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UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine, Health and Biological Sciences
Division of Infection, Inflammation and Repair

THE ROLE OF TRYPTASE AND ACTIVATION OF PROTEASE ACTIVATED RECEPTOR-2 (PAR-2) IN HUMAN AIRWAYS DISEASE

BY

AKHMED ASLAM

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ABSTRACT

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THE ROLE OF TRYPTASE AND ACTIVATION OF PROTEASE ACTIVATED RECEPTOR-2 (PAR-2) IN HUMAN AIRWAYS DISEASE

By Akhmed Aslam

Mast cell degranulation is an important process in allergic inflammatory conditions, such as asthma and rhinitis. The trypsin-like serine protease tryptase is a major secretory product released by activated mast cells. Tryptase is able to alter the behaviour of a number of cell types, but the mechanisms of action are not fully understood. Recently, tryptase has been found to activate protease activated receptor-2 (PAR-2), which has also been implicated in inflammation. Therefore, the activation of PAR-2 by tryptase may provide one mechanism by which tryptase can alter cell function. Eosinophils are also an important cell type in allergic inflammation, but the effects of tryptase on them and the extent to which these cells express PAR-2 is not fully understood. The aim of these studies was to investigate the role of tryptase and activation of PAR-2 in allergic airways disease, including bronchial asthma and allergic rhinitis.

The role of PAR-2 in inflammatory conditions has not been defined, due to the lack of investigational tools, such as PAR-2 monoclonal antibodies and antagonists. To address this, monoclonal antibodies which detect PAR-2 peptide fragments in direct ELISA, were produced. Monoclonal antibody P2A detected PAR-2 protein by flow cytometry in KNRK cells transfected with human PAR-2 ($KNRK_t$), by immunohistochemistry in lung, bronchial, nasal polyp and colon tissue embedded in GMA resin, and by Western blotting in $KNRK_t$ lysates. Rabbit antisera against peptide fragments spanning the cleavage site, peptides residing within the second extracellular loop and against a recombinant protein to the tethered ligand of human PAR-2 were also generated. The antibodies detected PAR-2 protein by flow cytometry in $KNRK_t$ cells, but not untransfected KNRK cells.

Levels of expression of PAR-2 in bronchial and nasal tissue from subjects with asthma and allergic rhinitis respectively were studied. Bronchial biopsies embedded in GMA resin from eight mild asthmatic subjects, from eight severe asthmatic subjects and from 15 non-asthmatic subjects were used. In addition, bronchial biopsies were also taken from six mild asthmatic subjects six hours following exposure to saline or house dust mite allergen. For a study of rhinitis nasal biopsies from eight perennial rhinitic subjects and six non-rhinitic subjects were used. Nasal biopsies were also collected from six patients with seasonal allergic rhinitis (out-of-season) and six hours following saline or grass pollen allergen treatment. Two micron sections were stained with P2A or an antibody raised against a peptide fragment of rat PAR-2 (B5 antiserum), and PAR-2 staining intensity quantified using computerised image analysis. PAR-2 expression was detect mainly on epithelial cells and found to be significantly higher in the bronchial epithelium of severe asthmatics compared to non-asthmatic subjects (Mann Whitney U test, $P < 0.05$). Although PAR-2 expression in the bronchial epithelium of mild asthmatics did not differ from that in subjects without asthma, expression was significantly increased following allergen provocation (Wilcoxon's test, $P < 0.05$). There was no change in the expression of PAR-2 in the nasal epithelium of perennial rhinitic subjects compared to those without rhinitis. However, expression was significantly reduced following allergen challenge (Mann Whitney U test, $P < 0.05$). The differences in expression of PAR-2 in the upper and lower airways suggest that its expression may be regulated by separate elements in the nose and the bronchi in rhinitis and asthma.

The expression of PAR-2 on eosinophils and the activation by tryptase on the function of these cells were investigated, using eosinophils purified from human peripheral blood. PAR-2 expression was assessed by flow cytometry using P2A and the B5 antiserum. PAR-2 was detected both at the surface and intracellularly on eosinophils using P2A. Expression was not detected with the B5 antiserum, suggesting that the two antibodies detect different epitopes on the PAR-2 receptor. Purified eosinophils were incubated with either purified lung tryptase, bovine trypsin, PAR-2 agonist peptide (SLIGKV-NH_2) or reverse peptide (VKGILS-NH_2), and measurements of EPO release determined. Tryptase inhibitors, benzamidine and leupeptin, were also employed for these studies. Tryptase (5 - 80 mU/ml) induced a concentration-dependent increase in the release of EPO from eosinophils, which was abolished by pre-incubation with either benzamidine or leupeptin. SLIGKV (10 - 320 μM) had no effect, nor did VKGILS (10 - 320 μM). Similarly, trypsin (10nM - 1 mM) had no effect on EPO release. Calcium flux measurements in response to the aforementioned PAR-2 agonists were also monitored. Purified lung and recombinant human tryptases, or the peptide agonists of PAR-2 failed to increase the intracellular calcium concentrations. Therefore tryptase may be activating eosinophils by a PAR-2 independent mechanism.

These studies demonstrate that tryptase activates eosinophils by a PAR-2 independent mechanism, and could involve activation of an as yet unidentified PAR. We have also shown that PAR-2 expression may be up-regulated in asthma. Thus, tryptase and PAR-2 could play key roles in allergic airways diseases, and as such can be considered as potential targets for therapeutic intervention.

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Abbreviations

AEC	3-amino-9-ethyl carbazole
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BAPNA	N-α-benzoyl-DL-argenine-*p*-nitroaniline
BCA	bicichiconic acid
BSA	bovine serum albumin
$[Ca^{2+}]_I$	intracellular calcium concentration
CGRP	calcitonin-gene related peptide
COPD	chronic obstructive pulmonary disease
COX-2	cyclo-oxygenase-2
DAB	diaminobenzadine
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
ECL	extracellular loop
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
ERK	extracellular-signal related kinase
FCS	foetal calf serum
FEV_1	forced expiratory volume in one second

FITC	fluoroscein isothiocyanate
GMA	glycol methacrylate
GM-CSF	granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HAT	Human airway trypsin-like enzyme
HES	hybridoma enhancing supplement
HRP	horse-radish peroxidase
ICAM-1	intercellular adhesion molecule-1
ICL	intracellular loop
Ig	immunoglobulin
IL	interleukin
IP-3	inositol trisphosphate
KLH	keyhole limpet haemocyanin
KNRK	Kirsten sarcoma-virus transformed normal rat kidney epithelial cells
$KNRK_t$	KNRK cells transfected with human PAR-2
LDH	lactate dehydrogenase
LTC_4	leukotriene C_4
MAP	mitogen activated protein
MBP	major basic protein
MC_T	mast cell containing tryptase, but not chymase
MC_{TC}	mast cell containing both tryptase and chymase
MEK	mitogen activated protein kinase kinase
MES	2-[*n*-morpholino]ethane-sulphonic acid
MMP	matrix metalloproteinase

PAGE	polyacrylamide gel electrophoresis
PAR	protease activated receptor
PBS	phosphate buffered saline
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PEG	polyethylene glycol
PHM	peptide histidine methionine
PIP-2	phophatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PMSF	phenyl methyl sulphonyl fluoride
PLC	phospholipase C
RPMI	Roswell Park Memorial Institute
SCF	stem cell factor
SDS	sodium dodecyl sulphate
tc-LIGRLO	*trans*-cinnamoyl-LIGRL-propylornithine
TGF	transforming growth factor
TNF	tumour necrosis factor
VIP	vasoactive intestinal peptide

CHAPTER 1

General Introduction

1 General Introduction

Mast cells have been implicated in many inflammatory conditions, and in particular in bronchial asthma and allergic rhinitis. The major secretory product released upon degranulation is the trypsin-like serine protease tryptase, and levels of this enzyme are elevated in the airways of asthmatics and rhinitics. Tryptase has a range of pro-inflammatory actions and is able to alter the behaviour of a number of cell types, but the mechanisms of its actions are little understood. Tryptase has been found to activate protease activated receptor-2 (PAR-2). This may be one means by which tryptase regulates the behaviour of a variety of cell types, though it is not known to what extent its actions are mediated by this receptor.

The accumulation of eosinophils is a prominent feature of allergic inflammation, but the effects of tryptase on these cells and the degree to which these cells express PAR-2 have been little investigated. Research in this area has been hindered to a large extent by the lack of investigational tools for PAR-2 with which cellular expression can be explored. It is unclear also to what extent levels of this receptor may be altered in disease.

1.1 Mast Cells and Bronchial Asthma

1.1.1 The Pathogenesis of Asthma

Asthma was the first name given to the condition, which affected patients who had difficult breathing of an episodic nature. This was an incorrect description since it was later shown that some individuals with cardiac arrhythmia also exhibited the same symptoms. Thus, asthma was sub-classified into two types – cardiac and bronchial. Over time, as the circulatory changes involved in cardiac arrhythmia became clearer, more terms were available and the name 'cardiac asthma' was no longer used. Therefore, bronchial asthma became simply known as asthma.

Asthma can be defined as a disease characterised by wide variations over short periods of time in resistance to flow in the airways of the lung' (American Thoracic Society, 1962). Individuals experience episodic attacks of breathlessness associated with wheezing. Whilst some variability in airflow remains, a certain component of reduced lung function cannot be reversed with bronchodilator or corticosteroid treatment. This fixed airway obstruction has been attributed to remodelling of the airways including sub-epithelial fibrosis and hyperplasia of the smooth muscle cell layer (Redington *et al*, 2001).

Another major clinical feature of asthma is airway hyperresponsiveness. Airway responsiveness is defined as the ease with which airways narrow in response to various non-allergic and non-sensitising stimuli (Hargreave *et al*, 1986). These stimuli include agonists such as histamine or methacholine, and natural physical stimuli, such as exercise and exposure

to cold air. The level of responsiveness is most commonly defined by assessing lung function (*e.g.* FEV_1; forced expiratory volume in one second) before and after inhaling increasing concentrations of an agent such as methacholine. The more responsive the airways, the lower the amount of drug needed to decrease lung function. Sensitivity is increased such that the threshold dose to trigger a set reduction in FEV_1 is lower is asthma (Juniper *et al*, 1981). Therefore, the airways of these individuals are 'hyperresponsive' to the given stimulus. Studies have shown that greater than 80 % of all subjects with a history of asthma and 98 – 100 % of subjects with current symptomatic asthma have increased responsiveness to histamine or methacholine challenge (Davé *et al*, 1990). Similarly, the level of airway responsiveness correlates with the severity of asthma symptoms and medication requirements in both adults (Juniper *et al*, 1981; Hargreave *et al*, 1986) and children (Murray *et al*, 1981; Amaro-Galvez *et al*, 1987). Stimuli that increase airway responsiveness in humans include viral respiratory infections, such as influenza A (Little *et al*, 1978), air pollutants such as ozone (Zwick *et al*, 1991) or cigarette smoke (Weitzman *et al*, 1990), inhaled allergens (O'Byrne *et al*, 1987), and occupational agents such as isocyanate (Chan-Yeung *et al*, 1990).

Another key feature of asthma is inflammation of the airways. This involves the participation of many cells including mast cells, eosinophils, neutrophils, T-lymphocytes, macrophages and epithelial cells. Indices of

airway inflammation (*e.g.* airway eosinophilia) may be associated with increased airway responsiveness (Chetta *et al*, 1996).

The examination of bronchoalveolar lavage (BAL) fluid and biopsy tissues from patients with asthma has provided valuable insights into the mechanisms of asthma and the features that may link altered lung function to a specific type of inflammation. Since the description of the technique by Reynolds and Newball (1974), BAL has been widely used to characterise pulmonary inflammation both in the large airways and parenchymal structures. More recently, BAL studies together with studies of bronchial/lung biopsy, bronchial brushings and induced sputum have provided evidence for airway inflammation associated with asthma. In healthy individuals, approximately 10-20 million cells can be recovered from BAL when the lavage volume exceeds 100 ml (Calhoun & Liu, 2000). Macrophages and lymphocytes are the dominant cell types, comprising 80-90 % and 10-20 % of cells recovered respectively. Mast cell numbers range from 0.02 % to 0.48 % (Adelroth *et al*, 1990; Liu *et al*, 1990). Eosinophils, mast cells, T-lymphocytes, basophils, neutrophils and macrophages are more numerous in the BAL of asthmatics compared to healthy controls (Calhoun & Liu, 2000). These cells well known to contribute to the inflammation associated with asthma (Beasley *et al*, 1989; Djukanovic *et al*, 1990; Jeffery *et al*, 1989).

Histopathological analyses in fatal asthma have indicated occlusion of the airway lumen by mucous plugs containing epithelial and inflammatory cells (Cardell & Pearson, 1959; Shimura *et al*, 1996), mucous gland hypertrophy or hyperplasia (Forstner *et al*, 1976) and goblet cell hyperplasia and metaplasia. There may also be extensive loss of the respiratory epithelium from the surface of the bronchial wall (Dunnill, 1960). Other characteristic features include basement membrane thickening (by increased collagen deposition), increased mass of smooth muscle layers, inflammatory cell infiltration, dilation of the capilliaries and post-capillary venules within the airway wall, airway oedema and microvascular leakage (Dunnill, 1960; Carroll *et al*, 1993; Synek *et al*, 1996; Beasley *et al*, 1989; Sharma & Jeffrey, 1990; Kuwano *et al*, 1993), commonly seen with airway remodelling.

Airflow obstruction, airway hyperresponsiveness and inflammation and remodelling of the tissue will be mediated by a complex interplay of many cell types. However, a cell that plays a particularly important contribution in these processes is the mast cell. This is discussed in more detail below.

1.1.2 Mast Cells in Asthma

Mast cells are widely distributed throughout the body, both in connective tissues and at mucosal surfaces. In the lungs of asthmatics, mast cells may be found in the epithelial lining (Djukanovic *et al*, 1990), are present

in large numbers in the bronchial mucosa, submucosa and lumen (Tomioka *et al*, 1984; Brinkman *et al*, 1968), alveolar walls (Fox *et al*, 1981) and can be found infiltrating bronchial smooth muscle (Brightling *et al*, 2002). These cells have been implicated as key participants in the early and late phase asthmatic reaction to allergens, chronic allergic asthma, intrinsic asthma, virus-induced asthma, occupational asthma, exercise-induced asthma and drug-induced asthma.

Following bronchial allergen challenge there is a rapid fall in pulmonary function (FEV_1) becoming maximal at 10-20 min and gradually recovering over the next 2 hr. This is termed the early asthmatic reaction (EAR). In some subjects there is a further fall in FEV_1 between 4-6 hr after allergen exposure termed the late asthmatic reaction (LAR) and this may last for 12 hr or more (Booij-Noord *et al*, 1972). Mast cell activation is a characteristic feature of the EAR. Mast cells may be activated when inhaled allergen cross-links immunoglobulin E (IgE) antibodies on their cell surface. IgE antibody binds to high affinity receptors ($Fc\varepsilon R_I$) on the mast cell membrane. When adjacent $Fc\varepsilon R_I$ molecules are aggregated by multivalent antigens, a signalling cascade is initiated, culminating in the secretion of an array of mediators. *In vivo* studies have demonstrated recovery of these mediators in BAL within 5-10 min of local bronchial allergen challenge (Murray *et al*, 1986; Casale *et al*, 1987; Wenzel *et al*, 1988, 1990, 1991; Liu *et al*, 1991; Sedgwick *et al*, 1991). The bronchoconstriction, mucosal oedema and mucus secretion produced by

histamine, prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) suggests that they may be responsible for the acute airway narrowing induced after allergen challenge. Mast cell tryptase levels are also rapidly increased within minutes following bronchial allergen challenge (Wenzel *et al*, 1988), supporting the idea that mast cell activation occurs in the EAR.

The LAR differs from the EAR in that it is characterised by inflammatory cell accumulation and activation, and thus the airway obstruction and bronchial hyperresponsiveness accompanying the LAR may be exacerbated by mediator release and tissue damage by infiltrating inflammatory cells. The LAR is associated with eosinophil influx into the bronchial mucosa and increased quantities of eosinophil granule proteins (De Monchy *et al*, 1985; Metzger *et al*, 1987; Bentley *et al*, 1993; Montefort *et al*, 1994). Tryptase levels do not increase during the LAR (Sedgwick *et al*, 1991) but increased levels of histamine, PGD_2 and LTC_4 are present (Liu *et al*, 1991; Sedgwick *et al*, 1991), suggesting that other inflammatory cells such as basophils may well be involved.

In chronic allergic asthma, increased numbers of mast cells in the bronchial epithelium can be found compared to non-asthmatics (Bradding *et al*, 1994), with evidence of continuous degranulation, as assessed by electron microscopy, in both the epithelium and submucosa (Beasley *et al*, 1989). Increased numbers of mast cells can also be recovered in BAL fluid from asthmatics compared to normal controls (Kirby *et al*, 1987)

together with increased concentrations of histamine and tryptase, providing further supporting evidence for on-going mast cell degranulation (Kirby *et al*, 1987; Wenzel *et al*, 1988). More recently, increased numbers of tryptase and chymase positive mast cells in the bronchial smooth muscle have been observed, which was inversely correlated with the degree of airway hyperresponsiveness. This suggests that the localisation of mast cells in bronchial smooth muscle and their interaction with these cells could be responsible for the development of the airflow obstruction associated with asthma (Brightling *et al*., 2002)

Intrinsic asthma is a condition in a sub-group of asthmatics to which no specific sensitivity to environmental allergens can be identified. Though this form is often more severe, later in onset and more persistent, the pattern of inflammation is more or less identical to that found in extrinsic asthma. Mast cells numbers are not different between the two groups, though numbers are increased in both groups compared to normal controls.

Occupational asthma is defined as asthma that develops after specific exposure in the workplace. Prevalence depends on the area and allergens to which there is exposure. An example of occupational asthma is toluene diisocyanate induced asthma, where numbers of activated mast cells are increased in the bronchial epithelium compared to normal controls (Saetta *et al*, 1992).

Eighty percent of asthmatics develop airway narrowing with exercise or hyperventilation of cold dry air. Bronchoconstriction occurs 5-10 min after exercise and lung function usually returns to normal within 30 min. Some studies have identified increased concentrations of histamine in the serum of asthmatics following exercise, indicative of a role for mast cell degranulation (Anderson *et al*, 1981; Barnes & Brown, 1981; Lee *et al*, 1982). This was not however found in BAL fluid taken before and after exercise, in another study (Broide *et al*, 1990). Later studies showed that the histamine H_1-receptor antagonists, terfenadine and clemastine, significantly attenuated exercise-induced bronchoconstriction (Hartley & Nogrady, 1980; Finnerty & Holgate, 1990).

As has been discussed, the mast cell appears to play a key role in the inflammation and remodelling of asthma in the lower airways. It follows then that mast cells may be actively involved in the pathogenesis of the upper airways diseases, such as rhinitis. The next section will discuss this in more detail.

1.2 Mast Cells and Rhinitis

1.2.1 The Pathogenesis of Rhinitis

Rhinitis is characterised by inflammation of the nasal mucosa and clinical symptoms include nasal congestion, rhinorrhea, itching/sneezing and impairment of smell. The international consensus report on the diagnosis

and management of rhinitis, classified the disease into three categories (Lund *et al*, 1994): (a) allergic rhinitis, which may be seasonal or perennial; (b) infectious rhinitis, acute or chronic, or specific or non-specific; and (c) other forms (non-allergic and non-infectious), such as idiopathic non-allergic rhinitis with eosinophilia syndrome (NARES), occupational, drug-induced, irritant-induced and food-induced.

Allergic rhinitis is an IgE-mediated inflammatory disease of the nasal mucosa that is frequently caused by exposure to perennial or seasonal allergens existing in the living environment, either indoors or outdoors. Common allergens include the pollens (grass, trees, weeds), which are the predominant cause of seasonal allergic rhinitis; and house dust mites, animal dander and moulds, which are the major cause of perennial allergic rhinitis. Occupational allergens (such as latex or laboratory animals) can also cause nasal allergy.

Inflammation is a common pathogenic feature in allergic rhinitis and can be shown in particular in allergen challenge studies. In the 'early allergic reaction' characteristic nasal symptoms such as itching, sneezing, increased nasal obstruction and watery discharge can be observed (Andersson *et al*, 2000). The inflammatory processes involved include plasma exudation (Akerlund *et al*, 1993); increased numbers of inflammatory cells in the nasal mucosa such as T-lymphocytes, mast cells and neutrophils (Fokkens *et al*, 1990); eosinophilia (Godthelp *et al*, 1996);

and increased absorption permeability of the nasal epithelium (Greiff *et al*, 1993).

1.2.2 Mast Cells in Rhinitis

Mast cells numbers are increased in the upper airways in allergic and non-allergic rhinitis, as well as other nasal diseases such as nasal polyposis and chronic sinusitis (Ohkubo, 1988; Yokoshima & Ohnishi, 1993). Mast cells can be seen in nasal mucosa, nasal epithelium, sub-epithelium and *lamina propria* of patients with allergic rhinitis, where they are most plentiful. Mast cells are rarely seen in the epithelial layers of patients with non-allergic rhinitis, though the numbers in the *lamina propria* do not differ to those in allergic rhinitic patients (Okuda *et al*, 1983). This suggests that mast cells in the epithelium may play important roles in barrier function in the allergic process associated with rhinitis.

Although mast cells numbers are increased, in both allergic and non-allergic rhinitis, they do differ in their function and character. For example, degree of expression of $Fc_{\varepsilon}RI$ located on the membrane surface of all mast cells, are increased in the nasal mucosa of patients with allergic rhinitis compared to non-allergic rhinitic subjects (Pawankar & Ra, 1996; Pawankar *et al*, 1997). The cytokine profile also differs, with increased numbers of mast cells staining positive for IL-4, IL-5, IL-6 and IL-13 allergic rhinitis and IL-8 and TNF-α in non-allergic rhinitis (Pawankar *et al*, 1995; Pawankar & Ra, 1996). The increase in mast cell numbers is also

associated with elevated levels of tryptase in the nasal lavage recovered from symptomatic seasonal allergic rhinitic subjects than when asymptomatic, and in subjects with perennial allergic rhinitics compared to non-rhinitic control subjects (Wilson *et al*, 1998).

Mast cells are evidently involved in progression of pathological processes of asthma and rhinitis. The next section will review the key characteristics of this cell type and the potential involvement of cellular constituents.

1.3 The Mast Cell

1.3.1 Origins and Maturation

In 1878, Ehrlich identified mast cells in human connective tissues on the basis of the metachromatic staining properties of their prominent cytoplasmic granules (Ehrlich, 1878). Mast cells are derived from CD34+ pluripotent progenitor cells in the bone marrow (Kirshenbaum *et al*, 1991). All mast cells express *c-kit*, the receptor for stem cell factor (SCF), a growth and maturation factor for mast cells (Tsai *et al*, 1991; Kirshenbaum *et al*, 1992). Mast cells reside in connective tissues and at mucosal surfaces, and increase in numbers at sites of tissue injury and inflammation. Little is known about the factors that are involved in the homing of mast cells to these sites, though it has been suggested that circulating levels of committed mast cell progenitors first adhere to endothelial cells *via* cell adhesion molecules (Thompson *et al*, 1992), then migrate through tissues to specific locations determined by the local mix of

connective tissue components and cellular elements. Mast cells do, however, express chemokine receptors (de Paulis *et al*, 2001) and evidence using mast cell lines suggests that chemotaxis may occur in response to SCF, Transforming growth factor β (TGFβ), eotaxin and RANTES (Frangogiannis *et* al, 1998; Quackenbush *et al*, 1998; de Paulis *et al*, 2001; Berger *et al*, 2003) Mast cells reside in tissues, where they acquire their mature phenotype. All mast cells can be characterised by the presence of the serine protease tryptase.

1.3.2 Functional Heterogeneity

Individual mast cells can vary in their phenotype and function, due to environmental and genetic influences. Human mast cells have been divided into two major sub-populations on the basis of their content of tryptase and chymase. Generally, tryptase-positive mast cells are referred to as MC_T cells, whereas chymase-positive mast cells are known as MC_{TC} cells (Irani *et al*, 1986). Subsequently it was found that carboxypeptidase and cathepsin G were present selectively in the MC_{TC} population. Mast cells are abundant in skin, conjunctiva, gut and lung. MC_T cells are the predominant, but not exclusive, type of mast cell found in the respiratory and gastrointestinal mucosa, whereas MC_{TC} cells predominate in skin, the submucosa of respiratory and gastrointestinal tracts, blood vessel walls and the heart (Irani *et al*, 1986; Sperr *et al*, 1994). The nasal and conjunctiva mucosa may contain substantial amounts of both sub-sets, though MC_{TC} usually predominate.

1.3.3 Cell Morphology

Mature mast cells have a nucleus without deeply divided lobes, numerous cytoplasmic granules and thin elongated folds of their plasma membranes (Dvorak, 1988). Cytoplasmic secretory granules constitute approximately 50 % of the total cell volume and are the most visible morphological feature (Lloyd *et al*, 1967; Dvorak *et al*, 1993). MC_T and MC_{TC} cells differ in their granular morphology at the ultrastructural level, as viewed by electron microscopy. Chymase-containing granules exhibit a lattice substructure, while regions lacking this protease exhibit scroll-like patterns (Craig *et al*, 1988; Craig & Schwartz, 1990).

1.3.4 Expression of Cell Surface Markers

Mature mast cells express various receptors for cytokines, including the *c-kit* proto-oncogene product, Kit the receptor for stem cell factor (SCF) (Lerner *et al*, 1991). Mast cells also express surface receptors for the cytokines IL-4 and TNF-α (Sillaber *et al*, 1991; Smith *et al*, 1990). Another characteristic surface marker for mast cells is $Fc_{\varepsilon}RI$ (Beaven & Metzger, 1993) and there is an increased degree of expression in mast cells from both atopic and non-atopic individuals. Other receptor types include glycosphingolipids – cell membrane associated molecules, and adhesion molecules – involved in cell migration, growth, differentiation and function.

1.3.5 Mediators

IgE-mediated activation of mast cells can cause the rapid release of granule contents (degranulation) and the generation of newly formed mediators (Fig. 1-1). This process begins and ends in minutes. Certain mediators, such as prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) are produced within minutes and their production may last for 30 minutes or more, whereas cytokine production may begin either very early or at some period after stimulation, and can last for hours. This section will review the major products of mast cell degranulation.

1.3.5.1 Histamine

Histamine (β-imidazolylethylamine) is a biogenic amine and the only one of its kind found in mast cells. Histamine is formed in mast cells, by the passive uptake of histidine (Bauza & Lagunoff, 1981), conversion to histamine by histidine decarboxylase (Schayer, 1963), and stored in secretory granules associated by ionic linkage with carboxyl groups of proteoglycans and/or proteins. Upon degranulation, histamine is externalised where it rapidly dissociates from the proteoglycan-protein complex. Once externalised, histamine is rapidly metabolised, within minutes of release, suggesting that it normally acts near the site of its release. Histamine has been implicated in the pathogenesis of both asthma and rhinitis. In asthma, histamine concentrations in BAL fluid are increased compared to healthy subjects (Broide *et al*, 1991; Crimi *et al*, 1991) and elevated within minutes of allergen provocation (Wenzel *et al*,

1988), with the ability to cause bronchoconstriction and increased bronchial hyperresponsiveness. In rhinitis, histamine is thought to be responsible for nasal blockage (McLean *et al*, 1977), and sneezing and rhinorrhea (Doyle *et al*, 1990), as assessed by nasal allergen challenge.

1.3.5.2 Proteoglycans

Proteoglycans are highly sulphated acidic macromolecules located in the secretory granules of mast cells and composed of glycosaminoglycan side chains covalently linked to a single-chain protein core. Two proteoglycans, heparin and chondroitin sulphate E, have been identified in purified human mast cells (Stevens *et al*, 1988; Thompson *et al*, 1988). These molecules serve to bind histamine, neutral proteases and acid hydrolases. Binding histamine maintains osmolarity and thereby prevents swelling of the granules. Interactions of heparin with pre-cursors of tryptase and chymase are essential for converting these enzymes to their active forms. Moreover, tryptase is stabilised as an enzymatically active tetramer bound to heparin and in its absence, it dissociates into inactive monomers (Schwartz & Bradford, 1986).

1.3.5.3 Neutral Proteases

Three proteases have been isolated from human mast cells to date, tryptase (Schwartz *et al*, 1981; Smith *et al*, 1984), chymase (Schechter *et al*, 1983, 1986), and a carboxypeptidase (Goldstein *et al*, 1987, 1989). There is also strong evidence for a cathepsin G-like protease in mast cells

(Meier *et al*, 1985; Schechter *et al*, 1990, 1994), though this has yet to be isolated from purified mast cells.

Proteases (also known as peptidases) are enzymes that hydrolyse peptide bonds. They can be sub-classified into those enzymes that cleave peptide bonds distant from the ends of polypeptide chains (referred to as endopeptidases) and those that cleave peptide bonds close to the ends of the chains (referred to as exopeptidases). Endopeptidases have been further classified into four groups based on the nature of their catalytic site; serine, cysteine, aspartic and metalloproteases (Hartley, 1960; Barrett, 1980). The serine proteases comprise the largest group of these enzymes and include tryptase, chymase and cathepsin G. Chymase and cathepsin G, cleave peptides at sites next to amino acids with aromatic residues, whereas tryptase cuts the peptide bonds next to amino acids with basic residues (lysine and arginine). Mast cell carboxypeptidase is a zinc-dependent metalloprotease exopeptidase. Proteases can also be classified according to the pH optima of the reactions they catalyse. The enzymes described in this section are the 'neutral proteases', which are those enzymes having optimal activity at neutral pH such as that of the respiratory tract.

The mast cell proteases, described above, are the dominant protein components of the secretory granules in human mast cells and have been used as selective markers for distinguishing mast cells from other cell

types. For example, mast cell tryptase is detected by monoclonal antibody AA1, which is specific for the enzyme (Walls *et al*, 1990) and currently used as the standard antibody for detection of mast cells. Mast cell tryptase and its biological actions will be discussed in more detail later in this chapter (section 1.4)

Human chymase is a monomer of 30 kDa and the principal enzyme accounting for the chymotrypsin-like activity present in human cutaneous mast cells. The enzyme is an endopeptidase that like tryptase, is stored fully active in association with proteoglycans in mast cell secretory granules, bound to heparin and/or chondroitin sulphate E. However, chymase resides on separate proteoglycan molecules from tryptase (Goldstein *et al*, 1992) and unlike tryptase, its stability is not affected by heparin (Sayama *et al*, 1987). A number of biological actions have been observed based on *in vitro* studies. Chymase is able to convert angiotensin I to angiotensin II much more efficiently than angiotensin-converting enzyme (Wintroub *et al*, 1984; Urata *et al*, 1990). Chymase cleaves endothelin and is a potent stimulus for mucus production from glandular cells (Nakano *et al*, 1997; Sommerhoff *et al*, 1989), suggesting a role for the enzyme in generating endothelins and hypersecretion of mucus associated with asthma and rhinitis.

Cathepsin G is a 30 kDa neutral protease also found in neutrophils and monocytes. The presence of a cathepsin G-like enzyme in MC_{TC} cells

was demonstrated using immunohistochemical, Western blotting and enzymatic techniques (Schechter *et al*, 1990). When mast cells are activated, chymase, cathepsin G and carboxypeptidase are released together in a 400-500 kDa complex with proteoglycan, and are likely to act in concert with other enzymes to promote proteolysis (Goldstein *et al*, 1992)

Mast cell carboxypeptidase is an enzyme with a molecular mass of 34.7 kDa. It appears to be selectively present in MC_{TC} cells (Irani *et al*, 1991), but not MC_T cells. As with cathepsin G, carboxypeptidase is co-released from mast cells in a complex with chymase and cathepsin G and has been shown to be bound to proteoglycans in the same macromolecular complex as chymase (Goldstein *et al*, 1992). Thus, carboxypeptidase may also act synergistically with the other mast cell enzymes to degrade peptides and proteins.

1.3.5.4 Lipid Mediators

Mast cell activation results in the cleavage of arachidonic acid from cellular phospholipids. The arachidonic acid is either re-incorporated into lipids or metabolised to generate lipid mediators. Arachidonic acid metabolism proceeds along one of two pathways, either the cyclooxygenase pathway to generate prostanoids, or the 5-, 12- or 15-lipoxygenase pathways to synthesise the leukotrienes (LT), LTB_4, LTC_4, LTD_4, LTE_4 and lipoxins. PGD_2 is generated by human mast cells after

they are activated *via* the IgE receptor or by calcium ionophore (Levi-Schaffer & Shalit, 1989). Purified human mast cells also produce LTC_4 in excess of LTB_4 (MacGlashan *et al*, 1982; Peters *et al*, 1984). LTC_4 is a potent bronchoconstrictor and can increase microvascular permeability, presumably by activating the single cysteinyl leukotriene receptor in the airways, $CystLT_1$ – located on airway smooth muscle and inflammatory cells (Lynch *et al*, 1999).

1.3.5.5 Cytokines

Mast cells are a source for a huge profile of cytokines. Human mast cells are capable of synthesising and releasing interleukin-3 (IL-3), IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and SCF (Bradding *et al*, 1992, 1994, 1996; Okayama *et al*, 1995; Klein *et al*, 1989; Castells *et al*, 1996). The ability of mast cells to produce those cytokines gives them the potential for interactions with other cell types to modulate homeostatic, allergic, and inflammatory and host defence functions. Mast cells may also generate remodelling cytokines, such as transforming growth factor (TGF-β) and basic fibroblast growth factor (bFGF) in substantial quantities.

1.4 Mast Cell Tryptase

Of the proteases produced by mast cells, tryptase has been found to be the most abundant, with approximately 10 pg per lung mast cell and 35 pg per skin mast cell (Schwartz *et al*, 1987). Until recently, tryptase was

thought of principally as a marker for mast cell activation, and its biological actions have long been neglected. Recent studies have indicated the importance of this mast cell protease as a mediator of inflammation, and as a new target for therapeutic intervention in asthma and other inflammatory conditions. The function and role of this major mast cell protease are reviewed below.

1.4.1 Biochemical Characteristics

Tryptase (Enzyme Commission (EC) code (EC 3.4.21.59)). Tryptase is a tetrameric serine endopeptidase with a molecular mass of approximately 130 kDa (Schwartz *et al*, 1981), composed of variably glycosylated subunits (28-38 kDa) (Schwartz *et al*, 1981; Smith *et al*, 1984; Walls *et al*, 1990; Benyon *et al*, 1993). Optimal catalytic activity is expressed when tryptase is in its tetrameric form.

Two tryptase cDNA sequences have been cloned from a human lung mast cell library (Miller *et al*, 1989, 1990) and termed α- and β-tryptase. In addition, three cloned independently from a human skin mast cell library (Vanderslice *et al*, 1990) have been named tryptases I, II and III. Tryptase II and β-tryptase were found to be identical and to share 98 % homology with tryptases I and III but only 90 % with α-tryptase. Consequently, tryptase I, II and III have been considered together as the β-tryptases but distinguished as βi, βII and βIII. Subsequent genome mapping identified a number of tryptase-like genes, termed γ-, δ- and ε-tryptase (Pallaoro *et al*,

1999; Caughey *et al*, 2000; Wong *et al*, 1999, 2001), which are not secreted by mast cells. Gamma-tryptase (also known as *trans*-membrane tryptase) is membrane bound (Caughey *et al*, 2000; Wong *et al*, 1999), δ-tryptase (also known as mMCP-7-like protease) appears to be a pseudogene (Min *et al*, 2001; Soto *et al*, 2002) and ε-tryptase is a product of foetal lung epithelial cells (Wong *et al*, 2002). Tissue mast cells, however, contain large amounts of mRNA encoding both α- and β-tryptase (Xia *et al*, 1995).

Alpha-tryptase may be released constitutively from mast cells in an inactive form, whereas β-tryptase is released upon mast cell degranulation (Schwartz *et al*, 1995). Alpha-tryptase is the major form detected in the plasma of patients with systemic mastocytosis, reflecting an increased body load of mast cells (Schwartz *et al*, 1995). Whereas the majority of tryptase, as detected by immunoassays in the serum of patients with systemic anaphylaxis is the β-form. Recent evidence has suggested that assays thought to detect α-tryptase may in fact have been detecting a pro-form (inactive) of β-tryptase (active) (Schwartz *et al*. 2003). It seems likely that β-tryptase will predominate in the airways of patients with asthma and rhinitis, since both these inflammatory conditions are associated with increased mast cell activation. Mast cell studies to date have used tryptase purified from lung or skin (β-like tryptases), as it appears that α-tryptase is not stored and is difficult to isolate from cells and tissues.

Determination of the crystallised structure of β-tryptase using X-ray crystallography (Pereira *et al*, 1998) shows that the four subunits of the tetrameric molecule are arranged in a square flat ring structure (Figure 1-2). The active site of each monomer is directed inwards to a central oval pore, which is liable to restrict access for large molecular weight enzyme inhibitors and substrates.

Tryptase is stored within mast cell granules tightly bound to heparin, thereby maintaining the tetrameric structure and stability of the enzyme (Schwartz & Bradford, 1986). Tryptase is enzymatically unstable in physiological buffers, with a half-life of less than three minutes (Alter *et al*, 1987). This involves conversion of the active tetrameric structure into the inactive monomers (Schwartz *et al*, 1990). Subsequently the rate of instability is reduced when tryptase is bound to heparin or other proteoglycans, but increased in the presence of divalent cations (Alter *et al*, 1987). Thus, the enzymatic actions of tryptase should be restricted to the immediate vicinity of mast cell degranulation. As tryptase is released bound to the active complex of proteoglycans, it follows then that the large size will limit diffusion away from the site of release.

1.4.2 Substrates and Inhibitors

Tryptase preferentially cleaves peptide and ester bonds on the carboxyl side of basic amino acids (Tanaka *et al*, 1983). Neuropeptides are

potential substrates, peptidergic nerves being present in the immediate vicinity of mast cells (Bienenstock *et al*, 1988; Hislop *et al*, 1990). Purified tryptase has been observed to cleave vasoactiveintestinal peptide (VIP), peptide histidine methionine (PHM) and calcitonin-gene related peptide (CGRP) (Tam and Caughey, 1990; Walls *et al*, 1992), where tryptase may play a role in the normal physiological control of these neuropeptides.

The acidic pH within the mast cell granules (De Young *et al*, 1987), is likely to suppress enzymatic activity (McEuen *et al*, 1996). In addition, within the granules, and in the vicinity of mast cells, high concentrations of histamine are known to reversibly inhibit tryptase activity (Alter & Schwartz, 1989). Evidence for the existence of endogenous inhibitors for tryptase in the extracellular environment is lacking. However, tryptase activity may be inhibited by leupeptin, diisopropyl fluorophosphate and phenylmethylsuphonyl fluoride (Alter *et al*, 1990).

1.4.3 Role of Tryptase in Airway Disease

In asthma, mast cell activation is a key feature of the inflammatory process. Beta-tryptase levels are increased in BAL fluid and bronchial biopsies from asthmatic subjects (Wenzel *et al.* 1988; Djukanovic *et al*, 1990). Tryptase levels also increase following endobronchial challenge with house dust mite allergen of atopic mild asthmatic subjects in the EAR, but not the LAR (Nocker *et* al, 1999). Tryptase can induce bronchoconstriction and increases bronchial hyperresponsiveness,

through the stimulation of histamine release from mast cells (He *et al*, 1998), degradation of bronchodilator neuropeptides and directly through potentiation of the inflammatory response (Clark *et al*, 1995). In seasonal allergic rhinitis, as in asthma, mast cell degranulation appears to be an ongoing process, because elevated β-tryptase levels are found during the season, but undetectable among the same patients out-of-season and among patients with chronic sinusitis (Rasp & Hochstrasser, 1993). In addition, following nasal challenge with grass pollen allergen tryptase levels are increased in subjects with seasonal allergic rhinitis (in-season) compared to when asymptomatic (Wilson *et al*, 1998)

The actions of tryptase are summarised in Figure 1-3. Neuropeptides such as vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM) and calcitonin gene related peptide (CGRP) are known to be natural substrates for tryptase (Tam and Caughey, 1990; Walls *et al.*, 1992). VIP and PHM are both potent relaxants of smooth muscle; therefore cleavage by tryptase may cause bronchoconstriction (Palmer *et al.*, 1987). CGRP is a potent vasodilator, hence cleavage by tryptase would result in attenuation of vasodilator activity (Walls *et al.*, 1992). Also, since mast cells reside close to peptidergic nerves, tryptase may make an important contribution to neurogenic inflammation by stimulating the release of substance P and CGRP (Steinhoff *et al.*, 2000). Fibrinogen is degraded by tryptase, such that it is no longer available as a substrate for thrombin in the blood clotting cascade (Ren *et al.*, 1997). Certain

components of the extracellular matrix (ECM) cleaved by tryptase include gelatinase, type VI collagen, and fibronectin (Lohi *et al.*, 1992; Kielty *et al.*, 1993). This involvement in the degradation of the ECM implicates tryptase in tissue destruction, and this along with tissue remodelling is an important process in chronic asthma.

Tryptase has been shown to stimulate the proliferation of various human cell types, including lung fibroblasts (Hartmann *et al.*, 1992), airway smooth muscle cells (Berger *et al*, 2001), epithelial cells (Cairns and Walls, 1996) and microvascular endothelial cells (Blair *et al.*, 1997). Other actions on cells include stimulating the synthesis and release of type I collagen from fibroblasts (Cairns and Walls, 1997) and fibroblast chemotaxis (Gruber *et al.*, 1997). This underlines the potential roles of tryptase and its participation in airway remodelling in the bronchial tissue of asthmatics. Since tryptase has been shown to cleave and activate a PAR-2 (Molino *et al.*, 1997), this may indicate a mechanism whereby tryptase can alter cell function, and this will be considered in more detail below.

1.5 Protease Activated Receptor-2 (PAR-2)

PAR-2 is a member of a larger family of seven transmembrane domain G-protein coupled receptors (GPCRs), with a unique mechanism of action (Fig 1-4)(Dery *et al.*, 1998). Protease activated receptors (PARs) possess

cleavage sites for serine proteases within their extracellular amino-terminal sequence (Bahou and Schmidt, 1996). Cleavage at these sites exposes a new amino-terminus which functions as a 'tethered ligand' and binds intramolecularly to the second extracellular loop, thereby initiating G-protein coupling and cell signalling (Fig 1-5)(Dery *et al.*, 1998; Coughlin, 1999). Four PARs have so far been identified and cloned. PAR-1 and PAR-3, which are cleaved and activated by thrombin; PAR-2, which is cleaved and activated by trypsin and mast cell tryptase; and PAR-4 which is activated by both thrombin and trypsin, but not tryptase (Hollenberg, 1999). Table 1-1 details the agonist of these receptors.

1.5.1 Characteristics of PAR-2

In 1994, Nystedt *et al.* first isolated a DNA sequence encoding a G-protein coupled receptor (GPCR) from a mouse genomic library, using oligoprobes based on the substance K receptor. The predicted protein was found to have ~30 % amino acid homology with the thrombin receptor (PAR-1), which has a similar mechanism of activation. The protein in question became known as PAR-2. Subsequently, PAR-2 has been cloned in humans and rats (Nystedt *et al.*, 1995; Bohm *et al.*, 1996; Saifeddine *et al.*, 1996). The human PAR-2 protein sequence was found to be 397 amino acids in length and have about 83 % homology with the mouse sequence. Fluorescence *in situ* hybridisation has mapped the human PAR-2 gene to chromosomal region 5q13 (Nystedt *et al.*, 1995), where previously the human PAR-1 gene has also been located (Bahou *et*

al., 1993). Similarly the PAR-3 gene was also located to the same chromosome (Kahn *et al.*, 1998).

1.5.2 Mechanisms of PAR-2 activation

The activation of most known GPCRs involves reversible activation by soluble agonist ligands and its receptors. This involves docking to extracellular and transmembrane domains of the receptor and cell surface. Protease agonists of PARs have a unique mechanism of activating the receptor, with the ligand physically part of the receptor sequence (Fig 1-5).

PAR-2 is cleaved by serine proteases including trypsin (Nystedt *et al.*, 1994) and mast cell tryptase (Corvera *et al.*, 1997; Molino *et al.*, 1997; Mirza *et al.*, 1997). Similarly other proteins have been shown to activate the receptor. Acrosin from mammalian spermatazoa has been shown to cleave PAR-2 (Fox *et al.*, 1997) and to activate PAR-2 on oocytes (Smith *et al.*, 2000), and gingipain-R from *Porphyromonas gingivalis*, which has been implicated in adult periodontal disease (Lourbakos *et al.*, 1998). More recently, coagulation factor Xa has been shown to activate PAR-2 on endothelial cells (Bono *et al.*, 2000).

PAR-2 contains a putative enzymatic cleavage site $SKGR^{34}\downarrow S^{35}LIGRL$ (Nystedt *et al.*, 1994) and is cleaved at the Arg^{34}-Ser^{35} bond, thus exposing the new tethered ligand (NH_2-SLIGRL...). Synthetic five to six amino acid peptides corresponding to this tethered ligand (SLIGRL in

mouse, SLIGKV in human) activate PAR-2 without the need for receptor cleavage (Fig 1-6)(Bohm *et al.*, 1996). Peptides composed of amino acids in the mouse or rat sequence, SLIGRL-NH_2 and the selective PAR-2 agonist *trans*-cinnamoyl-LIGRLO-NH_2 (tc-LIGRLO-NH_2) are more potent agonists than the peptide whose sequence corresponds to that of the tethered ligand of human PAR-2, SLIGKV-NH_2 (Al-Ani *et al.*, 1999). The human PAR-1 agonist peptide SFLLRN also activates human PAR-2 (Blackhart *et al.*, 1996). These short peptides have been shown to be useful tools for identifying the biological roles of PAR-2 *in vivo*. Site directed mutagenesis studies involving PAR-1 have shown that the molecular determinants for peptide agonist specificity reside within the N-terminal extension and the second extracellular loop (ECL_2) (Bahou & Schmidt, 1996). The ECL_2 of PAR-2 has also been shown to be critical for agonist specificity (Lerner *et al.*, 1996), and PAR-2 function can be altered by mutating the corresponding residues in the ECL_2 that were found to be of importance (Al-Ani *et al.*, 1999). Recently, a polymorphic form of PAR-2 has been cloned which is characterised by a Phe^{240} to Ser^{240} mutation within the ECL_2 (Compton *et al.*, 2000). The consequences of the mutation are that the polymorphic variant of the PAR-2 receptor is less sensitive to the PAR-2 agonists trypsin, SLIGKV and SLIGRL and more sensitive to tc-LIGRLO compared to the wild-type receptor. Human PAR-2 also possesses two glycosylation sites, one within the N-terminus at Asp^{30} (N^{30}RSSKGR↓SLIGKV^{42}) and the second located within the second extra cellular loop at Asp^{222} (LN^{222}ITTCHDV^{229}) (Compton *et al*, 2001,

2002). The human PAR-2 receptor is thought to exist in various glycosylated forms within a single cell type, since multiple bands were observed by Western blot analysis of human PAR-2 expressed in Pro5 cells (Compton *et al*, 2002). The consequence of N-linked glycosylation and sialylation of both sites is a reduced sensitivity to tryptase but not trypsin or SLIGRL.

PAR-2 is coupled to either $G_q\alpha$ or $G_o\alpha$ G proteins, resulting in activation of phospholipase Cβ, phosphoinositide hydrolysis and formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG), with eventual Ca^{2+} mobilisation and influx, and activation of protein kinase C (PKC) triggering the mitogen activated protein (MAP) kinase cascade of intracellular signalling events (Bohm *et al.*, 1996a). Trypsin, tryptase and PAR-2 activating peptides (PAR2-AP) can stimulate the generation of IP_3 and mobilisation of $[Ca^{2+}]_i$ in various cell types, including keratinocytes, smooth muscle cells, neurones and tumour cell lines (Dery & Bunnett, 1998). In enterocytes and transfected cell lines, Kong *et al.* (1997) showed that PAR-2 agonists could stimulate the release of arachidonic acid and rapid generation of the prostaglandins E_2 and $F_{1\alpha}$, implicating activation of phospholipase A_2 and cyclo-oxygenase-2 (COX-2). These signalling pathways may be involved in growth and inflammation (Dery & Bunnett, 1998). Figure 1-7 illustrates the intracellular events that follow PAR-2 activation.

1.5.3 Control of PAR-2 Expression

In addition to the cleavage site that leads to receptor activation, trypsin and tryptase at high concentrations can also cleave PAR-2 at the $Lys^{41}\downarrow Val^{42}$ site (Molino *et al.*, 1997). This would remove the tethered ligand and the receptor would become unresponsive to subsequent proteolytic activation, and this could be a mechanism for down-regulation of the receptor. However, little is known of attenuation of signal transduction by PAR-2. Cellular responses to activation of PARs are transient and homologous desensitisation is observed with repeated stimulation. This indicates that efficient mechanisms exist for termination of the signal.

Uncoupling of the activated receptor occurs as a result of receptor phosphorylation, involving the G-protein related kinases (GRKs) and β arrestins (agonist-dependent phosphorylation) and PKC (agonist-independent phosphorylation). PAR-2 is rapidly internalised after activation by its agonists. Both trypsin and PAR-2 activating peptide sequences can trigger rapid endocytosis of PAR-2 in transfected epithelial cells (Bohm *et al.*, 1996a). Internalisation of inactive PAR-2 is followed by formation into early endosomes and then lysosomes. Therefore, endocytosis and lysosomal degradation of PARs in lysosomes could contribute to desensitisation by removing the receptor from the cell surface. Resensitisation is dependent on the mobilisation of intracellular Golgi pools of PAR-2 (Bohm *et al.*, 1996a).

1.5.4 Biological Functions of PAR-2

PAR-2 has been localised by immunocytochemistry to the gut, pancreas, kidney, liver, airway, ovary and eye (D'Andrea *et al.*, 1998). In addition, PAR-2 has been shown to be present in epithelial and endothelial cells, smooth muscle cells, T-cell lines, neutrophils and epithelial tumour cell lines (D'Andrea *et al.*, 1998; Howells *et al.*, 1997; Dery *et al.*, 1998). Agonists of PAR-2 have been shown to cause vasodilation, which is endothelium-dependent and nitric oxide-mediated (Saifeddine *et al.*, 1996). Mitogenic effects of PAR-2 agonists depend on the cell type; stimulating proliferation of endothelial cells, lymphoid cells and smooth muscle cells (Mirza *et al.*, 1996; Mirza *et al.*, 1997; Bono *et al.*, 1997); yet inhibiting keratinocyte growth and differentiation (Derian *et al.*, 1997).

PAR-2, like PAR-1, has been shown to be involved in the regulation of blood haemostasis. PAR-2 is present in the human aorta and coronary artery smooth muscle cells, as well as in arteries traversing the walls of the small intestine (Molino *et al.*, 1998). Upregulation of PAR-2 on endothelial cells by inflammatory mediators, including tumour necrosis factor-α (TNF-α), promotes blood coagulation (Alm *et al.*, 1999). Proinflammatory cytokines, such as TNF-α and interleukin 1α (IL-1α) induce PAR-2 expression by endothelial cells (Nystedt *et al.*, 1996). As described, tryptase is mitogenic for this cell type, therefore PAR-2 may mediate some of the effects of tryptase. Furthermore, as tryptase is

involved in key processes in the inflammatory pathways of asthma and rhinitis, PAR-2 may provide clues to the mechanism of actions of this protease. In light of this, the next two sections will discuss potential pathophysiological roles of PAR-2 in disease and its potential involvement in the airways.

1.5.5 Pathophysiological Roles of PAR-2

PAR-2 activation on keratinocytes has been shown to stimulate the synthesis of the proinflammatory cytokines, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6 and IL-8 (Wakita *et al*, 1997; Hou *et al*, 1998), and to promote leukocyte rolling, adherence, and recruitment by a mechanism dependent on platelet activating factor release (Vergnolle, 1999). Further, Vergnolle *et al*. (1999a) showed, by injection of PAR-2 activating peptides into the rat hindpaw, that PAR-2 activation can cause a profound inflammatory response with oedema and granulocyte infiltration, which is independent of mast cell activation or prostanoid production. Lindner and co-workers (2000) found a delayed onset of inflammation in PAR-2 deficient mice, as measured by a reduction in inflammatory leukocyte rolling. More recently, Ferrell and co-workers (2003) have suggested a role for PAR-2 in the chronic inflammation associated with rheumatoid arthritis. Using PAR-2 deficient mice in a model of chronic inflammation, they showed that joint swelling was substantially reduced compared with wild-type mice, indicating that PAR-2 antagonists may have relevance in anti-inflammatory therapy.

Thrombin and mast cell tryptase have been shown to excite guinea-pig myenteric neurons through activation of PAR-1 and PAR-2, respectively. This may contribute to the abnormal neurotransmission and motility in the inflamed intestine (Corvera *et al.*, 1999). Steinhoff *et al.* (2000) have suggested a novel process of protease-induced neurogenic inflammation, whereby trypsin and tryptase, and PAR2-AP directly signal the release of the neuropeptides CGRP and substance P by activation of PAR-2, thereby mediating inflammatory oedema. More recently, it has been shown that activation of PAR-2 on peripheral neurones of the rat is pro-nociceptive, producing thermal hyperalgesia and triggering the pain sensation (Kawabata *et al.*, 2001).

It is evident then that PAR-2 is involved in a variety of inflammatory process. Thus, in light of this evidence the receptor may well contribute to the pathological processes involved in the diseased airways. This will be discussed in more detail below.

1.5.6 PAR-2 in the Airways

PAR-2 is abundantly expressed on airway epithelium and smooth muscle (D'Andrea *et al.*, 1998; Cocks *et al.*, 1999). There is evidence that PAR-2 activating peptides can initiate a PGE_2-dependent protection of airway function by preventing contraction of airway smooth muscle cells (Cocks *et al.*, 1999), by activation of epithelial PAR-2. Peptide agonists of PAR-1, PAR-2 and PAR-4 induced relaxant responses in mouse isolated tracheal

smooth muscle preparations (Lan *et al.*, 2000) by a mechanism found to be mediated by PGE_2 generated by COX-2 (Lan *et al.*, 2001). However, intravenous injection of PAR-2 activating peptides significantly inhibited the histamine-induced increase in lung resistance in guinea-pigs, by a mechanism that was independent of prostanoid or nitric oxide release (Cicala *et al.*, 2001). Moreover, Chow *et al.* (2001) showed that PAR-2 agonist peptides caused epithelium dependent-relaxations in the rat isolated airways. This supports the theory of a role for PAR-2 in preserving airway function lung disease by preventing bronchoconstriction.

Although some evidence suggests that PAR-2 activation may have anti-inflammatory consequences by preserving airway function, evidence also exists for the pro-inflammatory involvement of PAR-2. Activation of PAR-2 by Intratracheal instillation or intravenous injection of PAR-2 agonists in guinea-pig airways causes bronchoconstriction and other motor effects, partly mediated by a neural mechanism (Ricciardolo *et al.*, 2000). PAR-2 is expressed localised to mast cells (both the surface and on the membrane of secretory granules), and therefore activation of PAR-2 on mast cells by tryptase secreted from their granules may act as a positive feedback mechanism to stimulate further degranulation (D'Andrea *et al.*, 1999). Akers *et al.* (2000) stimulated the proliferation of human lung fibroblasts by mast cell tryptases via activation of PAR-2, but skin fibroblasts were unaffected. Matrix metalloproteinase-9 (MMP-9), a protease which is important in tissue remodelling and repair, was released

by human airway epithelial cells in response to stimulation by PAR-2 activating peptides (Vliagoftis *et al.*, 2000), suggesting that PAR-2 may be involved in tissue repair and remodelling. However, caution is needed when considering the precise roles of PAR-2 in allergic airways diseases, since PAR-2 has both pro-and anti-inflammatory roles in inflammatory disease. Therefore determining the regulation of this receptor's expression in airways disease may increase our understanding of the role of PAR-2 in these processes.

1.6 Eosinophils

Eosinophils have been recognised to play a major role in allergic diseases, such as bronchial asthma and atopic dermatitis (Kita, 1997). Moreover, eosinophils are a major cell constituent during the late reaction to allergen, and are present in proportion to the allergen dose (Sedgwick *et al*, 1991). However, the specific mechanisms by which eosinophils participate in, and contribute to, the airway obstruction, inflammation and hyperresponsiveness that occur in asthma and allergic disease are still to be determined.

1.6.1 Origins and Maturation

Eosinophils originate from bone marrow precursor cells, and following differentiation, mature eosinophils leave the marrow and circulates in the blood, before migration into tissues (Kroegel *et al.*, 1994). Eosinophils can

be distinguished from other cell types by their morphology; they have a bilobed nucleus and large intracellular granules (Miller *et al.*, 1966). Three types of granules have been described: primary, secondary and small granules (Seminario *et al.*, 1998). These granules contain a number of enzymes and basic proteins, some of which are cytotoxic (Kroegel *et al.*, 1994).

1.6.2 Eosinophil Granule Proteins

Four cationic proteins have been recognised within the secondary granules, which are released upon degranulation (Gleich and Adolphson, 1986): major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP). All four are capable of causing local tissue damage, and thus contributing to the pathogenesis of local inflammation (Seminario *et al.*, 1998). MBP promotes platelet aggregation (Rohrbach, 1990), and activates neutrophils (Moy, 1990). MBP is also toxic to many cell types, including human tracheal cells (Gleich *et al.*, 1979) and epithelial cells (Frigas *et al.*, 1980). Consistent with this, MBP can be detected in the sputum of asthma patients and has been has been localised to sites of epithelial injury by immunofluorescence (Filley *et al.*, 1982). ECP and EPO are also cytotoxic, with ECP forming pores in target cell membranes, causing cell damage (Young *et al.*, 1986), and EPO in combination with

hydrogen peroxide and halide, preferably bromide, kills mast cells with resultant release of granular contents (Seminario *et al.*, 1998).

1.6.3 Eosinophils and Allergic Airways Disease

Increased numbers of eosinophils have been reported in nasal lavage fluid (Lim *et al*, 1995) and biopsy tissue (Bradding *et al*, 1995) collected from subjects with perennial allergic rhinitis in comparison to healthy controls. In addition, nasal biopsy studies of symptomatic seasonal rhinitic subjects have identified increased eosinophil numbers both in the submucosa and in the epithelium (Okuda *et al*, 1983; Bentley *et al*, 1992). Furthermore, an increase is also observed in nasal lavage fluid collected from seasonal allergic rhinitics during the pollen season (Pipkorn *et al*, 1988).

Eosinophils produce mediators capable of producing many of the pathological features of asthma, and are present in large numbers in the airways of patients with asthma (Reed, 1994; Virchow *et al.*, 1994). MBP has been identified in the airways of patients who have died from asthma (Filley *et al.*, 1982). In addition, MBP and EPO have been shown to cause airway constriction and hyperresponsiveness (Gundel *et al.*, 1991), and degranulation of mast cells and eosinophils (Kumar and Busse, 1998); thus replicating some of the key features of asthma. Activated eosinophils secrete newly formed lipid mediators, including leukotriene C_4 (LTC_4) (Weller *et al.*, 1983), which causes smooth muscle contraction, bronchoconstriction and increased microvascular permeability (Kroegel *et*

al., 1994; Silberstein *et al.*, 1995). Moreover mast cell tryptase, when injected into the skin of guinea pigs, promotes the accumulation of eosinophils to the site, which appear to be in an activated state (He *et al*, 1997). Tryptase may well have similar effects on eosinophil recruitment in the airways.

Eosinophil activation in the airways may increase the degree of inflammation since they generate a number of cytokines, including interleukin-5 (IL-5), TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF) (Moqbel *et al.*, 1994). Some of these are involved in eosinophil recruitment and survival with the potential for positive feedback mechanisms to operate (Ebisawa *et al.*, 1994). IL-5 is present in significantly higher amounts in the airways of patients with symptomatic asthma, compared with asymptomatic asthma (Sur *et al.*, 1995).

Thus, there is the potential for eosinophils to play an important role in inflammatory processes in asthma and rhinitis. However, the means by which eosinophils may interact with and be regulated by other cells of the immune system, such as mast cells, has been little explored. The potential for eosinophil behaviour to be regulated by mast cells may depend in part on their expression of PAR-2 as a means of responding to tryptase. The expression of PAR-2 has yet to be identified on eosinophils,

but if this receptor is expressed then it could provide a mechanism for their regulation in allergic airways disease.

1.7 AIMS

The objectives of the study were to:

1. Prepare monoclonal and polyclonal antibodies specific for human PAR-2 and validate them as tools for the investigation of this receptor.
2. Examine expression of PAR-2 in the airways of asthmatic and rhinitic subjects.
3. Investigate the potential proinflammatory actions of tryptase and agonists of PAR-2 on eosinophil function.

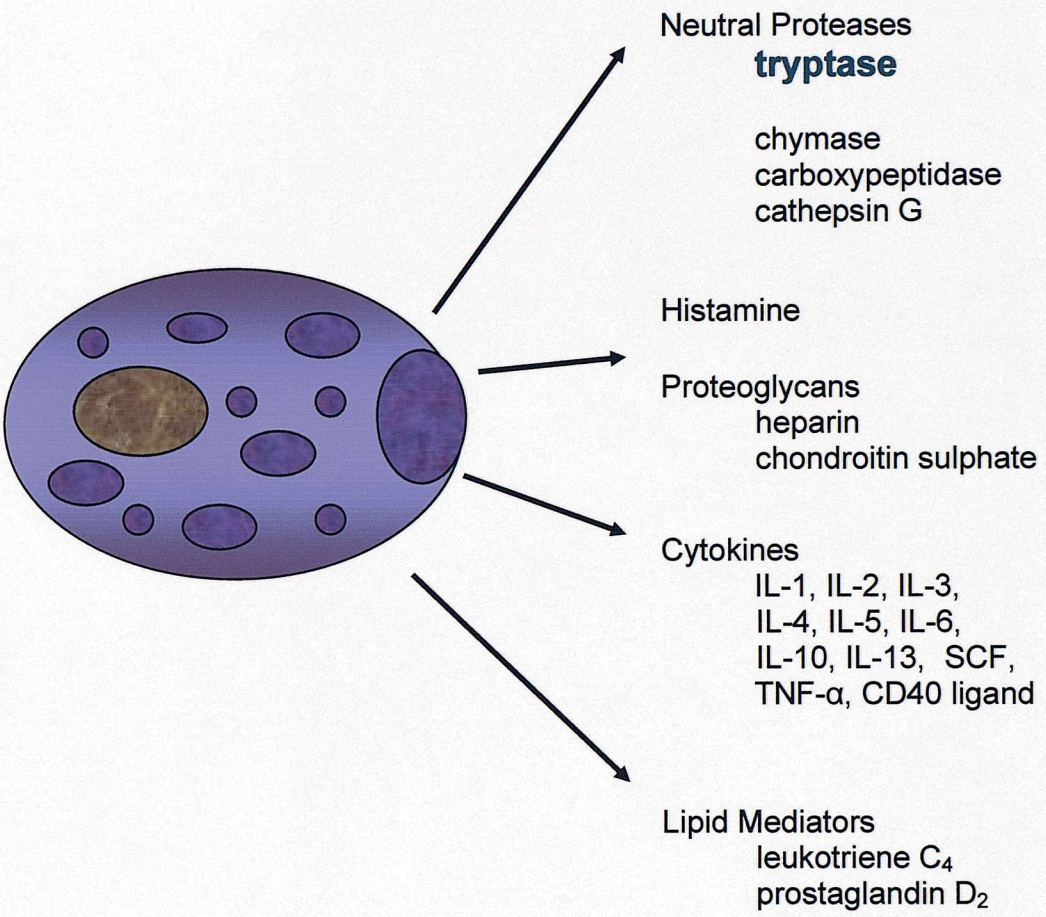


Figure 1-1 Mediators release by mast cells.

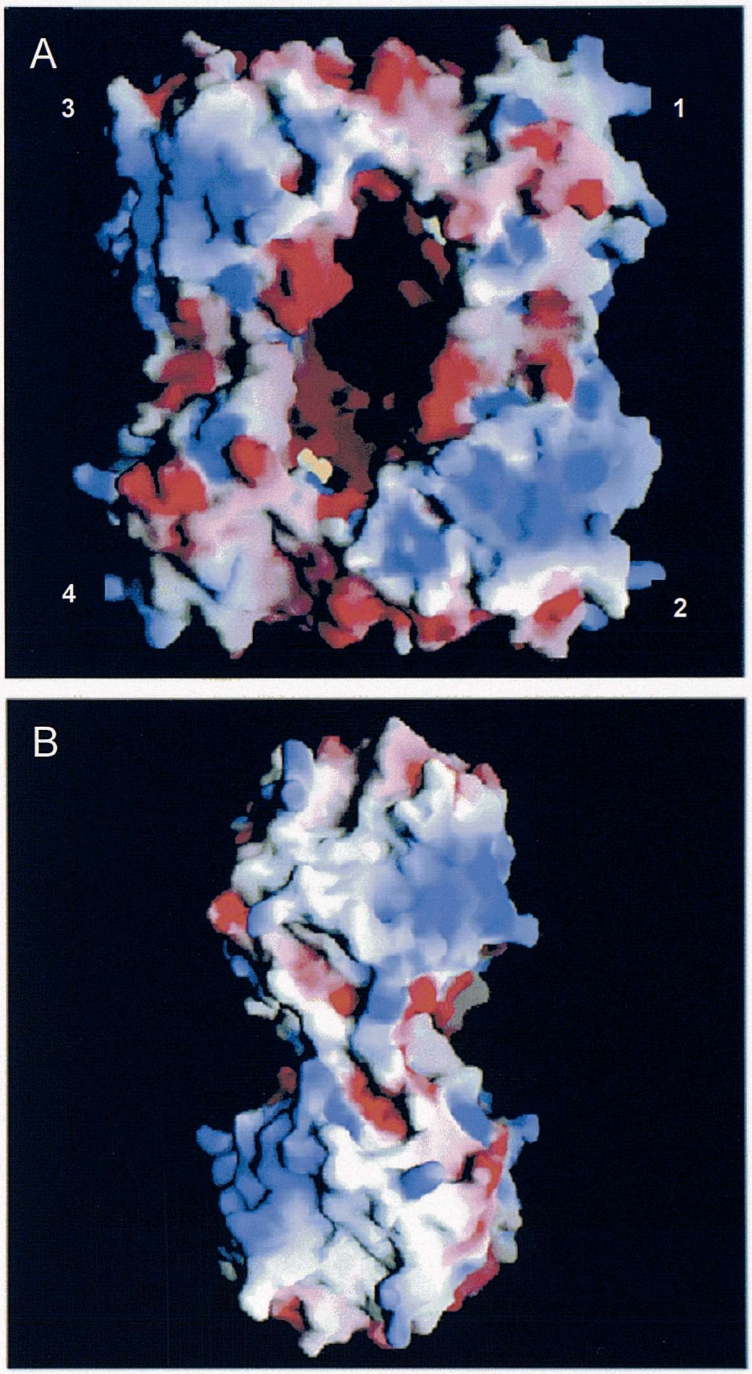


Figure 1-2 Solid surface representation of the tryptase tetramer. The colours indicate positive (blue) and negative (red) electrostatic potential at the molecular surface. A, front view. The four monomers (labelled 1 to 4) are arranged at the corners of a flat square, with a central pore. B, edge view towards the 3-4 dimer. The tetramer has been rotated around a vertical axis by 90° compared with A. Adapted from Pereira *et al*, 1998.

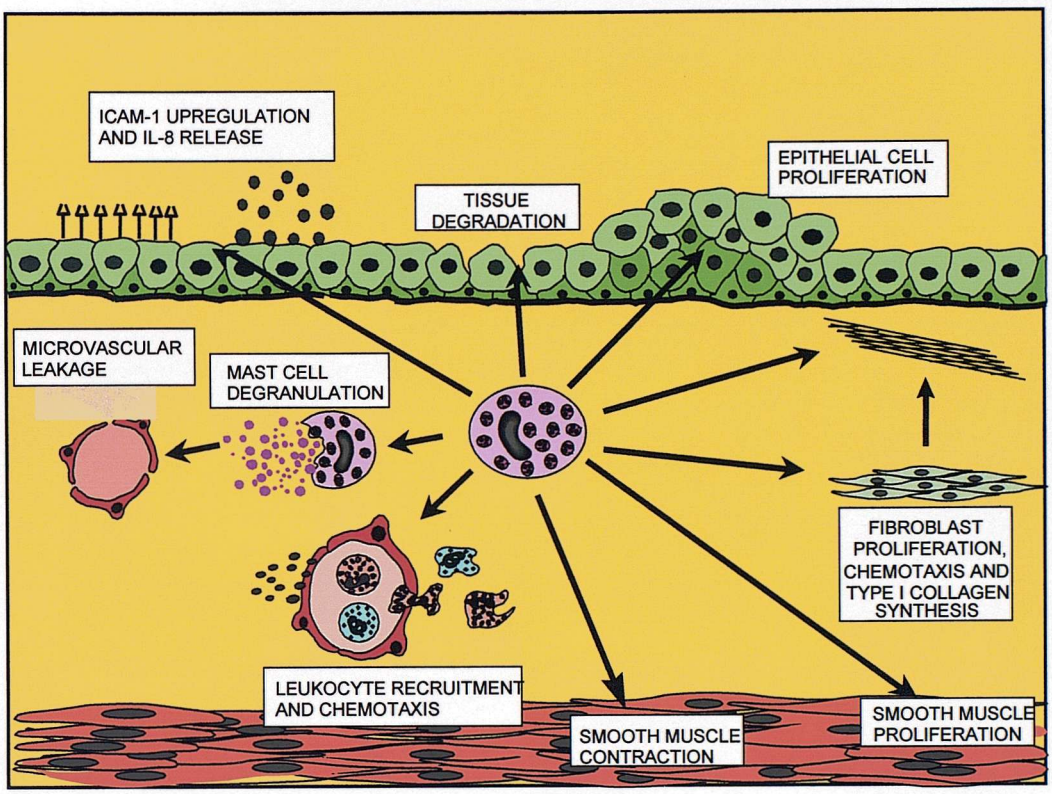


Figure 1-3 Summary of the actions of tryptase on cellular function.

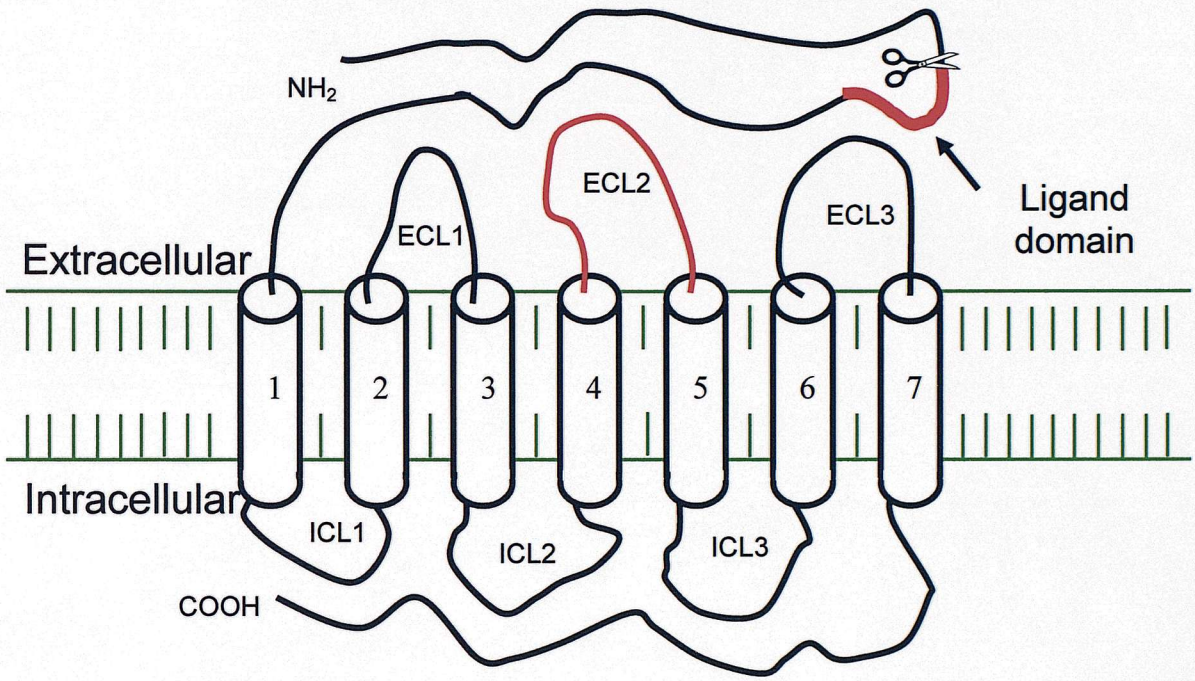


Figure 1-4 Diagrammatic representation of the PAR-2 receptor showing the 7 transmembrane domains, and the intracellular (ICL) and extracellular (ECL) loops. The proteolytic cleavage site is also shown (✂).

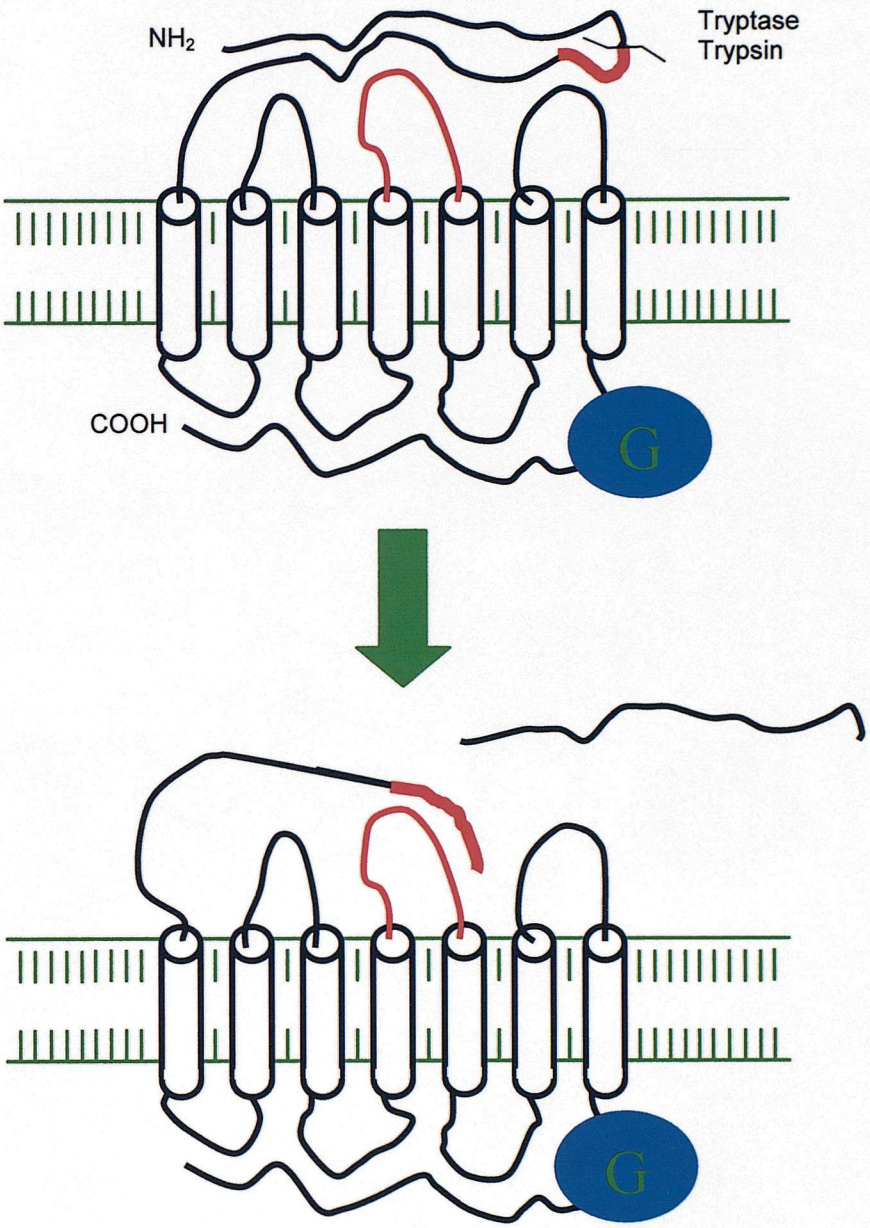


Figure 1-5 Mechanism of activation of PAR-2 by proteolytic cleavage. The G-protein (G) is shown.

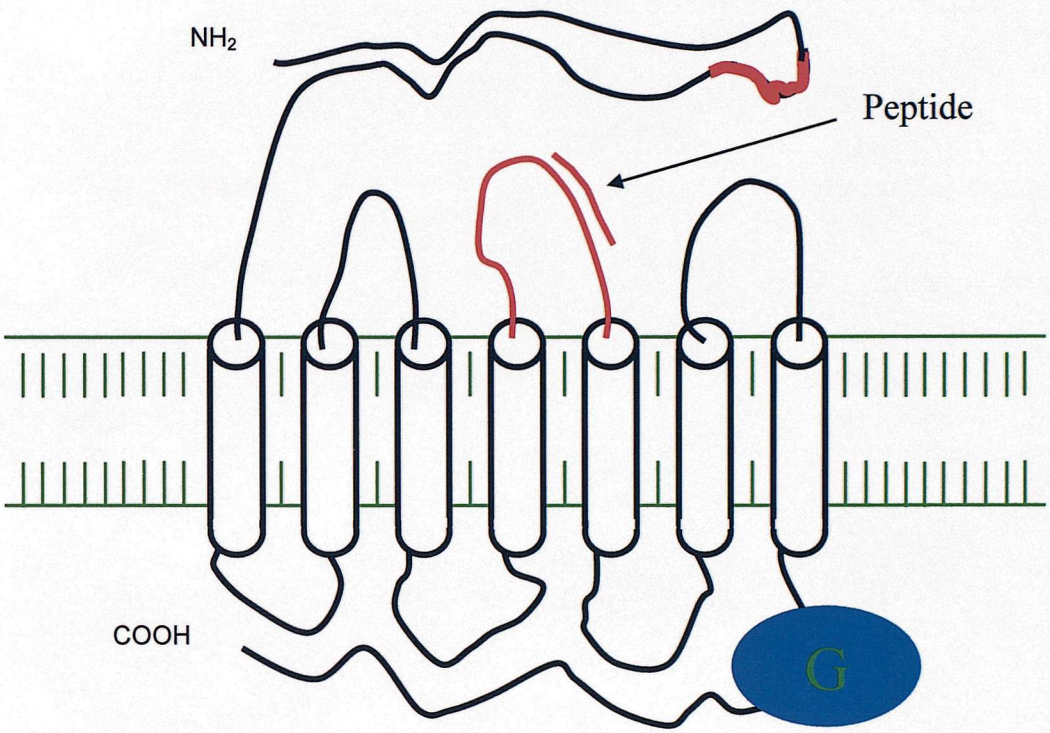


Figure 1-6 Mechanism of activation of PAR-2 by agonist peptides.

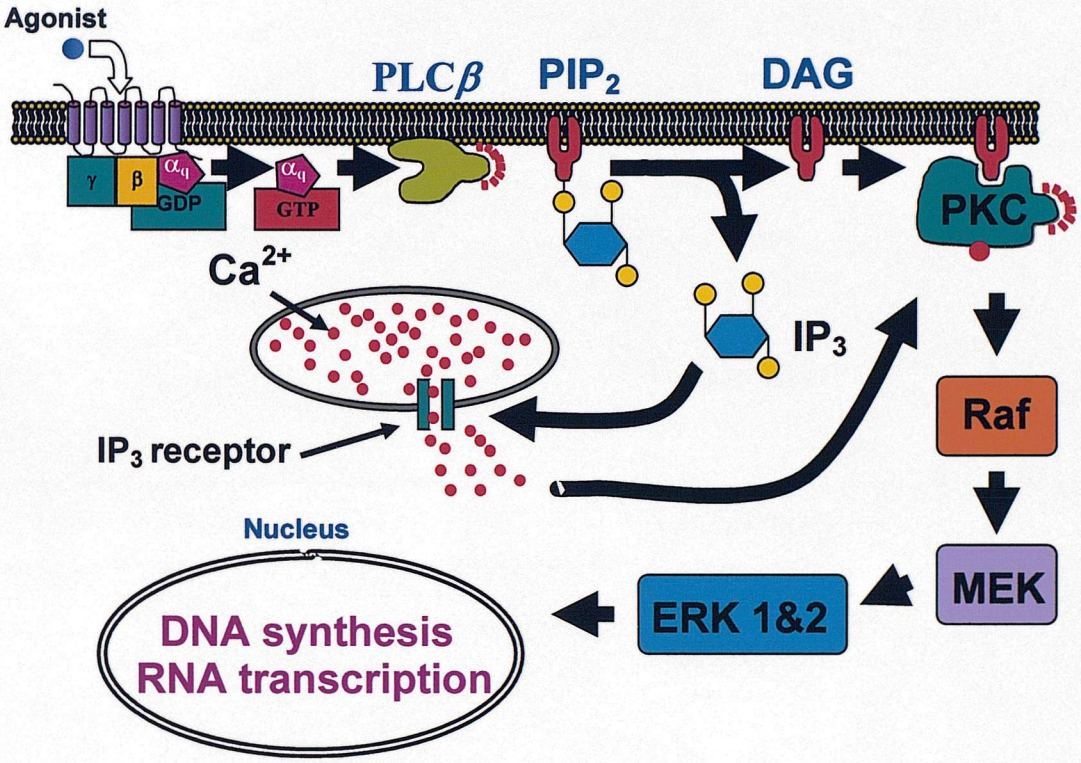


Figure 1-7 Intracellular signalling pathways for activated PAR-2.

DAG – diacylglycerol

ERK – extracellular signal-regulated kinase

IP_3 – inositol trisphosphate

MEK – mitogen activated protease kinase kinase

PIP_2 – phosphatidylinositol-4, 5-bisphosphate

PKC – protein kinase C

PLC – phospholipase C

	PAR-1		PAR-2		PAR-3		PAR-4	
	Human	***Rat***	***Human***	*Rat*	*Human*	*Rat*	*Human*	*Rat*
Major activating protease	Thrombin	Thrombin	Trypsin, tryptase	Trypsin, tryptase	Thrombin	Thrombin	Thrombin	Thrombin, Trypsin Cathepsin G
Tethered ligand	SFLLRN	SFFLRN	SLIGKV	SLIGRL	TFRGAP	ND	GYPGQV	ND
Selective agonist peptides	TFLLR	TFLLR	SLIGKV, SLIGRL	SLIGRL, SLIGKV	ND*	ND	GYPGQV, AYPGKV	GYPGKV, AYPGKV

Abbreviation: ND, not determined

* Application of TFRGAP peptide does not cause PAR-2 activation.

Table 1-1 Protease and peptide agonists for the four known protease activated receptors (PARs).

CHAPTER 2

Materials & Methods

2 Materials & Methods

2.1 Materials

Dulbecco's modified Eagles medium (DMEM), foetal calf serum (FCS), penicillin, streptomycin, glutamine, Hybridoma Enhancing Supplement (HES), geneticin and non-enzymatic cell dissociation solution were obtained from Invitrogen (Paisley, UK); heparin-agarose sepharose resin, Sephacryl S-200 resin, N-α-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride (BAPNA), benzamidine, leupeptin, trypsin (bovine and porcine), bovine serum albumin (BSA), dimethylsulphoxide (DMSO), complete and incomplete Freund's adjuvant, 3-amino-9-ethylcarbazole (AEC), Percoll and antibodies to mouse or rabbit immunoglobulins conjugated to FITC (fluoroscein isothiocyanate) were from Sigma (Poole, Dorset, UK); Amicon filters, ultrafiltration devices with a nominal 30 kDa cut-off, were from Millipore Bioscience (Watford, Herts., UK); Anti-CD16 microbeads and magnetic activated cell sorting (MACS) "C" columns were from Miltenyi Biotech (Surrey, UK); 3,3' diaminobenzadine and AEC staining kits were obtained from BioGenex (Berks., UK); glycol methacrylate (GMA) embedding kit was from Park Scientific (Northampton, UK); acetone and methyl benzoate were from Merck Ltd. (Lutterworth, UK); the peptides SLIGKV-NH_2, VKGILS-NH_2 and LSIGKV-NH_2 were obtained from SigmaGenosys (Cambridge, UK); the peptides SLIGRL-NH_2, *trans*-cinnamoyl-LIGRL-propylornithine (tc-LIGRLO) and tc-

LRGILO were obtained from the University of Calgary peptide synthesis facility (Calgary, Alberta, Canada); keyhole limpet haemocyanin (KLH) and cytotoxicity lactate dehydrogenase (LDH) assay kit were obtained from Pierce (IL, USA); Crystal Mount was obtained from Biomeda Biogenesis (Poole, UK); silver stain and Coomassie protein assay reagents were from Bio Rad Laboratories (Herts., UK). Synthetic clotting reagents were from Alpha Laboratories (Eastleigh, Hants., UK)

2.2 Cell Culture

2.2.1 Cells

The Kirsten sarcoma-virus transfected normal rat kidney epithelial (KNRK) cell line and KNRK cells transfected with human PAR-2 ($KNRK_t$) were a kind gift from Dr Steven Compton (University of Calgary, Alberta, Canada). $KNRK_t$ cells, permanent cell lines expressing high levels of the human PAR-2 receptor (Compton *et al*, 2000), were isolated by fluorescence-activated cell sorting using the B5 anti-PAR-2 polyclonal rabbit antiserum, raised against a peptide fragment of rat PAR-2 (Kong *et al.*, 1997). KNRK cells express very low levels of rat PAR-2, which are unlikely to interfere with our measurements (Kong *et al.*, 1997). Therefore, the $KNRK_t$ or KNRK system provided a means of studying PAR-2 function. Epithelial cells of the A549 line, derived from human alveolar carcinoma cells (Nardone & Andrews, 1979), were a gift from Dr.

SH Leir (University of Southampton, UK) and reported to express high levels of PAR-2 (Vliagoftis *et al.*, 2000).

2.2.2 Cell culture

$KNRK_t$ cells were grown in DMEM supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 600 μg/ml geneticin (to maintain positive selection pressure on the cells expressing the antibiotic resistance gene). KNRK cells were grown in the same medium, in the absence of geneticin. Both cell types were grown at a temperature of 37 °C and a humidified atmosphere of 95% air and 5% CO_2, passaged every three to four days.

A549 cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at a temperature of 37 °C and humidified atmosphere of 95% air and 5% CO_2. The cells were routinely passaged every three to four days.

2.3 Antibody Production

2.3.1 Monoclonal Antibody Production

Various strategies were employed in the attempt to produce a mouse monoclonal anti-PAR-2 antibody.

Two peptide sequences (greater than 75 % purity; SigmaGenosys, Cambridge, UK), termed peptide A and peptide B, were used as immunogens. Peptide A had a sequence corresponding to residues 37-52 of human PAR-2 within the tethered ligand, $\downarrow^{37}$SLIGKVDGTSHVTGK^{52}GC ($\downarrow$ denotes proteolytic cleavage site). A cysteine residue was added to allow coupling to a carrier protein. In theory, an antibody generated to this sequence should recognise both the cleaved and uncleaved forms of the receptor molecule. A second sequence, corresponding to residues 29-42 of human PAR-2 (greater than 75 % purity; ^{29}TNRSSKGR$\downarrow$SLIGK^{42}VC or peptide B) was selected as it was considered that an antibody produced to this sequence could recognise uncleaved molecules (as residues 1-36 are cleaved during proteolytic activation of the receptor). Also, antibodies directed against this sequence might prevent cleavage of the tethered ligand and signal transduction. Both peptide sequences were used together to improve the chances of obtaining antibodies to PAR-2. Figure 2-1 illustrates the part of the receptor molecule from which the peptides were selected. The peptides were designed to produce antibodies that would specifically bind to human PAR-2 and not mouse or rat PAR-2 or other PARs. Figure 2-2 illustrates the comparison of the peptide sequence of human PAR-2 with that of the corresponding rat or mouse sequences. The peptide sequences selected had very low homology with the corresponding sequences of PAR 1, 3 and 4 (not shown). Using the Blast database (http://www.ncbi.nlm.nih.gov/BLAST/), searches were

performed using the peptide sequences (peptides A-C), all three peptides produced hits showing 100 % identity with the human PAR-2 full protein sequence (Fig. 2-2). Epitope accessibility for antibody recognition was not established since the crystal structure for PAR-2 was unavailable and is as yet unknown.

The peptides were coupled to Imject® maleimide activated mariculture-keyhole limpet haemocyanin (mcKLH), according to the manufacturer's guidelines (Pierce, IL, USA). KLH is the most widely used carrier protein due to its large molecular mass, strong immunogenicity and many available lysines. Pierce mcKLH is isolated from the hemolymph of Giant Keyhole Limpets, which are not sacrificed during collection and therefore supply samples over an extended period of time. This differs from the previous methods to collect KLH, where limpets were harvested and then destroyed. The maleimide-activated mcKLH was used since the sulphydryl-reactive maleimide chemical group on the cross-linker is more resistant to hydrolysis than those of other similar cross-linkers, thereby allowing better conjugation of the carrier to the peptide. Two milligrams of maleimide-activated mcKLH (dissolved in distilled water to make a 10 mg/ml stock solution) were added to 2 mg of peptide (dissolved in PBS). The mixture was allowed to react for 2 hr and then dialysed against PBS for a further 2 hr to remove unconjugated peptide.

The immunogen was mixed repeatedly with complete Freund's adjuvant (containing heat-killed mycobaterium), using two Hamilton glass syringes connected by a plastic female to female luer®-lok connector with a small orifice (Fisher Scientific, Loughborough, Leics., UK), to form a stable emulsion. The emulsion was considered ready for use when a single drop did not disperse on the surface of water. Antigen was administered as an emulsion because these improve antibody generation by allowing a slow release of antigen from a depot, and thus provide continuous stimulation to the immune system. BALB/c mice (4-8 weeks old) were given subcutaneous injections, two sites in the back, with approximately 25 μg of each peptide per site. Three weeks later the same quantities of peptides were emulsified with incomplete Freund's adjuvant (lacking the mycobacterium) and injected, as before. Booster injections of emulsions in incomplete Freund's adjuvant were given up to four times. The final boost was administered in saline by intravenous injection (via the tail vein) of the usual amounts of peptides. Three to four days later the mouse was sacrificed by exposure to CO_2. The spleen was removed aseptically, and a single cell suspension prepared by dispersal of the tissue using a syringe plunger and sterile filter. The splenocytes were fused with the NS-1 myeloma cells by the slow addition of 48 % polyethylene glycol (PEG) solution in DMEM medium to promote membrane fusion. Fused cells were cultured in DMEM medium containing HAT (a mixture of hypoxanthine, aminopterin and thymidine). HAT medium was used to

promote the survival of thymocyte-myeloma hybridoma, since NS-1 cells have a metabolic defect in the presence of aminopterin, which inhibits the synthesis of certain nucleotides, rendering them unable to utilise the medium, B cells die in the absence of antigenic stimulation. Hybridomas retain the producing capacity of the B lymphocytes prior to fusion. Hybridomas were fed with fresh HAT medium every 2-3 days. Wells containing growing colonies were screened for the production of antibodies to PAR-2 by enzyme linked immunosorbent assay (ELISA) or by immunohistochemistry (IHC). After a positive result from the screening, hybridomas were cloned as soon as possible to prevent cells not producing antibodies to PAR-2 from overgrowing the productive ones. Hybridomas were seeded at 5, 1 and 0.3 cells per well and grown in 10% Hybridoma Enhancing Supplement (HES) conditioned medium from thymoma cells. Numbers of colonies growing within a single well were quantified to give an indication of the number of hybridomas to be screened further. Where possible, the supernatants of single colonies were cloned to minimise the risk of multiple clones in the same culture. The procedure is summarised in Fig. 2-3.

Three mice were injected and as described. Wells containing growing cells were tested for the production of anti-PAR-2 antibodies by ELISA (section 2.3.6). For each hybridoma culture plate, three plates were coated with antigen (500 ng). One with peptide alone, one with peptide

coupled to mcKLH (peptide-KLH) and one with mcKLH alone. In theory, any PAR-2 antibodies would detect the first two of these ELISA procedures, but not the third. However, it appeared that there were many wells positive in both the peptide-KLH and mcKLH ELISA plates. Hybridomas with peptide-KLH/mcKLH absorbance ratios of greater than 2.2 were selected for further screening by immunocytochemistry (section 2.3.7) since many wells were positive in both the peptide-KLH and KLH-alone plates with optical density ratios between 1.8 to 2.8.

A different approach involved immunizing mice with A549 lung alveolar epithelial cells (cells that express high levels of PAR-2) mixed with complete Freund's adjuvant. Hence mice would produce antibodies to the A549 cells, and there would be a chance of generating antibodies directed to human PAR-2, since the A549 cells express appreciable amounts of this receptor. The hybridomas were tested by immunocytochemistry (section 2.3.7). Another mouse was killed since the first attempt was unsuccessful, and the screening method adapted from section 2.3.7. This time lung tissue containing epithelial cells expressing PAR-2 were used in conjunction with the $KNRK_t$ (PAR-2 expressing) and KNRK (no PAR-2 expression) cell system.

The approach that eventually proved successful involved injecting with a combination of both peptides A and B, described above, and screening

hybridoma cells with ELISA procedures for the peptides coupled to BSA (section 2.3.6) and also for BSA alone. Hybridomas secreting antibody reacting with the peptide but not to BSA were cloned and subcloned as described in earlier in this section, to obtain a single population of cells secreting monoclonal antibodies.

2.3.2 Purification of monoclonal antibodies

Immunoglobulins in hybridoma culture supernatants were precipitated with 45 % saturated ammonium sulphate (278 mg/ml) at 4 °C for 24 h, to provide partial purification the antibodies. The precipitate was ultracentrifuged at 29, 000 *g* for 40 min and dialysed against PBS (pH 7.4) at 4 °C using 12, 000 kDa molecular weight cut-off (mwco) dialysis membrane (Sigma). Dialysis was repeated after 2 h against fresh PBS. Five millilitres of chromatography resin consisting of Protein-L cross-linked to 4 % agarose beads (Sigma) were packed into a glass scintillation column with a glass fibre filter at the bottom. The column was washed with 30 ml PBS at a flow rate of 0.75 ml/min. The dialysed antibody solution was loaded onto the column and washed through with 20 ml PBS. Immunoglobulins were eluted with 0.1 M glycine, pH 2.7 into fifteen fractions of one milliliter into tubes containing 100 µl of 2 M Tris buffer, pH 8, to neutralise the pH and limit denaturation of antibodies under acidic conditions. The fractions eluted were assayed for protein content using the BCA protein assay (see section 2.3.5).

2.3.3 Polyclonal Antibody Production

Peptide B was selected for injection into rabbits (K and L), as part of an attempt to prepare an antibody that could bind to the uncleaved form of PAR-2, and which could block PAR-2 activation. Two rabbits were injected with peptide B (section 2.3.1) that had been coupled to mcKLH.

Peptide C (greater than 75 %; VKQTIFIPALNITTCHDVLPEQL; SigmaGenosys, Cambridge, UK) was injected into two additional rabbits (M and N). This peptide sequence corresponds to residues 213 to 235 of human PAR-2 which are found within the second extracellular loop sequence (Figure 2-1). Antibodies to this sequence could recognise uncleaved PAR-2, since once activated, the new amino-terminus binds within the second extracellular loop.

Two further rabbits (O and P) were injected with a recombinant 90 amino acid protein corresponding to the first extracellular domain sequence of rat PAR-2, a kind gift from Dr. Steven Compton (University of Calgary, Canada). This peptide, which will be hereafter referred to as the P90 peptide, was selected primarily to produce an antibody that would resemble PAR-2 *in vivo* more closely. It was hoped that this antibody could be used to block activation of PAR-2.

Female New Zealand White rabbits were immunised with peptides as described above. On each occasion, 1ml immunogen was shared between four subcutaneous injections on the rabbit's back. The first immunisation was prepared in complete Freund's adjuvant, whereas incomplete Freund's adjuvant was used for boosts. Approximately three weeks later, blood was taken and the rabbit serum tested by ELISA (see below). The rabbits were immunised up to six times, and sera collected and tested after immunizations.

2.3.4 Purification of Polyclonal Antibodies

Polyclonal rabbit antibodies were purified in a similar manner to the monoclonal antibodies (section 2.3.2). However, protein A-agarose resin was used in the place of protein L resin.

2.3.5 Bicichoninic acid (BCA) protein assay

The BCA reagent was prepared by mixing 1ml of 4 % $CuSO_4$ with 50 ml BCA solution (Sigma). Ten microlitres of sample were added to 200 µl of BCA reagent in a 96 well plate, and the plate was then incubated at 37 °C for 30 to 60 min. The absorbance was read at 550 nm using a microtitre plate reader. The values were compared to a standard curve prepared using bovine gamma globulins, diluted between 25 µg/ml and 400 µg/ml using a mixture of elution and neutralisation buffers used in antibody purification.

2.3.6 Screening for PAR-2 Antibodies by ELISA

The monoclonal antibodies generated were tested in ELISA for the detection of PAR-2, peptides A and B and the P90 peptide. The isotype control antibody used was mouse IgM, since the monoclonal antibodies generated were all of the IgM class.

Polyclonal antisera were tested in ELISA procedures for the detection of peptides B and C, and the P90 peptide. Serum samples collected from the rabbits prior to immunisation and IgG from rabbits not immunised with PAR-2 peptides were used as negative controls. The six rabbits were boosted between 3 and 5 times (as described in 2.3.3) with bleeds taken at appropriate intervals after boosting (normally 3 to 5 weeks). Serum from each bleed was tested in the ELISA system to determine if the antibody titre had improved.

Nunc Maxisorp high protein binding ELISA microtitre 96-well plates (Fisher Scientific) were coated with the peptide used for immunising the mice or rabbits, overnight at 4 °C. However, the peptides were coupled to another carrier protein, to improve presentation of the peptide, and thus increase the sensitivity of the test. Wells were washed four times with wash buffer (PBS supplemented with 0.05% Tween 20) and non-specific binding was blocked by incubating the plates with 3% BSA in wash buffer for 30 min.

After further washes, diluted rabbit antiserum was applied for 90 min. Wells were washed, and a biotin conjugated secondary antibody against rabbit immunoglobulins was added for 60 min. Wells were washed, and Extravidin® peroxidase (a proprietary form of streptavidin peroxidase) applied for 30 min. All incubations were performed at room temperature. Colour was developed using 0.33 mg/ml *o*-phenyl diamine (OPD–a peroxidase substrate) in citrate-phosphate buffer, pH 5.5 supplemented with 0.01 % H_2O_2. The reaction was stopped by the addition of 3 M H_2SO_4, and absorbance values were read at 490nm using a plate reader.

2.3.7 Screening for PAR-2 Antibodies by Immunocytochemistry

A549, $KNRK_t$ and KNRK cells were embedded in synthetic fibrin clots (Sigma, Poole, Dorset) fixed in 85 % ethanol, dehydrated and processed into paraffin wax. Clots were cut into pieces and embedded, such that each block contained each of the three cell type. Sections were cut and collected on poly-L-lysine coated slides. Sections were dewaxed using Histoclear (Raymond Lamb, London, UK; histological cleaning agent) and rehydrated through ethanol solutions (100%, 100%, 90% and 70% for 5 min each), and distilled water for 5 min. Endogenous peroxidase activity was blocked was blocked using 0.1% sodium azide and 0.5% hydrogen peroxide in methanol for 10 min. Non-specific protein binding sites were blocked by application of 5% BSA in PBS for 30 min. Culture supernatants were added to the sections for 2 hr at room temperature,

followed by washes through each of three tris-tween wash buffers (high salt, low salt and no salt) for 5 min, and finally tap water for 5 min. Biotinylated antibodies to mouse immunoglobulins were diluted in 1% BSA (in PBS) and applied for 60 min at room temperature. Slides were washed as before, and then Extravidin®-peroxidase applied for 30 min in PBS supplemented with 1% BSA. Staining was developed using 3-amino-9-ethylcarbazole (AEC) substrate in acetate buffer pH 5.0, and sections were counterstained with Mayer's haemulum (BDH, Poole, Dorset).

2.3.8 Characterisation of Antibodies by Flow Cytometry

The different antibodies were characterised by their ability to bind to a transfected cell line expressing human PAR-2 ($KNRK_t$) compared to cells not expressing human PAR-2 (KNRK), using FACS (section 2.4). Mouse IgM and pre-immune rabbit serum were used as control antibodies. In addition, the antibodies were compared to a rabbit anti-rat PAR-2 antiserum (B5) kindly provided by Prof. Morley Hollenberg (University of Calgary, Canada; Kong *et al*, 1997). The experiments were repeated with each antibody after stimulation of the cells with 12 nM trypsin in the presence or absence of cytochalasin B (10 μg/ml) or the metabolic inhibitors, antimycin A (1 μM) and 2-deoxyglucose (10 mM) added in combination. Cytochalasin B, which prevents receptor internalisation, was added 5 min before the addition of trypsin, and the metabolic inhibitors

were incubated with the cells for 45 min in a 37 °C water bath, prior to stimulation.

2.3.8 Characterisation by Western Blotting

Proteins were separated on gels as described in section 2.6, and then transferred to nitrocellulose membranes (section 2.7). Immunolabelling of the blots for PAR-2 was performed after non-specific protein binding sites were blocked with 5 % milk powder for 45 min at room temperature with gentle orbital mixing. The blot was then washed 6 times for 2 min each with time with PBS-Tween (PBS supplemented with 0.1 % Tween 20). Monoclonal antibody P2A or mouse IgM raised against an irrelevant antigen was diluted (1/100 to 1/400) with 1 % BSA (in PBS) and applied for 1 hr at room temperature. After washing, a 1/20,000 to 1/50,000 dilution of anti-mouse IgM conjugated to horse-radish peroxidase (HRP; Sigma) was applied for 45 min. The blot was subjected to six further washes, and West Pico chemiluminescence substrate was applied for 5 min. Chemiluminescence was detected by exposure of ECL-2 film to the blot for 30 to 60 sec and development using an automated X-ray development machine.

2.3.9 Characterisation of Antibodies by Immunocytochemistry

The antibodies were tested by immunocytochemistry in lung, bronchi, nasal polyp and colon tissues embedded in GMA. Mouse IgM and pre-

immune rabbit serum were used as isotype control antibodies for the monoclonal and polyclonal antibodies respectively. As an additional negative control for P2A (1/400 dilution) antibodies were pre-adsorbed with 10 μM peptide B, for 24 hr at 4 °C prior to staining.

2.3.10 Effects of Antibodies on PAR-2 Mediated Calcium Signalling

Section 2.5 contains detailed information on the methodology for calcium flux measurements. Calcium signalling in $KNRK_t$ or KNRK cells was used as a marker for G-protein coupled receptor signalling of PAR-2. The initial peak fluorescence in $[Ca^{2+}]_i$ relates to the release of calcium from intracellular stores. Concentration dependent calcium response curves were produced for the PAR-2 agonists, tc-LIGRLO-NH_2, SLIGRL-NH_2 and trypsin. Effects of the purified monoclonal and polyclonal antibodies on calcium signalling were examined by pre-incubating the cells with Fluo-3 AM and each individual antibody for 30 min followed by washing and then challenge and fluorescence measurements. Sections 2.3.2 and 2.3.4 describe the purification of the monoclonal and polyclonal antibodies, respectively. Adenosine triphosphate (ATP; 300 μM) was used as a stimulus of G-protein coupled receptor-mediated calcium signalling which acts independently of the PAR-2 receptor.

2.4 Flow Cytometry

Cells were detached with non-enzymatic EDTA cell dissociation solution, diluted to 10^6 cells per millilitre and washed twice with ice cold PBS and centrifuged (300 g for 5 min at 4 °C) each time. Cell pellets were resuspended in ice cold 1% paraformaldehyde fixative for 15 min. The cells were washed once with PBS and centrifuged once more. Cell pellets were resuspended in 0.1 M glycine (in PBS) with incubation on ice for 10 min, followed by two washes in PBS. The cells were incubated with permeabilising solution (0.5 % saponin, 0.1 % Tween 20, 10 % FCS, in PBS) for 45 min on ice, followed by two washes with wash buffer (0.2 % Tween 20, 2 % FCS, in PBS). Cell pellets were resuspended in an appropriate volume of ice cold wash buffer and 400 µl cell suspension added to each challenge tube. The primary antibody was diluted 100 µl and added to the cell suspension, which was incubated on ice for 40 min. Cells were washed three times with wash buffer and cells pellets were resuspended in 500 µl diluted fluorophore-labelled secondary antibodies and incubated for 40 min on ice in the dark. Cells were washed twice with wash buffer and once with PBS. Finally, cell pellets were either resuspended in 0.5 ml PBS for immediate FACS analysis or in 1 % paraformaldehyde for deferred analysis within 24 hr. Analysis was performed using the fluorescent activated cell scanning (FACScan) system (Beckton Dickinson, CA, USA), with the CellQuest software supplied.

2.5 Measurement of Intracellular Calcium Concentrations

Cells were loaded with a dye that fluoresces only in the presence of calcium. The cells were loaded with the cell permeant dye Fluo-3 acetoxymethyl ester (Fluo-3 AM). This dye is de-esterified by the cells, limiting its ability to leave the cells by passive diffusion across the cell membrane, but since it can be actively pumped out of the cells, sulphinpyrazone was added to inhibit the transporter. This process is summarised in Fig. 2-4. Cells were lifted from the flask using non-enzymatic cell dissociation and the cell washed twice by resuspension in cell culture medium (as described in section 2.2.2) and centrifugation at 300 g for 5 min at room temperature. Cells were resuspended in 3 ml complete medium containing 10 mM HEPES buffer and 250 μM sulphinpyrazone (Sigma), and then 30 μl of 2.5 mg/ml Fluo3-AM. The tube containing the cells was covered with foil and rocked on an orbital platform for 35 min. Cells were diluted with 20 ml HBSS (containing calcium) supplemented with 20 mM HEPES pH 7.2, 0.1 % BSA and 250 μM sulphinpyrazone (challenge buffer), and then centrifuged at 300 *g* for 5 min at room temperature. Cells were resuspended in challenge buffer to give a suspension of approximately 330,000 cells per ml. A three millilitre aliquot (1 x 10^6 cells) of the cell suspension was transferred to a disposable or quartz cuvette for challenge, keeping the remainder in the dark to prevent fluorescent decay of the Fluo-3. The cuvette was placed into the spectrofluorimeter, with gentle continuous mixing using a

magnetic stirring bar added to the bottom of the cuvette. The cuvette was illuminated at 488 nm and emission was monitored at 530 nm using a fluorescence spectrophotometer (Photon Technologies Inc., Oxford, UK). Once a steady baseline was established (2-5 min), secretagogues were added at intervals of at least 2 min to allow monitoring of responses. Maximum fluorescence values (F_{max}) were established by lysing the cells in 0.1 % Triton X-100 detergent and minimum values in the absence of calcium (F_{min}) by the addition of 100 mM EDTA. Calcium concentrations were determined using the equation:

$$[Ca^{2+}] = K_D \times \left[\frac{F_x - F_{min}}{F_{max} - F_x} \right]$$

Where K_D is the calcium dissociation constant for fluorescent dyes and F_x the fluorescence of the cells in response to secretagogues. The K_D for Fluo3-AM has previously been shown to be 390 nM (Nair *et al.* 2002).

2.6 SDS-PAGE

Proteins were analysed by eletrophorectic separation on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels. Samples were prepared for loading on to the gels using Laemmli buffer containing 10 mM Tris-HCl (pH, 6.8), 2 % SDS, 10 % glycerol and 0.005 % bromophenol blue in water (Laemmli, 1970). Equal volumes of sample and buffer were mixed and heated to 100 °C for 5 min using a boiling

water bath. Ten microlitres of the diluted/treated sample were layered on to a 4 % polyacrylamide SDS stacking gel (0.125 M Tris-HCl, pH 6.8, 4 % acrylamide (with an acrylamide:bisacrylamide ratio of 19:1), 0.1% SDS, 0.05 % ammonium persulphate (APS) and 10 µl TEMED solution). Proteins were resolved on 10 % polyacrylamide separating gels (0.375 M Tris-HCl, pH 8.8, 10% acrylamide, 0.1 % SDS, 0.05 % APS, 10 µl TEMED solution) by subjecting them to electrophoresis for 1 hr at 100 V.

2.7 Transfer of Proteins to Nitrocellulose Membranes

Unstained SDS-PAGE gels were sandwiched with nitrocellulose membrane (Bio Rad, Herts., UK) between Scotch-Brite filter pads in a hinged cassette, and inserted into a blotting apparatus, containing transfer buffer with 48 mM Tris (pH 8.3), 3.9 mM glycine, 0.0375 % SDS and 20 % methanol in water. Proteins were transferred over 90 min by electrophoresis at 30 V and 4 °C. Blots were probed for specific proteins using antibodies as described in section 2.3.8.

2.8 Staining of Tissues Embedded in GMA

2.8.1 Processing of Tissue into GMA

Biopsy tissues (approximately 2 mm^3) were placed in ice-cold acetone containing protease inhibitors (2mM phenyl methyl sulphonyl fluoride (PMSF) and 20mM iodoacetamide) and fixed overnight at –20 °C. Protease inhibitors were washed out with acetone at room temperature for

15min, and then the biopsies were treated with methyl benzoate, to help infiltrate the tissue. Methyl benzoate was removed and the biopsies passed through three changes of glycol methacrylate (GMA) processing solution A, supplemented with 5 % methyl benzoate. Each incubation with GMA solution was conducted at 4 °C and lasted 2 hr. GMA embedding solution was prepared with benzoyl peroxide according to the manufacturer's instructions. Tissue specimens were embedded in flat-bottomed Taab capsules, placing the biopsy in the bottom and filling the capsule to the brim with resin and closing the lid tightly. The blocks were allowed to polymerise overnight at 4 °C. GMA embedded biopsies were trimmed with a razor, filed and stored dessicated at –20 °C until required for use.

2.8.2 Immunohistochemical Staining of Biopsies Embedded in GMA Resin

Sections were cut at a thickness of 2 μm using a Reichart Jung motorised microtome and floated out onto 0.5% ammonia solution for 1 to 1.5 min. The sections were collected onto poly-L-lysine coated slides and allowed to dry at room temperature for at least one hr, then wrapped in aluminium foil and stored at –20 °C for use within 2 weeks.

Endogenous peroxidase activity in the tissues was inhibited by application of a solution of 0.1 % sodium azide and 0.3 % hydrogen peroxide for 30 min. Sections were washed three times with Tris-buffered saline (TBS) between incubations. Slides were drained and non-specific protein binding sites blocked by applying blocking medium (containing 10 % FCS, 5 % BSA in RPMI 1640 culture medium). Diluted primary antibody was applied overnight, at 4 °C for polyclonal antibodies, and at room temperature for monoclonal antibodies. Biotinylated secondary antibodies were applied at appropriate dilutions for 2 hr at room temperature. Streptavidin biotin-peroxidase complexes were prepared in advance and were applied for 2 hr at room temperature. Substrate solutions containing DAB or AEC were prepared according to the manufacturer's instructions and applied to the sections for 10 or 20 min, respectively. After development of staining, the slides were rinsed with tris buffered saline (TBS) and washed in running tap water for 5 min. Sections were counterstained with Mayer's haematoxylin and blued in running tap water. Aqueous mountant, Crystal mount was applied and hardened by baking at 80 °C for 10 min. Slides were allowed to cool and coverslips were mounted using DPX.

2.9 Tryptase Preparation

2.9.1 Tryptase Purification

Tryptase was purified or extracted according to the method of Compton *et al*, 2000). Lung tissue (400 to 500g) was obtained after *post mortem*

examination of subjects, whose relatives given written consent for the removal of material for research purposes. The material was chopped finely, major blood vessels removed and the lung tissue washed with distilled water to remove excess blood. This material was then blended in an equal volume of low salt buffer consisting of 0.15 M NaCl, 20 mM 2-[*n*-morpholino]ethane-sulphonic acid (MES),1mM ethylenediaminetetraacetic acid (EDTA), pH 6.1 for 15 sec at low speed. The homogenate was centrifuged three times at 37,000 x g for 30 min at 4 °C to remove haemoglobin and other soluble material. After each extraction in low salt buffer, the precipitate was retained and the soluble material discarded. To solubilise tryptase and to dissociate the enzyme from proteoglycans, the homogenate was extracted three times using a high salt buffer consisting of 2M NaCl, 20mM MES, 1 mM EDTA, pH 6.1. The extract was ultracentrifuged as before and the soluble fractions retained. The supernatant fractions were pooled and filtered through paper filters with a pore size of 11 μm. The extract was dialysed against distilled water twice for 2 hr at 4 °C (total 4 hr) to reduce the salt concentration, dialysed against heparin-agarose column running buffer (0.4 M NaCl, 2mM $CaCl_2$, 10mM MES, pH 6.1) for 3 hr and then overnight at 4 °C. Insoluble material was removed from the extract using a series of Whatman paper and glass fibre filters (11 μm, 2.7 μm, 1.6 μm and 0.7 μm (Fisher Scientific). The filtered extract was applied to a heparin agarose column, and washed through with heparin agarose column running buffer. The

bound tryptase was eluted over a salt gradient of 0.4 M to 1.2 M NaCl in 10 mM MES, pH 6.1. Fractions containing tryptic activity (assessed by measuring the enzyme activity of tryptase as described in section 2.9.2), eluted at approximately 0.8 M NaCl, were pooled and diluted to 0.4 M NaCl by the addition of NaCl free buffer (2mM $CaCl_2$, 10mM MES, pH 6.1). The extract was then applied to a second heparin agarose column, to obtain greater resolution of protein in the extract. The fractions with tryptic activity, assayed by BAPNA cleavage, were pooled and concentrated to 1ml by ultrafiltration using an Amicon centrifugal concentrator with a YM-30 membrane (30 kDa cut off). Tryptase in the concentrated extract was separated from other proteins according to molecular weight using a Sephacryl S-200 gel exclusion chromatography resin. The partially purified tryptase was passed slowly through the Sephacryl column in high salt buffer (2 M NaCl, 10mM MES, pH 6.1) and the fractions collected over a period of 18 hr. Fractions with tryptic activity were pooled then concentrated, and finally diafiltered to a 1 M NaCl, 10 mM MES, pH 6.1. The procedure is summarised in Fig. 2-5.

2.9.2 Characterisation of Human Lung Tryptase by SDS-PAGE

The enzymatic activity of purified tryptase preparations was determined using the chromogenic substrate BAPNA, and expressed as units of activity (U). One unit is the amount of tryptase required to hydrolyse 1 μmol BAPNA substrate per minute at 25 °C. BAPNA was dissolved in

dimethyl sulphoxide (DMSO) to a concentration of 20 mM. Ten microlitres of tryptase solution were added to 90 μl of 1 mM BAPNA in 20 mM Tris buffer containing 1 M glycerol and absorbance was measured at 410 nm and 550 nm (correction wavelength) in a microtitre plate reader. The protein concentration of tryptase preparations was determined using the Coomassie Protein Assay, using BSA to prepare a standard curve. The specific activity of purified lung tryptase preparations was determined and expressed as units of activity/mg protein (Table 2-1). The purity of the tryptase was assessed by SDS-PAGE (section 2.6) and silver staining according to the manufacturer's protocols. Gels were fixed in 40 % methanol and 10 % acetic acid (v/v) for 30 min or overnight. Fixative concentration was reduced by immersion of the gel in 10 % ethanol and 5 % acetic acid (v/v) for 30 min (2 x 15 min). The fixative was removed and a 1 in 10 dilution of an oxidiser was added for 3 min with gentle orbital mixing. Oxidiser was removed by washing in high purity water for 2 min. This wash step was repeated a twice more. The gel was treated with silver stain reagent for 15 min, then this was removed and replaced with developer solution added until the solution turned yellow or smokey brown. This reagent was removed immediately and fresh developing solution added for 5 min. Development of staining was stopped by removal of the developer and addition of 5 % acetic acid. A single protein band corresponding to tryptase is clearly visible from the stained gel (Fig. 2-6A).

2.9.3 Characterisation of Human Lung Tryptase by Western Blotting

In addition to silver staining, proteins on an SDS-PAGE gel run with tryptase were transferred to nitrocellulose membrane (as described in section 2.6). The membrane was blocked with 3 % BSA and 10 % FCS in PBS with 0.05 % Tween 20 for 24 hr at 4 °C and washed 6 times for 2 min each using PBS with 0.05 % Tween 20. This was followed by incubation with tryptase-specific monoclonal antibody AA5 (Walls *et al.*, 1990), which was applied as 1/2000 diluted culture supernatant with PBS-Tween containing 1 % BSA for 60 min at room temperature. Following six two-minute washes in PBS-Tween 20, secondary antibody, biotin-conjugated antiserum to mouse immunoglobulins were added for a further 60 min. Extravidin® peroxidase was applied at a dilution of 1/10,000 for 60 min. After a further 6 washes in PBS-Tween, West pico chemiluminescence substrate (Pierce, IL, USA) was applied for between 1 and 5 min. The blots were exposed to ECL-2 film (Bio Rad, Herts. UK) for 1 min, then the film was developed using an automated X-ray development machine. The Western blot showed a single protein band (approx. 36 kDa), demonstrating the identity of the purified protein to be tryptase (Fig. 2-6B).

2.10 Eosinophil Isolation

Eosinophils were isolated using the MACS immunomagnetic separation technique, as described by Hansel *et al.* (1991). Peripheral heparinised

blood (mainly from atopic individuals, whose blood contained more eosinophils) was diluted with an equal volume of phosphate buffered saline (PBS, pH 7.4) containing 2% heat inactivated FCS. The blood/PBS mixture was layered on a Percoll density of 1.082 g/ml – 1.084 g/ml, and centrifuged at 500 x g for 30 min at 20°C. The blood was separated into layers by centrifugation, the mononuclear cell layer and the Percoll/ plasma above the red cell pellet were aspirated carefully and discarded, retaining the pellet (containing the granulocytes). The erythrocytes were lysed by addition of distilled water (200ml per initial 50ml blood taken) for 45 sec at room temperature before the addition of 1.8 % saline solution. The granulocyte suspension was washed twice with cold PBS/2% FCS, then resuspended in 0.5 ml PBS/ 2% FCS. Fifty microlitres of anti-CD16 MACS microbeads were added to the cells and incubated with them for 30 min on ice. One MACS "C" column was prepared for each 50 ml blood taken initially. Columns were washed once with 70% ethanol, then repeatedly washed with ice-cold PBS/2% FCS, before storage at 4 °C until used later in the day. Immunomagnetic removal of neutrophils was performed by passing the granulocyte suspension treated with magnetic anti-CD16 beads through a MACS "C" column placed inside a powerful magnet. Eosinophils were eluted in a total of 25 ml PBS/2% FCS passed through a 21 G needle, according to the manufacturer's instructions.

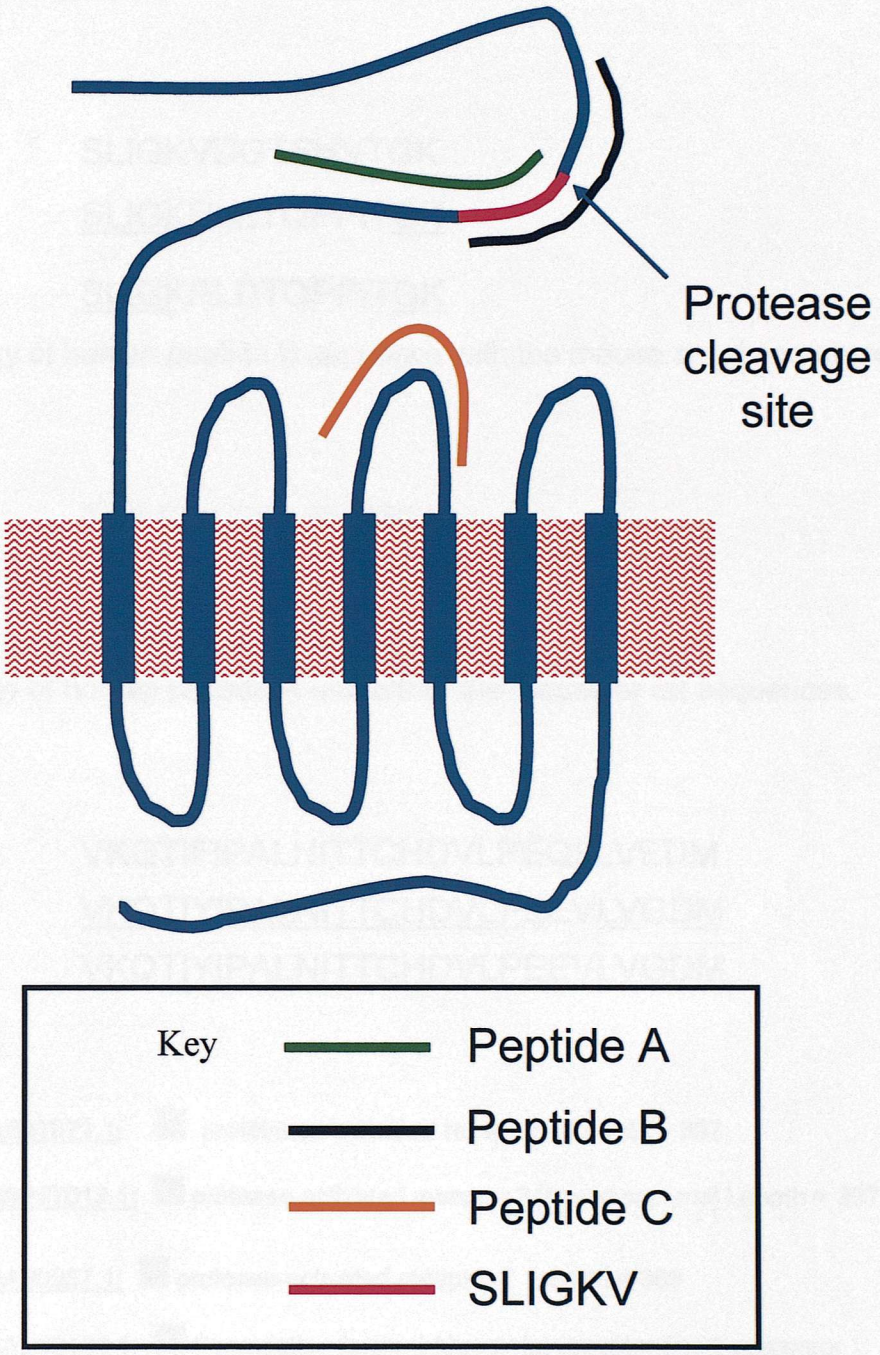


Figure 2-1 Diagrammatic representation of the PAR-2 receptor. The parts of the molecule where peptide A ($\downarrow^{37}$SLIGKVDGTSHVTGK^{52}G), peptide B (^{29}TNRSSKGR$\downarrow$SLIGK^{42}V) and peptide C (VKQTIFIPALNITTCHDVLPEQL) were selected as are indicated.

PEPTIDE A

Human PAR-2 SLIGKVDGTSHVTGK

Mouse PAR-2 SLIGKRLETQPPITGK

Rat PAR-2 SLIGKRLDTQPPITGK

36 % Homology of human peptide B sequence with the mouse or rat sequences

PEPTIDE B

Human PAR-2 TNRSSKGR↓SLIGKV

Mouse PAR-2 RPNSSKGR↓SLIGRL

Rat PAR-2 RPNSSKGR↓SLIGRL

71 % Homology of human peptide A with either the mouse or rat sequences.

PEPTIDE C

Human PAR-2 VKQTIFIPALNITTCHDVLPEQLLVEDM

Mouse PAR-2 VKQTIYIPALNITTCHDVLPEEVLVGDM

Rat PAR-2 VKQTIYIPALNITTCHDVLPEEVLVGDM

Blast results:

gi|1041729|gb|AAB47871.1| proteinase-activated receptor-2 Length = 397

gi|33149992|gb|AAP97012.1| protease-activated receptor 2 [*Homo sapiens*] Length = 397

gi|1208540|gb|AAA90957.1| protease-activated receptor 2 Length = 369

gi|17390292|gb|AAH18130.1| Coagulation factor II (thrombin) receptor-like 1 precursor [*Homo sapiens*] Length = 397

Figure 2-2 Amino acid sequences of the three peptides (A-C) corresponding to fragments of human PAR-2: comparison with the mouse and rat sequences. Amino acids are represented by their single letter codes. Identical residues between the three species are represented by letters underlined.

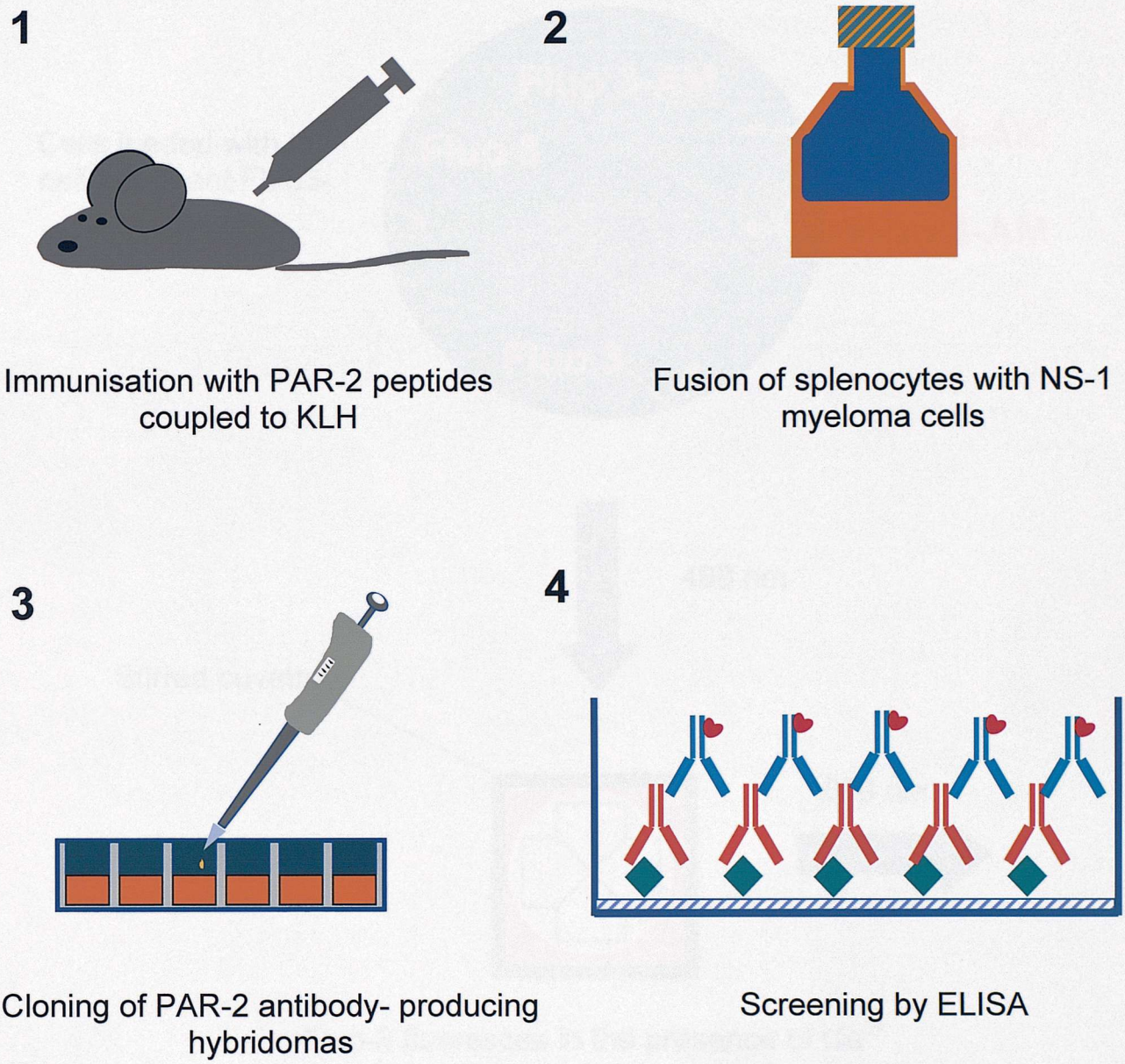


Figure 2-3 Production of monoclonal antibodies to PAR-2.

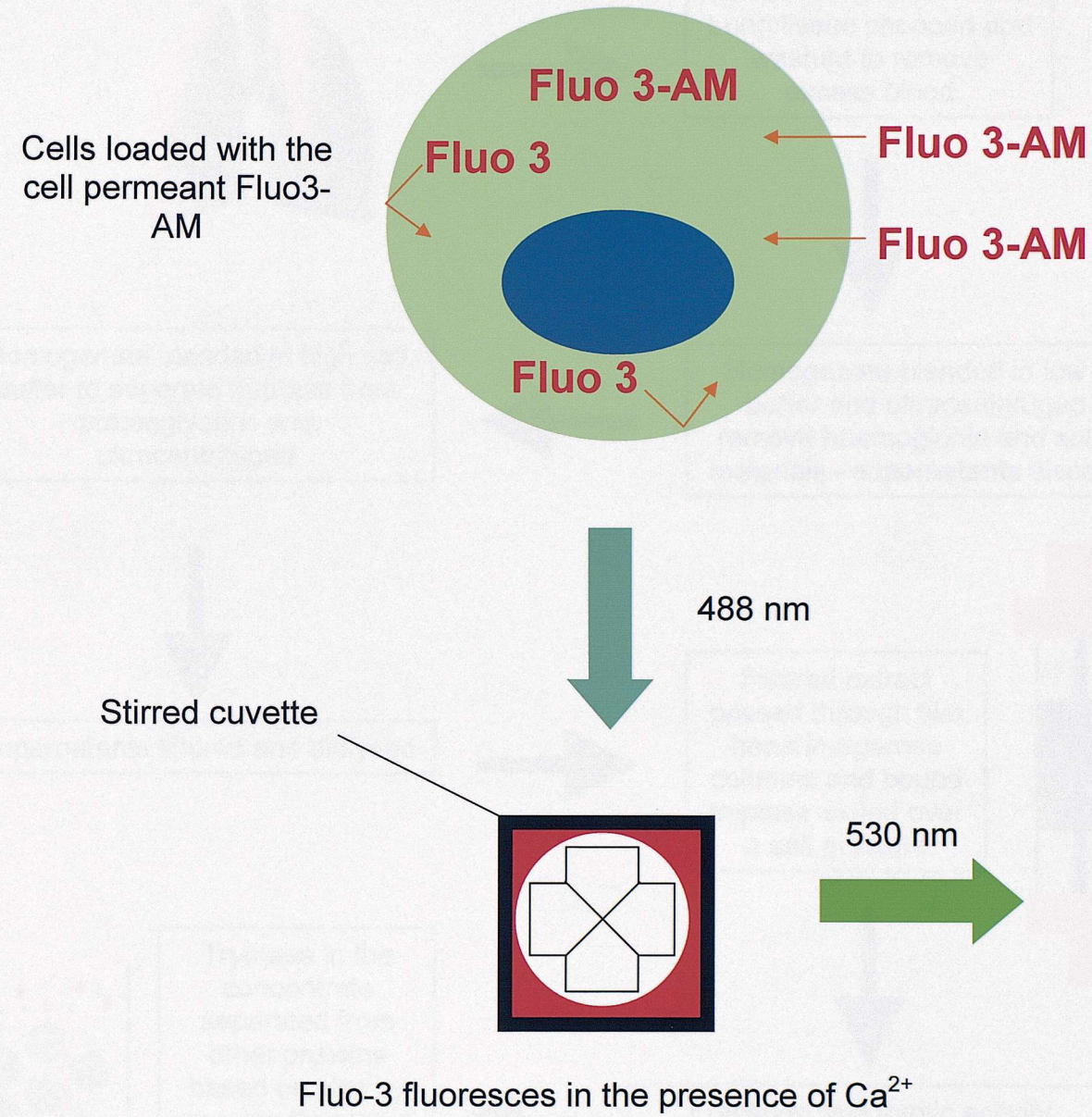


Figure 2-4 Diagrammatic representation of the method for measurement of intracellular calcium.

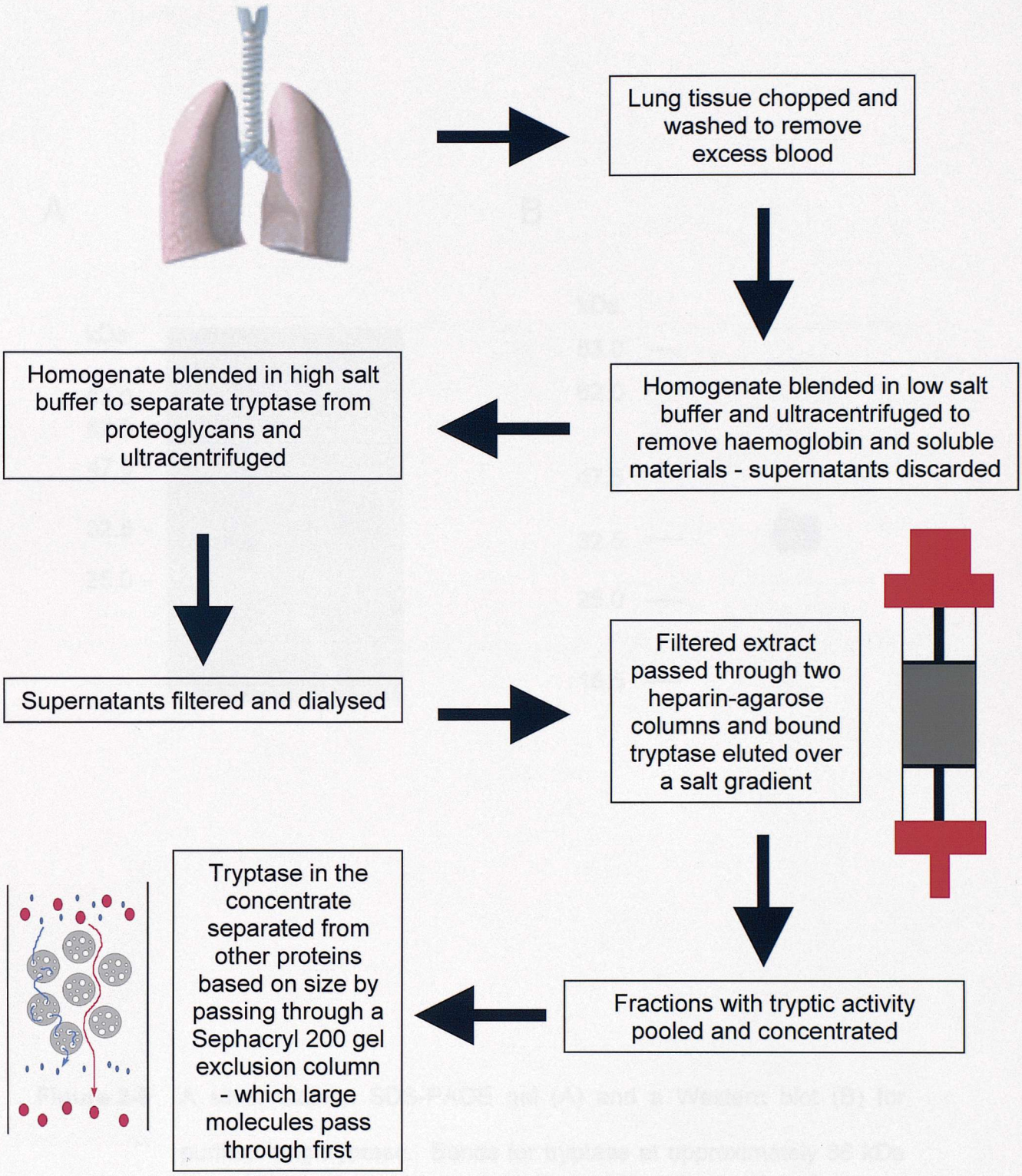


Figure 2-5 Purification of tryptase from human lung tissue.

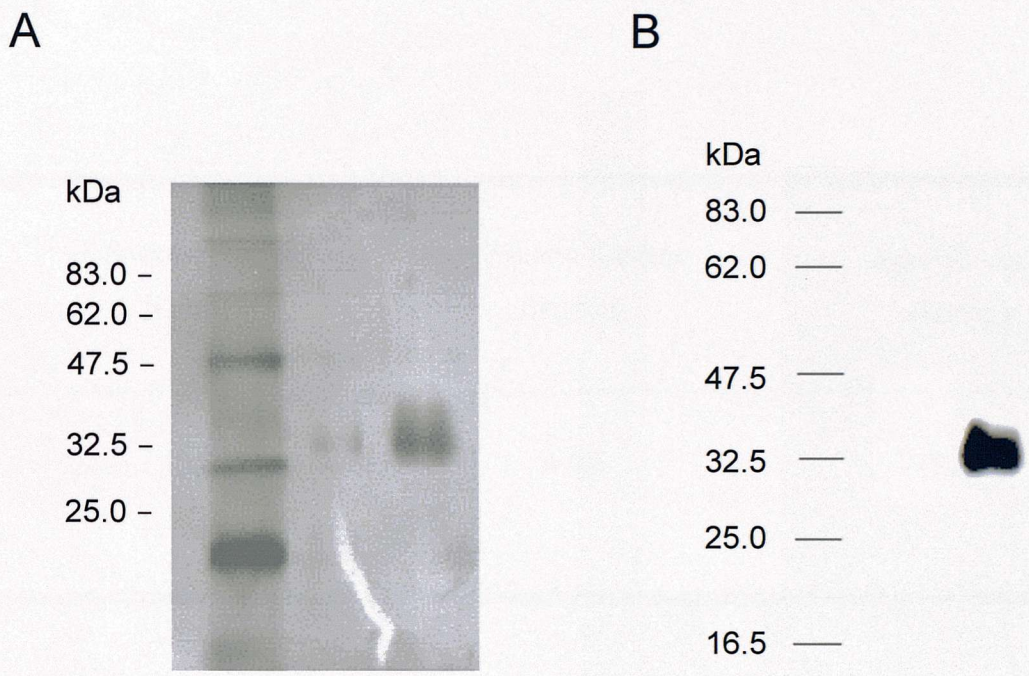


Figure 2-6 A silver stained SDS-PAGE gel (A) and a Western blot (B) for purified lung tryptase. Bands for tryptase at approximately 36 kDa are clearly visible.

Enzyme Activity (U/ml)	Protein Content (mg/ml)	Specific Activity (U/mg)
12.6	4.28	2.93

Table 2-1 Specific activity of purified human lung tryptase. One unit (U) of enzyme activity is the amount of tryptase required to hydrolyse 1 μmol of the chromogenic substrate BAPNA per minute at 25 °C

CHAPTER 3

Generation of Antibodies to PAR-2

3 Generation of Antibodies to PAR-2

3.1 Introduction

Levels of tryptase, a major mast cell protease, are elevated in the airways of patients with inflammatory conditions, such as bronchial asthma and allergic rhinitis (Wenzel *et al.*, 1988; Wilson *et al.*, 1998). Furthermore, tryptase has been found to have pro-inflammatory actions on a number of cell types. As well as proliferative effects on airway epithelial cells, tryptase can stimulate the synthesis and release of pro-inflammatory cytokines including IL-6 and 8 and GM-CSF, and up-regulate expression of intercellular adhesion molecule 1 (Cairns and Walls, 1996). Tryptase is also able to stimulate the proliferation of airway smooth muscle cells, lung fibroblasts and microvascular endothelial cells (Hartmann *et al.*, 1992; Blair *et al.*, 1997; Berger *et al*, 2001), underlying its potential roles in airway wall remodelling and inflammatory cell recruitment.

Although tryptase can alter the behaviour of epithelial and mesenchymal cells, and leukocytes in inflammatory disease, the mechanism of action of tryptase has been a mystery. The discovery that this protease can activate PAR-2 (Nystedt *et al*, 1994; Molino *et al*, 1997), suggests one possible pathway for the regulation of cell function. Furthermore, the role of PAR-2 in disease has yet to be defined, due to the lack of suitable tools for identifying the involvement of this receptor, such as specific antibodies and antagonists.

A rabbit anti-PAR-2 serum (B5) has been a useful tool in studying the expression of PAR-2 in disease (Kong *et al*, 1997). However, a peptide with the rat sequence rather than the human peptide sequence was used as an immunogen to raise this antiserum. Thus, there is some concern over antibody specificity when applied to human cells and tissues. Furthermore, the B5 antiserum contains polyclonal antibodies, which contain an assortment of immunoglobulins and therefore the antiserum may cross-react with other proteins. Monoclonal antibodies are made by hybridomas generated by the fusion of a B lymphocyte with a myeloma cell. Since the hybridoma cells are cloned, they produce antibody with a single specificity, recognising a single sequence and are therefore less likely to cross-react with other antigens.

In the studies described in this chapter, attempts were made to produce antibodies against PAR-2, which would allow identification of this receptor and if possible, to provide information on the activation state of the receptor. A secondary aim was to produce antibody reagents with the potential as tools to investigate PAR-2 function, possibly by inhibiting PAR-2 function.

3.2 Materials and Methods

Materials and methods employed in this section are described in Chapter 2 (monoclonal and polyclonal antibody production, ELISA, flow cytometry, Western blotting, immunocytochemistry, and calcium flux measurements). This work was carried out in conjunction with Dr. Mark Buckley, who conducted the studies involving injections of animals (which requires a license under the Animals (Scientific Procedures) Act, 1986).

3.3 Results

3.3.1 Generation of Monoclonal Antibodies

A number of strategies to produce monoclonal antibodies were attempted, but it was the use of purified peptides that resulted in the generation of four new monoclonal antibodies to PAR-2. These antibodies were termed P2A, P2B, P2C and P2D, and were subjected to further characterisation.

3.3.1.1 Detection of PAR-2 Peptide Fragments by Monoclonal Antibodies in ELISA

In direct ELISA, all four antibodies bound to peptide B (spanning the cleavage site of PAR-2) coupled to BSA (fig 3-1A) but not the peptide A-BSA conjugates (data not shown). In addition, P2A also detected the

recombinant N-terminal sequence of rat PAR-2, referred to as P90 peptide (fig 3-1B).

3.3.1.2 Detection of PAR-2 in $KNRK_t$ Cells by Flow Cytometry

Flow cytometric analyses revealed that P2A binds to PAR2 on $KNRK_t$ cells (expressing recombinant human PAR-2), as revealed by an increased fluorescence of this cell population compared to the untransfected KNRK cells (fig 3-2). P2B also revealed a shift in PAR-2 immunofluorescence on A5-1 cells compared to KNRK cells but P2C and P2D did not detect PAR-2 by FACS (data not shown).

Further characterisation of P2A involved stimulation of PAR-2 on the $KNRK_t$ cells prior to antibody labelling and flow cytometry. Trypsin was added to cleave and activate the receptor on the cells for 2 min or 10 min. Incubation with trypsin for more than 10 min tended to cause the cells to clump together, hampering FACS measurements. The degree of PAR-2 immunofluorescence did not differ 2 min (fig. 3-3C and D) or 10 min (fig. 3-3E and F) following stimulation with trypsin and analysis of intact or permeabilised cells when compared to the unstimulated cells (fig. 3-4A and B). In cells pre-treated with cytochalasin B, mean fluorescent intensity (MFI) was significantly increased at 2 and at 10 min following the addition of trypsin (fig. 3-3A - C). Pre-incubation of the cells with the

metabolic inhibitors, antimycin A and 2-deoxyglucose, did not alter the MFI of cells labelled using P2A (fig. 3-3B, D and F).

Monoclonal antibody (SAM11), a kind gift from Dr L Brass (University of Pennsylvania, PA, USA) which was raised against a peptide sequence corresponding to residues 37 to 54 of the tethered ligand of human PAR-2 ($\downarrow^{37}$SLIGKVDGTSHVTGKG^{54}H), did not label the $KNRK_t$ cells using FACS (data not shown). The B5 antiserum did, however, label $KNRK_t$ cells (fig. 3-4), with a significant shift in MFI compared to KNRK cells (data not shown). However, MFI was not affected by the addition of trypsin for 2 or 10 min either with or without prior incubation of the cells with cytochalasin B (fig. 3-5A, C and E) nor by the application of metabolic inhibitors (fig. 3-4B, D and F).

3.3.1.3 Western Blotting of $KNRK_t$ Cell Lysates using P2A

On Western Blotting, P2A detected three bands with molecular weights of 45, 65 and 170 kDa using densitometry (Quantity One image analysis software, Biorad), in $KNRK_t$ cell lysates but not in the untransfected KNRK cell lysates (fig. 3-5). Densitometry showed that the intensity of the 47 kDa band were increased after stimulation with trypsin for 2 min (fig. 3-7A and C, lane 2) compared to no stimulation (fig 3-6, lane 1). The intensity of this band was also increased after 2, 10, 30 and 60 min stimulation with SLIGKV-NH_2 (fig. 3-6, lanes 7-9, respectively) when compared to the

unstimulated cells (fig 3-6, lane 1). The intensity of the other bands was unaltered with PAR-2 stimulation.

3.3.1.4 Immunohistochemical Staining with P2A

P2A detected PAR-2 by immunohistochemistry in bronchial (fig. 3-7A), nasal (fig. 3-7B), lung and colon tissue embedded in GMA resin, but did not react with tissues embedded in paraffin wax (data not shown). Cells in all layers of the epithelium were stained (fig. 3-8A), but Staining intensity appeared to be greatest in more lower regions of the individual epithelial cells. A few cells underlying the epithelium, within the submucosa, also stained for PAR-2 (fig. 3-8B).

3.3.2 Preparation of PAR-2-specific Antisera

Antisera were generated in six rabbits: antisera K and L against peptide B, antisera M and N against peptide C, and antisera O and P sera against the P90 peptide. After boosting with the immunogen, antibody titre as assessed by ELISA, was improved with each successive bleed.

3.3.2.1 Detection of PAR-2 Peptide Fragments in ELISA

All six antisera reacted with their respective immunogens conjugated to BSA in direct ELISA (fig. 3-9). Antisera M and N were of higher titre than antisera K and L sera suggesting a more successful antibody generation. Titres for antisera O and P were considerably better than the other four

antibodies, although, the detecting antigen in ELISA was a protein rather than a fragment peptide.

3.3.2.2 Detection of PAR-2 in $KNRK_t$ Cells by Flow Cytometry

Antibody binding to whole PAR-2 was confirmed by FACS. The antibodies all labelled the PAR-2 expressing $KNRK_t$ cells and A549 cells but not the KNRK cells (data not shown). Following stimulation with trypsin at 2 and 10 min, in the presence or absence of cytochalasin B or antimycin A and 2-deoxyglucose, MFI was not altered using K, M and O sera (fig. 3-10 to 3-12). Antisera M did show a marginal shift (less than 10%) in expression at 2 min trypsin stimulation when pre-incubated with cytochalasin B (fig. 3-11A). However, this shift was not evident when comparing expression between unstimulated cells that had been pre-incubated with cytochalasin B (fig. 3-11E).

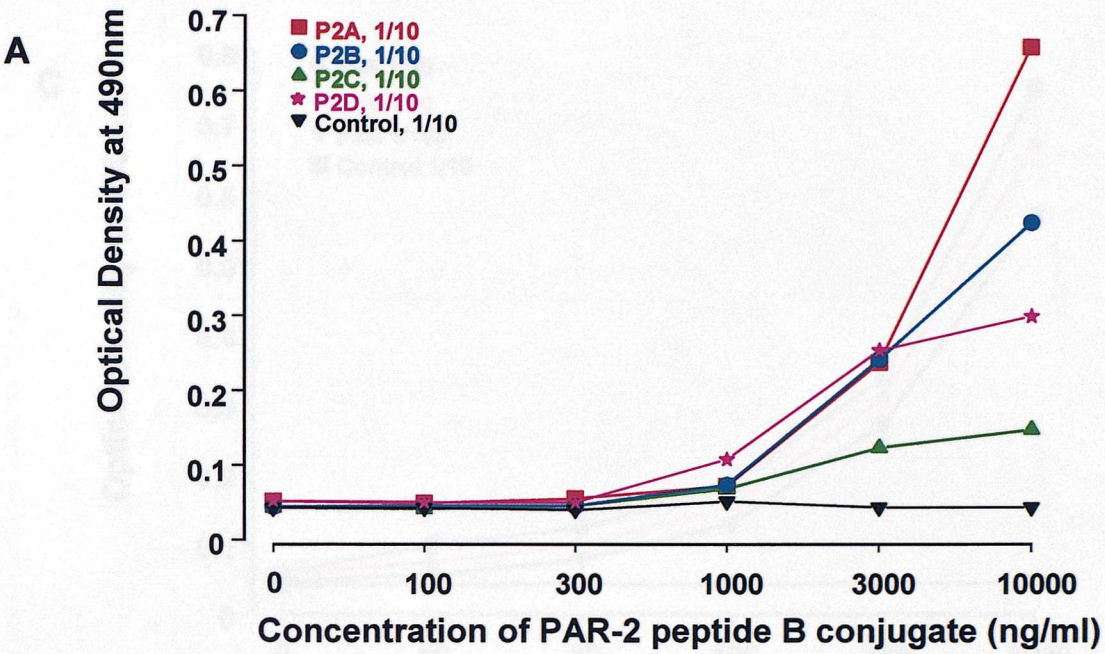
3.3.2 Antagonism of PAR-2 activation

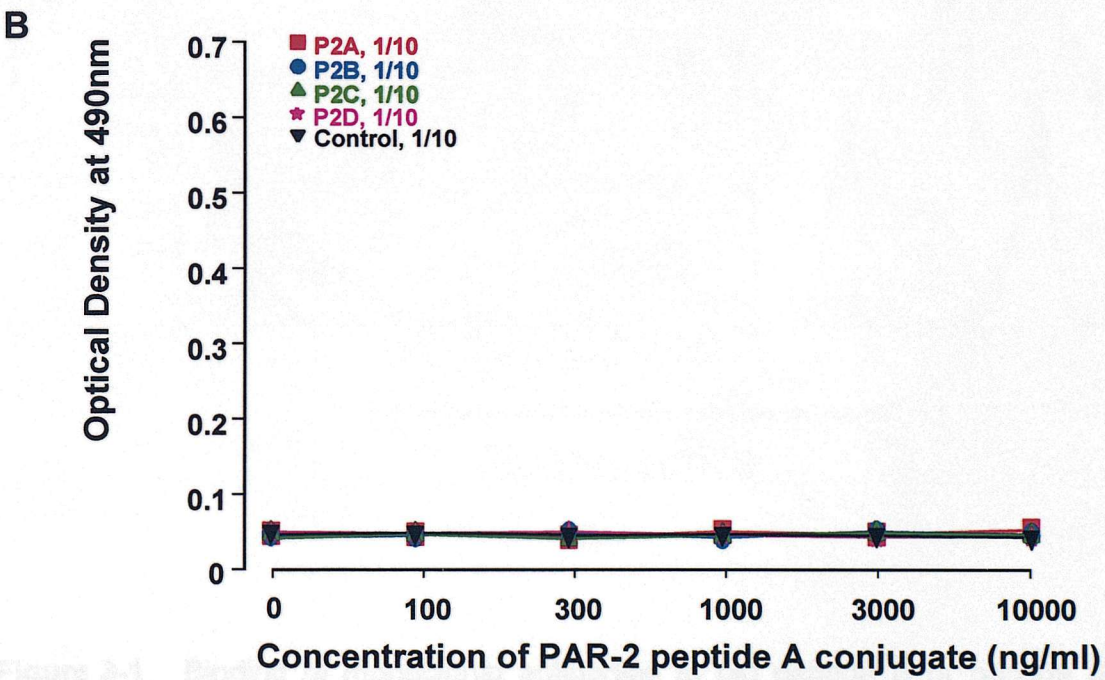
In order to investigate the potential of PAR-2-specific antibodies to act as antagonists for the receptor, a model was employed which involved measurements of $[Ca^{2+}]_i$ flux as a measure of G-protein coupled receptor signalling. The presence of functional PAR-2 on $KNRK_t$ cells was confirmed by stimulation by the agonists, tc-LIGRLO-NH_2, SLIGRL-NH_2 and trypsin (fig. 3-13A), which produced a characteristic initial peak in intracellular calcium concentrations. The receptor could be desensitised

to a second addition of the same agonist or other PAR-2 agonists, a phenomenon known as cross-desensitisation (data not shown). The same agonists did not stimulate calcium flux in untransfected KNRK cells (fig. 3-13B), though the positive control ATP did elicit a calcium response. These findings suggest that $KNRK_t$ cells but not untransfected KNRK cells expressed functional PAR-2 receptors.

Both the polyclonal and monoclonal antibodies when applied in this system caused a significant elevation in the baseline fluorescence when used unpurified (data not shown). For this reason, the antibodies were purified for use in the study of their potential to inhibit PAR-2 signalling (as described in section 2.3.2 and 2.3.4). Concentration response curves determined the agonist concentration to be used in the initial experiments, 30 μM tc-LIGRLO-NH_2, 30 μM SLIGRL-NH_2 and 40 nM trypsin (data not shown). The purified polyclonal anti-PAR-2 antibodies did not reduce the intracellular calcium response to any of the three agonists (fig. 3-14), although purified antibodies from antisera O (100 μg/ml) caused a slight reduction of approximately 10 % in the calcium response to trypsin (fig. 3-14C).

Purified P2A failed to block calcium responses induced by PAR-2 agonists under similar conditions (data not shown). Thus, it was not possible to inhibit PAR-2 activation using the new antibodies generated.


A
Optical Density at 490nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
P2A, 1/10
P2B, 1/10
P2C, 1/10
P2D, 1/10
Control, 1/10
0
100
300
1000
3000
10000
Concentration of PAR-2 peptide B conjugate (ng/ml)


B
Optical Density at 490nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
P2A, 1/10
P2B, 1/10
P2C, 1/10
P2D, 1/10
Control, 1/10
0
100
300
1000
3000
10000
Concentration of PAR-2 peptide A conjugate (ng/ml)

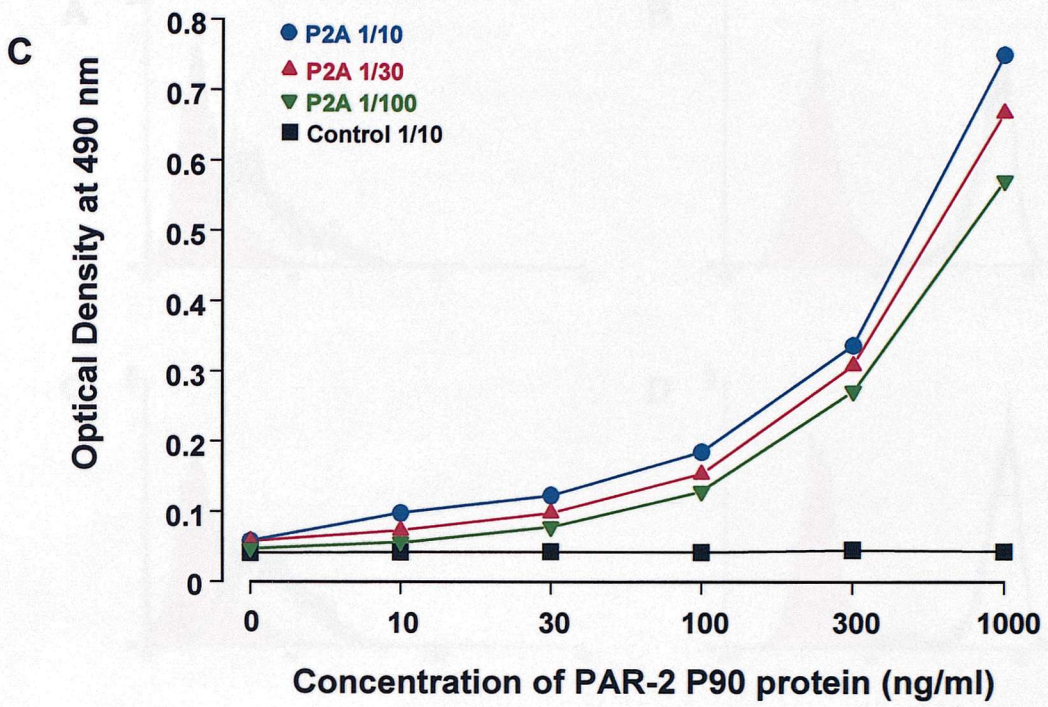


Figure 3-1 Binding of monoclonal antibodies to (A) peptide B or peptide (A) conjugated to BSA and (C) P90 peptide in direct ELISA. Culture supernatants were diluted as shown. The control monoclonal antibody was of the same class (IgM), but directed against an irrelevant antigen and used at 10 µg/ml.

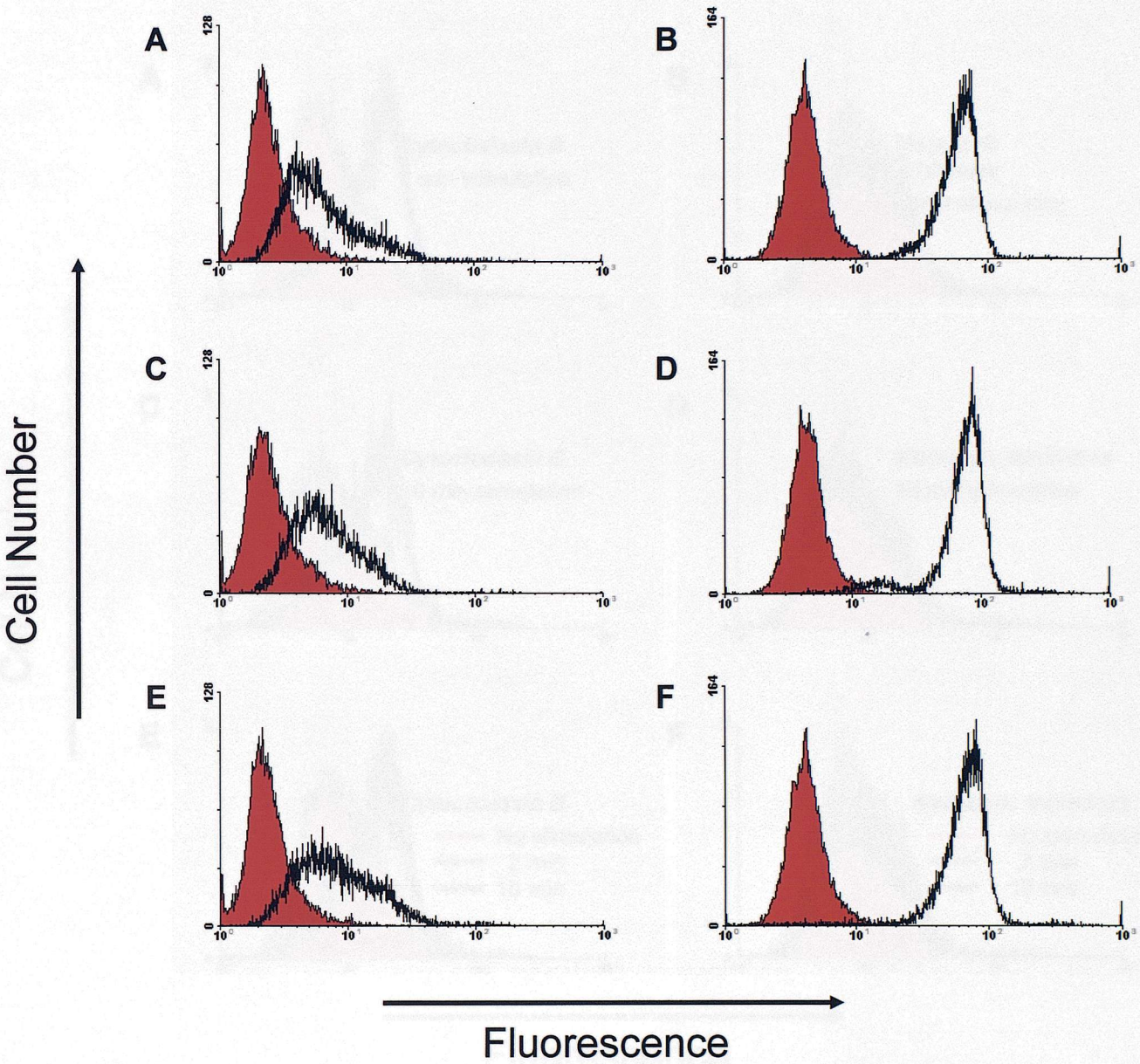


Figure 3-2 FACS analysis of $KNRK_t$ cells (open-black) and untransfected KNRK cells (filled-red) stained with monoclonal antibody P2A. Surface (A, C and E) and intracellular (B, D and F) expression of PAR-2 was examined before (A and B) or after stimulation with 12 nM trypsin for 2 min (C and D) or 10 min (E and F). Data shown is representative of three separate experiments.

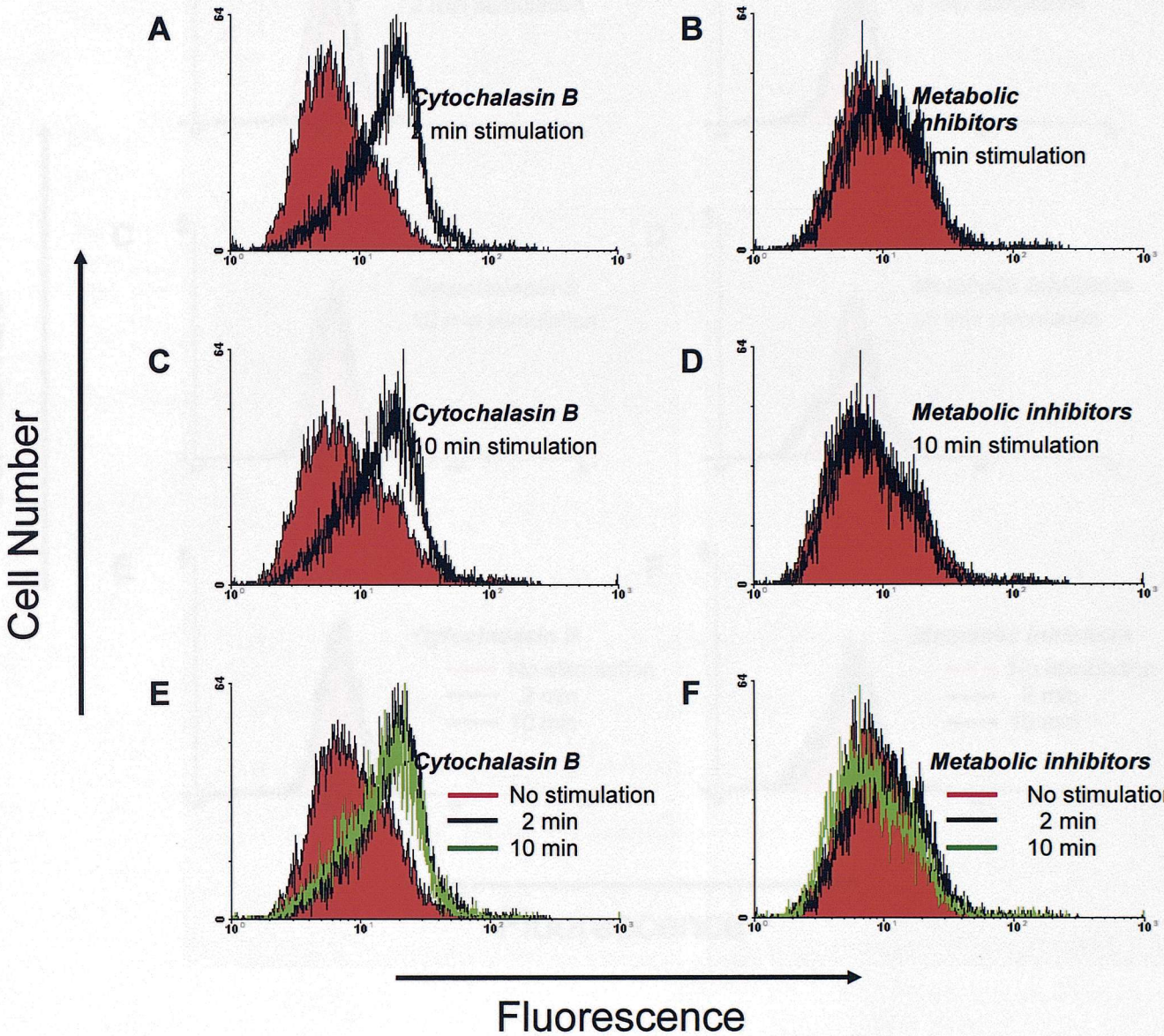


Figure 3-3 Surface PAR-2 Immunofluoresence on KNRK cells transfected with human PAR-2 ($KNRK_t$) using P2A, when exposed to cytochalasin B (A, C, E) or metabolic inhibitors (B, D, F). Cells were stimulated with 12 nM trypsin for 2 min (A and B) or 10 min (C and D). E and F, cytochalasin B and metabolic inhibitors were added to unstimulated cells (filled-red), compared to 2 min (open-black) and 10 min trypsin stimulation (open-green). Data shown is representative of three separate experiments.

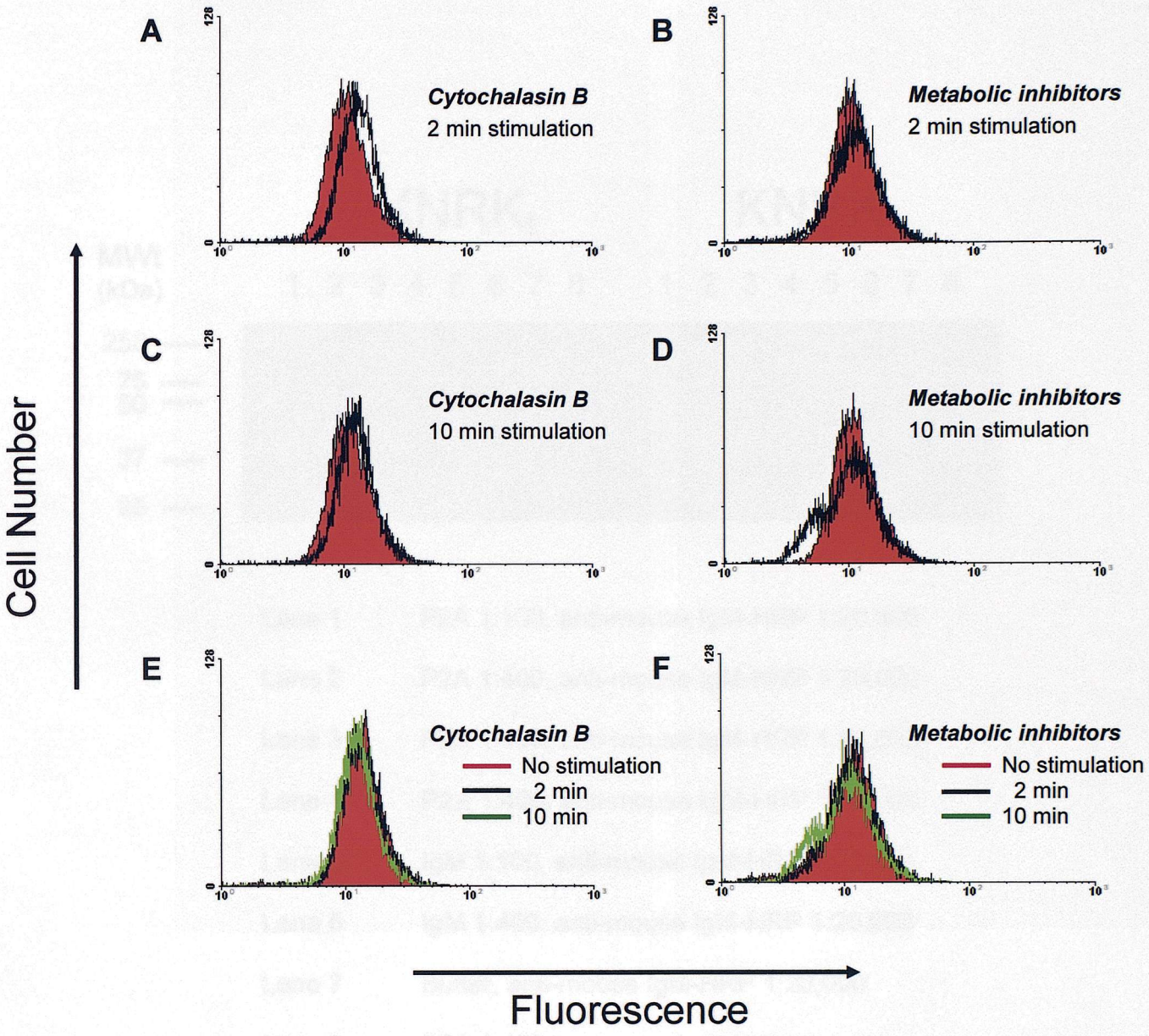


Figure 3-4 Surface PAR-2 immunofluorescence on KNRK cells transfected with human PAR-2 ($KNRK_t$) using B5 antiserum, when exposed to cytochalasin B (A, C, E) or metabolic inhibitors (B, D, F). Cells were stimulated with 12 nM trypsin for 2 min (A and B) or 10 min (C and D). E and F, cytochalasin B and metabolic inhibitors were added to unstimulated cells (filled-red), compared to 2 min (open-black) and 10 min trypsin stimulation (open-green). Data shown is representative of three separate experiments.

Lane 1	P2A 1:100, anti-mouse IgM-HRP 1:20,000
Lane 2	P2A 1:400, anti-mouse IgM-HRP 1:20,000
Lane 3	P2A 1:100, anti-mouse IgM-HRP 1:50,000
Lane 4	P2A 1:400, anti-mouse IgM-HRP 1:50,000
Lane 5	IgM 1:100, anti-mouse IgM-HRP 1:20,000
Lane 6	IgM 1:400, anti-mouse IgM-HRP 1:20,000
Lane 7	Buffer, anti-mouse IgM-HRP 1:20,000
Lane 8	P2A 1:100 (no secondary antibody)

Figure 3-5 Western blotting for PAR-2 using P2A in cells transfected with human PAR-2 (KNRK$_t$) and untransfected controls (KNRK).

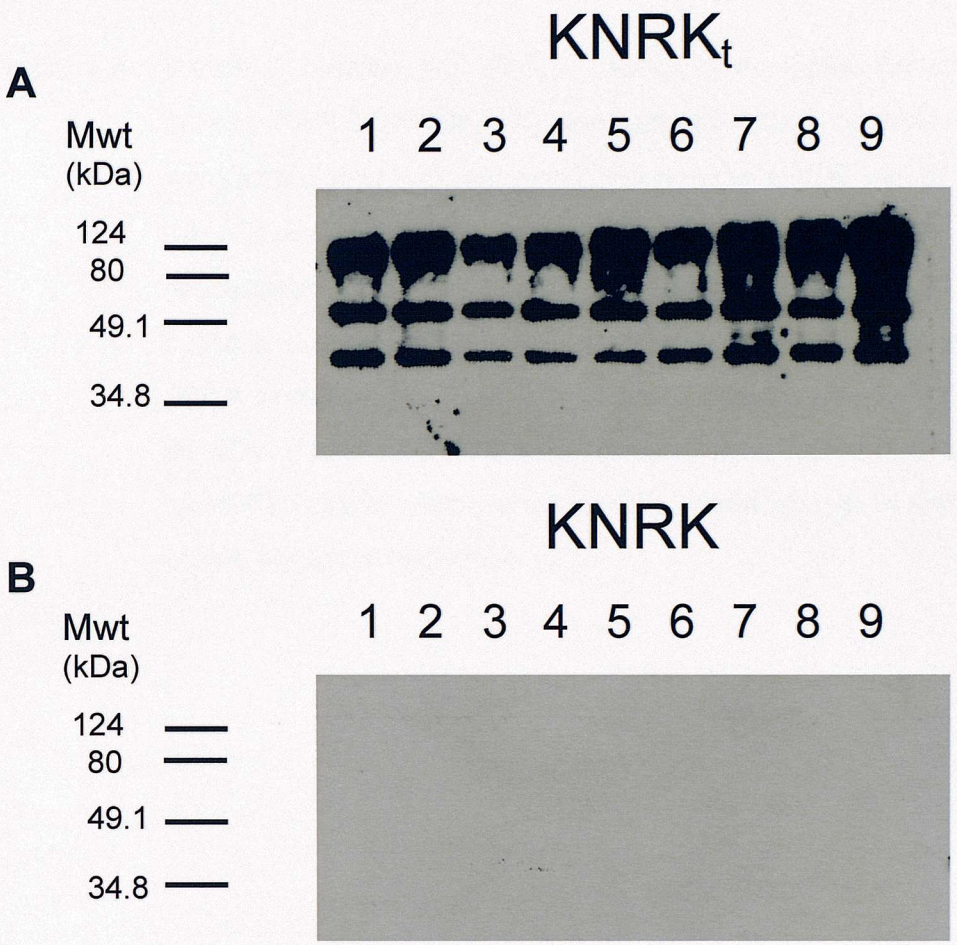

A
KNRK$_t$
Mwt
(kDa)
124
80
49.1
34.8
1 2 3 4 5 6 7 8 9
B
KNRK
Mwt
(kDa)
124
80
49.1
34.8
1 2 3 4 5 6 7 8 9
C
Arbitrary Units
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9
Lane Number

Figure 3-6 Western blotting for PAR-2 using P2A. Cells transfected with human PAR-2 ($KNRK_t$; A) and untransfected controls (KNRK; B) were stimulated with either 12 nM trypsin or 100 μM SLIGKV for 2 min (lanes 2 and 6, respectively), 10 min (lanes 3 and 7, respectively), 30 min (lanes 4 and 8, respectively) or 60 min (lanes 5 and 9, respectively), or left unstimulated (lane 1). Densitometry of blot A revealed three protein bands of approximately 45 KDa (red), 66 kDa (blue) and 174 kDa (green), respectively. Intensity was quantified using image analysis (C). Data shown is representative of two separate experiments.

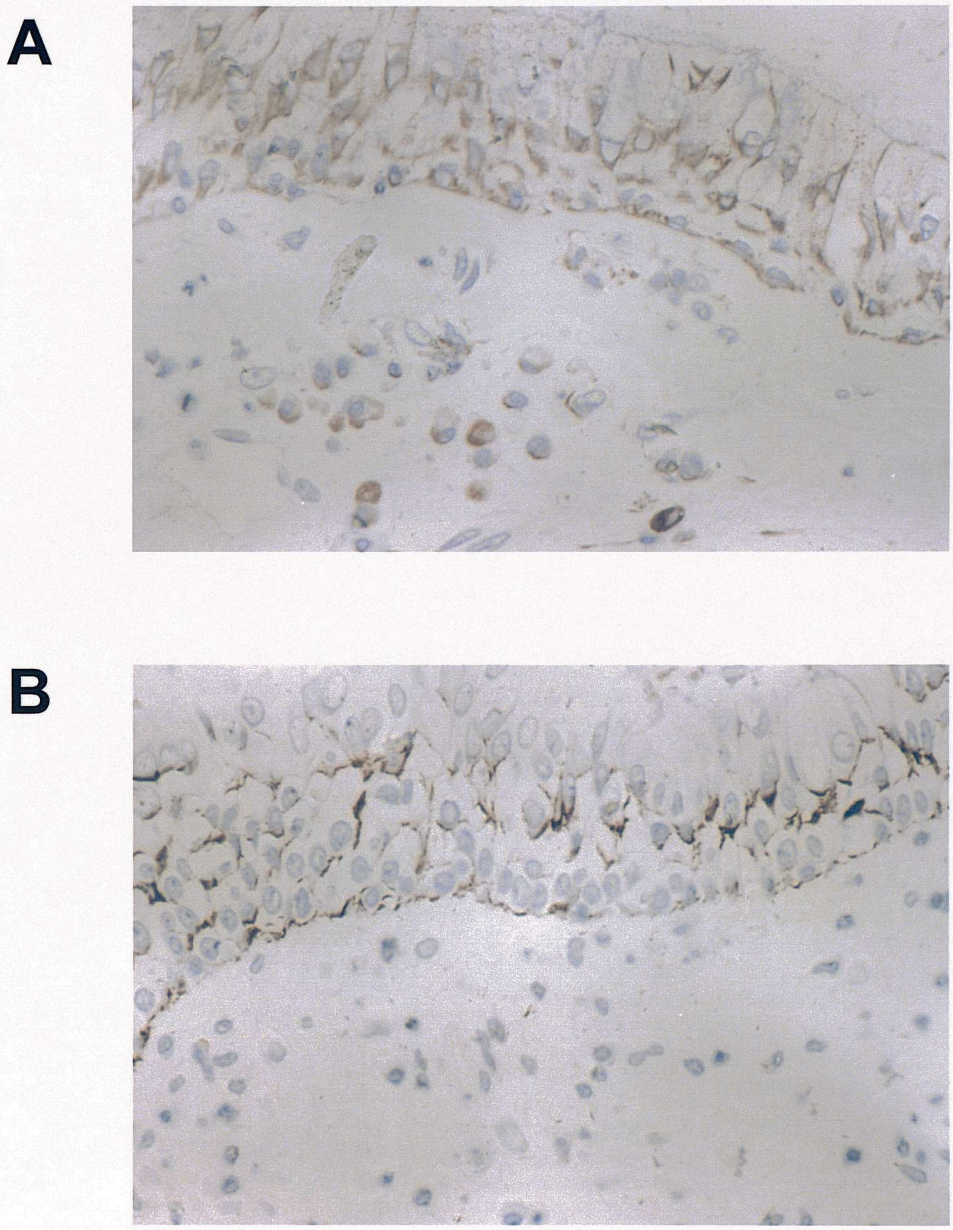


Figure 3-7 PAR-2 immunoreactivity in (A) human bronchial and (B) nasal polyp tissue, detected using monoclonal antibody P2A.

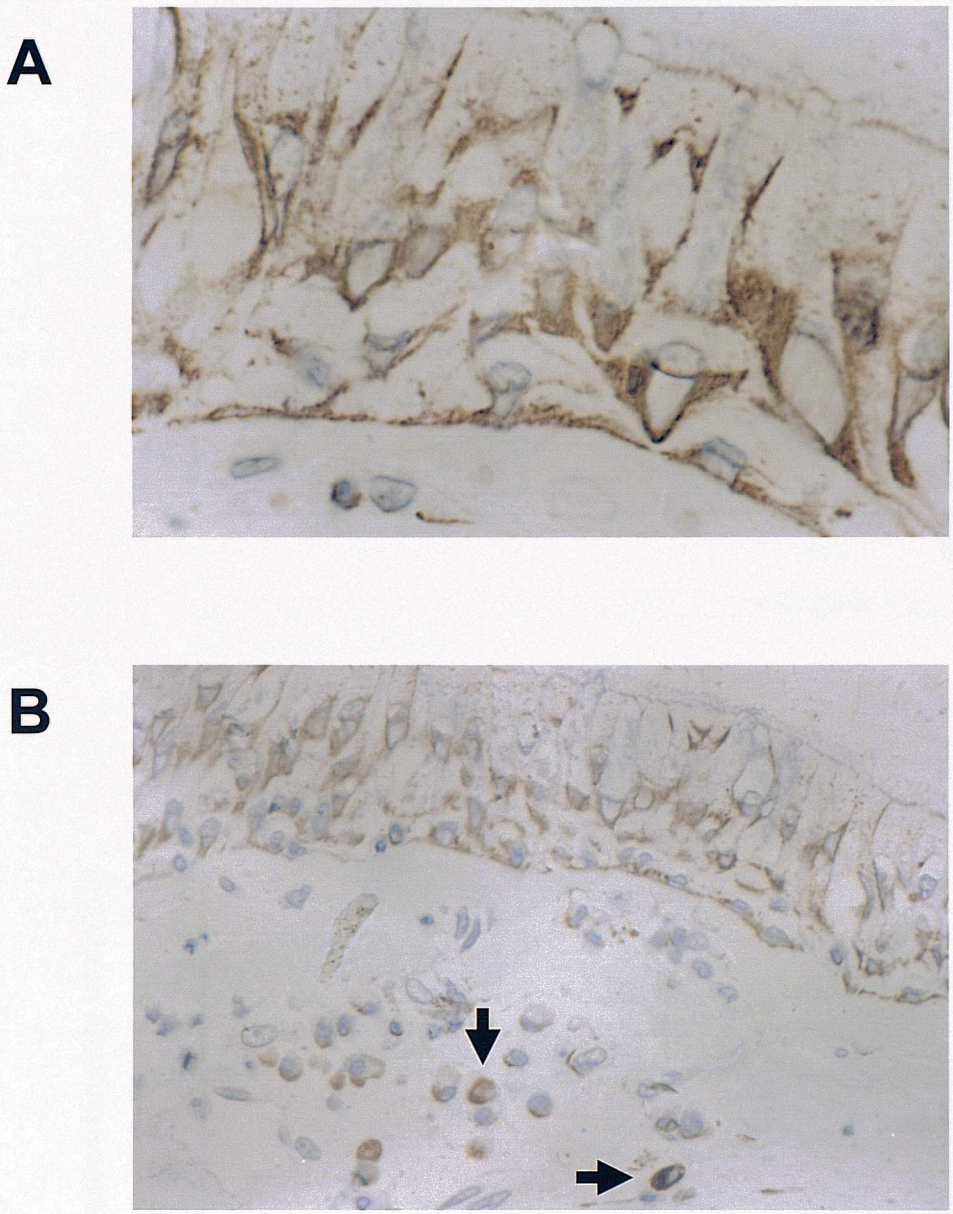
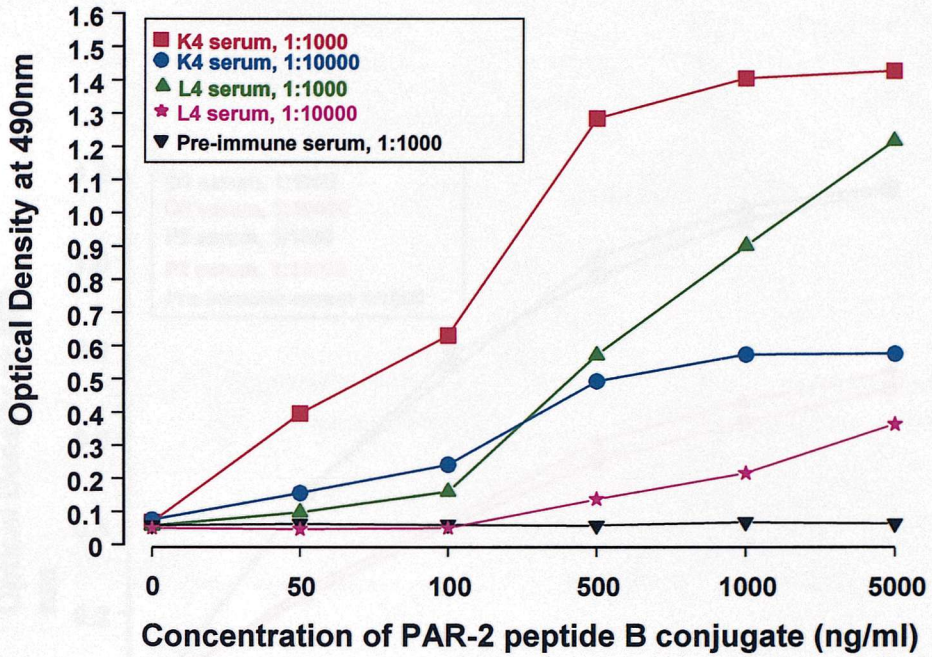
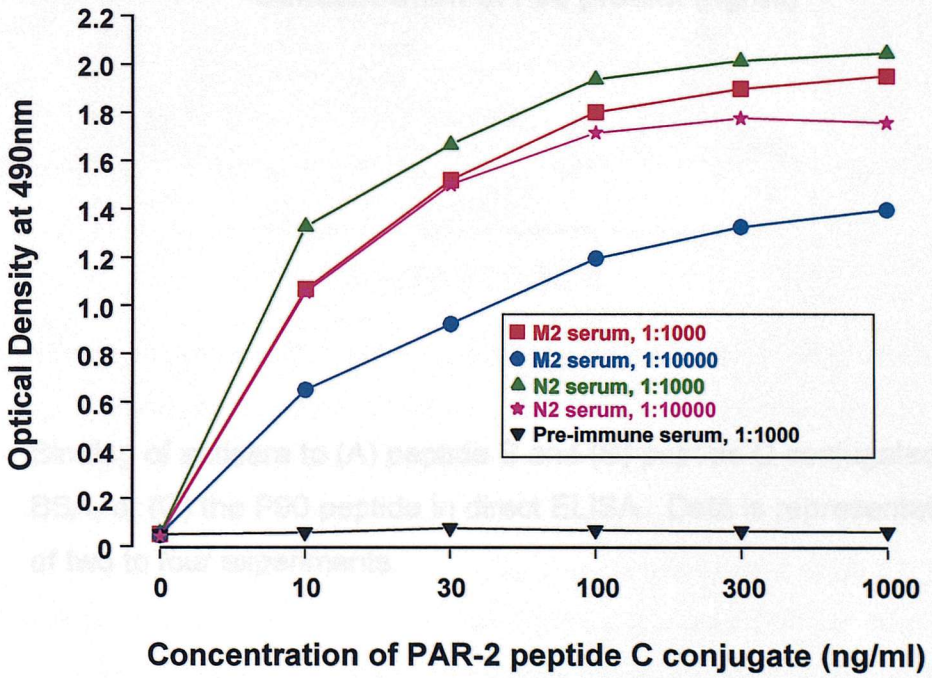


Figure 3-8 PAR-2 immunoreactivity (brown) in human bronchial tissue, using monoclonal antibody P2A, at (A) a higher and (B) a lower magnification. Cells in the *lamina propria*, expressing PAR-2 are indicated (arrows).

A


Optical Density at 490nm
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
K4 serum, 1:1000
K4 serum, 1:10000
L4 serum, 1:1000
L4 serum, 1:10000
Pre-immune serum, 1:1000
0
50
100
500
1000
5000
Concentration of PAR-2 peptide B conjugate (ng/ml)

B


Optical Density at 490nm
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
M2 serum, 1:1000
M2 serum, 1:10000
N2 serum, 1:1000
N2 serum, 1:10000
Pre-immune serum, 1:1000
0
10
30
100
300
1000
Concentration of PAR-2 peptide C conjugate (ng/ml)

C

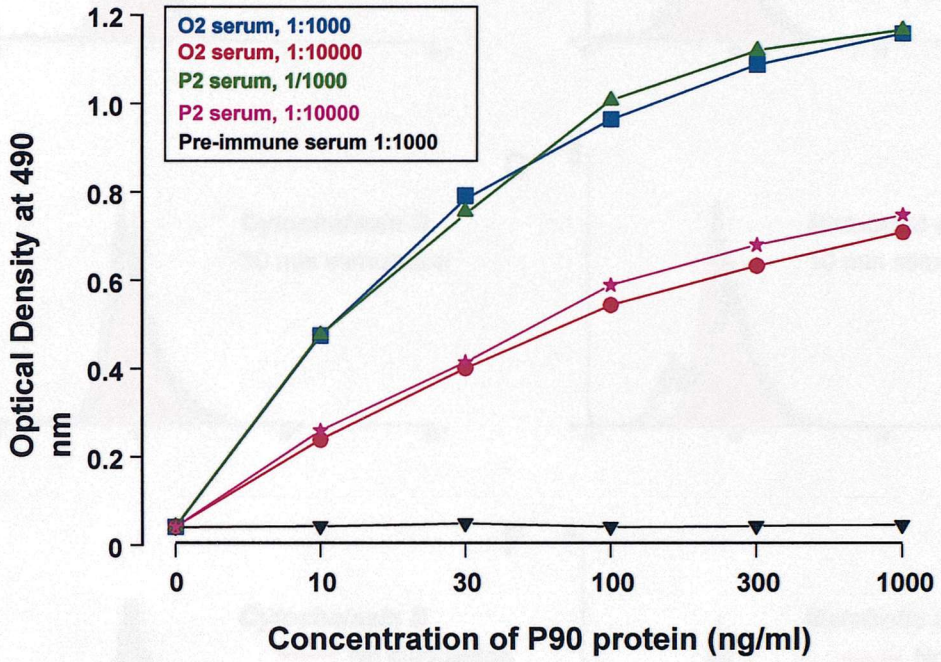


Figure 3-9 Binding of antisera to (A) peptide B and (B) peptide C conjugated to BSA, or (C) the P90 peptide in direct ELISA. Data is representative of two to four experiments.

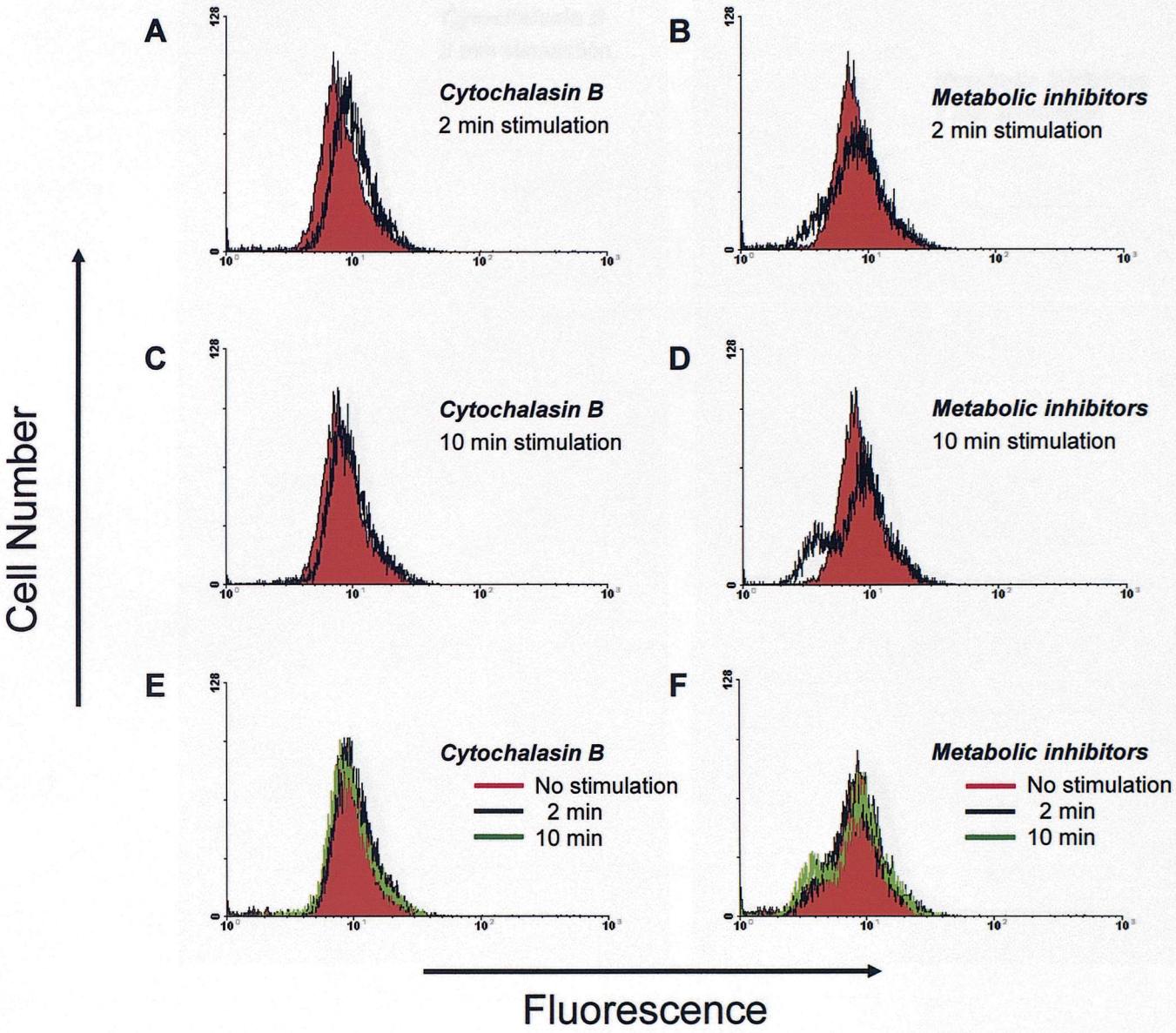


Figure 3-10 Effects of cytochalasin B and metabolic inhibitors on PAR-2 staining by antiserum K. PAR-2 immunofluorescence on the surface of KNRK cells transfected with human PAR-2 ($KNRK_t$) was labelled after treatment of the cells with cytochalasin B (A, C, E) or metabolic inhibitors (B, D, F). Cells were stimulated with 12 nM trypsin for 2 min (A and B) or 10 min (C and D). E and F, cytochalasin B and metabolic inhibitors were added to unstimulated cells (filled-red), compared to 2 min (open-black) and 10 min trypsin stimulation (open-green). Data shown is representative of two separate experiments.

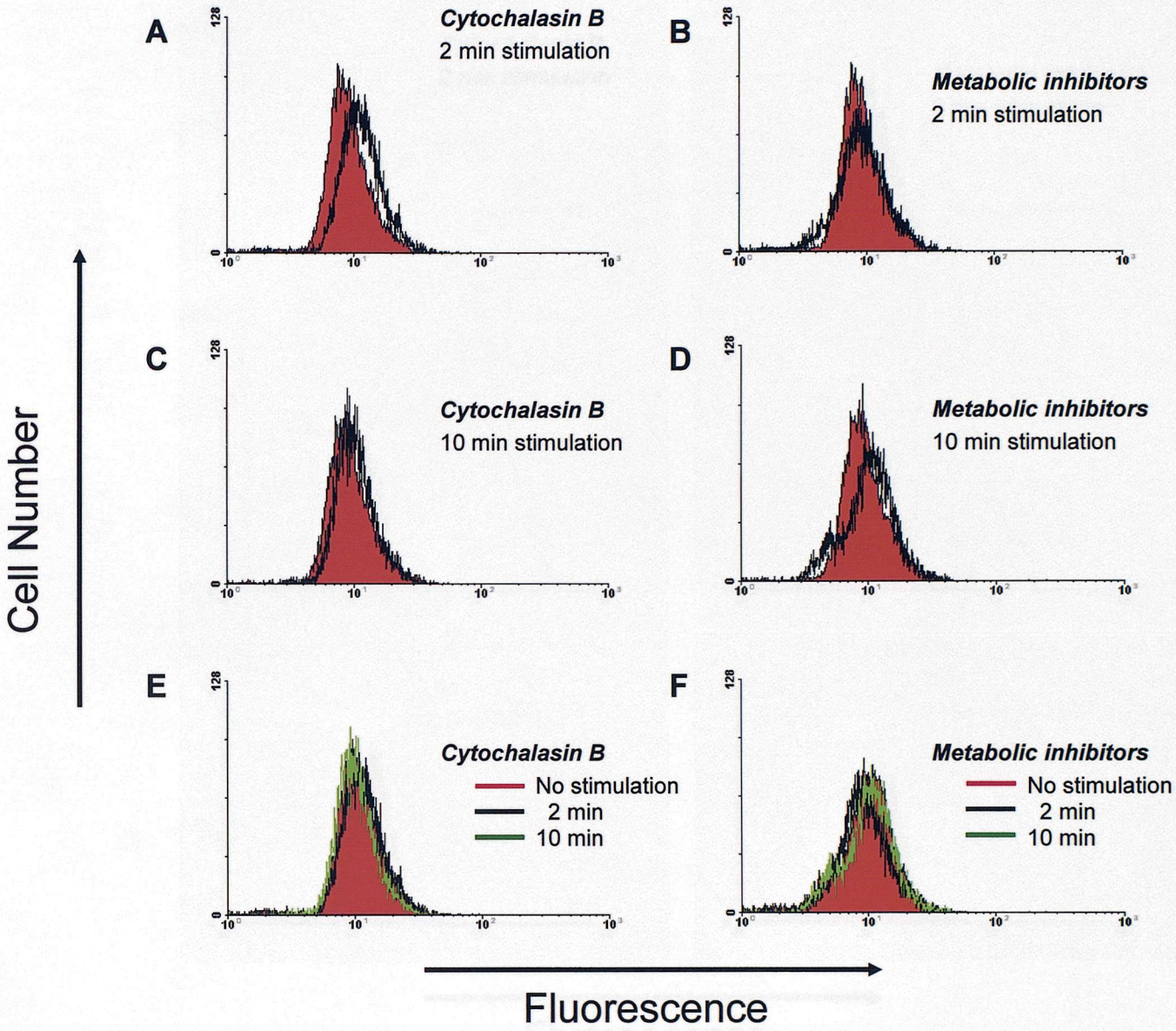


Figure 3-11 Effects of cytochalasin B and metabolic inhibitors on PAR-2 staining by antiserum M. PAR-2 immunofluorescence on the surface of KNRK cells transfected with human PAR-2 ($KNRK_t$) was labelled after treatment of the cells with cytochalasin B (A, C, E) or metabolic inhibitors (B, D, F). Cells were stimulated with 12 nM trypsin for 2 min (A and B) or 10 min (C and D). E and F, cytochalasin B and metabolic inhibitors were added to unstimulated cells (filled-red), compared to 2 min (open-black) and 10 min trypsin stimulation (open-green). Data shown is representative of two separate experiments.

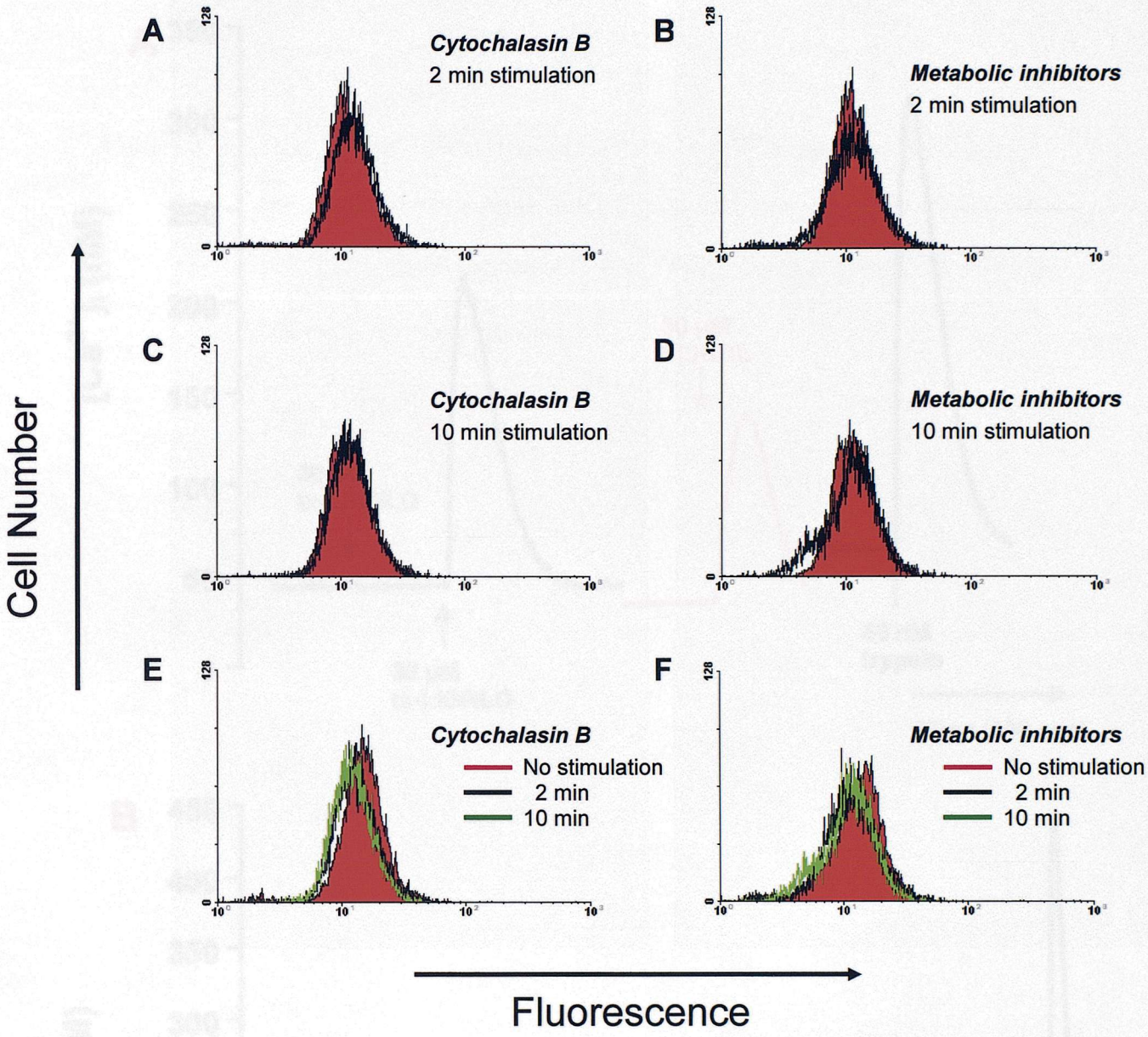


Figure 3-12 Surface PAR-2 immunofluorescence on KNRK cells transfected with human PAR-2 ($KNRK_t$) using antiserum O, when exposed to cytochalasin B (A, C, E) or metabolic inhibitors (B, D, F). Cells were stimulated with 12 nM trypsin for 2 min (A and B) or 10 min (C and D). E and F, cytochalasin B and metabolic inhibitors were added to unstimulated cells (filled-red), compared to 2 min (open-black) and 10 min trypsin stimulation (open-green). Data is representative of two experiments.

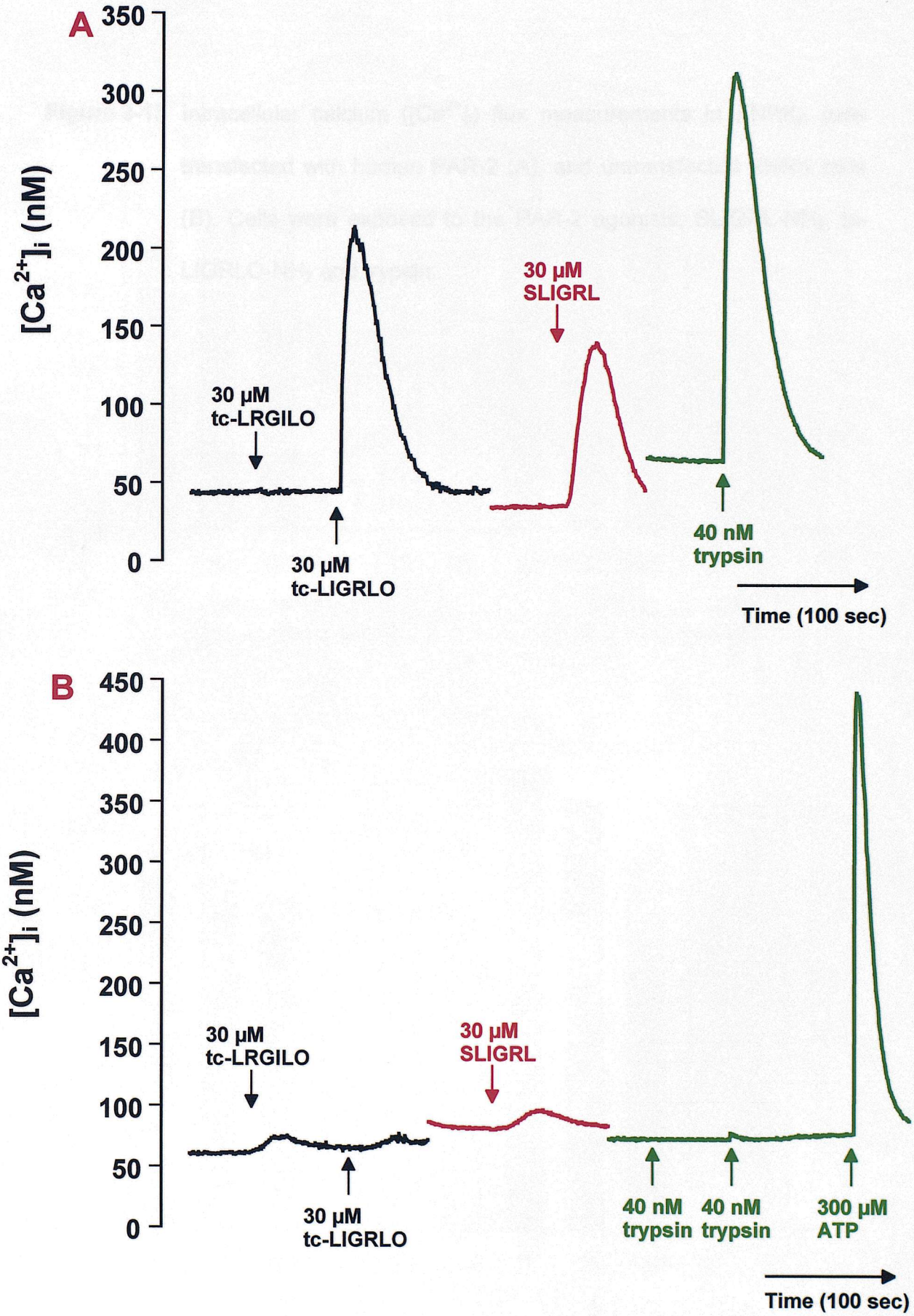
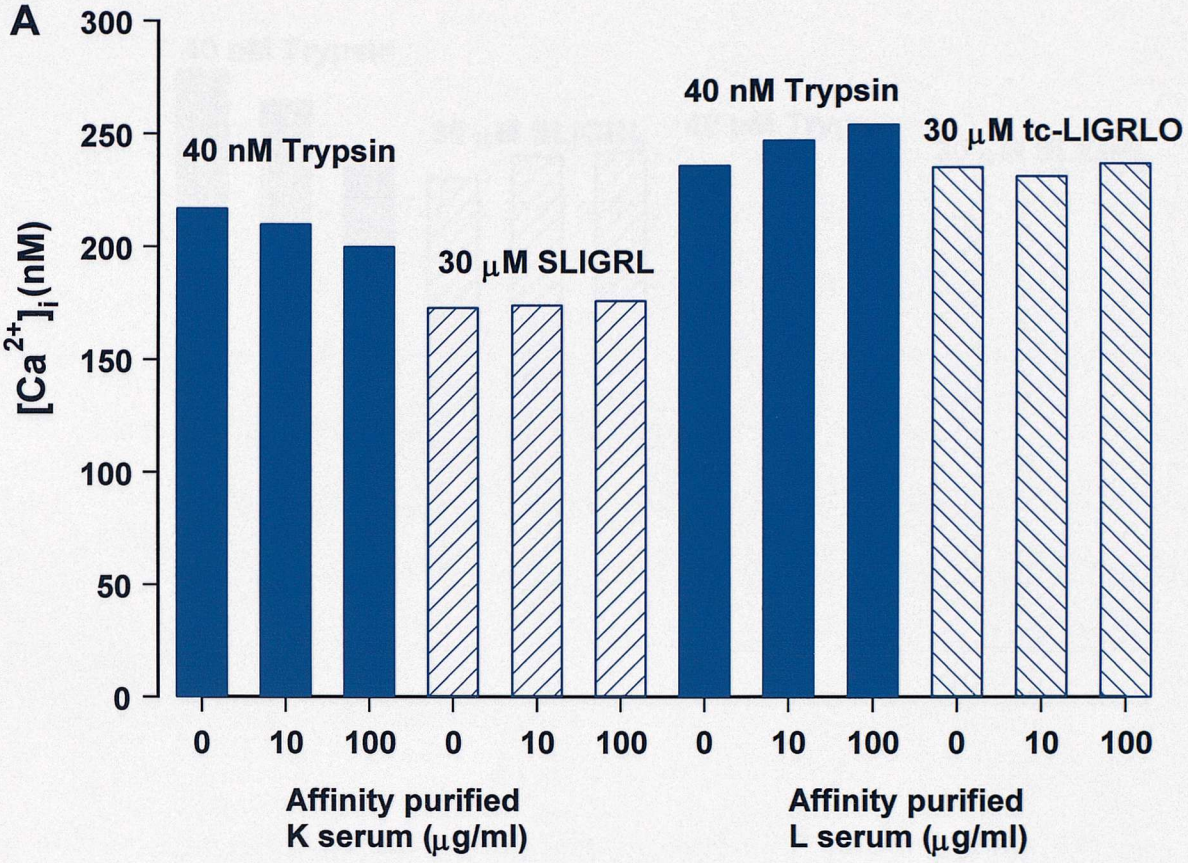
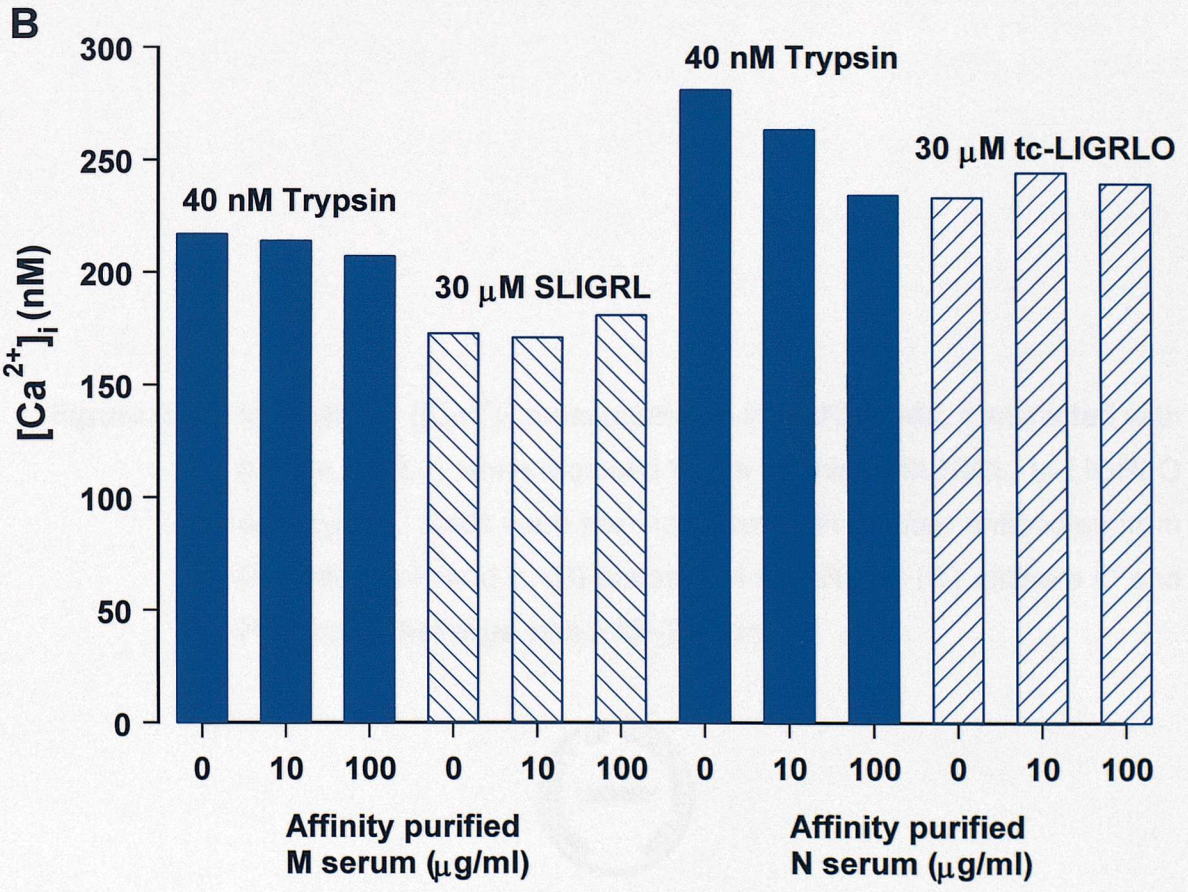

A
350
300
250
200
150
100
50
0
[Ca2+]i (nM)
30 µM
tc-LRGILO
30 µM
tc-LIGRLO
30 µM
SLIGRL
40 nM
trypsin
Time (100 sec)
B
450
400
350
300
250
200
150
100
50
0
[Ca2+]i (nM)
30 µM
tc-LRGILO
30 µM
tc-LIGRLO
30 µM
SLIGRL
40 nM
trypsin
40 nM
trypsin
300 µM
ATP
Time (100 sec)

Figure 3-13 Intracellular calcium ($[Ca^{2+}]_i$) flux measurements in $KNRK_t$, cells transfected with human PAR-2 (A), and untransfected KNRK cells (B). Cells were exposed to the PAR-2 agonists, SLIGRL-NH_2, tc-LIGRLO-NH_2 and trypsin.


A
300
250
200
150
100
50
0
$[Ca^{2+}]_i$(nM)
40 nM Trypsin
30 μM SLIGRL
40 nM Trypsin
30 μM tc-LIGRLO
0 10 100 0 10 100 0 10 100 0 10 100
Affinity purified
K serum (μg/ml)
Affinity purified
L serum (μg/ml)


B
300
250
200
150
100
50
0
$[Ca^{2+}]_i$(nM)
40 nM Trypsin
30 μM SLIGRL
40 nM Trypsin
30 μM tc-LIGRLO
0 10 100 0 10 100 0 10 100 0 10 100
Affinity purified
M serum (μg/ml)
Affinity purified
N serum (μg/ml)

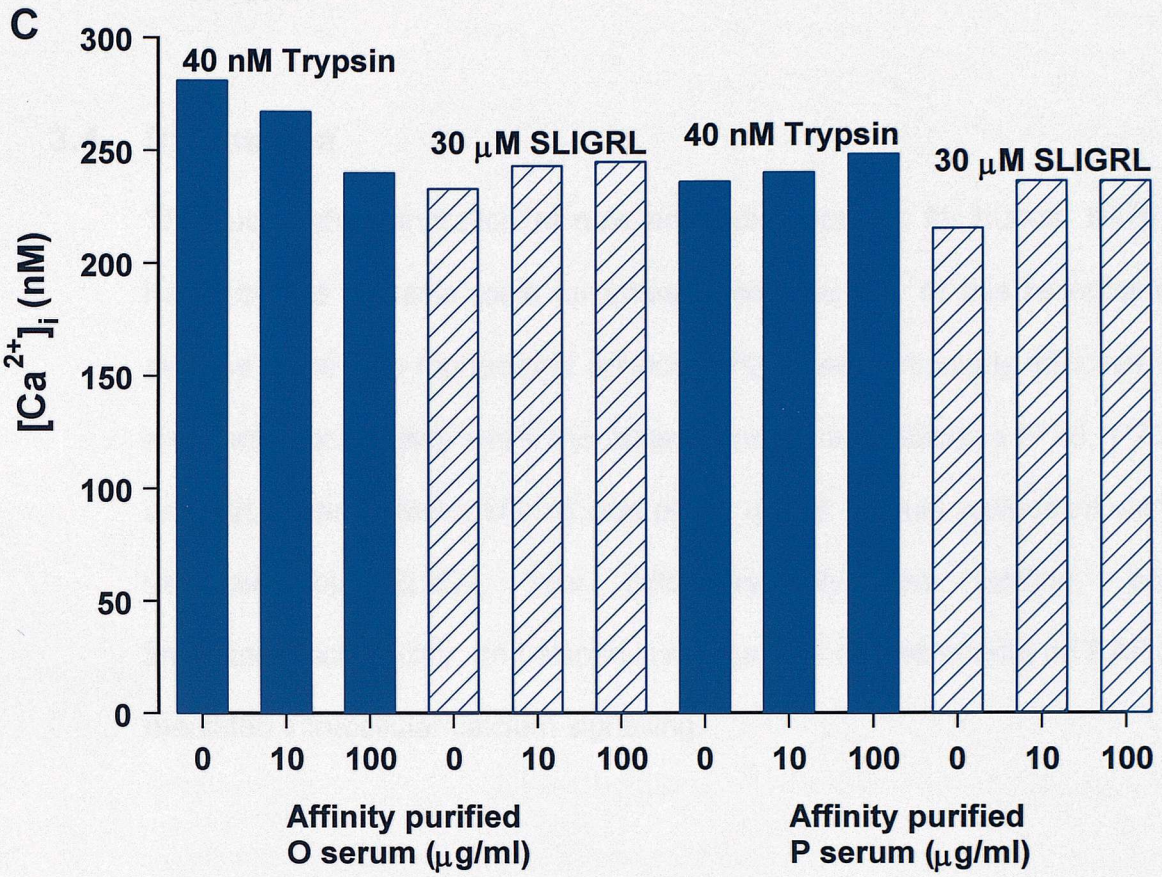


Figure 3-14 Intracellular ($[Ca^{2+}]_i$) measurements in $KNRK_t$ cells, transfected with human PAR-2, when exposed to the agonists, SLIGRL, tc-LIGRLO and trypsin. Cells were pre-incubated with purified antibodies from (A) antisera K and L, (B) antisera M and N and (C) antisera O and P, prior to challenge with PAR-2 agonists.

3.4 Discussion

The successful generation of new antibodies specific for human PAR-2 has provided valuable tools for investigating the role of this receptor in disease. The new monoclonal antibody, P2A was particularly effective in immunohistochemistry while the other monoclonal antibodies (P2B, P2C, and P2D), and antisera should also prove useful. These antibodies were validated by ELISA, flow cytometry, Western blotting, and immunohistochemistry, and also in investigations of their effects on PAR-2 mediated intracellular calcium signalling.

P2A is the first monoclonal antibody that permits reliable detection of PAR-2 expression in tissue specimens by immunohistochemistry, in flow cytometry and in Western blotting. The only other monoclonal antibody to have been used that was raised against a peptide fragment of human PAR-2 (SAM11), was not able to stain PAR-2 in tissue sections or to detect PAR-2 in $KNRK_t$ cells by Western blotting or flow cytometry. This may indicate that P2A reacts with a more robust epitope on PAR-2. P2A is also likely to have advantages over the B5 antiserum, as these antibodies were raised against a peptide fragment of rat PAR-2 (rather than human PAR-2). P2A should be more useful in studies of human disease.

Although the six antisera raised in the six different rabbits allowed detection of PAR-2 in ELISA or on $KNRK_t$ cells by flow cytometry, none of these antibodies stained PAR-2 in tissue sections. This suggests that the PAR-2 epitopes recognised may require application of an unmasking technique, as the polyclonal B5 has allowed staining of PAR-2 in tissue sections (Section 4.3.1.1). Pretreatment of tissues with proteases (such as trypsin or proteinase K) may be useful in future experiments using immunohistochemistry with these new antisera. However, the P2A antibody has proved very useful in immunohistochemistry, and was employed to investigate expression of PAR-2 in the diseased airways of subjects with asthma and rhinitis (Chapter 4).

Much of the PAR-2 detected in tissues or cells appeared to have an intracellular location. It is of interest to know if the antibodies detected cleaved or uncleaved PAR-2 receptor, whether on the surface or within the cell. Once the receptor is activated it may be rapidly internalised and further cell surface expression is dependent on golgi pools of new receptor (Dery *et al*, 1998).

After pre-treatment of $KNRK_t$ cells with cytochalasin B (which prevents receptor internalisation) and subsequent stimulation with trypsin, cell surface PAR-2 expression increased at 2 and 10 min compared to the unstimulated cells, suggesting that P2A may detect cleaved receptors

more readily. However, one must bear in mind that there was no difference between trypsin treatment for 2 min or 10 min, which suggests that although cleaved receptors are prevented from internalising, more intact receptors from the golgi pools may be expressed on the surface. Thus, the apparent change in expression may be a consequence of detecting both intact and cleaved PAR-2 receptors. It was not possible to treat $KNRK_t$ cells with trypsin for longer periods because this resulted in cell clumping. A study by Bohm *et* al, (1996) has suggested that PAR-2 shuttles between the membrane surface and cytoplasmic stores, and once cleaved the receptor is rapidly internalised, taking the cells between 30 and 60 min to recover sensitivity. In the present studies with $KNRK_t$ cells, it proved difficult to avoid cell clumping and so firm conclusions cannot be drawn on the movement of PAR-2 between the cytosol and cell surface in these cells.

Investigations of cleaved or uncleaved PAR-2 using Western blotting of $KNRK_t$ lysates were carried out with P2A only. The three bands observed were all related to PAR-2 expression and are likely to be different glycoforms. PAR-2 is known to be heavily glycosylated (Compton *et al*, 2001). Densitometric analyses showed that although intensity of PAR-2 expression did not change in the 65 and 170 kDa protein bands at any time-point after trypsin stimulation, the intensity was marginally increased at 2 min in the 47 kDa band, suggesting that P2A detects the cleaved

receptor more readily. Moreover, the intensity of the band was not different from that observed in the unstimulated cell lysates at 10, 30 or 60 min after stimulation. This indicates that once activated, receptors could be down-regulated as a consequence of receptor internalisation. Furthermore, the intensity of PAR-2 expression increased within the 47 kDa protein band at 2, 10, 30 or 60 min after SLIGKV-NH_2 stimulation (a process which will not cleave the tethered ligand). This further suggests that P2A detects the cleaved form of PAR-2 receptors more readily. These experiments would need to be carried out on a larger scale, and corroborative evidence obtained using the polyclonal antisera.

The antibodies produced against different regions of PAR-2 did not appear to have blocking actions against PAR-2 activation, and do not fulfil the long held need for specific receptor antagonists. P2A is an IgM sub-class of antibody, and due to the shear size of this pentameric large molecule, in theory it should be a very effective neutralising antibody. This lack of inhibition can be explained by the epitope recognition site that is detected by P2A, since this antibody detects peptide B in ELISA, but not peptide A, suggesting that the antibody does not bind to SLIGKV as these amino acids are the only sequences homologous to both peptides. This could then explain the reason for the inability of P2A in neutralising the activation of PAR-2, since the sequence may still be exposed to external cleavage even with bound antibody. Nonetheless, these antibodies

should prove valuable tools for investigating the extent of PAR-2 activation.

CHAPTER 4

PAR-2 Expression in the Lower (Asthma) and Upper (Rhinitis) Airways

4. PAR-2 Expression in the Lower (Asthma) and Upper (Rhinitis) Airways

4.1 Introduction

The respiratory epithelium was once thought of a relatively inert barrier to the outside environment. It is now clear that the epithelium has many functions in the airways other than its role as a barrier. The epithelium is involved in the regulation of fluid and ion transport across the airway and into the airway lumen, modulation of airway smooth muscle tone, and modulation of inflammatory cells and mediator secretion, in addition to its role in protection of airway cells and structures from the external environment (White & Leff, 2000). Disruption of these functions may contribute substantially to airway inflammation, oedema formation, mucus formation and airway constriction in airways' disease. Airway epithelial cells are the first line of defence, *i.e.* the first cell type in the respiratory mucosa to be exposed to environmental stimuli associated with airway inflammation. Activation of these cells by mediators derived from inflammatory cells or inhaled allergens can release a plethora of pro-inflammatory mediators (Holgate *et al*, 1999).

Endogenous and exogenous proteases are mediators known to be involved in inflammatory processes and are thought to play critical roles in the pathogenesis of asthma and rhinitis (Walls *et al*, 2000). Mast cell tryptase is one such enzyme, and levels of this mast cell protease are

elevated In BAL and sputum taken from asthmatics subjects compared to non-asthmatic control subjects (Broide *et al*, 1991; Jarjour *et al*, 1991), concentrations may be further increased following allergen challenge (Wenzel *et al*, 1988). Levels of tryptase are also increased in nasal lavage fluid from allergic rhinitic subjects compared to those without rhinitis (Wilson *et al*, 1998).

As mast cell tryptase is secreted in greater quantities into the airways of asthmatic and rhinitic subjects, there would be a potential for a greater degree of PAR-2 activation in these conditions. Further to this, there is evidence that PAR-2 may have pro-inflammatory roles in diseases of the airways. Activation of PAR-2 on airway epithelial cells have been shown to induce the release of MMP-9, GM-CSF, eotaxin, IL-6, IL-8 and PGE_2 (Vliagoftis *et al*, 2000 & 2001; Sun *et al*, 2001; Asokananthan *et al*, 2002). On the other hand, the possibilities of beneficial roles for PAR-2 have been proposed, particularly in preventing bronchoconstriction of the airways (Cocks *et al*, 1999). The extent to which PAR-2 may be expressed and regulated in the airways is not known, and answering these questions would help to evaluate the overall role of PAR-2 in the airways in asthma and rhinitis.

Therefore we investigated the expression of PAR-2 in the lower (asthma) and upper (rhinitis) airways by immunohistochemistry. We hypothesised

that PAR-2 activation may make an important contribution to the inflammation occurring in asthma and rhinitis and that its expression may be increased in these conditions.

4.2 Methods

4.2.1 Subjects

Bronchial biopsy tissue was collected from eight subjects with mild asthma (median age of 34; range 27-40), eight subjects with severe asthma (median age of 44; range 20-67) and from 15 control subjects without asthma (median age of 23; range 18-48). The mild asthmatics had normal lung function and though they were prescribed β-adrenoceptor agonists for use on demand, they were receiving no prophylactic treatment. The severe asthmatics had an FEV_1 of predicted value less than 80% and were receiving combination therapy with inhaled or oral corticosteroids.

A further six subjects with mild asthma (median age of 39; range 35-57) underwent bronchial challenge with saline or house dust mite allergen (*Dermatophagoides pteronyssinus*), and bronchial biopsy tissue was collected six hours post-challenge. For these subjects a skin-prick test dose-response series was undertaken with serial 10-fold dilutions of the allergen that produced the largest response. The final concentration used for endobronchial instillation was 1/10 of the lowest that produced at least a 3 mm wheal.

Nasal biopsy tissue was collected from eight subjects with perennial allergic rhinitis (median age of 38; range 27-44) and six control subjects without rhinitis (median age of 34; range 25-42). Patients with perennial

allergic rhinitis were house dust mite sensitive (*D. pteronyssinus*) with current moderate to severe symptoms and without prophylactic medication. Control subjects were non-atopic, with no history of rhinitis and no current symptoms.

A further six subjects with seasonal allergic rhinitis (out-of season; median age of 35; range 29-40) underwent nasal challenge with saline or grass pollen allergen. Nasal biopsy tissue was collected six hours post-challenge. These subjects had a history of seasonal allergic rhinitis for two years with no current therapy, exhibited positive skin-prick tests to grass pollen and positive immediate nasal response to grass pollen allergen challenge.

The studies were approved by the Southampton and Southwest Hampshire Local Research Ethics Committee.

4.2.2 Immunohistochemistry

Biopsies were processed into GMA resin (section 2.8.1) and then stained using the technique described in section 2.8.2. Sections were immunostained for PAR-2 using monoclonal antibody P2A and the B5 antiserum. Sections were also immunostained for specific monoclonal antibodies to mast cell tryptase (clone AA1, Walls *et al*, 1990), eosinophils

(clone EG2, Serotec, Oxford, UK), neutrophils (elastase, Serotec, Oxford, UK), myofibroblasts (vimentin, clone V9), macrophages (CD68, clone PG-M1), B lymphocytes (CD20, clone L26) and T lymphocytes (CD3, clone UCHT-1). Negative control experiments involved omitting the primary antibody and the inclusion of isotype-matched antibodies to irrelevant antigens. An additional negative control for PAR-2 staining was employed. This consisted of using the antibody which had been pre-adsorbed with 10 μM immunisation peptide for 24 hr at 4 °C prior to application to the sections.

Relative PAR-2 staining intensity was quantified by computerised image analysis (Zeiss, Oberkochen, Germany) performed while blinded to the codes. Epithelial DAB staining was expressed as a percentage of the total intact epithelium present. Cell counts for mast cells, eosinophils and neutrophils were compared to sequential sections stained for PAR-2 to determine whether the cells expressed the receptor, using a *camera lucida* system mounted on a microscope (Leica, Wetzlar, Germany).

4.2.3 Statistics

Differences between groups were analysed using non-parametric statistics. The Mann Whitney U-test was employed to compare unpaired data and Wilcoxon's-test for differences in paired data. Spearman's rank

correlation coefficient (r_s) was calculated to assess the extent of correlation between results obtained using the two antibodies and to compare PAR-2 expression with lung function measurements or inflammatory cell number. *P* values of less than 0.05 were taken as significant.

4.3 Results

4.3.1 Expression of PAR-2 in the Lower Airways

4.3.1.1 Cellular localisation of PAR-2

PAR-2 staining, using P2A antibody, was localised chiefly on the epithelium (Fig. 4-1A, B and C), with a small number of inflammatory cells stained in the underlying submucosa of a minority of sections. The epithelial PAR-2 staining was more apparent in the columnar epithelial cells and appeared to be located to the basolateral side of individual epithelial cells. In biopsies from subjects with severe asthma the PAR-2 immunoreactivity appeared to be on the apical side of columnar epithelial cells, in addition to the basolateral side (Fig. 4-1C; observed in eight of eight subjects). Staining was abolished by pre-adsorption of the antibody with the peptide (^{29}TNRSSKGR↓SLIGK^{42}VC) used for immunisation (Fig. 4-1D). Of the small number of cells stained for PAR-2 in the *lamina propria*, co-localisation studies showed that they were not mast cells (none of 120 AA1-staining cells in tissues analysed from 12 subjects), eosinophils (none of 106 EG-2-staining cells in tissues analysed from 12 subjects) or neutrophils (None of 240 elastase-staining cells in tissues analysed from 12 subjects).

Staining with the B5 antiserum also appeared to be on the basolateral surface of columnar epithelial cells (Fig. 4-2). In contrast to the staining

pattern seen with P2A antibody, PAR-2 staining with B5 antiserum was not observed on the apical surface in severe asthmatic biopsies (Fig. 4-2C).

4.3.1.2 Quantification of PAR-2 expression in the asthmatic epithelium

Using computerised image analysis, median expression of PAR-2 immunoreactivity stained with P2A antibody was found to be substantially greater in the bronchial epithelium of severe asthmatics than in that of control subjects ($p<0.05$, Fig 4-4A). This increase in PAR-2 expression did not correlate with measurements of lung function (Fig 4-5A), or with numbers of infiltrating mast cells or eosinophils (Fig 4-5B and C). When PAR-2 immunoreactivity using B5 antiserum was quantified, higher levels of expression were also found in the severe asthmatic epithelium than in the control or mild asthmatic epithelium ($p<0.05$, Fig. 4-4B), using this antibody. There was a significant correlation between PAR-2 immunostaining with the two antibodies ($r_s = 0.786$, $p < 0.05$) (Fig. 4-3).

4.3.1.3 PAR-2 expression following endobronchial allergen challenge

The distribution of PAR-2 in the epithelium did not appear to differ after house dust mite allergen challenge (Fig. 4-6), with staining located to the basolateral side of columnar epithelial cells. PAR-2 expression was approximately doubled in the allergen-challenged bronchial epithelium compared to the epithelium of the saline-treated lung ($p<0.05$, Fig. 4-7).

The increase in PAR-2 expression was accompanied by an elevation in the number of tryptase-positive mast cells infiltrating the underlying *lamina propria* ($p<0.05$, $n=6$), but not of neutrophil or eosinophil numbers (data not shown).

4.3.2 Expression of PAR-2 in the Upper Airways of Rhinitic and Healthy Subjects

4.3.2.1 Cellular localisation of PAR-2 in nasal tissue

PAR-2 was localised mainly to the nasal epithelium of rhinitic subjects (Fig. 4-8), using B5 antiserum. However, there were no significant differences between the staining intensity of nasal biopsies from control and subjects with perennial allergic rhinitis (Fig. 4-9). The staining pattern differed between the two groups in that PAR-2 was localised mainly to the columnar epithelial cells in the nasal epithelium of control subjects (Fig. 4-8a), whereas in tissue from subjects with perennial allergic rhinitis, PAR-2 was also observed on the basal cells (Fig. 4-8B). No association was found between PAR-2 staining intensity and numbers of infiltrating mast cells or eosinophils (Fig. 4-10). Co-localisation studies indicated that the mast cells or eosinophils did not express PAR-2 (0 out of 140 cells analysed, $n=14$).

4.3.2.2 PAR-2 expression following nasal allergen challenge

Epithelial staining was mainly restricted to columnar epithelial cells at the basolateral side and its distribution was not altered following challenge (Fig. 4-11). After challenge with grass pollen allergen, PAR-2 expression in the nasal epithelium was some 50% less than that following saline challenge ($p<0.05$, Fig. 4-12).

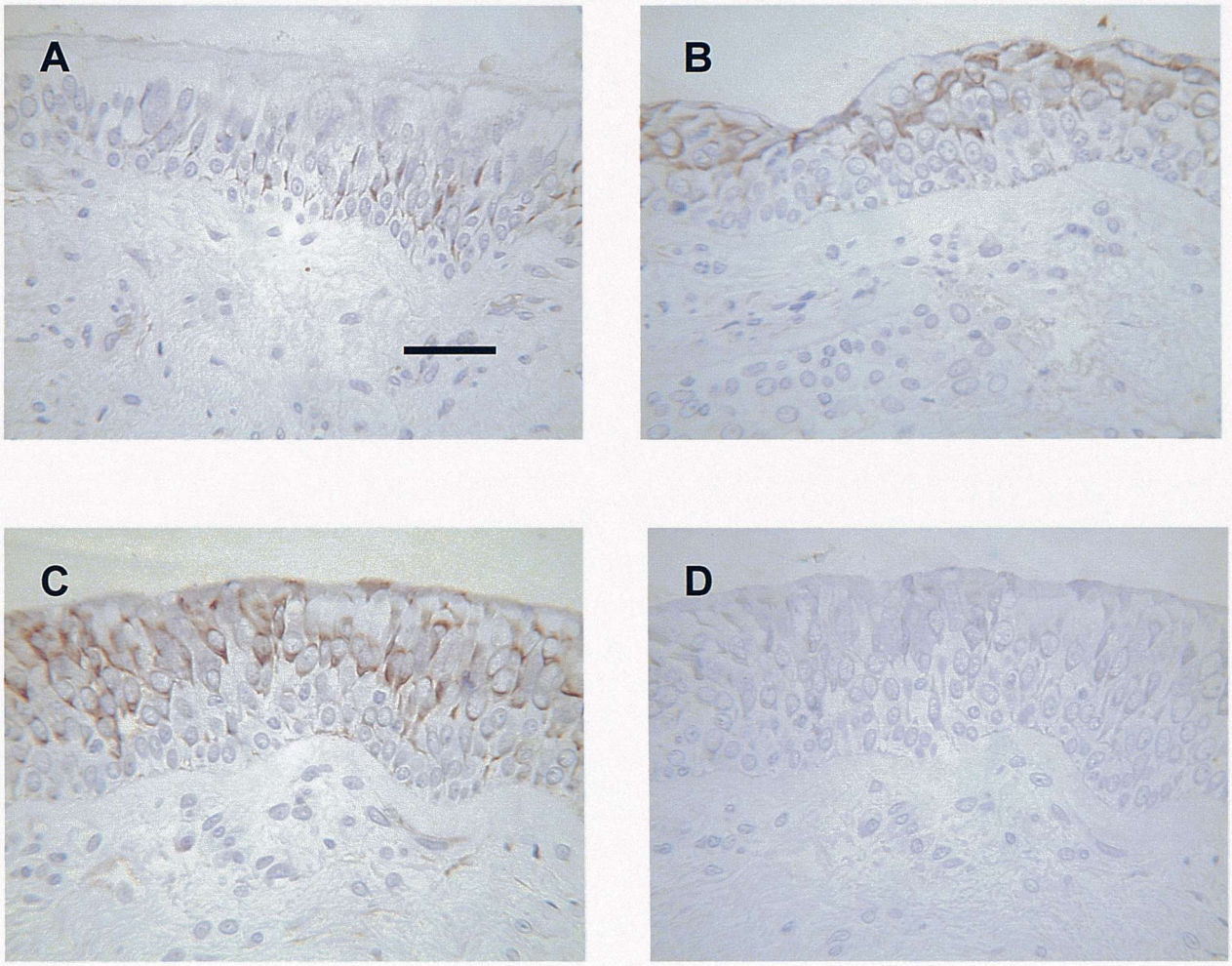


Figure 4-1 PAR-2 expression (brown staining) in the bronchial epithelium of a non-asthmatic control subject (A), a mild asthmatic (B) and a subject with severe asthma (C). Sections were stained using P2A antibody and intensity of staining was quantified using computerised image analysis. D, P2A antibody was pre-adsorbed with the peptide used for antibody generation, in tissue from the same severe asthmatic subject as in C. Scale bar represents 5 μm.

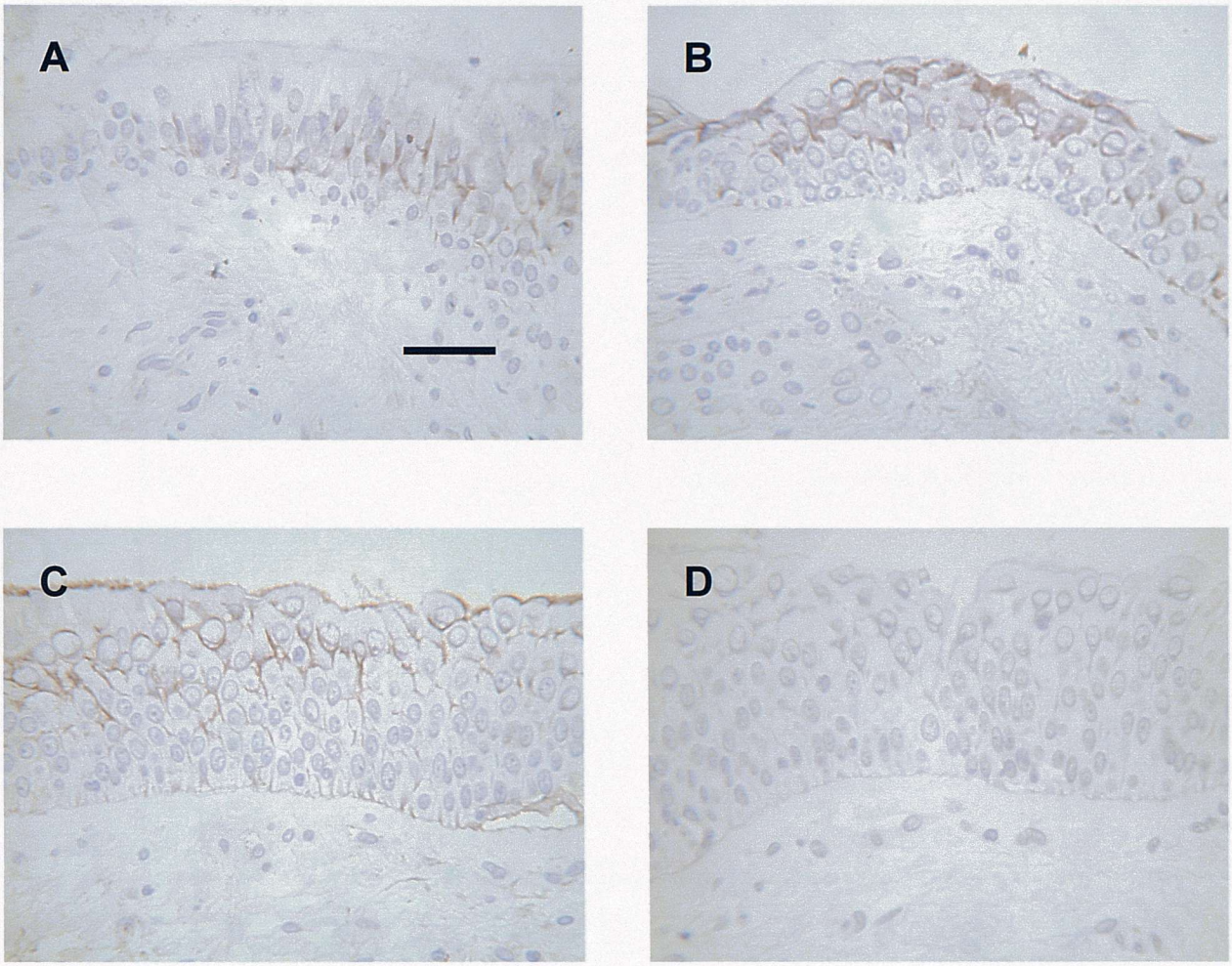


Figure 4-2 PAR-2 expression (brown staining) in the bronchial epithelium of a non-asthmatic control subject (A), a mild asthmatic (B) and a subject with severe asthma (C). Sections were stained using the B5 antiserum and intensity of staining was quantified using computerised image analysis. D, B5 antiserum was pre-adsorbed with the peptide used for antibody generation, in tissue from the same severe asthmatic subject as in C. Scale bar represents 5 μm. The sections of biopsy tissue used were from the same patients as shown in Fig. 4-1.

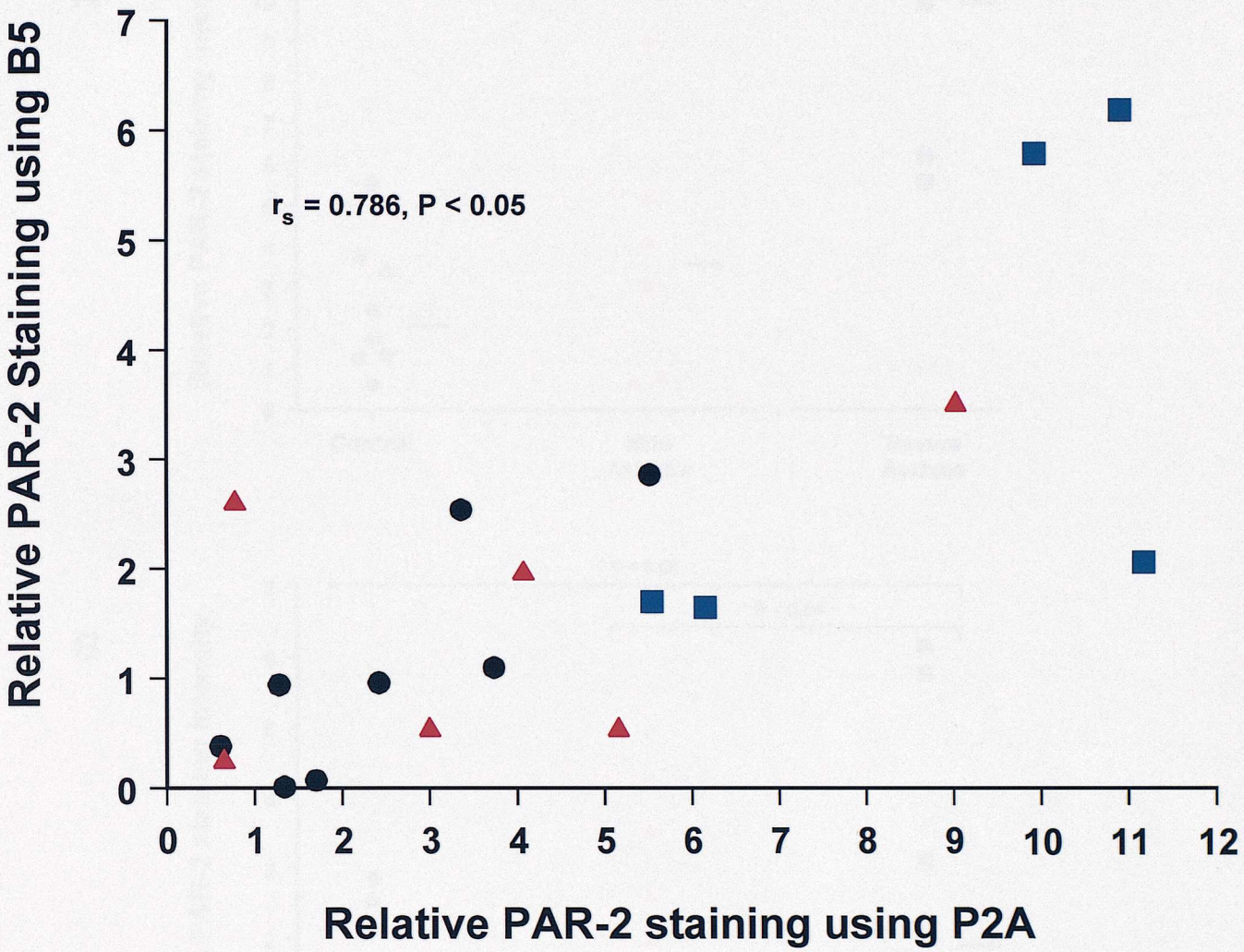


Figure 4-3 Comparison of the PAR-2 staining intensity in bronchial tissue from control subjects (●), mild asthmatics (▲) and subjects with severe asthma (■), using antibodies P2A and B5.

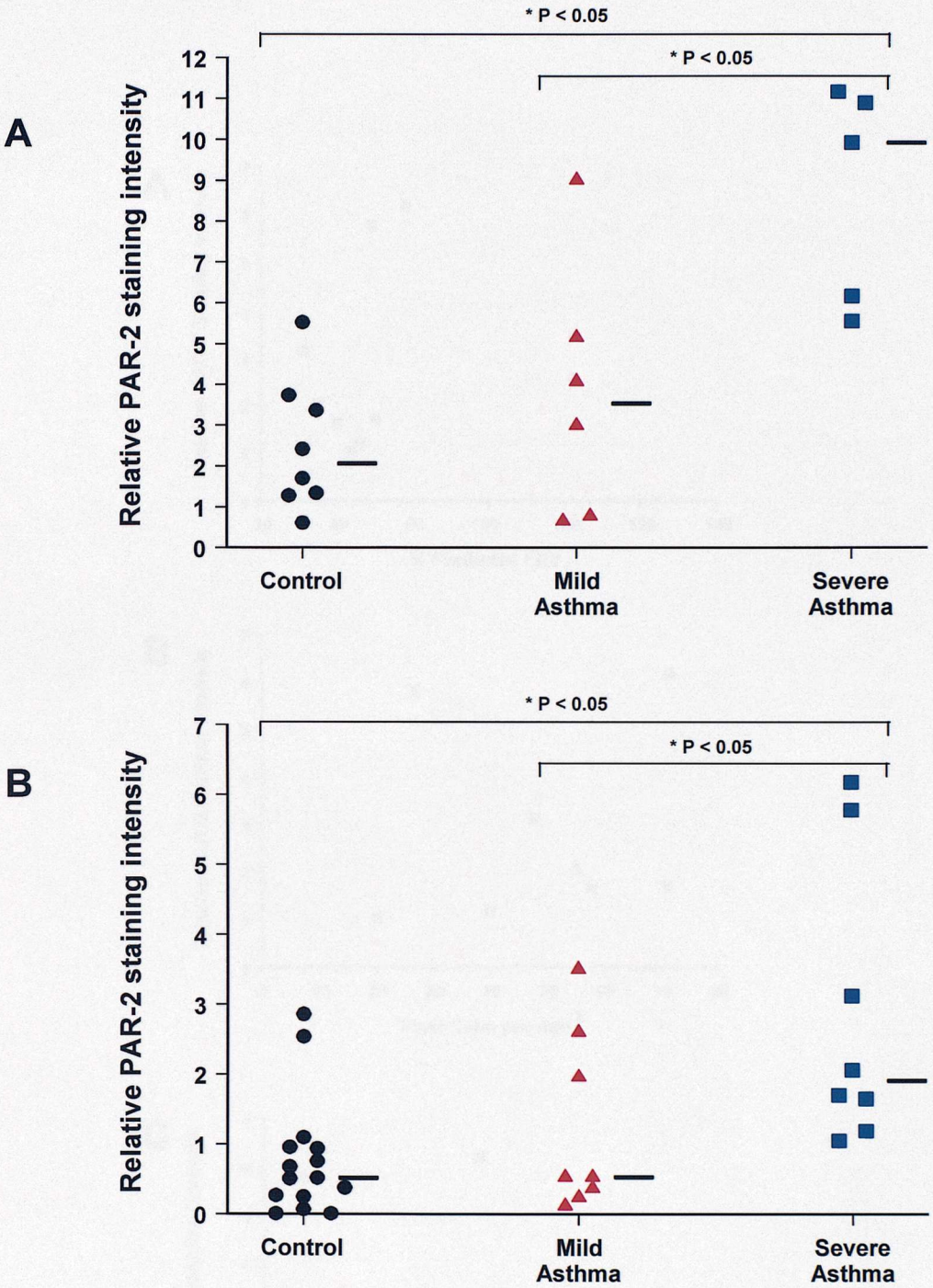


Figure 4-4 PAR-2 immunoreactivity in bronchial biopsy tissue taken from control subjects, mild asthmatics and subjects with severe asthma. Sections were stained with monoclonal antibody P2A (A) or with the B5 antiserum (B), and intensity of staining was quantified using computerised image analysis. Median values are indicated by bars.

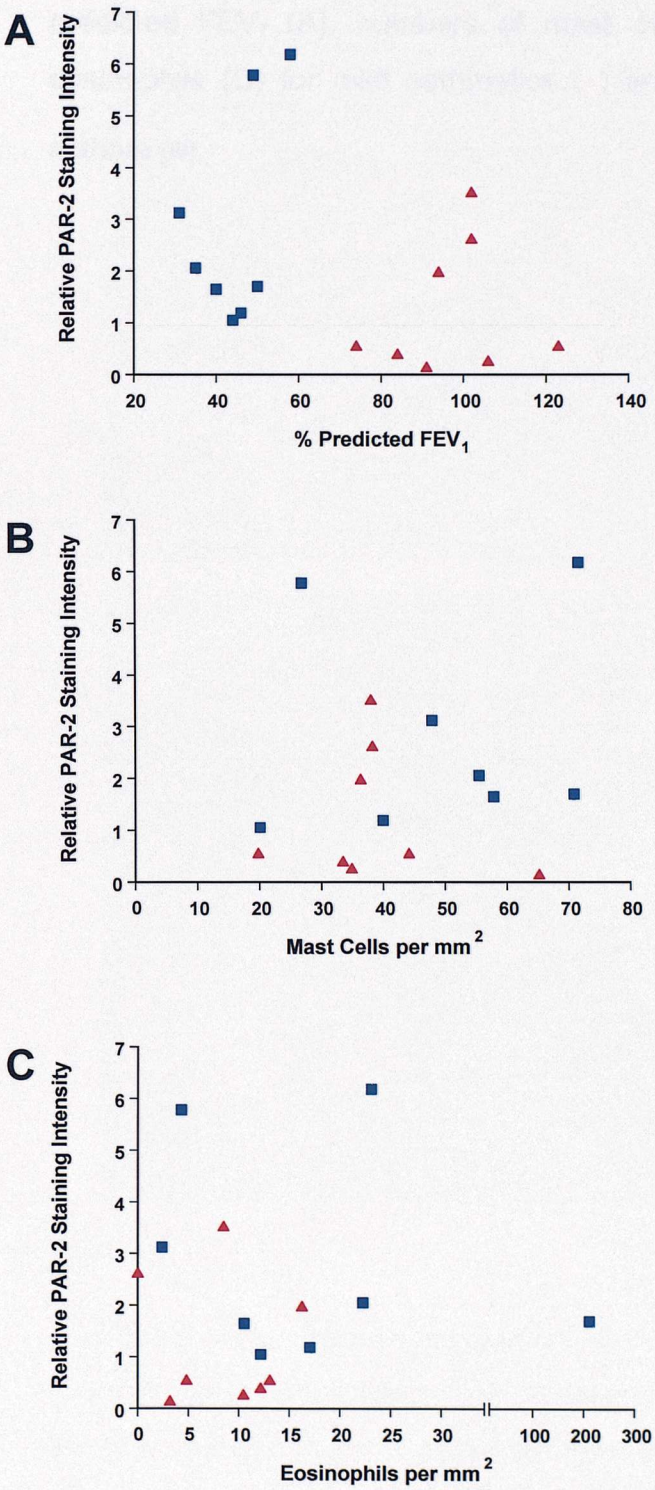

A
Relative PAR-2 Staining Intensity
0
1
2
3
4
5
6
7
20
40
60
80
100
120
140
% Predicted FEV1
B
Relative PAR-2 Staining Intensity
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
Mast Cells per mm2
C
Relative PAR-2 Staining Intensity
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
100
200
300
Eosinophils per mm2

Figure 4-5 Lack of association between PAR-2 immunoreactivity and % predicted FEV_1 (A), numbers of mast cells (B) or numbers of eosinophils (C) for mild asthmatics (▲) and subjects with severe asthma (■).

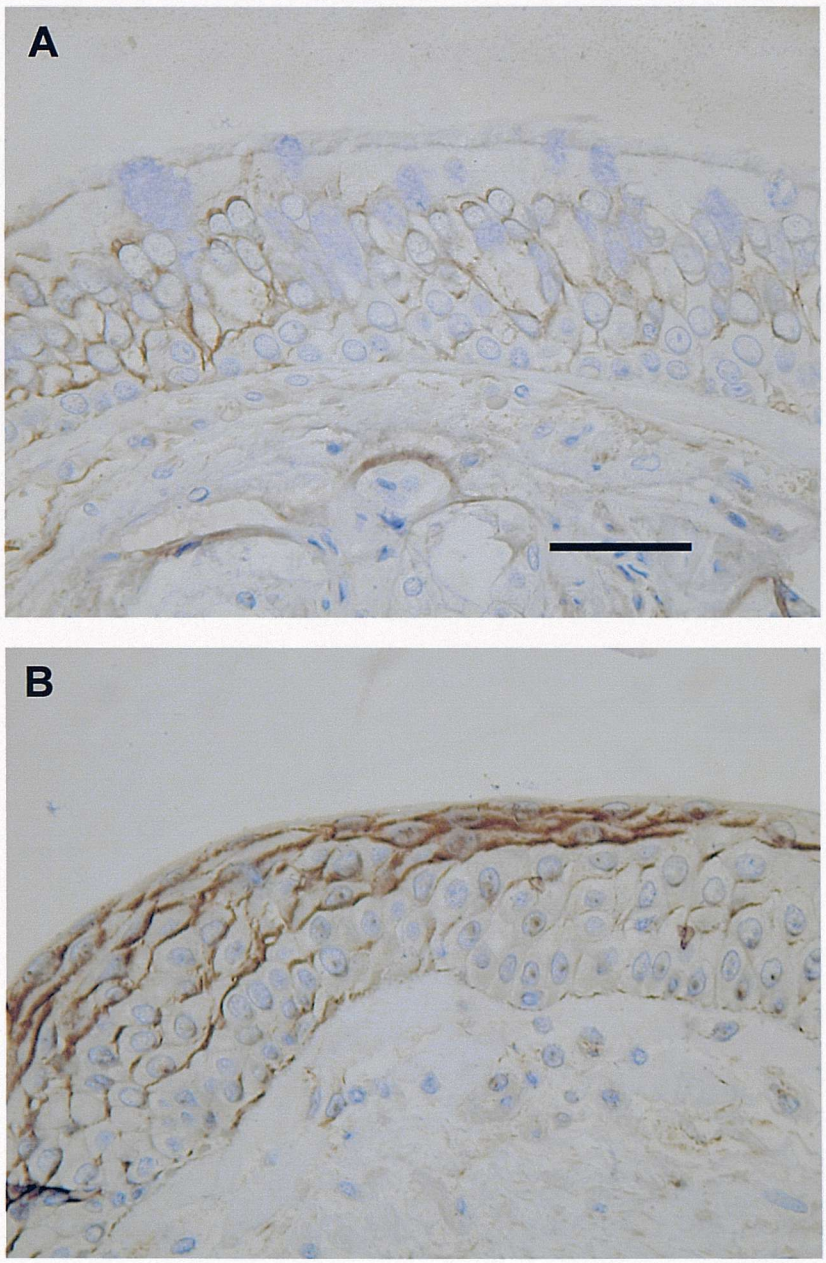


Figure 4-6 PAR-2 expression (brown staining) in the bronchial epithelium of a mild asthmatic subject six hours following challenge with either saline (A) or grass pollen allergen (B). Sections were stained using the P2A antibody and intensity of staining was quantified using computerised image analysis. Scale bar represents 5 μm.

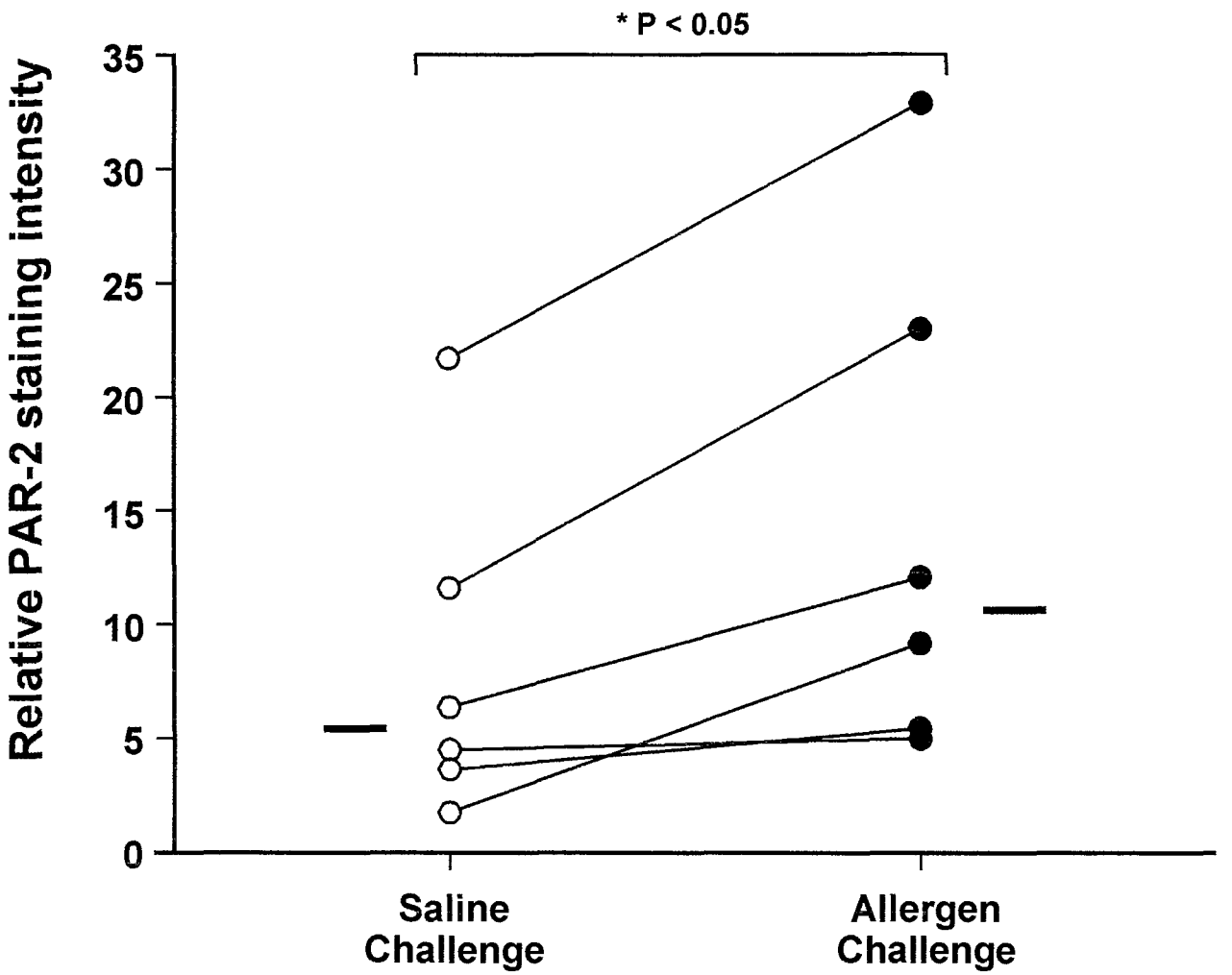


Figure 4-7 PAR-2 immunoreactivity in bronchial biopsy tissue taken from six subjects with mild asthma, six hours following challenge with either saline or house dust mite allergen. Sections were stained using P2A antibody and intensity of staining was quantified by computerised image analysis. Median values are indicated by bars.

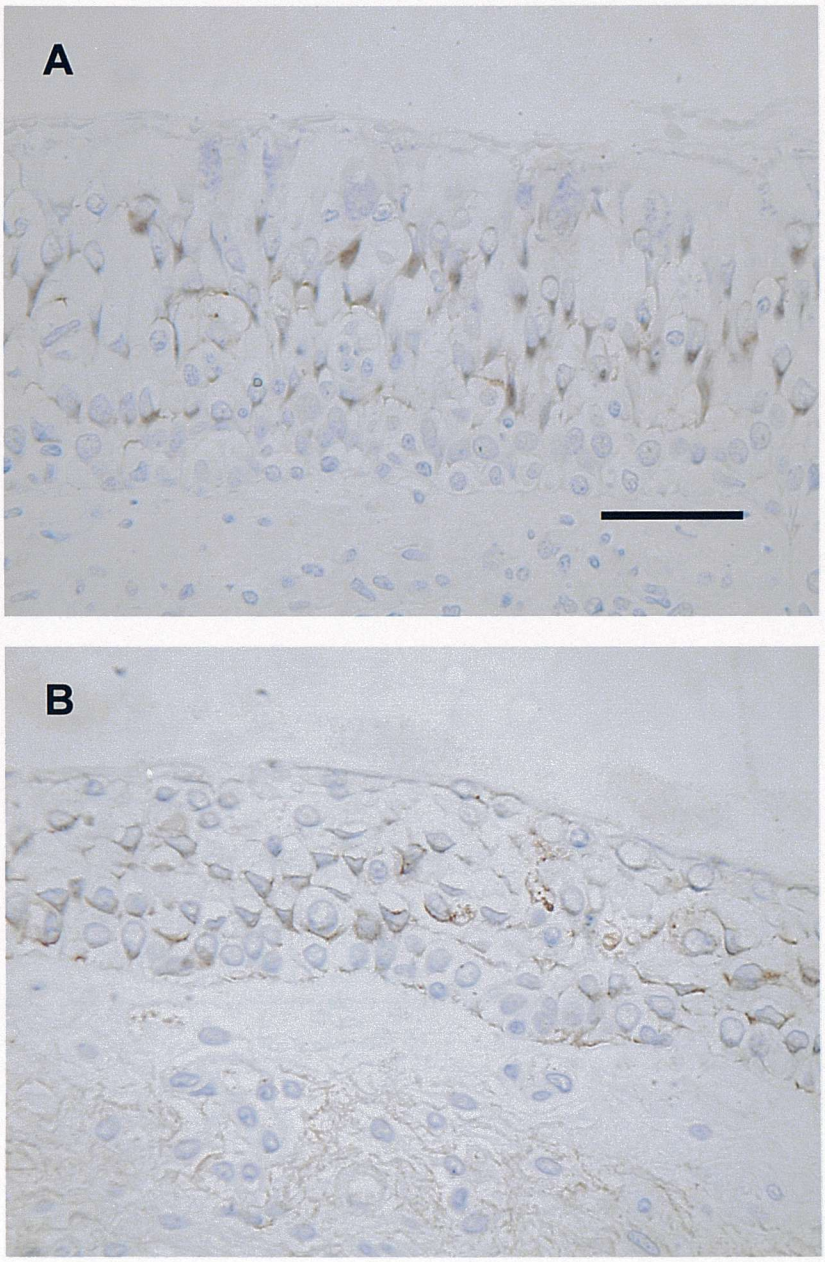


Figure 4-8 PAR-2 expression (brown staining) in the nasal epithelium of a control subject (A), and a subject with perennial allergic rhinitis (B) and a subject with severe asthma (C). Sections were stained using the B5 antiserum and intensity of staining was quantified using computerised image analysis. Scale bar represents 5 μm.

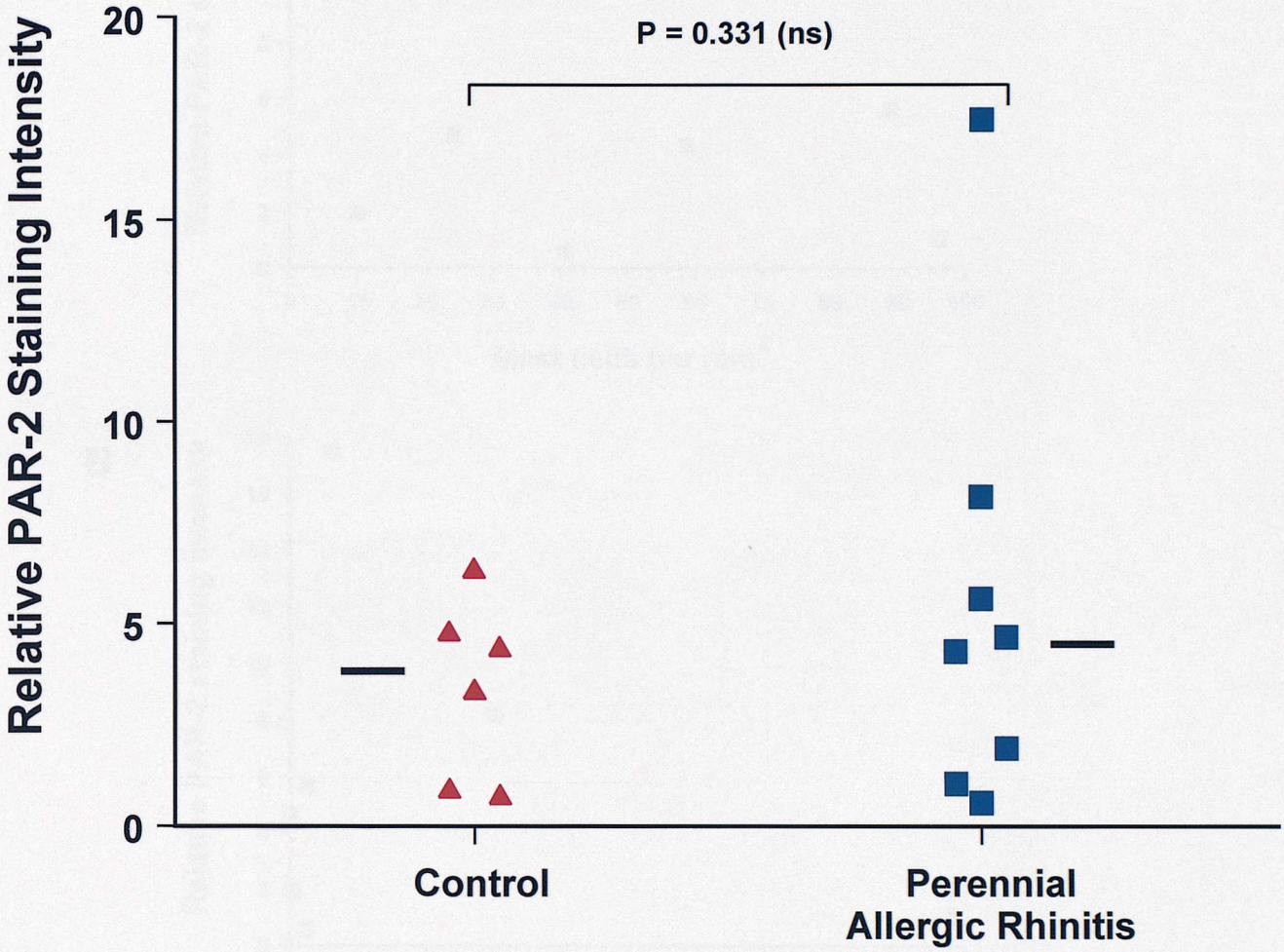


Figure 4-9 PAR-2 immunoreactivity in nasal biopsy tissue taken from control subjects, and subjects with perennial allergic rhinitis. Sections were stained with the B5 antiserum and intensity of staining was quantified using computerised image analysis. Median values are indicated by bars.

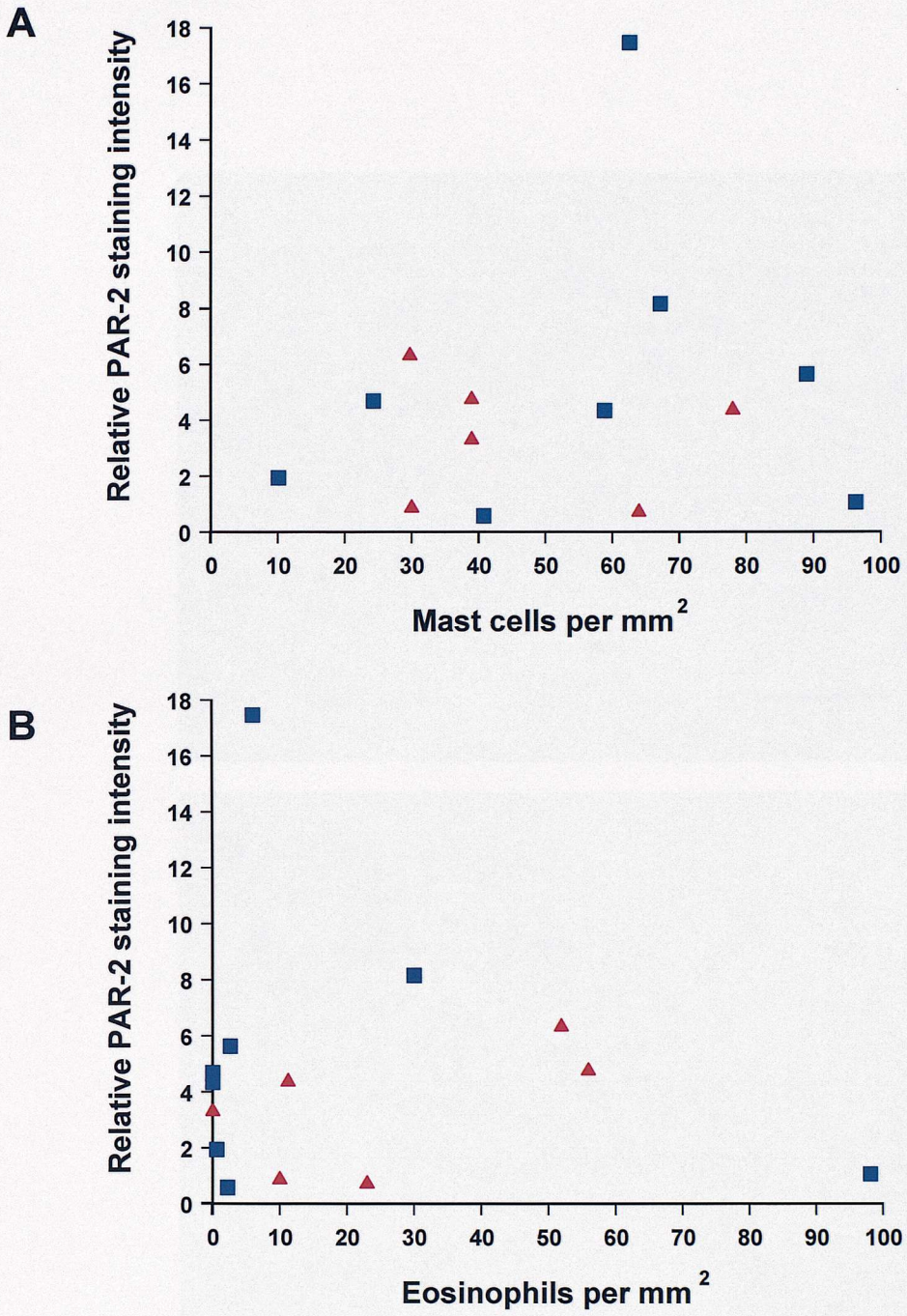


Figure 4-10 Lack of associations between PAR-2 immunoreactivity and numbers of mast cells (A) or eosinophils (B) for control subjects (▲) and subjects with perennial allergic rhinitis (■).

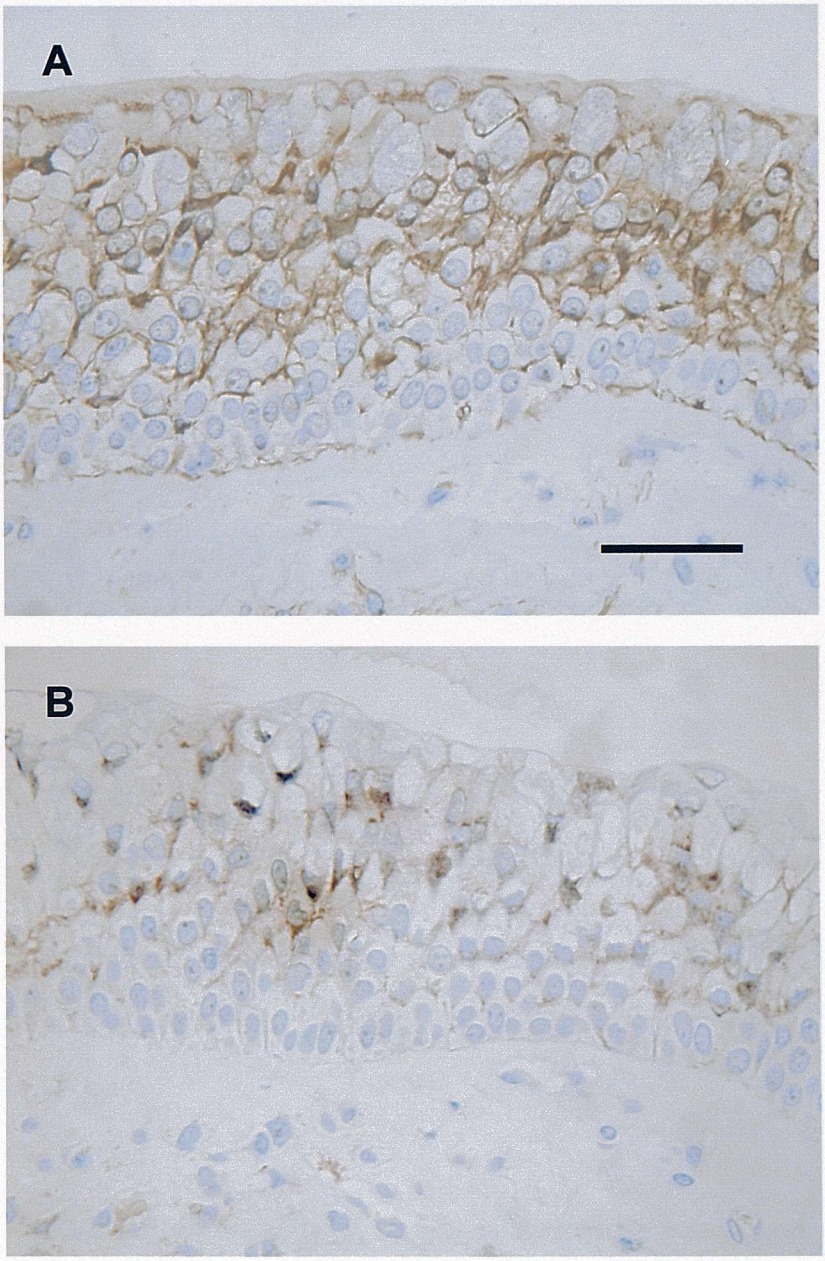


Figure 4-11 PAR-2 expression (brown staining) in the nasal epithelium of a subject with seasonal allergic rhinitis (out-of-season), six hours following challenge with either saline (A) or grass pollen allergen (B). Sections were stained using P2A antibody. Scale bar represents 5 μm.

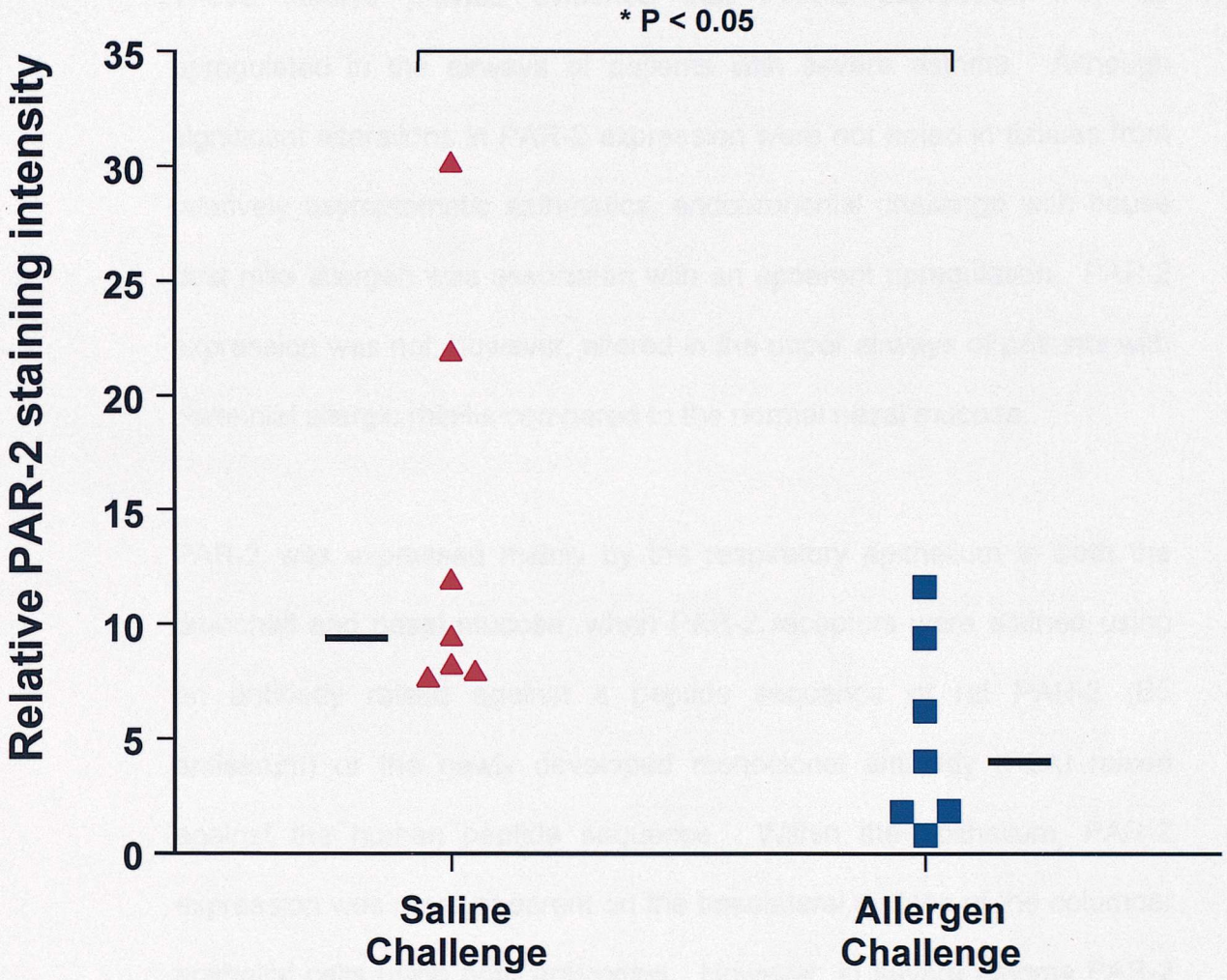


Figure 4-12 PAR-2 immunoreactivity in nasal biopsy tissue taken from six subjects with seasonal allergic rhinitis (out-of-season), seven hours following challenge with either saline or grass pollen allergen. Sections were stained using P2A antibody and intensity of staining was quantified using computerised image analysis. Median values are indicated by bars.

4.4 Discussion

These studies provide evidence that PAR-2 expression may be upregulated in the airways of patients with severe asthma. Although significant alterations in PAR-2 expression were not noted in tissues from relatively asymptomatic asthmatics, endobronchial challenge with house dust mite allergen was associated with an apparent upregulation. PAR-2 expression was not, however, altered in the upper airways of patients with perennial allergic rhinitis compared to the normal nasal mucosa.

PAR-2 was expressed mainly by the respiratory epithelium in both the bronchial and nasal mucosa, when PAR-2 receptors were stained using an antibody raised against a peptide sequence of rat PAR-2 (B5 antiserum) or the newly developed monoclonal antibody (P2A) raised against the human peptide sequence. Within the epithelium, PAR-2 expression was more apparent on the basolateral surface of the columnar epithelial cells using both antibodies. However, in severe asthma PAR-2 was also located on the apical surface using P2A antibody, but not B5 antiserum. The different staining patterns suggest that the antibodies recognise different epitopes on the receptor molecule, or possibly that P2A has a greater level of sensitivity. In addition, epithelial staining for PAR-2 with P2A revealed a small number of subepithelial cells expressing PAR-2 in some sections of bronchial and nasal tissue. Co-localisation studies performed showed that these cells were not mast cells,

eosinophils or neutrophils. These findings appear to be at variance with previous reports of PAR-2 expression by mast cells, eosinophils and neutrophils (D'Andrea *et al.*, 2000; Miike *et al.*, 2001; Howells *et al*, 1997), where the work had focused on isolated cells in culture as opposed to tissue sections. It is possible that the differences in findings could reflect differences in the activation state of cells, isolated cells may not be activated, whereas infiltrating cells are more likely to have been stimulated. Thus it may be that the PAR-2 antibodies employed bind activated receptors more readily.

The localisation of PAR-2 to the basal side of the epithelial cells in the airways suggests that the main activators are likely to be endogenous (*e.g.* tryptase) rather than exogenous (*e.g.* allergen proteases). This is in contrast to a report of PAR-2 expression on the apical surface of mouse bronchial epithelial cells (Cox *et al.*, 1999). Danahay and co-workers (2001) have shown that application to the basolateral surface of human bronchial epithelial cells of human airway trypsin-like (HAT) enzyme and trypsin, both tryptic proteases and agonists of PAR-2 activation, caused an increase in Ca^{2+} concentrations. The same agonists were without effect when applied to the apical surface, which consistent with our finding of PAR-2 localised to the basolateral surface.

The apparent upregulation of PAR-2 in the asthmatic airways suggests that the receptor could play a role in asthma. This is further supported by the observation of an apparent upregulation of PAR-2 in the bronchial airways following house dust mite allergen challenge. The increased PAR-2 expression in the bronchial epithelium six-hours following allergen challenge, was accompanied by an increase in the numbers of mast cells in the submucosa. A previous report has also shown increased numbers of mast cells in the airway mucosa of the late-phase allergen response (Crimi *et al.*, 1991).

Whether PAR-2 activation is likely to exacerbate or attenuate the symptoms of asthma is as yet undefined, as PAR-2 may exert complex effects in the airways. PAR-2 agonist peptides can induce the relaxation of human airway preparations through the release of PGE_2 from epithelial cells, leading to a powerful bronchodilatation (Cocks *et al.*, 1999). This is further supported by a report of PAR-2 mediated relaxation of mouse isolated tracheal tissue by the release of PGE_2 from airway smooth muscle cells (Lan *et al.*, 2002), pointing to the beneficial properties of PAR-2 activation in the airways. Also, PAR-2 agonist peptides were shown to reduce the histamine induced bronchial hyperresponsiveness in guinea pig isolated airway preparations (Cicala *et al.*, 2001). These reports are at variance with other studies with isolated tissue and *in vivo* studies in guinea pigs, which have highlighted the potential of PAR-2

activation to promote bronchoconstriction. Ricciardolo *et al.* (2000) reported that a PAR-2 agonist induced bronchoconstriction *in vivo*. A mixed bronchomotor effect was found with PAR-2 agonists *in vitro*, resulting in relaxation of isolated trachea and main bronchi and contraction of intrapulmonary bronchi. Saifeddine and co-workers (2001) on the other hand failed to elicit any contractile or relaxant responses to PAR-2 agonist peptides in guinea pig pulmonary strip preparations. These findings suggest that the effects of PAR-2 activation are highly dependent on the model employed and that caution is required in extrapolating these results to the airways of asthma patients.

The balance of the evidence has now swung in favour of PAR-2 activation contributing to features characteristic of asthma. Asokananthan *et al.* (2002) suggested an important role for PAR-2 in controlling inflammation of the lung. They showed that activation of PAR-2 on a number of human respiratory epithelial cell lines elicited the release of the pro-inflammatory cytokines IL-6, IL-8 and the prostanoid PGE_2, which may itself stimulate cytokine release. Other groups have observed the release of MMP-9 and GM-CSF from airway epithelial cells (Vliagoftis *et al.*, 2000 and 2001), contraction of isolated human bronchi leading to bronchoconstriction (Schmidlin *et al*, 2001), and human lung fibroblast (Akers *et al.*, 2000) and airway smooth muscle cell proliferation (Berger *et al.*, 2001). Thus, by acting on a variety of individual cell types, PAR-2 agonists may reproduce

some of the cardinal features of asthma, namely bronchoconstriction, inflammation and tissue remodelling.

Furthermore, Sun *et al.* (2001) have suggested that PAR-2 activation of lung epithelial cells by Der P 3 and Der P 9 can stimulate the release of the pro-inflammatory cytokines GM-CSF and eotaxin, which are known to be involved in the inflammatory process in asthma (Ackerman *et al.*, 1994; Ying *et al.*, 1997). As serine proteases from the house dust mite allergen faecal pellets (Smith *et al.*, 1994; King *et al.*, 1996), could activate PAR-2 on the airway epithelium and our findings of PAR-2 expression on the luminal surface of bronchial epithelial cells in severe asthma, this suggests a mechanism by which environmental stimuli may act directly on the epithelium to promote airway inflammation.

Although PAR-2 expression was increased in asthma, evidence for this was not found in perennial allergic rhinitis. Mast cell activation is a prominent feature in both allergic conditions and levels of mast cell tryptase in BAL and sputum are increased in allergic asthma (Broide *et al*, 1991; Jarjour *et al.*, 1991) and in subjects with perennial allergic rhinitis (Wilson *et al.*, 1998). Therefore one might expect that PAR-2 expression should be similarly increased these diseases. However, there is evidence that the regulation of PAR-2 expression in rhinitis may differ from that

observed in asthma. This is supported by our finding that PAR-2 expression may be downregulated after grass pollen challenge.

Although PAR-2 is increased in severe asthma, there was no association observed with FEV_1, used as an index for asthma severity. FEV_1 is a measure of airflow limitation and a decreased value compared to normal is suggestive of both inflammatory and remodelling processes. The severe asthmatic subjects in this study had lower than normal FEV_1 values but were on prophylactic medication to control their symptoms, and thus if FEV_1 was still decreased then this may have been due to remodelling processes only and not the underlying inflammation. It is well documented that PAR-2 has pro-inflammatory properties, though little is known of the actions of PAR-2 activation in remodelling processes. Thus, PAR-2 activation may not necessarily be linked to FEV_1 in the lower airways and may reflect more the control processes involved in inflammation.

The increased expression of PAR-2 in the lower airways suggests that these airways may become more sensitive to protease agonists of this receptor, such as tryptase and the house dust mite allergens. Inhibitors of PAR-2 function may therefore be beneficial agents in controlling the inflammatory, bronchoconstriction and remodelling processes associated with airways diseases, elicited by these enzymes.

CHAPTER 5

Regulation of Eosinophil Function by Tryptase and Activators of PAR-2

5 Regulation of Eosinophil Function by Tryptase and Activators of PAR-2

5.1 Introduction

There has long been evidence for the involvement of eosinophils in the inflammatory process of airway diseases. Tissue eosinophilia is a constant feature of allergic inflammation and correlates with high tissue levels of eosinophil granule proteins together with increased levels of eosinophil-derived cytokines (Sampson, 2000; Danahay *et al*, 1999). Eosinophils are present in large numbers in the airways of asthmatics and eosinophil degranulation with release of granule proteins is a prominent feature of asthma. Evidence for eosinophil activation in the asthmatic lung is provided by their morphological appearance (Beasley *et al*, 1989) and staining with EG2 monoclonal antibody (which recognises the secreted form of ECP) in bronchial biopsies (Djukanovic *et al*, 1990), and by increased levels of ECP and MBP in BAL and sputum (Djukanovic *et al*, 1990; Broide *et al*, 1991). Activated eosinophils generate lipid mediators, such as LTC_4, and a number of pro-inflammatory cytokines, including IL-5, TGFβ and TNF-α. Therefore, the mechanisms of activation of eosinophils are of particular importance in increasing our understanding of their involvement in inflammation.

During allergic inflammatory reactions mast cells and eosinophils can interact when they infiltrate into tissues in the late allergic response or

when the inflammation becomes chronic (Bochner *et* al, 2001; Gurish *et* al, 2001). Mast cells have been shown to enhance eosinophil survival *in vitro* by induction of GM-CSF autocrine production, which was subsequently found to be due to mast cell-derived TNF-α (Levi-Schaffer *et al*, 1998). Moreover, mast cell tryptase has been found to stimulate the release of ECP and to promote chemotaxis of eosinophils (Walls *et al*, 1995; He *et al*, 1997). Therefore an understanding of how eosinophils may become activated in the airways is important. Mast cell tryptase levels are also increased in the asthmatics airways, but the effects of tryptase on eosinophil function have been little explored.

We hypothesised that tryptase may represent a stimulus for the release of inflammatory mediators from eosinophils. In the present studies we have investigated the ability of tryptase to induce the release of EPO, a marker for eosinophil degranulation. In addition, the presence of PAR-2 on eosinophils was examined by flow cytometry using the new PAR-2 specific antibody. PAR-2 activation was also assessed by measurement of calcium flux.

5.2 Materials and Methods

5.2.1 Purification of Eosinophils

Eosinophils were isolated from blood as described in section 2.10. Cell viability was assessed using trypan blue exclusion. Percentage viability was expressed as the percentage of live cells in the total eosinophil preparation. Cells were counted after mixing with the Kimura stain (Kimura *et al.*, 1973), which contains the detergent saponin to lyse erythrocytes, making it easier to determine the total number of nucleated cells present.

5.2.2 Flow Cytometry

Expression of PAR-2 by eosinophils was evaluated by flow cytometry, as detailed in section 2.4. Cells were stained with either monoclonal antibody P2A (1/100 dilution) or the B5 antiserum (1/1000 dilution or 11.6 μg/ml). Mouse IgM (1/100 dilution; generated against irrelevant antigen) or rabbit IgG (11.6 μg/ml) were used in negative control experiments. The same experiment was repeated twice and compared to experiments with the KNRK PAR-2 expression system.

5.2.3 Measurement of EPO Release from Eosinophils

Purified eosinophils were challenged with varying concentrations of secretagogues for 30 min at 37 °C; reactions were stopped by incubating

on ice for 15 min. Cells were centrifuged at 300 x g and cell supernatants collected for the measurement of mediator release.

For assessment of EPO release, cell supernatants were added to 96-well microtitre plates with an equal volume of OPD (0.18 g/ml) in 0.05 M citrate-phosphate buffer pH 5.0 (containing 0.24 % H_2O_2). The peroxidase reaction was stopped by the addition of 3 M H_2SO_4 and the absorbance measured at 490 nm. The secretagogues investigated included: purified lung tryptase, bovine trypsin, porcine trypsin, SLIGKV, VKGILS (reverse PAR-2 activating peptide), tryptase with inhibitors (100 μM benzamidine and 100 μM leupeptin), heat-inactivated tryptase (heated to 100 °C for 5 min) and calcium ionophore (A23187). EPO release was expressed as a percentage of total, measured by cell lysis using 0.01 % CETAB (hexadecyltrimethylammonium bromide). Statistical differences were analysed using paired Student's t-test. Cytotoxicity was assessed using the Cytotox^{96} assay kit (Promega) which measures the release of lactate dehydrogenase (LDH), a marker of cell death.

5.2.4 Measurements of Calcium Flux

Section 2.5 describes the method for determining intracellular calcium concentrations from cells. A number of agonists were used to determine PAR-2 activation. Protease agonists included bovine trypsin, purified human lung tryptase (huL-tryptase), and recombinant human β-tryptase

(rhu-tryptase), expressed in the *pichia pastoris* expression system (Roche, UK) and purified using the method described in section 2.9.1.

5.3 Results

5.3.1 Purification of Eosinophils

Eosinophils were routinely isolated with greater than 99 % purity and viability. Figure 5-1 shows eosinophils stained using the Kimura method, with the characteristic bilobed nuclei are clearly visible (Fig. 5-1A). The cytoplasmic granules may also be seen when the cells are observed under green fluorescent light microscopy; these appear as red punctate dots due to the granules containing high levels of the pigment eosin.

5.3.2 Expression of PAR-2 on Eosinophils

PAR-2 was detected on eosinophils by flow cytometry using the generated monoclonal antibody P2A (Fig. 5-2). Cell surface immunofluorescence was observed in a sub-population of the cells (Fig. 5.2A), and a clear right shift in the fluorescence was seen when the cells were permeabilised (Fig. 5-2B). No immunofluorescence was observed when PAR-2 antibody was substituted with the same class of antibody (IgM) to an irrelevant antigen at the same concentration (Fig. 5-2C). Receptor expression was not seen on the cells when the B5 antiserum was used (Fig. 5-3).

5.3.3 Release of EPO from Eosinophils

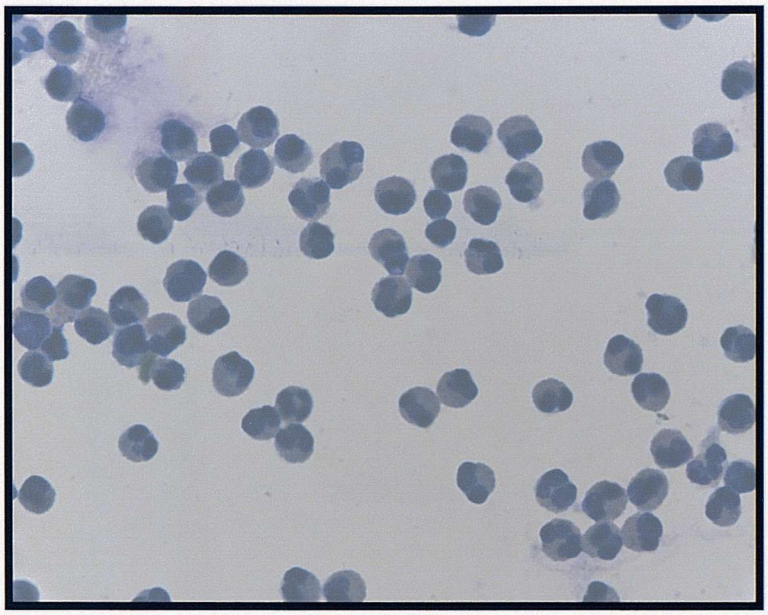
Tryptase (5-80 mU/ml) induced a concentration-dependent increase in EPO release which was significant at 40 and 80 mU/ml ($p<0.05$, $n=5$) and abolished by heat-inactivation (Fig. 5-4). The PAR-2 agonist peptide SLIGKV (10-320 μM) had no effect (Fig. 5-5), nor did the reverse peptide VKGILS (10-320 μM, data not shown). Another activator of PAR-2, trypsin failed to elicit EPO release when used over the range 10 nm – 1 μM, found to be effective in other studies (data not shown). The effects of tryptase 80 mU/ml were abolished by pre-incubation with the protease inhibitors leupeptin or benzamidine (Fig. 5-6). These same inhibitors did not inhibit EPO release induced by A23187 (data not shown). Cytotoxicity measurements were low and of similar values for all the supernatants tested (data not shown).

5.3.4 Measurement of Calcium Flux in Eosinophils

Addition of the PAR-2 protease agonists, purified lung tryptase (huL-tryptase) and recombinant human β-tryptase (rhu-Tryptase) to eosinophils was not associated with an increase the intracellular calcium concentration, when added at an activity of 40 mU/ml (Fig. 5-7A). Similarly trypsin was also without effect, though ATP did elicited a calcium peak (Fig.5-7A). The PAR-2 agonist peptides, SLIGKV-NH_2 and SLIGRL-NH_2, did not induce calcium flux (10-300 μM). Figure 5-7B shows that 300 μM SLIGKV or SLIGRL were without effect with prior addition of

amastatin. The peptide tc-LIGRLO, however, did induce a calcium peak response, which was desensitised to a second addition of agonist, the inactive reverse peptide (tc-LRGILO) was without effect (Fig. 5-7C).

A



B

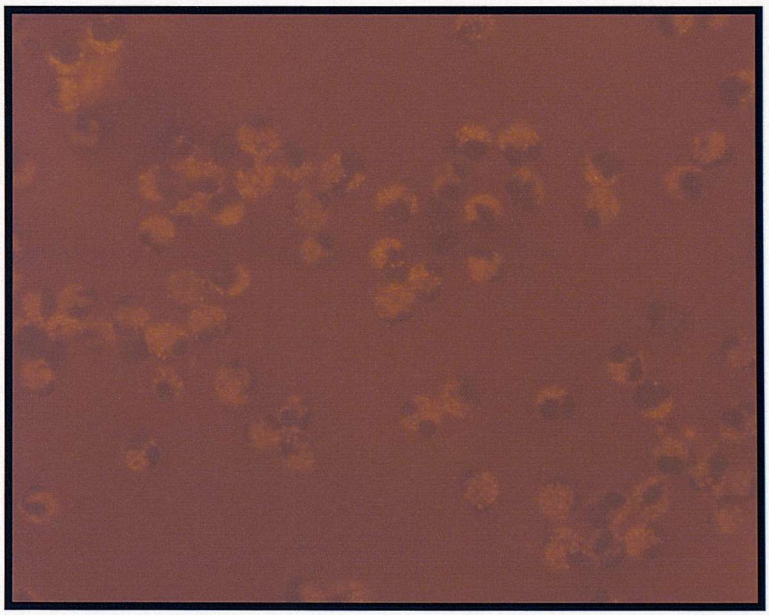


Figure 5-1 Purified eosinophils. The characteristic bi-lobed nuclei can be seen after staining using the Kimura method (A). Under green fluorescent light the eosin-rich cytoplasmic granules are clearly visible (B).

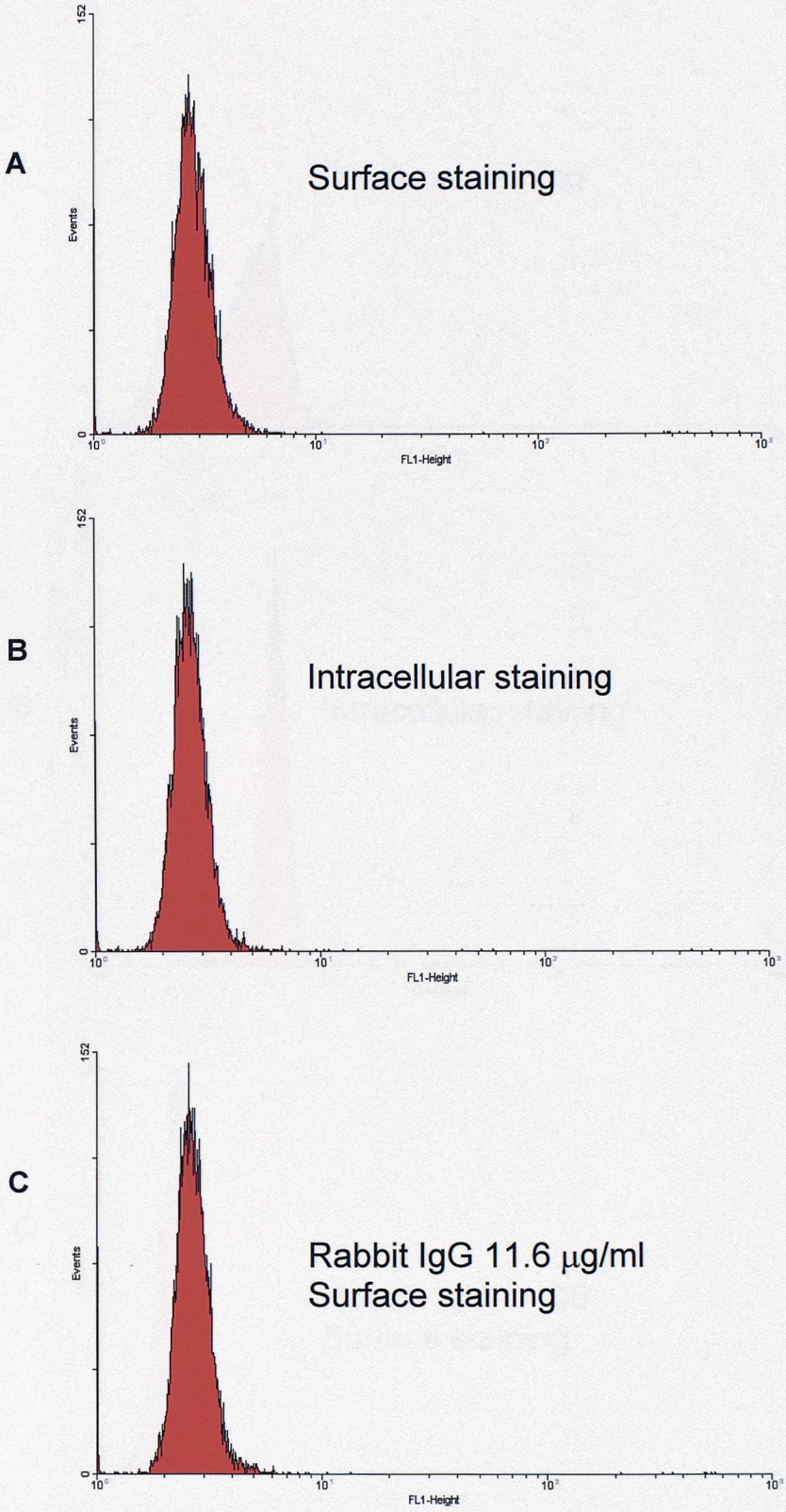


Figure 5-2 Lack of surface (A) or intracellular (B) PAR-2 expression on eosinophils employing the B5 antiserum at a dilution of 1/1000. (C) rabbit IgG was used in negative control experiments.

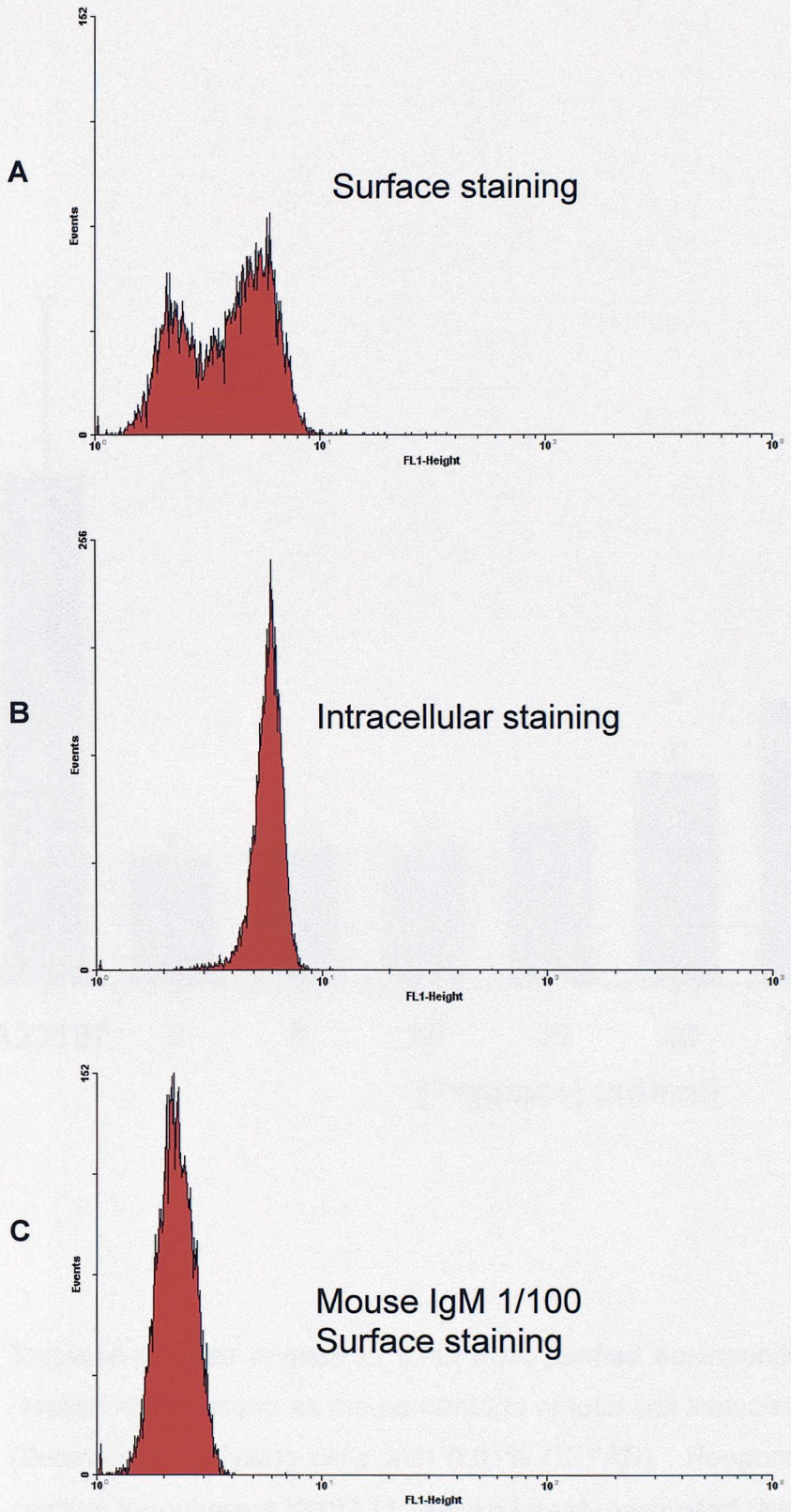


Figure 5-3 Surface (A) and intracellular (B) PAR-2 expression on eosinophils employing monoclonal antibody P2A at a dilution of 1/100, (C) mouse IgM was used as a negative control.

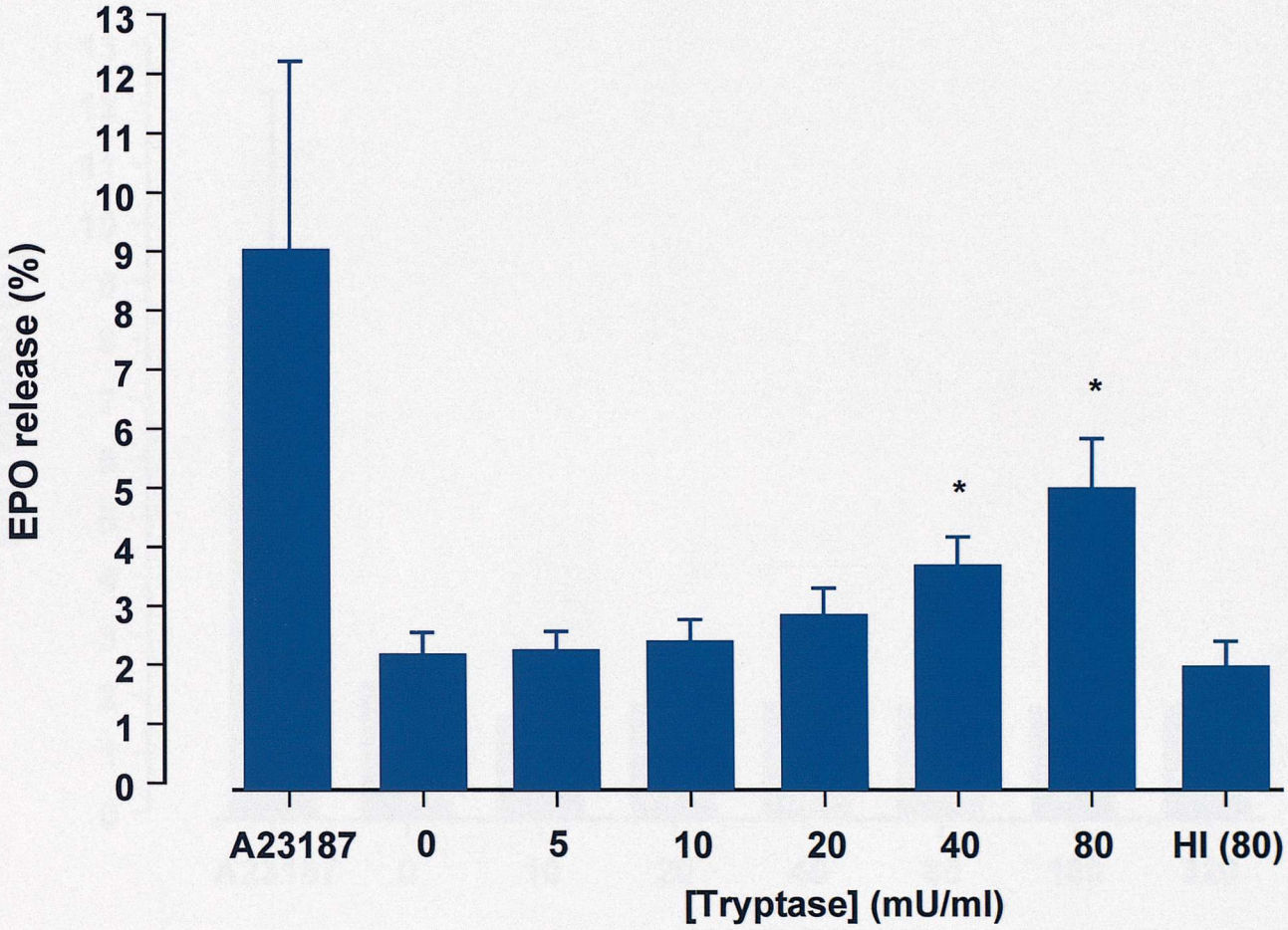


Figure 5-4 Tryptase-induced release of EPO from purified eosinophils. EPO release is expressed as the percentage of total cell associated EPO (determined by lysing cells with 0.01% CETAB). Responses with calcium ionophore A23187 (1 μM) and heat-inactivated (HI; 100 °C for 5 min) tryptase (80 mU/ml) are also shown. *P < 0.05 (Student's t-test. Mean values (± SEM) are shown for five experiments.

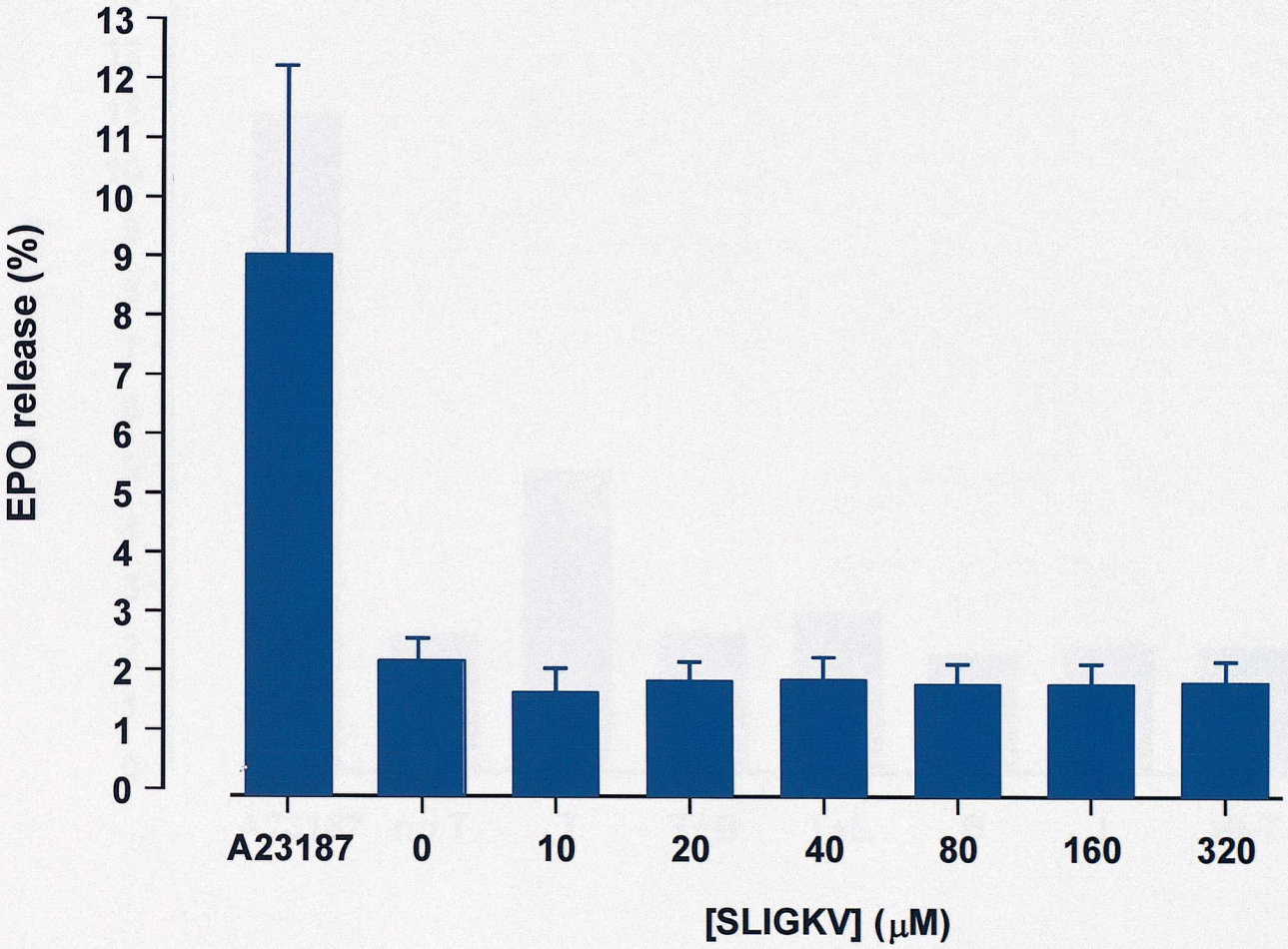


Figure 5-5 Effects of SLIGKV-NH_2 on the release of EPO from purified eosinophils. EPO release was expressed as a percentage of total cell associated EPO. Mean values (± SEM) are shown for five experiments.

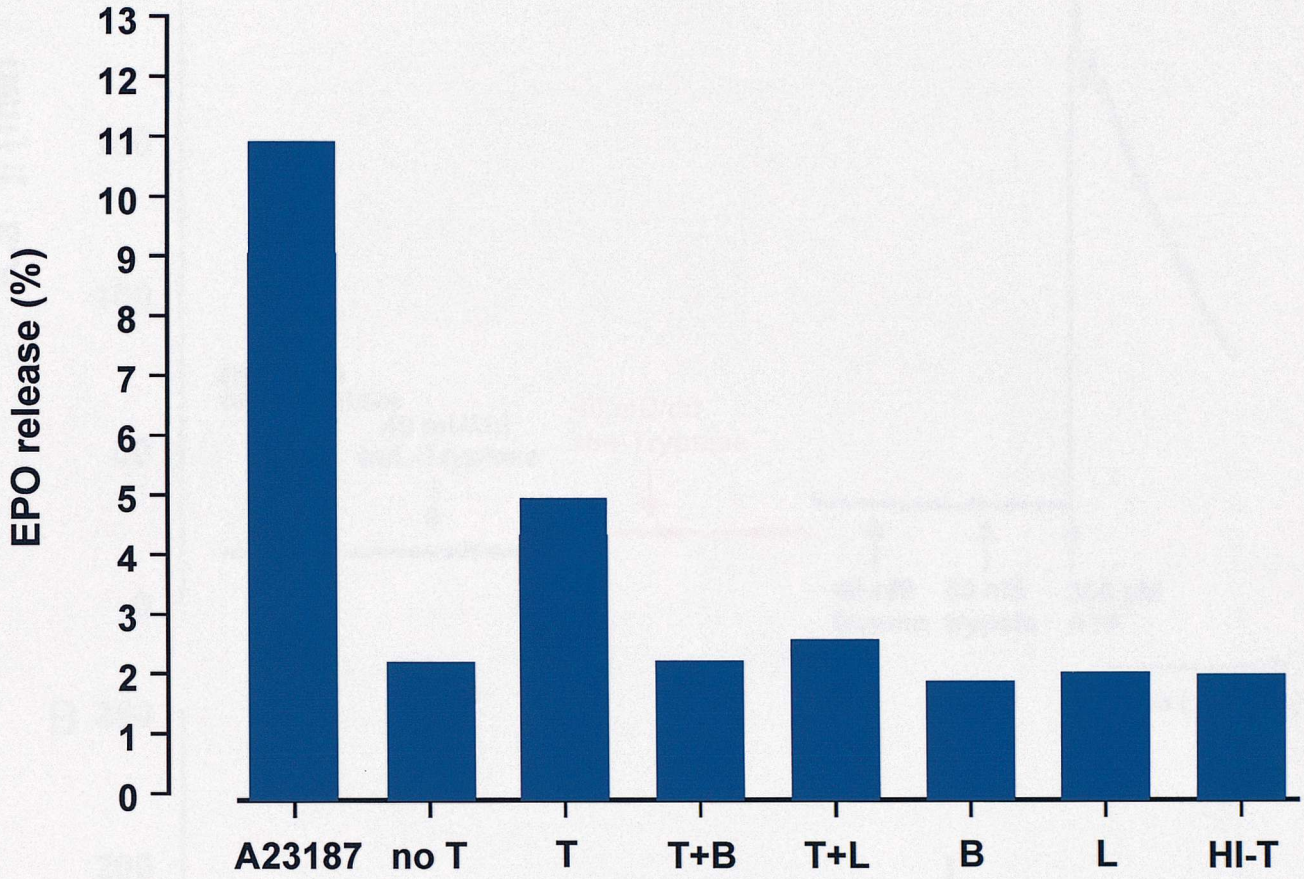
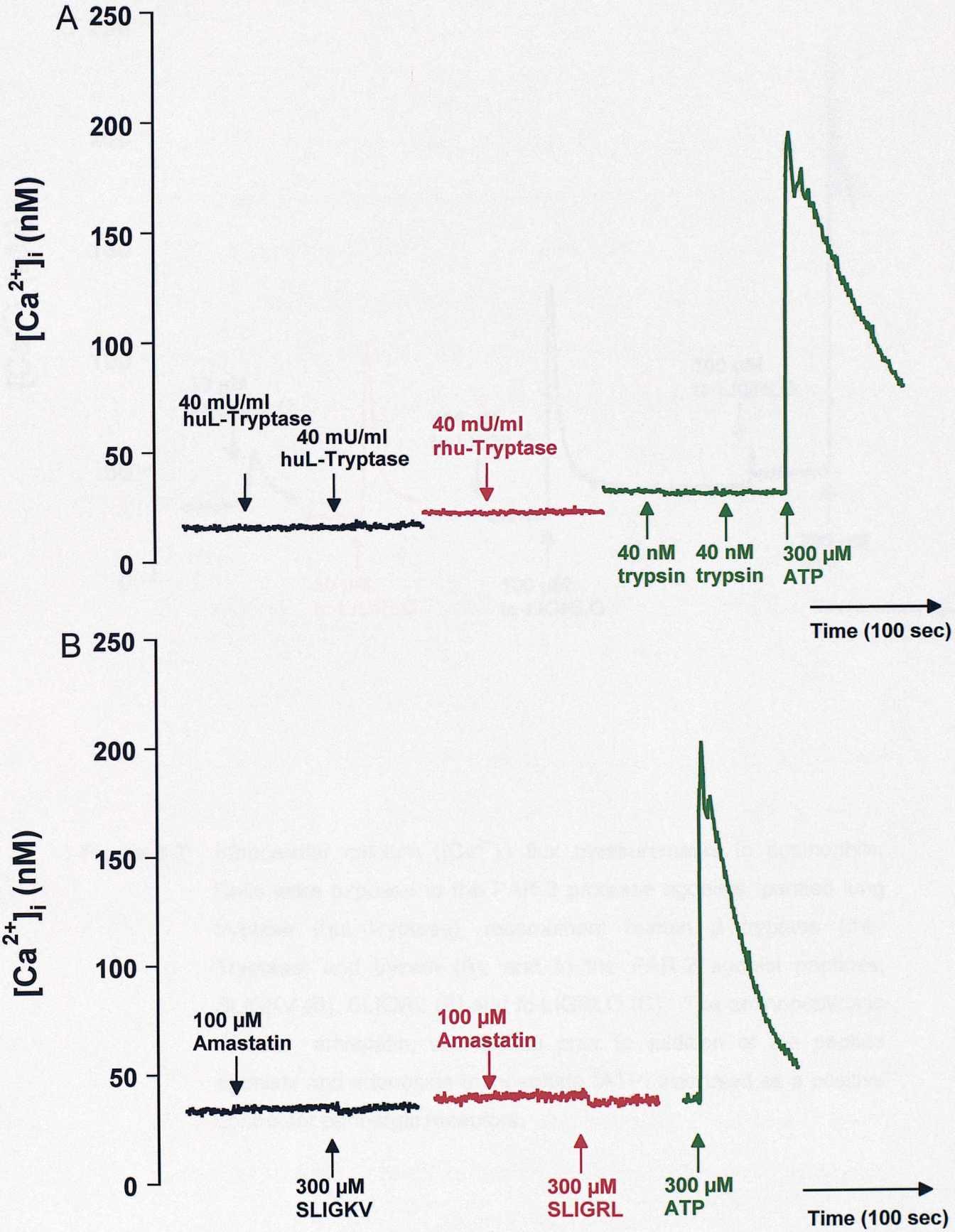


Figure 5-6 Effect on tryptase-induced EPO release from purified eosinophils of pre-incubating tryptase (T) with protease inhibitors. Benzamidine (T+B; 100 μM) and leupeptin (T+L; 100 μM) were each pre-incubated with 80 mU/ml tryptase or applied alone (B, L) for 1 hr on ice. EPO release in the presence of medium alone (no T), calcium ionophore A23187 (1 μM) and heat-inactivated tryptase (HI-T) are also shown. Mean values of two experiments are shown.


A
250
200
150
100
50
0
[Ca2+]i (nM)
40 mU/ml
huL-Tryptase
40 mU/ml
huL-Tryptase
40 mU/ml
rhu-Tryptase
40 nM
trypsin
40 nM
trypsin
300 µM
ATP
Time (100 sec)
B
250
200
150
100
50
0
[Ca2+]i (nM)
100 µM
Amastatin
100 µM
Amastatin
300 µM
SLIGKV
300 µM
SLIGRL
300 µM
ATP
Time (100 sec)

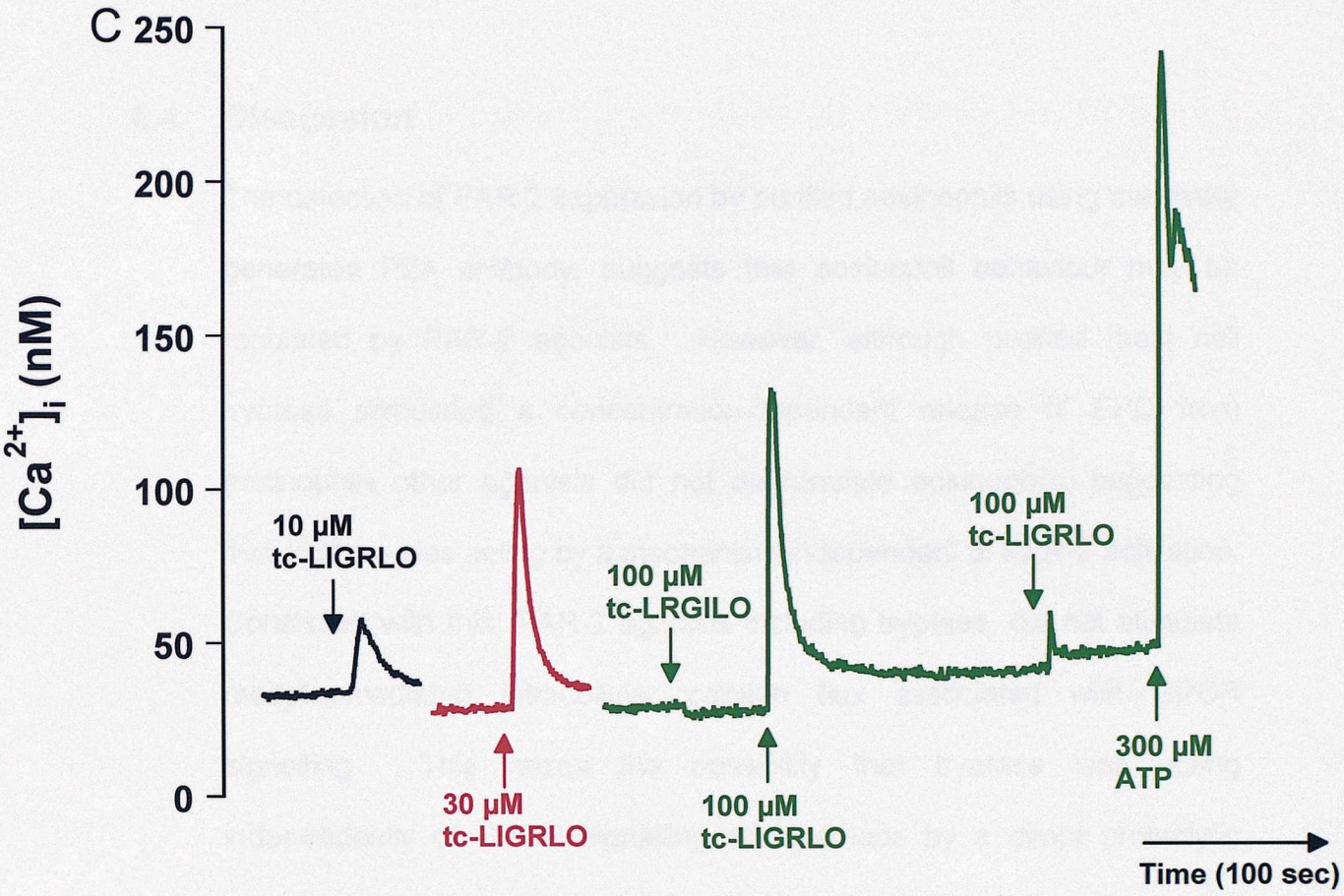


Figure 5-7 Intracellular calcium ($[Ca^{2+}]_i$) flux measurements in eosinophils. Cells were exposed to the PAR-2 protease agonists, purified lung tryptase (huL-Tryptase), recombinant human β tryptase (rhu-Tryptase) and trypsin (A); and to the PAR-2 agonist peptides, SLIGKV (B), SLIGRL (B) and tc-LIGRLO (C). The aminopeptidase inhibitor, amastatin, was added prior to addition of the peptide agonists and adenosine triphosphate (ATP) was used as a positive control for purinergic receptors.

5.4 Discussion

The detection of PAR-2 expression by purified eosinophils using the newly generated P2A antibody, suggests that eosinophil behaviour may be regulated by PAR-2 agonists. However, although purified mast cell tryptase stimulated a concentration-dependent release of EPO from eosinophils other agonists did not degranulate eosinophils, suggesting that tryptase was acting by a mechanism independent of PAR-2 activation. Consistent with this, PAR-2 agonists including tryptase, did not stimulate receptor-mediated intracellular calcium flux associated with GPCR signalling. This raises the possibility that tryptase was acting independently of GPCR signalling and perhaps by a direct proteolytic action.

Although flow cytometry with P2A antibody indicated cell surface and intracellular PAR-2 expression, B5 antiserum did not detect PAR-2 on eosinophils. However, the expression of PAR-2 by eosinophils is supported by a report showing that a rabbit antiserum raised against a peptide fragment of *human* PAR-2 was also able to label the receptor in FACS experiments (Miike *et al*, 2001). The lack of staining with the B5 antiserum, could reflect inability to bind an alternative form of PAR-2 expressed by eosinophils or a lack of binding B5 to either cleaved or uncleaved receptors. It is possible that the main epitope recognised by B5 antiserum is masked by another protein or proteoglycan on eosinophils

thereby blocking access to the antiserum, but not to P2A antibody, which may recognise another site on the PAR-2 molecule.

Tryptase induced a concentration dependent release of EPO from eosinophils after 30 min that was abolished by pre-treatment of the enzyme with an inhibitor or by heat-inactivation, suggesting that degranulation was associated with enzymatic activity. In contrast to tryptase, other agonists of PAR-2, such as trypsin and SLIGKV-NH_2, could not reproduce this effect. During the course of these studies, two groups have reported that PAR-2 induced mediator release from eosinophils, which are at variance with this study. Miike and co-workers (2001) investigated the release of eosinophil derived neurotoxin (EDN) and the generation of superoxide. They found a significant increase in EDN release and superoxide production in response to trypsin and $SLIGKV_2$, which was partially inhibited by pre-incubation with a PAR-2 blocking antiserum. Furthermore, Temkin and co-workers (2002) showed that tryptase elicited the release of IL-6 and IL-8 from eosinophils, and suggested that this may be due to PAR-2 activation since the PAR-2 blocking antiserum (as described by Miike *et al*, 2001) inhibited cytokine release in a concentration dependent manner. In the present study, tc-LIGRLO stimulated Calcium flux in the eosinophils, but there are doubts about the specificity of this peptide (McGuire *et al*, 2002).

The differences between the present study and those of Temkin and Miike are not a consequence of the type of blood donor, since both studies have isolated eosinophils from mildly allergic patients. Furthermore, all three studies used the method, as described by Hansel *et al* (1991). It could well be that different mechanisms of release exist for EPO, than for EDN and cytokines, and that tryptase may have had a direct proteolytic effect on the cell surface membrane releasing another mediator involved in triggering EPO release.

The finding that tryptase and other PAR-2 agonists failed to initiate changes in intracellular calcium concentrations also suggests tryptase-induced activation of eosinophils occurs independently of PAR-2 activation irrespective of PAR-2 activation. However, further experiments would need to be performed to adequately conclude this finding. It may be that eosinophils require priming to attain sensitivity to PAR-2 activators, since priming *via* pretreatment with cytokines (*e.g.* IL-5) strikingly enhances the production of eosinophilic mediator release (Takafuji *et al*, 1991). If time had permitted, these studies would have been extended to allow analysis of mediator release from eosinophils primed with cytokines, and the kinetics of responses to tryptase evaluated. In addition, experiments must also include studies of intracellular signalling pathways, since PAR-2 may also be coupled to inhibitory G-proteins and hence activation may not necessarily trigger the release of intracellular calcium. These experiments would utilise specific inhibitors of G-proteins, such as pertussis toxin.

Other experiments would involve the detection of PAR-2 messenger RNA (mRNA) using RT-PCR and primer sequences specific for PAR-2 and detection of the protein by Western blotting. Moreover, immunostaining of the PAR-2 protein using either electron or confocal microscopy would provide better evidence of the location of the receptors at the surface of the cell with respect to the numbers internalised and hence provide a better understanding of their activation in these cells. The antibodies generated thus far should be valuable tools for these experiments.

Although its mechanisms of action are uncertain at present the ability of tryptase to activate eosinophils provides a means by which it may regulate the behaviour of eosinophils which infiltrate into the tissues in the course of allergic inflammation.

CHAPTER 6

General Discussion

6 General Discussion

6.1 Introduction

The generation and validation of new monoclonal antibodies specific for PAR-2 should provide valuable insights into seeking the role of this receptor in disease. In the present studies, these were successfully employed to investigate expression of PAR-2 in the airways of asthmatics and rhinitics, and in eosinophils. The observations of alterations in PAR-2 expression could be important for understanding the function of PAR-2 in allergic disease.

PAR-2 expression was found to be greater in the bronchial epithelium of patients with severe asthma compared with non-asthmatic subjects, calling attention to the role of this receptor in the asthmatic airways. Moreover, although PAR-2 expression in the airways mild asthmatics did not differ from that in subjects without asthma, expression was significantly increased following allergen provocation. Expression of PAR-2 in the nasal epithelium of rhinitic subjects did not differ from that in those without rhinitis, but expression was significantly decreased in subjects with seasonal allergic rhinitis after grass pollen allergen challenge. The different patterns of PAR-2 expression in the upper and lower airways

following local allergen challenge raises the possibility of different mechanisms of control in those two compartments.

PAR-2 protein was detected both intracellularly and on the cell surface of eosinophils. In studies of eosinophil activation, tryptase elicited a concentration-dependent increase in the release of the eosinophil mediator EPO, which was inhibited by heat-inactivation and enzyme-inhibitors. Other agonists of PAR-2, including trypsin, SLIGKV-NH_2 and SLIGRL-NH_2, failed to stimulate EPO release. Moreover, although tryptase could stimulate the release of EPO from eosinophils, the enzyme failed to cause changes in calcium flux, associated with GPCR signalling, suggesting that tryptase may stimulate eosinophils by a mechanism that does not involve cleavage of PAR-2. These findings will be discussed further in this chapter.

6.2 PAR-2 in the Airways

Expression of PAR-2 was observed in the bronchial and nasal airways, mainly on the respiratory epithelium and in a small number of cells in the submucosa. On further examination, they were not found to be mast cells, eosinophils, neutrophils, macrophages, and fibroblasts, T- or B-lymphocytes. This suggests that the main targets for PAR-2 agonists in the airways may be respiratory epithelial cells.

Within the epithelium, PAR-2 was more apparent basolaterally (underneath the nuclei) within the cytoplasm of columnar epithelial cells of subjects with mild asthma, rhinitis and those without these diseases. Therefore, activation by substances generated in the tissue rather than those present in the airway lumen may be the main stimuli of PAR-2 on the epithelial cells. Stimulation of bronchial epithelial cells by PAR-2 agonists elicits the release of IL-6, IL-8, GM-CSF and eotaxin (Asokananthan *et al*, 2002; Sun *et al*, 2001) and the release of MMP-9, involved in tissue re-modelling processes (Vliagoftis *et al*, 2000). Therefore, endogenous PAR-2 activators may contribute to the pathogenesis of airway disease by releasing from epithelial cells mediators of inflammation and tissue remodelling.

Potential endogenous activators of PAR-2 include trypsin-like enzymes, such as human airway trypsin-like enzyme (HAT) and trypsin itself (Yasuoka *et al*, 1997, Cocks *et al*, 1999). Danahay and co-workers (2001), co-localised HAT and PAR-2 to human bronchial epithelial cells and reported that stimulation of PAR-2 caused an increase in intracellular concentrations of calcium ion and alterations in the permeability of the cell membrane to potassium ion when applied to the basolateral surface. They suggested that activation of PAR-2 may play a role in regulating sodium ion absorption and anion secretion, processes central to the control of airway surface liquid volume and composition. Cocks *et al*

(1999), co-localised trypsinogen and PAR-2 to epithelial cells and suggested that activation of PAR-2 on these cells prevented bronchoconstriction by causing the production of PGE_2. Thus in addition to its deleterious effects on airway function, PAR2 may have a secondary role in the maintenance of airway physiology and homeostasis.

In addition to the basolateral surface, PAR-2 was present on apical surfaces of bronchial epithelial cells in severe asthma patients, suggesting in severe asthma PAR-2 activators may arise from both the airway lumen and the underlying tissues. Mast cell tryptase is readily detectable in the BAL fluid of asthmatic patients (Wenzel *et al*, 1988), and therefore PAR-2 positioned apically may detect luminal tryptase that has permeated a disrupted barrier. It has also been suggested that the house dust mite proteases Der P1, Der P3 and Der P9 can activate PAR-2 on bronchial epithelial cell lines, triggering the release of IL-6, IL-8, GM-CSF and eotaxin (Asokananthan *et al*, 2002b; Sun *et al*, 2001). Under normal circumstances, PAR-2 in the epithelial cells are low in number and they may be involved in normal repair processes. However, in severe asthma the change in numbers and distribution of PAR-2 receptors on the bronchial epithelium may render the airways more sensitive to PAR-2 activators arising from the inflamed tissue or the airway lumen.

During the course of this study Knight and co-workers (2001) reported an increase in PAR-2 expression by the bronchial epithelium of asthmatics compared to controls, which supports our findings. A more recent study has investigated the expression of PAR-2 in the airways of smokers, where expression did not differ between smokers and non-smokers, but PAR-2 expression by bronchial blood vessels was increased in smokers with symptoms of chronic bronchitis and with normal lung function, compared with patients with chronic obstructive pulmonary disease (COPD) (Miotto *et al*, 2002).

Although PAR-2 expression may be upregulated in asthma, its expression was found to be unaltered in rhinitis, suggesting that there are differences between the epithelia of the lung and nose. It is possible that the epithelium of the healthy nose is quite inflamed due to its exposure to environmental stimuli and being the first cells of contact in the airways, and therefore if PAR-2 is linked to inflammation then it follows that PAR-2 expression might be less sensitive to increased inflammation in rhinitis. Alternatively, antibody binding may have been altered by PAR-2 activation. In this respect, attempts were made to produce antibodies, which may detect cleaved or uncleaved PAR-2. With the model used it was not possible to determine whether the antibodies produced detected activated or non-activated PAR-2, therefore further characterisation will be needed. An alternative explanation for the lack of an increase in nasal

epithelial expression in rhinitis is that other proteases might have inactivated PAR-2 and thus affected antibody reactivity. According to this scenario, the balance of proteases, which activate or inactivate PAR-2, may differ between asthma and rhinitis. Although direct evidence existing to support this theory is lacking, tryptase is thought to be involved in regulating PAR-2 expression since at higher concentrations it may cleave downstream of the activation site, thereby rendering it into an inactive state (Molino *et al*, 1997).

The reason for the decrease in PAR-2 expression by the nasal epithelium six-hours following intranasal administration of grass pollen is unclear at present. These findings may reflect a downregulation of PAR-2 as part of negative feedback to prevent over-stimulation of epithelial cells, and biopsies collected at other time points might have shown different results. Follow-up studies of PAR-2 expression in patients with seasonal allergic rhinitis, both in and out of season would help to increase our understanding of the role of PAR-2 in rhinitis.

6.3 PAR-2 and Eosinophils

Using P2A, we identified PAR-2 expression on the surface and intracellular of eosinophils. Purified lung tryptase caused a dose dependent increase in the release of the eosinophil granule protein EPO

from eosinophils. Other agonists of PAR-2 were without effect upon EPO release, including trypsin and SLIGKV-NH_2. Neither tryptase nor the other PAR-2 agonists affected Ca^{2+} flux measurements when used to stimulate purified eosinophils. This suggests that the tryptase-induced EPO release could be stimulated by PAR-2 independent mechanisms, probably by direct proteolysis of membrane proteins. However, one of the agonist peptides, tc-LIGRLO, elicited a calcium spike which was attenuated when a second concentration was applied to the eosinophils, demonstrating homologous desensitisation. This unusual finding suggests that the tc-LIGRLO peptide is activating another GPCR, and perhaps another PAR-like receptor.

Findings presented in this thesis of PAR-2 expression by eosinophils using flow cytometry are consistent with two recently published reports (Miike *et al*, 2001; Temkin *et al*, 2002). However, in contrast to these workers, in the present study eosinophils were not activated by PAR-2 agonist proteases, tryptase and trypsin, or with the PAR-2 peptides SLIGKV-NH_2 or SLIGRL-NH_2. Miike and co-workers (2001) isolated eosinophils either from normal individuals or from mildly atopic patients, and measured the release of EDN and the generation of superoxide in response to PAR-2 agonists. They found that trypsin or SLIGKV-NH_2 increased mediator release, but that tryptase was without effect. On the other hand, Temkin and co-workers (2002) investigated the activation and release of IL-6 and

IL-8 from eosinophils in response to recombinant tryptase, and showed concentration dependent increases in release, which halved when cells were pre-incubated with polyclonal anti-PAR-2 antisera. It could be that the differences observed in the effects and mechanism of tryptase activation could be related to the extent of receptor glycosylation (Compton *et al*, 2001).

6.3 Conclusions

Several antibodies were generated for the purpose of investigating PAR-2 involvement in airways disease. One of these, P2A, is the first monoclonal antibody against human PAR-2 that is suitable for detecting PAR-2 in tissue sections, western blotting and FACS analysis. Using this antibody, PAR-2 expression was found to be up-regulated on the epithelium of asthmatic airways. Furthermore, inhalation challenge with allergen increased PAR-2 immunoreactivity. Additional infiltrating cells stained for PAR-2, but it was not possible to identify them conclusively. However, they were found not to be mast cells or eosinophils.

However, peripheral blood eosinophils were found to express PAR-2 receptors by FACS analysis, and tryptase was found to stimulate eosinophil degranulation. Nonetheless, follow-up studies suggested that PAR-2 agonists did not stimulate eosinophil activation and that tryptase acted by a mechanism independent of PAR-2.

Taken together, these results indicate that PAR-2 expression is greatest on the epithelium and is up-regulated in asthma. The identities of the PAR-2 activators in disease are not yet clear, and although tryptase is one such candidate, the findings presented herein indicate that this enzyme may act by other means also. These findings are summarised in Fig. 6-1.

The challenge for the future remains to determine the nature of PAR-2 involvement in airway disease in order that therapies may be devised to limit the inflammation and remodelling associated with these conditions. The novel antibodies produced during this project should prove valuable tools in this quest, and the information concerning PAR-2 expression by eosinophils and the effects on them of mast cell tryptase provide a timely warning that not all of the actions of tryptase on cells are necessarily mediated by PAR-2, and that other physiological agonists of PAR-2 should continue to be sought. Much information on the various roles of PAR-2 has come to light during the course of this project, and it is likely that future research will contribute to our understanding of this interesting and unusual receptor and its involvement in airway disease.

The novel findings arising from this project are listed as follows:

1. A number of anti-PAR-2 antibodies have been generated including the first known monoclonal antibody (P2A) found to be useful in immunohistochemistry as well as in detecting PAR-2 by flow cytometry and Western blotting.

2. P2A was used to show that PAR-2 expression is increased in the bronchial epithelium of subjects with severe asthma and following allergen provocation, suggesting that PAR-2 activation may be involved in the inflammatory processes associated with the development of this disease.

3. PAR-2 expression was observed on eosinophils and found to be inactive in these studies, since agonists of this receptor (including trypsin, SLIGKV-NH_2 and SLIGRL-NH_2) failed to elicit the release of EPO or mobilisation of intracellular calcium.

4. Though PAR-2 agonists were ineffective in stimulating eosinophil activation, mast cell tryptase was found to elicit the release of EPO *via* mechanisms thought to be independent of PAR-2 activation. Moreover, the peptide (tc-LIGRLO) thought to activate an as yet unidentified PAR was able mobilise calcium release from

intracellular stores in a concentration dependent manner further supporting this theory.

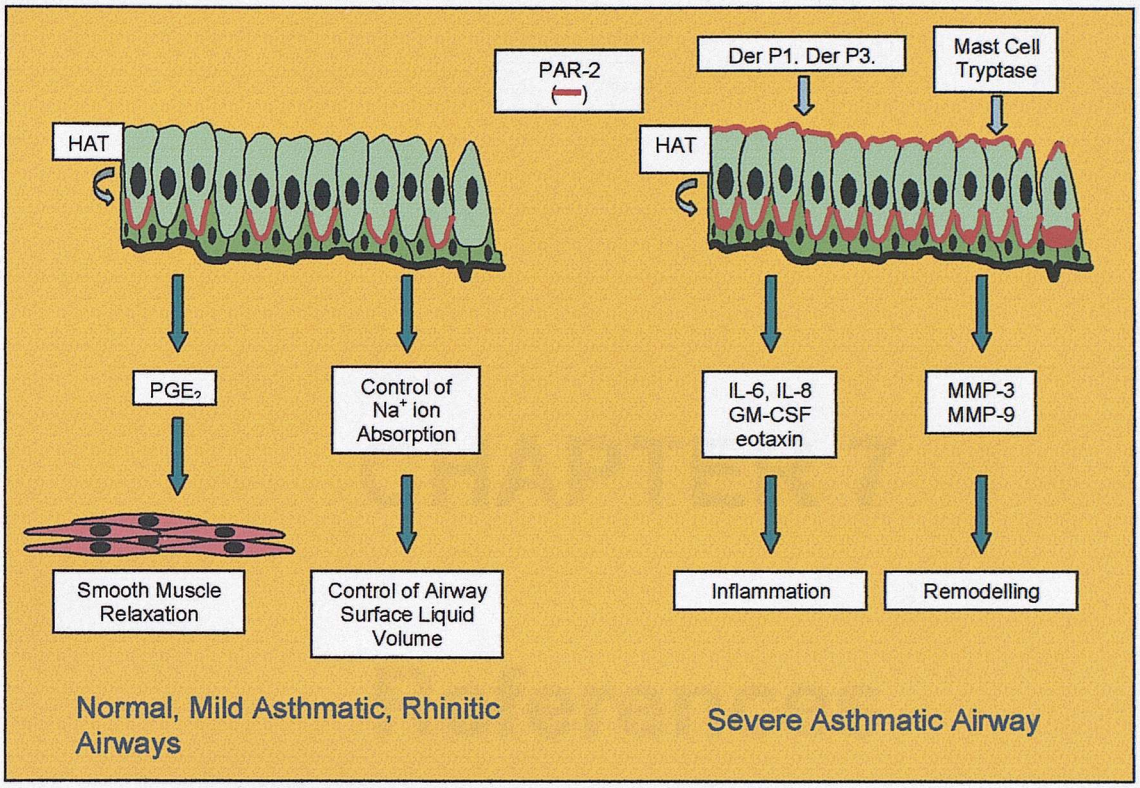


Figure 6-1 Consequences of PAR-2 activation in the airway epithelium. Potential activators of epithelial PAR-2 include mast cell tryptase, human airway trypsin-like enzyme (HAT), Der P1, Der P3 and Der P9.

CHAPTER 7

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7 REFERENCES

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