants gives cells the ability to finely control cAMP levels. The further range of properties related to these individual splice variants, including regulation by phosphorylation, sensitivity to inhibition and intracellular targeting, further refines this control.

1.8 Therapeutic agents that increase cAMP

The levels of intracellular cAMP can be increased pharmacologically by one of two mechanisms: either by activation of receptors linked to Gs proteins that stimulate adenylyl cyclase and cAMP production, or by the use of phosphodiesterase inhibitors which prevent its degradation.

1.8.1 β_2 adrenoceptor agonists

β_2 agonists are bronchodilators and one of the main stays of asthma treatment that exert their effects through β_2 adrenoreceptors whose endogenous ligand is adrenaline. β_2 receptors are present in many cells within the lungs, including bronchial smooth muscle cells, epithelial cells, presynaptic nerve terminals and many of the inflammatory cells, including eosinophils and mast cells (reviewed by Lipworth, 1998). Unfortunately β_2 agonists have no appreciable anti inflammatory actions, and thus are mainly used in conjunction with glucocorticosteroids for the effective management of asthma patients. The action of β_2 agonist is mediated through a chain of events, which are illustrated in figure 1.9. β_2 agonists, for example salbutamol or formoterol, bind to a pocket formed by the seven hydrophobic transmembrane spanning domains of the β_2 adrenoceptors. The intracellular loops of this receptor are bound to a stimulatory G protein (Gs). The Gs protein is composed of three subunits, termed the α, β and γ subunits. It is to the α subunit that the intracellular loops of the β_2 receptor bind and, on agonist occupation of the receptor, the α

subunit becomes activated (reviewed by Barnes, 1995a). The α subunit is activated by the dissociation of guanosine diphosphate (GDP), to which it was bound in its inactive state, and the subsequent re-association with a GTP molecule. The activated α subunit activates adenylyl cyclase, leading to the production of cAMP. As described in Section 1.4, cAMP can then bind to PKA, which results in its activation. Activated PKA in bronchial smooth muscle can phosphorylate large potassium conductance channels (maxi K^+ channels), causing a decrease in intracellular calcium levels by promoting Ca^{2+}/Na^+ exchange. This decrease in intracellular calcium causes smooth muscle cell relaxation, which is observed as bronchodilation. PKA can also inhibit phosphoinositide hydrolysis and myosin light chain phosphorylation within these smooth muscle cells.

Activated PKA can also translocate in the nucleus, where it can alter gene regulation. PKA phosphorylates a cAMP response element binding protein (CREB) in the nucleus. This phosphorylated CREB then binds a creb binding protein (CBP), which is bound to a cAMP response element (CRE). CRE is located within the promoter region of a number of target genes, and its association with CREB and CBP results in increased transcription of the target gene. This target gene can include the β_2 adrenoreceptor or phosphodiesterase enzymes (reviewed in Barnes, 1995b). It should also be noted that the promoter region of genes that contain a CRE sequence can also be negatively regulated if the CRE sequence is bound by a CRE modulator (CREM).

The bronchodilation caused by agonist induced activation of β_2 receptors may not, entirely be due to increased cAMP levels. It is hypothesised that activation of the maxi K channels may also result from direct association with the α subunit of Gs. This would explain why

higher levels of agonist are needed to increase cAMP levels than those required to elicit bronchodilation (reviewed in Barnes, 1995a).

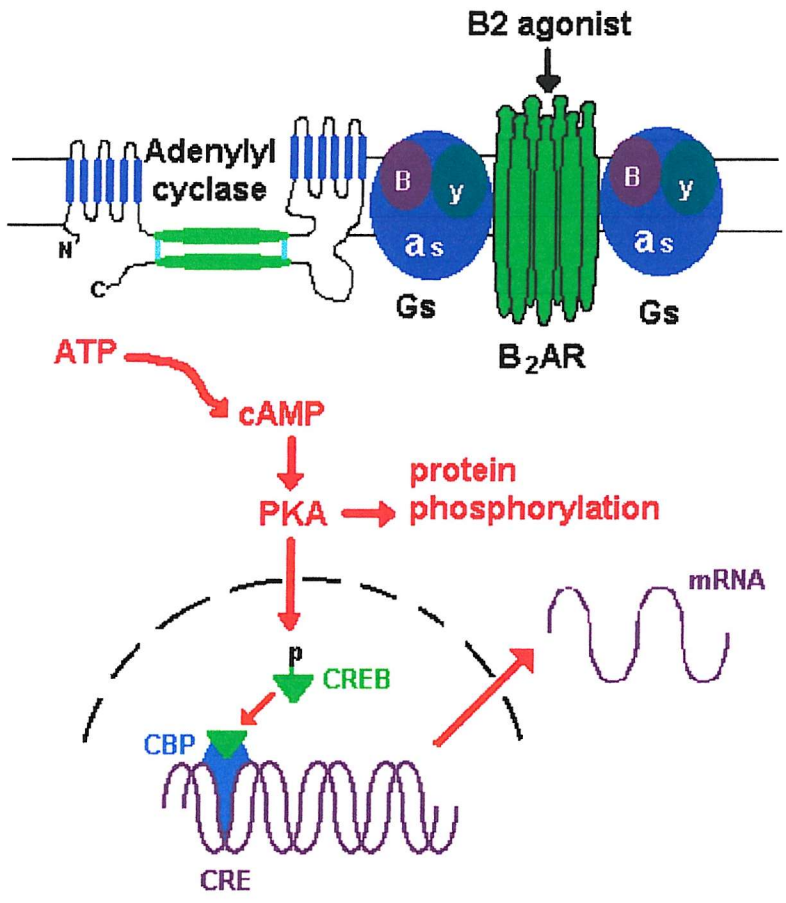


Figure 1.9 Schematic representation of the actions of β_2 agonists
A stimulus interacts with a β_2 adrenoreceptor (B_2AR) thus stimulating the dissociation and activation of heterotrimeric G proteins. This leads to adenylyl cyclase activation and the generation of cAMP which phosphorylates PKA. PKA, in turn, can then phosphorylate proteins or mediate effects within the nucleus.

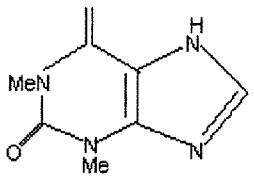
Long term β_2 agonist use is observed to lead to a desensitisation of β_2 receptors, resulting in reduced agonist effectiveness (Lipworth 1998). This desensitisation of β_2 receptors can be the result of numerous mechanisms. These include functional uncoupling of the receptors by G protein receptor kinases, PKA or PKC, receptor sequestration, changes in receptor turnover or receptor down regulation through degradation. Desensitisation can also occur

through changes in receptor gene expression. These changes can be mediated through regulation of; the gene promoter, gene transcription, mRNA stability or gene translation.

1.8.2 Phosphodiesterase type 4 Inhibitors

Henry Hyde Salter first prescribed strong coffee for the alleviation of asthma symptoms as early as 1860. Caffeine was the active component responsible for alleviation of these symptoms through its action as a bronchodilator (reviewed by Spina et al. 1998).

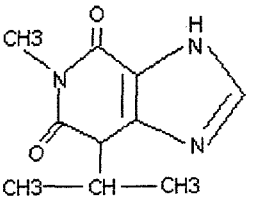
1.8.2.1 Theophylline (1,3-dimethylxanthine)



Theophylline has a similar chemical structure to caffeine, and was named in 1888 by Alfred Kossel who isolated a compound from tea which he called theophylline, meaning the divine leaf (Kjellin and Persson, 1985). Theophylline was first used in asthma treatment in the 1920s, primarily for its role as a bronchodilator. Its exact mode of action is still unclear, however; it is known to be a weak non specific phosphodiesterase inhibitor (Montana et al. 1998). The bronchodilatory actions of theophylline are thought to arise from inhibition of PDEs within the airway smooth muscle (reviewed by Giembycz, 2000). Whilst not fully understood, theophylline may also mediate its *in vivo* effects through mechanisms other than PDE inhibition. These include adenosine receptor antagonism, release of catecholamines or effects at calcium channels inhibiting calcium influx into cells (Giembycz, 2000). Theophylline is still one of the most used asthma drugs world wide (reviewed by Schmidt, 1999) and, although theophylline has been shown to have many therapeutic benefits, its use is also linked to severe side effects. These side effects are also generally linked to non specific PDE inhibition, and range from nausea, vomiting, gastrointestinal disorders, arrhythmias, seizures and, in extreme cases, strokes or even death

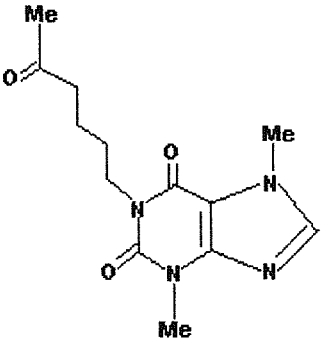
(reviewed by Makino, 1996). These side effects occur at concentrations less than twice the therapeutic dose of theophylline needed for bronchodilation, thus giving it a very narrow therapeutic index. More recently, the role of theophylline as an anti inflammatory agent has been described. It has been shown to be effective at reducing the late phase response after allergen exposure in allergic asthmatics at much lower concentrations than those known to cause bronchodilation (Giembycz, 2000).

1.8.2.2 IBMX (3-isobutyl-1-methyl xanthine)



IBMX, like theophylline, has a similar structure to caffeine and is also a broad-spectrum phosphodiesterase inhibitor. It is a potent bronchodilator, but is only used as a pharmacological tool in animal models.

1.8.2.3 Pentoxyfylline

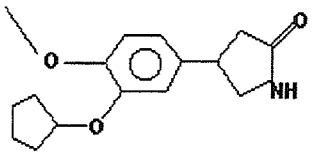


This, like denbufylline, is based on substitutions made onto the basic structure of theophylline, the natural xanthine.

1.8.2.4 Second generation PDE inhibitors

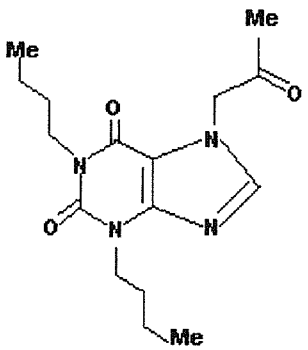
Theophylline exhibited a range of side effects due to its non specific inhibition of phosphodiesterases. Therefore, specific inhibitors of PDE family members were investigated with the hope of reducing the side effects. Phosphodiesterase 4 became the target isoform, as it was expressed most abundantly in inflammatory cells and also in smooth muscle.

1.8.2.4i Rolipram



Rolipram, a specific phosphodiesterase 4 inhibitor, was first described by Sheppard, Wiggan and Tsien in 1971 for its ability to preferentially inhibit cAMP degradation in brain preparation *in vitro*. From this initial observation, work progressed investigating the potential role of rolipram as an antidepressive agent. Wachtel reported in 1983 that rolipram had displayed antidepressive properties in preliminary human trials, and the hypothesis was proposed that rolipram exerted this effect by inhibiting phosphodiesterase enzymes (Wachtel, 1983). The antidepressive effects of rolipram are thought to be due to an increase in noradrenaline turnover and also an increased level of cAMP, due to inhibition of PDEs that break it down. These two actions lead to an enhanced central noradrenergic transmission. Rolipram is used widely as an *in vitro* tool to investigate the effects of PDE4 inhibition in inflammatory cells. These investigations found that rolipram bound PDE4 in rat brains with a high affinity that was not correlated to inhibition of the catalytic domain of the enzyme. Rolipram did however show binding to PDE4 with a lower affinity in other tissues that corresponded to inhibition of catalytic activity (reviewed by Barnette et al. 1996). The observation that rolipram had two distinct binding affinities for PDE4 led to the naming of a high affinity and low affinity site for this inhibitor on PDE4.

1.8.2.4ii Denbufylline (BRL30892)



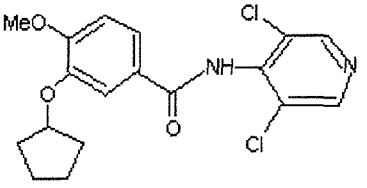
Along with rolipram, this was one of the second generation PDE4 selective drugs. Denbufylline exhibited cognition enhancing abilities by augmenting the excitability of the hippocampal neurones through increased cAMP levels that enhanced responses to glutamate (Nicholson et al. 1991).

Although these second generation inhibitors were an improvement upon theophylline, their use was limited as they produced side effects including increased gastric acid secretion, nausea and vomiting (Barnette et al. 1998).

1.8.2.5 Third generation PDE inhibitors

Research in the PDE4 field has indicated that rolipram binds PDE4 with two differing affinities. From this observation it was hypothesised that two distinct conformers of PDE4 existed. These two conformers were termed the high affinity rolipram binding site (HARBS or HPDE4) and the second lower affinity-binding site (LPDE4). The observation of these two distinct conformers caused speculation that binding at the HPDE4 site led to many of the neurological derived side effects. This was based on evidence that the HPDE4 conformer was most abundant in the CNS, where many side effects originated. Recent developments in inhibitor design have tried to target the LPDE4 conformer, in the hope of reducing the side effects seen with the second generation drugs. Drug development has also been targeted at the specific isoforms of PDE4, as they display differential tissue expression. It was reasoned that by targeting a specific PDE4 isoform, predominant in cells involved in the disease process of interest, might reduce side effects associated with inhibition of all PDE isoforms.

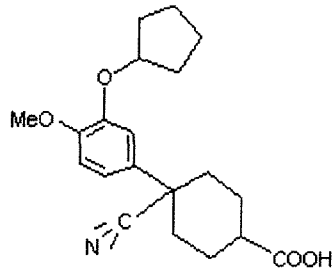
1.8.2.5i Piclamilast (RP 73401)



Piclamilast shows less potency as an inhibitor of PDE4C in comparison to the other PDE4 isoforms (Muller et al. 1996), and has also been shown to be between 3-14 times more potent than rolipram at increasing cAMP levels *in vitro* and inhibiting the guinea pig eosinophil generation of superoxide, MBP and ECP (Souness et al.

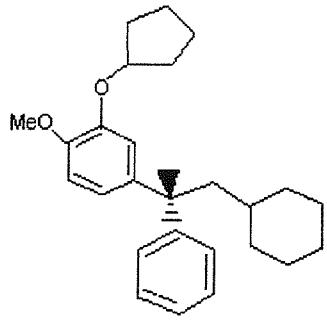
1995). However, piclamilast showed disappointing results in clinical trials. After a six week trial it showed no significant improvements to FEV_1, airway responsiveness to methacholine or level of exhaled nitric oxide in asthmatic subjects (reviewed by Giembycz, 2000).

1.8.2.5ii Ariflo (SB 207499)



Ariflo combines the two directions for development of third-generation inhibitors. It has a lower affinity for the HPDE4 conformer than rolipram, and is also 10 times more selective for the PDE4D isoform (Barnette et al. 1998). However, ariflo, like piclamilast, has not lived up to expectations in clinical trials. Over a six week trial, in asthmatic subjects, ariflo was only able to significantly improve their lung function, relative to placebo, at week two. In this trial ariflo was reported to be well tolerated at the dosage used, but affected less than 15% of patients (reviewed by Giembycz, 2000).

1.8.2.5iii CDP840



CDP840 shows less affinity for the HSPDE4 conformer than rolipram and, like piclamilast, is less potent in inhibition of the PDE4C isoform (Muller et al. 1996). This compound was extremely promising in early development, showing efficacy in clinical trials in asthmatics without the usual associated side effects. In these early clinical trials CDP840 reduced the late asthmatic response to allergen challenge by 30% (Harbinson et al. 1997). However, this only

occurred after 9.5 days of treatment, and CDP840 was ineffective when given in single doses. The failure of CDP840 to produce large beneficial responses may be due, in part, to its poor bioavailability and short half life, resulting from extensive first pass metabolism (reviewed by Giembycz, 2000).

1.8.2.6 Future drug development

The results of clinical trials for these third generation PDE4 inhibitors have proved disappointing in light of their initial promise in early *in vitro* and animal trials. Further drug developments incorporating reduced affinity for the HSPDE4 conformer and targeting of specific isoforms are still underway, with drugs such as D22888 (Dent et al. 1998b) and NCS 613 (Boichot et al. 2000). Other directions for PDE inhibitor development are also being considered. Further drug development of mixed PDE inhibitors, after theophylline and IBMX, was undertaken. Joint PDE3 and 4 inhibitors were developed, for example zardaverine, since PDE3 inhibition leads to relaxation of airway smooth muscle and PDE4 inhibition suppresses many responses of inflammatory cells. Therefore a compound capable of both would, theoretically, be a promising asthma treatment. A combination of these two inhibitory profiles may also reduce the cardiac and emetic side effects associated with PDE3 or PDE4 inhibition respectively, as they could be used a lower concentrations. Clinical trials of these joint PDE3/4 inhibitors are so far inconclusive (Giembycz, 2000).

The use of low doses of theophylline combined with a low dose of the steroid budesonide has proved as effective as high dose budesonide, thus giving a steroid sparing effect (Evans et al. 1997). It is therefore possible that specific PDE4 inhibitors may also have a similar steroid sparing effect in asthmatics, if combined in low doses with steroids.

Recent work investigating the two conformers of PDE4 with differing affinities for rolipram binding has further elucidated their structure. Laliberté et al. recently reported that the two conformational states of PDE4 may be associated with metal cofactor binding. The HPDE4 conformer has been hypothesised to be a holoenzyme version of PDE4 that is bound to a metal ion, for example Mg^{2+}. This interaction with a metal cofactor allows rolipram to bind with high affinity. The LPDE4 conformer represents the apoenzyme, which is not bound to Mg^{2+}, and thus has a lower affinity for rolipram (Laliberté et al. 2000). PDE4 enzymes are known to be magnesium dependent and, therefore, this lends credence to this hypothesis.

1.9 Disease states in which phosphodiesterase inhibitors may be therapeutic

Rolipram, one of the second generation phosphodiesterase inhibitors, was originally used in the treatment of depression. A combination of PDE3 and PDE4 inhibitors has given promising results in initial animal experiments in glomerulonephritis by preventing proteinuria, halting the proliferation of mesangial cells and decreasing the infiltration of macrophages (Dousa et al. 1997). Joint PDE3 and 4 inhibitors may also be therapeutic in preventing allograft rejection. Initial studies have indicated that a combination of cilostamide (a PDE3 inhibitor) and rolipram can significantly suppress the proliferative response of a mixed lymphocyte culture in response to contact with HLA-DR alloantigens from unrelated donors (Dousa et al. 1997). PDE3 specific inhibitors have been used in studies investigating the chloride permeability of the epithelial cystic fibrosis transmembrane conductance regulator (CFTR). Milrinone, a PDE3 selective inhibitor, was effective at increasing the chloride efflux *in vitro* in epithelial cells. This was somewhat surprising given that PDE3 is not the predominant PDE in these cells. The effects of milrinone are thought to be mediated through PKA and this increase in chloride efflux may

be relevant in the treatment of cystic fibrosis, which is characterised by defective chloride efflux in epithelial cells (Kelley et al. 1995).

Studies undertaken by Hanifin and colleagues have indicated that CP80,633, a specific PDE4 inhibitor, reduced PGE_2, IL-10 and IL-4 production in mononuclear cells *in vitro,* and demonstrated reductions in inflammatory parameters when tested on patients with atopic dermatitis (Hanifin et al. 1996). PDE4 inhibitors have also been suggested as a possible treatment for multiple sclerosis (MS) in order to prevent the central nervous system (CNS) inflammation widely associated with this disease. Initial experiments carried out in allergic encephalomyelitis, the animal model for MS, have shown that rolipram has proved very effective in its treatment and prevention (reviewed by Dinter et al. 1997). The potential benefits of rolipram for treatment of MS may stem from inhibition of TNF-α production. Monocytes are one of the most potent producers of TNF-α and one of the main effects of increasing cAMP, as rolipram does, is to reduce TNF-α production. Not only has TNF-α over production been implicated in MS, but over production may also be linked to disease states ranging from arthritis and AIDS to septic shock. (Souness et al. 1996).

Probably the most publicised PDE inhibitor, for its treatment of impotence, is Viagra® (Sildenafil). Sildenafil acts as a PDE5 inhibitor, increasing the intracellular levels of cGMP. Cyclic GMP levels are normally elevated in the penis by nitric oxide stimulation of guanylyl cyclase. These elevated cGMP levels then activate cGMP dependent protein kinases (PKG) which, in turn, reduce intracellular calcium levels by interaction with ion channels. Reductions in intracellular calcium leads to relaxation of the trabecular smooth muscles. This increases blood flow and expands the corporal volume. Subsequent exposure to systemic arterial blood pressures during sexual excitation leads to the sub tunical venules

of the penis being stretched and compressed, resulting in penile erection. Viagra® can only enhance this process by inhibition of the breakdown of cGMP, thus potentiating its actions. It does not function independently of initial sexual excitation (Moreland et al. 1998).

1.10 PDE profile and effects of PDE inhibitors in inflammatory cells

1.10.1 Eosinophils

It has been demonstrated that human eosinophils exhibit predominantly PDE4 cAMP hydrolysing activity (Hatzelmann et al. 1995), and mRNA has been detected in eosinophils for the splice variants PDE4A, B and D, but not C (Engels et al. 1994; Gantner et al. 1997a).

Non specific phosphodiesterase inhibitors, such as theophylline and IBMX, have demonstrated many anti inflammatory actions in eosinophils. *In vivo* studies in guinea pigs have shown that a 1 hour pretreatment with theophylline is able to decrease the eosinophil count in BAL at a 48 hour time point after IL-5 induced eosinophil recruitment (Lagente et al. 1995). *In vitro* studies have also shown anti-inflammatory properties of these drugs. In guinea pig eosinophils, IBMX was able to inhibit the opsonised zymosan stimulated respiratory burst (Dent et al. 1991). Also, high concentrations of theophylline were able to inhibit opsonised zymosan stimulated superoxide anion release in these cells. It is interesting to note that lower concentrations of theophylline, in fact, potentiated this release, probably due to the ability of theophylline to act as an antagonist at adenosine A_2 receptors (Yukawa et al. 1989). Studies of human eosinophils *in vitro* have also shown positive effects with these broad spectrum PDE inhibitors. Theophylline was able to suppress C5a or PAF induced eosinophil LTC_4 synthesis, as well as C5a or PAF induced eosinophil chemotaxis (Tenor et al. 1996). Release of GM-CSF and IL-8 stimulated by secretory IgA

coated beads was also inhibited by theophylline (Shute et al. 1998). Additionally, theophylline was also able to inhibit C5a, GM-CSF or PAF induced eosinophil degranulation, together with inhibiting eosinophil survival in the presence of IL-5 and eosinophil formation of reactive oxygen species (Hatzelmann et al. 1995; Momose et al. 1998).

There are many reports of isoform specific PDE inhibitors exerting anti inflammatory actions in eosinophils. In *in vivo* studies of guinea pigs, Banner et al. (1995), showed that in ovalbumin sensitised animals, rolipram and benzafentrine (a PDE3 and 4 inhibitor) failed to decrease eosinophil numbers in BAL, but did decrease EPO levels (Banner et al. 1995b). The same group have also shown, in the same model, that only chronic low dose pre treatment with RO-201724 (a PDE4 inhibitor) or zardaverine (a PDE3 and 4 inhibitor) is able to significantly reduce the eosinophil accumulation in BAL (Banner et al. 1995a). Eosinophil accumulation, in response to IL-5 treatment, was, however, inhibited by a 1 hour pretreatment with rolipram or RO-201724 (a PDE4 inhibitor) (Lagente et al. 1995). *In vitro* studies of isolated eosinophils from rats showed rolipram was able to suppress PAF or C5a induced chemotaxis (Alves et al. 1996). Several studies have also shown various PDE4 inhibitors such as rolipram, BRL-61063, RP73401 and denbufylline are able to inhibit guinea pig eosinophil superoxide production or respiratory burst in response to stimuli such as LTB_4, FMLP and opsonised zymosan (Barnette et al. 1995; Souness et al. 1995; Dent et al. 1991). In human eosinophils rolipram and RS-25344 (a PDE4 inhibitor) were able to inhibit C5a or PAF stimulated LTC_4 synthesis or eosinophil chemotaxis (Tenor et al. 1996; Kaneko et al. 1995). Opsonised zymosan stimulated superoxide generation by eosinophils was inhibited by rolipram, zardaverine and the PDE4 inhibitor D22888 (Dent et al.1994b

and 1998b). IL-5 mediated eosinophil survival and GM-CSF or PAF induced degranulation was also inhibited by the PDE4 inhibitor KF 19514 (Momose et al. 1998).

The ability of specific and non specific PDE inhibitors to produce the above regulation on the proinflammatory actions of eosinophils demonstrates the potential these inhibitors have as anti-inflammatory drugs.

1.10.2 Neutrophils

Human neutrophils are reported to express predominantly PDE4 (Muller et al. 1996). They weakly express mRNA for PDE4A and 4D, but show strong expression for the PDE4B isoform (reviewed by Giembycz et al. 1997).

Non specific PDE inhibitors have been reported to inhibit proinflammatory activities of neutrophils. In human neutrophils, IBMX inhibited the FMLP or C5a stimulated respiratory burst and theophylline was able to inhibit neutrophil superoxide generation, resulting from stimulation by FMLP, opsonised zymosan or calcium ionophore (Wright et al. 1990; Mahomed et al. 1998).

PDE isoform specific inhibitors were able to suppress several of the proinflammatory actions of neutrophils. In rat neutrophils, rolipram inhibited the FMLP stimulated release of arachidonic acid and activation of phospholipase A_2 and D (Nakashima et al. 1995). In human neutrophils, rolipram was able to inhibit the FMLP stimulated superoxide release and FMLP/thiomerosal elicited leukotriene biosynthesis (Schudt et al. 1991). FMLP stimulated LTB_4 release was also inhibited by a host of PDE4 inhibitors, including RC-14203, KF18280, RS-25344, RP73401, CDP840 and rolipram (Denis and Riendeau, 1999).

RP73401 and SB207499 were also able to inhibit zymosan induced IL-8 release from human neutrophils (Au et al. 1998). A very interesting report described how rolipram and RO-20-1724, both PDE4 inhibitors, were able to inhibit the FMLP stimulated adhesion of neutrophils to human endothelial cells. The same study also reported that rolipram was able to inhibit the FMLP induced neutrophil shape change and β_2 integrin expression (Derian et al. 1995). This report is particularly interesting as it implies that PDE4 inhibitors can suppress the functions that allow neutrophils to attach and migrate through the endothelium, the initial barrier to cell recruitment.

1.10.3 T cells

Messenger RNA for PDE3A, PDE4A, 4B, 4D and PDE7 has been detected in human T cells, and there is no apparent difference in expression between CD4+ and CD8+ cells (Sheth et al. 1997; Giembycz et al. 1996). Reports have shown that CD4+ and CD8+ T cells express PDE3, which is membrane bound, and PDE4, found in the soluble fraction. These cells also have a residual cAMP hydrolysing activity, which is speculated to be PDE7 (Giembycz et al. 1996). Work by Li et al. (1999) has shown that inhibition of PDE7, by PDE7 antisense oligonucleotides, in anti-CD3 and anti-CD28 stimulated peripheral T cells inhibits proliferation by 80%. Further inhibition of proliferation could reversed with a PKA inhibitor, suggesting that PDE7 regulates T cell proliferation via cAMP dependent PKA (Li et al. 1999). T cells have also been reported to express low levels of PDE1, 2 and 5 (Tenor et al. 1995b).

Phosphodiesterase inhibitors have been demonstrated to inhibit several proinflammatory actions of these cells. The non specific PDE inhibitor, theophylline, was able to inhibit T cells chemotaxis triggered by PAF and IL-8 (Hidi et al. 2000). Isoform specific PDE

inhibitors were also able to modulate some properties of clonal T cells. Rolipram was able to downregulate clonal T cells proliferation, and also synthesis of IL-13, IL-4 and INF-γ. It is interesting that, in the same experiment, a PDE3 inhibitor, siguazodan, was unable to downregulate these responses but increased the effects of rolipram when in combination (Essayan et al. 1997c and 1997b). In human peripheral T cells rolipram was able to inhibit T cell chemotaxis stimulated by PAF or IL-8 (Hidi et al. 2000). Rolipram was also able to inhibit the PHA or anti CD3 induced proliferation and IL-2 synthesis of both CD4+ and CD8+ cells. SK&F95654, a PDE3 inhibitor, had no effects on these responses, but was able to potentiate the effects of inhibition by rolipram (Giembycz et al. 1996). In another study investigating T cell proliferation by anti CD3 stimulation, rolipram inhibited 40% of this proliferative response. Motapizone, a PDE3 inhibitor, managed to inhibit this response by 30%. However, zardaverine, a mixed PDE3 and 4 inhibitor, inhibited virtually all of the response (Schudt et al. 1995). This finding implies that anti CD3 mediated proliferation involves both PDE3 and PDE4.

1.10.4 Monocytes

Human monocytes express mRNA for the PDE4 isoforms 4A, 4B and 4D (Souness et al. 1996). At the protein level, human peripheral monocytes contain predominantly PDE4 which is found in the soluble fraction, and PDE3 which is particulate (Gantner et al. 1997b). Groups investigating purified human monocytes have observed inhibition of proinflammatory activities by PDE inhibitors. Rolipram, RP 73401 and RS 25334 were all able to inhibit the LPS induced TNF-α production by these cells (Souness et al. 1996; Gantner et al. 1997b). Using a group of PDE4 inhibitors, which had differing potencies in their inhibition of PDE4A and 4B over 4D. Manning et al. were able to show that compounds with greater affinity for inhibition of the 4A and 4B isoforms were more potent

at inhibiting the LPS induced TNF-α production in human monocytes (Manning et al. 1999).

1.10.5 Peripheral blood mononuclear cells

Human peripheral blood mononuclear cells express mRNA for the PDE4 isoform 4D splice variants D1, D2 and D3 (Nemoz et al 1996). These cells express PDE3 and PDE4 at the protein level (Ekholm et al. 1999). PDE inhibitors were able to suppress many of the proinflammatory actions of monocytes. For example, in peripheral blood mononuclear cells (PBMC), rolipram was able to downregulate the antigen stimulated expression of IL-5, GM-CSF and IL-2 (Essayan et al. 1995 and 1997a). T440, a specific PDE4 inhibitor, was also able to suppress IL-5 production stimulated by allergen challenge in PBMC from atopic asthmatics (Kaminuma et al. 1996). Theophylline, zardaverine, CDP840 and RO-20-1724 were all able to suppress the PHA stimulated proliferation of PBMC derived from human umbilical cord blood (Banner et al. 2000).

1.10.6 Epithelium

Human airway epithelial cells express mRNA for PDE4A5, 4D3 and PDE7 (Wright et al. 1998). Fuhrmann et al. also reported the additional expression of mRNA for 4C1 and 4D2 (Fuhrmann et al 1999). Protein expression of PDEs has been demonstrated for PDE1, 3, 4 and 5, however reports are not in agreement as to whether or not PDE4 is the major form. It is agreed, though, that PDE3 and 5 are present in small amounts within these cells (Wright et al. 1998; Dent et al. 1998a). Fuhrmann et al. noted PDE activity that could not be inhibited by cAMP or cGMP specific PDE inhibitors. They have attributed this activity to PDE7, which is not unreasonable, as mRNA for PDE7 was found (Fuhrmann et al. 1999).

There have been few studies investigating the effect of PDE inhibitors on epithelial functions. However, in calu-3 cells, a cell line derived from airway epithelium, milrinone was able to increase the chloride efflux through CFTR channels, whilst PDE4 inhibitors had no effect (Kelley et al. 1995). ORG 9935 and rolipram, PDE3 and 4 inhibitors respectively, were able to reduce the IL-1β_1 stimulated release of GM-CSF from primary human airway epithelial cells (Wright et al. 1998). IBMX or rolipram, in combination with salbutamol, were not able to affect the TNF-α induced IL-8 release or bradykinin induced PGE_2 release. Only zardaverine, a joint PDE3 and 4 inhibitor, at a concentration of 100μM was able to increase the bradykinin induced PGE_2 release (Dent et al. 1998a). These initial reports seem to indicate that epithelial cell functions are under control of different PDE enzymes and, also, that some of their functions may well not be regulated by PDEs.

1.10.7 Mast cells and basophils

There are few reports of the actions of PDE inhibitors in either mast cells or basophils. However, theophylline and LY 186655 (a broad PDE inhibitor) were able to inhibit the IgE induced histamine release from human leukocytes and human lung fragments (Louis et al. 1992). Theophylline and IBMX were also reported to inhibit the IgE induced histamine release from human basophils and human lung mast cells. It is interesting that in this report rolipram, denbufylline, RO20-1724 and RP 73401, all PDE4 inhibitors, were able to suppress histamine release from basophils, but had no effect on mast cells. This would imply that PDE4 inhibitors can regulate basophil responses, but their effects on mast cells are unclear (Weston et al. 1997).

1.10.8 Macrophages

Macrophages are thought to express predominantly PDE1, together with PDE3, PDE4 and 5 (Tenor et al. 1995a). Few studies have investigated the effects of PDE inhibitors on macrophage functions. However, theophylline has been reported to inhibit the opsonised zymosan stimulated hydrogen peroxide release from human macrophages (Dent et al. 1994a). PDE3 (motapizone) and PDE4 (rolipram) inhibitors were reported to have little effect alone at inhibiting the LPS stimulated TNF-α release from human monocyte derived macrophages whilst, in combination, they were able to inhibit this response by 40%. With the additional cAMP stimuli, produced by PGE_2, they could completely block this response (Gantner et al. 1997b).

1.10.9 Endothelium

Reports in porcine pulmonary endothelial cells have demonstrated protein expression for PDE2, PDE3 and PDE4 (Suttorp et al. 1993). The PDE inhibitors, motapizone, rolipram or zardaverine, in combination with PGE_1 were able to inhibit the hydrogen peroxide induced vascular permeability in porcine endothelial cell monolayers (Suttorp et al. 1993). In endothelial cells derived from human lung microvasculature, rolipram, when in combination with salbutamol, was able to inhibit TNF-α stimulated E selectin expression. This combination of drugs, however, had no effect on ICAM 1 or VCAM 1 expression. The PDE3 inhibitor, ORG9935, only when in combination with rolipram, was able to inhibit endothelial cells expression of VCAM 1 and E selectin (Blease et al. 1998). These reports indicate that either PDE3 or PDE4 inhibition alone is not sufficient to inhibit these endothelial cell responses. However, when combined with an extra cAMP elevating agent, they are able to exert inhibition.

1.11 Associations between levels of PDE expression and inflammatory disease

In view of the inhibitory effect of cAMP on a range of responses of inflammatory cells, a number of studies have compared PDE expression in cells from normal donors with cells from patients with inflammatory diseases. There have been several reports suggesting increased PDE levels in cells from patients with atopic dermatitis, and it has been speculated that this may be a characteristic of atopy. These reports show that peripheral blood mononuclear cells (PBMC) from atopic dermatitis patients have decreased responses to cAMP elevating agents such as β_2 agonists, histamine and prostaglandins (PGE) (Safko et al. 1981). The decreased responsiveness to cAMP raising agents was linked to increased PDE levels in monocytes. Published work demonstrated that monocytes from patients with atopic dermatitis have higher PDE activity than corresponding cells from normal donors (Chan et al. 1993; Holden et al. 1986). It was also shown that leukocytes from patients with atopic dermatitis have higher PDE activity in comparison to those from normal donors (Grewe et al. 1982). This theory, however, is not supported by evidence from other investigators. Other published reports failed to show any increase in PDE activity levels in eosinophils, monocytes and T cells in cells from patients with atopic dermatitis, compared to normal cells (Gantner et al. 1997a). Whilst showing no increase in PDE levels in PBMC from atopic dermatitis patients, it is interesting to note that Banner et al.(1995) demonstrated that these PBMC have greater sensitivity to the PDE4 inhibitor rolipram (Banner et al. 1995c), perhaps suggesting a different enzyme conformation.

There have, as yet, been no reports to suggest that cells from asthmatic subjects have raised PDE levels. A published report has shown that alveolar macrophages from asthmatic subjects show no difference in levels of PDE activity in comparison to cells from normals (Tenor et al. 1995a).

1.12 Hypotheses

The hypotheses investigated in this thesis are that inflammatory cells from allergic asthmatics express a different profile, or a different subcellular distribution, of cAMP hydrolysing phosphodiesterase enzymes from the same cells from normal donors. Also that the intracellular location of individual PDE4 isoforms may be closely linked to the distribution of elements of the cAMP signalling cascade e.g. PKA. Finally, that agents capable of manipulating cAMP levels within cells e.g. PDE4 inhibitors, can effect the production of tPA and its inhibitor PAI-1 in bronchial epithelial cells thereby affecting the proteolytic/fibrinolytic balance within the airways.

1.13 Aims

The aims of this thesis are to investigate the profile of PDE enzymes in inflammatory cells and their intracellular location. Also to investigate the production of tPA and PAI-1 by bronchial epithelial cells. These investigations will use:

- RT-PCR to assess mRNA expression
- Immunohistochemistry to assess expression of PDE in bronchial tissue
- Western blotting to assess protein expression
- SPA to assess enzyme activity
- Confocal microscopy to assess intracellular localisation of PDE4 and PKA within primary bronchial epithelial cells.
- Specific ELISAs to measure production of tPA and PAI-1 in the cell culture supernatants of primary human bronchial epithelial cells.

Chapter 2

RT-PCR analysis of PDE mRNA expression

2.1 Introduction

PDE4 enzymes are of particular interest in the regulation of cell responses as they are the predominant cAMP hydrolysing PDE expressed in inflammatory cells. There are four distinct gene products that code for members of the PDE4 isoform family, and within these genes there is the ability, by alternative splicing, to generate individual splice variants. The published reports of the expression of these individual PDE4 isoforms within inflammatory cells are detailed in table 2.1.

Table 2.1 A summary of published findings for mRNA expression of the PDE isoforms in inflammatory cells or their related cell lines.

Cell Type	PDE4A	PDE4B	PDE4C	PDE4D	PDE7	Reference
Human eosinophils	+	+	-	+	n.d	Reviewed by Engels 1994, Gantner 1997a
Guinea pig eosinophils	-	-	-	+	n.d	Reviewed by Giembycz 1997a
Human neutrophils	+/-	+++	-	+	n.d	Reviewed by Giembycz 1997a
PBMC	n.d	n.d	n.d	D1,D2,D3	n.d	Nemoz et al 1996
Human monocytes	++	++	-	+/-	n.d	Manning et al 1996
U937	+	+	-	+	n.d	Reviewed by Engels 1994
Human T cells	A4	B1	-	D3	+	Seybold et al 1998
Human CD4+ cells	+	+	-	+	+	Giembycz et al 1996
Human CD8+ cells	+	+	-	+	+	Giembycz et al 1996
Jurkat T cells	+	-	-	-	-	Seybold et al 1998
Human B cells	+	B2	-	+	+	Gantner et al 1998
Epithelial cell line A549	+	-	-	D3	+	Wright et al 1998
Human airway epithelial cells	+	-	-	D3	+	Wright et al 1998
Human airway epithelial cells	A5	-	C1	D2,D3	+	Fuhrmann et al 1999

Experiment not done is represented by (n.d) and absence of a positive signal by (-).

Table 2.1 illustrates that mRNA expression can vary between different inflammatory cells and the same cell in different species. The difference in PDE4 mRNA splice variants, expressed by different inflammatory cells, might suggest that cells have a unique pattern of

PDE4 isoform expression that results in their varying biological functions. In addition, comparison of PDE expression between the same cell type derived from different species, guinea pig peritoneal eosinophils versus human blood eosinophils, for example, shows that eosinophils from guinea pigs express only PDE4D, while human eosinophils also express 4A and 4B isoforms (reviewed by Engels et al. 1994; Giembycz et al. 1997), indicating species-specific differences in expression of these enzymes.

It is clear that caution should also be taken when interpreting results using cell lines as models for primary cells. For example, whilst some cell lines such as U937 cells (Engels et al. 1994) express the same PDE4 isoforms as their primary monocyte counterpart, (Manning et al. 1996), others, such as jurkat T cells (Seybold et al. 1998), do not. These jurkat T cells express only 4A but their primary counterpart, human T cells, express 4A, 4B and 4D (Seybold et al. 1998). When cell lines are considered, it should be noted that the immortalisation process necessary for their production may influence the expression of numerous proteins, which could include the protein being studied.

Attempts to quantify levels of this mRNA expression by semi-quantitive PCR should also be treated with caution, as in some cases the expression of the 'control' mRNA used as standard for this procedure can show variability between donors or treatment. For example the expression of the housekeeping gene, G3PDH, did not remain at a constant level in epidermal samples undergoing different culture conditions (Wu and Rees, 2000). Therefore, in this Chapter, observations were made only in primary cells and in a non-quantitative manner.

The specific aims of this Chapter were to investigate the mRNA expression of the cAMP hydrolysing PDEs, PDE4A, 4B, 4C, 4D, PDE7 and PDE8, and to compare their expression in inflammatory cells derived from normal, atopic and asthmatic donors. Using the reverse transcription polymerase chain reaction (RT-PCR) to enhance mRNA transcripts, PDE mRNA was analysed in peripheral blood eosinophils, neutrophils and PBMC.

2.2 Materials

Microfast track kits were purchased from Invitrogen (Leek, Netherlands). 10mM dNTPs, 10X PCR buffer w/o $MgCl_2$, single strand cDNA kit, 100bp DNA ladder and Taq DNA polymerase were from Promega (Southampton, UK). Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Percoll, $MgCl_2$, Tris, N-[hydroxyethyl]piperazine-N-[2-ethanesulphonic acid] (HEPES) and DEPC were purchased from Sigma (Poole, UK). Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) primers were purchased from Clontech (Basingstoke UK). All other primers were synthesised by Oswell (Southampton, UK). Phosphate buffered saline (PBS 10X), Hank`s balanced salt solution (HBSS 10X), 100bp DNA ladder and heat inactivated foetal bovine serum (FBS) were purchased from Gibco-BRL (Paisley, Scotland). Anti CD16, CD3 and CD14 immunomagnetic beads were purchased from Miltenyi Biotech (Germany).

Patient groups

Local ethics authority approval was obtained for venepuncture from three subject groups. The first group were normals, who were characterised as being non atopic, i.e. having no reaction to skin prick testing with common aeroallergens. The second, atopic, group were characterised by development of a skin wheal >3mm after exposure to common allergens. The final group were asthmatic donors, who were patients attending Southampton General

Hospital for other asthma studies by Dr Andrew Zurek, Dr William McConnell and Edward Halsey. These asthmatic subjects were all taking β_2 agonists to control their condition. The provocative concentration of methacholine, giving a 20% drop in forced expiratory volume in 1 second (PC_{20}) in these patients was measured and found to be in the range 0.07>16 mg/ml. In some cases forced expiratory volume in 1 second (FEV_1) fell below 80% of that predicted and, therefore, PC_{20} measurement was not carried out for these patients.

2.3 Methods

Cell isolation

Cells were isolated from 100ml EDTA anticoagulated venous blood. This blood was diluted with an equal volume of PBS/FBS buffer (1X PBS with 2% heat-inactivated foetal bovine serum) at room temperature. 20ml of 1.082g/ml-1.084g/ml density Percoll (90ml of Percoll was diluted by the addition of 10ml 10X HBSS containing 200μM Hepes and adjusted to pH 7.4 with 10M NaOH. This solution was adjusted to a final density of 1.082-1.084g/ml by the addition of 50ml 1X HBSS containing 20mM Hepes with pH 7.4) was layered underneath the diluted blood (25ml) in 50ml Falcon tubes. The tubes were then centrifuged at 600g for 30min at 20°C. After this time there were two distinct layers, separated by a white layer of cells. The upper layer of plasma was discarded.

PBMC

The upper white cell layer, which contained the peripheral blood mononuclear cells (PBMC) was carefully removed by pipette and re-suspended in 20 ml PBS/FBS buffer. The suspension was centrifuged at 770g for 10 min at 4°C. The pellet was finally re-suspended in 5ml of PBS/FBS buffer and left on ice.

The lower pink layer of Percoll was removed by aspiration and discarded. The remaining pellet contained red blood cells and leukocytes. Red blood cells were lysed hypotonically with 400ml distilled water for 45secs, after which time an equal volume of 1.8% NaCl was added to restore isotonicity. The intact leukocytes were then re-pelleted by centrifugation at 770g, 10min, 4°C and the supernatant removed. Remaining red blood cells were removed by repeating the lysis step with centrifugation at 380g, 5min, 4°C. The white cell pellet was then resuspended in 1ml PBS/FBS buffer and incubated on ice for 30min with 100µl anti-CD16 beads, 10µl anti-CD3 and 10µl anti-CD14 immunomagnetic beads. The mixed leukocytes were applied to a MACS type C column equilibrated in ice cold PBS/FBS buffer.

Eosinophils

Eosinophils were eluted from the immunomagnetic column in 20ml PBS/FBS buffer. The cells were then pelleted by centrifugation at 770g for 5min at 4°C and resuspended in 1ml PBS/FBS buffer. Eosinophils routinely had a purity of between 97-99%, with the major contaminating cell being neutrophils.

Neutrophils

Neutrophils, which were bound via anti CD16 beads to the column, were eluted in 20 ml PBS/FBS buffer when the column was removed from the magnet. They were then pelleted and resuspended in 5ml PBS/FBS buffer. Their purity was between 98-99% with contaminating cells being T cells or monocytes bound to the anti CD3 or anti CD14 beads respectively.

Cell counts

Cell counts were performed using 10μl of the cell suspensions, diluted with 90μl Kimura stain, and counted on a haemocytometer at X 400 magnification.

Poly A Messenger RNA extraction

This was extracted from 1ml of each cell suspension, as in the protocol of the microfast track polyA mRNA kit from Invitrogen. Briefly, cells were lysed in a detergent based buffer containing RNase/protein degrader, then applied to a Oligo cellulose column. DNA, cell debris and degraded proteins were washed off this column with a high salt buffer, and a lower salt buffer washed off any non-polyadenylated RNA. Finally polyadenylated RNA was eluted from the column by a no salt buffer. RNA was quantified by measuring absorbencies at 260nm in a Pharmacia GeneQuant spectrophotometer. Calculations of RNA concentration assumed that 40μg/ml RNA had an OD of 1.0 at 260nm, OD 260nm/OD280nm ratios were used as an index of RNA purity. 0.1μg of poly A mRNA was transcribed to single stranded cDNA using the cDNA kit from Promega, method as in instructions. Briefly, an oligo (dT) primer hybridises to the polyA tail of the mRNA, and primes the synthesis of DNA by a reverse transcriptase, which, in this case, is an avian myeloblastosis virus reverse transcriptase. A recombinant RNasin ribonuclease inhibitor was present during cDNA synthesis to prevent mRNA degradation. Polymerase chain reaction (PCR), concentrations of constituents illustrated in table 2.2, was then carried out in a Perkin Elmer 9600 thermal cycler. Reactions were run for 40 cycles in a total volume of 25μl in 0.2ml thin wall PCR tubes. Annealing temperatures and $MgCl_2$ concentrations used for individual primers are shown in table 2.3. Contamination from genomic DNA was excluded by omission of reverse transcriptase in control experiments.

Table 2.2. Constituents of PCR reaction.

	1mM $MgCl_2$	1.5mM	2mM	2.5mM	3mM	3.5mM
cDNA	0.1μg					
10X PCR buffer	2.5μl					
DMSO	1μl					
Primers (5pmoles)	1μl each					
Taq polymerase	0.2μl					
10X dNTP's	2μl					
25mM $MgCl_2$	1μl	1.5μl	2μl	2.5μl	3μl	3.5μl
DEPC H_2O	16.3μl	15.8μl	15.3μl	14.8μl	14.3μl	13.8μl

Table 2.3 Sequences and conditions of reaction for PDE primers

Primer code	Sequence	Anneal temp (degrees Celcius)	MgCl2
4A 27129	AGGAGGGACAAGAGGACAAGAGT	60	3.5mM
4A 27130	GTCGTTGGAAGTTATGGCACAGG	60	3.5mM
4B2 6967	AGCGGTGGTAGCGGTGACTCTG	60	3.5mM
4B2 6968	GGTCGACTGGGCTACATCAGCAGC	60	3.5mM
4D 5205	CAGAGTTGTCTGGTAACCGGC	56	3.5mM
4D 2506	GTTACGTGTCAGGAGAACG	56	3.5mM
7 5123	CTTACCATAACGCAGTCCACG	56	3.0mM
7 5120	GAAAGCCAAATTCATATTGCTC	56	3.0mM
8 B7599	TCTGATGGTTTGCGAAGACTATCA	56	2.0mM
8 B7602	GCGCGCTCTAGATGGTGTCTCCCACTATTCAGG	56	2.0mM

Analysis of PCR products was carried out on 2% agarose gels containing 0.5 μg/ml ethidium bromide in TAE buffer (0.04M Tris acetate, 0.001M EDTA). Gel electrophoresis was carried out at 100volts for 60 min. The product bands were then visualised with ultra violet light on a transilluminator.

2.4 Results

The results from ethidium bromide stained PCR gels show that all preparations of neutrophil (n=21) and PBMC (n=23) cDNA were intact, as demonstrated by the expression of the G3PDH house keeping gene. The cDNA from eosinophil samples, however, was not always intact, with only 18 out of 42 samples tested giving a positive signal for G3PDH. This observation of loss of integrity of eosinophil cDNA was also observed using primers for adenine phosphoribosyltransferase (APRT), another housekeeping gene.

The results in tables 2.4-2.9 therefore only include sample numbers where a positive housekeeping signal was obtained. Figures 2.1-2.4 show the size, in base pairs, of the PCR products for the respective PDE isoforms.

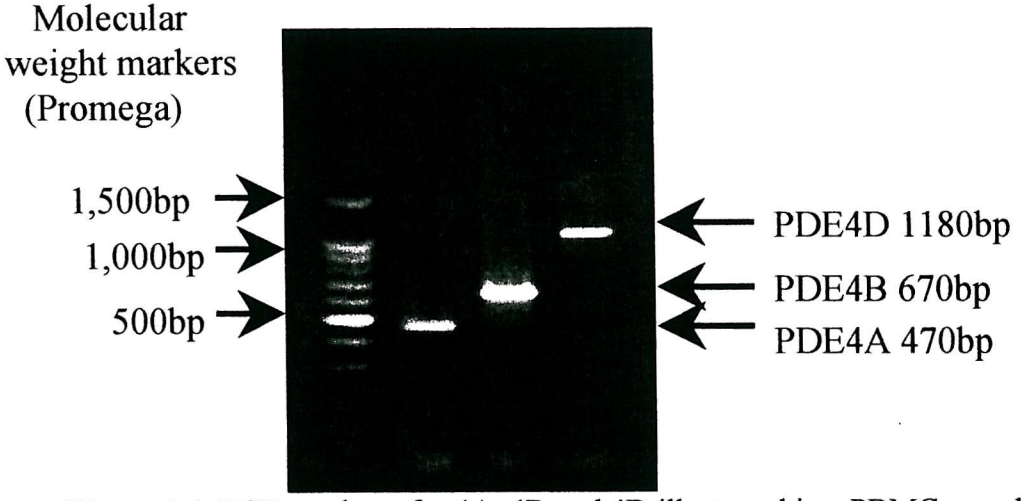


Figure 2.1 PCR products for 4A, 4B and 4D illustrated in a PBMC sample

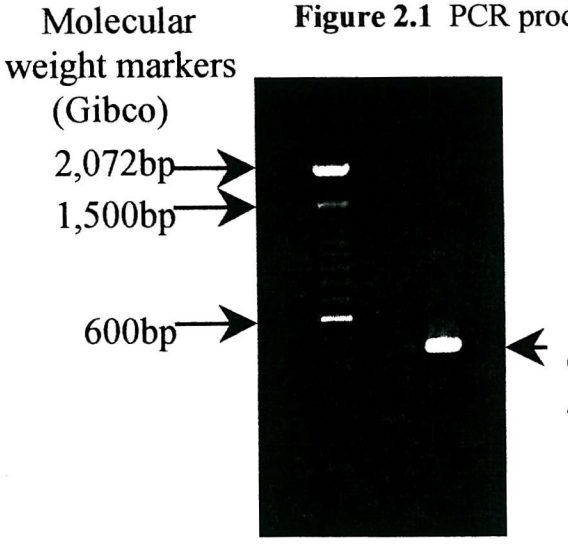


Figure 2.2 PCR product for G3PDH, illustrated in a eosinophil sample

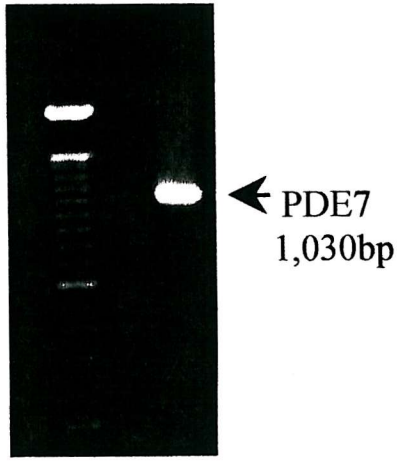


Figure 2.3 PCR product for PDE 7, illustrated in a PBMC sample

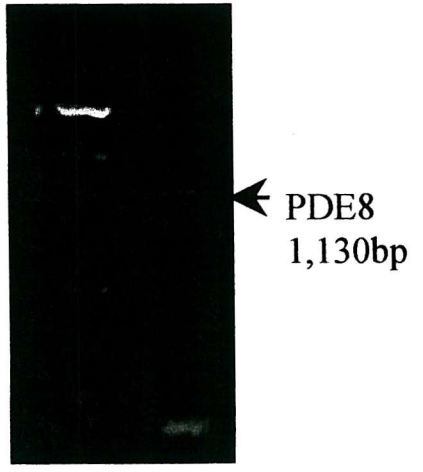


Figure 2.4 PCR product for PDE 8, illustrated in a PBMC sample

Table 2.4 Transcription of PDE4A genes in inflammatory cells

Cells	Normal	Asthmatic	Atopic
Eosinophils	2 of 3	7 of 10	4 of 5
Neutrophils	0 of 4	0 of 11	3 of 6
PBMC	0 of 5	7 of 12	5 of 6

The results in table 2.4 demonstrate that PDE4A mRNA is detected in the majority of eosinophil samples from normal, asthmatic and atopic donors. The detection of 4A within neutrophil samples is less uniform; there appears to be no expression in neutrophils from either normal or asthmatic subjects. However, half of the neutrophils from atopic patients did express 4A mRNA. Expression of 4A in PBMC, again, is not uniform, with no detection in PBMC from normals, detection in a half of asthmatic samples, and 5 out of 6 PBMC samples from atopic donors.

Table 2.5 Transcription of PDE4B2 genes in inflammatory cells.

Cells	Normal	Asthmatic	Atopic
Eosinophils	2 of 3	5 of 10	4 of 5
Neutrophils	0 of 4	1 of 11	5 of 6
PBMC	0 of 5	4 of 12	5 of 5

Detection of PDE4B2 mRNA is demonstrated in table 2.5. In at least half of all eosinophil samples from all donors mRNA for 4B2 was detected; however, the level of expression observed in neutrophils is less than that in eosinophils. In neutrophils from normal donors there is no evidence of expression, and only 1 out of 11 samples from asthmatic donors gave a positive signal. Neutrophils from atopic subjects showed 4B2 expression in 5 out of 6 cases. This pattern of no expression in normal donors, and low expression in asthmatic

donors, was repeated for PBMC samples and, again, samples from atopic donors expressed 4B2 in 5 out of 6 cases.

Table 2.6 Transcription of PDE4C genes in inflammatory cells

Cells	Normal	Asthmatic	Atopic
Eosinophils	0 of 4	0 of 5	0 of 1
Neutrophils	0 of 1	0 of 1	0 of 1
PBMC	0 of 1	0 of 1	0 of 1

Messenger RNA expression for PDE4C was not detected in any of the samples from eosinophils, neutrophils and PBMCs. The numbers for the analysis of this isoform are low because the initial experiments confirmed published results; namely, that PDE4C is not expressed in inflammatory cells and, thus, analysis of this isoform was discontinued.

Table 2.7 Transcription of PDE4D genes in inflammatory cells

Cells	Normal	Asthmatic	Atopic
Eosinophils	2 of 3	4 of 10	4 of 5
Neutrophils	0 of 4	0 of 11	3 of 6
PBMC	0 of 5	9 of 12	5 of 6

Table 2.7 demonstrates that 4D mRNA was detected in eosinophil samples from all donors. No expression of 4D was observed in neutrophils from normal or asthmatic donors, and only half of the atopic neutrophil samples gave a positive signal for PDE4D. Normal PBMC samples showed no expression of 4D; however, at least two thirds of asthmatic and atopic PBMCs did give a positive signal

Table 2.8 Transcription of PDE7 genes in inflammatory cells

Cells	Normal	Asthmatic	Atopic
Eosinophils	2 of 2	3 of 3	4 of 4
Neutrophils	0 of 4	0 of 11	4 of 6
PBMC	1 of 5	5 of 12	4 of 6

Table 2.8 clearly shows that whilst eosinophil samples from all donors express mRNA for PDE7, only neutrophils from atopic donors gave a positive signal for this PDE. Approximately a half of all PBMC samples from asthmatic and atopic donors expressed PDE7; however, little expression was observed in PBMC from normal donors.

Table 2.9 Transcription of PDE8 genes in inflammatory cells

Cells	Normal	Asthmatic	Atopic
Eosinophils	0 of 2	0 of 2	0 of 4
Neutrophils	0 of 4	0 of 11	0 of 4
PBMC	0 of 5	2 of 12	1 of 6

Expression of mRNA for PDE8, as shown in table 2.9, appears to be low for most cells. There is no evidence of PDE8 expression in eosinophils, and neutrophils and only a few cases of expression in PBMC from asthmatic and atopic donors.

2.5 Discussion

The results in this Chapter demonstrate that eosinophils express mRNA for PDE4A, 4B, 4D and PDE7 in cells from normal, asthmatic and atopic donors. The expression of PDE4 isoforms in these cells is in agreement with published reports (Engels et al, 1994; Gantner et al. 1997a) and the mRNA expression of PDE7 is a novel finding. The extraction of intact

mRNA from eosinophil samples proved to be challenging, with only 18 from 42 samples expressing a positive signal for a housekeeping gene. An integral function of eosinophils is the destruction of invading parasites and, for this purpose, they contain a large armoury of agents that can digest and degrade cells. Of this armoury, eosinophils are known to contain eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN), both of which are basic proteins with ribonuclease activity that is capable of completely degrading tRNA and rRNA (reviewed by Spry, 1988). The extraction of intact mRNA from these cells is therefore difficult. Whilst ribonuclease inhibitors were routinely used in the extraction of mRNA, degradation was still observed. It may therefore be the case that eosinophils contain higher levels of degradative enzymes than are ineffectively inhibited, or ribonucleases that are not affected by these inhibitory compounds. The challenge therefore is to neutralise these ribonucleases from eosinophils, thus allowing extraction of consistently intact mRNA.

Whilst the integrity of mRNA from neutrophils was consistently high, the expression of individual PDE isoforms showed variation within cells from different subject groups. PDE4A mRNA was not detected in neutrophils from normal or asthmatic donors, and in only a half of cells from atopics. This weak or absent expression links with published reports of either weak or no expression of this isoform in normal human neutrophils (reviewed by Giembycz et al. 1997; Engels et al. 1994). PDE4B was the most widely expressed PDE4 isoform in the cells examined in this study but even this isoform, was not detected in normal neutrophils, and in only 1 of 11 neutrophil samples from asthmatic donors. 4B was, however, expressed in 5 out of 6 neutrophil samples from atopics. Published reports have demonstrated that this 4B isoform is predominantly expressed in normal neutrophils (Giembycz et al. 1997; Engels et al. 1994); therefore, it is surprising that

we did not see expression in all samples. This might imply that, under the conditions of the PCR reaction, the number of PDE4B mRNA transcripts is greater in neutrophils from atopic donors than in either of the other subject groups. Neutrophil samples, again, showed weak expression of PDE4D, being detected in only half of the samples from atopics. However, weak expression has been previously reported in neutrophils; therefore this is as expected. Expression of PDE7 within neutrophils was only evident in two thirds of samples from atopics, and no visible bands were detected for samples from normal or asthmatic donors.

The number of positive PBMC mRNA samples was greater in cells from atopic and atopic asthmatic donors in comparison to those from normals, and PDE4A, 4B and 4D mRNA was observed in PBMC samples from asthmatic and atopic donors. PDE7 was, again, observed in PBMC from asthmatics and atopics, and even in 1 from 5 PBMC samples from normals. Message for PDE8, a more recently sequenced PDE, was observed in a few PBMC samples from asthmatics and atopics. This is a novel finding, and could represent weak expression of this PDE. The detection of these PDE4 isoforms, and of mRNA for PDE7, has previously been reported in individual cellular components of PBMCs. Manning et al 1996, reported PDE4A, 4B and 4D in human monocytes, and Seybold and Giembycz reported the same PDE4 isoforms as well as PDE7 in T cells (Seybold et al. 1998; Giembycz et al. 1996).

The apparent higher expression of PDE4 (in terms of number of positive samples), observed in atopic neutrophils and PBMC is intriguing. This may be linked to increased transcription of these PDE4 isoforms or increased stabilisation of already transcribed mRNA. It is interesting to note that Gantner et al reported increased mRNA expression for PDE4A and 4B2 in T cells from atopic dermatitis patients (Gantner et al. 1997a). This increased mRNA

expression in atopic T cells, however, was not related to increased protein expression or activation. Care must therefore be taken when extrapolating any findings from mRNA expression into actual enzyme levels. This is particularly true for PDE7 expression, whose mRNA is expressed at relatively high levels, whereas its protein is barely detectable or absent (Li et al. 1999).

Nevertheless, our reports of PDE7 mRNA expression in all eosinophil samples and PDE8 in some PBMC samples are novel, and investigations to see if these cells also express the corresponding proteins are necessary. The different distribution patterns of the mRNA for these cAMP hydrolysing PDEs, between inflammatory cells as reported in this chapter, also supports published reports detailed in table 2.1. As inflammatory cells appear to express individual mRNA profiles for PDE isoforms and even splice variants of these isoforms, this may suggest cells tailor their expression of these enzymes, depending upon the biological process within the cell that these PDEs regulate.

Chapter 3

Immunohistochemical analysis of PDE expression

3.1 Introduction

The airways of patients with allergic asthma are widely reported to express several morphological features characteristic of the disease. Tissue from asthmatic bronchi shows increased numbers of infiltrating eosinophils which, together with mast cells, also show an increased activation state. A thickening of the subepithelial basement membrane by collagen deposition is another characteristic (Djukanovic et al. 1992). Increased eosinophil numbers in asthmatics are often observed in the bronchoalveolar lavage (BAL), and their numbers show a relationship to asthma severity. However, it is also interesting to note that in some patients with mild asthma there are no increased eosinophil numbers in the BAL (reviewed by Liu and Calhoun, 1998). The ability to visualise these morphological changes in asthmatic tissue is made possible by the technique of fibreoptic broncoscopy, which allows small sections of bronchial tissue to be removed from living patients. Cells within these biopsies are then visualised by immunohistochemistry.

Together, these techniques presented the possibility of investigating the expression and localisation of the phosphodiesterase enzymes in the bronchi of asthmatic patients. It also enabled us to make direct comparisons of this expression with that seen in the bronchi of non atopic, non asthmatic volunteers (normal control subjects). Further, immunohistochemical investigation of bronchial biopsy specimens enabled a comparison of the expression of PDEs in inflammatory cells infiltrating airway tissues, with expression in the same cells in the blood.

Immunohistochemistry also allows visualisation within the tissue of individual cells at high magnification and, hence, permits the localisation of the PDEs to specific subcellular areas within these cells. For example, this feature of immunohistochemical analysis was

harnessed by Jin et al to localise PDEs to intracellular regions of a thyroid cell line. This group reported staining for PDE4D in distinct perinuclear areas, and in association with the plasma membrane of the quiescent thyroid cell line (FRTL-5) (Jin et al. 1998).

The technique of immunohistochemistry requires fixation of biopsies in acetone containing protease inhibitors in order to preserve the antigenicity of the proteins of interest. The fixed biopsies are subsequently embedded in glycol methacrylate (GMA), a resin which allows thin, 2μm, sections to be cut. Colocalisation of two antigens within a single cell is therefore possible, since cells are approximately 10μm in diameter, and sequential 2μm sections through the same cell may be cut and stained with different antibodies in order to determine if co-expression of different antigens occurs (Britten et al. 1993). There are, however, drawbacks to this technique. Bronchial biopsies are approximately1-2mm^3 in size and, as such, may not be representative of the whole organ of interest as, in this case of the airways, the surface area is comparable in size to half a tennis court. Another disadvantage of this technique is that it allows only a 'snap shot' of the disease process at a single time point, as the technique of biopsy retrieval is too invasive to be carried out at many sequential time points. However, even with these drawbacks, examination of bronchial biopsies by immunohistochemistry can yield valuable information on the *in vivo* expression of PDEs. This is of real importance when studying PDEs, as RT-PCR analysis yields information on the mRNA expression only, which is often unrepresentative of protein expression. Also, Western blotting and the enzyme activity assay (SPA) require lysis of the cells, and so intracellular localisation is limited.

This Chapter, therefore, aims to examine the *in vivo* expression of PDE, at the protein level, within bronchial tissue in normal and asthmatic patients using immunohistochemistry, in

order to test the hypothesis that within inflammatory cells expression may differ, when compared to that seen in peripheral blood cells.

3.2 Materials

Sodium chloride, tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid, hydrogen peroxide, DPX and sodium azide were all purchased from BDH (Poole,UK). Dulbecco's modified Eagle's medium and foetal calf serum were purchased from Gibco-BRL (Paisley Scotland). The diaminobenzidine (DAB) kit used was purchased from Biogenex (California,USA) and the streptavidin- biotin horseradish peroxidase reagents were purchased from Dako (High Wycombe UK). Bovine serum albumin was purchased from Sigma (Poole UK). Crystal mount was purchased from Biomedia (California USA).

3.3 Methods

3.3.1 Bronchial biopsies

Bronchial biopsies were obtained from patients with mild asthma, receiving treatment with β agonists only as required (forced expiratory volume in 1 second FEV_1 84-118% and provocative concentration of methacholine causing a 20% drop in this FEV_1 0.138->16) and normal non-atopic control subjects. These biopsies were obtained by fibreoptic broncoscopy as previously described ((Djukanovic et al. 1990) and National Institute Health guidelines), and were provided by Dr Will McConnell, Medical Specialities, Southampton General Hospital. These biopsies had been fixed in acetone containing protease inhibitors, and embedded in GMA. Sections of 2μm were cut on an ultramicrotome and floated out on 0.2% ammonia for 90 sec which allowed better presentation of antigens. The sections were then picked up on poly L-lysine coated slides, and dried at room temperature for 60 *min*,

before wrapping in aluminium foil and storage at -20°C for a maximum of 14 days, prior to staining.

3.3.2 Staining procedure

Endogenous peroxidase was blocked using a blocking solution containing 0.1% sodium azide and 0.3% hydrogen peroxide, which was applied to the slides for 30 *min*. After this time the slides were washed with Tris buffered saline (sodium chloride 0.15M, Tris 50mM and pH adjusted to 7.6) by covering the slides in buffer for 5 *min* then draining. This was repeated 3 times. Blocking medium (bovine serum albumin 1% in 80ml Dulbeccos modified eagles medium and 20ml foetal calf serum (supplemented with 5% swine serum when staining with polyclonal antibodies)) was applied for 30 *min*; then the slides were drained.

Primary antibody to the PDE isoenzyme or specific cell type was then applied at the concentration shown in Table 3.1 and left at room temperature overnight, covered with coverslips, for monoclonal antibodies or for 60 *min* for polyclonal antibodies. Slides were washed three times with TBS, as before, and drained. Appropriate biotinylated secondary antibodies (see Table 3.1) were then applied and incubated at room temperature for 90 *min* for monoclonal antibodies, and 60 *min* for polyclonal antibodies. Slides were washed with TBS, as before, and drained. Streptavidin-biotin-peroxidase complex (1:200 dilution in Tris-HCl buffer (0.2M Tris buffer, 0.1M HCl pH 7.6)) was then applied for 90 *min* for monoclonal, and 60 *min* for polyclonal primary antibodies. Slides were again washed with TBS and then drained. DAB chromagen (prepared as instructed in the kit, with addition of 0.3% sodium azide) was applied for 10 *min*. Subsequently, slides were rinsed with TBS and left in running tap water for 5 *min*, after which they were counterstained in Mayer`s haematoxylin to stain the nuclei for 1 min and then rinsed in running tap water for 5 *min*.

Crystal mount was applied after slides had been drained, and this was baked at 80°C for 10 *min.* Slides were then allowed to cool and mounted in DPX and coverslipped.

3.3.3 Titration of primary antibodies

This was particularly important as the PDE antibodies had been used previously for Western blotting, but not for immunohistochemistry. Primary antibodies were titrated on nasal polyp tissue and tonsil, as these two tissues were expected to contain a large number of inflammatory cells that express phosphodiesterases. Serial dilutions of primary antibodies were applied to tissue sections until a dilution gave clear staining with no background. Regarding the chromagen used, diaminobenzidine (DAB) was found to give less non specific staining than aminoethylcarbazole (AEC) for the antibodies used and also allowed image analysis. Stringent negative controls were required to assure the specificity of the polyclonal antibodies used to detect PDEs. Thus, a pool of total rabbit immunoglobulins purified from non immune serum were used at the same concentration of protein as the rabbit primary polyclonal antibody. Similarly, for the monoclonal antibodies, matched mouse immunoglobulins were used. It was imperative that these controls showed no staining in order to confirm that staining observed with antibodies was specific. Swine serum was added into the initial blocking solution for the polyclonal antibodies in order to block any non-specific staining resulting from the swine anti-rabbit secondary antibody. In order to reduce the non-specific staining of sections with affinity purified PDE7 antibody, 0.05% Tween 20 was added into the TBS wash buffer. This removed most of the non-specific staining associated with this antibody.

3.3.4 Quantification

The total mucosal area (excluding mucus glands, damaged tissue and epithelium) was measured using a Color Vision 164 SR image analysis system (Analytical Measurement

Systems, Cambridge). Positively stained nucleated cells were counted using a Zeiss light microscope at 400 fold magnification. Cell counts were expressed as positive cells per mm^2 of musosa. The percentage of the epithelial area staining positively for PDE was measured using the ColorVision image analyser, and expressed as a percentage of total epithelial area, also measured on the ColorVision system. Where possible, five different fields were analysed for each section and the mean of these values is reported.

3.3.5 Statistical analysis

Mann Whitney U, non- parametric analysis, was carried out to determine if there was a significant difference in numbers of inflammatory cells and staining for PDEs between biopsies from asthmatics, in comparison to those from normals.

Antibody	Polyclonal/ Monoclonal	Species raised in	Raised against	Purification	Dilution	Source
PDE4A	Polyclonal	Rabbit	Last 161αα of C terminal of 4A	Affinity purified	1:100	Celltech
PDE4B	Polyclonal	Rabbit	Peptide from C terminal of 4B	Serum	1:250	Celltech
PDE4C	Polyclonal	Rabbit	Peptide from C terminal	Serum	1:225	Celltech
PDE4D	Monoclonal	Mouse	Last 91 αα of C terminal of 4D	Affinity purified	1:150	Celltech
PDE7	Polyclonal	Rabbit	Peptide from C terminal of PDE7	Serum	1:1000	Celltech
PDE7	Polyclonal	Rabbit	Peptide from C terminal of PDE7	Affinity purified	1:30	Celltech
EG2 Eosinophils	Monoclonal	Mouse	ECP		1:200	Pharmacia
CD14 Monocytes	Monoclonal	Mouse	Monocyte endotoxin receptor		1:8	DAKO
CD3 Pan T cells	Monoclonal	Mouse	CD3 receptors		1:100	DAKO
AA1 Mast cells	Monoclonal	Mouse	Tryptase		1:1000	DAKO
Biotinylated rabbit anti-mouse	Monoclonal	Rabbit	Mouse immunoglobulins	Affinity purified	1:300	DAKO
Biotinylated swine anti-rabbit	Monoclonal	Swine	Rabbit immunoglobulins	Affinity purified	1:300	DAKO

Table 3.1 Panel of antibodies used to detect PDE isoenzymes and co-localise them to specific cell types

3.4 Results

Bronchial biopsy tissue samples were available from normal control subjects (n = 5) and patients with mild asthma (n = 6). Eosinophilia is often associated with asthma, with increasing numbers associated with more severe asthma. However, normal eosinophil numbers have also been reported in mild asthmatic patients. The FEV_1 indicated that the patient group had mild disease. Therefore, the number of eosinophils, as well as mast cells, T cells and monocytes in the bronchial submucosa, were counted in the biopsies to further estimate the severity of their asthma. Immunohistochemical staining for EG2 positive cells (figure 3.1) showed that eosinophils were present in the basal layer of the epithelium. There was no statistically significant difference in the number of eosinophils in the biopsies from the group of patients with asthma or the group of control subjects (figure 3.5).

CD3 positive T cells were present in the mucosa (figure 3.2) of all biopsy samples; however, their numbers were not statistically different between biopsies from asthmatics and normals (figure 3.6). Tryptase positive mast cells were present in the submucosa of biopsy samples (figure 3.3) and, again, their numbers were not statistically different between biopsies from asthmatic or normal donors (figure 3.7). Finally, a few CD14 positive monocytes were found in the submucosa of these biopsies (Figure 5.4), and they showed no difference in numbers between biopsies derived from asthmatic or normal donors (figure 3.8).

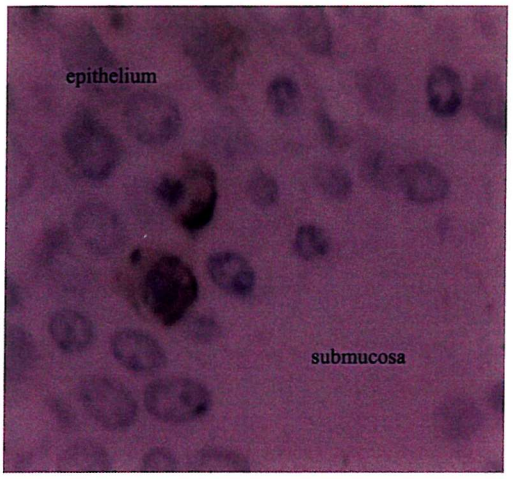


Figure 3.1 Eosinophils in bronchial epithelium of an asthmatic subject EG2 staining viewed at x 630 magnification.

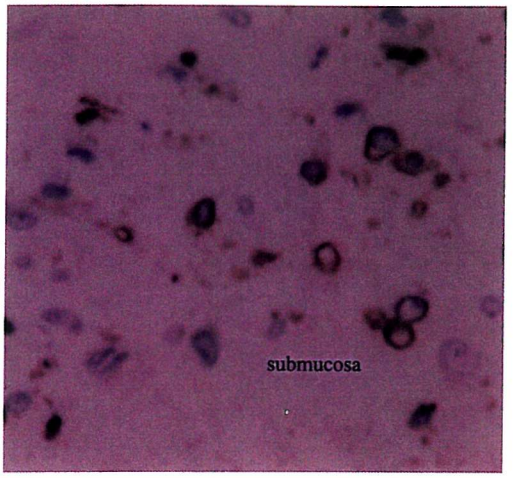


Figure 3.2 T cells in bronchial submucosa of an asthmatic subject CD3 staining of T cells viewed at x 400 magnification.

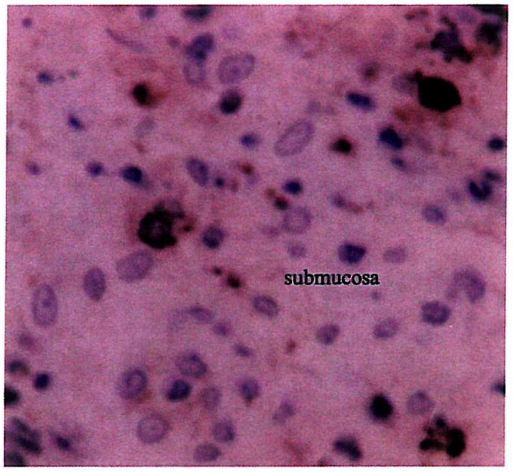


Figure 3.3 Mast cells in bronchial submucosa of an asthmatic subject. AA1 staining of tryptase +ve cells viewed at x 400 magnification.

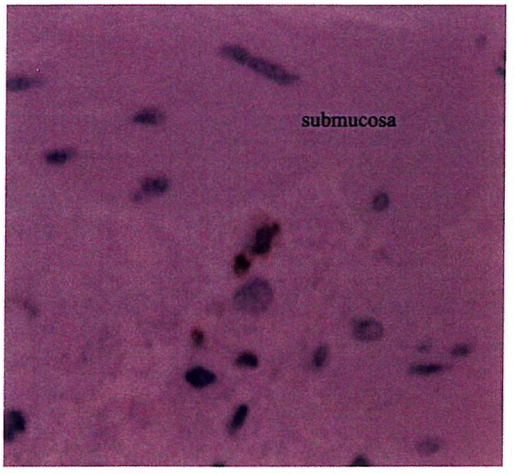


Figure 3.4 Monocytes in bronchial submucosa of an asthmatic subject. CD14 staining viewed at x 400 magnification.

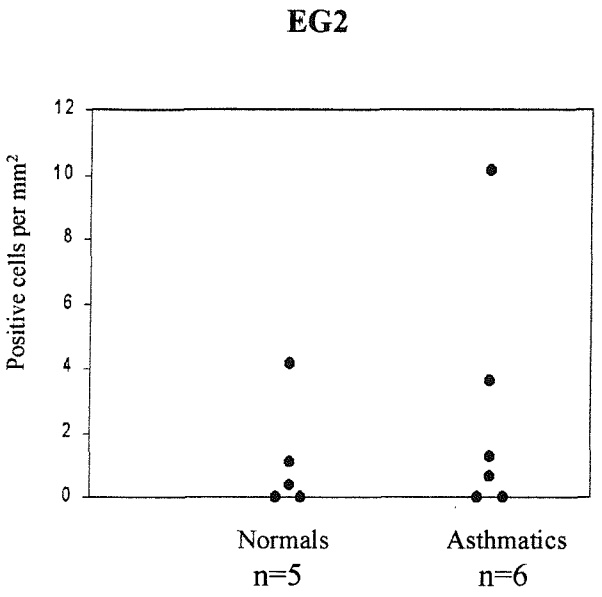


Figure 3.5 Eosinophils

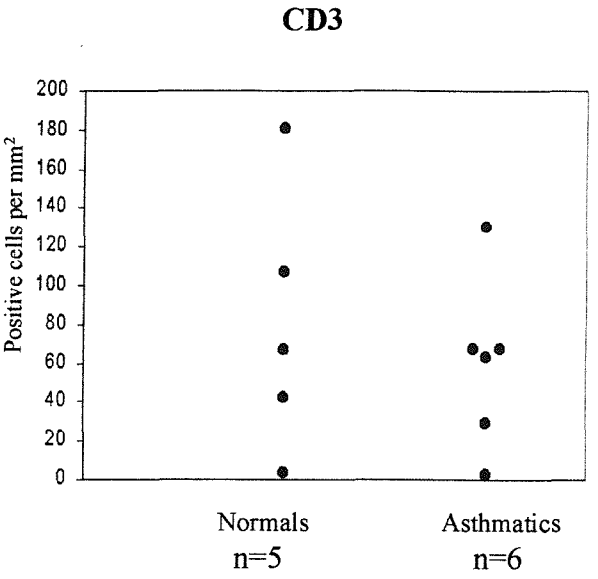


Figure 3.6 T cells

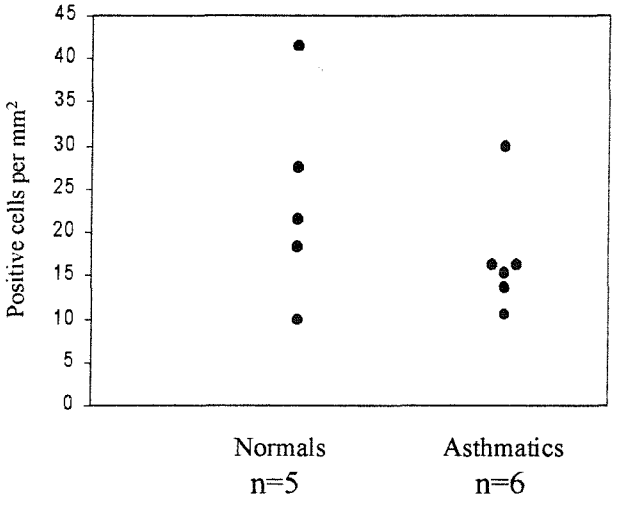


Figure 3. 7 Mast cells

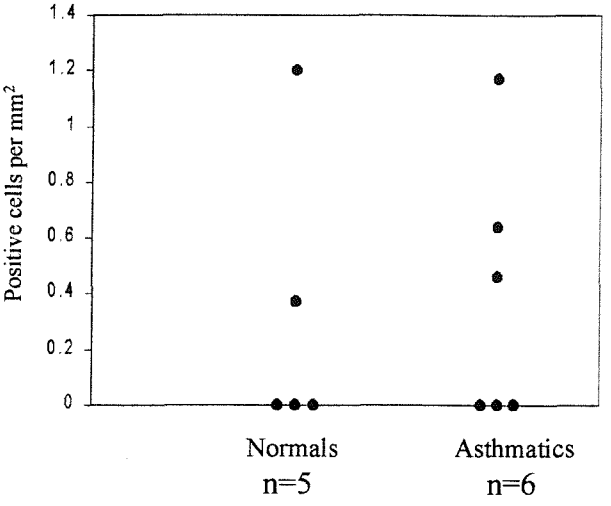


Figure 3.8 Monocytes

Figures 3.5-3.8 Counts of inflammatory cells in biopsies expressed as cells per mm^2. Significance was tested using the Mann Whitney U test. There were no significant differences in cell counts between normal and asthmatic patients for any of these inflammatory cells.

3.4.1 Expression of phosphodiesterase enzymes in bronchial biopsies

3.4.1.1 PDE4A

With the exception of a few cells in the submucosa that could not be colocalised to a particular cell type, all PDE4A staining was visualised in the epithelium (Figure 3.9). PDE4A staining was evident in basal epithelial cells and columnar epithelial cells. The cell staining appeared to be on the outer membrane of the cells, with some cells also showing staining on the nuclear membrane. This pattern of cell staining did not change between asthmatics or normals. There was no significant difference in percentage positive epithelial staining for PDE4A between normal and asthmatic subjects, as illustrated in figure 3.10

3.4.1.2 PDE4B

All PDE4B staining was observed in the epithelium, as illustrated in figure 3.11. Epithelial staining was predominantly in the columnar epithelial cells, and PDE4B staining was on the nuclear membrane of these cells, and was also visualised over the nucleus. There was a significantly higher percentage of positive epithelial cells staining for PDE4B in normals than asthmatics ($p<0.05$)

3.4.1.3 PDE4C

This antibody gave no staining above the preimmune serum control, when used at the same protein concentration. Therefore, it was assumed that no cell was expressing PDE4C at the protein level, in line with observations regarding mRNA expression of this enzyme.

3.4.1.4 PDE4D.

Figure 3.13 illustrates that PDE4D staining in the epithelium was consistently seen as a small distinct disc pattern of staining over the nuclei. This was quantified by counting the

number of positively stained cells per mm^2 area of epithelium. This PDE4D staining was present in both the basal and columnar cells of the epithelium. There is also evidence of cytoplasmic staining in some columnar cells. There are no significant differences in the number of epithelial cell staining for PDE4D between normal and asthmatic patients, as shown in figure 3.14. There was also evidence of positive staining of cells within the submucosa for PDE4D and, again, there were no differences in numbers of positive cells between normals and asthmatics.

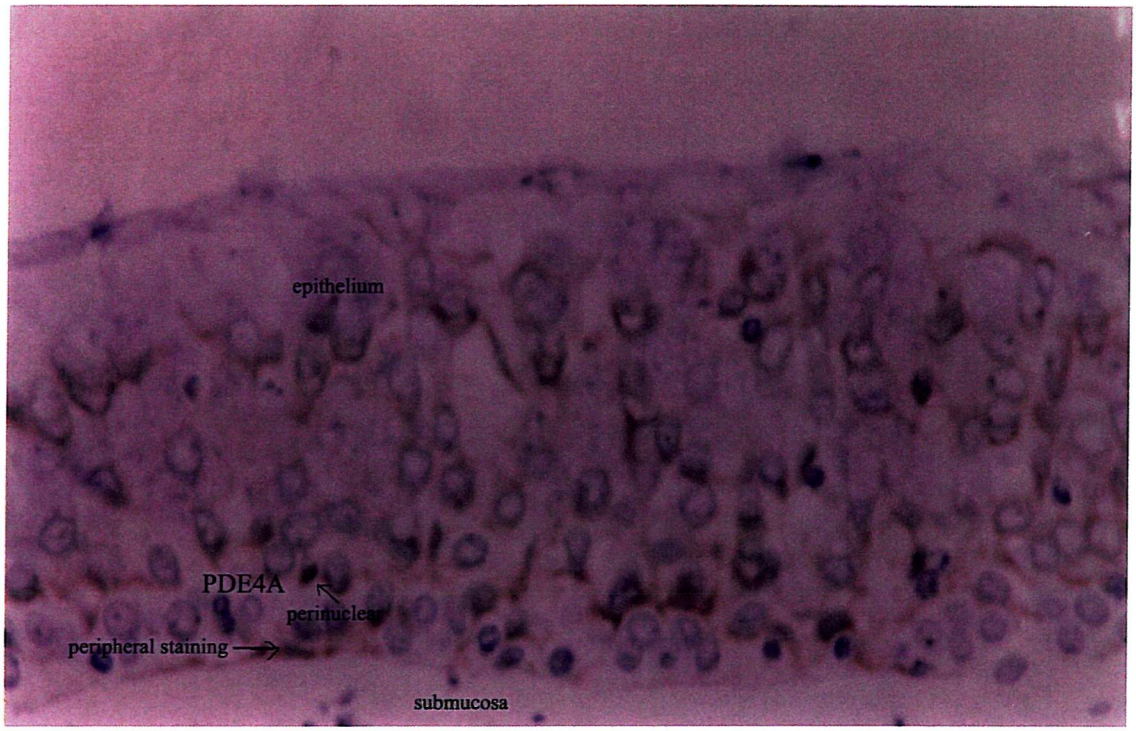


Figure 3.9 PDE4A staining of bronchial epithelium from a normal subject (x400 magnification). Arrows show PDE4A within the perinuclear region of epithelial cells and also peripheral epithelial cell staining.

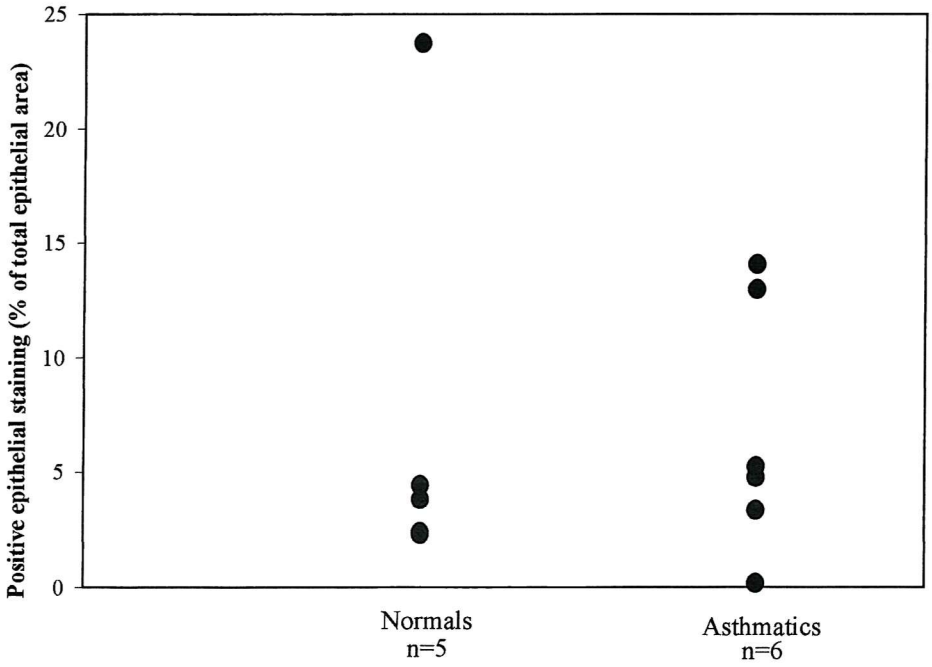


Figure 3.10 Epithelial staining for PDE4A. Significance was tested by Mann Whitney U. There were no significant differences in % of total epithelium stained positive for PDE4A between asthmatic or normal biopsies.

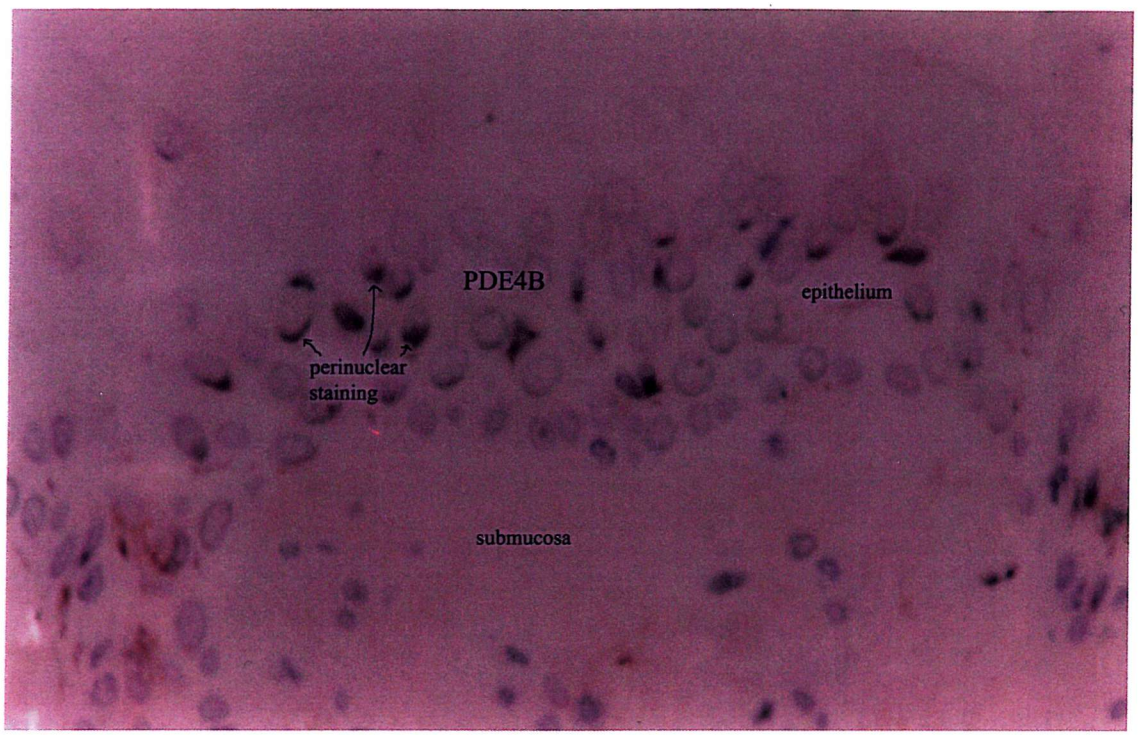


Figure 3.11 PDE4B staining of bronchial epithelium from a normal subject (x400 magnification). Arrows show PDE4B staining within the perinuclear region of the epithelial cells.

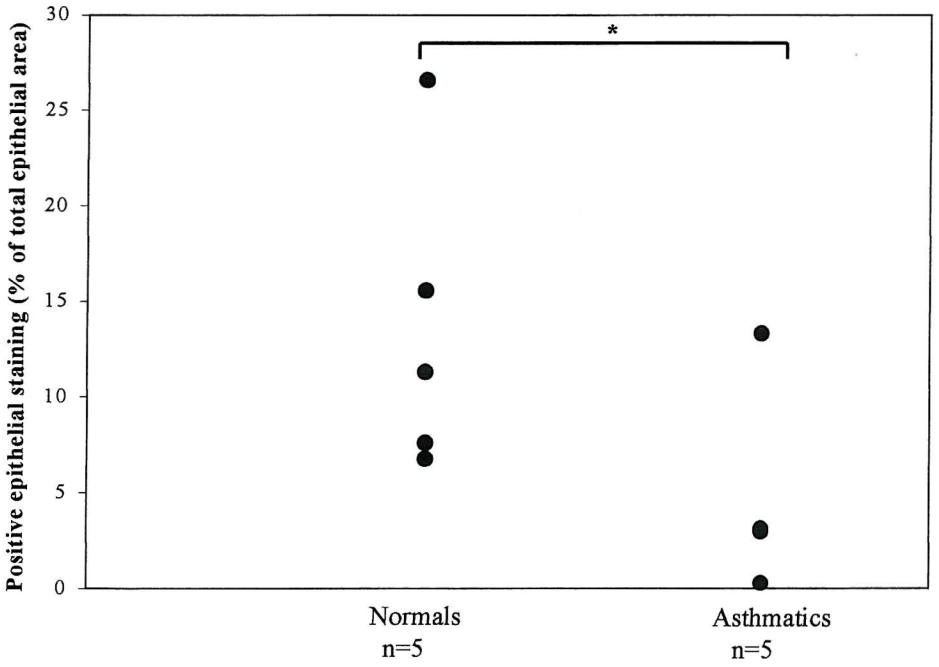


Figure 3.12 Positive epithelial staining for PDE4B. * represents $p<0.05$ Mann Whitney U test.

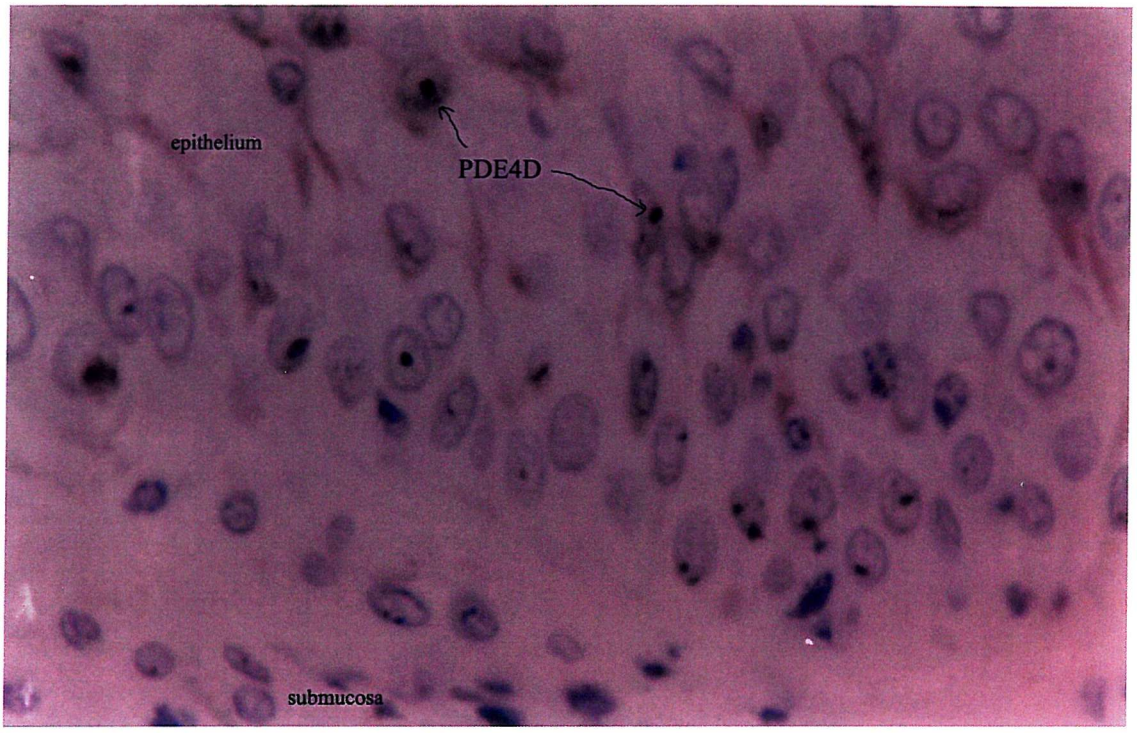


Figure 3.13 PDE4D staining in bronchial epithelium from a normal subject (x630 magnification). Arrows show PDE4D staining in a disc link pattern within the epithelial cells.

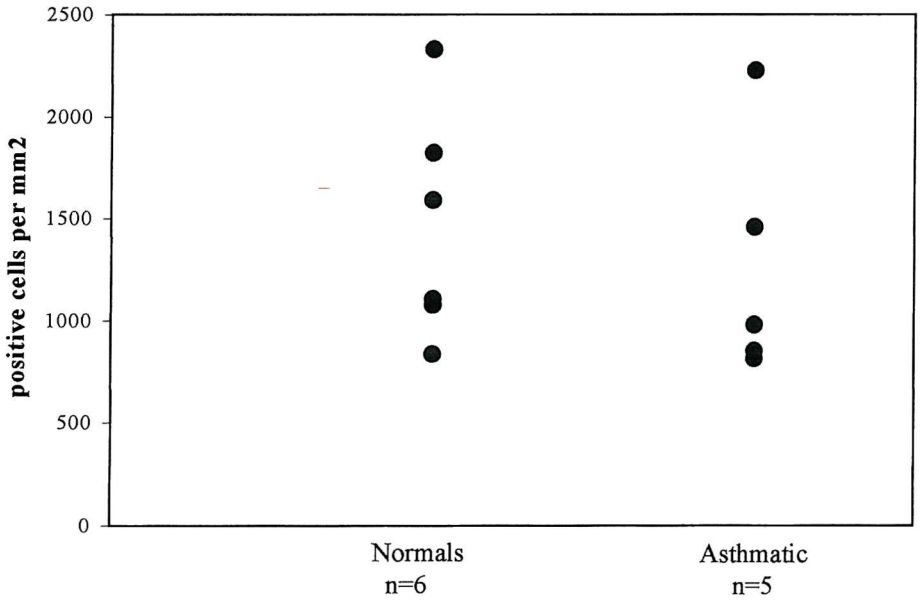


Figure 3.14 Number of PDE4D positive cells per mm^2 of epithelium. There was found to be no statistical significance between the number of PDE4D positive cells from asthmatic or normal biopsies and this was tested by the Mann Whitney U test.

3.4.2 Co-localisation of PDE4D expression with inflammatory cell markers.

In order to investigate the nuclear disc staining pattern observed with PDE4D in the submucosa of these bronchial biopsies, and to try and colocalise this staining to a specific inflammatory cell type, sequential sections were stained for PDE4D, and mast cell, eosinophil, T cell and monocyte markers. Biopsies were studied from normals (n=5) and asthmatics (n=10); however, figures 3.15-3.22 show that PDE4D co-localised only to T cells (figure 3.17a,b). The biopsies used initially, for which results are shown in figure 3.3-3.7, had low numbers of eosinophils per mm^2. However, PDE4D mRNA (Chapter 2) and protein (Chapter 4) was detected in eosinophils from peripheral blood; therefore biopsies with higher eosinophil counts were also investigated. It was again not possible to co-localise PDE4D staining to eosinophils in these biopsies. It is interesting to note that the co-localisation of PDE4D with T cells did not account for the total number of PDE4D stained cells within the submucosa. Therefore, further investigations are needed to identify what other cell types also express PDE4D.

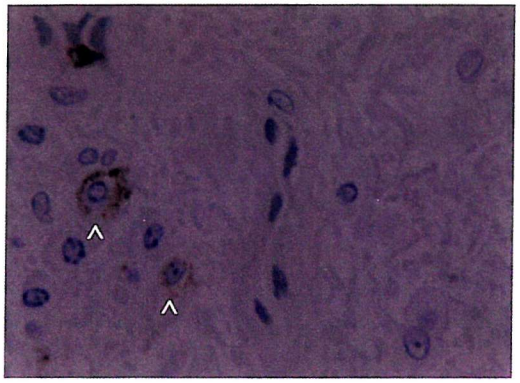


Figure 3.15a Eosinophil staining with EG2 in asthmatic tissue.

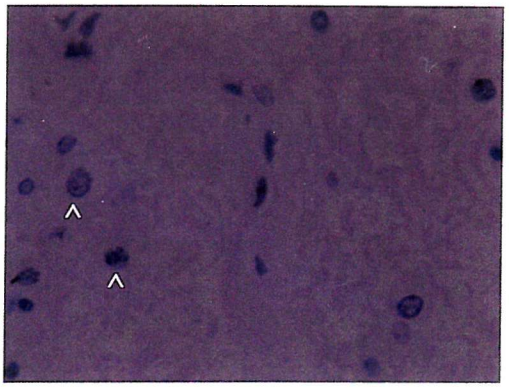


Figure 3.15b PDE4D staining of sequential section, adjacent to that shown in figure 3.17a

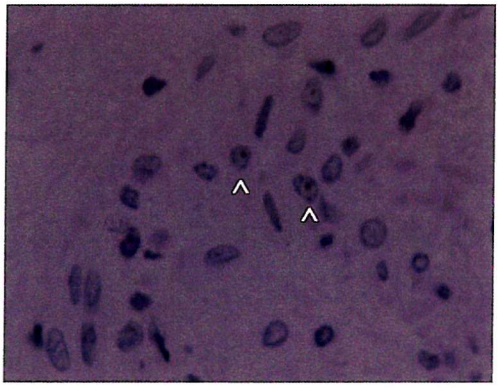


Figure 3.16a PDE4D staining

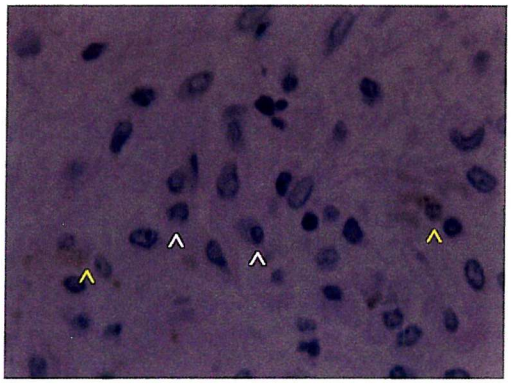


Figure 3.16b EG2 staining of sequential section, adjacent to that shown in figure 3.18a. White arrows represent matched cells and yellow arrows represent positive cells.

In tissue sections where EG2 positive eosinophils were clearly identified (figure 3.15a), staining for PDE4D, in the same cells, in the sequential section was not evident (figure 3.15b). Coversely, PDE4D positive cells in the submucosa (figure 3.16a) did not correspond to the EG2 positive cells (indicated with yellow arrow heads) in the adjacent section (figure 3.16b). All slides are shown at x 400 magnification.

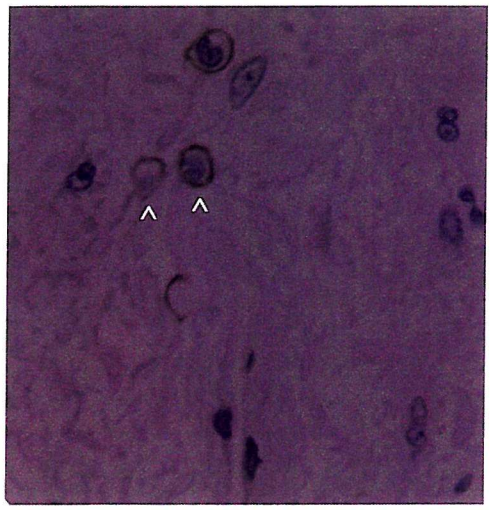


Figure 3.17a T cell staining with CD3 in asthmatic tissue. White arrows indicate positive cells

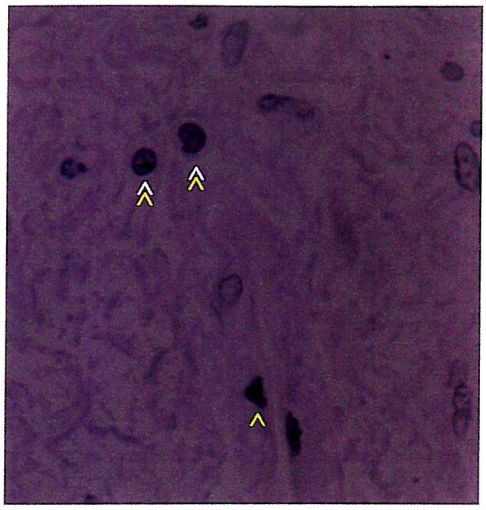


Figure 3.17b PDE4D staining of sequential section, adjacent to that shown in figure 3.19a. White arrows indicate matched cells and yellow arrows indicate positive cells.

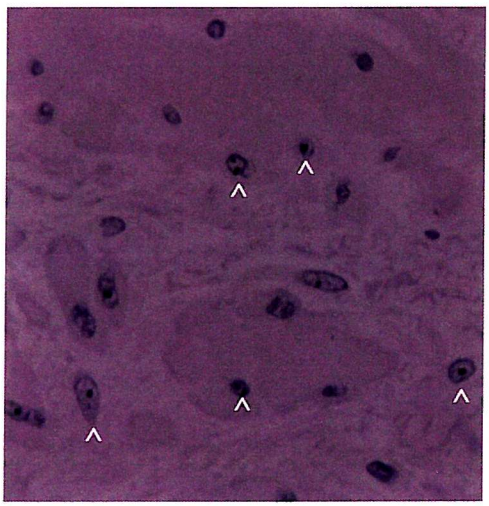


Figure3.18a PDE4D staining. White arrows indicate positive cells.

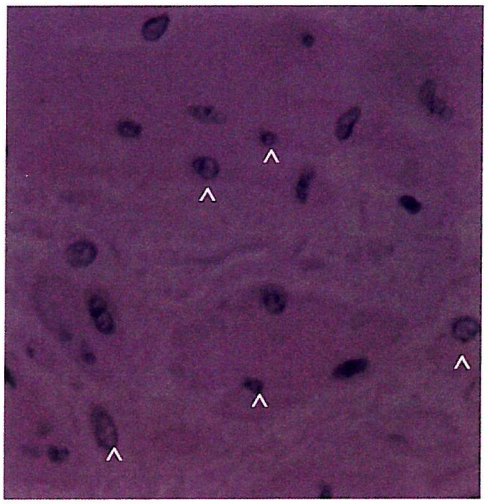


Figure 3.18b CD3 staining of sequential section, adjacent to that shown in figure 3.20a. White arrows indicate matched cells.

In tissue sections where CD3 positive T cells were clearly identified (figure 3.17a), staining for PDE4D in the sequential section indicated that some of these cells also expressed this enzyme (figure 3.17b). Ten asthmatic biopsies and five normal biopsies were examined for evidence of colocalisation between T cells and PDE4D expression, co-localisation was evident in biopsies from both groups.

Figure 3.18b demonstrates that not all PDE4D positive cells can be co-localised with CD3 positive T cells. All sections are shown at x 400 magnification.

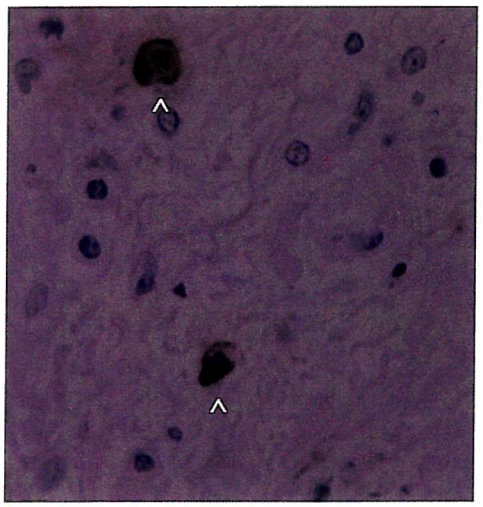


Figure 3.19a Mast cell staining with AA1, in asthmatic tissue.

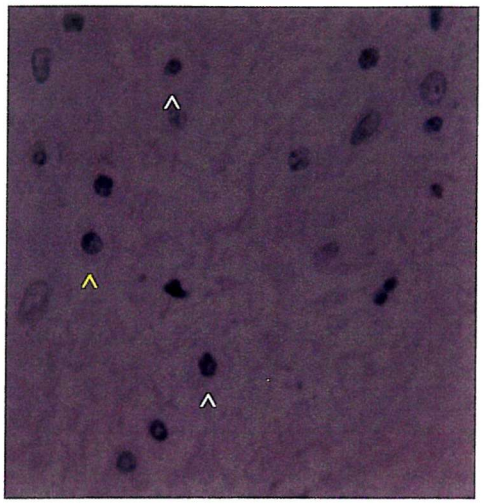


Figure 3.19b PDE4D staining of sequential section, adjacent to that shown in figure 3.19a. White arrows indicate matched cells and yellow arrows indicate positive cells.

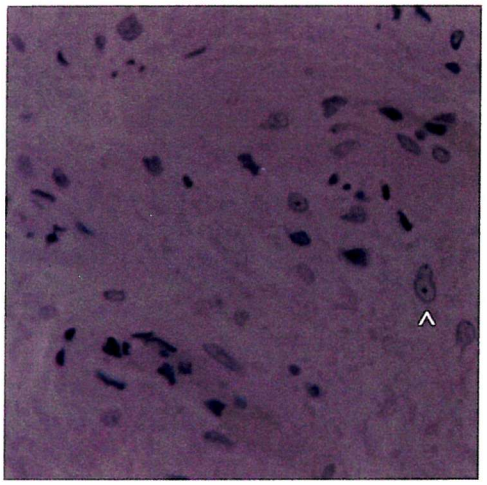


Figure 3.20a PDE4D staining

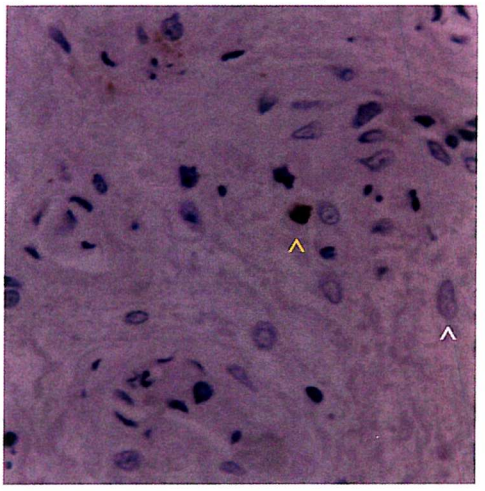


Figure 3.20b AA1 staining of sequential section, adjacent to that shown in figure 3.20a. White arrows indicate matched cells and yellow arrows indicate positive cells.

In tissue sections where AA1 positive mast cells were clearly identified (figure 3.19a), staining for PDE4D in the sequential section indicated that these cells did not express this enzyme (figure 3.19b). Conversely, PDE4D positive cells in the submucosa (figure 3.20a), did not correspond to the AA1 positve cells in the same section (figure 3.20b). Ten asthmatic and five normal biopsies were examined for co-localisation between mast cells and PDE4D and there was no evidence of co-localisation in any of these sections. All slides are at x 400 magnification.

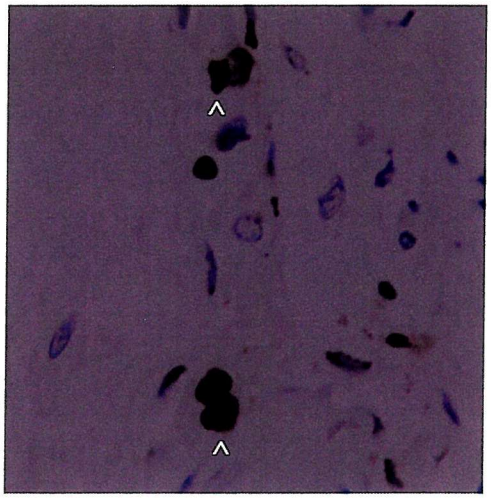


Figure 3.21a Neutrophil staining with anti-neutrophil elastase, in asthmatic tissue. White arrows represent positive cells.

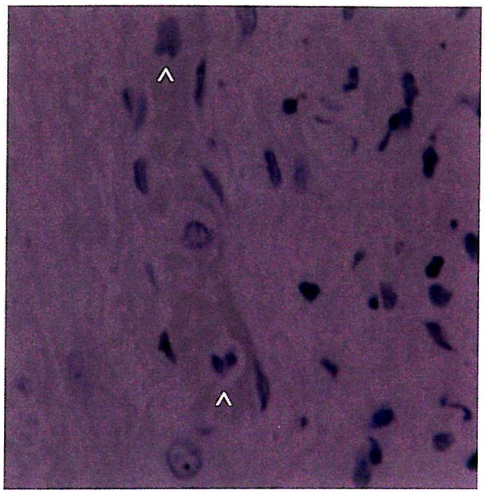


Figure 3.21b PDE4D staining of sequential section, adjacent to that shown in figure 3.21a. White arrows represent matched cells.

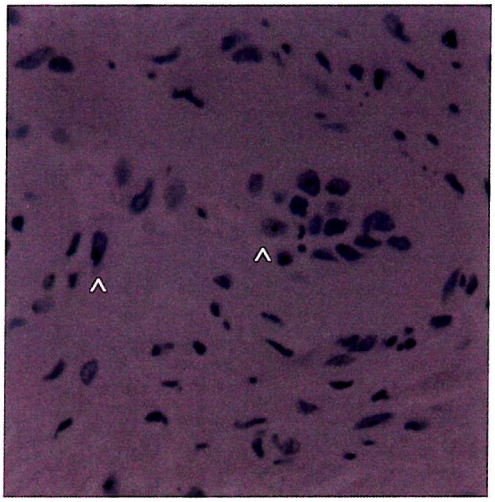


Figure 3.22a PDE4D staining. White arrows represent positive cells.

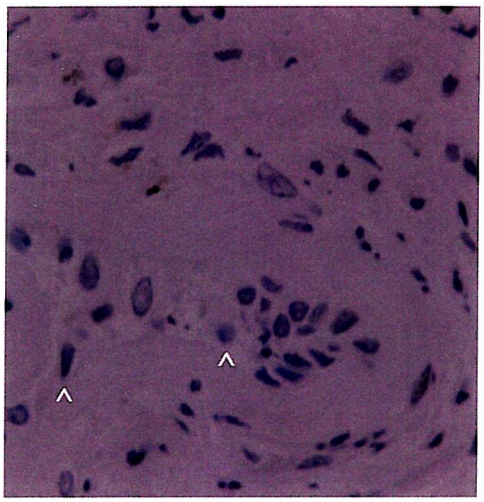


Figure 3.22b Anti-neutrophil elastase staining of sequential section, adjacent to that shown in figure 3.22a. White arrows represent matched cells.

In tissue sections where anti-neutrophil elastase positive neutrophils were observed (figure 3.21a), the absence of positive staining for PDE4D in the sequential section indicated that these cells did not express PDE4D (figure 3.21b). Conversley, PDE4D positive cells (figure 3.22a) did not show positive staining for anti-neutrophil elastase (figure 3.22b). Ten asthmatic and five normal biopsies were examined for evidence of any co-localisation. No evidence of co-localisation was found in any of these biopsies. All slides are shown at x 400 magnification.

A polyclonal antibody raised in rabbits was available to examine the expression of PDE7 in bronchial tissue. Early experiments using immune serum indicated that the staining of tonsil with this antibody was non-specific (fig 3.23) since an equal amount of staining was evident with pre-immune serum. During the course of this study an affinity purified form of the antibody became available, but no positive staining of normal or asthmatic bronchial tissue was detectable (fig 3.25). The lack of any suitable antibody to detect PDE7 meant this investigation could not be carried out.

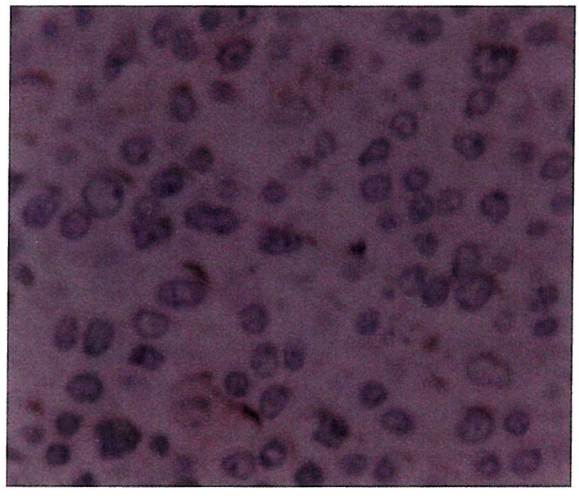


Figure 3.23 PDE7 antibody, immune serum, staining in tonsil (x400 magnification).

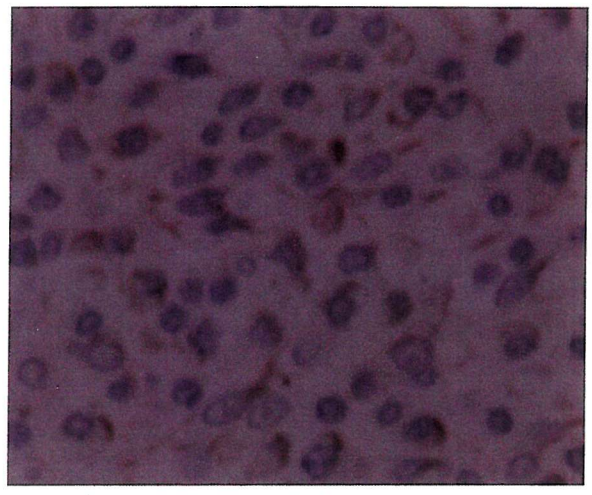


Figure 3.24 Staining with pre-immune rabbit serum at the same protein concentration as the PDE7 serum antibody in tonsil. (x400 magnification).

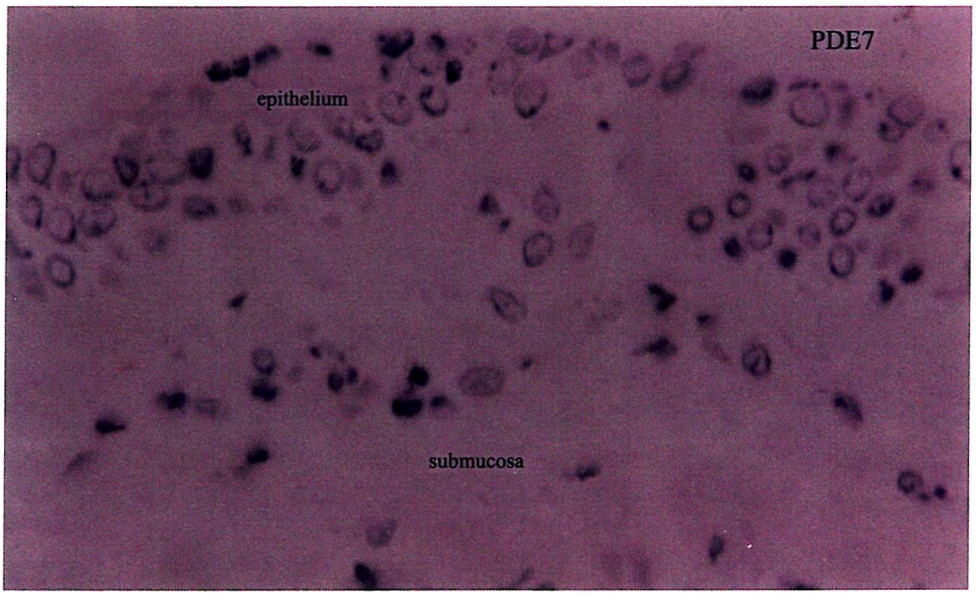


Figure 3.25 Staining with affinity purified PDE7 antibody in bronchial tissue (x400 magnification)

3.5 Discussion

The results obtained, using the immunohistochemical approach, demonstrate that the majority of PDE4A, 4B and 4D expression is localised to the epithelial layer. The relatively low numbers of submucosal inflammatory cells expressing PDE4A, 4B or 4D is surprising, as RT-PCR, Western blotting and activity assays have all demonstrated the presence of some of these isoforms within eosinophils, neutrophils or PBMC from the blood. This might suggest that these cells downregulate their expression of these PDE4 isoforms as they move into the tissue; however further investigations are necessary. For example, a comparison of blood and BAL fluid cells may confirm observations made in tissue.

PDE4 has previously been reported to be one of the major cAMP hydrolysing enzymes in human bronchial epithelial cells (Dent et al. 1998a); therefore the above results of PDE4 staining within the epithelium support these reported observations. Previous reports have indicated that PDE4 and PDE1 are present in equal amounts, and make up the majority of PDE expression. There are also small amounts of PDE3, PDE5 and what is suspected to be PDE7 expressed within these epithelial cells (Dent et al. 1998a; Wright et al. 1998). None of the published reports have investigated the expression in protein form of any of the isoforms of PDE4, and they have only reported mRNA expression. It is interesting to note that mRNA was reported in primary bronchial epithelial cells for PDE4A5, PDE4C1, PDE4D3 and PDE7, but not for any of the splice variants of PDE4B (Fuhrmann et al. 1999). This makes our observation of PDE4B staining within the epithelial cells in normals, and the lower expression in asthma, unique, and the expression of PDE4A and PDE4D observed is in agreement with these studies for mRNA expression.

The asthmatic patients used for this study were considered to have allergic asthma, as they showed bronchial hyperresponsiveness to methacholine. However, as they did not express significantly higher numbers of eosinophils within their bronchial submucosa than normal subjects, it is likely they had very mild disease. This is supported by the sole use of β agonists to treat their symptoms. More severe asthma normally also requires treatment with inhaled or oral corticosteroids.

The expression of PDE4 reported in primary human epithelial cells by Wright et al.(1998) was found to be largely cytoplasmic, with a lower percentage being expressed in the membrane associated fraction. The observed pattern of staining of PDE4A and 4B in epithelial cells, however, appears to be mainly perinuclear. This is true again for the staining for PDE4D, where the majority of 4D appeared in disc like staining pattern, associated with the nucleus.

With the exception of PDE4B, there are no significant differences in numbers of positive cells, or in the apparent distribution of the enzymes within these cells between biopsies derived from asthmatic or normal patients. This would suggest that these PDE enzymes are expressed constitutively, and may well have a role in house keeping activities within the cells. The significantly higher levels of PDE4B staining in normal biopsies, in comparison to asthmatic biopsies, may imply that this isoform in normal cells has tight control over a particular cAMP pool.

The PDE4D disc-like staining, observed in epithelial cells and in some cells in the submucosa of these biopsies, suggests an association with a structure within the nucleus,

perhaps the nucleoli. This finding supports the hypothesis that PDEs are compartmentalised and, therefore, responsible for regulation of cAMP in distinct subcellular organelles (Houslay and Milligan, 1997). There are previously published reports of the co-localisation of PDE4 subtypes to other compartments within cells. For example, Pooley et al demonstrated co-localisation of a splice variant of PDE4A (RD1) to the golgi complex within human follicular thyroid carcinoma cells, and Jin et al reported localisation of PDE4D within the perinuclear region of quiescent FRTL-5 thyroid cells (Pooley et al. 1997; Jin et al. 1998). Whilst there are no published reports of PDE4 localisation within the nucleoli, it is interesting to note that the RII regulatory subunit of PKA, a molecule which relies upon cAMP for activation, has been localised to the nucleoli by immunofluorescence in human breast cancer cells. This staining was also repeated when these human breast cancer cells were transfected into nude mice. Under these conditions it was evident that the RII molecule was moving from the cytoplasm into the nucleoli of these cells upon oestrogen withdrawal and subsequent regression of tumour (Kapoor et al. 1983,1984). Work by Jeong et al has also shown PKA can be colocalised to the nucleoli in rat liver cells, and has suggested that it has a role in `extra housekeeping activities` (Jeong et al. 1998). The nucleolus is known to be responsible for the processing and packaging of ribosomal proteins to form ribosomes that can then be exported (Alberts et al. 1989). If, indeed, PDE4D was colocalised to the nucleoli, this would implicate these enzymes as having a role in regulation of ribosome generation. As PKA is already implicated in having some involvement in regulation of this process, and PKA is activated by cAMP, which in turn is regulated by PDE4, the suggestion that PDE4D staining observed is linked to the nucleoli is worth further investigation.

As some cells within the submucosa were also found to have this disc-like staining with PDE4D, an attempt was made to identify these cells by co-localisation with respective inflammatory cell markers. The PDE4D staining was co-localised to some CD3 stained T cells, suggesting this PDE4 isoform is also involved in regulation of cAMP levels within this location, possibly the nucleolus of T cells, as well as epithelial cells. This co-localisation, however, was not found in all T cells, and T cell staining did not account for all the PDE4D positive cells within the submucosa. PDE4D staining was not co-localised to mast cells, neutrophils or eosinophils in these experiments. The inability to co-localise PDE4D staining to eosinophils is surprising, as it was shown that mRNA and protein, analysed by Western blotting, for PDE4D is present in eosinophils within the peripheral blood. Initially, this lack of co-localisation was thought to be attributed to the use of mild asthmatics, with low eosinophil numbers, and the fact that significantly higher numbers of eosinophils were not detected in asthmatic biopsies in comparison to normals, which has been widely reported to be characteristic of asthmatics (Djukanovic et al. 1992). However, further investigation using biopsies with higher eosinophil counts also failed to yield any evidence of co-localisation. This lack of PDE4D staining within eosinophils in these biopsies may suggest that the expression of PDEs changes as the cells migrate from the blood into the tissue, possibly as they become activated; further investigations would be necessary to clarify this, however. If PDE4D "disappears" from eosinophils, future work using in situ hybridisation would be needed to reveal whether expression of PDE4D mRNA had also disappeared in cells in tissue.

It is particularly interesting that the majority of PDE4 expression observed in these experiments was associated with the epithelium. The epithelium, as described in Chapter 1, is vitally important in protecting the underlying tissue from the environment, as well as

participating in biological responses, as it has both anti inflammatory and pro inflammatory actions (Polito and Proud, 1998). As PDE inhibitors have been used in asthma treatment to suppress accumulation and activation of inflammatory cells, the effects of these inhibitors on epithelial cells have been investigated. Wright et al reported that inhibition of PDE4, by rolipram, increased human airway epithelial cells formation of the bronchodilator, PGE_2, and broad PDE inhibition by IBMX and ORG-9935 (a PDE3 inhibitor) reduced IL-1β stimulated GM-CSF release from these cells. However, rolipram had less effect on GM-CSF release, and no effect on IL-8 or 15 HETE release (Wright et al. 1998). Fuhrmann et al 1999, also reported that rolipram, through PDE4 inhibition, was able to increase PGE_2 formation in airway epithelial cells, whilst having no effect on IL-8 or 15HETE production. Dent et al (1998a), reported that human bronchial epithelial cells express low levels of PDE activity, and only a high concentration of zardaverine (a combined PDE3 and PDE4 inhibitor), and not rolipram, was able to increase basal PGE_2 release. PDE inhibition in these cells was again unable to affect IL-8 release (Dent et al. 1998a).

It would appear from these reports that different PDE subtypes are responsible for regulation of different events within these cells. Also, that whilst PDEs regulate cAMP levels responsible for release of some mediators, for example, PGE_2, they alone do not regulate release of other mediators, for example IL-8. The expression of PDE4A, 4B and 4D within these cells may therefore regulate cAMP levels associated with specific cellular processes. If future work was able to identify which intracellular process these individual PDE4 subtypes regulated, the possibility of response specific-inhibition within epithelial cells may be attainable.

Chapter 4

Detection of spliced variants of PDE by Western blotting

4.1 Introduction

There are four members of the PDE4 enzyme family, 4A, 4B, 4C and 4D. All of these individual PDE4s can be further characterised on the basis that they are expressed as splice variants, thus leading to further diversity. Splicing of the PDE isoforms occurs at the N terminal of the protein, and serves several purposes. For example, it allows variants of the same isoform to be targeted to different locations within the same cell. The presence of two splice variants of 4B in T cells is an example of this. 4B2 is associated with the TCR/CD3 complex, and is phosphorylated upon receptor occupation. This linkage of 4B2 to the TCR complex may be associated with the presence of four myristylation sites in the N terminus of this splice variant. The other 4B splice variant, 4B1, found in these cells, lacks these myristylation sites and has no association with the TCR complex (Baroja et al. 1999).

The presence of splice variants may also allow cells to tailor individual cell responses, by manipulating cAMP gradients within the cells. This manipulation may protect protein complexes from phosphorylation by PKA, or even control the threshold at which anchored PKA are activated by cAMP originating from adenylate cyclase activity (Scotland et al. 1998). The ability of individual splice variants to change their activation state, or susceptibility to inhibitors, may aid in this tailoring. The expression of PDE4D splice variants in quiescent thyroid cells is an example of this; thyroid stimulating hormone (TSH) is able to activate splice variants of this isoform in a spatial and temporal way. For example, the PDE splice variant 4D3, present in both the particulate and soluble fractions, is activated by phosphorylation after a short incubation with TSH. Incubation with TSH over a longer time period causes the upregulation of another PDE4, 4D2, which is only found in the soluble fraction (Jin et al. 1998).

Splicing of members of the PDE4 isoform family also allows them to adapt their conformation by removal of an inhibitory domain. This is demonstrated by 4D3 expressed in human thyroid cells. This splice variant contains an inhibitory region that, when removed, increases enzyme activity. The inhibitory region is thought to hold the catalytic site in a less active conformation, which is reversed by phosphorylation of the regulatory region of this protein; thus removing this inhibitory constraint (Lim et al. 1999).

Therefore, it is not only the expression of the individual PDE isoforms that is important in regulation of individual cellular events, but their splice variants, and their individual properties, that allows cells to finely manipulate cAMP gradients and thus respond to extracellular signals.

In Chapter 2 PDE expression in inflammatory cells was examined at the mRNA level. Although mRNA analysis indicates the potential for translation and protein synthesis in cells, there is often great disparity between expression of message and the coded protein. Therefore, the aim of this Chapter was to analyse the protein expression in inflammatory cells of the PDE4 enzymes by Western blotting. This technique not only allowed the identification of PDE4 proteins, but also their respective splice variants by separation according to molecular weight. The localisation of these PDE4 enzymes within cells was investigated by the separation of inflammatory cell homogenates into supernatant and pellet fractions after cell lysis.

4.2 Materials

Glycine, glycerol, Kodak scientific imaging film and Tris were purchased from BDH, (Poole UK). Sodium dodecyl sulphate (SDS), bovine serum albumin (BSA), Tween-20,

biotinylated goat anti-mouse, and bromophenol blue were purchased from Sigma, (Poole UK). Phosphate buffered saline (PBS) (X10) was purchased from Gibco BRL, (Paisley, Scotland). Kaleidoscope prestained markers and nitrocellulose membrane were from BioRad, (California USA). Precast 4-20% SDS-page gels were from Novex, (California USA). Complete protease inhibitors were from Boehringer Mannheim GmbH (Germany) and inhibited a broad spectrum of serine, cysteine and metalloproteases, as well as calpains. StreptABComplex/HRP, biotinylated $F(ab')_2$ fragment of swine anti-rabbit immunoglobulins were purchased from Dako (High Wycombe UK). Lymphoprep was purchased from Nycomed (Buckinghamshire,UK). Supersignal chemiluminescent substrate was from Pierce (Chester UK). Primary antibodies to the PDE4 isoenzymes were gifts from Celltech (Slough UK), and are detailed in table 3.1.

4.3 Methods

4.3.1 Cell purification

Eosinophils, neutrophils and PBMC were purified, as described in Chapter 2, and cell counts were recorded.

4.3.2 Cell lysis

A 1ml suspension of eosinophils (8.6 (3.7-20.6) x 10^6 cells/ml) was pelleted by centrifugation at 380g for 5 min at 4°C. The pellet was resuspended in 200µl lysis buffer (PBS containing 0.1% Triton X 100 and 5% of a 25X stock of protease inhibitors) and vortexed to cause cell lysis. Lysed cells were centrifuged at 1,500g for 10min at 4°C. The supernatant was aspirated and kept on ice, and the pellet resuspended by gentle pipetting in 200µl lysis buffer. Samples were diluted with 200µl of SDS buffer (80ml 1 X PBS, 20ml glycerol, 2mg SDS and 2mg diothiothreitol (DTT)), and then stored as 20µl aliquots at

-20°C until use. The above procedure was repeated using 2ml of neutrophil cell suspension (35 (20.4-52.6) x 10^6 cell/ml) and 2ml of PBMC cell suspension (26.1 (20.5-48.5) x 10^6 cell/ml). Cells were lysed in 400µl lysis buffer, centrifuged and separated into pellet and supernatant fractions. The pellet fraction was then re-suspended in 400µl lysis buffer and both fractions were diluted by the addition of 400µl SDS buffer. The 20µl samples were heated at 100°C in a heating block for 10 minutes before addition of 5µl loading buffer (20% glycerol 0.1% SDS, 192mM glycine, 25mM Tris-HCl and a trace of bromophenol blue), and the 25µl was then loaded onto Laemmli Tris-Glycine SDS page 4-20% precast gels.

4.3.3 Western blotting

Electrophoresis was carried out in electrophoresis buffer (25mM Tris, 192mM glycine and 0.1% SDS) at 60mA (30mA for a single gel) for 90 minutes in the Novex Xcell II gel system. Prior to Western blotting the gels were pre-soaked in transfer buffer (electrophoresis buffer with 20% methanol added), together with the nitrocellulose membrane and filter paper. Proteins were transferred to 0.45 µm pore size nitrocellulose (BioRad trans-blot) using a semi dry blotter (BioRad) at 150mA for 180 min.

After transfer, the nitrocellulose membranes were incubated with blocking buffer (1X PBS, 2% Tween 20 and 1% BSA) for 60 min at room temperature, and then overnight at 4°C with the primary mouse monoclonal antibody to phosphodiesterase 4D, at a dilution of 1:2000 in wash buffer (1X PBS with 0.05% Tween). When investigating expression of PDE4A and 4B, membranes were incubated in blocking buffer overnight at 4°C. The membrane was then incubated with primary antibodies, rabbit polyclonal anti-PDE4A or anti-PDE4B at

dilutions of 1:1000 and 1:2500 respectively for 90 min. Membranes were washed with wash buffer 3 times for 5 minutes. Secondary antibody, biotinylated goat anti-mouse at a dilution of 1:10,000 for PDE4D detection was used. For detection of PDE4A and 4B, biotinylated swine anti-rabbit was used as a secondary antibody, at a dilution of 1:5000. The membranes were incubated for 90 min at room temperature with their respective secondary antibodies. The same washing procedure as above was repeated before addition of streptavidin biotin complex, at a dilution of 1:5000, which was incubated with the membranes for 90 min at room temperature. Washing was repeated, and the membranes developed with the chemiluminescent Supersignal® system by mixing of equal 1ml volumes of luminol/enhancer solution and stable peroxide solution, and applying to the membranes for 5min. This solution was rinsed off gently in running tap water, to minimise background, and bands were visualised by exposure of autoradiography film in the darkroom. Finally, Western blots were analysed quantitatively by scanning densitometry, using a Hewlett Packard scanjet 4100C with Quantiscan software. Statistics were carried out using values obtained from scanning densitometry using non parametric Mann Whitney U tests.

4.3.4 Method development

The possible activation of neutrophils, via positive selection with anti-CD16+ beads, and its subsequent effect on their PDE4B expression was investigated. The effect of the addition of a broad acting cocktail of protease inhibitors, which inhibit serine, cysteine and metalloproteases, was also investigated. Neutrophils were purified from normal venous blood as total granulocytes, including a < 4% contamination with eosinophils. Briefly, 10ml of venous blood was diluted in 5ml of PBS/FBS buffer (1x PBS, 2% foetal bovine serum). This was then layered onto an equal volume of Lymphoprep in a 50ml

centrifuge tube and centrifuged at 770g for 30min at 20°C. The upper layer was aspirated, leaving a cell pellet. Erythrocytes were removed by hypotonic lysis of the cell pellet. Distilled water (50ml) was added to the pellet for 45sec at, which time, an equal volume of 1.8% NaCl was added to restore isotonicity. Cells were pelleted by centrifugation at 770g for 10min at 4°C, and the supernatant again aspirated. The pellet was then resuspended in 1ml of PBS/FBS buffer and cells were counted on a haemocytometer.

Equal numbers of neutrophils(14×10^{-6} cells) were separated into four (3.5×10^{-6} cells), 1.5ml eppendorf tubes and placed on ice. Tubes A and B were left on ice for 30min and anti-CD16+ magnetic beads (10µl) were added to tubes C and D which were again left on ice for 30min. After this time cells in tubes B and D were lysed in 100µl lysis buffer, vortexed and centrifuged at 1,500g for 10min at 4°C. Cells in tubes A and C were lysed in PBS with 0.1% Triton (lysis buffer without protease inhibitors), and then vortexed and pelleted as above. Finally, all tubes were treated as detailed for cell samples, section 4.3.2, by separation of pellet and supernatant fractions, followed by addition of 100µl SDS buffer. Samples prepared in this way were then analysed by Western blotting as detailed above.

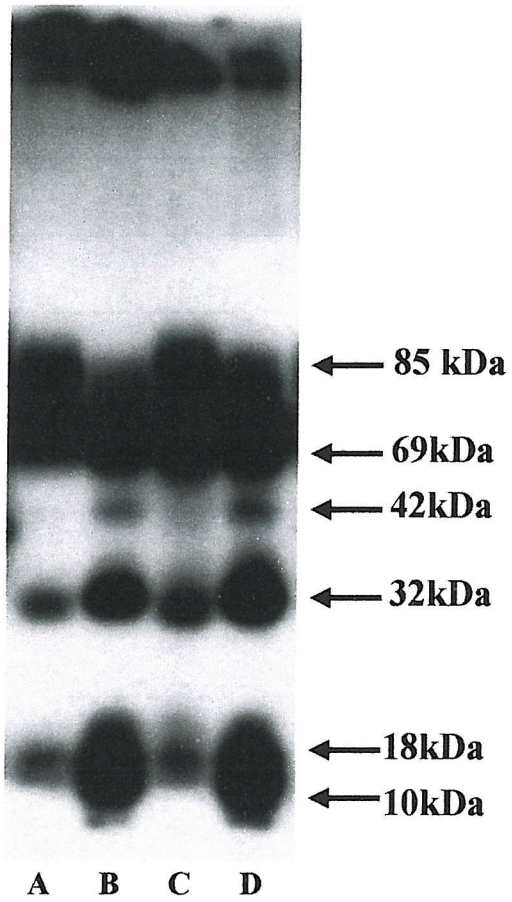


Figure 4.1 The effect of CD16+ selection or addition of protease inhibitors has on neutrophils expression of PDE4B. Lane A is neutrophil sample without CD16+ or protease inhibitors. Lane B has protease inhibitors but no CD16+. Lane C has CD16+ but no protease inhibitors and lane D has CD16+ and protease inhibitors.

Figure 4.1 demonstrates that isolation of neutrophils by CD16+ selection does not affect expression of PDE4B. Both lanes A and C have bands of molecular weights 85kDa, 69kDa, 32kDa, 18kDa and 10kDa. What is evident from figure 4.1 is that addition of protease inhibitors, as shown in lanes B and D, leads to an extra band with molecular weight 42kDa, and increased intensity of bands with molecular weights 32kDa and 10kDa. This may suggest that, without protease inhibitors, these bands are digested to smaller fragments that are not recognised by the antibody. This experiment shows that the addition of protease inhibitors is not sufficient to prevent all proteolytic cleavage of the PDEs, as low molecular weight fragments are still present, even with protease inhibitor treatment.

4.4 Results

PDE4A

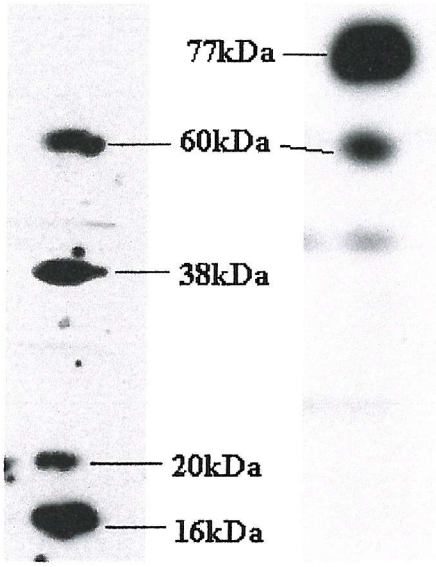


Figure 4.2 Western blots illustrating molecular weights of PDE4A bands. Lane 1 represents an eosinophil pellet sample and lane 2 represents an eosinophil supernatant sample.

Distribution of the PDE4A bands, illustrated in figure 4.2, was varied between eosinophils, neutrophil and PBMCs, and also between their cellular location. Whilst eosinophil pellet samples contained bands at 60kDa, 38kDa, 20kDa and 16kDa, supernatant samples from the same cells had bands at 77kDa, 60kDa and 30kDa. Neutrophil pellet and supernatant samples both contained two bands with low molecular weights of 28kDa and 16kDa. PBMC pellet and supernatant samples, again, had two bands, but with molecular weights of 77kDa and 44kDa. In all cells there was no difference in expression of these varying molecular weight proteins between samples from normal, atopic or asthmatic donors. The intensity of these molecular weight bands for 4A was not assessed by densitometry, because varying exposure times were used to optimise visualisation of different bands; therefore

comparison between bands is impossible.

PDE4B

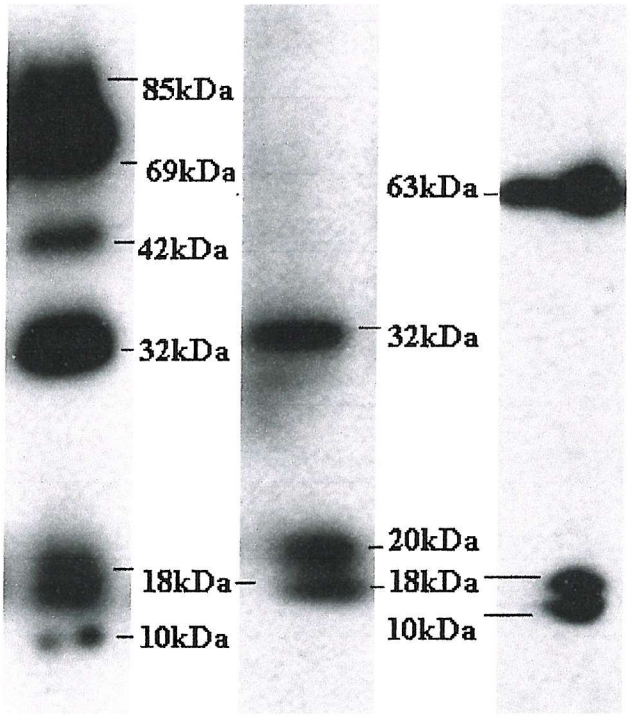


Figure 4.3 Western blots illustrating molecular weights of PDE4B bands. Lane 1 represents molecular weight bands found in a neutrophil pellet sample. Lane 2 represents a PBMC supernatant sample and lane 3 is an eosinophil pellet sample.

As was the case for expression of PDE4A, there is variation in the expression of PDE4B molecular weight bands between different cells and different cellular compartments. A high molecular weight band at 180kDa was observed in some eosinophil pellet and supernatant samples from the same donors. There was, however, no obvious link between the expression of this band and the subject group of the donor. Eosinophil pellet and supernatant samples routinely expressed bands with molecular weights 63kDa, 42kDa, 32kDa, 18kDa and 10kDa. Whilst neutrophil pellet samples expressed bands with molecular weight 85kDa, 69kDa, 42kDA, 32kDa, 18kDa and 10kDa, neutrophil

supernatant samples expressed bands with only the smaller molecular weights 42kDa, 32kDa, 18kDa and 10kDa. This was again the case for PBMC samples; PBMC pellet samples gave molecular weight bands of 85kDa, 69kDa, 32kDa, 20kDA, 18kDa and PBMC supernatant samples had bands of lower molecular weights 42kDa, 32kDa, 20kDa and 18kDa. There were no differences in the expression of any of these molecular weight bands between cells from different donors, and bands were not quantified for the same reason as PDE4A.

PDE4D

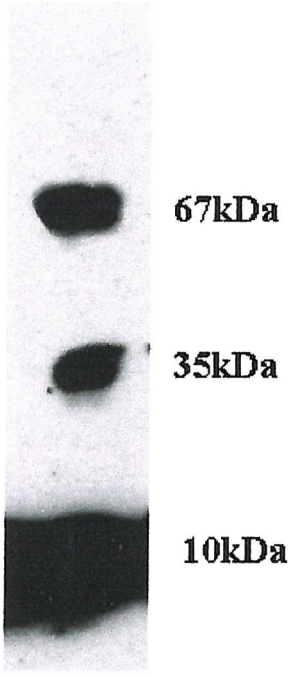


Figure 4.4 Western blot illustrating the molecular weights of PDE4D bands present in eosinophil pellet samples from an asthmatic donor.

The Western blot shown in figure 4.4 shows that, under the conditions used, specific staining for PDE4D indicated the presence of two bands in the membrane fractions of eosinophil samples from atopic and atopic asthmatic patients. These bands had estimated molecular weights of approximately 67kDa and 35kDa, by comparison with molecular

weight standard proteins. In atopic and atopic asthmatic samples there was also detected a low molecular weight band, estimated with a molecular weight approximately 10kDa. No bands were apparent in normal eosinophil samples. However, SPA analysis (Chapter 5) confirmed PDE4 in normal eosinophils and, therefore, X-ray film exposure time was increased for comparison of subject groups. Scanning densitometry was performed to quantify and compare the density of the 35kDa and 67kDa bands from the different subject groups on membranes developed and exposed to film for 120 min, and the results shown in figure 4.5 and 4.6.

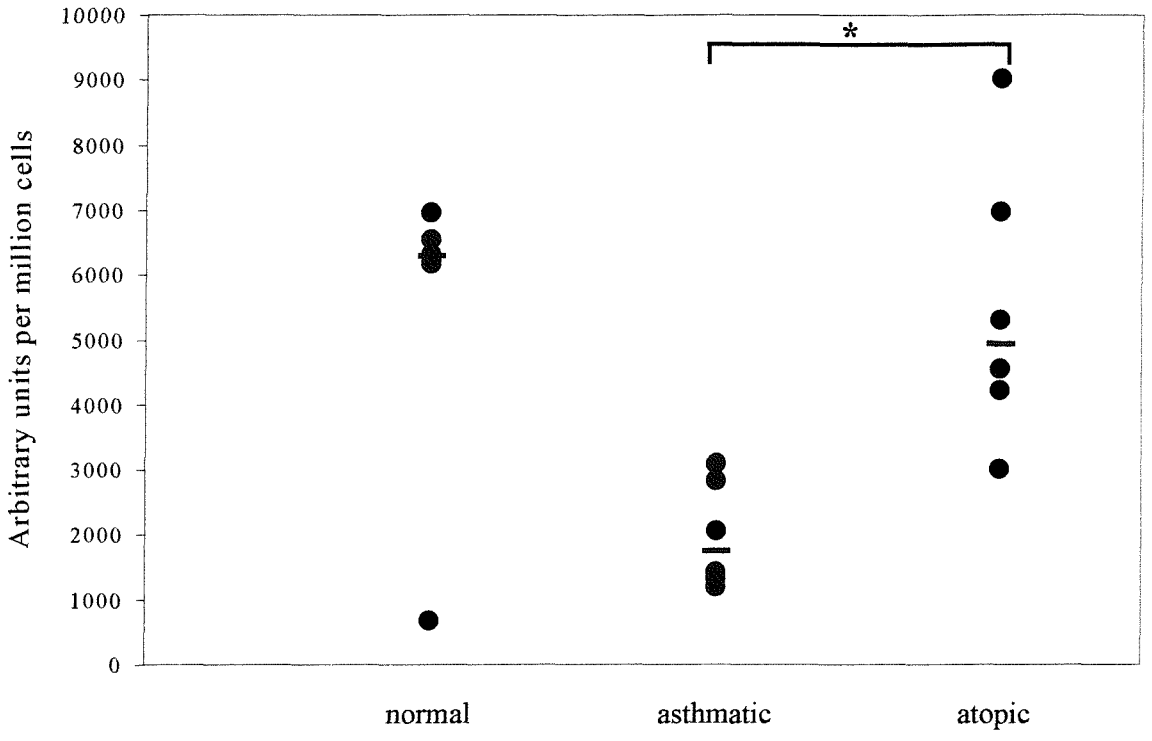


Figure 4.5 PDE4D 35kDa band present in Western blots of eosinophil membrane fractions. Units are arbitrary units given by scanning densitometry. * represents $p < 0.01$, $n = 6$, Mann Whitney U Test.

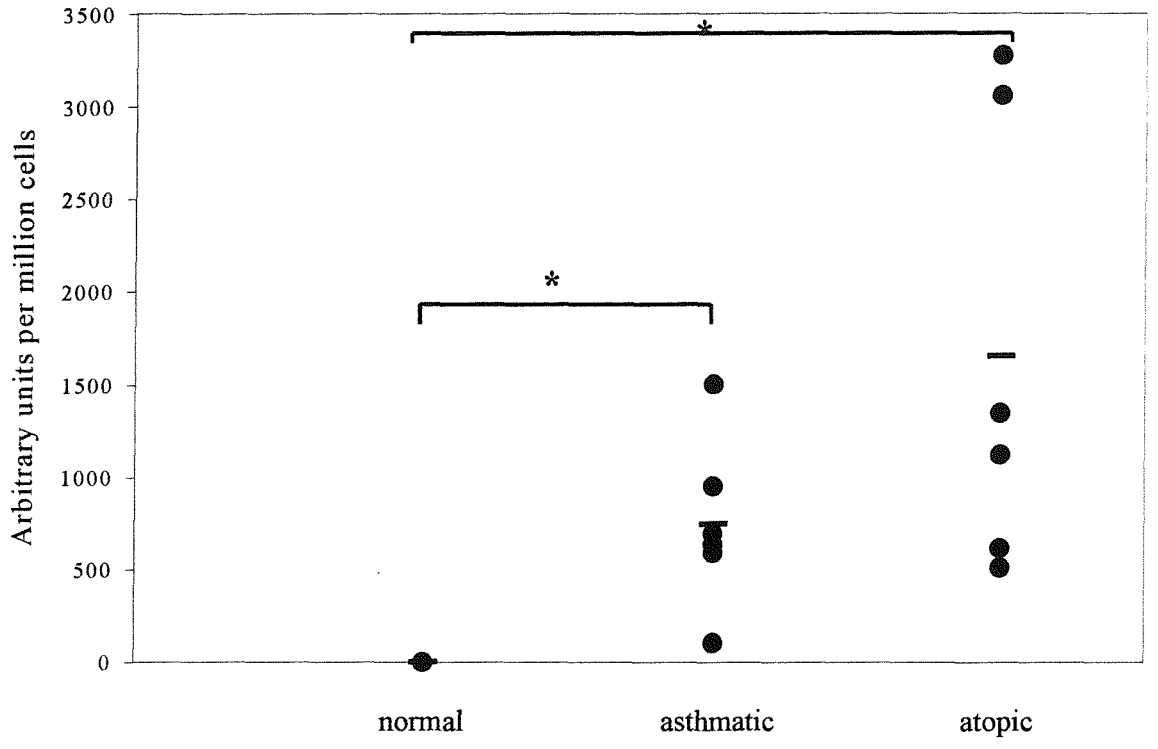


Figure 4.6 PDE4D 67kDa band present in Western blots of eosinophil membrane fractions. Units are arbitrary units given by scanning densitometry. * represents $p < 0.01$, $n = 6$, Mann Whitney U test.

Table 4.1 Ratio of the 67kDa PDE4D band to that of the 35kDa band (density units).

Atopics	Asthmatics	Normals
0.62:1	0.67:1	0:1
0.29:1	1.23:1	0:1
0.26:1	0.4:1	0:1
0.53:1	0.12:1	0:1
1.04:1	0.77:1	0:1
3.13:1	0.64:1	0:1

Figure 4.5 shows that there is a significantly higher level of expression of the 35kDa form of PDE4D in atopics (4935 AU range 3030-9020) than asthmatics (1745 AU range 1210-3100). There were no significant differences in levels of expression between the normal subjects and either the atopics or asthmatics. Figure 4.6 shows that there is significantly

higher expression of the 67kDa form of PDE4D in atopics and asthmatics than normals, for whom no protein of this Mw was detected. Table 4.1 shows that there is no significant difference in the ratio of the 67kDa form to the 35kDa in either atopic or asthmatic subjects. There was no evidence for the 10kDa band in the samples from healthy subjects, under any conditions

Neither of the 67kDa or 35kDa forms were detected in any eosinophil supernatants (n = 18), neutrophil pellets or supernatants (n = 18), and PBMC pellets or supernatant fractions (n = 18), after exposure of Western blots to film for up to 16 hours.

4.5 Discussion

The results in this chapter have clearly demonstrated that different inflammatory cells have individual profiles of PDE4 expression, and that this expression also differs in its location within these cells.

Expression of splice variants of PDE4A within eosinophils, neutrophil and PBMC in our study would suggest that no intact forms of 4A are present in any of the cell types. This is implied by the fact that previous reports attribute molecular weights of between 110-130kDa to the 4A splice variants (Jacobitz et al. 1996), and no bands in this size range were observed in our experiments. Two separate reports, investigating deletions within this isoform, have shown that by no means all of the full length protein is necessary for catalytic activity. Owens et al noted that deletions in a recombinant 117kDa 4A protein still yielded activity when the protein was 71kDa in size (Owens et al. 1997). Jacobitz et al, also working with a recombinant 4A protein of 110kDa noted that the minimum size of this protein needed for catalytic activity was as little as 45kDa (Jacobitz et al. 1996). It

would, therefore, not be implausible to suggest that, whilst no full length 4A proteins were observed in any of our samples, the bands of molecular weights 77kDa and 60kDa found in eosinophil and PBMC samples may represent truncated versions of PDE4A that still retain catalytic activity. The presence of low molecular weight bands in all samples, especially neutrophil pellet and supernatant samples which had only bands of 28 and 16kDa, suggests that these are the products of proteolytic cleavage of larger 4A proteins. There would appear to be no distinction in the expression of these truncated 4A proteins between the supernatant and pellet fractions of any of these cells, in our results. This may suggest these proteins are involved in the more generalised control of cAMP levels, rather than cAMP levels in a distinct location, within the cell.

The assignment of PDE4B immunoreactive bands, found in this Chapter, to individual splice variants is difficult, as published reports widely differ in molecular weights assigned to each 4B splice variant. Huston et al 1997 reported a molecular weight of 104kDa for the 4B1 splice variant, when expressed in Cos 7 cells (Huston et al. 1997). However, Baroja et al (1999) reported a 4B1 protein with molecular weight between 75-80kDa in human T cells. For the purposes of assignment of individual splice variants to molecular weight bands the reported values of Baroja, PDE4B1, molecular weight 75-80kDa, and PDE4B2, molecular weight 65-67kDa, when expressed in human peripheral blood T cells, were used as these represent proteins from primary human cells. Using these molecular weights, there is evidence in this Chapter that intact forms of PDE4B are expressed within inflammatory cells.

The presence of a molecular weight band of 180kDa, in some eosinophil pellet and supernatant samples, may represent a PDE4B isoform complexed to another molecule,

thus resulting in a much higher molecular weight. As this high molecular weight protein was only detected in a few samples, and even this detection was not associated with the subject group of these samples, its presence does not represent the predominant 4B expression. Neutrophil pellet and PBMC pellet samples displayed 4B proteins with molecular weights 85kDa and 69kDa; it is likely that these proteins correspond to the 4B1 and 4B2 splice variants respectively. The expression of a protein with molecular weight 63kDa in eosinophil pellet and supernatant samples may represent a slightly clipped 4B2 splice variant. Smaller 4B proteins were present in all cells, and are probably the products of proteolytic cleavage. It is interesting to note that, in both neutrophil and PBMC samples, the pellet fraction contains the intact 4B proteins, together with cleavage fragments; however, the supernatant fraction contains only cleavage fragments. This observation would indicate that only pellet fractions from these cells have 4B activity, and supernatant fractions are more susceptible to proteolysis; which may imply increased expression of proteases within this fraction.

The results in this chapter have also shown that eosinophils from both atopics and atopic asthmatics contain two PDE4D proteins, with estimated molecular weights of 67kDa and 35kDa, and that these are present in the pellet fraction of eosinophils, but absent in the soluble fraction. In view of the reported molecular weight of 67±2kDa for the PDE4D1 splice variant in transfected Cos cells (Erdogan et al. 1997), the 67 kDa band reported in this Chapter may reflect expression of this spliced variant. Normal subjects do not express the 67kDa protein, although the 35kDa was detectable. There is significantly higher expression of the 67kDa form in atopics (4935 AU range 3030-9020) than asthmatics (1745 AU range 1210-3100). All three subject groups, including normal subjects, contain a 35kDa PDE4D form. In atopics and asthmatics the dense band of PDE4D, with

molecular weight approximately 10kDa, is not present in normals, and this band may be cleavage products of the larger PDE4D proteins. No PDE4D protein was detected in eosinophil supernatant fractions or pellet and supernatant fractions from neutrophils and PBMCs. These results are in agreement with the results from PDE activity assays, described in Chapter 5. The results of the SPA activity assay, and the results in this Chapter, indicate that the majority of the cAMP hydrolysing activity is in the pellet fraction of eosinophils. In parallel with the results from the activity assays, which showed that PDE enzyme activity was not detected in supernatant fractions from eosinophil samples, no PDE4D protein was detected in the supernatant of eosinophils by Western blotting.

One of the major PDE4D bands that was evident in the Western blots in the pellet fraction from the eosinophils has a molecular weight between 60-70kDa, which corresponds to PDE4D1 previously reported to have a molecular weight of 67kDa (Erdogan et al. 1997). PDE4D1 is one of the shorter splice variants of 4D, and lacks the UCR1 region. A PDE4D protein, with an estimated molecular weight of 35kDa, was found in eosinophils in all subject groups. This protein appears to be the only spliced variant of PDE4D expressed in normal subjects. It is interesting that there has been a previous report of a PDE4D protein with this Mw expressed in human monocytes (Truong and Müller, 1994). It was speculated that this 35kDa form represents one of the monomers of a form of PDE4D, which may exist as a dimer, or that this 35kDa is a cleaved version of a PDE4D isoform that still retains activity.

The smaller cleavage products detected in atopics and asthmatics, with estimated molecular weight of 10kDa, suggest non specific proteolysis of PDE, possibly to inactive fragments, despite the presence of protease inhibitors. The different profiles between normals,

atopics and asthmatics may reflect relatively lower amounts of proteases in this subcellular fraction in normal cells.

From the results in this Chapter it appears that eosinophils express a clipped variant of 4A, which is possibly active, and intact PDE4B2 in both pellet and supernatant fractions, however, PDE4D1 is only expressed in the pellet fractions. Results in Chapter 5 have indicated that over 80% of cAMP hydrolysing PDE activity is found in the pellet fraction of eosinophils. Therefore, the supernatant PDE4A and 4B bands found in this Chapter may represent enzymes with low activity or, alternatively, PDE4D, found only in the eosinophil pellet fractions, may largely account for the observed activity in the pellet fraction. These results complement RT-PCR studies that have demonstrated presence of mRNA for 4A, 4B and 4D in eosinophils. Neutrophils, however, have no intact 4A or 4D protein and express only intact forms of 4B1 and 4B2 in their pellet fraction. This is, again, in agreement with RT-PCR analysis showing neutrophils express nearly exclusively PDE4B, and have a very weak/ absent mRNA signal for PDE4D in PCR analysis (Müller et al. 1996). It is, however, puzzling that SPA enzyme activity analysis in Chapter 5 has demonstrated that supernatant fraction of neutrophils possess higher cAMP hydrolysing activity than the corresponding pellet fraction. The Western blotting results in this chapter would imply that more PDE4 protein is found in the pellet fraction of these cells. An explanation for this maybe that the PDE4 proteins are held in a less active conformation in the pellet, possibly due to their association with membrane components. Therefore, whilst they may contribute a higher proportion of protein expression, their low activity means they do not contribute the significant proportion of total cell activity.

PBMCs have a clipped and possible active version of 4A in both cellular compartments but only intact 4B1 and 4B2 in the pellet fraction. In PBMCs there is also no evidence of any 4D protein. This absence of 4D bands in PBMCs is not as expected. Other published work has detected the presence of mRNA by PCR and protein by Western blotting of PDE4D1 and D2 in human blood mononuclear cells (Nemoz et al. 1996). T cells are the major component of PBMCs, and the major PDE isoform in T cells is PDE3. It may therefore be the case that these cells also express 4D, but at a level that is not detected in our experiments. It is also surprising that the pellet fraction of these PBMC has more intact 4B protein expression as, by SPA analysis, the supernatant fraction of these cells possessed most cAMP hydrolysing activity; again this could be the results of different enzyme conformation.

Whilst there is a clear variation in the protein expression of PDE4 isoform and splice variants between the inflammatory cells in this Chapter, it is difficult to attribute regulation of any specific functions within these cells to expression of these proteins, as this area is largely un-investigated, and any proposed roles appear to be cell specific. It would, however, appear that, as cells do have such individual complements of these enzymes, they must be involved in regulation of intracellular processes in a cell specific manner.

Chapter 5

Analysis of PDE enzyme activity

5.1 Introduction

The activity of the cAMP hydrolysing phosphodiesterases is determined by their ability to degrade cAMP to AMP. Members of this cAMP hydrolysing PDE group include PDE1, 2, 3, 4, 7 and 8, and their expression within inflammatory cells is detailed in Table 5.1, from which it is clear that PDE4 is the predominant cAMP hydrolysing PDE expressed in these inflammatory cells. It is also evident that the intracellular distribution of these enzymes varies between different reports, with 75% of PDE4 being reported as soluble in eosinophils (Hatzelmann et al. 1995), whilst another report claims 95% of total activity (of which PDE4 is 90%) to be particulate (Dent et al. 1994b). Reports also differ as to the contribution of each enzyme to cAMP hydrolysing activity. Gantner et al 1997, reports 80% of activity in monocytes to be attributable to PDE4, whilst Verghese et al 1995, attributes only 50% of total cAMP hydrolysing activity to PDE4 in the same cells. These anomalies may be the result of different cell isolation techniques or variations in methods for determining activity. Indeed, the method of cell disruption is known to influence the activity of PDEs as, reported by Truong and Muller 1994, detergent lysis of PBMC yielded an activity level two fold lower than observed when cells were lysed by sonication.

Both Holden et al 1986, and Grewe et al 1982, reported a higher PDE activity in monocytes from atopic dermatitis patients in comparison to normals. Grewe et al speculated that the increased PDE activity was a characteristic of atopy, and may account for the observation by Safko et al 1981, that cAMP elevation, produced by isoprenaline, histamine and PGE1, appeared to be blunted in atopics (Safko et al. 1981; Grewe et al. 1982; Holden et al. 1986). However, subsequent investigations have not confirmed these findings. For example, Gantner et al, 1997, showed that there is no difference in the PDE activity of monocytes from patients with atopic dermatitis when compared to cells from normal donors. This study

noted a slight increase in the mRNA expression of PDE4A and 4B2 in T cells from atopics. However, it was concluded that there was no overall difference in PDE activity or protein expression between monocytes, B lymphocytes, T cells and eosinophils from atopic dermatitis patients and normal controls (Gantner et al. 1997a). Work by Tenor et al 1996, also failed to show any different levels of PDE activity in eosinophils from atopic donors, in comparison to cells from normals. Reports detailed in Table 5.1 indicate that there are variations in cell expression, intracellular localisation and activity of the cAMP hydrolysing enzymes even for reports examining the same cell type. Our own investigation of this PDE cAMP hydrolysing activity was necessary to link activity with protein expression from identical cells detailed in Chapter 4.

The expression of PDE mRNA in peripheral blood cells described in Chapter 2 has indicated that eosinophils and PBMC express PDE4A, PDE4B2 and PDE4D, whilst only PDE4B2 mRNA can be detected for neutrophils. The expression of PDE protein in these peripheral blood cells, investigated by Western blotting in Chapter 4, has indicated that eosinophils have PDE4A, 4B and 4D, neutrophils have only PDE4B, and PBMC have PDE4A and 4B. The aim of this Chapter is to investigate the cAMP hydrolysing activity of PDEs expressed in these peripheral blood cells. The use of specific PDE inhibitors will enable this cAMP hydrolysing activity to be attributed to individual members of the PDE family. By separation of membrane and soluble fractions of eosinophils, neutrophils and PBMC, the intracellular localisation of the PDE activity will be examined. Finally, comparison of cells from normal, atopic and mild atopic asthmatic individuals will examine whether changes in PDE activity are associated with these conditions.

	METHOD OF DISRUPTION	MAIN PDE FORM	DISTRIBUTION	ROLIPRAM IC_{50} μM	ACTIVITY PMOL/MIN/ 10^6 CELLS
Eosinophils (Hatzelman et al 1995)	sonication	95% PDE4 5% PDE7	75% PDE4 in cytosol		normal 2.35+/-0.26
Eosinophils (Dent et al 1994b)	freeze/thaw sonication	PDE4	95% total activity particulate	0.55μM	uncharacterised donors 1.73+/- 0.37
Eosinophils (Gantner et al 1997)	sonication	PDE 4 small amount of PDE 3	80 % PDE 4 soluble		normal 2.18+/- 5 atopic 2.46+/- 14
Eosinophils (Tenor et al 1996)	sonication	80% PDE4 20% PDE7	not determined	normals 0.2 atopic 0.16	normal 2.50+/-0.05 atopics 2.91+/- 0.18
Neutrophils (Schudt et al 1991)	sonication	PDE4	30% PDE 4 membrane bound	0.13μM	
Neutrophils (Wright et al 1990)	homogenisation	PDE 4	80% total activity membrane bound soluble fraction probably PDE 3	membrane bound 1.7 μM	
Neutrophils (Mahomed et al 1998)	no separation	PDE4			normal 1.5
PBMC (Truong and Müller 1994)	detergent lysis		90% cytosolic	uncharacterised donors 1.3μM	
Monocytes (Gantner et al 1997)	sonication	80% PDE4 20% PDE3	PDE4 exclusively soluble	0.17 normal 0.14 atopic	normal 0.59+/- 0.05 atopic 0.65+/- 0.12
Monocytes (Verghese et al 1995)	digitonin permeabilisation	50% PDE4 25% PDE3	PDE4 cytosolic PDE3 membrane bound		

	METHOD OF DISRUPTION	MAIN PDE FORM	DISTRIBUTION	ROLIPRAM IC_{50} μM	ACTIVITY PMOL/MIN/ 10^6 CELLS
Monocytes (Holden et al 1986)	freeze/thaw sonication	not established	not established	not established	plate adhered normal 0.042+/- 0.02 atopic dermatitis 0.207+/-0.05
Monocytes (Souness et al 1996)	homogenisation	cytosolic 75% PDE4 24% PDE3 particulate 50% PDE3	60% cytosolic	cytosolic 0.313 +/- 0.0067	
T- Lymphocytes (Gantner et al 1997a)	sonication	50% PDE3	PDE 4 soluble		normal 0.29+/- 0.13 atopic 0.38+/- 0.11
Lymphocytes (Sheth et al 1997)	freeze/ thaw	70% PDE3 30% PDE4	only soluble assessed		
T-lymphocytes (Tenor et al 1995b)	sonication	80% PDE3 + PDE4 20% possibly PDE7	PDE4 soluble PDE3 membrane bound	soluble CD4 0.12 +/- 0.01 CD8 0.13+/- 0.005	normal CD4 soluble 0.56+/- 0.023 membrane 0.177 +/- 0.013 CD8 soluble 0.51+/- 0.032 membrane 0.182+/- 0.021
T-lymphocytes (Seybold et al 1998)	osmotic lysis	80% PDE3 + PDE4 20% possibly PDE7			normal 0.22+/- 0.01
T-lymphocytes (Giembycz et al 1996)	osmotic lysis	65% PDE4	PDE4 soluble	0.1-0.3	

Table 5.1 Summary of published reports investigating cAMP hydrolysing PDE activity levels in inflammatory cells

5.2 Materials

[8 ^{3}H] Adenosine 3`,5`,cyclic phosphate, ammonium salt (cAMP), [^{3}H] AMP and scintillation proximity assay (SPA) beads were purchased from Amersham (Buckinghamshire,UK). N-Tris [hydroxymethyl] methyl 2 aminoethanesulfonic acid (TES), cGMP, ADP and Triton X-100 were purchased from Sigma (Poole UK). 96 well Optiplates were purchased from Canberra Packard (Pangborne UK). Rolipram (racemate) and the PDE7 inhibitor CT 5651 were gifts from Celltech (Slough, UK), Phosphate buffered saline (PBS) was purchased from Gibco life technologies (Paisley Scotland). Complete protease inhibitor tablets were purchased from Boehringer Mannheim GmbH (Germany) which inhibited a broad spectrum of serine, cysteine and metallo-proteases, as well as calpains.

5.3 Methods

5.3.1 Drug preparations

Rolipram and CT 5651 were diluted in DMSO to a stock concentration of 20mM. Further dilutions from the stock were made using the TES buffer (50 mM TES, 10mM $MgCl_2$ in distilled water, adjusted to pH 7.6 with NaOH).

5.3.2 Sample preparation

Eosinophils, neutrophils and PBMCs were isolated from the venous blood of normal (n=5), atopic (n=6) and asthmatic (n=6) patients by the methods described in Chapter 2.
After cell counts were recorded, a 1ml suspension of eosinophils (8.6 (3.7-20.6) x 10^{6} cells/ml) was pelleted by centrifugation at 380g for 5min at 4°C. The pellet was re-suspended in 200µl lysis buffer (PBS containing 0.1% Triton X 100 and 5% of a 25X concentration of protease inhibitors) and vortexed to cause cell lysis. The lysate was then

centrifuged for 10min at 4°C at 1,500g, the supernatant was aspirated and kept on ice, and the pellet resuspended by gentle pipetting in 200µl lysis buffer. Finally, both pellet and supernatant fractions were diluted with a further 200µl of lysis buffer, before being stored at -20°C in 20µl aliquots. The above procedure was repeated using 2ml of neutrophil cell suspension (35 (20.4-52.6) x 10^6 cell/ml) and 2ml of PBMC cell suspension (26.1 (20.5-48.5) x 10^6 cell/ml). Cells were re-suspended in 400µl lysis buffer, and membrane and supernatant fractions diluted with a further 400µl of lysis buffer.

5.3.3 Scintillation proximity assay (SPA)

Figure 5.1 illustrates the principle behind the SPA technique. The activity of the cAMP hydrolysing PDEs is measured by their ability to hydrolyse cAMP to AMP, which is then captured by yttrium silicate SPA beads and quantified.

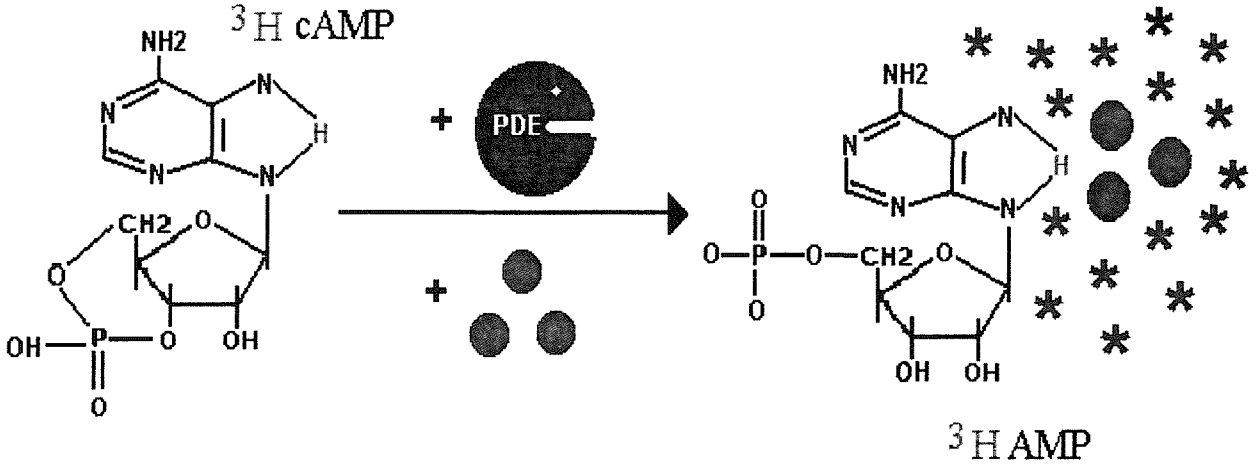


Figure 5.1 The basis of the SPA assay. Tritiated cAMP is hydrolysed by phosphodiesterases (PDE) to produce labelled AMP, which binds to yttrium silicate beads and causes them to scintillate.

Yttrium silicate has a high affinity for AMP, the product of cAMP hydrolysis by the phosphodiesterase enzymes. Using [^{3}H] cAMP as the substrate, the tritiated AMP product was captured on the SPA beads and its close proximity then allowed β-radiation from the

tritium to excite the scintillant within the bead. The emitted light was quantified using a scintillation counter.

Cell lysates were diluted 1 in 10 in TES buffer, and 50µl was incubated for 30min at room temperature (20°C) with 100µl 0.15 µM [^{3}H] cAMP to give a final concentration of 0.1µM substrate. After this time 50µl of SPA beads were added in order to bind the [^{3}H] AMP hydrolysis product and, together with the zinc sulphate also present with the beads, this terminated the reaction. The beads were then left to settle in the plate for 30 min before counting in the Top Count scintillation counter (Canberra Packard). All assays were carried out in triplicate. The effect of rolipram, a PDE4 inhibitor, on enzyme activity in the soluble and particulate fractions was determined in the final concentration range of 10^{-7}M to 10^{-5}M. Final drug dilutions were prepared in the 100µl [^{3}H]cAMP solution added at the start of the experiment. Cyclic GMP was added at a final concentration of 10^{-4}M. This high level of cGMP will activate the PDE2 enzyme to hydrolyse cAMP. However, this PDE2 activity is unlikely to contribute to cAMP hydrolysing activity, as there are no reports of this form of PDE in eosinophils, neutrophils or PBMC. Such a high concentration of cGMP also ensured that any PDE3 would utilise this substrate preferentially over cAMP and, hence, PDE3 activity would not contribute to the observed cAMP hydrolysing activity. The addition of cGMP thus left the cAMP hydrolysing activity only attributable to either PDE4, PDE7 or PDE8. The contribution of PDE7 enzyme to this activity was assessed by the addition of CT5651, a PDE7 inhibitor, at a concentration of 5 X 10^{-7}M.

Number of wells	Dilution of cell extract	cGMP	rolipram	CT 5651
1-3	1:10			
4-6	1:10	100μM		
7-9	1:10	100μM	10-7M	
10-12	1:10	100μM	5x10-7M	
13-15	1:10	100μM	10-6M	
16-18	1:10	100μM	5x10-6M	
19-21	1:10	100μM	10-5M	
22-24	1:10	100μM	5x10-5M	
25-27	1:10	100μM	10-5M	5x10-7M

Table 5.2 Plate layout for SPA assay. All concentrations are given as the final concentrations they are at in the assay.

5.3.4 Assay development

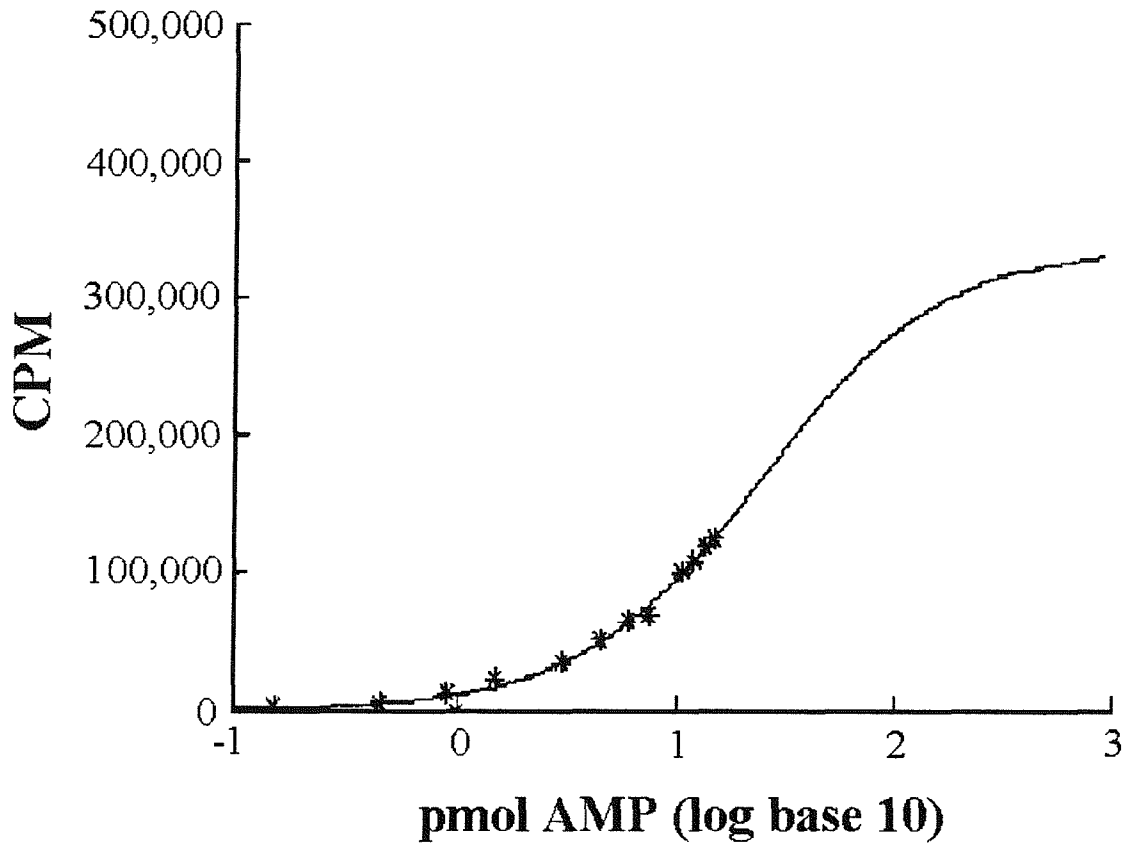


Figure 5.2 Sigmoid standard curve for ^{3}[H] AMP.

Enzyme activity was measured as counts per minute (CPM) and then converted into fmol/min/million cells. This conversion was possible using the AMP standard concentration curve in Figure 5.2, constructed by adding a range of concentrations of [^{3}H]AMP prepared in TES buffer at the same final volume as that stated for the enzyme assays. This data was transformed to give a sigmoid curve using the InPlot™ (GraphPad Software, San Diego, CA, USA) software package, and this standard curve is shown in figure 5.2.

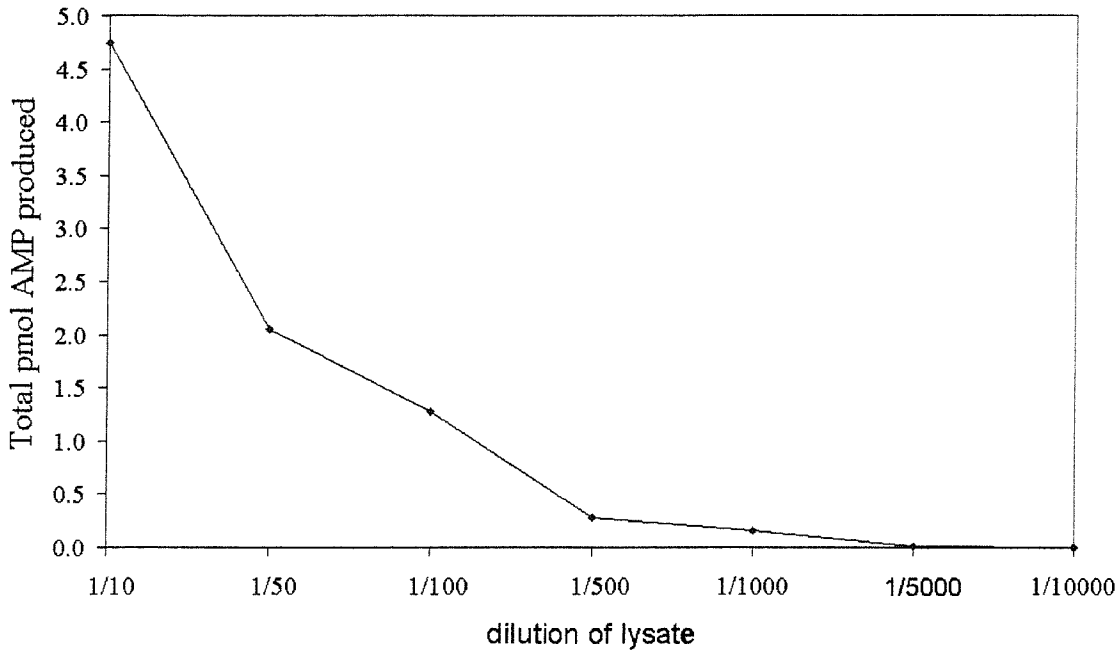


Figure 5.3 Dilution curve of the particulate fraction of a preparation of human eosinophils

In preliminary experiments dilution curves of the lysate fractions were performed in order to determine the optimal dilution of cell lysate that would give approximately 10% substrate conversion, i.e. which gave 1.5 pmol ^{3}H[AMP]. From eosinophil, neutrophil and PBMC data a dilution of 1:10 was determined to be optimum.

5.3.5 Statistics

Initially Kruskal-Wallis tests were carried out and if these gave a significant difference between groups, the Mann Whitney U test was used to investigate in which two groups the data was significantly different.

5.4 Results

Using the standard assay conditions that were developed for this study, the activity of cAMP hydrolysing enzymes in the soluble and particulate fraction of eosinophils, neutrophils and PBMC were compared.

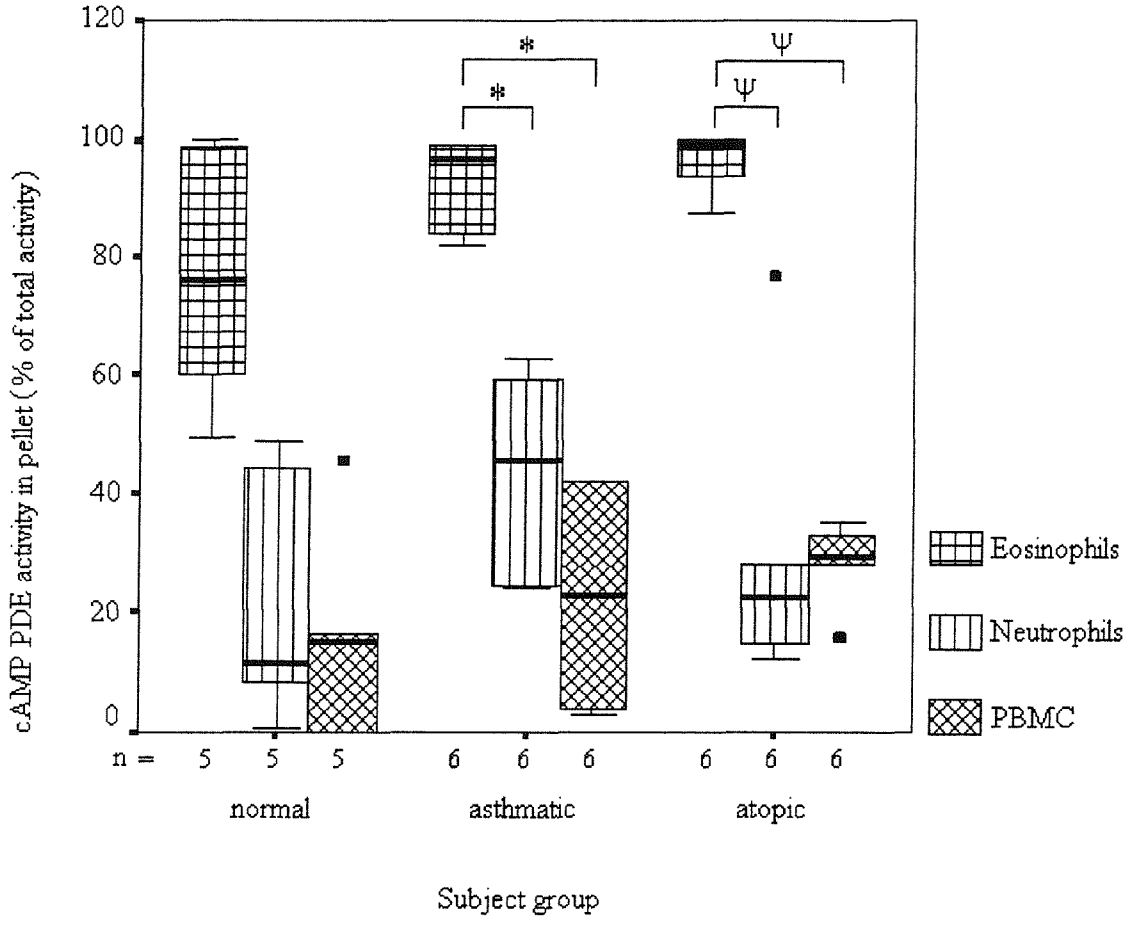


Figure 5.4 cAMP hydrolysing activity in cell pellets expressed as % of total.
Box and Whisker plot, interquartile range represented by solid box, median by solid bar and actual range by whiskers. Solid circles represent outliers.
ψ represents p = 0.0039 and * represents p= 0.0037 (Mann Whitney U test).

Figure 5.4 clearly demonstrates that the majority of cAMP hydrolysing activity is present in the membrane fraction of eosinophils, although this is not true of neutrophils and PBMC, where more activity is found in the soluble fraction.

Total activity was measured as a combination of the results obtained from the pellet and supernatant samples; activity in the pellet fraction was then expressed as a percentage of this. The level of PDE activity in the soluble fraction of eosinophils from normal donors was barely detectable at a 1:10 dilution; therefore a 1:2 dilution was used to determine total activity. This, unfortunately, meant that not enough sample was available to assess how much of this total activity was attributable to individual PDE family members

The percentage activity in the membrane fraction was significantly higher in eosinophils (96.6 (82-99.5) %) than in either neutrophils (45.45 (24.4-62.82) %) or PBMCs (22.95 (2.7-42.36) %) in asthmatics donors. The same was also true of atopic donors, eosinophils (99.35 (87.42-100) %), neutrophils (22.49 (12.3-76.86) %) and PBMC (29.12 (15.94-35.03) %). The percentage activity in the membrane fraction of eosinophils was not significantly higher in normal subjects than in other subjects; however it did follow the same trend, eosinophils (99 (49.6-100) %), neutrophils (11.17 (0.53-48.92) %) and PBMC (15 (0-45.48) %). There was no difference in the percentage of PDE activity that was membrane bound between eosinophils from the different subject groups. This observation is also true of the other cells types neutrophils and PBMC. There was also no significant difference in the percentage activity that was membrane bound between neutrophils and PBMC for the different subject groups.

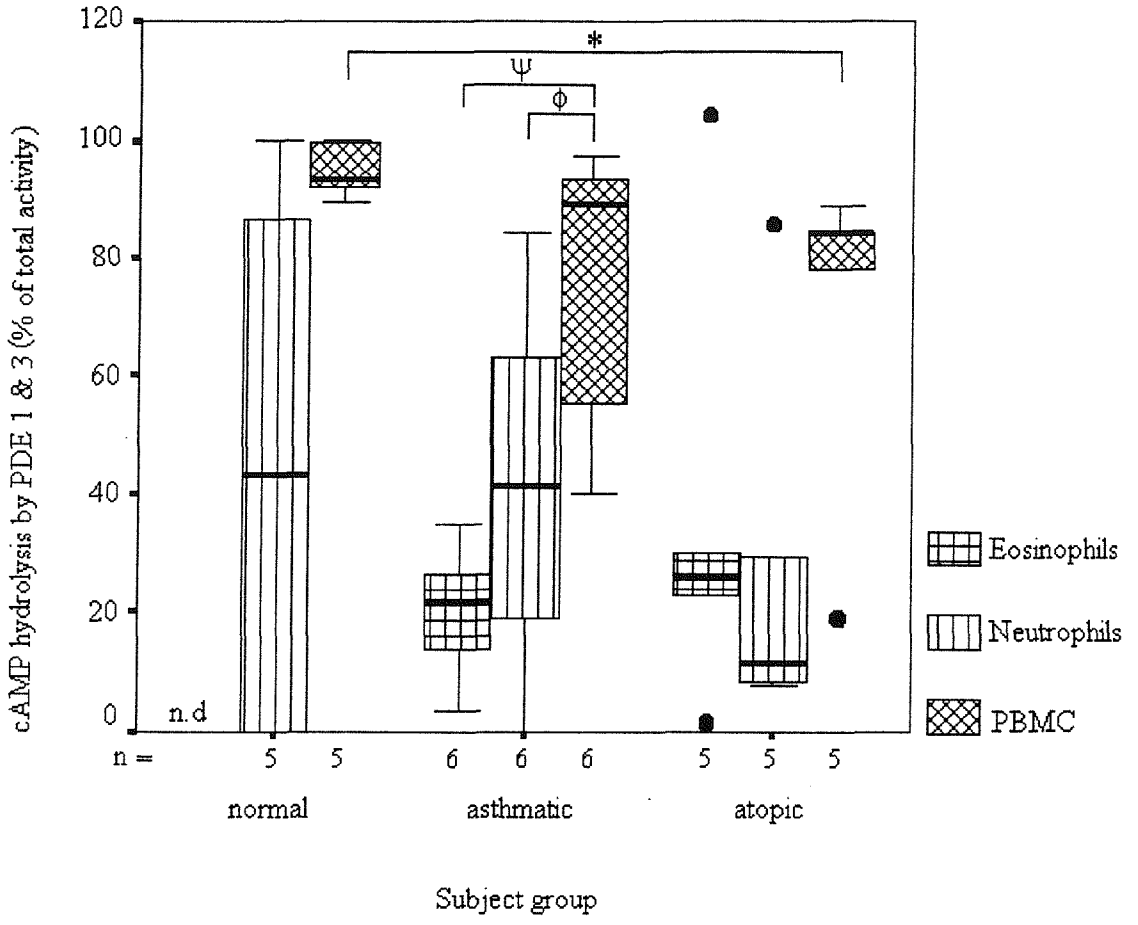


Figure 5.5 % cAMP hydrolysis by PDE3 as a % of total activity (sum of pellet and supernatant)
Activity that was inhibited by addition of 100μM cGMP was determined to PDE3.
* represents p=0.0062, ψ represents p= 0.0039, ϕ represents p=0.0374. (Mann Whitney U test). Solid circles represent outliers. (n.d) represents experiments not done.

In the presence of 100μM cGMP, PDE3 does not hydrolyse cAMP as the high concentration of cGMP acts as a competitive inhibitor at its catalytic site. Therefore the activity inhibited by 100μM cGMP is attributed to PDE3, and the remaining cAMP hydrolysing activity is due to PDE4, 7 and 8. As Figure 5.5 illustrates, a high percentage of total cAMP hydrolysing activity in PBMC is due to PDE3. There was a significantly higher percentage of total cAMP PDE activity attributable to PDE3 in PBMCs from normal subjects (93.26 (89.44-100) %) than atopics (83.85 (19.08-88.76) %). However, there was no difference in PDE3 in PBMC between normal subjects and asthmatics (88.39 (40.3-

97.26) %). A large variation in the percentage PDE3 between donors was seen in the group of asthmatics. Also, a significantly higher percentage of PDE3 activity was observed in the PBMC from asthmatics (88.39 (40.3-97.26) %) compared to neutrophils (31.46 (0-84.14)%) and eosinophils (21.94 (3.5-34.7)%), from the same donors.

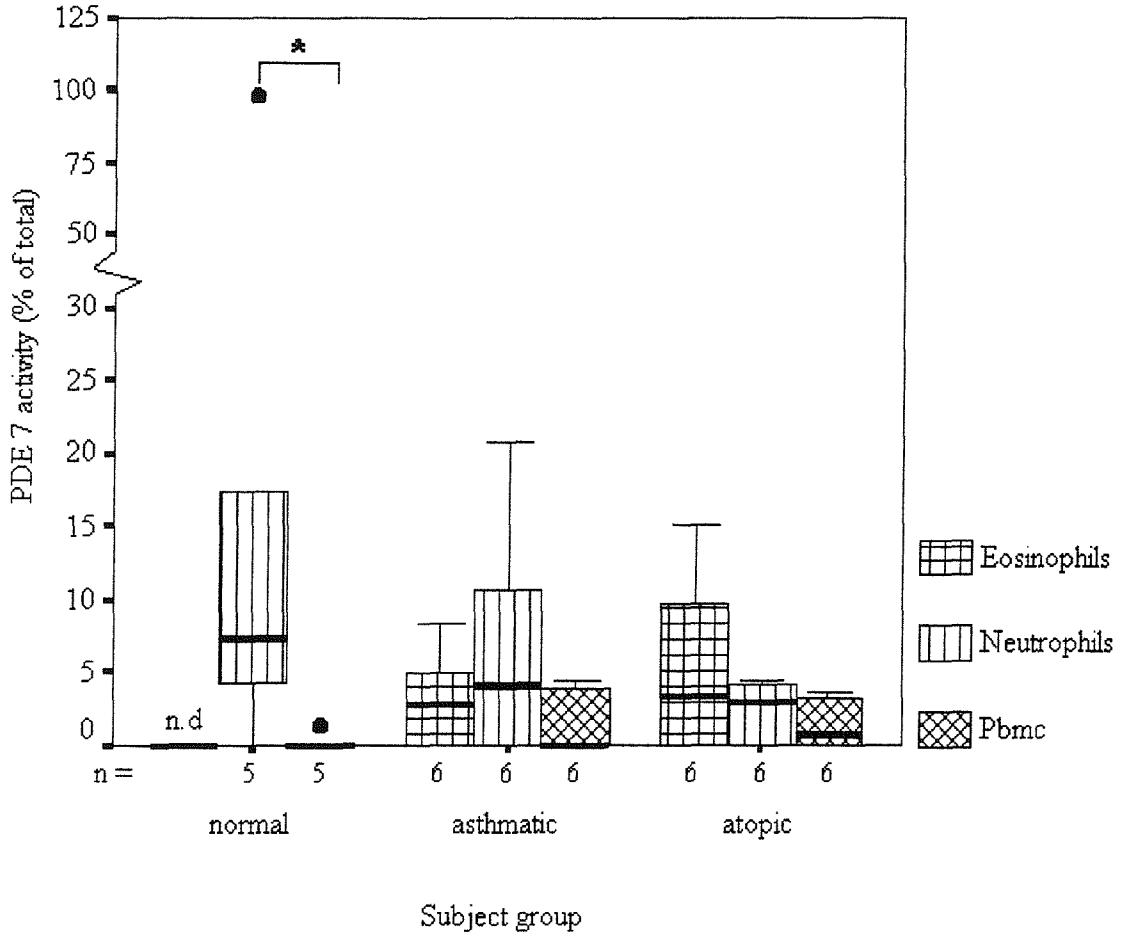


Figure 5.6 PDE7 activity as a % of total activity (sum of pellet and supernatant).
PDE7 activity was determined as activity that could be inhibited by CT 5651 a selective PDE7 inhibitor. * represents p= 0.0343 (Mann Whitney U test).
Solid circle represent outliers. (n.d) represents experiments not done.

PDE7 was assayed as the percentage of total activity (pellet and supernatant) that could be inhibited by CT5651, a specific PDE7 inhibitor, developed by Celltech. As can be seen in figure 5.7, cAMP hydrolysing activity due to PDE7 is a minor component of total cAMP hydrolysing activity in eosinophils, neutrophils and PBMCs from all subject groups. A significantly higher percentage of PDE7 activity was observed in neutrophils (7.3 (0-100) %) than PBMC (0 (0-1.2) %) for normal donors.

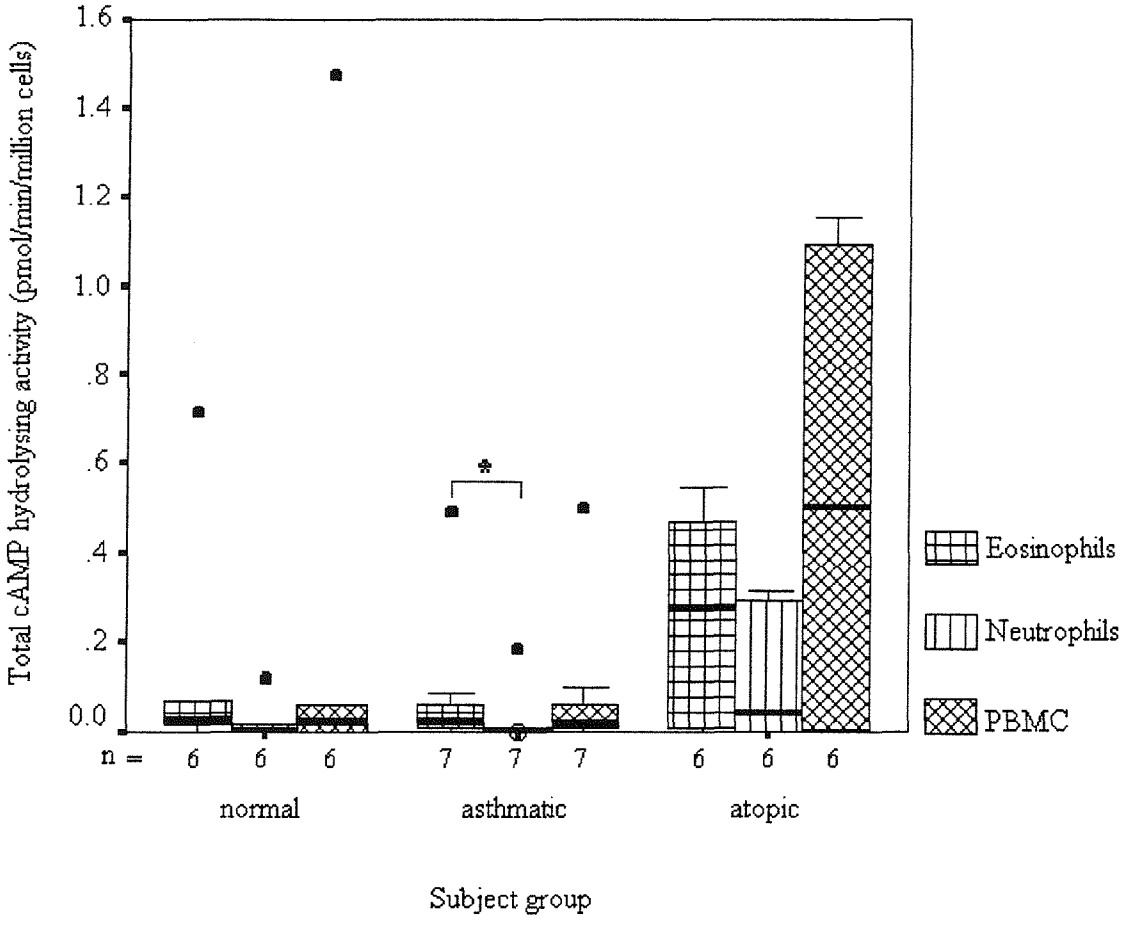


Figure 5.7 Total cAMP hydrolysing activity
Total activity is that of the combined activity of pellet and supernatant fractions.
* represents p=0.022 (Mann Whitney U test)

The SPA assay gave total cAMP hydrolysing activity considerably lower in these results than those previously published. There was significantly higher PDE activity in eosinophils from asthmatics (26.8 (4.5 - 489.5) fmol/min/10^6 cells) than neutrophils (3.4 (0-183.8) fmol/min/10^6) from the same group. This trend was also repeated in atopic and normal donors. However, it did not reach significance. Whilst not significant, it would also appear that eosinophils, neutrophils and PBMC from atopic donors showed a trend for higher levels of cAMP hydrolysing activity than cells from asthmatics or atopics.

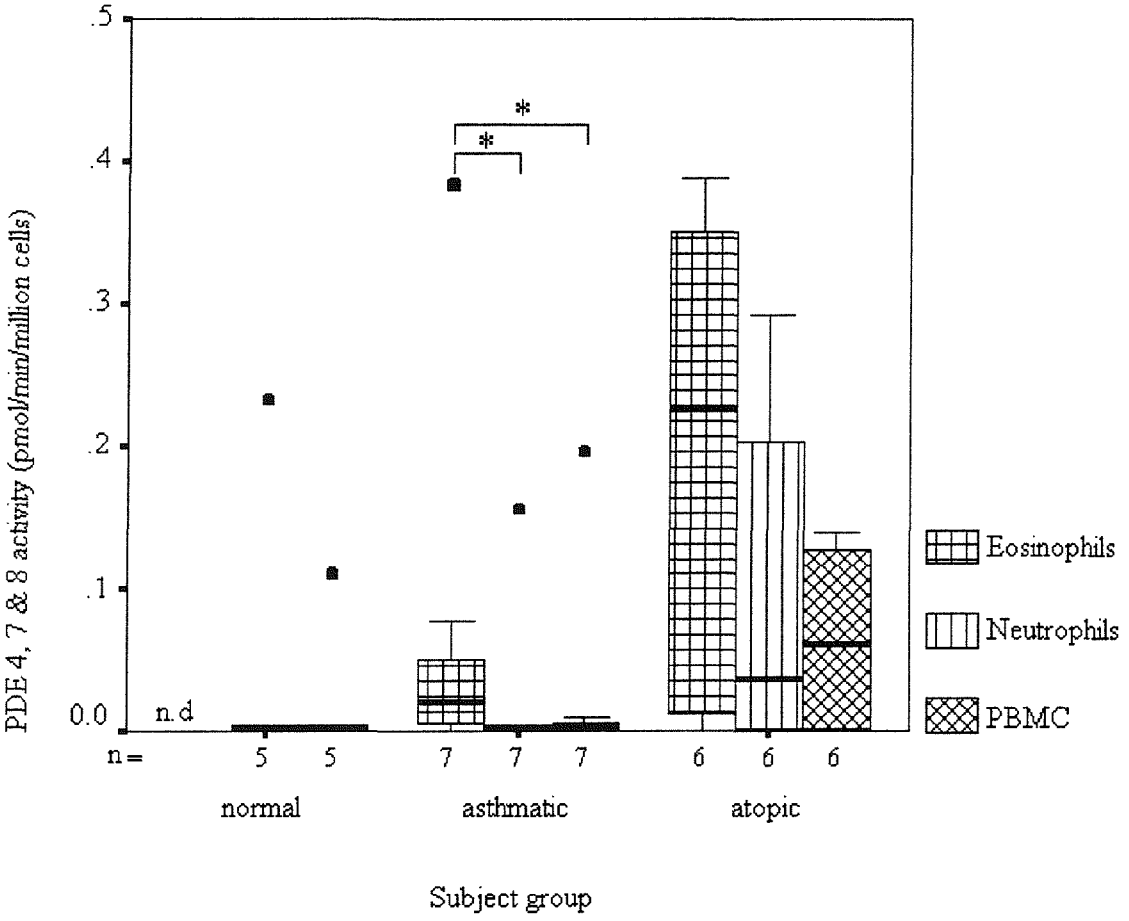


Figure 5.8 PDE 4, 7 and 8 activity.
PDE4, 7 and 8 activity is that which is not inhibited by addition of 100μM cGMP.
* represents p=0.035 (Mann Whitney U test). (n.d) represents experiments not done.

Reflecting the low total cAMP hydrolysing PDE activity levels, the levels of activity attributable to PDE4, 7 and 8 were also lower than previous reports. Significantly higher levels of PDE4, 7 and 8 cAMP hydrolysing activity were detected in eosinophils from asthmatics (20.8 (3.3-383.8) fmol/min/10^6 cells) in comparison to neutrophils (2 (0-154.4) fmol/min/10^6) and PBMC (1.7 (0.3-195.2) fmol/min/10^6) from the same donors. A similar trend for cells isolated from atopic subjects was observed, eosinophils (347.2 (11.2-387.1) fmol/min/10^6), compared to neutrophils (35.5 (1.6-203.2) fmol/min/10^6 cells), and PBMC (59.8 (0.6-139) fmol/min/10^6 cells). The values from normal donors were not significantly lower, due to outliers in neutrophils and PBMC. However, there is s trend towards lower values. Data was not obtained for normal eosinophils; as discussed earlier in the methods section, this was due to the limited number of cells that could be isolated from 100ml of normal blood.

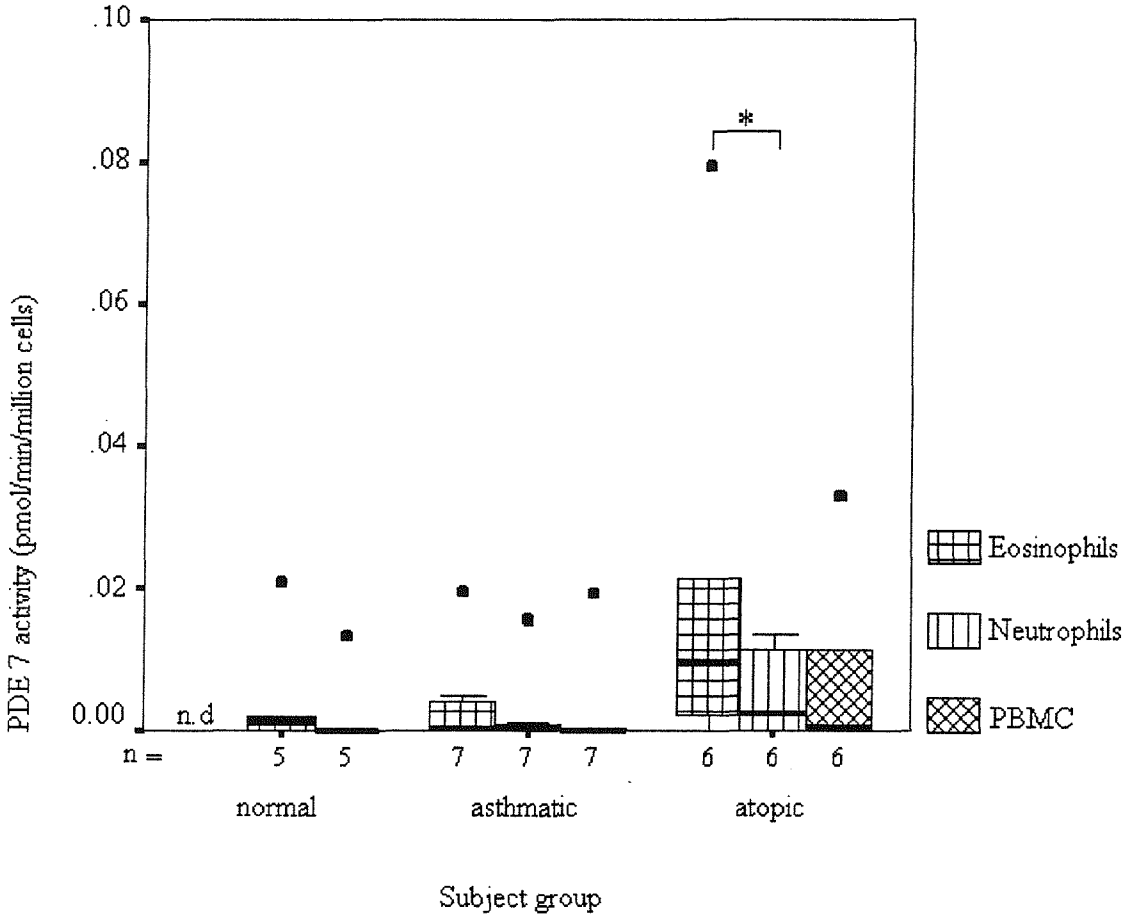


Figure 5.9 PDE 7 activity
PDE7 activity is determined as total activity in the pellet and supernatant that is inhibited by CT 5651, a selective PDE7 inhibitor.
* represents p=0.048 (Mann Whitney U test). (n.d) represents experiments not done.

As Figure 5.9 demonstrates, PDE7 activity is very low in eosinophils, neutrophils and PBMC from all subject groups. The results indicate higher levels of PDE7 activity in eosinophils from atopic subjects (14.6 (1.9-79.3) fmol/min/10^6 cells), in comparison with neutrophils from the same donors (2 (0-11.2) fmol/min/10^6 cells). It is also noticeable, though not significant, that cells from atopic patients have higher levels of PDE7 activity than cells from normal or asthmatic donors.

	Subject group	Membrane μM	number	Soluble μM	number
Eosinophils	Normal	n.d	1	n.d	1
	Asthmatic	0.95	2	n.d	2
	Atopic	0.56	2	n.d	2
Neutrophils	Normal	1	1	1	1
	Asthmatic	0.87	1	0.6	1
	Atopic	1.24	3	0.37	3
PBMC	Normal	0.9	1	0.23	1
	Asthmatic	0.46	1	0.1	1
	Atopic	0.35	3	0.73	3

Table 5.3 IC 50 values for inhibition with rolipram
n.d represents data not available as activity was un-detectable.
The above numbers are representative of the number of samples that had sufficient activity and sample volume to give data for the whole rolipram inhibition curve.

Unfortunately, few of the tested samples had sufficiently high levels of PDE4 activity to give a full rolipram inhibition curve. Therefore, the n values for this analysis are too low for statistical analysis. However, a significantly higher IC_{50} for rolipram was observed in the membrane bound PDE4 in neutrophils from atopics than the soluble form from the same cells ($p= 0.05$). The membrane bound PDE4 in neutrophils from atopic donors also had a significantly higher IC_{50} for rolipram than that observed for the membrane bound PDE4 from PBMC of the same donors ($p=0.05$).

5.5 Discussion

It is illustrated, by the results in this Chapter, that the cAMP hydrolysing PDEs show different cellular distributions between inflammatory cells. There is a particularly striking association of cAMP hydrolysing PDEs in eosinophils with the membrane fraction, and this fraction accounts for approximately 80% of total activity. This membrane localisation has also been noted by Dent et al 1994, in human eosinophils. However Hatzelmann et al 1995,

and Gantner et al 1997, reported the majority of cAMP PDE to be in the soluble fraction. Cells were disrupted by Triton X-100, a neutral detergent, in the experiments reported in this Chapter. The pellet fraction, obtained by centrifugation at 1500g, contained intracellular structures, such as the nuclei, nucleoli, and storage granules, as well as membrane structures such as the endoplasmic reticulum and the golgi apparatus. The association of PDE activity with this fraction implies that the PDEs are linked by some mechanism to these structures. This scenario is reported in work published investigating a recombinant form of PDE4A within transfected thyroid carcinoma cells. RD1, the recombinant 4A, contains membrane anchoring regions that locate it exclusively with the membrane fraction (Pooley et al. 1997).

This association of PDE with membranes in eosinophils is not observed in neutrophils or PBMC; instead, approximately 60% of total activity was found in the soluble fraction in these cells. Whilst this soluble location for PDE activity is in agreement with published reports for PBMCs (Truong and Müller, 1994), and for the individual cellular components of T cells (Tenor et al. 1995b; Giembycz et al. 1996) and monocytes (Souness et al. 1996; Gantner et al. 1997a), there are again conflicting reports for neutrophils. Schudt et al 1991, reported only 30% of activity to be membrane bound which our results are in agreement with, however Wright et al 1990, reported 80% of activity to be membrane bound. These conflicting reports for PDE localisation within eosinophils and neutrophils may stem from the method or cell disruption. Detergent lysis by Triton X-100, the method utilised in these experiments, liberates proteins into the soluble fraction that are bound by hydrophobic interactions, or to structures that themselves are solubilised by Triton. However, this method will not affect any enzymes bound by ionic interactions and only strong salt concentrations can break these bonds (Huston et al. 1997). It is likely that individual splice

variants use different anchoring methods to attach to different intracellular structures. Huston et al 1997, reported that PDE4B2 could be liberated from the membrane fraction of COS 7 cells (isolated by a high speed centrifugation) by Triton X 100, whilst the same splice variant was only liberated from the membrane fraction (isolated by low speed centrifugation) by 2M NaCl, suggesting ionic interactions were in place. Many published reports have disrupted cells by sonication; however the energy output of sonication may differentially affect the distribution of the PDEs (Hatzelmann et al. 1995). These observations illustrate that the method of separation is likely to influence the localisation of some forms of PDE, depending upon their strength of binding to various structures within the cells.

The percentage of total cAMP activity attributable to PDE3, which was inhibited by high concentrations of cGMP, also differs between cells. Eosinophils express the smallest percentage of PDE3, which is as expected since PDE4 is widely documented to be the predominant PDE form in these cells (Hatzelmann et al. 1995; Dent et al. 1994b, Tenor et al. 1996). Neutrophils also express a relatively small percentage of PDE3 and, again, PDE4 is reported to be the predominant PDE. It is interesting that, in published reports investigating PDE expression in eosinophils and neutrophils, PDE3 activity was not consistently reported. However, the results in this Chapter, of low levels of cGMP inhibitable PDE activity, probably due to PDE3, are confirmed in eosinophils by Gantner et al 1997,and in neutrophils by Wright et al. (Gantner et al. 1997a; Wright et al. 1990). PBMCs expressed a significantly higher percentage of total cAMP hydrolysing activity that could be attributed to PDE3, in comparison to eosinophils and neutrophils in asthmatics, and a similar trend was observed in normals and atopics. This is as expected, considering T cells are the predominant component of PBMC (68%) and PDE3 is the most abundantly

expressed isoform in these cells (Sheth et al. 1997). It would also appear from our results that PBMC from normal donors (median 93.2%) had a higher percentage PDE3 activity than from atopic donors (median 83.8%). This difference, however, was not large enough to be reflected in a significant decrease in another PDE form in normal PBMC, in comparison to atopics.

The percentage of total cAMP hydrolysing PDE activity attributable to PDE7 was below 20% in all cells, and this is in agreement with other observations in eosinophils and T cells (Tenor et al. 1996; Hatzelmann et al. 1995; Tenor et al. 1995b; Seybold et al. 1998).

From our results it was noted that eosinophils from asthmatics have significantly higher levels of total cAMP hydrolysing activity (median 26.8 fmol/min/10^6 cells) than neutrophils (median 3.4 fmol/min/10^6 cells) from the same donors. A similar trend was observed in atopics. This may imply that eosinophils, through higher PDE levels, have a tighter regulation on cAMP levels, and any increases in cAMP levels, following stimulation, are likely to be short lived. As cAMP elevations are likely to be short lived in eosinophils, this would suggest that rises in cAMP levels within eosinophils are responsible for regulation of immediate early responses to stimulation in these cells.

Eosinophils also appear to have higher levels of cAMP PDE activity attributable to PDE4, 7 and 8 than either neutrophils or PBMC. This is significant in asthmatics, (eosinophils (median 20.8 fmol/min/10^6 cells) neutrophils (median 2 fmol/min/10^6 cells) and PBMC (median 1.7 fmol/min/10^6 cells)), and follows a similar trend in atopics, (eosinophils (median 347.2 fmol/min/10^6 cells) neutrophils (median 35.5 fmol/min/10^6 cells) and PBMC (median 59.8 fmol/min/10^6 cells)). One would have expected higher levels of PDE4, 7 and

8 activity in eosinophils, in comparison to PBMC, as PDE4 is the predominant PDE in eosinophils, whilst PDE3 is predominant in T cells. Eosinophils also have higher PDE4, 7 and 8 levels than neutrophils, where again PDE4 is the predominant PDE. These higher activity levels in eosinophils may result from increased PDE expression in these cells or increased activity of the PDE enzymes, as a consequence of their membrane association. Changes in activity levels depending upon intracellular localisation are reported by Huston et al 1997, who showed that particulate associated forms of PDE4, 4B1 and 4B2, expressed in COS 7 cells, had activity levels 40% lower than their cytosolic counterparts. Whilst opposite from these results, it is feasible that in the membrane fraction of eosinophils, a PDE form (either PDE4, 7 or 8) is able to adopt a conformational change that increases its activity, in comparison to the same soluble enzyme in neutrophils.

Although not significant, there is a general trend for atopic donors to have higher cAMP hydrolysing activity, in comparison to normal or asthmatic subjects in all cell types. Previous reports have differed on this issue. Holden et al 1986, reported higher PDE levels in monocytes from atopic dermatitis patients, whilst Gantner et al 1997, observed no differences in PDE levels in monocytes, eosinophils or T cells from patients with atopic dermatitis in comparison to normal subjects (Gantner et al. 1997a). The trend observed in our results is also associated with a wide range in activity values within the atopic group, in comparison to either asthmatics or normals. This might suggest that patients characterised as atopic do not consistently exhibit raised PDE activity levels. It is interesting, also, that patients characterised as asthmatic in this study are also atopic, and do not show any trend towards raised PDE levels; therefore suggesting that any trend in increased PDE activity levels is not associated with atopy.

The values for total cAMP PDE activity obtained in this Chapter are lower than those published for all cell types. Eosinophils gave ten fold lower values (average 0.1708 pmol/min/10^6 cells) than those reported by other groups (2.35 pmol/min/10^6 cells) (Hatzelmann et al. 1995). Whilst the results for neutrophils (average 0.027 pmol/min/10^6 cells) are approximately 50 times lower than those published (1.5 pmol/min/10^6 cells) (Mahomed et al. 1996). The values for PBMC (average 0.3149 pmol/min/10^6 cells) are closest, being only half those observed in monocytes (0.59 pmol/min/10^6 cell) and equal to those in T cells (0.29 pmol/min/10^6 cells) (Gantner et al. 1997a). These comparisons are for normal donors, where sonication was used as the method of cell lysis. There may be several explanations to account for the low levels of activity we observed in neutrophils and eosinophils. Firstly, the method of quantification was different. We used the SPA technique to measure PDE cAMP hydrolysing activity by the production of AMP from cAMP. All other studies employed a technique, first described by Thompson, which measures the production of adenosine, the result of further breakdown of AMP (Thompson and Applemann, 1971). This technique utilises the ability of snake venom (a 5′ nucleotidase) or alkaline phosphatase to break down AMP (the first breakdown product of cAMP) to adenosine. 5′ nucleotidases are also present endogenously in cells, and could account for the low values observed in our experiments. The breakdown of AMP to adenosine in our assays by these 5` nucleotidases would lead to an underestimation of activity levels, since only AMP is measured in our system as the SPA beads are unable to bind adenosine. ADP may be used in other assays to competitively block the activity of these 5′ nucleotidases; however, ADP itself can bind non specifically to the SPA beads and, hence, could not be used. Another explanation for the low activity levels may be the proteolytic cleavage of the PDEs by proteases in eosinophils and neutrophils. This cleavage was indicated by the Western blot results presented in Chapter 4, where several smaller cleaved products of

PDE4D, 4A and 4B were observed. If PDEs underwent cleavage near their active site, it may render them inactive and, hence, activity assays would give lower estimates of total protein present. It could also be possible that the substrate concentration of cAMP used in our assays affected the activity levels. Tenor et al 1995, clearly demonstrated that changes in substrate concentration affect the activity of PDE (Tenor et al. 1995a). Therefore, the use of 0.1μM cAMP in our experiments, in comparison to 0.5μM or 1μM used by other investigators, may have decreased our activity levels (Dent et al. 1991; Tenor et al. 1995a). A final explanation may rest in the observation by Truong and Muller 1994, that sonication results in activity levels two fold higher than those observed when detergent lysis was used. Therefore, the sonication lysis used in all comparable reports may in fact activate these PDEs.

The IC_{50} for rolipram inhibition in the membrane fraction of neutrophils from atopic donors (mean 1.24 μM) is significantly higher than that for the soluble enzyme (mean 0.372 μM) from the same cells. This would imply that the PDE4 enzyme found in the soluble fraction has a higher affinity for rolipram and, hence, lower IC_{50}. This difference in IC_{50}s may be due to a different distribution of PDE4 splice variants between the soluble and particulate fraction. Indeed, work by Bolger et al 1997, has demonstrated that PDE4D isoforms in transfected COS cells have different IC_{50}s for rolipram, for example, PDE4D5 has an IC_{50} at least 10 fold higher than that for 4D4 (Bolger et al. 1997). It is also possible that the same splice variant expressed, in both fractions, adopts a conformation in the soluble fraction that is more susceptible to rolipram inhibition. This has again been shown in COS cells, where PDE4D3 and 4D5 have an IC_{50} in the particulate fraction at least double in value to that when they are in the soluble fraction. The membrane bound PDE4 in atopic neutrophils (mean 1.24 μM) also has a significantly higher IC_{50} for rolipram than the membrane bound

PDE4 from atopic PBMCs (mean 0.354 μM); again, any of the above explanations may account for this. Unfortunately, comparisons of IC 50 values for rolipram in the membrane and soluble fractions of eosinophils were not possible, as the majority of PDE activity was membrane associated and soluble fractions had undetectable levels of PDE activity.

IC 50 values for rolipram inhibition obtained in our studies (mean 0.56 μM) are in the same order of magnitude as those previously published for atopic eosinophils (0.16 μM) (Tenor et al. 1996), whilst neutrophils from normal donors in our studies (1 μM) were less sensitive to rolipram inhibition than in published data (0.13 μM) (Schudt et al. 1991). However, PDE inhibition by rolipram in the soluble fraction of PBMC s (0.097-0.731 μM) was again within limits of published reports (monocytes 0.17 μM and T cells 0.1-0.3 μM) (Gantner et al. 1997a; Giembycz et al. 1996).

Due to small numbers of samples giving high enough activity levels to obtain rolipram IC_{50} values, it was not possible to compare the sensitivity of PDE 4 to rolipram between different donor groups within our experiments. It is interesting to note that published reports differ again in this issue, Banner et al 1995, reported a significantly lower IC_{50} value for rolipram in PBMC from atopic dermatitis patients, in comparison with normals (Banner et al. 1995c), whilst Gantner et al 1997, reported no differences in IC_{50} values in monocytes between these two subject groups (Gantner et al. 1997a).

It would appear, from the results in this Chapter, that there are differences in the contribution of individual PDEs to the total cAMP hydrolysing PDE activity between different inflammatory cell types. It is also the case that there are differences in distribution of this activity to subcellular compartments between these cells. Through the expression of

different PDEs, or differential compartmentalisation of the same PDE, it is possible for these cells to exhibit differences in sensitivity to rolipram inhibition. The numerous combinations of PDE expression and localisation will allow these cells to regulate a huge variety of intracellular processes reliant upon cAMP elevations. Results in this Chapter have failed to show any significant difference in PDE activity levels between cells derived from atopic, asthmatic or normal donors, implying that this is not a feature of atopy or asthma.

Chapter 6

Release and synthesis of tPA and PAI-1 from primary human bronchial epithelial cells

6.1 Introduction

In asthma it would appear that the fine balance between fibrosis of the airways, caused by excess ECM synthesis, and proteolysis caused by degradation of ECM, is not in equilibrium as seen in normal tissues. Bronchial biopsies from asthmatics show subepithelial fibrosis, due to increased deposition of fibronectin and collagen type 3 and 5 deposition, by myofibroblasts (Calhoun and Liu, 1995). The presence of fibrosis would suggest that protease inhibitors are in molar excess, and that there is a decrease in net proteolytic activity in asthmatics. There is evidence from asthmatic bronchial tissue to support this theory. Increased levels of the proteinases elastase and MMP-9 have been reported in asthmatics; however, levels of their inhibitors, α2 antitrypsin and TIMP-1 respectively, are even further elevated (Kips and Pauwels, 1999). There are no reports of plasminogen activator or plasminogen activator inhibitor-1 levels in asthma and, for this reason, this Chapter has focussed on assessing their production by bronchial epithelial cells in culture.

Tissue plasminogen activator (tPA) is a serine protease, whose human form has a molecular weight of 70kDa (Saskela and Rifkin, 1988; Parmer et al. 1997). It is synthesised as a single chain polypeptide containing 527 amino acids, but can be converted, by plasmin, into a more active two chain variant (Elhasade et al. 1998; Johnsen et al. 1998). tPA can be acutely secreted from endothelial cells, and this corresponds to its release from small dense vesicles, which are unique from Weibel Palade bodies within these cells (Emeis et al. 1997). Over a longer time period tPA is synthesised by *de novo* protein synthesis within these endothelial cells (Rydholm et al. 1995).

Plasminogen activator inhibitor (PAI-1) is produced by endothelial cells, epithelial cells and vascular smooth muscle cells (Emeis et al. 1997; Gerwin et al. 1990; Bouchie et al. 1998).

PAI-1 circulates in the plasma, and is a single 379 amino acid chain polypeptide with a molecular weight of 45kDa (Mignatti and Rifkin, 1996). Unlike tPA, there are no stored intracellular pools of PAI-1 and, hence, there is a lag phase, after stimulation, of several hours to allow transcription, translation and processing to occur before any PAI-1 is secreted (Rydholm et al. 1995). PAI-1 forms a 1:1 complex with tPA, inhibiting it (Sartori et al. 1998). It is interesting to note that, if tPA is complexed with fibrin it is immune to the inhibitory effects of PAI-1, and fibrin promotes the activity of tPA (Elhasade et al. 1998).

Agents that increase cAMP levels within cells are known to influence the release and synthesis of both tPA and PAI-1. Dibutyryladenosine 3`,5` cyclic monophosphate (db cAMP), a cell permeant analogue of cAMP, stimulated an increase in tPA mRNA expression in rat hepatocytes. The broad acting PDE inhibitor, IBMX, was able to mediate the same effect (Uno et al. 1998). Isoprenaline, a beta adrenoceptor agonist, was able to stimulate the rapid release of tPA in the human forearm vasculature (Stein et al. 1998). The stimulatory effects of cAMP elevating agents upon baseline tPA release and synthesis, however, have not been reported by all investigators. Santell and Levin reported that forskolin, an adenylyl cyclase activator, IBMX or db cAMP had no significant effect on tPA secretion from human umbilical vein endothelial cells (HUVECs) (Santell and Levin, 1988). The effects of cAMP elevating agents on baseline PAI-1 levels are less disputed. IBMX, db cAMP and forskolin all decreased PAI-1 synthesis in human endothelial cells (Santell and Levin, 1988; Rydholm et al. 1995). The same effect was observed with these drugs in rat hepatocytes (Uno et al. 1998).

If, as it appears, the net effect of cAMP elevating agents upon the tPA/PAI-1 balance is a decrease in PAI-1, a shift in the balance in favour of proteolysis is predicted. There are no

previous reports of the effects of theophylline, a broad acting PDE inhibitor, and rolipram, a specific PDE4 inhibitor, on constitutive tPA and PAI-1 release from bronchial epithelial cells. PDE3 has been reported in human epithelial cells (Dent et al. 1998a) and Kelley et al.(1995) reported that milrinone, a specific PDE 3 inhibitor, increased chloride efflux through the cystic fibrosis conductance regulator (CFTR) channel in an epithelial cell line. This effect was through the cAMP dependent activation of PKA. These observations suggest that PDE3 has an important role in regulation of cAMP levels in these cells, and the effects of milrinone were therefore investigated. The effects of salbutamol, a short acting β_2 agonist, were studied as β receptors are present on bronchial epithelial cells (reviewed by Liggett, 1998), and this agent is widely used as a treatment in asthma. Histamine and methacholine both elicit bronchoconstriction, and are used to determine the airways responsiveness in asthmatic patients. Both of these agents have also been reported to increase release of tPA in human endothelial cells, and human forearm vasculature respectively (Hanss and Collen, 1987; Wall et al. 1998), and were therefore investigated.

Aims

The aims of this chapter are two fold. Firstly, to investigate if human primary bronchial epithelial cells released tPA or PAI-1 in culture, as has been previously demonstrated in human endothelial cells (Rydholm et al. 1995). Secondly, to investigate the effects of agents that modify intracellular cAMP levels upon the production and release of tPA and PAI-1.

6.2 Materials

Primary bronchial epithelial cells and basal epithelial growth medium (BEGM) were purchased from BioWhittaker (Wokingham, UK). Heat inactivated foetal bovine serum and phosphate buffered saline (PBS) were purchased from Gibco BRL (Paisley, Scotland).

Tissue culture 24 well plates and 75cm^2 flasks were purchased from Greiner (Stonehouse UK). Gelatin, trypsin-EDTA solution (10X), insulin-transferrin-sodium selenite media supplement, salbutamol, histamine diphosphate and methacholine chloride were purchased from Sigma (Poole UK). Theophylline, rolipram and milrinone were gifts from Celltech (Slough UK). tPA and PAI-1 enzyme immunoassays (ELISAs) were purchased from American Diagnostica Inc (Greenwhich USA).

6.3 Methods

6.3.1 Cell culture

Primary bronchial epithelial cells, derived from normal donors, which are certified to have normal morphology, were re-suspended in 1ml BEGM supplemented media (bovine pituitary extract 25μg/ml, human recombinant epidermal growth factor 5ng/ml, hydrocortisone 0.5μg/ml, adrenaline 0.5μg/ml, transferrin 10μg/ml, insulin 5μg/ml, retinoic acid 0.1ng/ml, triiodothyronin 6.5ng/ml, gentamycin 50μg/ml and amphotericin B 25μg/ml) with 1% foetal bovine serum that had been warmed to 37°C. The re-suspended cells were seeded at a density of 3500cells/cm^2 in 75cm^2 flasks and 24 well plates. Flasks and plates were previously coated with 1% gelatin, in a humidified atmosphere, for 1 hour at 37°C. The cells were allowed to grow to 80% confluency, and then used in experiments or passaged and split into further flasks. Passaging was carried out by removing all medium, then washing the cells three times with 10ml 1X PBS. Trypsin (50μg/ml), EDTA (20μg/ml) solution (5ml) was added to the flasks, and the cells incubated at 37°C until they had rounded up and started to detach from the flask. This took approximately 3-5 min and was observed under a microscope. Flasks were gently rapped to aid this detachment. 1ml of foetal bovine serum was then added to neutralise the trypsin, and the solution containing

the cells was transferred to a 15ml centrifuge tube and centrifuged at 220g for 5 min at 20°C. The supernatant was removed, and the cells were resuspended in 1 ml BEGM serum supplemented media and counted using a haemocytometer. Cells were then re-seeded as above. All primary cells used in these experiments were between passage 2 and 5.

6.3.2 Challenge of cells

24 hours prior to stimulation, the cells were serum starved by removing serum supplemented media, washing twice with 1X PBS, and then culturing for 24 hours in BEGM basal media with serum free supplements containing 5μg/ml insulin, 5μg/ml transferrin and 5ng/ml sodium selenite. Experiments were carried out at time courses, between 15min and 16 hours, using concentrations of drugs previously shown to elicit biological responses in other cell culture experiments. Theophylline was used at 10^{-3}M, rolipram at 10^{-4}M, milrinone at 10^{-4}M, histamine diphosphate and methacholine chloride both at 10^{-4}M. Theophylline, rolipram and milrinone were initially made up at a 100X concentration in DMSO, and then further diluted in serum free basal media. Histamine and methacholine were dissolved in serum free basal media. Control wells for these experiments contained serum free basal media with no drugs. Cell supernatants were collected by aspiration during the time course, and stored in aliquots at -20°C until use.

6.3.3 tPA and PAI-1 measurements

Undiluted samples of culture supernatants for tPA ELISAs were assayed. Samples for PAI-1 analysis were initially analysed at a 1 in 5 dilution in basal medium, and then further dilutions were made, dependent upon initial results. ELISAs were carried out as indicated in the manufacturers' instructions, with minor modifications. Briefly, the culture supernatants were incubated for 1 hour at room temperature for tPA, and overnight at 4°C

for PAI-1, in the wells of an ELISA plate coated with primary antibodies directed against tPA or PAI-1. In the tPA assay greater than 95% of tPA binds to this antibody, and in the PAI-1 ELISA both latent and active forms of PAI-1, as well as complexed forms, are detected. After this incubation, wells were washed three times and then incubated with the secondary antibody, an anti-tPA HRP conjugate or anti-PAI-1 biotin conjugate. Streptavidin HRP is then added to the PAI-1 ELISA, and peroxidase substrate added to both assays to give a colourimetric read out. Stop solution was added to both ELISAs, and they were read at 450nm for PAI-1 and 490nm for tPA on a Dynatech MR5000 plate reader. Concentrations were calculated automatically by the plate readers own software against tPA and PAI-1 standard curves.

6.3.4 Statistical analysis

Unpaired t tests were carried out to determine if there were any statistical differences. However, as the number of repeat experiments is small, n=4, the use of statistics has a high risk of failing to pick up trends, that with increased n numbers would be significant. Therefore the statistics used in this chapter are mearly an indication of trends that require further experiments to support.

6.4 Results

Constitutive release of PAI-1 and tPA from primary epithelial cells

The results in figure 6.2 clearly demonstrate that human primary bronchial epithelial cells constitutively produce PAI-1, when in culture. PAI-1 levels in tissue culture supernatants were significantly higher ($p<0.05$) at 16hours (43.03 ± 9.69) in comparison with those after a 15 min culture (0.72 ± 0.16). Constitutive levels of tPA, in culture supernatants, did not

significantly change after longer incubation periods (figure 6.3); however, there was a biphasic trend for increased levels in comparison to the 15min incubation.

As the net proteolytic activity is dependent upon the balance of tPA and its inhibitor, PAI-1, which binds in a 1:1 complex, the molar ratio of tPA to PAI-1 was calculated. This calculation was based on a molecular weight of 70kDa for tPA and 45kDa for PAI-1. At all time points, the molar ratio of tPA to PAI-1 was less than 1.0, which indicated that the epithelial cells produced more PAI-1 than tPA. Molar ratios of tPA/PAI-1 were not significantly different with time; however, there was a trend for values to decrease as length of culture time increased, indicative of the relative increase in PAI-1 synthesis and release, figure 6.4.

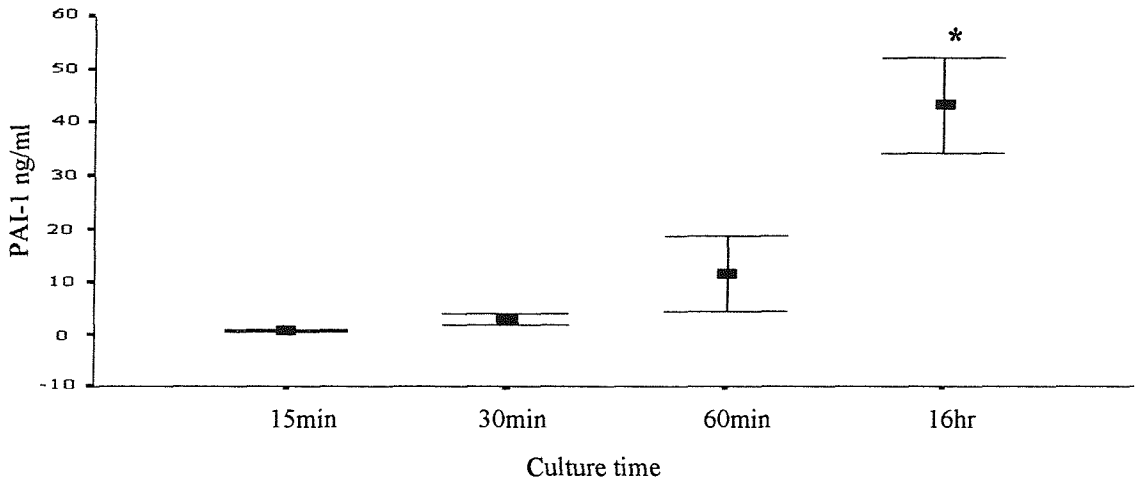


Figure 6.1 Constitutive release of PAI-1 from bronchial epithelial cells. Graphs represent mean ± SEM, n = 4. * represents $p<0.05$ in comparison to values at 15min.

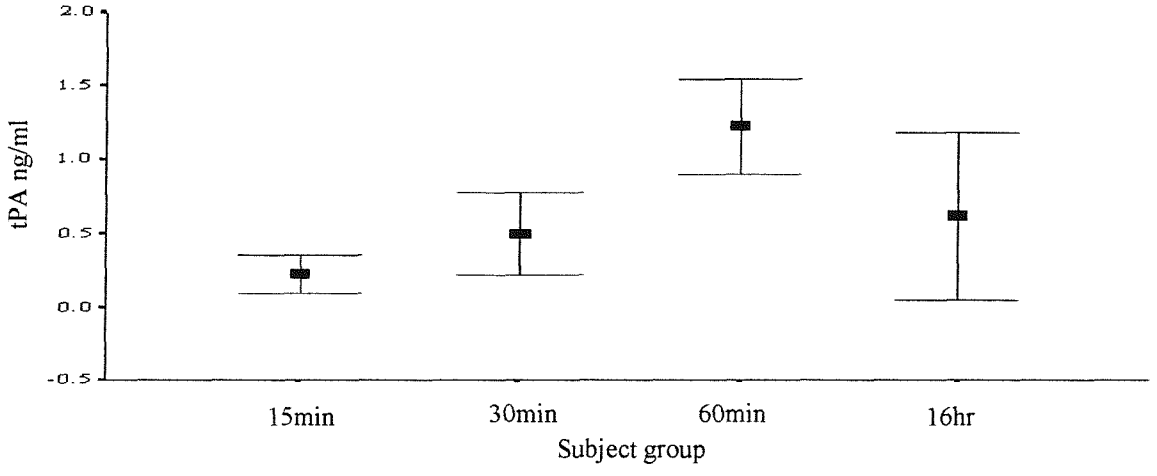


Figure 6.2 Constitutive release of tPA from bronchial epithelial cells. Graphs represent mean ± SEM, n = 4

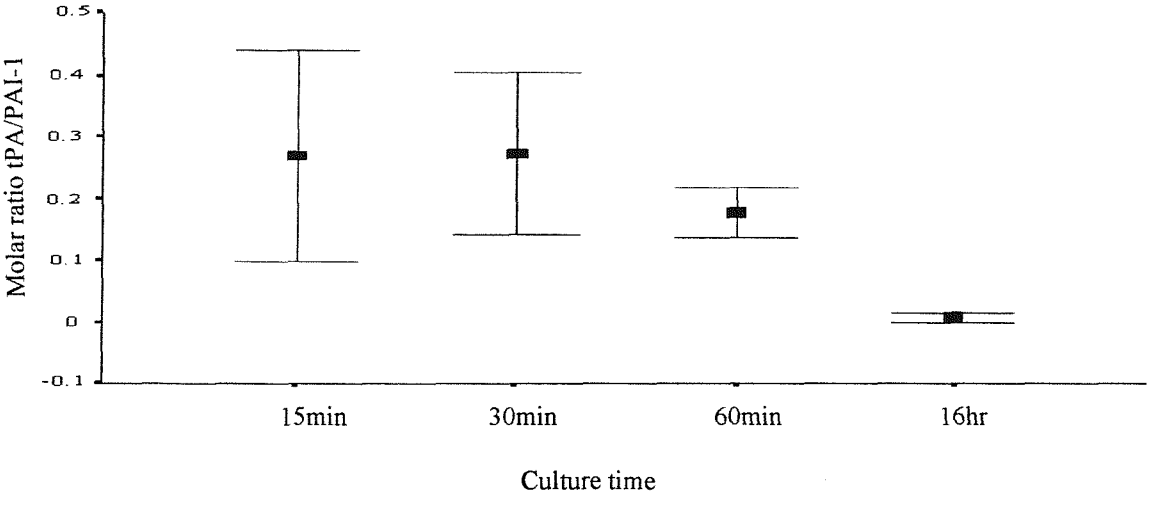


Figure 6.3 Constitutive molar ratio of tPA/PAI-1. Graphs represent mean ± SEM, n = 4

	Cone^n	**tPA(ng/ml)** mean ± SEM	**PAI-1(ng/ml)** mean ± SEM
15 min			
control		0.224 ± 0.129	0.718 ± 0.163
theophylline	10^{-3}M	0.183 ± 0.183	1.394 ± 0.474
rolipram	10^{-4}M	0.108 ± 0.108	0.405 ± 0.033
methacholine	10^{-4}M	0.000 ± 0.000	0.631 ± 0.150
histamine	10^{-4}M	0.055 ± 0.056	0.481 ± 0.040
30 min			
control		0.497 ± 0.276	2.886 ± 0.994
theophylline	10^{-3}M	0.205 ± 0.108	3.492 ± 0.928
rolipram	10^{-4}M	0.000 ± 0.000	6.275 ± 3.177
methacholine	10^{-4}M	0.058 ± 0.054	4.280 ± 2.096
histamine	10^{-4}M	0.441 ± 0.263	4.156 ± 2.004
milrinone	10^{-4}M	0.995 ± 0.850	2.757 ± 0.059
60 min			
control		1.226 ± 0.320	11.372 ± 7.064
theophylline	10^{-3}M	0.881 ± 0.422	5.074 ± 1.032
rolipram	10^{-4}M	0.119 ± 0.079 *	6.172 ± 2.770
methacholine	10^{-4}M	0.716 ± 0.380	6.192 ± 3.274
histamine	10^{-4}M	1.348 ± 0.416	5.562 ± 2.287
milrinone	10^{-4}M	4.125 ± 4.024	8.300 ± 1.518
16 hr			
control		0.615 ± 0.566	43.025 ± 8.938
theophylline	10^{-3}M	1.205 ± 0.704	100.988 ± 26.097
rolipram	10^{-4}M	0.931 ± 0.931	33.150 ± 32.800
methacholine	10^{-4}M	0.644 ± 0.054	38.025 ± 3.775
histamine	10^{-4}M	1.025 ± 1.025	13.500 ± 13.500
milrinone	10^{-4}M	1.757 ± 0.144	33.333 ± 6.201

Table 6.1 tPA and PAI-1 release from bronchial epithelial cells (mean ± SEM)
* represents $p =< 0.05$, unpaired t test, n = 4, comparing each drug with the control value

Pharmacological modulation of tPA and PAI-1 levels.

The concentrations of tPA and PAI-1 under each set of culture conditions, and at each time point, are shown in Table 6.1. The data in this table shows that no drug treatment produced a significant change in release of tPA or PAI-1 in short, 15 and 30 minute incubations, when compared to control. When this data was converted to the molar ratio of tPA/PAI-1 in culture supernatants there was no significant change in molar ratio at either time point (Figures 6.5 and 6.6).

A 60 minute incubation with rolipram led to a significantly ($p<0.05$, $n=4$) lower molar ratio of tPA to PAI-1 (0.010 ± 0.009) in comparison to control(0.176 ± 0.040), as demonstrated in figure 6.7. This is linked with a significantly ($p<0.05$, $n=4$) lower level of tPA produced with rolipram treatment (0.119 ± 0.079) in comparison to control (1.226 ± 0.320), as shown in table 6.1.

Figure 6.8 illustrates that a 16 hour incubation with milrinone, a PDE3 inhibitor, gave a significantly ($p<0.05$, $n=3$) higher molar ratio of tPA to PAI-1 (0.036 ± 0.006) in comparison to control (0.009 ± 0.007). This increase in molar ratio is the result of increased tPA release and decreased PAI-1 release by these cells (Table 6.1).

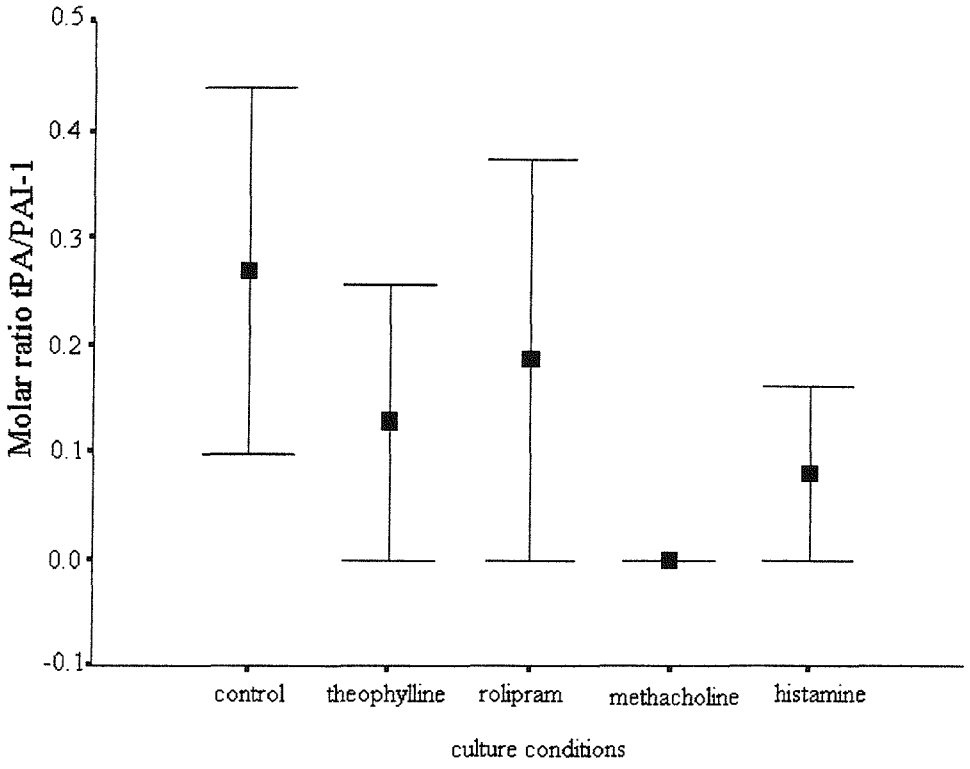


Figure 6.4 Molar ratio of tPA/PAI-1 (mean ± SEM) in culture supernatants of bronchial epithelial cells. Cells were incubated for 15 minutes with and without drugs at the concentrations shown in Table 6.1

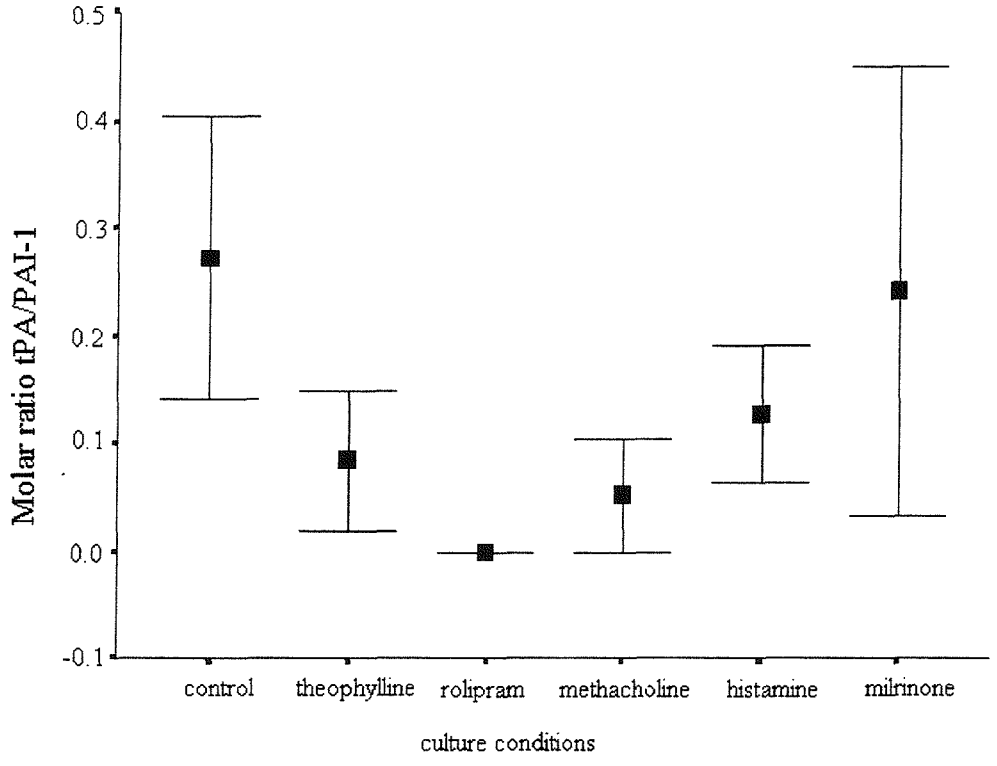


Figure 6.5 Molar ratio of tPA/PAI-1 (mean ± SEM) in culture supernatants of bronchial epithelial cells. Cells were incubated for 30 min with and without drugs at the concentrations shown in Table 6.1.

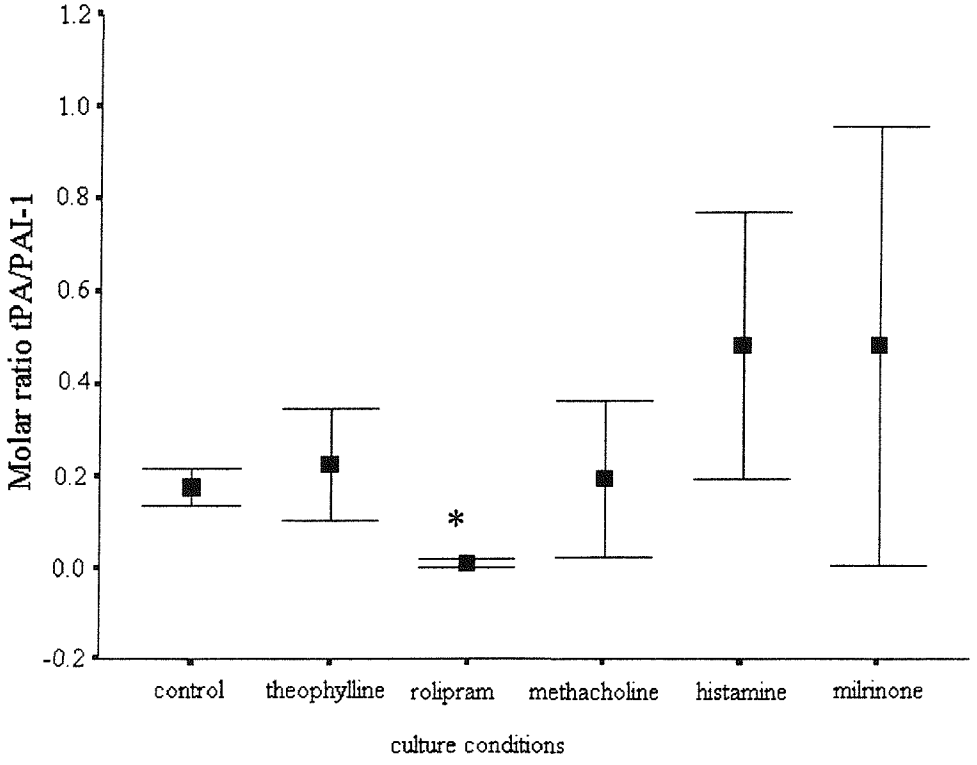


Figure 6.6 Molar ratio of tPA/PAI-1 (mean ± SEM) in culture supernatants of bronchial epithelial cells. Cells were incubated for 60 min with and without drugs at the concentrations shown in Table 6.1. * represents $p < 0.05$, unpaired t test, $n = 4$.

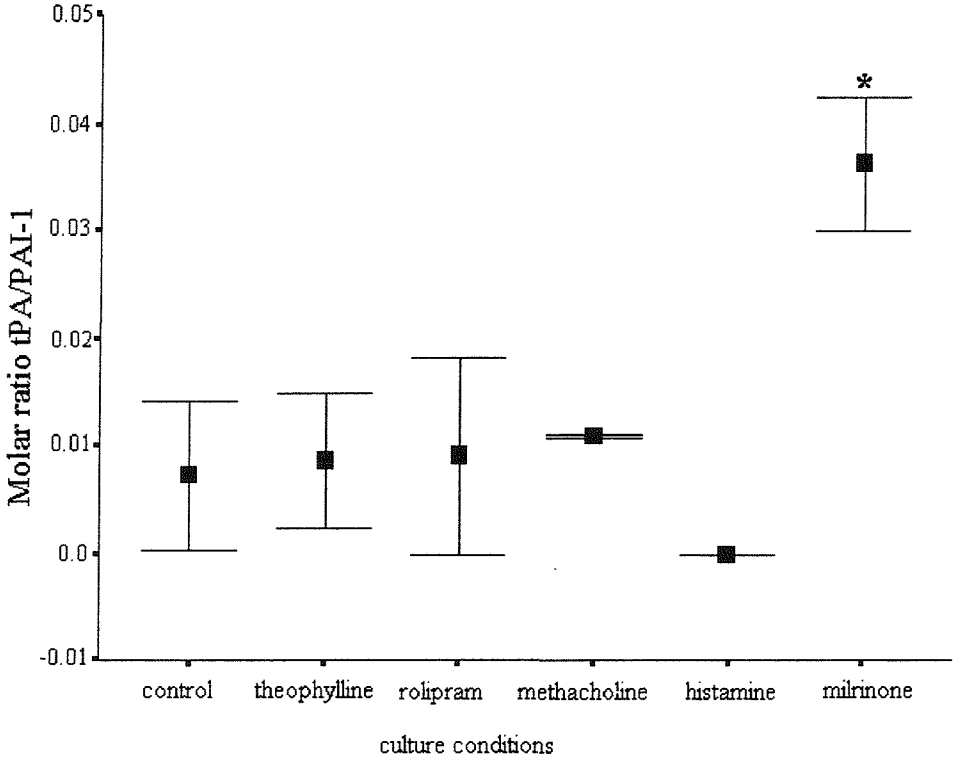


Figure 6.7 Molar ratio of tPA/PAI-1 (mean ± SEM) in culture supernatants of bronchial epithelial cells. Cells were incubated for 16hr with and without drugs at the concentrations shown in Table 6.1. * represents $p < 0.05$, unpaired t test, $n = 3$.

Synergism between the effects of a β agonist, salbutamol, and a PDE inhibitor, theophylline.

tPA

Previous experiments (Table 6.1) had shown that the PDE inhibitor rolipram significantly inhibited tPA release from bronchial epithelial cells in 60min cultures. In these experiments the effect of a β-agonist, salbutamol, alone and, in combination with theophylline, were investigated to test for synergism between these drugs. Control values for tPA in culture supernatants were 1.052 ± 0.352, which is not different to values found in other experiments. Salbutamol alone decreased tPA release significantly in a biphasic manner at 10^{-12}M and 10^{-8}M (figure 6.9). This biphasic effect was also seen for the effects of salbutamol on PAI-1 synthesis (Figure 6.11). Theophylline alone at 10^{-3}M, similarly, decreased tPA release significantly. However, although not significant, theophylline appeared to antagonise the effects of salbutamol, stimulating release of tPA, again in a biphasic fashion.

PAI-1

In a short 30 min incubation with salbutamol, or salbutamol in the presence of 10^{-3}M theophylline, there were no significant differences in PAI-1 levels in the culture supernatants, when compared with control. In general, there was a trend for theophylline, when in combination with salbutamol, to increase the levels of PAI-1 in culture supernatants, in comparison to those observed with the same concentrations of salbutamol alone. This was significant at 10^{-8}M salbutamol, where the combination of 10^{-3}M theophylline and 10^{-8}M salbutamol led to significantly ($p< 0.05$) higher levels of PAI-1 (1.541 ± 0.050), in comparison to treatment with 10^{-8}M salbutamol alone (0.908 ± 0.062).

A longer incubation period of 16 hours with salbutamol led to a biphasic concentration dependent response. Treatment with salbutamol at concentrations of 10^{-11}M, 10^{-10}M and 10^{-9}M led to significantly higher levels of PAI-1 (94.90 ± 2.65), (101.43 ± 4.75), (104 ± 0.35) in the culture supernatants, in comparison with no drug treatment (68.60 ± 0.65). Treatment with a combination of theophylline 10^{-3}M and salbutamol 10^{-9}M also led to significantly higher PAI-1 levels (114.20 ± 4.00) than no drug treatment. Treatment with 10^{-3}M theophylline, in combination with salbutamol, led to no changes in PAI-1 levels, when compared to salbutamol treatment alone.

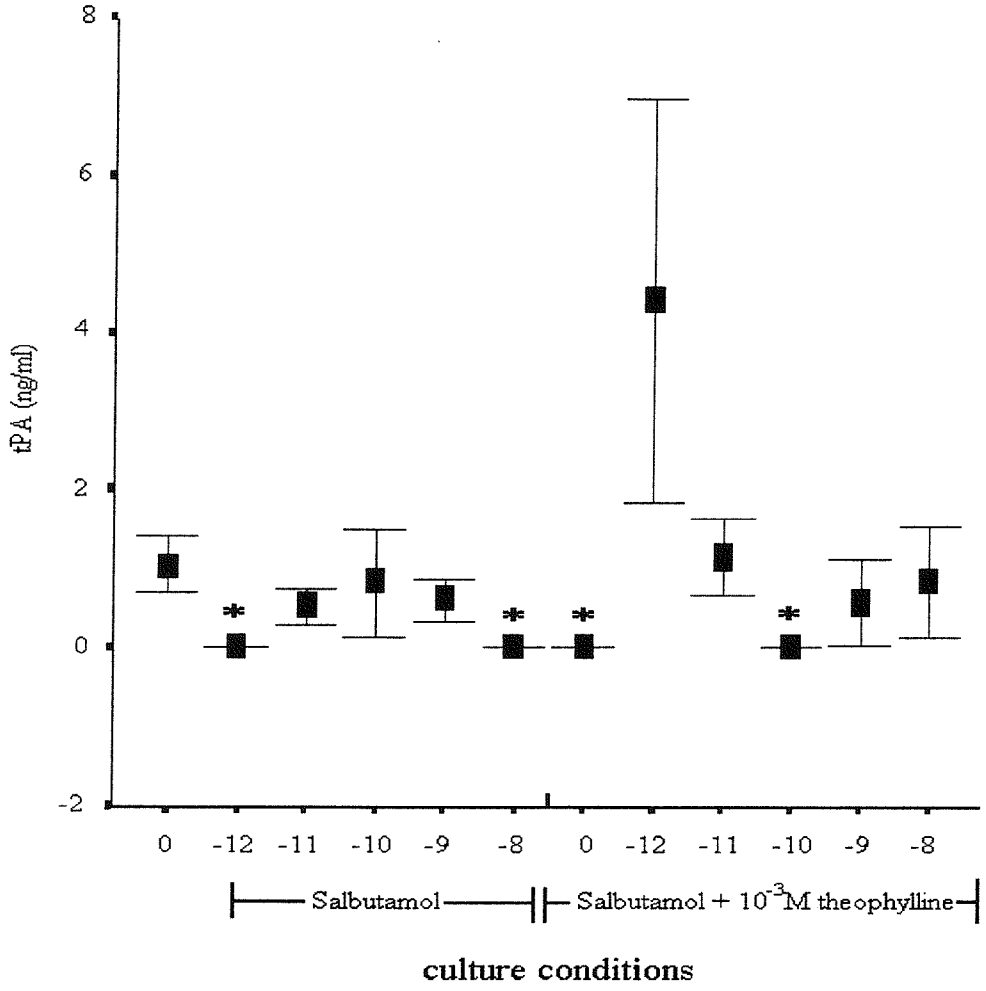


Figure 6.8 The effect of salbutamol, alone and in combination with theophylline, on tPA concentrations in supernatants from bronchial epithelial cells. Graph represents mean ± SEM. Cells were incubated for 60min with salbutamol at the concentrations shown, in the absence and presence of 10^{-3}M theophylline. * represents $p< 0.05$, unpaired t test, n = 4, when compared with no drug control. Statistical analysis was also carried out to compare if the addition of 10^{-3}M theophylline significantly enhanced the responses observed to salbutamol alone. No significant synergism of theophylline with the effect of salbutamol was found

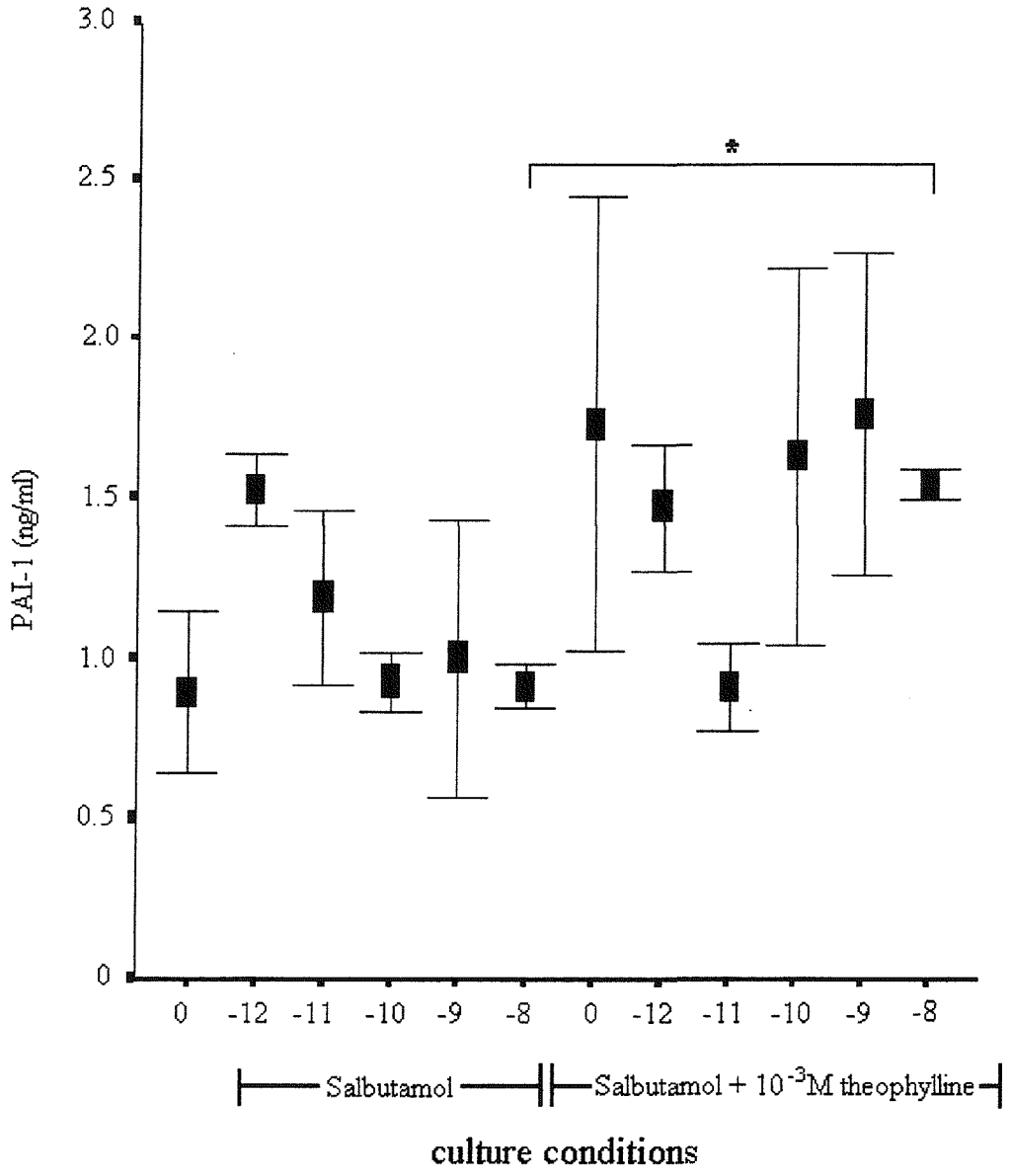


Figure 6.9 The effect of salbutamol, alone and in combination with theophylline, on PAI-1 concentrations in supernatants from bronchial cells. Graph represents mean ± SEM. Cells were incubated for 30min with salbutamol at the concentrations shown, in the absence and presence of 10^{-3}M theophylline. * represents $p < 0.05$, unpaired t test, $n = 3$ when comparing the effect of theophylline at a set concentration of salbutamol. Values were also compared against the control, where no drug was added, but there were no significant differences.

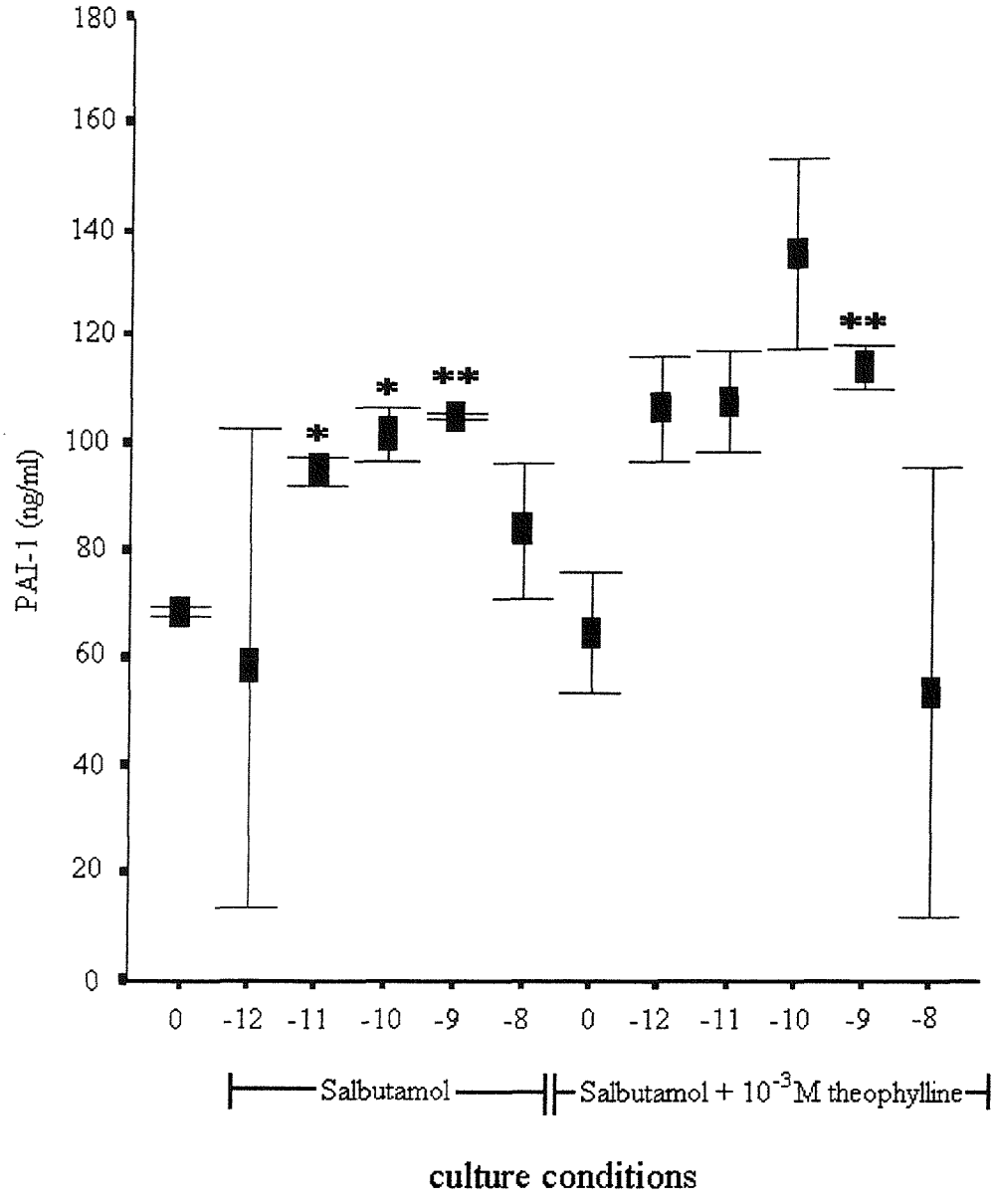


Figure 6.10 The effect of salbutamol, alone and in combination with theophylline, on PAI-1 concentrations in culture supernatants from bronchial epithelial cells. Graph represents mean ± SEM. Cells were incubated for 16hr with salbutamol at the concentrations shown, in the absence and presence of 10^{-3}M theophylline. * represents $p < 0.05$, ** represents $p < 0.01$, unpaired t test, $n = 3$. When compared with no drug control. Statistical analysis was also carried out to compare if the addition of 10^{-3}M theophylline significantly enhanced the responses observed to salbutamol alone. No significant synergism of theophylline with the effects of salbutamol was found.

6.5 Discussion

The results in this chapter demonstrate that primary bronchial epithelial cells constitutively synthesise and release PAI-1, which supports findings by Gerwin et al. (1990) who demonstrated that normal bronchial epithelial cells constitutively express PAI-1 mRNA. Tissue plasminogen activator (tPA) levels in culture supernatants increased up to 60min, indicating that primary bronchial epithelial cells constitutively release tPA. It is puzzling to note that levels of tPA in culture supernatants did not increase over a longer incubation period of 16 hours. This may suggest these bronchial epithelial cells are able to release stored tPA, but do not synthesise it constitutively, and a further stimulus is necessary for these cells to produce tPA by *de novo* protein synthesis. The molar ratio of tPA to PAI-1 in the supernatants from these bronchial epithelial cells never reached equilibrium and there was always a molar excess of PAI-1. This would suggest that there is no net fibrinolytic activity in these cells, and this would also limit the tPA activated proteolytic cascades that degrade elements of the ECM. These results are from epithelial cells derived from normal donors, and it would therefore be relevant to investigate if the same results are found in asthmatic subjects.

Constitutive mRNA expression for PAI-1 has been reported in primary bronchial epithelial cells; however there are no previously published findings of tPA release or synthesis in these cells (Gerwin et al. 1990). It is interesting to note that, whilst Gerwin et al. (1990), did not measure tPA levels, they did report the levels of uPA, another plasminogen activator, and found that constitutive uPA mRNA expression was higher than that for PAI-1. This would imply that at the mRNA level there would be a net excess of uPA in these cells. Reports in human tracheal epithelial cells have indicated a constitutive net fibrinolytic activity, which they have attributed largely to uPA (Idell et al. 1994). Reports

investigating tPA/PAI-1 production in human umbilical vein endothelial cells (HUVECs) have shown a constitutive excess of PAI-1, and in culture supernatants they reported no net fibrinolytic activity (Rydholm et al. 1995; Santell and Levin, 1988; Hanss and Collen, 1987). These observations may imply that endothelial cells and epithelial cells show a difference in their constitutive balance of fibrinolytic and proteolytic activities. It would therefore be interesting to examine the fibrinolytic activity of the culture supernatants from the bronchial epithelial cells in my experiments, to investigate if there was net fibrinolytic activity resulting from the imbalance of molar amounts of uPA, tPA and PAI-1. If fibrinolytic activity was observed, it may imply that the suggested molar excess of PAI-1 to tPA, found in the results in this Chapter, is re-balanced or even shifted towards fibrinolysis by uPA production.

Agents that increase intracellular cAMP through inhibition of PDEs, such as theophylline and milrinone, have little or no effect upon the release of tPA or PAI-1 observed at 15, 30 and 60 min. Rolipram, however, significantly decreased tPA release at 60 min suggesting a block in release of intracellular stores. Milrinone, after a 16 hour culture increased the synthesis of tPA, whilst apparently decreasing the synthesis of PAI-1; therefore, suggesting that PDE3, when not inhibited, is involved in the stimulation of PAI-1 synthesis and the inhibition of tPA synthesis. Drugs that inhibit PDE3 may, therefore, have some use in the treatment of fibrotic diseases involving the epithelium. Theophylline and rolipram had no appreciable effects upon the synthesis of either tPA or PAI-1 observed over longer culture periods of 16hours.

It is interesting to note that different members of the PDE enzyme family appear to regulate tPA levels in different manners. PDE4 inhibition, achieved by rolipram, leads to an initial

inhibition of tPA store mobilisation, thus suggesting the PDE4 enzyme is stimulatory in release of tPA from intracellular stores. PDE3 inhibition, however, achieved by milrinone leads to an increase in tPA synthesis and release, thus suggesting the PDE3 enzyme may be involved in inhibition of tPA *de novo* synthesis. It may not be surprising that theophylline, a broad PDE inhibitor, therefore has no significant net effect on either tPA or PAI-1 levels, when used alone, as it would encompass both PDE3 and PDE4 inhibition. This apparent difference in the regulation of tPA levels by different members of the PDE family may relate to their control of distinct intracellular cAMP pools that regulate the production and release of tPA. Through regulation of these distinct cAMP pools the PDEs are able to orchestrate specific responses within the cells. It may be that PDE3 is responsible for regulating a cAMP pool, which in turn activates the transcription of tPA, by producing higher cAMP levels through inhibition of PDE3. It is also possible that PDE4 regulates a cAMP pool that in some way prevents the release of pre-formed stores of tPA, possibly by stabilising the cell membrane.

Treatment of bronchial epithelial cells with the beta-adrenoreceptor agonist salbutamol led to a significant decrease in tPA release, and this response occurred in a concentration dependent biphasic manner. This would suggest that salbutamol is able to inhibit the intracellular mobilisation of tPA stores. A previous report has shown that in the human forearm vasculature isoprenaline (a β agonist) was able to stimulate a rapid release of tPA (Stein et al. 1998), and this would suggest the release of tPA is differently regulated depending on the tissue or cells. Treatment with theophylline had no effect on PAI-1 release from these cells, and this is not particularly surprising, given the fact that PAI-1, unlike tPA, is not found in any intracellular stores. PAI-1 synthesis, however, was significantly increased by the action of salbutamol, again in what appeared to be a

concentration dependent biphasic manner. This result implies that enhancement of cAMP levels, mediated by the affects of salbutamol on β_2 receptors, are able to stimulate the *de novo* protein synthesis of PAI-1. Theophylline had little effect upon the salbutamol mediated decrease in tPA release and, whilst there was some evidence of it antagonising the effects of salbutamol by increasing tPA levels, this was not significant. Theophylline was able to significantly increase PAI-1 release when in combination with 10^{-8}M salbutamol in comparison with that seen with 10^{-8}M salbutamol alone. This is most likely attributable to the stimulation by theophylline of PAI-1 release which, whilst not significant when used alone or with other concentrations of salbutamol, causes a trend for increased PAI-1 release. As there are no intracellular stores of PAI-1, how theophylline increases PAI-1 release in such a short time period of 30 min is somewhat puzzling. The significant stimulation by salbutamol of PAI-1 synthesis and release, observed after a 16hr culture, indicates that this drug, through increases in cAMP levels, is able to stimulate de novo synthesis of PAI-1.

In human endothelial cells cAMP elevating agents, such as forskolin, IBMX or db cAMP, all decreased PAI-1 synthesis (Santell and Levin, 1988; Rydholm et al. 1995), and it would again appear that production of PAI-1 is regulated differently in different cells. Theophylline, alone, had no effect upon PAI-1 synthesis, and did not enhance or reduce the stimulation observed with salbutamol. This implies that theophylline does not affect PAI-1 synthesis. Salbutamol and theophylline are both able to increase cAMP levels within cells; therefore, the apparent differences in their effects upon PAI-1 synthesis are not as expected. An explanation for these varying results may lie in the cAMP elevation level needed to stimulate PKA activation, and its subsequent control of PAI-1 gene transcription. This PKA activation threshold hypothesis has been proposed by Houslay and Milligan (Houslay and Milligan, 1997). The PKA pool that regulates PAI-1 gene expression may require

cAMP levels to be above a certain threshold limit before it will activate transcription. If basal adenylyl cyclase activity is low in bronchial epithelial cells, it would appear that theophylline alone is not able to increase cAMP levels sufficiently to cross this threshold, and so no increase in PAI-1 was observed. However, salbutamol was able to increase cAMP levels high enough to cross this threshold and, therefore, lead to increased PAI-1 production. As the cAMP threshold is already achieved by the actions of salbutamol alone, the subsequent addition of theophylline, which may further elevate cAMP levels, has no additive effect upon PAI-1 production.

From the results in this Chapter, it would appear that bronchial epithelial cells constitutively synthesise an excess of PAI-1. No fibrosis is observed in normal airways, which may be because other cells within the airways express higher levels of tPA, and there is an overall balance in the tissue between tPA and PAI-1. Or alternatively, uPA levels, which were not measured in this Chapter, may also redress this PAI-1 excess. Levels of uPA were not measured in this Chapter, as previous reports from our laboratory (B Dahlen, unpublished work) have shown that there is no positive staining for uPA within the epithelium of bronchial biopsies, whereas, there is positive staining for tPA. When considering the tPA/PAI-1 balance, it should be taken into account that tPA and PAI-1 levels were only measured in the culture supernatants of epithelial cells in our experiments. Tissue plasminogen activator (tPA) is known to bind to proteins, for example annexin within the cell membrane of endothelial cells (Hajjar et al. 1998). It is therefore feasible that tPA levels are an underestimate of the total amount released by the cell, and a proportion of tPA may be bound to the cell surface of the epithelial cells and, hence, not measured in the assay. It would be interesting to investigate this hypothesis by removing the epithelial cells from the culture plate and assaying the homogenate for tPA.

Current thinking is that tPA is partly responsible for the fibrosis observed in asthma, as tPA generates plasmin which activates and releases growth factors from the ECM. These released growth factors stimulate fibroblast proliferation and deposition of the collagens associated with the observed subepithelial fibrosis (personal communication, Shute,J.K). Under normal conditions this fibrosis would be removed by the actions of MMPs and other proteases; however, as previously mentioned, asthmatics show higher levels of protease inhibitors, for example TIMP-1 and α_2 antitrypsin, and therefore the actions of these proteolytic enzymes are inhibited (Kips and Pauwels, 1999).

The fine balance between fibrosis and proteolysis is key in the regulation of the lung tissue architecture. If the above hypothesis that tPA contributes to fibrosis is true, agents that decrease tPA synthesis or release, or alternatively, increase PAI-1 synthesis may have therapeutic potential in preventing the subepithelial fibrosis observed in asthma. The net effect on this fibrosis-proteolytic balance of all drugs used in asthma therapy should therefore be fully evaluated.

Chapter 7

The subcellular localisation of PDE and PKA isoenzymes in primary bronchial epithelial cells

7.1 Introduction

Cyclic AMP is part of a fundamental signalling cascade that operates within most cells. The cascade is initiated by the occupation of a cell surface receptor, for example a β adrenoreceptor by a ligand. This is then relayed into the cell to a stimulatory G protein, which then becomes susceptible to guanosine triphosohate (GTP) that, on binding, causes a conformational change in the G protein. This conformational change enables the activation of adenylate cyclase, which can then convert ATP into cAMP (reviewed by Berridge, 1985).

The formation of the second messenger, cAMP, enables the initial signal to be transmitted across the cell membrane. Cyclic AMP then must activate molecules further down the signalling pathway to translate this message into events within the cells (Berridge, 1985). Protein kinase A (PKA) is activated by cAMP, and is therefore the next step in this cascade. PKA is composed of four subunits, two catalytic subunits (C) and two regulatory subunits (R). The binding of two cAMP molecules to each R subunit causes this $R_2(cAMP)_4$ unit to dissociate from the two catalytic subunits. The catalytic dimer is then free to phosphorylate threonine and serine residues on specific proteins (Corbin et al. 1975; Døskeland, 1978). The phosphorylation of these proteins triggers events within the cytoplasm. The catalytic units can also translocate into the nucleus and phosphorylate proteins, such as CRE binding protein (CREB) or cAMP responsive element modulator (CREM). Both of these molecules can then bind to genes that contain a cAMP response element (CRE) in their promoter sequence and regulate the transcription of these genes (Delmas et al. 1994).

There are three potential forms of the catalytic subunits of PKA, which are termed, α, β or γ. The regulatory subunits are more complex and form two alternative PKA types, namely

PKA I, which has RI subunits, and PKA II, which has RII subunits. These RI subunits can be either RIα or RIβ, and the same is true for RII as RIIα and RIIβ (McKnight et al. 1988). This diversity in composition of the PKA tetramer may serve to localise PKAs to specific cell sites, depending upon their subunits. This would go someway to explaining how a molecule, such as PKA, is present in most cells, where it activates numerous proteins, yet is able to respond to a specific stimulus by phosphorylating only one of these individual proteins.

The subunits for PKA have been reported to be expressed in specific locations within many cells, for example in B lymphocytes RIα is thought to associate with the antigen receptor during activation (Levy et al. 1996). RIα has also been reported to be able to bind a Grb protein, which then allows it to interact with the epidermal growth factor receptor (EGF-R) in MCF-7 cells (Tortora et al. 1997). Constantinescu et al. (1999), reported that the alpha catalytic subunit (Cα) translocates from the golgi to the nucleus in response to ethanol in NG108-15, a neuroblastoma cell line. RII levels are reported to dramatically increase in the nucleoli in MCF-7 cells during tumour regression (Kapoor et al. 1984). It would, however, appear that the majority of PKA RI in cells is found in the cytosol whilst the majority of PKA RII is associated with the particulate fraction (Houslay and Milligan, 1997). This association of the PKA RII isoform to the particulate fraction is mediated via its hydrophobic association with A kinase anchoring proteins (AKAPs). The AKAPs have been identified on numerous cytoskeletal and organelle associated elements, including mitochondria, golgi, microtubules and actin binding proteins (Lin et al. 1995; Rios et al. 1992; Lohmann et al. 1984). It should also be noted that, more recently, AKAPs have been identified that also bind PKA RI (Huang et al. 1997). These examples illustrate that

components of the PKA tetramer are expressed in different locations of cells, and this may vary dependent upon their isoform variation or the cell type in which they are expressed.

This cell and isoform specific localisation is not unique to PKA. Similarly, there have been numerous recent reports regarding members of the PDE family, illustrating that the huge diversity in enzyme and isotype forms may allow them to regulate distinct events within the cell. Different members of the PDE family are present within many different cell types and exert control over different functions of that cell. For example, in human mesangial cells, PDE4 regulates a pool of cAMP that activates PKA, which in turn inhibits the release of reactive oxygen metabolites. Within the same cells PDE3 regulates a separate pool of cAMP, which activates a separate PKA, and is able to suppress proliferation of these cells (Chini et al. 1997). Additionally, it has been observed that different isoforms of the same PDE can control very different cellular processes in the same animal. For example, PDE3A is found mostly in adipose tissue of rats and is responsible for hormonal regulation of glycogenolysis and lipolysis, whilst PDE3B is found mainly in cardiac tissue within these animals and controls myocardial and smooth muscle contractility (Reinhardt et al. 1995). The exact localisation of these PDEs within cells is hypothesised to be determined in some part by their N terminal regions. PDE46, a PDE4A expressed in COS 7 cells, is able to associate with SRC and LYN kinases through SH3 region linkage, and was colocalised with LYN to an area surrounding the nucleus and cortical regions of the periphery of these cells (McPhee et al. 1999). It is also possible that members of the PDE family are associated with scaffold proteins that recruit other proteins to form a signalling complex at a specific location within a cell. Yarwood et al.(1999) demonstrated that, in yeast hybrids, PDE4D5 is bound to a receptor for activated C kinase (RACK 1), and this RACK 1 has the potential to bind other signalling molecules and form a signalling complex.

Thus, localisation of these PDEs within individual cells may be the first step in investigating the cellular process controlled by each PDE, or any of its splice variants. As immunohistochemical analysis indicated, in Chapter 3, there appear to be pools of PDE4D enzyme present in distinct locations within epithelial cells. A key factor in the generation of a non uniform intracellular distribution of cAMP is the degradative system provided by PDE activity, in the absence of which intracellular cAMP levels would rapidly become uniform. The specific subcellular localisation of PKA within individual cells lends credence to the hypothesis, postulated by Houslay and Milligan, that PDE isoforms are responsible for regulation of cAMP levels within distinct cellular pools (Houslay and Milligan, 1997). Therefore, this Chapter aims to try to co-localise the pools of PDE4 isoenzymes with PKA, and identify the regulatory subunits of PKA in epithelial cells. The hypothesis investigated in this chapter is that PDE isoenzymes responsible for the degradation of cAMP are found in the locality of specific PKA pools.

7.2 Materials

Tetramethylrhodamine (TRITC) goat anti-mouse IgG (H+L) conjugate was purchased from Molecular Probes (Eugene USA). Mouse anti protein kinase A RII subunit was purchased from Serotec (Oxford UK). Mouse anti protein kinase A RI subunit and mouse anti protein kinase C subunit were purchased from Transduction laboratories (Lexington USA). Theophylline was a kind gift from Celltech (Slough UK). Lab Tek II chamber slides were purchased from Gibco-BRL (Paisley Scotland)

7.3 Methods

7.3.1 Primary cell culture

Primary bronchial epithelial cells were cultured as described in Section 6.3. Cells were plated into gelatin coated Lab Tek II, 8 well slides, and cultured until approximately 50% confluent to allow individual cells to be visualised easily. The effects of theophylline upon PKA distribution were investigated by washing cells twice with culture medium, and then adding theophylline (10^{-3}M) in culture medium for a 60 minute incubation prior to fixation. Cells were washed twice in 1X PBS buffer before this fixation.

7.3.2 Staining of primary cells

Buffer was removed, and cells were fixed by immersion in ice cold methanol for 10 min. Slides were then blocked, to remove non specific staining, and stained as in Section 3.3. Primary antibodies are detailed in Table 7.1. TRITC labelled goat anti-mouse was used as a secondary antibody to label cells with a TRITC stain that has an emission wavelength of 580nm. Slides were incubated with this TRITC labelled secondary antibody for 1 hour in the dark. Cells were labelled with a FITC stain by initial incubation with a biotin labelled secondary antibody for 2 hours, followed by 5x 5min washes, and subsequent incubation with a FITC labelled avidin for 20min in the dark. The FITC stain has an emission wavelength of 518nm. Slides were washed with PBS for 5x 5min washes before counterstaining of cell nuclei, which was achieved by addition of propidium iodide final concentration (25μg/ml) in PBS for a 30second incubation. The slides were again washed with PBS for 5x 5min washes before being mounted in Moviol and coverslipped and kept at 4°C, in the dark until visualisation. Slides were visualised on a Leica TCS4D confocal laser scanning microscope, which uses a laser light source to excite the fluorophore. Light emitted from this fluorophore then passes through a pin hole aperture in the microscope,

which removes a lot of `out of focus' fluorescence. The confocal microscope also only uses a shallow depth of field and these features allow greater contrast and clarity of pictures. A succession of optical sections are taken by the microscope through the specimen and this allows a three dimensional picture of the cell to build up. A representative field of each slide was analysed, and these three dimensional images were downloaded for printing. Control slides incubated with PBS, and then secondary antibodies, showed no immunofluorescence.

Antibody	polyclonal/monoclonal	species	raised against	dilution	source
PKA C	monoclonal	mouse	C α, β and γ	1/500	Transduction laboratories
PKA RI	monoclonal	mouse	RI α and RI β	1/1000	Transduction laboratories
PKA RII	monoclonal	mouse	RII α and RII β	1/1000	Serotec
Biotinylated rabbit anti mouse	monoclonal	rabbit	mouse immunoglobulins	1/300	DAKO
Biotinylated swine anti rabbit	monoclonal	swine	rabbit immunoglobulins	1/300	DAKO
TRITC congugated goat anti mouse	monoclonal	goat	mouse IgG (H+L)	1/500	Molecular probes
PDE4A	polyclonal	rabbit	C terminal of 4A	1/100	Celltech
PDE 4B	polyclonal	rabbit	C terminal of 4B	1/250	Celltech
PDE4D	monoclonal	mouse	C terminal of 4D	1/150	Celltech
FITC labelled avidin				1/50	Sigma

Table 7.1 Panel of antibodies used to detect PDE isoforms and PKA

7.4 Results

The observations of this chapter are based on a single experiment that was carried out in duplicate, and as such should be regarded as initial observations that require further repeat experiments to support the findings.

7.4.1 Localisation of phosphodiesterase enzymes

FITC staining for PDE4A indicates that this enzyme is evenly distributed throughout the cytoplasm, with little nuclear staining evident (Figure 7.1). PDE4B staining is more closely associated with the plasma membrane, with little cytoplasmic or nuclear staining (Figure 7.2). The PDE4D distribution within the cell appears to be more widespread (Figure 7.3), with FITC labelling evident within the cytoplasm and nucleus.

7.4.2 Distribution of PKA subunits

The distribution of the catalytic subunit (C) of PKA, as shown in figure 7.4A, appears weak throughout the cell. Since local changes in cAMP concentrations will affect the spatial cAMP gradient within cells, and possibly downstream events, the effect of theophylline on the distribution of PKA C and R subunits was investigated. However, there was no increase or change in location of the PKA C subunit after a 60 min incubation with 10^{-3}M theophylline (figure 7.4B). The RI subunit staining displays a clear ring staining pattern around the nucleus, and this staining becomes more diffuse moving towards the plasma membrane (Figure 7.5). Thus, there appears to be a concentration gradient of RI, being more abundant in the perinuclear areas and weak or absent at the cell membrane. There is also some evidence of staining within the cell nucleus. This pattern of staining is not affected by pre incubation for 60 min with theophylline. It is noticeable that staining of this RI subunit is more intense than that for either RII or the catalytic subunit, suggesting

stronger expression. The expression of the alternative regulatory subunit RII, shown in figure 7.6A, also appears as a ring surrounding the nucleus, but much weaker than that observed for RI. There is again diffuse staining moving out from this `ring` and evidence of plasma membrane staining, but no evidence of nuclear staining. It is interesting to note that a 60 min incubation of these cells with 10^{-3}M theophylline leads to a change in the predominant staining pattern for this isoform. Figure 7.6B illustrates that theophylline causes RII to move outwards from the perinuclear ring exhibited in figure 7.6A to a cytoplasmic and plasma membrane staining pattern.

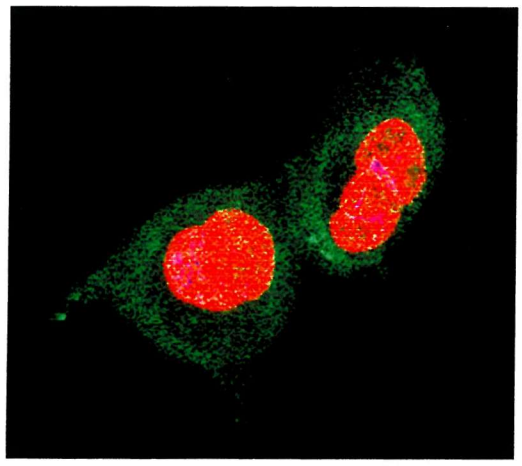


Figure 7.1 Confocal image of PDE 4A immunostaining in bronchial epithelial cells. Nuclei are stained red with propidium iodide and PDE 4A stained green with FITC.

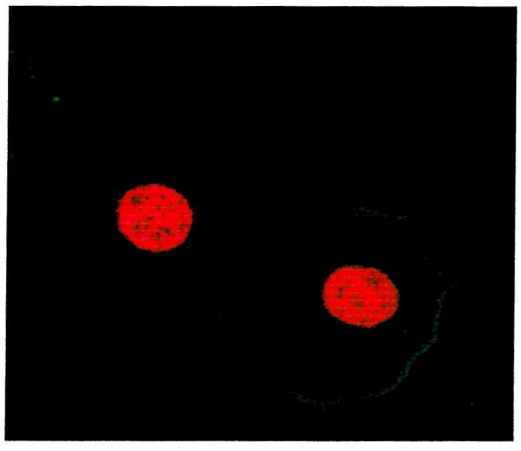


Figure 7.2 Confocal image of PDE 4B immunostaining in bronchial epithelial cells. Nuclei are stained red with propidium iodide and PDE 4B green with FITC

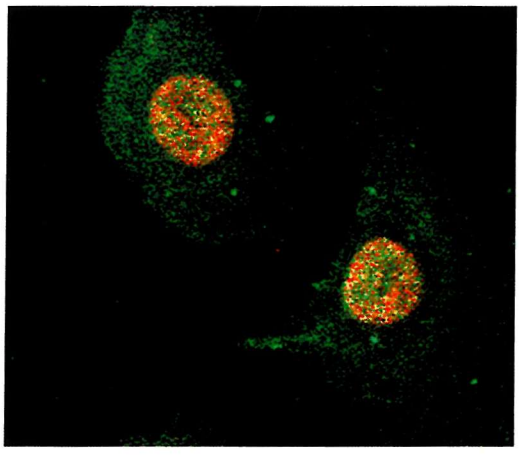


Figure 7.3 Confocal image of PDE 4D immunostaining in bronchial epithelial cells. Nuclei are stained red by propidium iodide and PDE 4D green by FITC.

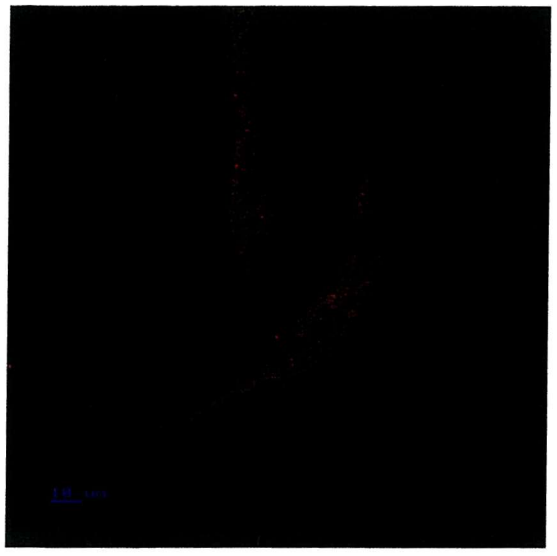


Figure 7.4A Confocal image of the catalytic subunit of PKA stained red with TRITC in bronchial epithelial cells.

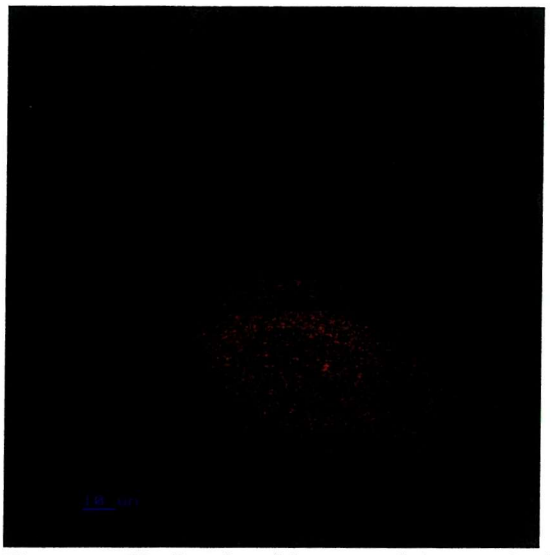


Figure 7.4B Confocal image of the catalytic subunit of PKA after a 60 minute incubation with theophylline.

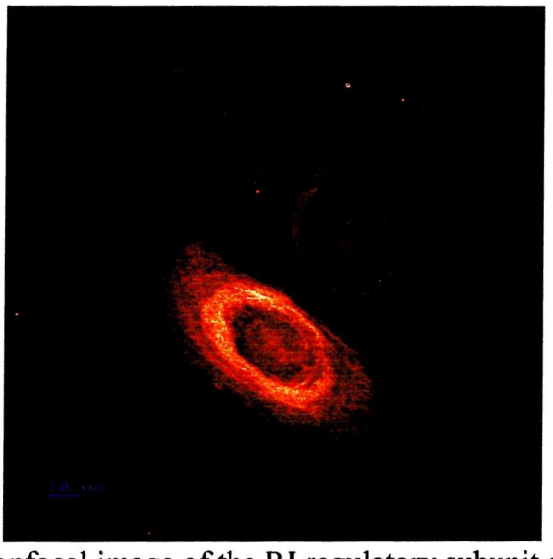


Figure 7.5 Confocal image of the RI regulatory subunit of PKA stained red with TRITC in bronchial epithelial cells.

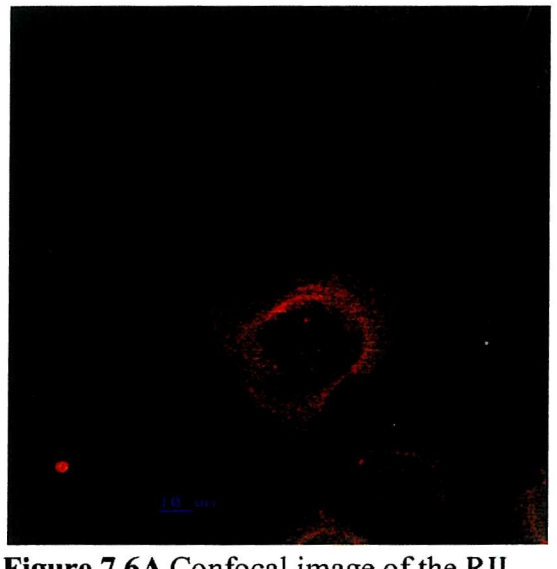


Figure 7.6A Confocal image of the RII regulatory subunit of PKA stained red by TRITC in bronchial epithelial cells.

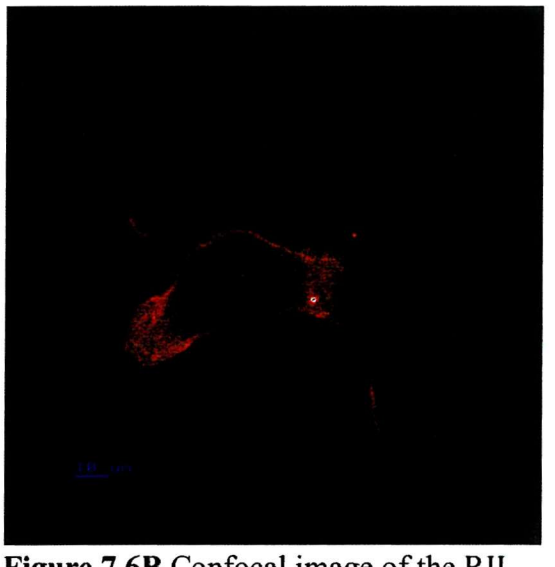


Figure 7.6B Confocal image of the RII regulatory subunit of PKA after a 60 minute incubation with theophylline.

7.5 Discussion

The results for subcellular localisation of the PDE4 isoforms in primary epithelial cells are in agreement with those discussed in Chapter 3 for immunohistochemical staining of bronchial tissues. PDE4A and 4B both showed cytoplasmic staining within the epithelial cells of bronchial biopsies, and it was noticeable that more PDE4A staining was present in comparison to staining with 4B. Using confocal analysis, we were unable to repeat our initial observation of discrete PDE4D staining of nucleoli within epithelial cells. PDE4D did however, unlike 4A and 4B, show nuclear staining, suggesting that this isoform is present in or over the nucleus.

The presence of 4B staining close to the plasma membrane may suggest that this isoform has a role in regulating export/import mechanisms within the cells, and could possibly affect membrane ion channels, for example the CFTR chloride channel, which has been reported to be stimulated by PKA RII (Steagall et al. 1998). It is possible that PDE4B is localised in this position by attachment to structures, such as AKAPs, within this membrane. The more generalised distribution of 4D may suggest that this enzyme is responsible for regulating basal cAMP levels within the cells. Expression of 4A within the cytoplasm, and not in the nucleus, suggests that this isoform is not involved in the regulation of gene transcription. Its presence in the cytoplasm may imply a role of modulating processes occurring in this area, which include post translational protein modification and export. Distribution of the catalytic subunit of PKA within these bronchial epithelial cells manifested as a weak staining throughout the cell, and indicates that there is weak expression of the subunit. It is possible that this could be due to rapid degradation of this catalytic unit once it has phosphorylated its substrate. There also appears weak staining for this unit within the

nucleus, implying that it is able to cross the nuclear membrane and phosphorylate target proteins within. This is in agreement with previous reports of the catalytic unit crossing into the nucleus (Constantinescu et al. 1999).

The very distinct expression of the RI subunit of PKA, in a ring like staining pattern around the nucleus, suggests that this unit is anchored to a structure close to the nuclear membrane. Also, that it has a specific role in regulating a process occurring at or near this membrane. It is possible that this subunit may be attached to the endoplasmic reticulum, or Golgi apparatus, and is closely involved in protein synthesis and its subsequent processing within the Golgi. Although there are no reports of RI localisation with the Golgi, Martin et al. (1999) observed a close link between the Golgi apparatus and the RII subunit.

Expression of the second form of regulatory subunit of PKA, RII, shows relatively weaker staining than for RI within these epithelial cells. It is also noticeable that there is no staining within the nucleus for this subunit, suggesting it does not cross the nuclear membrane. The staining for this subunit does, however, like RI, indicate its presence around either the nucleus or golgi apparatus within cells. It is also very interesting that this subunit, unlike both RI and the catalytic subunit, displays a shift in distribution after a 60 min treatment of the cells with theophylline. This implies that theophylline, probably through the inhibition of PDE mediated degradation of cAMP, triggers a response that causes this RII subunit to move away from the nuclear membrane/ golgi apparatus towards the cell plasma membrane. It is possible that this movement is associated with protein transport, from the golgi, where it is processed, to the plasma membrane ready for export, which is stimulated by the action of theophylline. The secretion of many proteins by bronchial epithelial cells is regulated by cAMP, including tPA and PAI-1, as demonstrated by experiments reported in Chapter 6.

There is also a possibility that the actions of theophylline are mediated via its action on adenosine receptors, which have been reported in human airway epithelium (Lazarowski et al. 1992).

The experiments presented in this Chapter suggest distinct roles within bronchial epithelial cells for the PDE4 enzymes, and also the individual units of PKA. These initial studies indicate further work to investigate the effect of other stimuli upon their distribution is needed, together with the ability to use a 3 channel confocal microscope to investigate the co-localisation of these PDE4 isoforms with the PKA subunits under various conditions of stimulation. It would also be interesting to investigate the distribution of these enzymes/subunits of PKA in bronchial epithelial cells derived from asthmatic donors. Immunohistochemical results indicated that there are no differences in distribution of the PDE4 isoforms between biopsies from normal or asthmatic patients. It would therefore be interesting to investigate if this was true for the subunits of PKA and, also, if any different distribution of the PDEs/PKA subunits occurred in asthmatics after drug treatment.

Chapter 8

General Discussion

8.1 General discussion

The hypothesis on which this thesis was based was: **Inflammatory cells from asthmatics express a different profile of cAMP hydrolysing phosphodiesterase enzymes than the same cells from normal donors.** To investigate this hypothesis it was necessary to examine the gene expression of the PDEs and their subsequent translation, indicated by cellular protein expression, the functional activity of the PDE enzymes and their relative contribution to total cellular cAMP hydrolysing activity, and, finally, the subcellular distribution of this activity and protein expression.

The results have indicated that there is no evidence for different cAMP hydrolysing phosphodiesterase expression, activity or subcellular localisation in inflammatory cells from asthmatics, in comparison to those from normal subjects. This is the first such study to comprehensively investigate this hypothesis, and this is a novel observation. The implications of these findings are that whilst broad acting PDE inhibitors, for example theophylline, have proven beneficial effects in the treatment of asthma (reviewed by Schmidt et al. 1999), the development of isoenzyme or even splice variant specific inhibitors to target a notional specific PDE, unique to asthmatics, is unlikely to be as successful as first hoped, at least in inflammatory leukocytes in mild asthmatics. The epithelium may, however, present a novel target for the development of PDE inhibitors. The results in this thesis have shown that PDE4A, B and D are present within the epithelial layer of bronchial biopsies, and in primary human bronchial epithelial cells when in culture. The ability of rolipram, a PDE4 inhibitor, to modulate release of tPA and salbutamol, a β agonist, to increase PAI-1 synthesis suggests that a combination of these drugs may have potential in controlling the tissue remodelling occurring in asthma.

The results in this thesis do provide evidence to suggest individual inflammatory cells have a unique profile of intracellular targeting of PDE family members. These intracellular profiles of PDE protein and activity expression differ between eosinophils, neutrophils and PBMC; therefore suggesting that they tailor their expression of PDE enzymes to regulate their unique biological functions. There is an increasing body of published evidence using cell lines to support this theory.

Through the intracellular targeting of individual PDE forms, cells may be able to manipulate cAMP stores in discrete subcellular regions. This theory of discrete targeting, both of PDE forms and elements whose activity is regulated by cAMP namely PKA, has been largely championed by Houslay and his research group in Glasgow.

Evidence in this thesis would support the theory of targeting, and the confocal images in Chapter 7 clearly illustrate distinct cellular localisation patterns individual to the PDE4A, 4B and 4D members in bronchial epithelial cells. This intracellular targeting can also affect the activity of the PDEs, or the affinity of inhibitors for them, through altering their conformation. Such diversity, not only in individual PDE isoform or splice variant expression, but also in their subcellular localisation and the subsequent effects on their activity or susceptibility to inhibitors, allows cells to manipulate their cAMP levels with great subtlety, and in association with specific responses.

The evidence provided in this thesis that subcellular localisation of PDEs occurs within eosinophils, neutrophils, PBMC and primary bronchial epithelial cells suggests that, in these

cells, future investigations may be able to associate PDE enzymes with discrete signalling pathways involved in regulation of specific cellular responses. Therefore, it may become a matter of selecting a cellular process, for example LTC_4 synthesis in eosinophils which is known to be inhibited by PDE inhibitors (Tenor et al. 1996), and examining which PDE is found associated with elements of the leukotriene synthetic machinery. In this way individual intracellular processes may be modulated by the delivery of a specific inhibitor to a PDE at a functionally important site. Delivery of PDE inhibitors directly to the PDE responsible for regulating a biological response would mean much lower concentrations of inhibitors were needed. As general PDE inhibition would not be necessary, any side effects associated with these drugs may be diminished. The targeting of PDE inhibitors to intracellular localisations, for example the cell membrane, may be possible by making these drugs more lipophilic, an approach that was used in the development of the long-acting β agonist salmeterol from its short acting counterpart, salbutamol. The epithelium should also be considered for targeting of inhibitors to individual isoforms present in distinct locations, particularly as it has both pro inflammatory and anti inflammatory actions, many controlled by cAMP. A widespread PDE inhibitor would most likely also knock out some beneficial actions of the epithelium, as well as its pro inflammatory actions. Therefore, subcellular targeting of PDE inhibitors to PDEs identified to regulate pro inflammatory actions would be therapeutically very useful.

8.1 Future Work

The results of this project have identified several potential areas for further investigation.

Co-localisation of PDEs in inflammatory cells

Co-localisation of the PDE4 isoenzymes with the subunits of PKA, by three channel confocal microscopy, would complete initial work undertaken in this thesis. It would also be interesting to investigate the effects of cAMP elevating agents, or stimuli that activate the cells, upon intracellular localisation of these molecules. The observed movement of the RII subunit of PKA upon theophylline treatment should be investigated in order to identify if this effect of theophylline is mediated through PDE inhibition, and if the movement can be reproduced by specific PDE inhibitors.

The identification of anchoring molecules, possibly AKAPs, structural components, cytoskeletal actin or tubulin, or even cell signalling components i.e. RACK1 or SRC family kinases, to which the PDEs bind and colocalise in eosinophils, neutrophils, PBMC or bronchial epithelial cells would allow significant developments in elaborating how cells utilise these PDE enzymes to control their biological functions. This in turn should provide information for further drug development.

Expression of elements of the fibrinogenic cascade in bronchial epithelial cells

There is scope to investigate the levels of tPA and PAI-1 synthesis and release from primary bronchial epithelial cells derived from asthmatic donors, in order to ascertain if asthmatics show different regulation of these molecules in comparison to those obtained in this thesis from normal donors. As relatively high concentrations of PDE inhibitors were used, the effects of lower concentrations of these drugs, by concentration curve experiments, should be investigated. The effects of these PDE inhibitors on stimulated epithelial cells, for example cells stimulated by house dust mite allergen, would be interesting as this is likely to occur within the bronchial epithelium of allergic asthmatics. As tPA and PAI-1 are

important mediators in the regulation of fibrinolysis, the effects of PDE inhibitors upon levels of these molecules in the endothelium should also be investigated alongside those in the epithelium. This is particularly important, as dysregulation of the tPA/PAI-1 balance at the endothelium may precipitate a stroke, tPA being necessary to prevent clot formation.

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