THE SKIN MAST CELL: A COMPARISON WITH MAST CELLS FROM VARIOUS TISSUES IN MAN AND OTHER SPECIES

A Thesis presented to the University of London
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy
in the Faculty of Science

by

Katrina Robertson Tainsh

The Christopher Ingold Laboratories
University College London

June 1991
To My Family

Beware you be not swallowed up in books!
An ounce of love is worth a pound of knowledge

John Wesley
Acknowledgements

I would like to extend my sincere gratitude to my supervisor, Dr. Frederick L. Pearce, for his advice and support. In addition, it is with the deepest appreciation that I acknowledge my colleagues for their fellowship and encouragement throughout this work. I would especially like to thank Dr. Alaster Lau and Dr. Steve Liu for their unfailing patience and guidance and many helpful discussions during the writing of this thesis.

The following are also acknowledged; The Science and Engineering Research Council and Fisons p.l.c, for the provision of a CASE award; The Department of Chemistry and The Joint Animal House, University College London, for the use of their facilities; The surgeons and staff of University College, The Middlesex, The Elizabeth Garrett Anderson and St. Bartholemew’s Hospitals for their co-operation in the supply of human tissue samples.

Finally, I would like to dedicate this thesis to my family, in particular my Mother and Father and my sister, Marion, for their constant love, understanding and support.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>Introduction</td>
</tr>
<tr>
<td>1.1</td>
<td>The mast cell - an historical perspective</td>
</tr>
<tr>
<td>1.2</td>
<td>A role for the mast cell in physiology and pathology</td>
</tr>
<tr>
<td>1.3</td>
<td>The basis of the immediate hypersensitivity reaction</td>
</tr>
<tr>
<td>1.3.1</td>
<td>IgE production</td>
</tr>
<tr>
<td>1.3.2</td>
<td>The IgE receptor and triggering of mast cell degranulation</td>
</tr>
<tr>
<td>1.4</td>
<td>Exocytosis and mediator release from mast cells</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Preformed constituents of mast cell granules</td>
</tr>
<tr>
<td>1.4.1.1</td>
<td>Autacoids</td>
</tr>
<tr>
<td>1.4.1.2</td>
<td>Serotonin</td>
</tr>
<tr>
<td>1.4.1.3</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>1.4.1.4</td>
<td>Granule enzymes</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Newly synthesized mediators</td>
</tr>
<tr>
<td>1.4.2.1</td>
<td>Arachidonic acid metabolites</td>
</tr>
<tr>
<td>1.4.2.2</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Chemotactic factors</td>
</tr>
<tr>
<td>1.4.4</td>
<td>The cytokines</td>
</tr>
<tr>
<td>1.5</td>
<td>Non-immunological histamine release</td>
</tr>
<tr>
<td>1.6</td>
<td>Biochemical events involved in mediator secretion</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Evidence for the role of Ca(^{2+}) as a second messenger in mast cell stimulus-secretion coupling</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Calcium pools involved in the secretory process</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Calcium channels in the mast cell</td>
</tr>
</tbody>
</table>
1.6.4 Calmodulin - the intracellular Ca\(^{2+}\) receptor 24
1.6.5 Proposed mechanisms for the mobilization of intracellular calcium 25
1.6.5.1 The phosphoinositide cycle 25
1.6.5.2 Phospholipid methylation 29
1.6.5.3 Arachidonic acid metabolism 30
1.6.6 Regulatory components of signal transduction mechanisms 32
1.6.6.1 GTP-regulatory proteins 32
1.6.6.2 Serine esterases 33
1.6.7 The role of cAMP 34
1.7 Mast cell heterogeneity 36
1.7.1 Mast cell ontogeny 37
1.7.1.1 The origin of mast cells 37
1.7.1.2 Mast cell culture 38
1.7.1.3 The role of microenvironment in the regulation of mast cell differentiation 40
1.7.2 Morphological and histochemical differences between mast cells 42
1.7.3 Biochemical and functional heterogeneity 44
1.8 Aims of the present study 46

Chapter 2 Materials and methods 54
2.1 Animals 54
2.2 Human tissues 54
2.3 Buffers 54
2.4 Isolation of mast cells 54
2.4.1 Rat peritoneal mast cells 55
2.4.2 Rat mesenteric and lung mast cells 55
2.4.3 Rat and guinea pig cutaneous mast cells 55
2.4.4 Guinea pig uterine mast cells 56
2.4.5 Human lung and uterine mast cells 56
2.4.6 Human cutaneous mast cells 57
2.4.7 Human bladder mast cells 57
2.4.8 Human basophil leukocytes 57
2.5 Immunological sensitization of rat and human mast cells 58
2.5.1 Active sensitization of rat mast cells with Nippostrongylus brasiliensis 58
2.5.1.1 Preparation of the third stage larvae 58
2.5.1.2 Preparation of allergens from Nippostrongylus brasiliensis 59
2.5.2 Passive sensitization of human mast cells 59
2.6 General procedure for histamine release experiments 59
2.6.1 Effect of secretagogues 59
2.6.2 Effect of metabolic inhibition on histamine release 60
2.6.3 Effect of neuraminidase on histamine release 60
2.6.4 Inhibition of histamine release 61
2.7 Histamine assay 61
2.7.1 Preparation of samples for manual assay using a spectrofluorimeter 61
2.7.2 Preparation of samples for assay using an autoanalyser 62
2.7.3 Calculation of percentage histamine release or percentage inhibition of histamine release 63
2.8 Mast cell characterisation 63
2.8.1 Cell counting and viability 63
2.8.2 Evaluation of mast cell histamine content 64
2.8.3 Fixation and staining 64
2.9 Statistical analysis 65
2.10 Materials 66
2.10.1 Histamine liberators 66
2.10.2 Inhibitors of histamine release 67
2.10.3 Materials for buffers 68
2.10.4 Materials for fixation and staining 68
2.10.5 Other materials 69

Chapter 3 Mast cell heterogeneity in the rat and guinea pig: the effect of secretagogues 75

3.1 Introduction 75
3.2 Methods 76
3.3 Results 76
3.3.1 Basic characteristics of rat and guinea pig mast cells 76
3.3.2 Histamine release in the rat induced by immunologically directed ligands 77
3.3.3 Effect of the calcium ionophores on histamine release from rat mast cells 77
3.3.4 Histamine release from rat mast cells by polybasic compounds 78
3.3.5 Histamine release induced by the neuropeptide substance P and other structurally unrelated basic compounds 78
3.3.6 The effect of neuraminidase on the histamine release from rat peritoneal cells with Co. 48/80, substance P and mastoparan 79
3.3.7 Histamine release induced by immunological ligands from guinea pig mast cells 79
3.3.8 Histamine release induced by calcium ionophores from isolated guinea pig mast cells 80
3.3.9 Histamine release induced by polybasic compounds and the neuropeptide substance P from isolated guinea pig mast cells 80
3.3.10 Histamine release from guinea pig mast cells induced by a variety of basic compounds of divergent structure

3.4 Discussion

Chapter 4 Mast cell heterogeneity in the rat: effect of anti-allergic compounds

4.1 Introduction
4.2 Methods
4.3 Results
4.3.1 Inhibition of histamine release by anti-asthmatic compounds and flavonoids
4.3.2 Inhibition of histamine release by agents which affect cyclic AMP levels
4.3.3 Inhibition of histamine release by H₂ and β₂ receptor directed agents
4.4 Discussion

Chapter 5 A comparison of the response of human skin, uterine and lung mast cells to secretagogues

5.1 Introduction
5.2 Methods
5.3 Results
5.3.1 Basic characteristics of mast cells isolated from human skin, lung and uterus
5.3.2 Histamine release from human mast cells induced by immunologically directed ligands
5.3.3 Histamine release from human mast cells induced by the ionophores A23187, ionomycin and chlortetracycline
5.3.4 The effect of a number of polybasic secretagogues on
5.3.5 Histamine release induced by the detergents triton X-100 and tween 20 from human mast cells

5.3.6 Some structure-activity studies using human skin mast cells

5.3.7 The effect of a number of polybasic compounds on histamine release from the human basophil leukocyte

5.4 Discussion

Chapter 6 The effect of anti-allergic compounds on histamine release from human skin and uterine mast cells

6.1 Introduction

6.2 Methods

6.3 Results

6.3.1 Inhibition of histamine release from human mast cells by some anti-asthmatic compounds and flavonoids

6.3.2 Inhibition of histamine release from human mast cells by agents which affect cyclic AMP levels

6.3.3 Inhibition of histamine release from human mast cells by H₂ and β₂ receptor-directed agents

6.4 Discussion

Chapter 7 Characterisation of mast cells isolated from the bladder mucosa and submucosa/muscle of normal individuals and one patient with interstitial cystitis (IC)

7.1 Introduction

7.2 Methods
7.3 Results

7.3.1 Some basic characteristics of mast cells isolated from normal human bladder mucosa and submucosa/muscle compared with those from a patient with IC

7.3.2 Histamine release from mast cells isolated from normal bladder induced by immunologically directed stimuli and calcium ionophores

7.3.3 Histamine release from isolated, normal bladder mast cells induced by polybasic compounds

7.3.4 Inhibition of histamine release from the normal bladder submucosa/muscle mast cells by anti-asthmatic compounds, the cAMP-directed agent, theophylline and the β2 agonist, isoprenaline

7.3.5 Histamine release induced by anti-human IgE, anti-human IgG, the lectin, Con A, and the calcium ionophores from mast cells isolated from the bladder submucosa/muscle and mucosa of a patient with IC

7.3.6 Histamine release induced by a variety of polybasic agents from bladder mast cells isolated from a patient with IC

7.3.7 Inhibition of histamine release from submucosa/muscle and mucosal mast cells isolated from a patient with IC using a variety of inhibitory agents

7.4 Discussion

Chapter 8 General conclusions and future directions

Bibliography

Appendix 1 Structures

Appendix 2 List of abbreviations

Publications
ABSTRACT

In the present study, the histochemical and functional characteristics of the skin mast cell of the rat, guinea pig and man have been assessed and compared with those of mast cells from other connective tissue locations in these species. Of particular interest was the functional heterogeneity displayed by different mast cell subtypes to a number of polycationic compounds, the range of which was extended, in this study, to include a wide variety of both endogenous and synthetic, structurally diverse agents which shared a predominantly basic nature. In addition, the isolation and characterisation of a number of novel mast cell types are described; the uterine mast cell from both the guinea pig and man, and mast cells from the human bladder mucosa and submucosa/muscle from both normal specimens and one from a patient with interstitial cystitis (IC).

The existence of a range of mast cell phenotypes in rat connective tissues has been demonstrated using histochemical and functional criteria. The peritoneal mast cell responded most strongly to both immunological and polybasic ligands, while the skin and lung mast cells were, in general, the least responsive. Variation in responsiveness to a variety of inhibitory agents was, however, less striking. The guinea pig skin and uterine mast cells were indistinguishable on the basis of their formalin sensitivity but the former cell displayed marked reactivity to polybasic compounds to which the latter were essentially refractory.

In man, the skin mast cell was exquisitely responsive to all the polycationic agents tested which was in contrast to the relative quiescence of mast cells from other connective tissue sites such as the uterine myometrium and lung parenchyma. Preliminary findings have also suggested functional similarities between the skin mast cell and bladder mast cells from both the submucosa/muscle and mucosa in their reactivity to polybasic compounds. These cell types were also similarly refractory to the effects of DSCG and nedocromil sodium although, in general, differences were not marked amongst human mast cells in their responses to a spectrum of other inhibitory agents.
The results of this study have highlighted the inadequacy of the present system of nomenclature (CTMC and MMC) which is in widespread use in the field of mast cell heterogeneity. In addition, while the rat does not provide a good test model for the human system, the guinea pig shows potential in this area. In conclusion, the present study has furthered our understanding of the extent and nature of mast cell heterogeneity in a variety of species.
CHAPTER 1: INTRODUCTION

1.1 The mast cell - an historical perspective

The mast cell has been the object of scientific fascination for well over a century.

In 1863 von Recklinghausen [1] discovered the presence of granular cells in frog mesentery. However, the first clear description of the mast cell was not until 1877, when Ehrlich demonstrated metachromatic staining of highly granulated cells in the connective tissues of animals with certain basic dyes such as toluidine blue [2]. The presence of such large numbers of granules in these cells prompted him to name them *Mastzellen*, from the German *masten* meaning to fatten or feed well. Two years later he also identified a similar type of cell circulating in the blood, the basophil.

In 1902 Portier and Richet [3] were first to describe the phenomenon of anaphylaxis (from the Greek *phulaxis*, meaning *guarding*) from their work in dogs with sea-anemone toxin. A number of dogs survived an initial injection of the toxin, but died with symptoms of acute shock following subsequent administration of the substance, to which they had been sensitized.

The involvement of histamine in anaphylaxis was proposed by Dale and Laidlaw [4] in 1919 following their discovery that injection of histamine into animals induced symptoms similar to those seen during anaphylactic shock. Histamine was seen also to be released from sensitized guinea pig lung *in vivo* when it was challenged with the appropriate antigen. A role for the amine in local immediate hypersensitivity reactions was suggested in 1927 when Lewis [5] first reported that injection of histamine into human skin produced a local wheal, flare and erythema, which he named the *triple response of inflammation*.

In the 1940s Jorpes [6] and his co-workers in Sweden were involved in investigations of
the anticoagulant properties of heparin, isolated from dog liver. Following their discovery that staining of heparin with toluidine blue produced metachromasia, they showed that a good correlation existed between the mast cell count of a given tissue and the amount of heparin that could be extracted from it. In 1952 Rocha e Silva [7] noted that both histamine and heparin were released simultaneously from the liver of dogs suffering anaphylactic shock and even proposed that both substances might co-exist in the same cell.

However, it was not until the early 1950s that Riley and West provided the important evidence that histamine was located in tissue mast cells. They noted in 1951 that intravenous injection of stilbamidine into the rat resulted in disruption of tissue mast cells and subsequent histamine release. Following Jorpes’ lead they undertook to study the distribution of histamine in a variety of animal and human tissues and tumour samples [8,9,10]. Using normal skin from a variety of animals they found mast cell numbers correlated well with the histamine content of the tissues and therefore concluded that the mast cell was the main tissue repository of histamine.

Initially, measurement of histamine was by classical pharmacological assay using isolated guinea pig ileum. However, in 1959, Shore et al. [11] published work on a fluorimetric assay for histamine based on the condensation product of its reaction with o-phthalaldehyde (OPT).

The idea that hypersensitivity could be passively transferred between individuals was demonstrated by Prausnitz and Küstner [12]. Serum from the latter, who was sensitive to fish protein, when injected intradermally into a normal individual was capable of mediating a local immediate hypersensitivity reaction following antigen challenge some 24 hours later.

However, it was not until much later, in the late 1960s, that the nature of this transfer factor or reagin present in the serum of hypersensitive individuals was established by the
Ishizakas [13,14]. It was found to be a new class of immunoglobulin named IgE.

It was later discovered [15,16] that both mast cells and basophils possess high affinity surface receptors for the Fc portion of the immunoglobulin E molecule, hence providing unequivocable evidence for the combined involvement of IgE, mast cells and histamine in the phenomenon of anaphylaxis.

1.2 A role for the mast cell in physiology and pathology

Mast cells are distributed throughout normal connective tissue, in particular adjacent to blood and lymphatic vessels [17] or near to or within nerves [18-20]. They are to be found in the mucosa and submucosa of the respiratory, gastrointestinal and urinary tracts [21-23], in the conjunctiva of the eye [24,25], free in the bronchial lumen and at the dermo-epidermal junction of the skin [26] as well as in the synovium [27] and in physiological transudates such as peritoneal fluid. In short, mast cells are to be found in locations which are particularly exposed to environmental antigens, suggesting they play a role in host defence.

One of the most striking examples of this is the involvement of mast cells in combating helminth parasitic infestations [28, 29]. Evidence for this comes from reports of elevated serum IgE, mast cell hyperplasia and eosinophilia coincident with such infestations. Entry of the parasite stimulates the production of specific IgG and activation of the complement cascade by both classical and alternative pathways. Tissue mast cells primed by anti-helminth IgE are attached to the parasite via their membrane complement receptors and are stimulated by helminth antigen to release a number of mediators including eosinophil chemotactic factor of anaphylaxis (ECF-A) and histamine. These are chemotactic for eosinophils, which are thus recruited to the site of infection and are stimulated to carry out parasite killing via IgG and complement mediated release of granular constituents such as major basic protein and eosinophil cationic protein.
In addition to this, mast cells have been postulated to be involved in certain aspects of mammalian physiology. The uterine mast cell has been implicated in menstruation [30] and embryo implantation [31] and a putative role for the stomach mast cell in gastric acid secretion has been suggested [32,33] for certain species.

Mast cell numbers have been shown to increase in a large number of different conditions [34] and, as such, mast cells have been implicated in the aetiology of a variety of allergic disorders such as asthma [35], allergic rhinitis [36-38], conjunctivitis [39], inflammatory bowel disease [40], interstitial cystitis [41-43], rheumatoid arthritis [44,45] and skin disorders like urticaria, dermatitis and mastocytosis [46].

1.3 The basis of the immediate hypersensitivity reaction

1.3.1 IgE production

Allergy is a disorder of the immune system in which entirely innocuous substances such as animal dander, certain grass pollens or the house dust mite, *Dermatophagoides*, are mistaken as harmful, resulting in the production of IgE against epitopes of these antigens [47]. Individuals who produce IgE under these circumstances are said to be atopic.

Immunoglobulin E, like other immunoglobulins, is secreted by plasma B cells (mature B cells). Foreign antigen, on invasion, is phagocytosed by macrophages which act as accessory cells, processing the foreign antigenic determinants and presenting them along with MHC Class II on their cell surfaces [48]. This combination of foreign antigen plus MHC Class II is recognised by specific helper T cells (T<sub>H</sub> cells), which thereby become activated to secrete soluble factors which induce specific B cell clones (stem cells) to proliferate and secrete IgE (plasma cells) which has a recognition site against an epitope on the original invading antigen.

IgE production is under the regulation of antigen-specific helper and suppressor T cells
Isotype-specific $T_H$ cells which express the low affinity receptor for IgE (FceRII) release IgE binding factors (cleavage products of FceRII). These up or down regulate IgE secretion by activated IgE-bearing B cells [52, 53]. It has been demonstrated in mice that the T cell-derived lymphokines, interleukin 4 (IL4) and interferon $\gamma$ (IFN$\gamma$), are also involved in regulation of the differentiation of B cells to IgE forming cells [54, 55]. IL4 is responsible for "isotype switching", which is switching sIgM or sIgD B cells (s=stem) to express sIgG1 and/or sIgE. It may also induce the production of IgE binding factors. The effect of IL4 is counteracted by IFN$\gamma$ [56,57]. Two subsets of helper T cell, $T_H$1 and $T_H$2 have been shown to exist [58] which secrete IL2/IFN$\gamma$ and IL4/IL5, respectively, when activated by antigen. The $T_H$2 T cell subset is hence essential for IgE synthesis which is under exquisite regulation by these two T helper cell subsets. Indeed, it has been suggested that atopy may result from an imbalance in the relative proportions of these T cell subsets (increased numbers of $T_H$2), defective production of IFN$\gamma$ or an ability of certain allergens to activate preferentially $T_H$2 T cell subsets [59].

### The IgE receptor and triggering of mast cell degranulation

IgE antibody molecules, when free in the circulation, bind via their Fc portions to the high affinity IgE receptor (FceRI) found exclusively on mast cells and basophils [60,61]. Once coated in IgE, mast cells and basophils are said to be sensitized. The number of FceRI on human basophils [62], rat mast cells [63] and rat basophilic leukemia cells (RBL cells) [64] have been estimated as being 40,000-100,000, 300,000 and 1,000,000 per cell, respectively.

The FceRI on RBL cells [65,66] and rat mast cells [67] has been extensively characterized. A model for the receptor has been proposed in which each FceRI consists of a tetrameric complex of one 40-60 kDa $\alpha$ subunit, one 33 kDa $\beta$ chain and a disulphide linked dimer of 7-9 kDa $\gamma$ peptides (Fig. 1.1). The IgE binding site is contained in the surface-exposed, transmembrane $\alpha$ subunit. Recent studies by Helm et al. [68] using a series of overlapping recombinant Fce gene products have located the FceRI binding site...
on human IgE to a peptide comprising the amino acid sequence 301-376 in the ε chain.

It is now generally accepted that bridging and cross-linking of cell-bound IgE molecules by multivalent antigen provides the initial stimulus to trigger mast cell degranulation with concomitant mediator release. This conclusion is based on the findings of a number of workers [69-72]. The elegant and independent experiments of Ishizaka et al. [73,74] and Isersky et al. [75] using antibodies raised to the FcεRI on RBL cells (anti-RBL), showed that whereas divalent anti-RBL antibodies and their dimeric (Fab′)₂ fragments were capable of producing histamine release from rat serosal mast cells and inducing positive skin reactions in normal rats, monovalent Fab′ fragments could only bind to the receptors without inducing histamine release (see Fig. 1.2). This suggested that dimerization of IgE molecules attached to their receptors on the mast cell surface by antigen generates the signal for secretion.

Subsequently, a number of studies have investigated the ability of monomeric Fab′ fragments of monoclonal antibodies (mabs) raised to the FcεRI on RBL cells to block immediate type hypersensitivity reactions [76, 77].

1.4 Exocytosis and mediator release from mast cells

Triggering of mast cells by immunological stimuli results in granule exocytosis and/or the release of granule contents. Ultrastructural differences exist between mast cells and basophils from different species and locations and in their granule morphology, but this will be reviewed later in section 1.7.2. Widespread variation also exists in the mechanism of anaphylactic degranulation [78] although solubilization of histamine is via a ubiquitous simple ion exchange reaction between histamine bound to the granule matrix and cations normally excluded from the cell [79,80].

In the human basophil, single granules are extruded to the exterior through multiple individual openings in the plasma membrane [81,82], whereas in the guinea pig basophil,
individual granule membranes fuse to form a large central cytoplasmic degranulation sac containing membrane-free granules [83, 84]. The sac opens to the exterior at a single point and progressively widens, distilling the granular contents into the extracellular milieu.

In human mast cells, granules swell and fuse with each other forming an elaborate labyrinth or chain connected by multiple degranulation channels, which merge with and open out to the plasma membrane at one point [82,85]. Such channel formation is accompanied by changes in the granule ultrastructure of human lung [86], nasal mucosal and skin [87] mast cells, from an initial densely packed, scroll-like organization through to a final uniform amorphous appearance, suggesting that preformed mediators are partially solubilized prior to their eventual secretion.

The involvement of actin filaments in granule exocytosis from rat peritoneal mast cells has been investigated by Tasaka et al. [88-90] using elaborate electron microscopic techniques. A complex, dense network of filaments which interconnects the granules has been observed. Microelectrophoretic application of compound 48/80 (Co. 48/80) to mast cells loaded with rhodamine-phalloidin, an actin-specific fluorescent dye, resulted in a local increase in fluorescence in the membrane, suggestive of a circular actin structure which might be effective in expelling granules from the cell. Extruded granules were connected to the cell surface by filaments up to 5 μm in length which progressively shortened indicating a reincorporation of granules into the cell - a post-degranulation repair process.

From electron microscopic studies of human lung mast cells [91,92] there is evidence that these cells also have the ability to reuse their granular membrane following degranulation and, where partial degranulation has occurred, certain elements of the granular matrix. In cells where the granules had been completely exteriorized, the Golgi apparatus swelled with concomitant development of numerous small cytoplasmic vesicles. Repackaging of the granules occurred where the contents became more concentrated and dense, a process
described as condensation, and a variety of crystalline patterns began to appear.

1.4.1 Preformed constituents of mast cell granules

1.4.1.1 Autacoids

Histamine is the major preformed mediator or autacoid stored in mast cell and basophil granules (5 μg/10⁶ cells and 1 μg/10⁶ cells, respectively) and comprises 5-10 % of the human granule by weight. It is synthesized in the Golgi apparatus on decarboxylation of the amino acid histidine by the enzyme histidine decarboxylase and stored bound to heparin. Extracellularly, histamine is rapidly metabolized by methylation (N-methyltransferase) and to a lesser extent oxidation (diamine oxidase or histaminase) and secreted in the urine.

Three receptors for histamine have been classified using specific agonists and antagonists; H₁ [93], H₂ [94] and most recently H₃ [95]. The discovery and use of a specific agonist and antagonist for the H₃ receptor [96] has lead to its location in rat cerebral cortical slices and a presynaptic autoreceptor role has been ascribed to it both in the CNS and the periphery [97]. In addition, H₃ receptors present on mast cells in rat lung, spleen and skin may control the synthesis and release of histamine and perhaps other mediators of allergy and inflammation [96].

Dale and Laidlaw [98] were the first to recognise the bronchospastic and vasodilator properties of histamine. Contraction of bronchial and intestinal smooth muscle, increased vascular permeability [99], stimulation of nasal mucus production [100] and induction of eosinophil and neutrophil chemokinesis are mediated by histamine via its H₁ receptors.

H₂-mediated effects include stimulation of T suppressor cells, IFNγ production, bronchodilatation, stimulation of airway mucus production, mediation of acid secretion [94] and inhibition of IgE-mediated basophil [101] and skin mast cell histamine release.
The overall effect of histamine therefore is clearly defined by the nature of the receptor subtypes present in the local tissue bed. Thus, the wheal and flare induced by intradermal injection of histamine for example is mediated by both $H_1$ and $H_2$ effects.

1.4.1.2 Serotonin

5-Hydroxytryptamine (5HT) or serotonin, as it was named following the coincident discovery of its vasoconstrictor activity with its presence in serum, is stored with histamine in the granules of rodent mast cells but not those of man [103]. In the rat, mast cells from a number of connective tissue locations have been shown to contain varying amounts of 5HT (up to 22 pg per cell) in varying proportions to histamine, whereas those from mucosal sites contain much smaller quantities [104,105]. 5HT is formed by 5-hydroxylation of tryptophan followed by subsequent decarboxylation of the resultant intermediate and is catabolised by monoamine oxidase.

Despite the greater relative proportion of 5HT compared to histamine in certain mast cell types, comparatively less 5HT than histamine is detected following degranulation. Given that both amines share the same storage location, this would suggest that post secretory mechanisms, such as the selective reuptake of 5HT, an active process inhibitable by tricyclic antidepressants, [106,107] are involved.

In human skin, 5HT is a potent vasoconstrictor whereas in rodents it induces increased postcapillary venular permeability. It is also a potent bronchoconstrictor in rodents while having no effect in human airways.

1.4.1.3 Proteoglycans

Proteoglycans are major components of tissue ground substance, bone and cartilage. They form the structural basis of lysosomal granules and, in mast cell granules, act as storage matrices for preformed mediators such as histamine and, in rat mast cells, 5HT.
It is the property of heparin to shift the colour absorption and emission spectrum of certain basic dyes such as azure A (metachromasia) which results in the ability to stain and hence visualize mast cells by light microscopy.

Mast cells are the sole source of extracellular heparin, liberated on degranulation, which has a wide variety of biological properties; as an anticoagulant and an inhibitor of complement activation and eosinophil cytotoxicity [108,109].

Proteoglycans consist of a common peptide core of repeating serine and glycine residues substituted at every second or third serine residue with covalently linked glycosaminoglycan (GAG) side chains of varying degrees of sulphation (see Fig. 1.3) [110]. The GAGs are attached via a unique sequence of sugars comprising xylose-galactose-galactose-glucuronic acid.

There are two families of GAG side chain, the heparin-heparan sulphate family and the chondroitin sulphate family. The former consists of repeating $\alpha_{1,4}$-linked disaccharide units of glucuronic acid or iduronic acid in a $\beta_{1,4}$ linkage with glucosamine. One carboxyl group and from 1 to 4 sulphate groups are attached to the nitrogen and hydroxyl groups of glucosamine and hydroxyl groups of iduronic acid. Chondroitin sulphates are less highly sulphated but structurally similar to heparin. The repeating disaccharide unit of this GAG consists of galactosamine and glucuronic acid in a $\beta_{1,3}$ linkage, linked together via $\beta_{1,4}$.

As a result of their acidic properties and capacity to exclude water, proteoglycans are able to inactivate and package other stored mediators in the mast cell granule [111]. Approximately 10% of granule histamine is bound ionically to the ester sulphate and carboxyl groups of GAGs. A further small proportion is complexed to zinc but the majority of histamine is bound via carboxyl groups on highly basic polypeptides which are found complexed with heparin.
1.4.1.4 **Granule enzymes**

There are three main classes of enzyme in the mast cell granule; the neutral proteases, the acid hydrolases and the oxidative enzymes.

The neutral proteases are a group of proteolytic enzymes or serine esterases of optimal activity at neutral pH, which cleave peptide and ester bonds on the carboxyl side of basic (tryptase), aromatic (chymase) or terminal aromatic (carboxypeptidase) amino acids.

The distinct distribution of these enzymes throughout rat and human mast cells is an indication that mast cells from different locations are morphologically heterogeneous (see section 1.7.2). This is exemplified in the rat where two immunologically distinct forms of chymase have been identified. Mast cells from connective tissue locations contain a chymotryptic enzyme, designated rat mast cell protease I (RMCPI) [112], which is a single chain protein whose substrates include fibrinogen and components of the basement membrane, fibronectin and type IV collagen. Those from mucosal sites, however, contain a more soluble form of the enzyme (RMCPII) [113], a dimeric peptide with a subtle function in cellular events associated with activation and proliferation, particularly of lymphocytes. Although chymases are the major neutral protease in rodent granules, some tryptase is also present [114].

In man, two distinct types of mast cell have been classified on the basis of their neutral protease composition [115]. Tryptase has been found in all human mast cells investigated but chymase is found in mast cells from the skin, intestinal submucosa and to a lesser extent lung [116]. Very small amounts of tryptase (< 1 % that in mast cells) may be found in basophils [117]. The presence of this enzyme in serum can be used as a specific marker for mast cell activation in certain disease states [118].

The catalytic form of tryptase is tetrameric and is inactive as a monomer. It is involved in complement activation by cleavage of C3 into its component parts C3b and the
anaphylatoxin, C3a [119]. In the presence of heparin, however, which has a modulatory effect on the enzyme, tryptase further cleaves and inactivates C3a. The protease also has a local anticoagulant action through degradation of fibrinogen and high molecular weight kininogen [120] and is involved in the breakdown of connective tissue [121,122]. In addition, tryptase from human lung mast cells may play a role in bronchial hyperresponsiveness in asthma through its ability to degrade and inactivate vasoactive intestinal peptide (VIP), the main bronchorelaxant neurotransmitter of non-adrenergic, non-cholinergic (NANC) nerves in the lung [123]. This is selective since substance P (SP), a bronchoconstrictor, is not affected by tryptase.

Chymase in human mast cells is quite distinct from that in rodents. It is a monomeric enzyme which efficiently catalyses the conversion of angiotensin I to angiotensin II [116], inactivates bradykinin and kallidin, degrades basement membrane components [124] and cleaves both VIP and substance P which may be of importance in attenuation of neurogenic inflammation in skin [125].

Carboxypeptidase A is found in association with RMCP in the rodent whereas in human mast cells a different isomer, carboxypeptidase B, is associated with tryptase. These enzymes complement the actions of the other neutral proteases in protein degradation. Other neutral proteases include dipeptidase which catalyses the conversion of leukotriene D4 (LTD4) to LTE4, kininogenase, kallikrein, which liberates bradykinin from kininogen, and plasminogen activator.

The neutral proteases are found tightly complexed to proteoglycans in the acidic environment of the granule which serves to stabilize and inactivate them. On degranulation, they remain bound and hence cell-associated.

The acid hydrolases comprise a group of enzymes with optimal activity at acid pH which includes the exoglycosidases, β-hexosaminidase, β-glucuronidase and β-galactosidase, which degrade glycoproteins and proteoglycans, and aryl sulphatase which hydrolyses
aromatic sulphate esters. All of these enzymes have been found in the granules of both rat and human mast cells in different isomeric forms. β-Hexosaminidase is useful as a marker of mast cell degranulation in vitro.

The oxidative enzymes present in rat peritoneal and human lung mast cell granules include superoxide dismutase [126] and peroxidase [127]. Both enzymes function to remove superoxide anions and, in the case of peroxidase, inactivate dihydroxy and sulphidopeptide leukotrienes.

1.4.2 Newly synthesized mediators

1.4.2.1 Arachidonic acid metabolites

Rat [128], human [129-133] and cultured mast cell lines [134, 135] when stimulated in vitro with anti-IgE or Ca\(^{2+}\) ionophore produce newly synthesized, pro-inflammatory mediators, the products of arachidonic acid (AA) metabolism, namely the eicosanoids prostaglandin D\(_2\) (PGD\(_2\)) and leukotrienes C\(_4\) and D\(_4\) (LTC\(_4\), LTD\(_4\)). AA, generated by the action of phospholipase A\(_2\) (PLA\(_2\)) on membrane phospholipids, is metabolized via two major enzymic pathways (see Fig. 1.4). Cyclooxygenase catalyses the formation of PGE\(_2\), PGF\(_{2\alpha}\), PGI\(_2\), PGD\(_2\) and the thromboxanes, TXA\(_2\) and TXB\(_2\), via the unstable cyclic endoperoxidase intermediates PGG\(_2\) and PGH\(_2\) and 5-lipoxygenase gives rise to LTB\(_4\), LTC\(_4\), LTD\(_4\) and LTE\(_4\) via 5-HPETE and the unstable peroxide LTA\(_4\). Recently, the mast cell granule has been identified as the site of eicosanoid production in rat serosal mast cells by the localization of cyclooxygenase and its product PGE\(_2\) to the granule matrix by immunocytochemical techniques [136]. Once formed, the eicosanoids act as local hormones and are thus metabolized close to their sites of generation. TXA\(_2\) and PGI\(_2\) in particular have very short half-lives.

LTC\(_4\), LTD\(_4\) and LTE\(_4\) have been identified as the components of the slow reacting substance of anaphylaxis (SRS-A) which induces pronounced bronchoconstriction and
oedema in the lungs of experimental animals and dose-related erythema and wealing on injection into human skin [137]. PGD$_2$ is also a potent spasmogen, a peripheral vasodilator, but a pulmonary and coronary vasoconstrictor.

Elevated levels of PGD$_2$ metabolites have been observed in the plasma and urine of patients during attacks of mastocytosis [138] and some evidence also suggests a role for newly formed mediators in generation of the late phase reaction (LPR) in man [139].

1.4.2.2 Platelet activating factor

Alkylglyceryletherphosphorylcholine (AGEPC), originally named platelet activating factor (PAF) because of its ability to aggregate and degranulate rabbit platelets, consists of a family of naturally occurring molecules containing a variety of fatty alcohol chains at the sn-1 position of the 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine molecule. Biosynthesis of PAF is a two-stage process in which 1-0-alkyl-2-acyl-glyceryl-3-phosphorylcholine is hydrolized by phospholipase A$_2$ to yield an intermediate, lyso-AGEPC, which is then further acetylated.

PAF has been shown to be produced by a wide variety of inflammatory cells including several murine mast cell lines [140,141] and more recently, purified preparations of isolated human lung mast cells [142] following challenge of these cells by anti-IgE and the calcium ionophore, A23187.

PAF also activates a number of inflammatory cells. It is one of the most potent eosinophil chemotaxins and activators in vitro [143] and also induces neutrophil chemotaxis and activation. In the lungs it causes pulmonary oedema, vasoconstriction and bronchoconstriction and systemically produces hypotension. On injection into human skin, PAF produces a wheal and flare reaction and is 100 - 1000 times more potent on a molar basis than histamine [144,145].
1.4.3 Chemotactic factors

Chemotactic factors stimulate directed migration of cells, usually polymorphonuclear (PMN) leukocytes, towards sites of inflammation at which these chemotaxins are liberated. They also enhance the cell surface expression of various receptors (e.g. C3b and IgG) thereby reducing the threshold at which these cells will respond to other stimuli. Chemotactic factors are responsible for the second wave of inflammation or LPR, which occurs 3-6 hours after immediate type hypersensitivity and is characterized by the influx of neutrophils and eosinophils. Such LPRs dominate more chronic allergic reactions such as asthma, eczema and urticaria.

Mast cell chemotaxins include ECF-A and inflammatory factor of anaphylaxis (IF-A), which stimulates neutrophil (2-8 hours) and monocyte (24 hours) infiltration [146]. LTB4 and PAF, newly generated mediators, also have chemotactic activity.

1.4.4 The cytokines

Recently, much attention has focused on the production of cytokines by mast cells. The cytokines are a group of glycoproteins secreted by lymphocytes, monocytes/macrophages, fibroblasts, endothelium and other cells. They largely affect the growth and differentiation of lymphoid and haemopoietic cells from their progenitors with a range of specificities. Recently, tumor necrosis factor-α (TNF-α) or cachectin was shown to be produced by certain cultured murine mast cell lines [147,148], mast cell/basophils from human bone marrow cultures [149] and peritoneal mast cells from the mouse [150]. The use of in situ hybridization techniques located TNF-α mRNA in the cytoplasm and TNF-α protein in the granules of individual cells [149], suggesting this cytokine is both inducible and preformed in the mast cell. Mast cells are involved in a variety of processes including tumour cell cytotoxicity [151-153], bone remodelling [154] and fibroblast growth [155, 156] in which TNF has also been shown to play a role [157].
Murine mast cell lines also produce an impressive array of other polypeptide cytokines following immunologic or Ca\(^{2+}\) ionophore-induced stimulation. These include IL3 (a mast cell growth factor), IL4 (IgE switch factor), IL5 (eosinophil differentiation factor), IL6 (controls IgG secretion) [158, 159], GM-CSF [160], IL1, IFN\(\gamma\), MIP\(\alpha\) and MIP\(\beta\) (macrophage inflammatory protein) [161]. Mast cells may therefore modulate their own growth and functional activity by the secretion of IL3 and IL4, respectively, and be involved in the asthmatic LPR, characterized by the presence of large numbers of eosinophils, through their production of IL5.

In addition to their role in mast cell growth and differentiation, cytokines may also modulate the functional responsiveness of basophils and perhaps also mast cells. Recently, recombinant interleukin 3 (rIL3) has been shown to release histamine from some human basophils from atopic donors, a process dependent on the presence of surface IgE [162]. RIL3 [163] and rIL1 [164] also have the ability to potentiate the histamine release response of basophils to a variety of immunological stimuli and hence these cytokines may have a pro-inflammatory role by upregulating the effects of IgE-related signals in such cells.

1.5 **Non-immunological histamine release**

In addition to immunological stimuli, mast cells are susceptible to releasing histamine with a wide variety of structurally diverse substances [165,166].

The lectin, concanavalin A (Con A) is believed to exert its histamine-releasing activity by binding to glucose and mannose moieties on the Fc region of mast cell-bound IgE [167,168] and is therefore considered to act similarly to antigen, by cross-linking Fc\(\varepsilon\) receptors.

Other compounds which induce histamine release can be classified into two groups [169]; selective, non-cytotoxic liberators which act without release of the cytosolic marker lactate
dehydrogenase (LDH), and non-selective, lytic agents typified by the detergents melittin, isolated from bee venom, and triton X-100 [170]. Amongst the selective liberators are Co. 48/80 [171], the anaphylatoxins, C5a and C3a [172], ATP [173], calcium ionophores [174], vasoactive and neuro-peptides [175], polycations [176], a number of structurally unrelated drugs [177, 178], the plasma substitute, dextran [179], and simple salts such as NaF [180].

It is, however, important to mention that the action of many of these secretagogues on mast cells is highly tissue and species specific.

1.6 **Biochemical events involved in mediator secretion**

In 1961, Douglas and Rubin coined the term *stimulus-secretion coupling* to denote the concept of calcium (Ca\(^{2+}\)) linking the receptor-mediated events at the plasma membrane with the process of exocytosis [181]. Since this time much research effort has been devoted to elucidating the mechanisms involved in this coupling process in the context of the mast cell. It is important to mention, however, that the main body of work has been conducted using a line of rat leukemia cells, the RBL 2H3 cells. Although these cells provide an adequate *in vitro* model of mucosal type mast cells, results generated from this cell type cannot easily be extrapolated to human cells.

1.6.1 **Evidence for the role of Ca\(^{2+}\) as a second messenger in mast cell stimulus-secretion coupling**

Receptor-mediated responses such as IgE-mediated stimulation of mast cells require systems for transducing cell surface signals across the plasma membrane. This is commonly achieved by activation of biochemical second messenger cascades within the cell, one of the most ubiquitous of which uses Ca\(^{2+}\) as the second messenger [182].

The pioneering work of Mongar and Schild in 1958 [183], using fragments of guinea pig
lung, provided the initial evidence that anaphylactic secretion of histamine from mast cells was dependent on the presence of \( \text{Ca}^{2+} \) ions in the bathing solution. Uptake of extracellular \( \text{Ca}^{2+} \) by mast cells following antigen challenge was subsequently demonstrated by Foreman, Hallett and Mongar [184] using radioisotopic \( ^{45}\text{Ca}^{2+} \). It was thus concluded that entry of this cation from the extracellular milieu induced exocytosis. These observations were later extended using human lung mast cells [185].

The most direct evidence, however, for the involvement of calcium in mediator secretion was provided by Kanno and coworkers [186] who succeeded in inducing rapid degranulation of rat mesenteric mast cells by microinjection of the cation into these cells. Such degranulation was specific for \( \text{Ca}^{2+} \) and could not be reproduced by injection of magnesium or potassium or by direct mechanical insult.

A variety of other methods have been employed to introduce \( \text{Ca}^{2+} \) ions into mast cells in order to study the effect on exocytosis, including fusion of the cell with \( \text{Ca}^{2+} \)-containing liposomes [187] and use of calcium ionophores [188]. The latter are organic molecules comprising a hydrophilic interior, capable of binding specific metal ions, and a hydrophobic exterior which facilitates partition of the complex into the cell membrane. This unique structure confers on these molecules the ability to transfer cations across biological membranes down their concentration gradient.

The dependency of the exocytotic process on calcium has been demonstrated by the use of lanthanum ions (\( \text{La}^{3+} \)) [189,190] which, by virtue of a similar ionic radius to that of calcium, coupled with a higher valency, are able to compete with and hence displace \( \text{Ca}^{2+} \) from its superficial binding sites in the cell membrane. Thus \( \text{La}^{3+} \) can be employed as a specific \( \text{Ca}^{2+} \) antagonist and has been shown to inhibit histamine release induced by various secretagogues [190].

More detailed investigations of the role played by \( \text{Ca}^{2+} \) have been facilitated by the development of the fluorescent tetracarboxylate calcium chelator, quin-2. Antigen
challenge of quin-2-loaded rat peritoneal mast cells resulted in a rapid and transient increase in cytosolic fluorescence indicative of the involvement of intracellular Ca\(^{2+}\) in cell signalling [191].

1.6.2 Calcium pools involved in the secretory process

The establishment of Ca\(^{2+}\) as the second messenger in mast cell stimulus-secretion coupling has lead to investigation of the various mast cell-associated pools of this cation.

Levels of intracellular Ca\(^{2+}\) in the non-secreting mast cell are strictly regulated. The presence of strong inward concentration and electrochemical gradients ([Ca\(^{2+}\)]\(_i\) is of the order of 0.1 μM compared with 1 mM extracellularly) is maintained by the limited permeability of the plasma membrane to the cation and the existence of Na\(^+\)/Ca\(^{2+}\) antiporters and Ca\(^{2+}\) ATPases which extrude the ion or promote its sequestration into internal stores [192, 193].

Optimal anaphylactic secretion of histamine requires the presence of Ca\(^{2+}\) in the extracellular medium. This observation has given rise to the hypothesis that on triggering of the cell by antigen, the Ca\(^{2+}\)-permeability of the mast cell membrane is transiently increased by the opening of specific gates or membrane channels, allowing influx of the cation and hence stimulation of exocytosis [189,190,194]. Evidence for this has been derived from a number of investigations which have uncovered superficial binding sites for Ca\(^{2+}\) on the cell membrane, where the cation is in rapid equilibrium with the extracellular environment and from which it can migrate into the cell via such channels [195-197]. After dilution of rat mast cells incubated in a calcium-containing medium to a medium devoid of calcium, the cells responded to histamine-releasing agents like antigen [195], the ionophore A23187 [196] and Con A [197], only if the cells were exposed to these agents immediately and even then in a reduced fashion. When the releasing agents were added at increasing intervals following dilution, the secretory response gradually diminished. This observation was consistent with the presence of
loosely bound and rapidly dissociating pools of \( \text{Ca}^{2+} \) in the membrane utilized by the above agents. The slow kinetics of release characterized by IgE-directed ligands and suboptimal ionophore concentrations compared with the fast dissociation of the cation from these sites enabled observation of this dilution effect.

Agents like Co. 48/80, polylysine and peptide 401 [188,198,199], however, released considerable amounts of histamine from rat mast cells in the absence of extracellular \( \text{Ca}^{2+} \). Long exposure of these cells to the calcium-chelating agent ethylenediaminetetraacetic acid (EDTA) [200, 201] and preincubation with the ionophore, A23187 [196] in the absence of extracellular calcium rendered them unresponsive to Co. 48/80. The cellular response was restored, however, following reintroduction of \( \text{Ca}^{2+} \) to the medium. The implication from these observations was that such agents mobilized intracellular stores of the cation which were depleted by the above treatments and replenished by addition of extracellular \( \text{Ca}^{2+} \).

Another indication that mobilization of the cation was from an intracellular location came from studies using quin-2-loaded rat mast cells [191]. On incubation of these cells with EDTA and subsequent antigen challenge, an increase in fluorescence was observed and, although this was smaller than that induced in the absence of the chelator, it was nonetheless substantial. Such stores may be buried deep within the cell membrane, attached to the inner surface of the membrane or sequestered in the endoplasmic reticulum or mitochondria, the latter two of which constitute the major \( \text{Ca}^{2+} \) pools in most cells.

Brief exposure to EDTA enhances the secretory response to many inducers [198]. Such treatment is believed to remove \( \text{Ca}^{2+} \) ions from regulatory sites in the cell membrane leading to membrane destabilization and thus facilitating release of the cation from internal stores. Conversely, supramaximal concentrations of extracellular \( \text{Ca}^{2+} \) depress the response to Co. 48/80 slightly [198], presumably through saturation of these regulatory sites resulting in membrane stabilization and restricted uptake of the cation.
1.6.3 Calcium channels in the mast cell

Recently, the question of the existence of Ca\(^{2+}\) channels in the non-excitatory mast cell membrane has been addressed by Penner and Neher [202].

Earlier observations had indicated that there were no voltage-operated Ca\(^{2+}\) channels in the mast cell membrane [203]. However, the existence of some sort of channel in non-excitatory cells which was sensitive to elevated [Ca\(^{2+}\)]\(_i\) had been suggested [204]. Patch clamp studies of rat peritoneal mast cells stimulated with either Co. 48/80 or substance P, revealed the existence of a sustained plateau of elevated [Ca\(^{2+}\)]\(_i\) which was dependent on external calcium and which followed the initial, large, transient increase in [Ca\(^{2+}\)]\(_i\) [202]. This Ca\(^{2+}\) plateau, in contrast to the early Ca\(^{2+}\) rise, was ideally timed to influence the secretory response.

Three ionic mechanisms were identified which explained this calcium influx. A chloride channel, activated by internally applied cAMP, hyperpolarizes the membrane (holds the membrane potential at a negative value) and provides the electrical driving force necessary for the uptake of Ca\(^{2+}\). This hyperpolarization facilitates the opening of Ca\(^{2+}\)-specific channels in the membrane, which are also sensitive to intracellular applied IP\(_3\). Finally, there is a non-specific channel through which divalent cations can permeate. Such channels are not voltage-gated but are potential-dependent insomuch as the Ca\(^{2+}\) influx depends on an electrical driving force. Thus it would appear that Ca\(^{2+}\) influx in the mast cell is regulated by membrane channels sensitive to the two intracellular messengers IP\(_3\) and cAMP (see sections 1.6.5.1 and 1.6.7). Indeed, where depolarization in excitable cells results in the opening of voltage-gated Ca\(^{2+}\) channels, hyperpolarization has often been associated with Ca\(^{2+}\) influx in non-excitatory cells [205,206].

These exciting findings have stimulated interest in the use of calcium channel blockers, such as nifedipine and verapamil in clinical inhibition of immunological histamine release [207,208]. However, the concentrations of these drugs required to be effective in the
treatment of asthma are very much higher than the concentrations active in smooth muscle.

1.6.4 Calmodulin - the intracellular Ca\(^{2+}\) receptor

Elevation of intracellular calcium is only one step in the second messenger transduction system. The mechanism by which the signal is propagated through the cytosol to elicit eventual secretion is thought to be by the interaction of Ca\(^{2+}\) ions with the polypeptide, calmodulin (CaM), which is generally accepted as being the universal intracellular receptor for the cation [209-211].

CaM is ubiquitously distributed in eukaryotic cells and its structure has been found to be highly conserved [198,209,212]. It is an extremely acidic molecule with a molecular weight of 17,000 and possesses the ability to bind a maximum of four Ca\(^{2+}\) ions with high affinity. Binding of Ca\(^{2+}\) to CaM confers a more stable structure on the protein and the resultant conformational changes reveal active sites on the surface of the molecule which allow interaction with and activation of apoenzymes or effector proteins.

A number of key enzymes involved in important cellular processes such as secretion and proliferation are regulated by CaM; cyclic nucleotide phosphodiesterase, adenylate and guanylate cyclases, methyltransferase, phospholipase A\(_2\), the ATPase and calcium pump of the plasma membrane and sarcoplasmic reticulum and a number of kinases, including myosin light chain kinase [198,210,211]. The use of CaM antagonists, naphthalene sulphonamide (W7) and the phenothiazines, trifluoperazine and chlorpromazine, has uncovered a putative role for CaM in the process of exocytosis. However, although these agents inhibited histamine release from both mast cells and basophils [213-215], their inhibition was not specific for CaM as they displayed binding to membranes, cell surface receptors and also protein kinase C.

Studies of protein phosphorylation have revealed that on challenge of rat mast cells with
anti-IgE, Co. 48/80 or A23187, three proteins of molecular weight 42, 59 and 68 kD are phosphorylated prior to histamine secretion, in a calcium dependent fashion [216]. Termination of exocytosis was also concomitant with the phosphorylation of a 78kD protein [216]. The phosphorylation of specific proteins may then be important for both the induction and termination of histamine secretion and although the precise involvement of CaM in this process is as yet unknown, it is certainly tempting to speculate that it has a role to play through its action on cellular kinases.

As previously discussed in section 1.4, there is also considerable evidence to suggest that microtubules are centrally involved in histamine secretion [86,217]. CaM, both in vitro and in vivo is known to promote the disassembly of microtubules [218] and thus activation of this enzyme in the mast cell may be involved in termination of secretion. CaM activation may also have profound effects on the regulation of cyclic nucleotides levels and in Ca^{2+} homeostasis. CaM has been implicated in mediation of the characteristic transient increase in cAMP noted following immunological stimulation of the mast cell (see section 1.6.7) [219].

Until such times, however, as the myriad of enzyme activation and deactivation processes within the cell are identified, the exact sequence of events subsequent to the rise of intracellular Ca^{2+} and activation of CaM in the mast cell will remain an enigma.

1.6.5 Proposed mechanisms for the mobilization of intracellular calcium

1.6.5.1 The phosphoinositide cycle

In the early 1950s, Hokin and Hokin [220,221] discovered that cholinergic stimulation of slices of pigeon pancreatic tissue resulted in the rapid incorporation of $[^{32}\text{P}]$ phosphate into membrane phospholipids. The subsequent interest which this discovery generated, lead to the inspired proposal by Michell in 1975 that the enhanced metabolism of membrane phospholipids following receptor stimulation in some way caused the
mobilization of Ca\textsuperscript{2+} ions [222].

A number of investigations using rat peritoneal mast cells, human basophils and a variety of mast cell lines including RBL-2H3 cells, have subsequently demonstrated the uptake of [\textsuperscript{32}P] phosphate label into phospholipids on stimulation with Co. 48/80, chymotrypsin and IgE-directed ligands [223-227]. This phospholipid hydrolysis was associated with a rise in [Ca\textsuperscript{2+}]\textsubscript{i} and secretion of histamine.

Inositol phosphate metabolism can be followed by incubation of intact tissues and cells with radiolabelled precursors such as [\textsuperscript{32}P] phosphate or D-myo [\textsuperscript{3}H] inositol in the presence of Li\textsuperscript{+} which allows the accumulation of labelled breakdown products. Following simple extraction, these products can be identified using high pressure liquid chromatographic techniques [228].

Highly complex pathways exist for the cycling of phosphoinositides and readers are referred to a number of excellent reviews for a more detailed description of this [229-231]. In brief, there are three inositol phospholipids in the plasma membrane, the most abundant of which is the parent compound, phosphatidylinositol (PI). This is rapidly converted by sequential addition of two phosphate groups by the action of ATP-dependent kinases to phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}). In addition, corresponding phosphomonoesterases exist to convert PIP\textsubscript{2} back to PI and hence there is a constant and futile cycling of these molecules in the plasma membrane. Occupation of a receptor by its agonist, however, diverts PIP\textsubscript{2} out of this futile cycle towards a phosphodiesterase, phospholipase C (PLC), which catalyses its hydrolysis, producing the two second messengers, inositol-(1,4,5)-triphosphate (IP\textsubscript{3}) which is released into the cytosol and diacylglycerol (DAG), which remains in the membrane.

A series of inositol phosphatases hydrolyse IP\textsubscript{3} to free inositol, generating an array of breakdown products which have their own messenger functions and hence potentially diverse effects on cellular activity. IP\textsubscript{3} is rapidly converted to either inositol 1,4-
bisphosphate (IP$_3$) or inositol-(1,3,4,5)-tetrakisphosphate (IP$_4$) [232]. An isomer of IP$_3$, inositol-(1,3,4)-triphosphate [233] is generated as a result of the dephosphorylation of IP$_4$ at the 5 position.

Evidence that physiologically relevant concentrations of IP$_3$ (0.2-0.5 μM) are able to induce half maximal release of Ca$^{2+}$ from non-mitochondrial intracellular sites has been provided by a variety of studies using permeabilized cells [234-236]. These effects were specific for IP$_3$, since other inositol phosphates had little or no effect on mobilization of intracellular Ca$^{2+}$ [234]. IP$_4$ together with IP$_3$ have, however, recently been implicated in regulation of the opening of specific channels in the plasma membrane thereby allowing the influx of extracellular Ca$^{2+}$ [237,238], which would result in an amplification of the Ca$^{2+}$ signal (see section 1.6.3). According to a model proposed by Putney [239], influx of extracellular Ca$^{2+}$ occurs in order to replenish intracellular stores and persists as long as IP$_3$ is being produced. Once production ceases, and stores of the cation are refilled, entry of Ca$^{2+}$ from the cell exterior stops.

Results from studies using RBL 2H3 cells are consistent with mobilization of intracellular Ca$^{2+}$ ions by IP$_3$; the phosphoinositide response in these cells is neither affected by the blockade of Ca$^{2+}$ influx by La$^{3+}$ [240] nor induced by direct mobilization of calcium ions using the ionophore, A23187 [241]. However, in contrast to earlier studies of PI turnover in mast cells [224], hydrolysis of inositol phospholipids induced by antigen stimulation of RBL cells was shown to be dependent on extracellular Ca$^{2+}$. This caused considerable controversy since, if IP$_3$ is involved in releasing Ca$^{2+}$ from intracellular stores, its generation should not be calcium dependent. Such a discrepancy is now thought to be due to the slow kinetics of receptor aggregation displayed by the particular antigen utilised in these studies. Subsequent efforts by independent investigators using an antigen with higher binding affinity have corroborated the results from other mast cells regarding extracellular Ca$^{2+}$ requirements and have shown that the generation of IP$_3$ in the RBL cell indeed precedes the onset of the Ca$^{2+}$ signal [242].
The other product of PIP$_2$ hydrolysis, DAG, is now established as an activator of protein kinase C (PKC) [243], a calcium and phospholipid-dependent enzyme which catalyses the phosphorylation of protein serine and threonine residues. PKC is present in a number of tissues and has been discovered also in mast cells [244,245]. Binding of DAG to PKC in the presence of phospholipids, reversibly increases the affinity of the enzyme for calcium ions, thus facilitating its activation at submicromolar concentrations of the cation [246]. Activation induces translocation of the protein from the cytosol to the membrane.

Production of DAG has been demonstrated in mast cells [223] and using cultured murine mast cells challenged with antigen, White et al. [191,247] have observed a rapid increase in membrane associated PKC activity which reached a maximum after 30 sec and preceded histamine release. More recently work from this author’s laboratory has been concerned with studying the effects of two potent PKC inhibitors, staurosporine and K-252a, on the secretory process in rat serosal mast cells [248,249]. Both compounds strongly suppressed liberation of histamine and PGE$_2$ from these cells, and LTC$_4$ from mouse bone marrow-derived mast cells (mBMMC), when this was induced either by anti-IgE or PMA, but were only partially effective when the stimulus was Co. 48/80. This suggests that the mechanism by which Co. 48/80 induces secretion is not linked to the activation of PKC.

DAG may therefore function as a second messenger to activate phosphorylation of enzymes involved in the exocytotic process. Indeed, some evidence exists to suggest that phosphorylation of the light chain of myosin in RBL cells is mediated in part by PKC [250]. Phosphorylation of a PKC substrate protein (45K), which has been identified in mBMMC, was observed on IgE stimulation of these cells [249] but was dramatically reduced following treatment of these cells with PKC inhibitors.

Use of the exogenous activators of PKC, namely the phorbol ester PMA and oleoyl acetylgllycerol, in studies of human basophils [251], rat mast cells [245] and RBL cells [240,252,253], has shown that low concentrations of A23187 and PMA which do not elicit
secretion individually, synergize with one another to do so. The concentrations of A23187 and PMA employed were, however, sufficient to induce substantial increases in $[Ca^{2+}]_i$ and translocation of PKC, respectively. Thus, these events would appear to require to act in concert for histamine secretion to be induced, the so-called dual signal hypothesis proposed by Berridge [254].

In addition to its synergistic effects, PKC may also provide an inhibitory signal to, or display feedback modulation of, the secretory process. Evidence for this has come from studies using RBL cells [240,245,252,255] and various other cell types [256] where concentrations of PMA as low as 1 nM caused a rapid decline in antigen induced hydrolysis of membrane inositol phospholipids and the resultant $Ca^{2+}$ signal and higher PMA concentrations (25-50 nM), added either before or after antigen, completely abrogated the signal. However, although these early stimulatory signals were suppressed, histamine secretion was only modestly reduced. Thus, as yet, the exact contribution of both IP$_3$ and DAG to the generation of exocytosis is unclear.

1.6.5.2 Phospholipid methylation

An alternative mechanism for membrane signal transduction was proposed in 1980 by Hirata and Axelrod [257], following their discovery of two unique membrane bound methyltransferases (I and II) in rat erythrocyte membranes [258]. These enzymes are responsible for the sequential methylation of the membrane phospholipid phosphatidylethanolamine (PE) to form phosphatidylcholine (PC) using the methyl donor, S-adenosyl-L-methionine (S-AM), a process termed phospholipid methylation (see Fig.1.5). The spatial organization of the enzymes - methyltransferase I on the cytoplasmic face, methyltransferase II towards the external side of the membrane - results in simultaneous translocation of the methylated phospholipids across the membrane and hence increased membrane fluidity. These changes in microviscosity may be associated with increased permeability to $Ca^{2+}$ ions [259] or may facilitate activation of an ecto-ATPase, thought by some to be responsible for $Ca^{2+}$ transport into the mast cell [260].
Bridging of IgE receptors resulted in phospholipid methylation in murine mast cells [261,262], purified human pulmonary mast cells [185], cultured basophils [227] and RBL cells [263]. In each case this preceeded an increase in $^{45}\text{Ca}^{2+}$ uptake and histamine release, suggesting phospholipid methylation is an obligatory step in calcium translocation and secretion. Further evidence for this comes from the use of the inhibitors of S-AM-mediated methylation, 3-deazaadenosine (3-DZA) and L-homocysteine thiolactone, which inhibited the IgE-mediated increase in $[\text{Ca}^{2+}]_i$ and release of both histamine and arachidonic acid as well as phospholipid methylation [263,264].

The establishment of two variants of the RBL 2H3 cell lines, which each lack one of the two methyltransferase enzymes, lent further support to the importance of phospholipid methylation in these cells [265]. Neither variant was capable of releasing histamine on bridging of their IgE receptors, although they did so on exposure to the calcium ionophore, A23187, indicating that all mechanisms distal to the mobilization of $[\text{Ca}^{2+}]_i$ were intact. On fusion of these two mutant cell lines, a hybrid was created which possessed both enzymes and a normal secretory response was restored on IgE stimulation.

Despite the above compelling evidence, however, exhaustive studies by a number of independent investigators using both mast cells [266,267] and other cell types [268] have failed to reproduce the results. It has also become apparent that the inhibitor 3-DZA is non-specific and interacts with other biochemical processes distal to the methylation step [267]. On this basis therefore it is impossible to assess the relative importance of phospholipid methylation in induction of secretion in the mast cell.

1.6.5.3 Arachidonic acid metabolism

The metabolism of membrane phospholipids, either by the enzyme phospholipase A$_2$ (PLA$_2$) or a combination of PLC and diacylglycerol lipase, generates free arachidonic acid (AA) which in turn can be metabolised via the cyclooxygenase or lipoxygenase pathways giving rise to a variety of prostaglandins, leukotrienes and thromboxanes (see section
1.4.2.1). As well as the inflammatory properties of these compounds and their intermediates they may also play a role in modulation of the release process.

This hypothesis has arisen from the observations that highly purified PLA₂ preparations induced non-cytotoxic histamine release from rat peritoneal mast cells and human basophils [269-272] and that, in addition, potent PLA₂ inhibitors, such as p-bromophenacyl bromide (p-BPB) [271,273,274] or mepacrine [270,275], suppressed antigen- and A23187- induced release from these cells.

DAG, which has been discovered in mast cells following stimulation with Co. 48/80 [276], is a known fusogen, as are its cleavage products generated by the action of diacylglycerol lipase, the free fatty acid (AA) and monoacylglycerol [277,278]. These products of PLC activation may therefore directly promote conjunction of the plasma and perigranular membranes. Indeed, exogenously applied AA potentiated anaphylactic histamine secretion in rat mast cells and human basophils [279,280].

The products of the lipoxygenase pathway, 5-hydroperoxy and 5-hydroxy-eicosatetraenoic acid (5HPETE and 5HETE) have also been implicated in modulation of exocytosis. These compounds have been demonstrated to enhance antigen-induced histamine release from human basophils [281,282]. Moreover, the AA analogue, eicosa-5,8,11,14-tetraynoic acid (ETYA), which inhibits both cyclooxygenase and lipoxygenase enzymes, and the more specific lipoxygenase inhibitor, 5,8,11-eicosatriynoic acid (ETI), suppressed the secretion of histamine induced by a variety of stimuli [279,283]. More recently, the highly specific 5-lipoxygenase inhibitor, L651-392 (4-bromo-2,7-dimethoxy-3-H-phenothiazine-3-one), although it inhibited the release of both LTC₄ and 5-HETE from human lung mast cells and basophils, was found to have no effect on histamine release even at high doses [284].

The non-steroidal anti-inflammatory drugs, such as aspirin, indomethacin and diclofenac, were, in contrast, ineffective at inhibiting secretion and in fact may even enhance release [280,285].
1.6.6 Regulatory components of signal transduction mechanisms

1.6.6.1 GTP-regulatory proteins

An essential component in the transduction machinery which, in the case of PI turnover, links receptor occupancy with PLC activation, is a guanine nucleotide-dependent regulatory protein or G protein, designated $G_p$ [286]. G proteins are a large homologous family of trimeric proteins comprising an $\alpha$ subunit that binds guanine nucleotides and $\beta$ and $\gamma$ subunits which are always tightly associated and link the $\alpha$ subunit to the receptor [287]. The G protein cycles between an active GTP-bound form, catalysed by receptor occupancy, and an inactive GDP-bound conformation resulting from an intrinsic GTPase activity of the protein itself (see Fig. 1.6). The activated receptor has a high affinity for a G protein conformation in which the $\alpha$ and $\beta\gamma$ subunits are associated and the single guanine nucleotide binding site is empty. Binding of the activated receptor to the G protein in turn induces GTP binding to the $\alpha$ subunit, resulting in dissociation of the G protein into $\alpha$-GTP and $\beta\gamma$ complexes. The $\alpha$-GTP complex in some way activates the effector system although the exact mechanism by which this occurs is largely unknown.

The importance of G proteins in mast cell exocytosis (for a review see Ref. 288) was indicated from work using mast cells permeabilised with ATP$^4^-$. Permeabilisation allows control over the precise composition of the cytosol and cells are able to be resealed by conversion of ATP$^4-$ to its salt by addition of Mg$^{2+}$, thus trapping exogenous compounds within the cell. Introduction of non-hydrolysable analogues of GTP, GTP$_\gamma$S or guanylyl-5'-$\beta\gamma$-imido)triphosphate (Gpp$\cdot$NH$_p$), into these cells induced Ca$^{2+}$-dependent exocytosis [289]. In addition, pertussis toxin (PtX), which inactivates the inhibitory G protein, $G_i$, of the adenylate cyclase transduction machinery by ADP-ribosylation of the $\alpha$-subunit, was an effective inhibitor of GTP-induced secretion from permeabilised RPMC and also that induced by Co. 48/80 in intact cells [290,291]. Interestingly, PtX was ineffective against IgE-mediated stimuli, suggesting that G proteins are not involved in transduction of the signal induced by bridging of IgE receptors [274,291].
Recent work in the mast cell and neutrophil has suggested that there may be two distinct G proteins involved in exocytosis; one at the receptor level, linked to PLC, and a second, directly activated G protein, designated $G_E$, which exists at a level distal to the $\text{Ca}^{2+}$ signal [292,293]. Initial patch-pipette studies of the mast cell [294], where an increase in membrane capacitance was taken as a measure of exocytosis, indicated that even in the presence of high concentrations of EGTA, infusion of $\text{GTP}^\gamma S$ induced exocytosis, which was evidence that G protein activation was taking place independently of the $\text{Ca}^{2+}$ signal under these conditions.

Subsequently, studies using streptolysin-O permeabilised cells, have shown that $\text{GTP}^\gamma S$ together with $\text{Ca}^{2+}$ induced secretion even in the presence of low concentrations of neomycin ($< 1$ mM) [295], which by binding to inositol phospholipids blocks their metabolism [296], thus ruling out any effects of $\text{GTP}^\gamma S$ on $G_p$. The oncogenic transformant of $\text{H-ras}^{\text{val}(12)}$ is a constitutively activated G protein which has the ability to effect degranulation on microinjection into the mast cell cytosol; it is thought to act by mimicking $G_E$ [297]. More detailed investigations are now awaited in order to fully uncover the role of $G_E$ in regulation of the terminal steps of exocytosis.

1.6.6.2 Serine esterases

The question has also been raised whether, as with PI turnover, some sort of regulatory mechanism, activated on receptor occupancy, is operative for phospholipid methylation.

One possible candidate for the regulation of the methyltransferase enzymes has been proposed by Austen and Brocklehurst [298] and involves activation of proteolytic enzymes. Diisopropylfluorophosphate (DFP), which is a potent, irreversible serine esterase inhibitor, blocked IgE-dependent histamine release from rat serosal mast cells [299,300], and fragments of guinea pig and human lung [298,301]. In detailed studies of rat peritoneal mast cells, DFP also inhibited a number of related biochemical events such as phospholipid methylation, $^{45}\text{Ca}^{2+}$ uptake and the transient cAMP rise [302]. This effect
was only apparent when the inhibitor was present during challenge and not merely on pretreatment of the cells, indicating involvement of the serine esterase following initiation of the secretory process by receptor bridging.

Further evidence for the involvement of serine esterases in cell signalling derives from the observation that α-chymotrypsin [303,304] and rat mast cell chymase [305] induced exocytosis in rat mast cells. In addition, substrates and inhibitors of trypsin and chymotrypsin also inhibited immunologically mediated phospholipid methylation and the cAMP spike [114,306].

1.6.7 The role of cAMP

Adenosine 3'-5'-(cyclic) monophosphate, or cAMP, constitutes another variety of intracellular second messenger involved in signal transduction. Generation of cAMP is analogous with that of Ca\(^{2+}\); receptor occupancy activates the membrane bound enzyme adenylate cyclase, via a GTP regulatory protein, which catalyses the dephosphorylation and cyclisation of ATP to produce its cyclical derivative.

Although cAMP may act as the exclusive second messenger in some systems, its potential interaction with Ca\(^{2+}\)-modulated systems has been resolved into monodirectional and bidirectional by Berridge [192]. In monodirectional systems, generation of the two messengers is typically via the one agonist, and cAMP enhances secretion induced by Ca\(^{2+}\) by promoting its influx or mobilization from internal stores. In contrast, the two messengers are produced by the action of two different agonists in bidirectional systems and antagonise one another. It is thought that cAMP might activate calcium ATPases via cAMP-dependent protein kinases to promote extrusion of the cation.

Initial studies of the mast cell and basophil leukocyte indicated that a bidirectional system was in operation in the induction of histamine secretion. As early as 1936, Schild [307] demonstrated that adrenaline, which was subsequently shown to raise intracellular levels
of cAMP, inhibited anaphylactic release of histamine from sensitized guinea pig lung. Subsequent investigations revealed that pharmacological agents which elevated intracellular cAMP such as sympathomimetic amines, analogues of the nucleotide - the dibutyryl or bromo derivatives, Bu$_2$cAMP and 8-bromo-cAMP -, the methylxanthines, cholera toxin and certain prostaglandins (PGs), inhibited release of histamine from human basophils [308,309], lung fragments [310] and isolated lung mast cells [311,312].

There were conflicting reports, however, concerning the role of cAMP in rat serosal mast cells. Despite raising cAMP levels, β agonists, such as isoprenaline, failed to inhibit IgE-dependent histamine release [313] and were even reported to enhance secretion [314]. Suboptimal concentrations of Bu$_2$cAMP or 8-bromo-cAMP have also been reported to induce histamine release from RPMC [315]. On the other hand, the methyxanthines, theophylline and 3-isobutyl-1-methylxanthine (IBMX), which prevent cAMP breakdown by inhibition of phosphodiesterases, and Bu$_2$cAMP decreased mediator secretion induced by both immunological and non-immunological stimuli [316-318] in this cell type.

An initial rise and fall in cAMP levels some 15-45 seconds following IgE receptor bridging was observed by a number of workers in human lung mast cells [185], human basophils [319,320] and rat peritoneal mast cells [266,321-323]. In the rat peritoneal cells this was followed by a second, later rise within 3-5 min of cell activation which was thought to be due to the release of prostaglandins of the E$_1$ class [324]. Consistent with this, Lewis et al. showed that the early rise was inhibited by indomethacin [325]. Indomethacin, however, failed to affect the initial cAMP rise and was also without effect on histamine release. PGD$_2$ and PGI$_2$ raised cAMP levels substantially without having any effect on IgE-mediated secretion [326]. Theophylline inhibited histamine release, however, in a concentration dependent manner and this was unchanged by the presence of PGD$_2$. The effects of cyclic nucleotides in eukaryotic cells are mediated by activation of protein kinases [327] and immunologic stimulation of rat serosal mast cells was concomitant with an increase in protein kinase activity [328,329]. In agreement with this, theophylline, but not PGD$_2$, was seen to activate protein kinases [330]. These results
suggested the existence of distinct pools of adenylate cyclase, and hence cAMP, in rat mast cells, which were differentially regulated by stimuli and drugs. PGD$_2$ stimulated a pool of cAMP which was not linked to an inhibitory mechanism whereas the theophylline-sensitive cAMP pool was involved in termination of the secretory response.

Evidence also exists from studies using adenosine analogues to suggest that the early rise in cAMP is associated with triggering rather than termination of the secretory process. Holgate et al. [331] have shown that a close correlation exists between the ability of ribose and purine (2',5'-dideoxyadenosine or DDA) adenosine analogues to inhibit and enhance IgE-dependent cAMP increases with their ability to inhibit and enhance mediator secretion. This was also subsequently verified using DDA in human basophils [332]. In addition, recent patch clamp studies of rat serosal mast cells [202] (see section 1.6.3), have indicated that cAMP may provide a stimulatory signal for exocytosis by regulating the opening of chloride channels in the membrane.

It is also possible, in the light of a number of studies, that cAMP has no important role to play at all in the mast cell release process. Despite the occurrence of other changes associated with signal transduction, no accompanying rise in cAMP levels was seen in the RBL cell which possesses a functional membrane adenylate cyclase [265,306,333] or in human lung mast cells [334], on immunological stimulation. This latter observation was contrary to previous findings [185] in the same cell type. In addition, histamine release induced by a variety of diverse, non-immunological stimuli such as Co. 48/80, A23187, polymixin B, nerve growth factor (NGF) and peptide 401 failed to result in intracellular cAMP changes [335,336]. Thus, if cAMP is in any way involved in signal transduction of immunological stimuli, its action must almost certainly be at an early stage that is common to the IgE release mechanism but bypassed by the above agents.

1.7 Mast cell heterogeneity

Mast cells were thought to represent a homogeneous population, until the studies of
Enerbäck and coworkers in the mid 1960s on the distribution of mast cells in the gastrointestinal tract of the rat [21,337-339] uncovered two distinct types of mast cell. Those in the intestinal submucosa resembled mast cells found in other connective tissues (connective tissue type mast cells or CTMC), whereas those present in the mucosa (named mucosal mast cells or MMC) differed in terms of their size, fixation requirements, dye-binding capacity and reactivity to Co. 48/80 (table 1.1).

Work in this area has been greatly facilitated by the development of efficient techniques for the isolation of mast cells from a diversity of tissues, most notably the intestine [133,340], lung [341,342] and skin [343,344]. It is now firmly established that mast cells from different species and indeed from different tissues within the same species, as well as also within the same mast cell population, exhibit marked variation in their biochemical, morphological, histochemical and functional properties.

In particular, the existence of functionally heterogeneous populations of mast cells in man as well as rodents, has raised a number of important questions concerning the suitability of animal models in predicting the reactivity of their human mast cell counterparts to various secretagogues and, more importantly, their susceptibility to anti-allergic compounds. Consequently, much research effort is currently devoted to the further understanding of the extent of human mast cell heterogeneity and gaining insight into its developmental basis.

1.7.1 Mast cell ontogeny

Before discussing the various manifestations of mast cell heterogeneity it is important to understand something of the origin and development of mast cells.

1.7.1.1 The origin of mast cells

Mammalian mast cells are derived from precursors which originate in the bone marrow.
This was established from an elegant series of experiments by Kitamura et al. [345-348] (for a review see Ref. 348) using genetically mast cell deficient mutant mice and their normal (+/+) or wild-type littermates. A double dose of mutant genes either at the W (white spotting) or Sl (steel) locus of the mouse results in a macrocytic anemia, sterility, lack of hair pigmentation and profound mast cell deficiency [345,346,349]. WBB6F1-W/W\textsuperscript{v} (W/W\textsuperscript{v}) mice, however, could develop mast cell populations if they received bone marrow cells from their normal littermates [346], indicating that mouse mast cells develop from circulating bone marrow precursors [350] which have since been shown more precisely to be multipotential haemopoietic stem cells of the myeloid lineage [351]. Mast cells could not be reconstituted however in WCB6F1-Sl/SI\textsuperscript{d} (SI/SI\textsuperscript{d}) mice by injection of normal bone marrow, but bone marrow from Sl/SI\textsuperscript{d} mice repaired the mast cell deficiency of W/W\textsuperscript{v} mice. These results indicated that mutations at the W locus result in abnormalities in the bone marrow precursors whereas mast cell deficiency of Sl/SI\textsuperscript{d} mice reflects an abnormality of tissue factors which regulate mast cell development [352].

In support of this, it has been discovered recently that the W locus in the mouse encodes the putative tyrosine kinase receptor (c-kit) [353] which has significant homology to a number of growth hormone receptors. In addition, in the past year, Zsebo et al. have mapped the Sl locus of the mouse to the murine stem cell factor (SCF) [354], which has also been shown to be a growth factor for mast cells [355] and is a ligand for the c-kit receptor.

1.7.1.2 Mast cell culture

The haematopoietic origin of mast cells suggested from the above in vivo reconstitution experiments was subsequently confirmed by in vitro work. In 1981, several groups discovered that apparently pure populations of cells with many features of mast cells could be generated from cultures of mouse bone marrow in the presence of medium derived from mitogen-activated T cells or WEHI-3B tumor cells [356-358]. These cells are known as mouse bone marrow-derived mast cells (mBMMC).
The growth factor on which such cultures depended was identified as IL3 [359] and it was later found that IL4 could act in synergy with IL3 to stimulate increased outgrowth of mast cells [360]. Proliferation of mast cells with properties of MMC in cultures of rat bone marrow was also later found to be dependent on a soluble T cell factor which was mimicked by rIL3 [361,362].

Growth of cells in such large numbers has greatly facilitated the study of many aspects of mast cell biology [363,364]. In studies of rat bone marrow-derived mast cells (rBMMC) these cells appeared to be broadly similar to rat MMC [134], although subtle differences did occur (see sections 1.7.2 and 1.7.3). A variety of mast cell lines have since been developed by transfection of IL3-dependent cultures with Harvey sarcoma virus [365] or Abelson murine leukemia virus (Ab-MuLV) [366] or coculture of mouse haematopoietic cells with fibroblasts which produce Kirsten murine sarcoma virus (KiSV) [367]. The latter cells resemble mature peritoneal mast cells more closely than classical growth factor-dependent mouse mast cell lines.

Mature CTMC purified from mouse peritoneal cells were shown to proliferate in vitro in methylcellulose culture and maintain their morphological, biochemical and functional characteristics [368]. The viability of rat CTMC was also maintained for at least 30 days [369] when they were co-cultured with Swiss albino mouse skin 3T3 fibroblasts and, in addition, these cells exhibited full responsivity to a variety of agents [135].

Varying degrees of success have been obtained in trying to culture human mast cells from bone marrow [370,371], umbilical cord blood [372] and human foetal liver cell precursors [373,374]. These cells, however, resembled basophils more closely than mast cells although small numbers of mast cells have been demonstrated when bone marrow was cultured on agarose or on layers of 3T3 fibroblasts [375,376]. Simple extrapolation of the culture conditions which proved effective for the growth of rodent mast cells was, however, largely ineffective for the human system and the soluble growth factors responsible for the selective proliferation of human mast cell precursors have not yet been
identified [377].

1.7.1.3 The role of microenvironment in the regulation of mast cell differentiation

There are two main lines of evidence to suggest that microenvironmental factors play a profound role in the regulation of mast cell differentiation.

The first of these comes from the transdifferentiation experiments conducted by Kitamura et al. [378-381] using genetically mast cell deficient mice. Injection of mBMMC, which have characteristics of MMC, into the peritoneal cavity of W/W<sup>Y</sup> mice resulted in acquisition by these cells of properties of CTMC of +/+ mice [378]. Similarly, if mBMMC were injected into the glandular stomach and skin of W/W<sup>Y</sup> mice these cells gave rise to CTMC in the skin and intestinal submucosa, or MMC in the mucosa [379]. Conversely, if a single peritoneal cell or clonal colonies of either mouse skin or peritoneal mast cells of +/+ mice, maintained in methylcellulose, were injected into the stomach wall of W/W<sup>Y</sup> mice, CTMC appeared in the muscularis propria and MMC in the mucosa [378,380,381]. Thus, mast cells appeared to have the ability to dedifferentiate, migrate to a new site or different microenvironment and there proliferate and redifferentiate [378]. This provides strong evidence to suggest that mast cells do not have a committed phenotype and that CTMC and MMC are not derived from distinct lineages but from a common precursor.

The second indication that microenvironmental factors are involved in mast cell differentiation was the discovery that when mBMMC were co-cultured with mouse skin-derived 3T3 fibroblasts in the presence of WEHI-3 conditioned medium, they underwent phenotypic changes and acquired characteristics of CTMC [382]; they became safranin positive, synthesized heparin instead of chondroitin sulphate E and, as a result of the IL3 present in the WEHI-3 medium, generated increased cellular histamine. Adherence of mast cells to the fibroblast layer was concomitant with a heightened cellular reactivity.
The physical effect therefore of the juxtaposition of mast cells and fibroblasts may be to enhance secretion by allowing diffusion of a short-lived chemical mediator produced by the fibroblast.

This possibility was addressed recently by Jarboe et al. [384] using mast cell committed progenitors (MCCP) from the rat. MCCP are non-granulated cells harvested from the mesenteric lymph nodes of mice infected with *Nippostrongylus brasiliensis* and represent a late committed stage in the differentiation of mast cell progenitors just prior to granulation. When cultured on a fibroblast monolayer, MCCP differentiated into mast cells of the CTMC type. These cells also formed colonies of CTMC in methylcellulose when supplemented with fibroblast conditioned medium (CM). Fibroblasts produce a diverse array of cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, IL1 and IL6. However, none of these factors in addition to IL2, IL4, IFN, NGF and epithelial growth factor (EGF) were able to induce phenotypic change in the progenitors. Thus, the fibroblast-derived factor which regulates the differentiation of haematopoietic cells into CTMC remains to be determined. Indeed, the participation of a soluble factor is questionable since such a fibroblast-derived CM had previously been shown to be ineffective in maintaining rat CTMC in culture [369].

After differentiation, there is some evidence to suggest that further growth or maturation of the mast cell may also influence its properties [385-387]. When populations of rat serosal [385] and purified human lung mast cells [386,387] were fractionated according to density by countercurrent elutriation, it was found that different sizes of cell varied in their mediator content and secretory activity. Histamine content and PGD₂ generation was positively correlated with mast cell size [386], but density subsets varied randomly in their response to secretagogues [387].

In summary, heterogeneous subpopulations of mast cells arise as a result of the influence of specific growth factors, environmental stimuli and the stage of cellular maturation, but are not thought to be due to distinct mast cell lineages.
1.7.2 **Morphological and histochemical differences between mast cells**

Mast cells and basophils from man and other species are heterogeneous in terms of their overall and granular morphology [78].

Mucosal type mast cells in the rat are smaller in size and more variable in shape than CTMC, and have a lower histamine (10-30 µg/10^6 cells in CTMC compared with 0.1-1 µg/10^6 cells in MMC) and serotonin content [104,105]. MMC have a shorter lifespan and proliferate in a thymus-dependent fashion in response to certain parasitic infections [388-390]. Ultrastructurally, mBMMC are very similar to MMC [391] although their granules are less electron-dense because they contain approximately only one tenth of the histamine content of MMC [357].

In man, the basophil leukocyte is found circulating in the blood and comprises a mere \(~0.5\%\) of total leukocytes [392]. It is typically of between 5-7 µm in diameter with a segmented nucleus, as with other granulated leukocytes, and a few large round cytoplasmic granules containing electron-dense particles and rarely large crystals [393]. Its surface is covered with irregular, short, thick processes. Human mast cells are somewhat larger in size (9-12 µm) with a non-segmented, usually round or oval nucleus, which occupies an eccentric location. Granules are smaller, more numerous and generally more variable in shape than in the basophil [394] and mast cells contain numerous lipid bodies in their cytoplasm [395]. The surface is covered in many, relatively uniformly distributed, elongated, thin processes.

As previously discussed in section 1.4.1.4, mast cells of the rat can be distinguished on the basis of differences in their chymase content; CTMC contain RMCPI [112] whereas the chymase in MMC is RMCPII [113]. In accordance with their similarity to MMC, rBMMC also synthesize RMCPII [134,396].

Within the human mast cell population two distinct types of cell have been described on
the basis of their neutral protease composition [115]. In addition, heterogeneity in the 
granule morphology of various human mast cells has been correlated with their neutral 
protease content by Craig et al. [397,498] and others [399]. Those cells containing 
tryptase alone (TM.mc), in the lung and intestinal mucosa, and those containing tryptase and 
chymase (TC.mc), in the skin and intestinal submucosa, corresponded to mast cells whose 
granules either contained predominantly discrete scrolls or grating and lattice 
substructures, respectively. Granules in the TC.mc were larger and more uniformly 
electron-dense than those in TM.mc. Occasionally, however, granules existed with both 
types of morphology and hence a strict dichotomy of two distinct mast cell types in 
humans on the basis of granule morphology is an oversimplification although trends in 
predominant granule morphology are apparent.

The unique histochemical properties of mast cells are conferred by their proteoglycan 
content. Rat mucosal mast cells contain a non-heparin, chondroitin sulphate proteoglycan 
[400] which has been reported to consist of chondroitin sulphates di-B, A and E in 
varying proportions [401,402]. BMMC of the rat produce chondroitin sulphate di-B as 
their major proteoglycan [367], whereas mBMMC synthesize chondroitin sulphate E [403]. 
In the rat, CTMCs synthesize heparin proteoglycans [403,404] and also contain 
appreciable but lesser amounts of preformed chondroitin sulphate di-B and E in their 
secretory granules which may have been synthesized at an earlier stage of development 
[405].

Such differences in predominant proteoglycan structure are reflected in the staining 
properties of these mast cell subsets with cationic dyes. Both heparin and chondroitin 
sulphate proteoglycans stain with thiazine dyes such as toluidine blue. However, using 
a staining sequence of alcian blue and safranin O, the granules of MMCs and rBMMCs 
colour blue whereas those of CTMCs counterstain red [134,338]. The physico-chemical 
basis for this selectivity is not understood but it has proved a powerful tool in the 
identification of rat mast cell subtypes. The fluorescent dye berberine sulphate may also 
be used in differential staining, as it forms a strongly fluorescent complex with heparin.
but not chondroitin sulphate proteoglycans [406]. In addition, MMC are sensitive to reversible blockage of cationic dye binding by aldehyde fixation [337,407].

The glycosaminoglycans of human mast cells from the nasal [408,409] and intestinal mucosa and submucosa [409], skin [409,410] and lung [411,412] are degraded by nitrous acid and therefore appear to contain heparin. However, chondroitin sulphates have also been reported to be produced by these cells in differing amounts [410-412] and indeed this has been reported to be the predominant proteoglycan produced in the human colonic and gastric mucosae [413,414].

Subpopulations of human mast cells from the gastrointestinal tract [415,416], skin [417], and lung [418] have been demonstrated to be sensitive to formaldehyde fixation. The relative sensitivity of various mast cell populations to aldehyde fixation can be quantitatively assessed by measuring the critical electrolyte concentration (CEC) which blocks cationic dye binding. This method was devised by Scott and Dorling [419] and CEC is defined as the electrolyte concentration at which 50% of staining is extinguished. Thus, human nasal mucosal mast cells are more sensitive to blockage of dye binding by aldehydes (CEC = 0.49 M MgCl₂) than their counterparts in skin (CEC = 0.64 M MgCl₂) [408]. Since the CEC of rat MMC is much lower than that of CTMC [409] this measure is indicative of a more mucosal-like nature of human nasal mast cells compared to human skin mast cells.

1.7.3 Biochemical and functional heterogeneity

The elaboration of arachidonic acid metabolites by subtypes of both human and rat mast cells constitutes another parameter in which heterogeneity is evident. In the rat, CTMCs isolated from the peritoneal cavity responded to immunological activation by producing PGD₂ in the absence of significant amounts of leukotrienes [128,420]. In total contrast, mast cells isolated from the rat intestinal mucosa liberated substantial quantites of LTC₄ and LTB₄ but considerably lesser amounts of PGD₂ [128]. Cultured mBMMC resembled
MMC by producing large quantities of LTC₄ [383], while rBMMC, on the other hand, synthesized LTC₄ and PGD₂ in a ratio of 1:1 and in this respect differed markedly from MMC [134].

PGD₂ appeared to be the major cyclooxygenase metabolite produced on immunological challenge of isolated human mast cells from lung [129,421], bronchoalveolar lavage (BAL) [130], intestinal mucosa [133] and skin [132]. The latter cells also produced PGD₂ following non-immunological challenge by a variety of agents [422,423] with lesser elaboration of LTC₄. In lung and BAL mast cells LTC₄ was the major sulphidopeptide leukotriene [421], although the quantity of this released was up to 4 times less than PGD₂. On the other hand, LTE₄ predominated in intestinal mast cell mediator release, although this could have been the result of increased metabolism of LTC₄ in these cells [133]. In each case, when impure populations of cells were used for measurements, the release of arachidonic acid metabolites correlated well with histamine, indicating that the mast cell was the major source of these products.

Early studies using the polyamine Co. 48/80 [339] indicated that MMC of the rat were less responsive than those of RPMC to the histamine releasing properties of this stimulus. Since that time a range of compounds have been tested, and it has become apparent that fundamental differences exist between these two mast cell subtypes in their reactivity to a number of histamine liberators [340,424,425] and anti-allergic compounds [426,427]. Responsivity of MMC to these agents was mirrored by rBMMC [134].

A similar situation exists in the human mast cell population. Although all human mast cells release mediators in response to IgE-directed stimuli in a Ca²⁺- and ATP-dependent manner [428-430], the skin mast cell appears to be unique in being additionally responsive to a variety of structurally diverse non-immunological stimuli [344,431,432], an effect which is partially dependent on Ca²⁺. Marked variation also occurs in the response of human mast cells to anti-allergic compounds [433,434].
1.8 **Aims of the present study**

This study was undertaken in order to investigate further the extent of mast cell heterogeneity in man, the rat and the guinea pig with particular reference to the characteristics of the skin mast cell in these species.

This was investigated in two ways. Firstly, the histochemical properties of skin mast cells from these species were compared with those from other tissues, notably the lung, mesentery and uterus and, in the case of the rat, the peritoneal cavity. Secondly, an extensive range of structurally diverse secretagogues which shared a predominantly basic nature was used to assess the relative functional characteristics of each mast cell type. Likewise, a variety of anti-allergic drugs were employed to establish the relative susceptibility of the biochemical secretory process in these cells to inhibition. These studies are of use in underscoring the unique functional properties of the skin mast cell, particularly in man. In addition, it was hoped that by understanding something of the nature of the differences in functional reactivity between mast cell subsets some insight could be gained into how best rational tissue-specific treatments for allergic disorders could be sought in the future.

In addition, procedures for the isolation of two novel mast cell types from the human uterus and bladder are described. Functional characterisation of these cells will help to assess their relative contribution to uterine and bladder pathophysiology.
Fig. 1.2 Schematic representation of the triggering of histamine release (HR) by anti-receptor antibodies and their divalent (F(ab')2) and monovalent (Fab) fragments. Anti-RGG = anti-Fab. Source: Ref. 15.
Fig. 1.3 The structure of heparin. Xyl = xylose; Gal = galactose; GlcUA = glucuronic acid; IdUA = iduronic acid; GlcNH$_2$ = glucosamine; SO$_4$ = sulphate. Source: Ishizaka T. (1988) in Allergy, Principles and Practice (eds. Middleton, Reed, Ellis, Adkinson and Yunginger), The C.V. Mosby Co., vol. 1, p71.
Fig. 1.4  Arachidonic acid metabolism.
Fig. 1.5 Sequential reactions involved in phospholipid methylation.
Fig. 1.6  Conformational changes in G proteins following receptor interactions.

Table 1.1 Some properties of mucosal and connective tissue mast cells from the gastrointestinal tract of the rat.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Mucosal mast cells</th>
<th>Connective tissue mast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Small, variable shape sparsely granulated</td>
<td>Large, uniform, densely granulated</td>
</tr>
<tr>
<td>Size</td>
<td>9.7 μM</td>
<td>19.6 μM</td>
</tr>
<tr>
<td>Thymus dependency of proliferation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Fixation and staining</td>
<td>Formalin sensitive</td>
<td>Formalin insensitive</td>
</tr>
<tr>
<td></td>
<td>Safranin and berberine negative</td>
<td>Safranin and berberine positive</td>
</tr>
<tr>
<td>Histamine content</td>
<td>1.3 pg cell(^{-1})</td>
<td>15 pg cell(^{-1})</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Low content</td>
<td>High content</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>Soluble granular matrix composed of chondroitin sulphate</td>
<td>Less soluble granular matrix composed of heparin</td>
</tr>
<tr>
<td>Serine protease</td>
<td>RMCP II</td>
<td>RMCP I</td>
</tr>
<tr>
<td>Lifespan</td>
<td>&lt; 40 days</td>
<td>&gt; 6 months</td>
</tr>
<tr>
<td>IgE</td>
<td>Present in the cytosol</td>
<td>Absent from cytosol</td>
</tr>
<tr>
<td></td>
<td>Low density of surface receptors</td>
<td>High density of surface receptors</td>
</tr>
<tr>
<td>Histamine release by Co. 48/80</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Effect of cromoglycate</td>
<td>Insensitive</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

Compiled from Refs. 21 and 29.
CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Throughout the study, male Sprague-Dawley rats (150-250 g) and female Dunkin Hartley guinea pigs (300-800 g) were used. These animals were obtained from closed, random-bred colonies kept at the Joint Animal House, University College London.

2.2 Human tissues

Samples of lung tissue were obtained following surgery for bronchial carcinoma at The Middlesex Hospital. Infant foreskin was obtained following circumcision performed either by Dr J. Cohen or Miss Wright, paediatricians at University College Hospital. Human uterine samples were obtained following hysterectomy performed by surgeons at both University College Hospital and The Elizabeth Garrett Anderson Hospital, London. Samples of human bladder were kindly provided by Dr. T. J. Christmas, Urology Department, St. Bartholemew’s Hospital.

2.3 Buffers

Unless otherwise stated in the text, release and inhibition experiments were carried out in Full Hepes Tyrode’s (FHT) buffer which is a modified Tyrode’s solution buffered with N-2-hydroxyethyl-piperazine-N’-2-ethane sulphonlic acid (HEPES). The pH of buffers was adjusted by the addition of 4M NaOH or 3M HCl as required. In general, the pH was set to between 7.2-7.4.

The composition of the Full Hepes Tyrode’s buffer and details of modifications made to it for individual experiments are laid out in Table 2.1.

2.4 Isolation of mast cells
2.4.1 Rat peritoneal mast cells

Rats were asphyxiated with CO$_2$ in an enclosed chamber, and their spinal cords broken. The abdominal skin was removed and heparinized FHT buffer (10-15 ml) was injected into the peritoneal cavity through the exposed abdominal wall. The abdomen was then massaged gently for 2 min, an incision made along the midline to expose the peritoneal cavity and the peritoneal exudate carefully recovered using a plastic liquipipette (Elkay). The cells were transferred into a polypropylene centrifuge tube and any sample heavily contaminated with blood at this stage was discarded. The cells were pelleted by centrifugation at room temperature at 100 g for 2 min, washed twice in prewarmed Tyrode buffer and resuspended to the required volume in the appropriate buffer. Cells from more than one animal were pooled if required.

2.4.2 Rat mesenteric and lung mast cells

The animals were sacrificed as in section 2.4.1 above. The mesentery was dissected from the small intestine and any attached lymph nodes removed. The lung was removed from the chest cavity and the parenchyma was dissected free of major airways and blood vessels. After washing in FHT, the tissues were cut into small fragments using scissors and further chopped using a McIlwain automated tissue chopper. The tissue was then incubated at 37 °C in prewarmed BSA-Tyrode buffer (25 ml) containing collagenase at 160 U/ml for 60 min in a shaking water bath. Following enzymic dispersion the tissue was further disrupted by repeated expression through a syringe. The final suspension was then filtered through moistened gauze to remove tissue debris and the cells were recovered by centrifugation (100 g, 3 min). Cells were then washed twice in FHT and resuspended to the required volume in the appropriate buffer.

2.4.3 Rat and guinea pig cutaneous mast cells

Guinea pigs were killed by cervical dislocation and rats sacrificed as in section 2.4.1 above. An area of abdominal skin on the animals (approximately 5 cm$^2$) was trimmed
of fur with scissors and then shaved closely using a razor blade. The skin was then cut from the underlying abdominal wall and any attached fat was removed. The tissue was cut into small fragments (1 mm$^2$) and incubated in BSA-Tyrode (100 ml) containing collagenase (160 U/ml) and hyaluronidase (500 U/ml) in a 37 °C hot room with constant stirring for 4 hours. After this period, the tissue was further disrupted by repeated expression through a syringe and the resulting suspension was filtered through moistened gauze into 50 ml centrifuge tubes (Elkay). The cells were recovered by centrifugation (200 g for 5 min), washed twice in FHT and resuspended to the required volume in the appropriate buffer.

2.4.4 Guinea pig uterine mast cells

Guinea pigs were killed as in section 2.4.3 above. Both uterine horns were dissected out and trimmed free from any attached mesentery. The tissue was then chopped finely using a McIlwain automated tissue chopper. The dispersion procedure was essentially identical to that outlined in section 2.4.2, except that incubation was for 90 min. In addition, the final cell suspension required filtration through a porous plastic filter (Vyon F) to remove strands of mucus and cellular aggregates which had formed.

2.4.5 Human lung and uterine mast cells

Macroscopically normal human lung parenchyma, obtained following resection for bronchial carcinoma, was dissected free of major airways and blood vessels. Human uterine myometrium was separated by blunt dissection from samples of normal uterine tissue obtained following hysterectomy. Both tissues were cut into fragments in a laminar flow biological safety cabinet and, where possible, chopped further using a McIlwain automated tissue chopper. The approximately 1 mm$^2$ fragments were washed for 15 min in Tyrode's solution in order to reduce mucus formation in and erythrocyte contamination of the final cell suspension. The tissues were digested in BSA-Tyrode solution (20 ml/g tissue) containing collagenase (120 U/ml lung, 160 U/ml uterus) for 2 x 90 min at 37 °C with constant stirring. Between digests, the supernatant fluid was recovered through
moistened absorbent gauze, the cells recovered by centrifugation at 150 g for 3 min and the enzyme-containing buffer replaced to continue the digest of remaining tissue fragments. After the final digest, the tissue was further disrupted by repeated expression through a syringe and the resulting suspension filtered through two layers of moistened absorbent gauze. The cells were washed twice in large volumes of Tyrode's buffer (in order to dilute any mucus that may have formed), resuspended in BSA-Tyrode's solution containing heparin (5 iU/ml) (30-40 ml) and kept at 4 °C until use (this was regularly overnight).

2.4.6 Human cutaneous mast cells

Infant foreskin, obtained following circumcision, was dissected free from fat and cut into 1 mm² fragments using scissors in a laminar flow biological safety cabinet. The tissue was digested in Tyrode's solution containing collagenase (160 U/ml) and hyaluronidase (500 U/ml) for 3 x 2 hours, or longer if after this time substantial tissue was left undigested. Thereafter, cutaneous cells were recovered following the same procedure as outlined in section 2.4.5 for human lung and uterine cells.

2.4.7 Human bladder mast cells

Bladder samples were separated into mucosa and submucosa/muscle by blunt dissection. Preparation of the cells was essentially the same as in section 2.4.5, for uterine mast cells, the only difference being that the dispersion time was 2 x 60 min.

2.4.8 Human basophil leukocytes

Venous blood (20 ml) was collected into 5 ml of clinical isotonic saline with dextran (6 %) containing glucose (30 mg/ml) and heparin (50 U/ml) to prevent coagulation. The solution was mixed well and then allowed to stand for 60-90 min at room temperature or until the erythrocytes had formed rouleaux and descended to the bottom of the tube, leaving a platelet-leukocyte-rich supernatant.
The supernatant was aspirated using a plastic disposable liquipipette and the leukocytes pooled by centrifugation (100 g, 5 min). The platelet-rich supernatant was discarded and the cell pellet washed twice in FHT before being resuspended in the required volume of the appropriate buffer.

2.5 **Immunological sensitization of rat and human mast cells**

2.5.1 **Active sensitization of rat mast cells with *Nippostrongylus brasiliensis***

The natural or experimental infection of rats with the nematode parasite *Nippostrongylus brasiliensis* is a particularly effective stimulus for the production of serum IgE (anaphylactic antibody) in these animals, thereby sensitizing the mast cells [390].

Specific pathogen free Sprague Dawley rats were infected with the nematode by subcutaneous injection of the third stage larvae (L3) (2,500 larvae, equivalent to 0.2-0.4 ml) into the hind leg on day 0 (Fig. 2.1). Mast cell sensitization occurs on day 21 following injection and the animals remain in a sensitized state for a further 3-4 weeks.

2.5.1.1 **Preparation of the third stage larvae**

Faeces from the injected rats were collected on day 7. These were moistened and gently ground with an equal weight of activated granular charcoal (BDH) until a fine mash was formed. This mixture was then transferred to petri dishes (~15 g/dish), as illustrated (Fig. 2.2), which were kept in the dark at 20 °C for a minimum of 7 days to a maximum of 21 days.

The larvae were isolated from the mixture by tipping the contents of the petri dish onto layers of gauze and lens tissue in the apparatus which is illustrated in Fig. 2.2. The larvae sedimented through the warm tap water into the graduated glass tube within 60 min, were then washed thoroughly to rid them of any residual charcoal, counted and resuspended in sterile saline at the required concentration for injection (6,500-12,500 larvae/ml).
2.5.1.2 Preparation of allergens from *Nippostrongylus brasiliensis*

Rats were injected subcutaneously with 4,000-6,000 L₃ larvae on day 0 and asphyxiated on day 6-8 using CO₂ in a closed chamber. The duodenum and part of the upper jejunum, which were coloured red (indicative of infection with adult worms), were removed. This section of gut was opened along its midline and the contents containing worms filtered through gauze into sterile saline using the apparatus in Fig. 2.3. The worms were collected after approximately 60 min, and washed twice with saline. They were then incubated at 37 °C for 5 hours in 10 times their volume of sterile saline after which time the supernatant was collected and aliquoted to be kept at -20 °C until required for challenge of sensitized rat mast cells. Further supernatants could be collected on reincubation of the worms with saline as before. The allergen fractions were then tested for histamine releasing potency on RPMC from sensitized animals and assigned an arbitrary concentration of 1 worm equivalent/µl (WE/µl).

2.5.2 Passive sensitization of human mast cells

Suspensions of dispersed human lung, uterine and skin mast cells in BSA-Tyrode’s containing heparin (5 iU/ml) were passively sensitized using high IgE titre serum from atopic patients, generously provided by the Immunology Department of the UCMSM or obtained commercially from the Binding Site, University of Birmingham. The serum was mixed with the cells at a final concentration of 150-200 units/ml, left for 2 hours at 37 °C, followed by an overnight incubation at 4 °C. The cells were washed free of excess serum before use in experiments.

2.6 General procedure for histamine release experiments

2.6.1 Effect of secretagogues

Cells were made up to the required volume with the appropriate buffer and left to equilbrate to 37 °C in a water bath for 10 min. Prior to this, a pilot study was conducted
in order to find a cell concentration which would give approximately 40-50 ng of histamine per tube. Aliquots of cells (250 μl) were added to polypropylene tubes containing 200 μl of appropriate buffer and 50 μl of secretagogue (at 10 x the final concentration), dilutions of which were also made in the same buffer. A tube without secretagogue (250 μl cells + 250 μl buffer) was also set up to measure the spontaneous histamine release from the cells. Incubation was for 10 min at 37 °C, except in the case of basophil leukocytes where incubation was for 30 min. The reaction was terminated by the addition of ice-cold Tyrode's solution (1.5 ml peritoneal, 500 μl others). Cells were pelleted by centrifugation at 100 g for 2-3 min and the supernatant (containing the released histamine) was recovered by decanting into a corresponding polypropylene tube. The cell pellets were resuspended in the required volume of buffer (2 ml peritoneal, 1 ml others) and both pellet and supernatant tubes were then prepared for assay (see section 2.7).

2.6.2 Effect of metabolic inhibition on histamine release

Buffers for metabolic inhibition experiments were made as outlined in Table 2.1. Antimycin A inhibits oxidative phosphorylation and 2-deoxy-D-glucose is a glycolysis inhibitor. In these experiments cell washing, final resuspension and drug dilutions were performed using glucose free buffer. Cell suspensions (180 μl) were incubated for 20 min at 37 °C with equal volumes of one of the four buffers before challenge with 40 μl of secretagogue (at 10 x the final concentration). After a 10 min incubation, the reaction was terminated with 600 μl of ice-cold buffer. The subsequent procedure was as outlined in section 2.6.1.

2.6.3 Effect of neuraminidase on histamine release

Aliquots of RPMC were resuspended in appropriate volumes of Full Tyrode's buffer and left to equilibrate to 37 °C for 10 min. Nine parts of cells were incubated with one part of neuraminidase V solution (1 U/ml) for 60 min along with control cells (1 ml) (no neuraminidase V solution). After the incubation, cells were washed twice and
resuspended in the appropriate volume of Full Tyrode's buffer. Cells (250 μl) were added to polypropylene tubes containing 200 μl of buffer and 50 μl of secretagogue (at 10 times the final concentration). After a 10 min incubation, the reaction was terminated with 1.5 ml of ice-cold buffer. The remaining procedure was otherwise identical to that outlined in 2.6.1.

2.6.4 Inhibition of histamine release

Aliquots of cells (180 μl) were added to an equal volume of inhibitor (at 2 x the final concentration) for a defined period of preincubation (usually 10 or 30 min) according to the specific inhibitor. After preincubation, 40 μl of allergen (rat cells) or anti-human IgE (human cells) at an appropriate concentration to give approximately 20-30% histamine release was added to stimulate secretion. Some inhibitors did not require preincubation and in this case cells were added simultaneously to both the inhibitor and secretory stimulus. A tube containing buffer instead of inhibitor was set up for each period of preincubation to assess the unblocked histamine release induced by the secretory stimulus alone. After a 10 min incubation with the secretory stimulus, the reaction was terminated with 600 μl ice-cold buffer and the subsequent procedure carried out as before (section 2.6.1).

2.7 Histamine assay

Two methods are outlined below for the measurement of the fluorescence generated by the alkaline condensation product of histamine with o-phthaldialdehyde (OPT) (Fig. 2.4), as first described by Shore et al. [11].

2.7.1 Preparation of samples for manual assay using a spectrofluorimeter

Histamine release from rat peritoneal mast cells was measured by the following manual procedure. Tubes containing the cell pellets in buffer (2 ml) were allowed to stand in a boiling water bath, appropriately stoppered, for 10 mins in order to fully disrupt the cells,
hence releasing all their residual histamine. NaOH (1 M, 267 μl) was added to each sample, followed by OPT (1% in methanol, 100 μl) which was incubated for 4 min before the addition of HCl (3 M, 133 μl) to quench the reaction. The tubes were vortexed after each addition to ensure adequate mixing.

Fluorescence was measured with a Perkin-Elmer LF 5B fluorescence spectrophotometer, using an emission wavelength of 440 nm and excitation wavelength of 360 nm. The fluorimeter was first zeroed to background fluorescence using a blank tube, containing buffer alone. The limit of sensitivity of detection was ca. 5 ng/ml of histamine.

2.7.2 Preparation of samples for assay using an autoanalyser

For all tissue mast cells and human basophil leukocytes, where the samples contained much cellular debris, and rat peritoneal mast cells where the secretagogues or inhibitors used interfered with the fluorescence generated in the manual method, a commercial autoanalyser (Technicon Autoanalyser 11 or Chemlab Autoanalyser) was used, according to the method of Siraganian et al. [435].

Samples (1 ml) were prepared for this assay by the addition of perchloric acid (72%, 33 μl) to a final concentration of 0.4 M (2.4%) (to release residual histamine and precipitate any proteins present) followed by vortexing to ensure mixing. The tubes were then centrifuged (200 g, 15 min at 4 °C) and decanted into autoanalyser cups for automatic sampling.

Briefly, the histamine in the samples was concentrated and purified by a series of sequential phase separations in the autoanalyser between organic solvents and aqueous solutions. The histamine was finally concentrated into alkaline NaOH solution (1 M) before being condensed with OPT followed by the addition of HCl to create a stable fluorescent adduct which could be measured using the attached fluorimeter. The limit of sensitivity of this procedure was ca. 0.5 ng/ml of histamine.
2.7.3 Calculation of percentage histamine release or percentage inhibition of histamine release

In the above methods, histamine was assayed in both the supernatant and pellet fractions and release was expressed as a percentage of the total cellular content of the amine as follows.

\[
\text{% Histamine release} = \frac{\text{Histamine in supernatant}}{\text{Histamine in supernatant} + \text{histamine in pellet}} \times 100\%
\]

Each value was corrected for the spontaneous release occurring in the absence of the secretagogue.

Inhibition of histamine release was expressed as the percentage inhibition of the control or unblocked release occurring in the absence of the inhibitor. As above, all values were corrected for spontaneous release before calculation.

\[
\text{% Inhibition} = \frac{\text{Histamine release in the (absence - presence) of inhibitor}}{\text{Histamine release in the absence of inhibitor}} \times 100\%
\]

2.8 Mast cell characterisation

2.8.1 Cell counting and viability

The total number of nucleated cells and their percentage viability were evaluated by exclusion of the dye Trypan Blue. Viable cells exclude the dye whereas dead cells take up the stain. Three parts of the cell suspension were added to one part of this dye (0.1 %) and incubated at 37 °C for 5 mins. The cells were loaded onto an improved Neubauer haemocytometer by capillary action for counting under x 40 magnification. All 25 squares were counted.
The number of cells was determined as follows:

1. \[ \text{cells/ml} = \frac{\text{Total N}^2 \text{ of cells in 25 squares} \times 10^4 \times 4/3}{1} \]

For an estimate of the percentage of mast cells/ml of a sample, live cells could be stained with alcian blue (0.1 %) or toluidine blue (0.15 %) and counted on a haemocytometer. Nine parts of the cells were incubated with 1 part of the dye for 5 mins in a water bath (37 °C).

2. \[ \text{N}^2 \text{ of mast cells/ml} = \frac{\text{Total N}^2 \text{ of stained cells in 25 squares} \times 10^4 \times 10/9}{1} \]

\*, to correct for volume

\†, dilution factor

This can be expressed as a percentage of the number of cells/ml from 1. above. More accurate mast cell counts were achieved, however, by fixation and staining of cytospin preparations of the cells as outlined in section 2.8.3.

2.8.2 Evaluation of mast cell histamine content

Knowing the number of mast cells/ml of the cell suspension and therefore the number of mast cells per tube in the experiment, the histamine content (ng/ml) of the tube and hence per mast cell could be worked out by comparison with known histamine standards on the autoanalyser.

2.8.3 Fixation and staining

The cells were counted and resuspended to a volume of $10^6$ cells/ml in the case of RPMC or $5 \times 10^4 - 10^4$ cells/ml for the other tissue mast cells and cytospin preparations were made using a Shandon Cytospin 2 (50 g, 5 min, 100 μl). These were air-dried for a
minimum of 30 min and fixed by one of the following methods.

Camoy’s Solution: a mixture of absolute ethanol: chloroform: glacial acetic acid in the ratio 6:3:1: v/v, made fresh daily - for 30 min.

Formol Saline: formalin 10% v/v in 0.9 % saline - overnight.

The slides were washed in distilled water (1 min) after fixation and allowed to dry before staining as follows.

1. Alcian Blue (0.1 %, w/v) 30 min
2. GDW 2 min
3. 7 % HCl 5 min
4. Safranin O (0.5 % in 0.125 M HCl) 7-10 min
5. GDW 2 min

The cells were air-dried before being examined at x 100 magnification. It should be noted that in step 4., the prior use of slides of RPMC as a control to assess the uptake of the safranin dye, establishes the preferred staining time for each fresh batch of dye.

A minimum of 3 slides for each fixative were prepared for each mast cell suspension and 10 fields, or a minimum of 1,000 cells, were counted from each slide to give an average value of the percentage mast cells present in that tissue.

2.9 Statistical analysis

All values given are means ± standard error of the mean (S.E.M.) for the number (n) of observations noted. The points on the graphs represent the means and the vertical error bars show S.E.M.. Error bars have been omitted for ease, where multiple points converge. A paired Student’s "t" test was used for related measures and the symbol, *, on the graphs denotes a significance level of p < 0.05.
2.10 Materials

2.10.1 Histamine liberators

Anti-sera
- Rabbit-anti-human IgE
- Rabbit-anti-human IgG (H+L chains)
- Sheep-anti-rat IgE
- Sheep-anti-guinea pig IgG₁
- Sheep-anti-guinea pig IgG₂

Basic secretagogues
- ACTH₁₂₄
- Chlortetracycline hydrochloride
- Complement fragment, C3a and analogues, C3a₁₂₈R and 21R
- Compound 48/80
- Codeine hydrochloride
- Formyl-methionine-leucine-phenylalanine (FMLP)
- Histone
- Mastoparan
- Morphine sulphate
- Poly-L-arginine hydrochloride
- Poly-L-lysine hydrobromide
- Polymixin B sulphate
- Protamine sulphate
- Substance P
- SP₁₄(CH₂)$_{11}$CH₃
- d-Tubocurarine chloride

Dako
Sigma, London
ICN Biomedicals
ICN Biomedicals
ICN Biomedicals

Gift from T.E. Hugli, La Jolla, CA
Gift from Dr.H. Repke, Academy of Drug Research, Berlin, GDR
Sigma, London
Calcium ionophores
A23187 Calbiochem
Ionomycin Calbiochem

Lectins
Concanavalin A (Con A) (Jack Bean) Sigma, London
Phytohaemagglutinin (PHA) (Phaeseolus vulgaris) Sigma, London
Wheat Germ Agglutinin (WGA) (Triticum vulgaris) Sigma, London

Miscellaneous
Triton X-100 Sigma, London
Tween 20 Sigma, London

2.10.2 Inhibitors of histamine release

β Agonists
Isoprenaline bitartrate Sigma, London
Salbutamol sulphate Gift from Glaxo Group Research

H₂ Antagonists
Cimetidine Gift from Dr. J. Skidmore, SKB
Ranitidine Gift from Dr. J. Skidmore, SKB

Anti-allergic compounds
Disodium cromoglycate (DSCG) Gift from Mr P. Sheard, Fisons
FPL 59002K (Nedocromil sodium) Gift from Dr T.S.C. Orr, Fisons

Flavonoids
Chrysin Sigma, London
Quercetin Sigma, London
Phosphodiesterase inhibitors and agents which raise cAMP levels

Dibutyryl cAMP Sigma, London
Isobutylmethylxanthine Sigma, London
Theophylline Sigma, London

2.10.3 Materials for buffers

Calcium chloride Hopkins and Williams
Ethylenediaminetetraacetic acid (EDTA) BDH Chemicals
Glucose BDH Chemicals
HEPES BDH Chemicals
Hydrochloric acid Fisons
Potassium chloride Fisons and BDH Chemicals
Sodium chloride Fisons
Sodium dihydrogen orthophosphate Hopkins and Williams
Sodium hydroxide BDH Chemicals

2.10.4 Materials for fixation and staining

Absolute alcohol (99.8 %) James Burrough (F.A.D.) Ltd
Alcian blue BDH Chemicals (Gurr)
Chloroform BDH Chemicals
Formalin Hopkins and Williams
Glacial acetic acid BDH Chemicals
Microscope slides BDH Chemicals
Safranin O BDH Chemicals (Gurr)
Toluidine blue BDH Chemicals
Trypan blue BDH Chemicals
2.10.5 Other materials

Activated charcoal (particle size: 0.85-1.70 mm)  BDH Chemicals
Bovine serum albumin  Sigma, London
Butan-1-ol  Fisons
Carbon dioxide  British Oxygen Company
Collagenase (type 1A)  Sigma, London
Dextran (mwt 70,000 and 110,000) (6 %, w/v) in saline  Fisons
Dimethylsulphoxide (DMSO)  Hopkins and Williams
Heparin  C.P. Pharmaceuticals
n-Heptane  Fisons
Hyaluronidase (type 1S)  Sigma, London
Lens tissue  Whatman
Methanol  BDH Chemicals
Neuraminidase type V  Sigma, London
Nitrous oxide  British Oxygen Company
Perchloric acid (72 %)  May and Baker
Phosphatidylserine  Sigma, London and Lipid Products
o-Phthaldialdehyde (OPT)  Sigma, London
Triton X-405  Sigma, London
**Fig 2.1** Life cycle of *Nippostrongylus brasiliensis* in the rat.
Mashed mixture of faeces containing eggs and equal amount of charcoal

Moist filter paper

Aluminium foil

Petri-dishes

Faeces, L₃ and charcoal

2 Layers of gauze interleaved with 1 layer of lens tissue

Tap water; 37°C

Rubber tube

Graduated test tube

Larvae, L₃ stage

Fig. 2.2 Preparation of third stage larvae.
Chapter 2

Fig. 2.3 Preparation of allergen.
Fig. 2.4 The reaction of histamine with OPT.

Fluorescent adduct

Histamine

\[ \text{C-CH}_2\text{-CH}_2\text{-NH}_2 + \text{OPT} \]
<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Full Tyrode’s</th>
<th>Glucose Free Buffer</th>
<th>2x Glucose Buffer</th>
<th>2x Antimycin A Buffer (Anti A)</th>
<th>2x 2-deoxy-D-glucose Buffer (2-DG)</th>
<th>2x 2-DG + Anti A Buffer</th>
<th>Phosphate buffered saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>154</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.6</td>
<td>---</td>
<td>11.2</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>---</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>15</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>---</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.002</td>
<td>---</td>
<td>0.002</td>
<td>---</td>
</tr>
<tr>
<td>2-deoxy-D-Glucose</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>10</td>
<td>10</td>
<td>---</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>67</td>
</tr>
</tbody>
</table>

BSA-Tyrode’s - Full Tyrode’s containing BSA (1 mg/ml)
Heparin-Tyrode’s - Full Tyrode’s containing heparin (5 iU/ml)
CHAPTER 3: MAST CELL HETEROGENEITY IN THE RAT AND GUINEA PIG: THE EFFECT OF SECRETAGOGUES

3.1 Introduction

The initial observation of the existence of two mast cell subtypes was by Maximow in 1906 [435]. However, the concept of mast cell heterogeneity was not firmly established until the mid 1960s by Enerbäck through detailed morphological and histochemical studies on the gastrointestinal tract of the rat [337-339]. He observed that although mast cells in the deeper layers of the intestinal wall or submucosa were very similar to those found in other connective tissues (termed CTMC), those in the mucosa, confined mainly to the lamina propria, displayed numerous differences. These cells, designated mucosal mast cells (MMC) were smaller, more variable in shape than their counterparts in the submucosa with a lower content of histamine, negligible amounts of 5HT and fewer granules (see table 1.1). MMC were refractory to the effects of Co. 48/80 and also displayed a dramatic proliferative response to infection with the nematode Nippostrongylus brasiliensis [390].

The subsequent development of techniques for the enzymic dispersion of free mast cells from a variety of tissues in the rat and guinea pig [340,343,437,438] has lead to more detailed investigation of the histochemical, functional and biochemical properties of mast cells from a variety of locations and species. In particular, the rat intestinal mast cell has been studied vigorously in this regard. These cells showed a much weaker response than peritoneal cells to immunologically directed ligands and calcium ionophores and were totally refractory to Co. 48/80 and peptide 401 from bee venom [340,426]. In addition, rat intestinal mast cells were markedly unresponsive to a wide variety of neuroenteric peptides such as somatostatin, substance P, vasoactive intestinal peptide (VIP), neurotensin and bradykinin in contrast to the strong response of serosal mast cells to these agents [425].

It is the aim of the present study to investigate further the functional and histochemical
heterogeneity of mast cells from a variety of connective tissue locations in the rat and
guinea pig, such as the mesentery, lung, skin and uterus and extend previous
investigations by the use of a wide range of structurally diverse polybasic compounds.
Of particular interest is the response of the skin mast cell in both species since there is
evidence that this cell type in man is unique in its reactivity [439].

3.2 Methods

The methods pertaining to this section can be found in full in chapter 2.

3.3 Results

3.3.1 Basic characteristics of rat and guinea pig mast cells

Cell suspensions containing free mast cells were obtained following enzymic dispersion
of tissue samples from rat lung, mesentery and skin and the skin and uterus of the guinea
pig. Mast cells comprised 4.1±0.5 (rat skin), 4.7±0.6 (rat mesentery), 2.8±0.1 (rat lung),
2.2±0.3 (guinea pig skin) and 2.1±0.6 % (guinea pig uterus) of the total nucleated cells
(table 3.1). The rat skin and lung mast cells contained comparable amounts of histamine
while that of the mesenteric cell was marginally greater. The rat peritoneal cell, however,
contained the most histamine (24.3±2.3 pg). In the guinea pig, the skin mast cell had a
remarkably low histamine content as compared with the uterine cell.

The cells obtained were structurally intact as assessed by light microscopy and highly
viable as judged by their exclusion of the dye trypan blue and their low basal histamine
release (< 7.8 %). Use of formol saline as the fixative distinguished two mast cell
subtypes in the rat and one in the guinea pig following a staining sequence of alcian
blue/safranin O. Mast cells from both the guinea pig skin and uterus were exquisitely
sensitive to formaldehyde fixation and those from the rat lung were also predominantly
formalin sensitive and exhibited little or no staining with safranin. In contrast, mast cells
from rat mesentery, skin and peritoneum were mainly formaldehyde insensitive and
stained positive with safranin.

3.3.2 **Histamine release in the rat induced by immunologically directed ligands**

Anti-rat IgE induced a dose-dependent histamine release from all four mast cell subtypes. Those from rat lung and mesentery responded strongly over the entire concentration range to this agent. In contrast, the response of the skin cell was weak (Fig. 3.1).

The lectin concanavalin A (Con A) elicited a concentration-dependent histamine release from all four tissue mast cells (Fig. 3.2). The cells from the peritoneum were the most responsive to this agent, releasing a maximum of 41.7±9.1 % of their total cellular histamine compared with only 13.4±2.5 % from the rat skin mast cell. A gradation in responsiveness of the different cell types was observed. The response of the rat peritoneal and mesenteric mast cells to Con A was approximately doubled in the presence of phosphatidylserine (PS) (table 3.3) and marginally enhanced for the skin and lung cells.

Mast cells from the mesentery were moderately responsive to the lectins PHA (table 3.4) and WGA (table 3.5), releasing a maximum of 21.8±9.7 and 22.1±7.4 % histamine, respectively, in the absence of PS. In contrast, cells from the peritoneum, lung and skin were largely refractory to these compounds. In the presence of PS, however, the responsiveness of the peritoneal cells to both lectins was greatly enhanced and that of the mesenteric cells approximately doubled. The response of the rat skin and lung mast cells was largely unaffected by PS.

3.3.3 **Effect of the calcium ionophores on histamine release from rat mast cells**

The calcium ionophores A23187 (Fig. 3.3a), ionomycin (Fig. 3.3b) and chlortetracycline (Fig. 3.4) induced a dose-related histamine release from all four tissue mast cells. The secretion was suppressed at high concentrations of A23187 and ionomycin. Maximal secretion occurred at 0.5 μM A23187 for the peritoneal and mesenteric cells and at 1 μM for the lung and skin. Optimal secretion was at 3 μM ionomycin for the peritoneal cells.
in contrast to 1 μM for the other three tissues. A clear gradation in responsiveness was evident with the peritoneal cells being the most responsive for all three ionophores, the skin cell the least responsive to A23187 and ionomycin and the lung cells the least responsive to chlortetracycline.

3.3.4 **Histamine release from rat mast cells by polybasic compounds**

The classic histamine liberator, Co. 48/80 (Fig 3.5), the polyamino acids polyarginine (Fig 3.6a) and polylysine (Fig 3.6b) and the antibiotic polymixin B (Fig 3.7) induced a pronounced histamine release from rat peritoneal mast cells and had a substantial histamine-liberating effect on the mesenteric mast cells. Cells from the lung and skin responded to these agents, but to a much lesser extent. Indeed, the skin cell was largely refractory to the effects of Co. 48/80.

3.3.5 **Histamine release induced by the neuropeptide substance P and other structurally unrelated basic compounds**

Both the neuropeptide substance P and its synthetic analogue, SP$_{1-4}$C$_{12}$ (Fig 3.8) elicited a dose-dependent liberation of histamine from all four cell types. The peritoneal cell gave the strongest response to substance P but the lung and skin cells were only weakly active to this compound at top concentrations. This profile of responsiveness was matched when SP$_{1-4}$C$_{12}$ was the secretagogue. However, SP$_{1-4}$C$_{12}$ was more potent than its parent compound on all four cell types. In the rat peritoneal mast cell this agent was approximately 50 times more potent, with an EC$_{50}$ of 0.1 μM compared with 10 μM for substance P. The analogue was also able to differentiate the response of the skin and lung cells, the former being more responsive to this compound.

The polypeptides ACTH$_{1-24}$ (Fig. 3.9a), mastoparan (Fig. 3.9b) and the large cationic polypeptides protamine sulphate (Fig 3.10a) and histone (Fig. 3.10b) were all potent histamine liberators from the rat serosal mast cell and the mesenteric mast cell albeit to a reduced extent.
Protamine sulphate and histone also induced a modest histamine release from both the lung and skin mast cells which were largely refractory to ACTH$_{1-24}$ and mastoparan.

The alkaloid, d-tubocurarine (Fig. 3.11a) and the H$_2$ antagonist, ranitidine (Fig. 3.11b) were strong histamine releasers from rat peritoneal and to a lesser extent mesenteric mast cells. The lung mast cells were almost totally refractory to d-tubocurarine but were more active than the skin mast cells when ranitidine was the secretagogue.

All the above secretagogues induced a graded response from the four mast cell types.

3.3.6 The effect of neuraminidase on the histamine release from rat peritoneal cells with Co. 48/80, substance P and mastoparan

The effect on the secretory response of rat peritoneal mast cells of a 60 min preincubation with a concentration range of the enzyme neuraminidase which strips cells of their membrane-associated sialic acid was tested (Fig. 3.12). Concentrations of Co. 48/80 (0.25 μg/ml), substance P (10 μM) and mastoparan (3 μM) which induced an unblocked release of 37±3.6 %, 52.7±3.4 % and 23.2±2.5 %, respectively, were used. Neuraminidase treatment of the cells inhibited the release induced by these secretagogues in a dose-dependent fashion. The optimal inhibitory concentration of neuraminidase was 0.05 U/ml and this reduced the secretory response to these compounds by as much as 78.3±5.0 % (Co. 48/80), 52.7±15.6 % (substance P) and 89.3±7.2 % (mastoparan). Given the variation in the magnitude of the unblocked release, the extent to which neuraminidase treatment ablated the secretory response in these cells was comparable for all three compounds.

3.3.7 Histamine release induced by immunological ligands from guinea pig mast cells

Mast cells from both the skin and uterus responded in a dose-dependent manner to anti-guinea pig IgG$_1$ and anti-guinea pig IgG$_2$ with optimal histamine secretion occurring at a dilution of 1/3000 (Fig. 3.13a and b). With each ligand the skin mast cells released
around 40% of their total cellular histamine but the uterine cells were considerably less responsive releasing only around 15% histamine.

3.3.8 **Histamine release induced by calcium ionophores from isolated guinea pig mast cells**

Both the calcium ionophores A23187 and ionomycin induced a marked dose-dependent release of histamine from guinea pig skin and uterine mast cells (Fig. 3.14a and b). A23187 appeared to be equally active against both cell types whereas the skin cells appeared less active in response to ionomycin compared with their counterparts in the uterus.

3.3.9 **Histamine release induced by polybasic compounds and the neuropeptide substance P from isolated guinea pig mast cells**

Mast cells from the guinea pig skin were highly reactive to Co. 48/80 (Fig. 3.15a) and exhibited a maximum histamine release of 10.9±1.4% when challenged with the neuropeptide substance P (Fig. 3.15b). The uterine mast cells were only marginally reactive to Co. 48/80 and completely refractory to substance P.

Both the skin and uterine mast cells responded to the polyamino acid polyarginine which induced a release of 9.2±1.7 and 5.1±0.7% of the total cellular histamine, respectively (Fig. 3.16a). In contrast, both cell types were refractory to polylysine (Fig. 3.16b). The antibiotic polymixin B, on the other hand, elicited as much as 24.7±4.4% histamine release from the guinea pig skin mast cells and 6.1±1.5% from mast cells of the guinea pig uterus, albeit at high concentrations (Fig. 3.17).

3.3.10 **Histamine release from guinea pig mast cells induced by a variety of basic compounds of divergent structure**

Mast cells isolated from guinea pig skin responded vigorously to the large cationic
polypeptide histone and somewhat less strongly to protamine sulphate, whereas cells from the uterus were largely refractory to these compounds (Fig. 3.18a and b). In addition, high concentrations of d-tubocurarine elicited a moderate histamine release from the guinea pig skin cells whilst having no effect on the uterine mast cells (Fig. 3.19a). Neither cell type was responsive to the wasp venom peptide mastoparan (Fig. 3.19b).

3.4 Discussion

The present study indicates that viable and functionally intact mast cells can be obtained by the enzymic dispersion of rat lung, mesentery and skin and guinea pig uterus and skin using the enzyme collagenase. Pearce et al. [440] found this enzyme to be a highly specific protease whose activity was unaffected by the presence of serum proteins such as bovine serum albumin in the dispersion mixture, added to stabilize the cells in suspension. The heteroglycan, hyaluronic acid, synthesized by fibroblasts, is one of the main components of the ground substance of skin and, hence, hyaluronidase which acts by depolymerization of this substance, was added for more efficient dispersion of this tissue.

In addition, in studies comparing the functional properties of mast cells from the rat intestine and peritoneum, the latter cells have been subjected to incubation with collagenase to recreate dispersion conditions [426]. The function of these cells was not in any way compromised by the presence of the enzyme and, hence, the widespread differences observed in functional responsiveness of rat mast cells cannot be attributed to the procedures employed in obtaining the cells.

Mast cells from all four locations in the rat were well preserved by Carnoy’s fixative and stained strongly and differentially with both alcian blue and safranin O under these conditions. Safranin O binds to the heparin proteoglycan in mast cell granules and hence the safranin positivity of cells can be taken as an indication of their CTMC phenotype [338]. Cells from the peritoneum were all of the CTMC subtype whereas those from the lung stained mainly with alcian blue and hence approximated more closely to the MMC
phenotype [401]. These findings were in agreement with those of Bachelet et al. [441] who found cells from rat lung were mainly alcian blue positive. Cells from the rat mesentery and skin were of intermediate phenotype, containing a mixture of both subtypes. Those from the mesentery were predominantly safranin positive; those from the skin, however, comprised roughly equal quantities of both subtypes.

Cells from the peritoneum, skin and mesentery were predominantly insensitive to the dye blocking properties of the aldehyde fixative, formaldehyde. In contrast, cells from the lung were mainly formaldehyde sensitive like those of the intestinal mucosa [337,407] indicating that their proteoglycans were of a less highly sulphated nature than heparin. Indeed, rat lung mast cells have been described as responding in a similar fashion to those of the intestine to infection by *Nippostrongylus brasiliensis* [442]; after two weeks of infection the lung mast cells showed a dramatic proliferative response in addition to staining positive for alcian blue but negative for berberine sulphate. The content of RMCPII was also seen to increase [443] in these cells.

All four rat mast cell types released histamine in response to anti-rat IgE indicating that these cells possess functional IgE receptors. The reason for the relatively low release from rat skin mast cells may be due to a lower receptor number or a predominance of a lower affinity receptor on these cells as has been documented for the rat intestinal mast cell [444,445].

The plant lectins Con A, PHA and WGA bind to specific carbohydrate residues on membrane glycoproteins and it has been suggested that Con A elicits histamine secretion from mast cells by binding to glucose and mannose residues in the carbohydrate portion of adjacent IgE molecules thereby cross-linking them and initiating release [167,168,446,447]. The relatively weak response of the rat skin mast cell to Con A may then correlate with a reduced number of IgE receptors as suggested above.

Although Con A released histamine from the rat peritoneal cells in the absence of phosphatidylerine (PS), and was moderately effective at releasing histamine from rat
mesenteric mast cells, addition of PS greatly enhanced both the responses of the peritoneal and mesenteric cells to this compound. PHA and WGA were effective in the absence of PS only in the mesenteric cells. The response of these lectins in the peritoneal cells was strongly dependent on the presence of PS which is in agreement with the findings of Ennis et al. [448] but in contrast to the work of Lansman et al. [449]. It has been suggested that rat peritoneal cells are unusual in being deficient in PS [450]. PS was shown to potentiate release only in the presence of exogenous Ca\(^{2+}\) which is consistent with the view that the lipid may promote the influx of the cation from the extracellular environment [450] by the formation of Ca\(^{2+}\)-PS bridges between the granule and the plasma membrane.

The effect of lectins can be antagonised by the addition of the sugar residues to which they bind. The effect of Con A can be inhibited by the addition of glucose or mannose residues to the reaction mixture and that of WGA by the addition of N-acetylglucosamine (NAG) and N-acetylneuraminic acid (NANA) [451]. The ability of a lectin to initiate secretion in a particular cell type seems then to be dependent on the presence and accessibility of the specific carbohydrate residue to which it binds in the membrane proteins involved in histamine release. This would suggest by inference that the important residues are lacking in rat skin or lung membrane glycoproteins and that fundamental differences in membrane structure may form the basis of mast cell heterogeneity in the rat.

Moreover, the marked gradation in responsiveness observed with rat mast cells to the ionophores A23187, ionomycin and chlortetracycline may reflect variations in the membrane compositions of the target cell leading to differences in the lipid solubility and rate of diffusion of the ionophore across the membrane. However, the histamine releasing effect of the ionophores in these cells, although variable, suggests that mobilization of internal calcium is a necessary and sufficient trigger for exocytosis [198]. However, the differential effect of the ionophores may be an indication that internal Ca\(^{2+}\) is utilized to varying degrees in each mast cell type to modulate the secretory process.
Extensive use of polybasic compounds such as Co. 48/80, the polyamino acids polyarginine and polylysine and the neuropeptide substance P has been made in order to study mast cell heterogeneity within and across species [452-457]. In rat tissue mast cells Co. 48/80, polyarginine, polylysine and polymixin B have their most pronounced effect on cells from the rat peritoneum and the response gradually diminishes, the weakest effect being on the skin cells. The polyamino acids and polymixin B induced a maximum of around 20% histamine release from rat skin and lung mast cells which were essentially refractory to Co. 48/80. Rat skin mast cells have, however, been reported to release up to 25.9±2.7% histamine with much higher concentrations of this agent [343]. The lack of reactivity of the lung mast cells to Co. 48/80 observed here is in contrast to published results [438]. However, this is perhaps not unexpected given the predominance of the alcian blue positive mast cell phenotype in this tissue which is probably a result of active sensitization of the animals with Nippostrongylus brasiliensis [442].

The mechanism of release induced by polylysine, polyarginine and Co. 48/80 appears to be distinct from that utilized by immunologically directed ligands [458-460] and several characteristics of histamine secretion are shared by these compounds such as the ability to induce secretion in a Ca²⁺ free medium [461] and competitive antagonism by benzalkonium chloride [462].

Histamine release induced by a variety of neuropeptides such as neurotensin [463,464], somatostatin [465], bradykinin [466] and most recently, neuropeptide Y (NPY) [467] from rat serosal mast cells has been well documented and indeed it has been suggested that these compounds are potential endogenous mast cell activators. In the present study, both substance P and its structural analogue SP₁₄C₁₂, which is synthesized by formation of a peptide bond between the N terminal Arg-Pro-Lys-Pro sequence of the parent molecule and dodecylamine [468], were potent releasers of histamine from the rat peritoneal mast cell and, to a lesser extent, the mesenteric mast cell.

The relatively poor reactivity of the skin mast cells to both substance P and SP₁₄C₁₂ would seem to indicate that mast cell degranulation is not directly involved in the initial
phase of neurogenic inflammation in rat dermis as also suggested by the in vivo studies of Kowalski et al. [469,470]. In addition, it seems unlikely, given the poor response of the pulmonary cells to these agents, that direct activation of mast cells by NANC nerves in the lung is of physiological relevance [471,472].

Indeed, a number of lines of evidence point to a non-specific interaction between basic peptides and the mast cell membrane to initiate secretion. Firstly the observation that substance P antagonists act as mast cell liberators [466] and analogues such as physalaemin and eleodoisin are inactive in this regard [459] suggests that the action of substance P is not via classical P or E type receptors. In addition, the concentration range over which this agent displays histamine-liberating properties in rat mast cells is some three orders of magnitude greater than those at which it has effects on smooth muscle, making it unlikely to be acting through a receptor dependent mechanism.

Jasani et al. [473] have shown using a variety of peptides derived from ACTH that the essential structural features of histamine releasing peptides were basic amino acids such as Arg and Lys at the N terminus and hydrophobic amino acids at the C terminus. In addition, a more recent study by Devillier et al. [474] has indicated that the N terminal Arg of both substance P and bradykinin was essential to induce histamine release. A hypothesis relating to the mechanism of action of basic peptides on mast cells has been put forward by Repke et al. [475]. The C terminal lipophilic end of the compound anchors in the membrane via formation of an α-helix. This alters the membrane conformation, opening up pockets of negative charge with which the N terminal basic amino acids then interact to initiate secretion.

The results obtained in the present study are in support of such a mechanism since the SP$_{1-4}$C$_{12}$ analogue with its long aliphatic hydrocarbon chain and core of N terminal basic residues (Appendix 1) was more active against all rat tissue mast cells tested when compared with the parent compound. In addition, from this study it is evident that an extensive range of compounds of diverse structure which share a predominantly basic nature induced a histamine release from rat peritoneal mast cells which was comparable
to that of substance P. It is difficult to reconcile this fact with the presence on one cell type of specific receptors for each one of these compounds or indeed with cross-reactivity of these agents to a small number of receptors. Hence, the concept of a non-specific membrane binding site seems a more favourable one.

Amongst the numerous and varied compounds which have been reported to cause histamine release from the rat serosal mast cell are the anaphylatoxins C3a and C5a [172], LHRH antagonists [476] and most recently the 28 amino acid residue of the α human atrial natriuretic factor which contains five Arg residues [477]. In the two latter cases the histamine releasing properties of these compounds severely limit their clinical utility.

The first 24 amino acids of the human adrenocorticotropic hormone ACTH contains three Arg and four Lys residues (for structure see Appendix 1) and is a potent histamine liberator from rat peritoneal and to a lesser extent mesenteric mast cells although is is inactive against the skin and lung cells. Such a gradation in responsiveness was also observed with the large cationic polypeptides, protamine sulphate and histone. The structure of protamine sulphate comprises approximately 69 % Arg residues whereas Arg and Lys residues constitute around 24 % of the histone molecule. Interestingly, both compounds elicited a substantial histamine release of around 37 % and 20 % from the normally unreactive rat lung and skin mast cells, respectively. This may be in part due to the strong hydrophobicity of these compounds. Histone in particular contains around 25 % hydrophobic amino acids and, as such, may interact more effectively with the membranes of the rat lung and skin mast cells thereby eliciting a quite considerable release, in much the same way as SP$_{1-4}$C$_{12}$, with its long aliphatic hydrocarbon chain, is a more effective histamine liberator in these cells than substance P.

In a previous study comparing the effects of both substance P and histone on rat serosal mast cells [478] it was suggested that these compounds in fact interacted with an identical site on the mast cell membrane and, in common with substance P [459], histone was shown to induce histamine release in the absence of extracellular calcium [479]. Indeed, the addition of Ca$^{2+}$ to the bathing medium reduced the release. This was postulated to
be due to the cation competing with histone for binding to negatively charged sites in the membrane.

The alkaloid, d-tubocurarine possesses basicity by virtue of its quaternary ammonium group and also has intensely hydrophobic regions conferred by an extensive ring structure (see Appendix 1). Again, this structure appears to be effective for the induction of a pronounced degranulation of rat peritoneal mast cells and in a graded manner from the other tissue mast cells tested.

The H$_2$ antagonist, ranitidine, proved to be a potent liberator of histamine from the rat peritoneal mast cell, in accordance with the findings of Lau and Pearce [480], and in a decreasing fashion from the mesenteric, lung and skin cells. Release was reported to be rapid and blocked by metabolic inhibitors and extremes of temperature [480]. The proposed mechanism for the histamine releasing action of this compound is by intercalation into the membrane resulting in changes in the conformation of membrane proteins which in some way triggers secretion [481]. However, given the structure of this anti-histamine (see Appendix 1) it is certainly tempting to speculate that its mode of action is similar to the other agents discussed here; that following diffusion into the lipid bilayer, interaction of its tertiary positive nitrogen atoms with negative sites in the membrane occurs to effect release. Indeed, in common with the action of Co. 48/80 and other polybasic compounds, the histamine release induced by ranitidine was blocked by BDTA [480].

The tetradecapeptide mastoparan, a toxin from wasp venom, contains three Lys residues (Appendix 1) and proved to be an effective histamine releaser from rat serosal and mesenteric mast cells in this study. This compound has been reported to induce histamine release in rat mast cells by direct activation of a G protein [482]. It has also been reported that histamine release induced by the polyamines Co. 48/80 and somatostatin from rat serosal mast cells was markedly inhibited by prior exposure of the cells to a small amount of the islet-activating protein, pertussis toxin, which is a specific modifier of G proteins [290,483,484].
Subsequent detailed investigations by Mousli et al. [485] demonstrated that both substance P and Co. 48/80 were able to stimulate directly G proteins purified from calf brain which had been reconstituted into phospholipid vesicles by increasing the rate of binding of GTP<sub>Y</sub>S. In addition, this phenomenon was sensitive to pretreatment with pertussis toxin [486]. Such evidence has resulted in a refinement of the hypothesis regarding the mechanism of action of polybasic compounds [487]; that G proteins constitute the binding site for these compounds, in much the same way as the intracellular loop of the activated neurokinin receptor interacts with the C-terminal domain of the G protein α subunit [488], and this interaction results in mast cell triggering.

Sialic acid residues may play a role in the initial interaction of polybasic compounds with the membrane. In accordance with previous findings [489,490], pretreatment of rat peritoneal mast cells in the present study with neuraminidase, which strips them of their sialic acid residues, significantly reduced the response of these cells to Co. 48/80, substance P and mastoparan. This provides strong evidence in favour of a non-specific, electrostatic interaction [491] between these basic compounds and negative sites in the mast cell membrane. Although no negative control was included here, it has been reported that neuraminidase pretreatment has no effect on the response of rat serosal mast cells to the calcium ionophore A23187 [490], suggesting that this mechanism of action was specific for peptide histamine releasers. Given an initial attachment to the membrane, the peptide could then span the lipid bilayer by virtue of its hydrophobic residues [492] which would facilitate the interaction of remaining basic residues with G proteins in the cytosolic compartment.

This theory has interesting implications concerning the fundamental basis of mast cell heterogeneity. It may be that the anionic charge of the cell might contribute significantly to the selectivity of cellular responsiveness to receptor-independent effects of cationic peptides. Whether such apparent membrane differences amongst rat mast cells are somehow a function of the cellular microenvironment is a source of some speculation.

From the results presented in this study it would appear that Enerbäck’s concept of the
existence of two distinct mast cell phenotypes in the rat is an oversimplification. Mast cells from the mesentery, lung and skin investigated here are derived from connective tissues and, on the basis of this classification, would be expected to behave in a similar if not identical fashion to the classical CTMC, the serosal mast cell. This is, however, clearly not the case since mast cells from these four tissues showed a marked gradation in responsiveness to the lectin Con A, calcium ionophores and a wide range of polybasic compounds. On histological examination it was evident that the cells from the mesentery, skin and lung comprised mixed populations of both CTMC and MMC and the order of responsivity of these cells correlated well with their relative proportion of safranin positive cells. Those from the lung parenchyma and skin contained significant proportions of cells which stained positive with alcian blue and were much less responsive than the peritoneal cells which were all of the safranin positive phenotype.

These observations emphasize the inappropriate usage of the terms CTMC and MMC which define a cell on the basis of its site of origin. Clearly, such a classification does not accurately reflect the widespread variation in functional reactivity that exists between cells from a number of connective tissues in the rat.

In the guinea pig, mast cells from the skin and uterus were indistinguishable on the basis of their histochemistry. In accordance with previous studies using guinea pig lung mast cells [441] and cells from a variety of connective tissue and mucosal locations in this species [493], only alcian blue positive cells were present in these tissues. In addition, all cells were sensitive to formaldehyde fixation, strongly suggesting that guinea pig mast cells contain chondroitin sulphate rather than heparin proteoglycan.

Functionally, however, the guinea pig skin mast cell appears to be exquisitely sensitive to a range of polybasic agents. In particular this cell released up to $34.2 \pm 3.7\%$ of its total cellular histamine in response to the cationic polypeptide, histone, to which the uterine cells were essentially refractory. As suggested by Lagunoff et al. [166], this may be due in part to the large proportion of Arg residues present in this compound since protamine sulphate and polyarginine which are rich in this amino acid similarly proved
to be highly effective histamine releasers from the skin cells and in the latter case also the uterine cells. The diaminobutyric acid moieties in polymixin B also proved to be quite potent against the skin cell. Interestingly, however, compounds which contain Lys as their predominant basic amino acid, such as polylysine and mastoparan, proved to be ineffective histamine releasers from either cell type.

Indeed, the skin cell appears to be unique amongst guinea pig mast cells in its reactivity to polybasic compounds since, despite releasing histamine in response to both antigen and calcium ionophores, mast cells from guinea pig lung and mesentery were also reported to be completely refractory to the effects of Co. 48/80, polylysine [438] and substance P [457].

IgG is the major anaphylactic immunoglobulin in the guinea pig and both the skin and uterine cells released histamine in response to anti-guinea pig IgG1 and IgG2. Those from the skin, however, responded more vigorously which may indicate a higher level of endogenous sensitization in these cells. The strong response of both cell types to the calcium ionophores A23187 and ionomycin provided further evidence that these cells were functionally intact following enzymic dispersion.

It is evident from the present study that clear differences exist between species in the reactivity of cells from the same tissue location. While in the rat the skin cell was almost totally quiescent, this cell type in the guinea pig was unique in being exquisitely responsive to a range of polybasic compounds. In addition, although use of the alcian blue/safranin O staining system was adequate in the rat to discern mast cell subtypes it did not transfer well to the guinea pig system. In this species, cells which appeared to be histochemically identical were in fact functionally quite distinct. The assumption that cells which stain positive for alcian blue (MMC) are functionally inactive, derived from use of these stains in the rat, is also clearly inappropriate in the guinea pig as is evident from the highly responsive nature of the skin cell in this species.
Fig. 3.1  Histamine release induced by anti-rat IgE from isolated rat mast cells (n=5).

Fig. 3.2  Histamine release induced by concanavalin A from isolated rat mast cells (n=4).
Fig. 3.3 Histamine release induced by the calcium ionophores (a) A23187 and (b) ionomycin from isolated rat mast cells (n=4-6).

(a)

% histamine release

0.1 1 10
A23187 (µM)

peritoneal  skin  lung  mesentery

(b)

% histamine release

0.1 1 10
Ionomycin (µM)

peritoneal  skin  lung  mesentery
Fig. 3.4 Histamine release induced by chlortetracycline from isolated rat mast cells (n=3-5).

![Graph](image1)

Fig. 3.5 Histamine release induced by compound 48/80 from isolated rat mast cells (n=3-6).

![Graph](image2)
Fig. 3.6  Histamine release induced by the polyamino acids (a) polyarginine and (b) polylysine from isolated rat mast cells (n=4-7).

(a)

% histamine release

0 1 10 100
Polyarginine (μg/ml)

peritoneal  skin  lung  mesentery

(b)

% histamine release

0 1 10 100
Polylysine (μg/ml)

peritoneal  skin  lung  mesentery
Fig. 3.7  Histamine release induced by polymixin B from isolated rat mast cells (n=4-5).
Fig. 3.8 Histamine release induced by the neuropeptide (a) substance P and its analogue (b) SP$_{1-4}C_{12}$ from isolated rat mast cells (n=4-6).

(a)

(b)
Fig. 3.9 Histamine release induced by (a) ACTH<sub>1-24</sub> and (b) mastoparan from isolated rat mast cells (n=4-6).

(a) % histamine release

[Graph showing histamine release in response to varying concentrations of ACTH<sub>1-24</sub> in different tissues.]

(b) % histamine release

[Graph showing histamine release in response to varying concentrations of mastoparan in different tissues.]
Histamine release induced by (a) protamine sulphate and (b) histone from isolated rat mast cells (n=4-6).

(a)

![Graph showing histamine release induced by protamine sulphate.](image)

(b)

![Graph showing histamine release induced by histone.](image)
Fig. 3.11  Histamine release induced by (a) d-tubocurarine and (b) ranitidine from isolated rat mast cells (n=4-5).

(a) % histamine release

![Graph showing histamine release induced by d-tubocurarine.]

(b) % histamine release

![Graph showing histamine release induced by ranitidine.]

- peritoneal  ◦ skin  ▲ lung  □ mesentery
Fig. 3.12  Inhibition of histamine release induced in rat peritoneal mast cells with Co. 48/80 (0.25 μg/ml), substance P (10 μM) and mastoparan (3 μM) by preincubation of the cells with neuraminidase (n=3).

% inhibition

Neuraminidase (U/ml)

- Co. 48/80, 0.25μg/ml
- Substance P, 10 μM
- Mastoparan, 3 μM
Fig. 3.13  Histamine release induced by (a) anti-guinea pig IgG$_1$ and (b) anti-guinea pig IgG$_2$ from isolated guinea pig mast cells (n=5-6).

(a)

% histamine release

(b)

% histamine release
Fig. 3.14  Histamine release induced by the calcium ionophores (a) A23187 and (b) ionomycin from isolated guinea pig mast cells (n=4).

(a) % histamine release

(b) % histamine release
Fig. 3.15  Histamine release induced by (a) compound 48/80 and (b) substance P from isolated guinea pig mast cells (n=4-6).

(a)

% histamine release

48/80 (µg/ml)

- uterus  - skin

(b)

% histamine release

Substance P (µM)

- uterus  - skin
Fig. 3.16  Histamine release induced by the polyamino acids (a) polyarginine and (b) polylysine from isolated guinea pig mast cells (n=4).

(a)

% histamine release

Polyarginine (µg/ml)

--- uterus  --- skin

(b)

% histamine release

Polylysine (µg/ml)

--- uterus  --- skin
Fig. 3.17  Histamine release induced by polymixin B from isolated guinea pig mast cells (n=4).
Fig. 3.18  Histamine release induced by (a) protamine sulphate and (b) histone from isolated guinea pig mast cells (n=4).

(a)

% histamine release

(b)

% histamine release

Protamine sulphate (nM)

Histone (µM)

uterus  skin

uterus  skin
Fig. 3.19  Histamine release induced by (a) mastoparan and (b) d-tubocurarine from isolated guinea pig mast cells (n=4-5).

(a)

% histamine release

\begin{center}
\begin{tabular}{c}
0.01 & 0.1 & 1
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
0.01 & 1 & 10
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
d-Tubocurarine (mM)
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
\rightarrow \text{uterus} & \rightarrow \text{skin}
\end{tabular}
\end{center}

(b)

% histamine release

\begin{center}
\begin{tabular}{c}
0.01 & 0.1 & 1
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
0.01 & 1 & 10
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
Mastoparan (\mu M)
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
\rightarrow \text{uterus} & \rightarrow \text{skin}
\end{tabular}
\end{center}
Table 3.1  Some basic characteristics of mast cells derived from a variety of locations in the rat (n=3-6).

<table>
<thead>
<tr>
<th></th>
<th>Peritoneum</th>
<th>Skin</th>
<th>Lung</th>
<th>Mesentery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells (% total)</td>
<td>5.3±0.6</td>
<td>4.1±0.5</td>
<td>2.8±0.1</td>
<td>4.7±0.6</td>
</tr>
<tr>
<td>Histamine (pg/mast cell)</td>
<td>24.3±2.3</td>
<td>2.6±0.2</td>
<td>3.8±0.3</td>
<td>7.6±0.8</td>
</tr>
<tr>
<td>Viable cells (% total)</td>
<td>98.1±0.7</td>
<td>90.2±2.3</td>
<td>74.4±6.8</td>
<td>98.6±1.4</td>
</tr>
<tr>
<td>Spontaneous histamine release (%)</td>
<td>6.2±0.4</td>
<td>6.2±0.4</td>
<td>7.8±1.3</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>Formaldehyde sensitive (%)</td>
<td>0.4±0.2</td>
<td>20.2±7.5</td>
<td>78.0±4.9</td>
<td>23.0±9.3</td>
</tr>
<tr>
<td>Safranin O positive</td>
<td>99.7±0.1</td>
<td>38.5±0.2</td>
<td>13.4±0.2</td>
<td>68.2±1.3</td>
</tr>
</tbody>
</table>
Table 3.2 Some basic characteristics of mast cells derived from the skin and uterus of the guinea pig (n=2).

<table>
<thead>
<tr>
<th></th>
<th>Skin</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells (% total)</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Histamine (pg/mast cell)</td>
<td>0.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Viable cells (% total)</td>
<td>91.2</td>
<td>83.1</td>
</tr>
<tr>
<td>Spontaneous histamine</td>
<td>1.7±0.03</td>
<td>2.7±0.07</td>
</tr>
<tr>
<td>release (%) (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde sensitive (%)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Safranin O positive</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.3  Histamine release from rat mast cells induced by the lectin concanavalin A in the absence (a) or presence (b) of phosphatidylserine (15 μg/ml).

(a)

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Peritoneum n=4</th>
<th>Skin n=4</th>
<th>Lung n=3</th>
<th>Mesentery n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>37.4±9.2</td>
<td>12.8±3.0</td>
<td>8.4±3.3</td>
<td>22.8±6.1</td>
</tr>
<tr>
<td>50</td>
<td>41.7±9.1</td>
<td>13.4±2.5</td>
<td>9.5±4.0</td>
<td>22.1±5.1</td>
</tr>
<tr>
<td>10</td>
<td>23.7±8.8</td>
<td>12.3±3.0</td>
<td>13.7±5.4</td>
<td>19.8±3.3</td>
</tr>
<tr>
<td>5</td>
<td>23.7±8.5</td>
<td>10.7±3.4</td>
<td>17.0±6.6</td>
<td>19.6±2.9</td>
</tr>
<tr>
<td>1</td>
<td>2.5±1.2</td>
<td>7.4±2.9</td>
<td>17.0±8.5</td>
<td>8.4±4.9</td>
</tr>
<tr>
<td>0.5</td>
<td>0.4±0.4</td>
<td>5.4±1.9</td>
<td>10.3±4.5</td>
<td>6.5±3.6</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1±0.1</td>
<td>3.9±1.4</td>
<td>1.2±0.8</td>
<td>1.8±1.6</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Peritoneum n=4</th>
<th>Skin n=4</th>
<th>Lung n=3</th>
<th>Mesentery n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>79.6±2.0</td>
<td>17.8±2.7</td>
<td>9.7±4.3</td>
<td>39.5±6.3</td>
</tr>
<tr>
<td>50</td>
<td>80.2±2.0</td>
<td>16.3±1.5</td>
<td>10.8±5.4</td>
<td>40.5±7.6</td>
</tr>
<tr>
<td>10</td>
<td>75.9±2.2</td>
<td>14.7±3.4</td>
<td>14.5±7.3</td>
<td>36.7±9.2</td>
</tr>
<tr>
<td>5</td>
<td>70.1±4.7</td>
<td>14.7±4.2</td>
<td>17.2±7.6</td>
<td>36.6±10.8</td>
</tr>
<tr>
<td>1</td>
<td>25.2±7.2</td>
<td>7.9±3.8</td>
<td>19.3±8.7</td>
<td>20.3±10.3</td>
</tr>
<tr>
<td>0.5</td>
<td>7.6±3.5</td>
<td>7.1±3.0</td>
<td>15.3±5.7</td>
<td>14.6±8.6</td>
</tr>
<tr>
<td>0.1</td>
<td>0.8±0.6</td>
<td>4.5±1.8</td>
<td>1.3±0.9</td>
<td>5.9±3.0</td>
</tr>
</tbody>
</table>
Table 3.4  Histamine release from rat mast cells induced by the lectin phytohemagglutinin (PHA) in the absence (a) or presence (b) of phosphatidyliner (15 μg/ml).

(a)

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Peritoneum (n=3)</th>
<th>Skin (n=6)</th>
<th>Lung (n=3)</th>
<th>Mesentery (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3.4±1.2</td>
<td>5.7±0.6</td>
<td>4.5±0.8</td>
<td>19.2±5.4</td>
</tr>
<tr>
<td>33</td>
<td>1.7±1.0</td>
<td>6.7±1.1</td>
<td>5.5±1.1</td>
<td>19.8±7.3</td>
</tr>
<tr>
<td>10</td>
<td>1.6±1.2</td>
<td>8.7±1.3</td>
<td>4.8±1.1</td>
<td>21.8±9.7</td>
</tr>
<tr>
<td>3.3</td>
<td>2.5±1.3</td>
<td>9.3±1.6</td>
<td>2.4±1.2</td>
<td>18.9±8.1</td>
</tr>
<tr>
<td>1</td>
<td>3.2±1.2</td>
<td>3.8±1.3</td>
<td>0.5±0.5</td>
<td>6.3±3.0</td>
</tr>
<tr>
<td>0.1</td>
<td>4.2±1.2</td>
<td>0.1±0.1</td>
<td>1.0±1.0</td>
<td>2.2±0.2</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Peritoneum (n=4)</th>
<th>Skin (n=6)</th>
<th>Lung (n=3)</th>
<th>Mesentery (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>61.6±4.2</td>
<td>7.4±1.6</td>
<td>7.2±2.1</td>
<td>41.0±3.2</td>
</tr>
<tr>
<td>33</td>
<td>62.0±4.4</td>
<td>8.4±1.5</td>
<td>7.1±2.0</td>
<td>39.9±2.7</td>
</tr>
<tr>
<td>10</td>
<td>59.2±5.5</td>
<td>10.2±1.7</td>
<td>6.4±1.9</td>
<td>38.5±3.1</td>
</tr>
<tr>
<td>3.3</td>
<td>55.2±4.8</td>
<td>12.3±2.0</td>
<td>3.0±1.4</td>
<td>32.4±4.4</td>
</tr>
<tr>
<td>1</td>
<td>28.0±5.0</td>
<td>8.4±1.6</td>
<td>0.5±0.3</td>
<td>19.0±6.1</td>
</tr>
<tr>
<td>0.1</td>
<td>1.2±0.6</td>
<td>2.2±1.8</td>
<td>0.4±0.4</td>
<td>0.7±0.8</td>
</tr>
</tbody>
</table>
### Table 3.5

Histamine release from rat mast cells induced by the lectin wheat germ agglutinin (WGA) in the absence (a) or presence (b) of phosphatidyserine (15 µg/ml).

(a)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Peritoneum n=3</th>
<th>Skin n=6</th>
<th>Lung n=3</th>
<th>Mesentery n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.1±0.1</td>
<td>3.5±0.7</td>
<td>4.6±1.6</td>
<td>17.2±5.1</td>
</tr>
<tr>
<td>50</td>
<td>2.0±0.7</td>
<td>3.2±0.7</td>
<td>3.8±1.2</td>
<td>20.1±6.1</td>
</tr>
<tr>
<td>10</td>
<td>2.9±0.9</td>
<td>3.0±0.7</td>
<td>3.2±2.0</td>
<td>22.1±7.4</td>
</tr>
<tr>
<td>5</td>
<td>1.9±0.3</td>
<td>4.2±1.0</td>
<td>2.2±1.3</td>
<td>21.9±8.1</td>
</tr>
<tr>
<td>1</td>
<td>1.0±0.5</td>
<td>3.9±0.9</td>
<td>1.1±1.0</td>
<td>12.7±5.3</td>
</tr>
<tr>
<td>0.5</td>
<td>1.4±1.4</td>
<td>3.5±0.9</td>
<td>0</td>
<td>6.5±3.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.2±0.2</td>
<td>1.1±0.3</td>
<td>1.0±0.9</td>
<td>4.9±1.2</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Peritoneum n=3</th>
<th>Skin n=6</th>
<th>Lung n=3</th>
<th>Mesentery n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>66.4±3.8</td>
<td>6.4±1.3</td>
<td>6.0±1.2</td>
<td>37.5±2.5</td>
</tr>
<tr>
<td>50</td>
<td>66.4±2.1</td>
<td>5.5±1.2</td>
<td>6.1±1.6</td>
<td>34.9±1.6</td>
</tr>
<tr>
<td>10</td>
<td>62.7±3.4</td>
<td>5.4±1.1</td>
<td>4.5±1.6</td>
<td>35.6±2.6</td>
</tr>
<tr>
<td>5</td>
<td>57.6±2.6</td>
<td>5.9±1.3</td>
<td>4.0±1.3</td>
<td>34.5±3.5</td>
</tr>
<tr>
<td>1</td>
<td>30.6±5.7</td>
<td>8.2±1.5</td>
<td>1.3±0.3</td>
<td>27.9±6.2</td>
</tr>
<tr>
<td>0.5</td>
<td>21.2±8.0</td>
<td>6.7±1.1</td>
<td>1.3±0.3</td>
<td>19.0±7.3</td>
</tr>
<tr>
<td>0.1</td>
<td>3.2±1.8</td>
<td>0.4±0.3</td>
<td>0.9±0.5</td>
<td>1.6±1.0</td>
</tr>
</tbody>
</table>
CHAPTER 4: MAST CELL HETEROGENEITY IN THE RAT - EFFECT OF ANTI-ALLERGIC COMPOUNDS

4.1 Introduction

The rat peritoneal mast cell has been used extensively in studies to determine the efficacy of a wide range of anti-allergic compounds since these cells can be obtained readily in large numbers by direct lavage [318].

Active sensitization of rats with the nematode *Nippostrongylus brasiliensis* induces a generalised mast cell hyperplasia in the whole animal, most dramatically in the lamina propria of the small intestine [389]. The life cycle of the nematode involves migration of the larvae from the skin where penetration occurs, through the lung, before maturation and settling of the adult worms in the lumen of the small intestine [494]. During this infection, massive production of IgE antibody, directed against soluble antigens derived from the adult worm, occurs in the host. Mast cells from the gut and also the connective tissues become sensitized with this antibody. This is accompanied by a dramatic, thymus-dependent mast cell hyperplasia around day 12-14 with numbers reaching five times their normal level in the lamina propria and a simultaneous expulsion of the worms in the faeces [390], which is suggestive of a causal relationship.

The production of large numbers of sensitized mast cells in the rat by the above method has facilitated detailed study of the activity of anti-allergic drugs against the intestinal mast cell [426,427,452]. Results from these studies suggest that marked heterogeneity exists between isolated rat mast cells from connective tissue (RPMC) and mucosal sites in response to a number of anti-allergic compounds. The present study aims to extend this work to include isolated mast cells from rat skin, thereby comparing the susceptibility of two mast cells from connective tissue locations to inhibition by a range of anti-allergic compounds.
4.2 Methods

The methods pertaining to this section can be found in full in chapter 2.

4.3 Results

4.3.1 Inhibition of histamine release by anti-asthmatic compounds and flavonoids

Both the anti-allergic chromone disodium cromoglycate (DSCG) (Fig. 4.1a) and its more potent congener nedocromil sodium (Fig. 4.1b) proved to be highly effective inhibitors of histamine release from rat peritoneal mast cells. These compounds reduced release from the serosal cells by as much as 69.7±9.9 and 64.1±8.7 % at 1 mM and 100 μM, respectively. In contrast, however, the rat skin mast cells were totally refractory to the inhibitory effects of DSCG (Fig. 4.1a) and were markedly less sensitive than their counterparts from the peritoneum to nedocromil sodium (Fig. 4.1b), although this agent inhibited release by 48.4±10.1 % at top concentrations.

Preincubation of both DSCG and nedocromil sodium with the cells for 10 min, resulted in a dramatic reduction in their inhibitory effect. Such a tachyphylactic phenomenon was apparent for nedocromil sodium with both rat skin and peritoneal cells (Fig. 4.2b) and inhibition of histamine release from rat peritoneal cells with 1 mM DSCG was almost totally abrogated following a 10 min preincubation (Fig. 4.2a).

The flavonoids, quercetin and chrysin, whose structures are similar to those of the chromone, were, in contrast, effective inhibitors of histamine release from both the rat skin and peritoneal cells (Fig. 4.3). Indeed, the behaviour of both cell types in the presence of each agent was largely indistinguishable except at low concentrations of chrysin where the peritoneal cells were less sensitive to its inhibitory effects. In general, however, quercetin proved to be a more potent inhibitor than chrysin. Histamine release was reduced from the rat skin and peritoneal cells by as much as 98.1±1.9 and 89.1±4.9 % in the presence of 100 μM quercetin compared with only 38.4±5.6 and 39.1±6.2 %
with the same concentration of chrysin.

4.3.2 Inhibition of histamine release by agents which affect cyclic AMP levels

The methylxanthines, theophylline and isobutylmethylxanthine (IBMX), raise intracellular cyclic AMP levels by the inhibition of a specific phosphodiesterase which hydrolyzes this second messenger to adenosine monophosphate. Both compounds induced a significant dose-dependent inhibition of histamine release from rat skin and peritoneal mast cells (Fig. 4.4). At 1 mM these agents were equiactive against both cell types, inhibiting release by between 56±5.2 - 67±8.0 %. IBMX was more active than theophylline over a lower concentration range; the effect of theophylline was negligible at 0.1 mM whereas that of IBMX persisted at 0.01 mM.

Dibutyryl cyclic AMP is an analogue of cyclic AMP which enters the cell by virtue of its lipid solubility. This compound also induced a strong concentration-dependent inhibition of histamine release from rat peritoneal and to an even greater extent rat skin mast cells (Fig. 4.5).

4.3.3 Inhibition of histamine release by H₂ and β₂ receptor directed agents

The H₂ receptor antagonist, cimetidine, elicited a dose-dependent inhibition of histamine release from rat skin and peritoneal mast cells (Fig. 4.6). The top concentration of this agent, 10 mM, was equiactive against both cell types, inhibiting release by 86.3±8.1 and 86.0±3.8 % from the skin and peritoneal cells, respectively. Cimetidine was, however, marginally more effective against the skin cell over the entire concentration range.

In contrast, the β₂ agonists, salbutamol and isoprenaline were ineffective inhibitors of histamine release from both rat skin and peritoneal cells (Fig. 4.7). A maximum inhibition of only 22.4±5.1 and 24.3±5.8 % was observed from rat skin cells with 10 µM salbutamol and isoprenaline, respectively, compared with a mere 7.5±5.1 and 6.6±5.7 % from peritoneal cells.
4.4 Discussion

A range of animal models were employed in initial studies to test the efficacy of disodium cromoglycate as an anti-allergic compound. In the rat, this agent proved to be effective in models of passive respiratory [495], peritoneal [496] and cutaneous anaphylaxis (PCA) [497,498]. However, although DSCG was a potent inhibitor of histamine release from isolated peritoneal mast cells in the present study, the effect of this compound against isolated rat skin mast cells was clearly not as predicted from the results obtained using in vivo models; this agent was devoid of anti-allergic activity in the rat skin mast cell.

Such striking differences may be attributed to the varying level of control release between in vivo and in vitro systems. For purely practical reasons of measurement the control release in in vitro studies is necessarily in the range 10-15 %. Secretion of this magnitude probably never occurs in vivo. Indeed, it has been estimated that the amount of histamine release in even the most severe asthmatic attack is likely to be less than 1 % of the total lung content of the amine. Given this and the well-known fact that the activity of cromoglycate and other similar drugs varies inversely with the magnitude of the anaphylactic response [198], it is therefore not surprising to observe that where DSCG proved to be a potent inhibitor of histamine release in vivo, it failed in this regard in vitro. These results therefore highlight the discrepancies inherent in extrapolating between in vitro and in vivo systems.

Nonetheless, the finding that isolated mast cells from the skin varied markedly in their response to DSCG when compared with cells from the peritoneum serves to underly that important functional differences exist between mast cell subtypes. Indeed, isolated mast cells from the rat intestine have also been reported to be refractory to inhibition by this agent [426].

In addition, nedocromil sodium was reported to be ineffective against the rat intestinal cell [434]. However, in accordance with previous investigations using the rat PCA model [499] and isolated serosal mast cells [426,500], this compound was active against isolated
cells from both the rat peritoneum and skin. The activity of the skin cell to nedocromil sodium and its concomitant refractoriness to DSCG make it difficult to envisage a common mechanism of action for these two compounds. However, the activity of both compounds was markedly reduced following preincubation in each cell type. This was not readily apparent in the rat skin cells with DSCG which was undoubtedly due to the low level of inhibition observed even in the absence of preincubation in these cells, since tachyphylaxis has been reported in the rat PCA reaction with this compound [501].

Many anti-allergic compounds exert their effect distal to histamine release from mast cells. Disodium cromoglycate, however, has been proposed to interfere with the exocytotic process itself [502] although the exact mechanism remains an enigma. Pecht and his coworkers [503-510] in a series of elegant experiments have identified a 60 kD glycoprotein, the cromolyn binding protein (CBP), in rat basophilic leukemia cells (RBL). This protein was proposed to constitute the calcium channel in the mast cell membrane and binding of the chromone to this site was in turn proposed to interfere with the calcium gating mechanism in these cells, thereby suppressing degranulation. However, the RBL cell is the only mast cell in which a CBP has been identified. Moreover, this cell type is entirely resistant to the inhibitory effects of DSCG [456] and as such the relevance of such a mechanism of action for cromoglycate is under question.

It has also been suggested that the mechanism of action of cromoglycate may result from activation of an endogenous secretory control mechanism. It has been observed that within 10 secs of challenge of rat peritoneal mast cells with anti IgE, Co. 48/80 or A23187, three proteins of relative molecular mass 68, 59 and 42 kD were phosphorylated [216,511]. This was followed some 30-60 secs after challenge by phosphorylation of a fourth protein of 78 kD. It was proposed that the early phosphorylation of the three proteins was involved in initiating secretion whereas that of the 78 kD protein some time later was responsible for the termination of secretion. DSCG itself induced specific phosphorylation of this 78 kD protein [511,512]. Interestingly, treatment of the cells with dibutyryl cyclic GMP also resulted in profound phosphorylation of this protein and it has been proposed that DSCG may inhibit degranulation in this manner via a cyclic GMP-
dependent protein kinase although as yet no direct evidence for this exists [513].

Indeed, an automatic depletion or dephosphorylation of the phosphorylated 78 kD protein by the cell, despite the continued presence of the drug, would account for the observed tachyphylaxis of cromoglycate in the present study. This therefore reinforces a mechanism of action of the drug on protein phosphorylation. Further studies to elucidate fully the definitive mechanism of action of disodium cromoglycate are, however, eagerly awaited.

The flavonoids quercetin and chrysin, are structurally similar to the chromones. Cromoglycate and quercetin exhibited cross-tachyphylaxis in rat peritoneal mast cells [514] which is often taken as evidence that a compound has a similar mechanism of action to the chromone [515]. There is also some evidence to indicate that quercetin as well as cromoglycate stimulates the phosphorylation of a 78 kD protein in rat mast cells [216].

However, the results of the present study make it difficult to propose a common mechanism of action for the two classes of compound. In fact, these findings indicate that the two types of agent clearly differ in their mode of action since, unlike DSCG, both quercetin and chrysin were effective inhibitors of histamine release from the rat skin mast cell. Such a difference in activity between quercetin and disodium cromoglycate has also been documented for the rat intestinal mast cell [427]. In addition, where the response of rat mast cells was abrogated following a 10 min preincubation with DSCG and nedocromil sodium, quercetin and chrysin in fact required such a preincubation for optimal inhibitory activity and did not exhibit tachyphylaxis.

Quercetin proved to be a more potent inhibitor than chrysin against both cell types in this study which is in agreement with the order of potency of these compounds on the rat MMC [427]. However, in contrast to the results presented here, chrysin has been reported to be more active than quercetin against the serosal cell [427,516].
The mechanism of action of quercetin has been proposed to be by increasing the efficiency of ATP-dependent ion pumps such as the Ca\textsuperscript{2+}-ATPase which pumps calcium out of cells [517]. Histamine release, which is dependent on a rise in Ca\textsuperscript{2+}, would hence be inhibited. At high concentrations, quercetin may inhibit oxidative phosphorylation which would have obvious inhibitory activity on secretion [518].

Results from the present study have shown that agents which raise intracellular cyclic AMP levels such as the methylxanthines theophylline and IBMX and the cAMP analogue dibutyril cyclic AMP strongly inhibited antigen-induced histamine release from both rat peritoneal and skin mast cells. These results were consistent with published reports on the action of these agents in the rat peritoneal mast cell [316-318]. Although initial studies found that theophylline was inactive against cells from the rat intestine when secretion was induced by antigen [426] or neuropeptides [519], this has subsequently been refuted in recent studies with this cell type [Liu W.L. PhD Thesis, University of London, 1990] where theophylline proved to be a potent inhibitor.

The weaker inhibitory activity observed for dibutyril cyclic AMP against the peritoneal cell, may reflect underlying differences in the membrane composition of this cell compared with its counterpart in the skin, such that this agent was less soluble in the rat peritoneal mast cell membrane and hence its rate of diffusion into the cell was slower.

In conflict with the above findings, the β\textsubscript{2} adrenoceptor agonists salbutamol and isoprenaline, which also raise intracellular cAMP, by activation of adenylate cyclase, were without inhibitory activity on either cell type. The existence of large numbers of high affinity β\textsubscript{2} receptors on rat peritoneal mast cells has been demonstrated, however, using radioligand binding studies [314] but, in the light of the findings of the present study and others [313,314,328], the functional significance of these receptors is unclear. It may be that the β\textsubscript{2} receptors present on rat peritoneal and skin mast cells are in a modified form or are partially uncoupled from membrane bound adenylate cyclase. It has also been suggested that these agents generate intracellular cAMP which exists in a discrete pool, not directly involved in inhibition of secretion [198].
In contrast to the histamine releasing properties of the H$_2$ antagonist, ranitidine (see chapter 3), cimetidine has a potent inhibitory action on mast cells from the peritoneum and skin of the rat. Such a dual action of anti-histamines has been well documented [480] and is thought to be related to the particular physicochemical features of each compound. Unlike the $\beta_2$ agonists, cimetidine does not elevate intracellular cyclic AMP levels [480] and, as such, its action is not thought to be via histamine receptors linked to adenylate cyclase. Indeed, it has been proposed that the action of this agent is via a direct effect on the cell membrane; that by intercalation and expansion in the membrane cimetidine prevents fusional changes associated with exocytosis [481]. In the present study mast cells from the peritoneum were less susceptible to inhibition by cimetidine than those isolated from skin and this may indeed be as a result of differences in membrane structure and fluidity between these cells.

In summary, the results of this investigation indicate that where marked differences were observed between mast cells from the peritoneum and skin in response to a range of secretagogues (see chapter 3), the difference in the susceptibility of these cell types to a diversity of anti-allergic compounds was less striking or simply not apparent, except perhaps for the chromone disodium cromoglycate and nedocromil sodium. Where variation between the two cell types did occur, with dibutyryl cAMP and cimetidine, this was subtle and possibly represented differences in membrane composition resulting in variation in the solubility and/or diffusion of compounds across the plasma membrane, rather than fundamental, biochemical differences in the cellular regulation of exocytosis.
Fig. 4.1  Inhibition of histamine release from isolated rat peritoneal and skin mast cells by the anti-asthmatic compounds (a) disodium cromoglycate (DSCG) and (b) nedocromil sodium (n=5) (no preincubation). A concentration of allergen was chosen which gave control release of 8.7±1.6 % (skin) and 25±4.5 % (peritoneal).

(a)

```
% inhibition
100 80 60 40 20 0 -20 -40

DSCG (µM)
```

- — peritoneal
- — skin

(b)

```
% inhibition
100 80 60 40 20 0

Nedocromil sodium (µM)
```

- — peritoneal
- — skin
The effect on inhibition of histamine release from rat mast cells by (a) disodium cromoglycate (DSCG 1 mM) and (b) nedocromil sodium (0.1 mM) of a 10 minute preincubation with the drug (n=5).

(a)

![Graph showing inhibition by DSCG (1 mM)]

(b)

![Graph showing inhibition by nedocromil sodium (0.1 mM)]
Fig. 4.3  Inhibition of histamine release from isolated rat peritoneal and skin mast cells by
the flavonoids (a) quercetin and (b) chrysin (n=4-7) (10 min preincubation). A concentration of
allergen was chosen which gave control release of 12.9±2.4 % (skin) and 21.2±3.6 % (peritoneal).

(a)

% inhibition

120
100
80
60
40
20
0

100
10
1

Quercetin (µM)

peritoneal  skin

(b)

% inhibition

120
100
80
60
40
20
0

100
10
1

Chrysin (µM)

peritoneal  skin
Fig. 4.4 Inhibition of histamine release from isolated rat peritoneal and skin mast cells by the methylxanthines (a) theophylline and (b) isobutylmethylxanthine (IBMX) (n=5) (10 min preincubation). A concentration of allergen was chosen which gave control release of 12.9±2.4 % (skin) and 23.3±4.5 % (peritoneal).

(a)

% inhibition

0.1 1 10

Theophylline (mM)

peritoneal  skin

(b)

% inhibition

0.01 0.1 1

Isobutylmethylxanthine (mM)

peritoneal  skin
Fig. 4.5  Inhibition of histamine release from isolated rat peritoneal and skin mast cells by the cyclic AMP analogue, dibutyryl cyclic AMP (n=4) (10 min preincubation). Control release was 13.4±2.1 % (skin) and 32.1±6.5 % (peritoneal).

![Graph showing inhibition of histamine release by dibutyryl cyclic AMP](image)

Fig. 4.6  Inhibition of histamine release from isolated rat peritoneal and skin mast cells by the H₂ antagonist, cimetidine (n=5) (10 min preincubation). Control release was 12.9±2.4 % (skin) and 23.3±4.5 % (peritoneal).

![Graph showing inhibition of histamine release by cimetidine](image)
Inhibition of histamine release from isolated rat peritoneal and skin mast cells by the β₂ agonists (a) salbutamol and (b) isoprenaline (n=4) (10 min preincubation). A concentration of allergen was chosen which gave control release of 12.9±2.4 % (skin) and 23.3±4.5 % (peritoneal).

(a) 

(b)
CHAPTER 5: A COMPARISON OF THE RESPONSE OF HUMAN SKIN, UTERINE AND LUNG MAST CELLS TO SECRETAGOGUES

5.1 Introduction

In the skin, mast cells have been implicated in disorders such as dermatitis and urticaria [46,520] and it has also been suggested that they are involved in the control of local blood flow [521] and angiogenesis [522]. The lung mast cell is important in the pathogenesis of asthma [35].

While mast cell heterogeneity in the rodent is well established [523], the existence of distinct mast cell subpopulations in man has been less extensively investigated. Evidence exists to show that mast cells from the skin and lung differ in their neutral protease content [115] and early reports showed that dispersed lung mast cells were unresponsive to Co. 48/80 [428] whereas histamine release from skin mast cells was demonstrable in vivo following intradermal infusion of this agent [524] and from skin slices [525]. Subsequent investigations have since uncovered marked variations in functional responsiveness between human mast cells, in particular to polybasic compounds [430,431].

The aim of the present study is to extend these investigations to include a wide range of structurally diverse compounds which are essentially cationic, in order to investigate the extent of mast cell heterogeneity in man and the existence and nature of the putative polybasic receptor on human mast cells.

The isolation of a novel mast cell type, the human uterine myometrial mast cell, is also described here. The precise role of the uterine mast cell in mammalian physiology is as yet unclear. There is a large body of evidence to suggest that it plays a central role in blastocyst implantation. Changes in mast cell number during the oestrous cycle have been observed in the rat [526] and hamster [527], with the greatest numbers occurring during ovulation, prior to potential implantation. These fall sharply following blastocyst attachment [528,529]. In addition, administration of oestrogens in both the mouse [530]
and rat has been associated with increases in mast cell number. It has been postulated that histamine release from uterine mast cells at the time of blastocyst attachment mediates the local increase in vascular permeability which is observed coincident with implantation. Indeed, consistent with this hypothesis, systemic administration of anti-histamines in the rat prior to implantation was found to inhibit this local oedema.

More recently, human embryos of the 2-4 cell stage have been shown to produce a factor which induces histamine release from the human basophil and isolated rat uterine mast cells, named embryo-derived histamine-releasing factor (EHRF). Histamine has a well known immunosuppressive action and it is possible that EHRF, via degranulation of uterine mast cells, may, in part, mediate the response mounted by the embryo to evade maternal immune rejection. However, although the mast cell may play an important role in implantation, it does not appear to be essential for this process, since blastocyst attachment and gestation of offspring proceeded unaffected in mast cell deficient W/W^v mice.

In man, increased numbers of uterine mast cells are found premenstrually, and it has been postulated that their degranulation, and concomitant release of heparin, following the onset of menstruation maintains menstrual blood in a fluid state.

In the present study, it was hoped that some insight might be gained into the physiological role of the human uterine mast cell through its histochemical and functional characterization. Of particular interest was the response of this cell type to a variety of secretagogues compared with its counterpart in the skin since, given their similar connective tissue origin, the functional properties of these cells may be expected to resemble one another.

5.2 Methods

A full account of the relevant methods for this section can be found in chapter 2.
5.3 Results

5.3.1 Basic characteristics of mast cells isolated from human skin, lung and uterus

The enzymic dispersion of tissue samples of human skin, lung and uterus yielded suspensions of free mast cells which comprised 8.7±1.6, 3.5±0.8 and 3.1±0.7 % of the total cellular content, respectively (table 5.1). The histamine content of these three cell types was comparable, with the lung cell containing the most histamine (2.6±0.1 pg/cell) and the skin cell the least (1.2±0.3 pg/mast cell).

In each case, the cells were highly viable as judged by their exclusion of the dye trypan blue and their low spontaneous histamine release (< 6.7 %). The cells could be distinguished on the basis of their sensitivity to staining with alcian blue after fixation with formol saline; those from the lung and uterus were similar in being predominantly formalin sensitive, whereas the skin mast cells were mainly insensitive to this procedure and retained their ability to stain with alcian blue (ca 80 %).

5.3.2 Histamine release from human mast cells induced by immunologically directed ligands

The response of passively sensitized human mast cells to anti-human IgE is shown in Fig. 5.1. The human lung mast cells were the most responsive, releasing up to 27.7±1.9 % of their total cellular histamine with a 1/100 dilution of anti-IgE. The skin cells displayed markedly less reactivity to this agent with top concentrations inducing a release of only 13.2±1.3 % histamine.

In contrast, none of the three human mast cell types was sensitive to release by anti-human IgG (table 5.2) or to the lectins Con A, PHA and WGA either in the presence or absence of PS (table 5.3).
5.3.3 **Histamine release from human mast cells induced by the ionophores A23187, ionomycin and chlortetracycline**

All three mast cell types exhibited a dose-dependent release of histamine with the ionophores A23187 (Fig.5.2a) and ionomycin (Fig.5.2b). However, the response of the human skin mast cell to these agents was significantly weaker than that of either the lung or uterus, with a maximum histamine release of 29.3±8.2 % (A23187) and 25.7±6.3 % (ionomycin). However, in comparison with the relatively weak effect of chlortetracycline on the human lung mast cells, the response of the human skin mast cells to this agent was pronounced (Fig. 5.3). These cells released 32.0±4.9 % of their total cellular histamine with 1 mM of the antibiotic.

5.3.4 **The effect of a number of polybasic secretagogues on human mast cell degranulation**

The classic histamine releaser, Co. 48/80 (Fig. 5.4a) and the neuropeptide substance P (Fig. 5.4b) both proved to be strong histamine liberators from human skin mast cells whilst having only a weak effect at high concentrations on those cells from the human lung and uterus. At top concentrations these agents released up to 11.4±1.5 % and 17.5±2.8 % histamine from the skin cells, respectively. Release for both compounds was a non-cytotoxic and energy-dependent process since the effect of 10 μg/ml Co. 48/80 and 50 μM substance P was dramatically reduced by a 20 min preincubation of the cells with the metabolic inhibitors 2-deoxy-D-glucose and antimycin A (Fig. 5.5a and b).

The polyamino acid polylysine (Fig. 5.6b) induced a concentration-dependent histamine release from the human skin mast cell, the maximum release being 8.8±2.0 % at 100 μg/ml of the compound. Mast cells from human lung and uterus were, however, refractory to this agent. In contrast, polyarginine (Fig. 5.6a) induced a moderate histamine release from both human skin and uterine mast cells and also to a lesser extent the lung cells. The antibiotic polymixin B (Fig. 5.7) was also most effective against the human skin mast cells at high concentrations.
Protamine sulphate (Fig. 5.8a) was also a selective and dose-dependent histamine releaser from the human skin mast cell, those from the lung and uterus being largely refractory to its histamine releasing properties. In contrast, another large polycationic peptide, histone (Fig. 5.8b), induced a comparable histamine release in all three cell types at top concentrations.

Fragment 1-24 of the human adrenocorticotrophic hormone, ACTH (Fig. 5.9a), and mastoparan, a peptide extracted from wasp venom (Fig. 5.9b), also displayed pronounced and selective histamine liberating properties from the human skin mast cell, releasing a maximum of 7.6±2.9 and 8.9±1.2 % of total cellular histamine at top concentrations, respectively. ACTH\textsubscript{1-24} was largely ineffective at liberating histamine from the human uterine and lung mast cells and cells from the human uterus were essentially refractory to mastoparan.

D-Tubocurarine (Fig. 5.10a), the H\textsubscript{2} antagonist ranitidine (Fig. 5.10b) and the narcotic analgesics morphine (Fig. 5.11a) and codeine (Fig. 5.11b) were tested for their ability to degranulate human mast cells. All four compounds showed selective histamine releasing properties against the human skin mast cell, although the uterine mast cell showed mild reactivity towards top concentrations of both ranitidine and morphine.

It is of interest to note, however, that on no occasion does the extent of degranulation from the human skin mast cell match that observed in the rat serosal or even mesenteric mast cells to any of these compounds, where upwards of 80-90 % total cellular histamine was liberated (see chapter 3).

5.3.5 Histamine release induced by the detergents triton X-100 and tween 20 from human mast cells

The detergents triton X-100 (Fig. 5.12a) and, to a lesser extent, tween 20 (Fig. 5.12b) were potent, cytotoxic histamine releasers from both human skin and uterine mast cells. Triton X-100 was approximately five-fold more potent against the human skin mast cell
compared with the uterine cell. The profile of histamine release with tween 20 was, however, comparable for the two cell types, although the human skin mast cells appeared more sensitive than the uterine cells to degranulation at lower concentrations of the detergent.

5.3.6 Some structure-activity studies using human skin mast cells

The neuropeptide, substance P, and its synthetic derivative, SP_{1-4}C_{12}, showed very similar patterns of histamine release from the human skin mast cell, although the parent compound was marginally more potent at all concentrations tested (Fig. 5.13).

The anaphylatoxin, C3a, induced a dose-dependent release of histamine from the human skin mast cells, releasing a maximum of 9.7±4.1 % histamine (Fig. 5.14). Its synthetic structural analogue, 21R, was a less potent histamine liberator than the parent molecule and C3a_{desArg}, which lacks a carboxy Arg residue, was totally devoid of any histamine-releasing activity.

5.3.7 The effect of a number of polybasic compounds on histamine release from the human basophil leukocyte

The human basophil leukocyte was tested for its ability to release histamine in response to top concentrations of a number of polybasic secretagogues (Fig. 5.15). These cells were totally refractory to Co. 48/80 (10 µg/ml), substance P (50 µM) and protamine sulphate (10 µM) and only weakly active to 100 µg/ml of both polymixin B and polylysine. However, the basophils released 47.1±4.5 % histamine with 100 µg/ml polyarginine and 57.0±3.6 % with 100 µM histone which was comparable to that induced by 1 mM of the bacterial tripeptide, FMLP.

5.4 Discussion

The enzymic dispersion of human lung parenchyma, uterine myometrium and foreskin in
the present study resulted in the isolation of viable and functionally reactive mast cells. The skin appeared to contain a greater percentage of mast cells than the other two tissues, but the mast cells themselves were indistinguishable on the basis of their content of the biogenic amine, histamine.

Marked differences did exist, however, in the formaldehyde sensitivity of the skin mast cell compared with that of mast cells from the lung and uterus. The human skin mast cell, like the archetypal CTMC in the rat, the peritoneal cell (see chapter 3), was almost exclusively resistant to formalin fixation, and metachromasia with alcian blue persisted. This was in complete accordance with Olafsson et al. [538] who found that staining of only around 20% of human dermal mast cells was lost following conventional aldehyde fixation. In contrast, mast cells from both the lung and uterine myometrium, despite their connective tissue origin, were similar to mast cells from the human intestinal mucosa [415,416] and bronchoalveolar lavage (BAL) [539] in being predominantly formalin sensitive. Results with the uterine cell were in keeping with a recent study by Crow et al. [540], who found that mast cells from both the human uterine myometrium and endometrium stained poorly following formalin fixation. Conversely, those from dispersed rat uterus appeared to be resistant to aldehyde fixation [31].

It has been suggested that formaldehyde may induce cross-linking of the protein shell around the mast cell proteoglycan, thus restricting dye binding [407]. Since neutral proteases comprise a substantial proportion of the protein bound to the proteoglycan in human mast cells, it has been proposed that the differences in protease composition which have been observed in human mast cells [115] may contribute to differences in susceptibility to blockage of metachromatic staining following formaldehyde fixation [539]. Indeed, cells from both the lung and intestinal mucosa which are mainly tryptase-containing (MCₜ) were almost exclusively formaldehyde sensitive and hence there appears to be a good correlation between the presence of chymase in the mast cell and resistance to blockage by formalin, both of which are properties of the human skin mast cell.

However, the relative proportion of formalin sensitive subtypes in both rat and man can
be manipulated by altering the periods of fixation and staining [407,538,541]; shorter periods of fixation and longer staining times result in a greater proportion of visible mast cells. Typing of neutral proteases and proteoglycans therefore remain the most reliable histochemical methods for examining mast cell heterogeneity in man and the rat, respectively.

Isolated human mast cells were functionally responsive as judged by their ability to release histamine with anti-human IgE. It is of interest to note, however, that the response of the human skin mast cells, which were isolated predominantly from infants and young children (see chapter 2), was considerably lower than that in mast cells isolated from locations in the adult, despite routine passive sensitization. This is consistent with results from a number of studies which compared the responses of isolated adult and neonatal skin mast cells to anti-IgE [430,431] and may be due to a lower density of IgE receptors present on these cells compared with their adult counterparts. Indeed, the circulating levels of serum IgE in young children is much less than in adults [542] and it would appear that, at least in the human basophil, there is a good correlation between FcεRI number and the serum IgE level [543].

There is also some evidence to suggest that antibodies of the IgG4 subclass play an important role in human allergic and inflammatory processes, since raised circulating levels of this antibody have been detected in patients with asthma, atopic eczema [544] and ulcerative colitis [545,546], and that these may act synergistically with IgE in mast cell sensitization [547,548]. However, in the present study, whole anti-human IgG failed to elicit a significant release from any of the three mast cell types studied. This may be because tissue samples were not explicitly drawn from atopic populations and hence sensitization with IgG4 would perhaps not be expected.

In contrast to the strong response of mast cells from the lung, uterus and skin to anti-human IgE, cross-linking of surface FcεRI with the plant lectin Con A was an insufficient trigger for histamine release. Similarly, PHA and WGA were also without effect and addition of PS was unable to enhance the cellular response to any of the three lectins.
The differences observed between the reactivity of human mast cells to these lectins, when compared with that observed in RPMCs (see chapter 3), may reflect variation in the structure, degree of glycosylation and/or accessibility of carbohydrate moieties of the FceRI between the rodent and human systems.

The profile of responsiveness of the human mast cells to the ionophores, A23187 and ionomycin, was similar to that with anti-IgE; the human skin mast cell was considerably less responsive to these agents compared to mast cells isolated from the lung and uterus. This would again suggest that infant mast cells are generally less functionally mature than those isolated from other locations in the adult.

Both A23187 and ionomycin had distinctive release characteristics, with secretion falling dramatically at high ionophore concentrations. Concentrations of ionophore greater than 3 μM may be damaging to the cell or lead to sufficiently large increases in intracellular calcium so as to inhibit rather than evoke the secretory response.

Chlortetracycline or aureomycin, an antibiotic produced by *Streptomyces aureofaciens*, has also been demonstrated to have ionophoretic properties [549]. However, this agent was an ineffective histamine liberator from human lung mast cells, whilst it, in contrast, released up to 32 % histamine from the human skin mast cell. In fact, the profile of activity of this agent was so dissimilar to that of the ionophores, A23187 and ionomycin in these cell types, that it would therefore seem unlikely that the mechanism of action of chlortetracycline is as an ionophore, at least in man. Indeed, a recent unpublished study by Mustafa et al. (personal communication) has shown that histamine release from the RPMC by chlortetracycline was inhibited when lanthanum, as well as calcium ions, were present in the external medium. This provided strong evidence to suggest that the mechanism of action of this agent was via the opening of calcium-specific membrane channels, following initial binding, which could be blocked by lanthanum ions, and not as an ionophore. It is perhaps more likely that chlortetracycline, by virtue of its basic structure which contains a tertiary nitrogen atom (Appendix 1), triggers secretion by direct interaction with the skin mast cell membrane in a similar non-specific manner to other
polybasic compounds, as discussed later, with the resultant influx of calcium via membrane channel-opening.

The results of the present study have shown that human skin mast cells are unique, compared with those from human lung and uterus, in their ability to release histamine in response to a wide variety of non-immunological stimuli, which is consistent with the findings of others [344, 430-432].

Co. 48/80 is a mixed polymer of 3-methoxy-N-methyl phenylethylamine cross-linked by formaldehyde (Appendix 1). It was synthesized in 1948 and characterized as a histamine releaser in 1951 [550]. In the present study, the skin mast cell was reactive to this agent, releasing histamine in comparable amounts to that in other studies [344, 430], whereas mast cells from the lung and uterus were similar to isolated mast cells from the intestinal mucosa [133, 551, 552] in being essentially resistant to Co. 48/80. In addition, the human skin mast cell was unusual, compared to its counterparts in the lung and uterus, in releasing histamine with the undecapeptide, substance P.

The location of mast cells in skin, in the dermis, just below the dermal-epidermal junction [26], in close association with blood vessels and nerves [553, 554], and their degranulation following in vitro challenge with a number of neuropeptides [433], including substance P, make it tempting to speculate that they are under neuronal regulation.

The concept of neurogenic inflammation was first suggested by Lewis in 1927 [5]. He described the triple response to histamine in skin and suggested that the flare component was mediated by a neurogenic mechanism involving antidromic impulses conducted along primary afferent neurones. Indeed, in the rat, the flare component of the triple response was abolished by section of these sensory unmyelinated C fibres [555] whilst in man, application of local anaesthetics or topical capsaicin reduced the spread of the flare [556, 557].

A variety of neuropeptides have been localized in mammalian sensory nerves including
substance P, somatostatin and VIP [558-560]. Substance P injected intradermally into humans was approximately 100-fold more potent at inducing wheal and flare than histamine [561] and structural analogues of substance P also showed a close association between their ability to produce flare and their ability to release histamine from mast cells in vitro [556]. There is also some evidence to suggest that the flare following intradermal injection of substance P is mediated by release of histamine from mast cells since the H₁ anti-histamine, chlorpheniramine, reduced this whilst leaving the wheal unaffected [556,562]. However, other workers have been unable to repeat these findings [563,564] and their validity has been disputed [565].

A number of more recent detailed investigations of mast cell-nerve interactions have also shown that, at least in rat skin, such associations are not readily apparent [566] and arise as frequently with other bone marrow-derived cells, such as eosinophils and plasma cells, as with mast cells [567]. In addition, the high concentrations of substance P required to trigger release in vitro (>1 μM), observed in the present study, makes it hard to envisage that neurogenic inflammation is a physiological process. However, until the concentrations reached by substance P released at the neuroeffector junction have been estimated or indeed, an indication of what constitutes a physiologically relevant level of histamine release from mast cells calculated, the participation of mast cells in neurogenic inflammation cannot be wholly ruled out, at least in human skin. Whilst skin cells may be under neurogenic control, it is not clear why this should be confined exclusively to mast cells from this location.

In addition to the effects of Co. 48/80 and substance P, a diversity of compounds, including small and large peptides, narcotic analgesics, alkaloids and antibiotics, were all selective in releasing histamine from the human skin mast cell but not those from the uterus or lung in the present study. The widespread degranulating properties of such a variety of structurally diverse compounds, which share a predominantly basic nature, would therefore suggest that non-immunological release is a non-specific, receptor-independent event. Such a mechanism has been described in detail by Repke et al. [475] and is discussed more fully in chapter 3. In brief, it has been proposed that polybasic
compounds bind initially to the mast cell membrane by an ionic interaction with negatively charged sialic acid residues [568,569]. Following this primary interaction, these agents intercalate in the membrane by virtue of their hydrophobicity and this allows the further interaction of clusters of positive charge in the molecule with negative sites in the membrane, recently postulated in rat mast cells to be G proteins [487,570,571], which triggers secretion. In particular, the effectiveness of SP_{1,4}C_{12} as a histamine liberator from human skin mast cells supports such a mechanism of action in this cell. The structure of this compound comprises the fundamental requirements for such a non-specific interaction, namely a long chain hydrocarbon covalently linked to the amino acid sequence, Arg-Pro-Lys-Pro (Appendix 1).

The characteristics of non-immunological release differ from those of IgE-dependent stimuli in being more rapid (complete in < 20 sec) and only partially dependent on the presence of extracellular calcium [344]. In addition, the two types of stimuli have been reported to differ in their ability to induce eicosanoid release from human skin mast cells; substance P and a variety of other non-immunological stimuli released similar amounts of histamine to anti-IgE but some 12 to 21 fold less PGD_{2} and LTC_{4} [423].

The polyamino acids, polyarginine and polylysine, released up to 9% histamine at top concentrations from human skin mast cells, in accordance with other reports [430,431]. The lung and uterine mast cells, like human tonsillar, adenoidal and colonic mast cells [430], were, however, refractory to the effects of polylysine. Polyarginine, on the other hand, induced a comparable histamine release from human uterine and, to a lesser extent, pulmonary mast cells. A similar, more generalised histamine-releasing property was also displayed by the large cationic polypeptide, histone. Interestingly, in the present study, both polyarginine and histone also induced substantial histamine release from the normally quiescent human basophil leukocyte which, aside from its reactivity to FMLP, which is a highly specific basophil secretagogue [430], was refractory to Co. 48/80, substance P, polymixin, polylysine and protamine sulphate. This confirms the unique potency of histone and polyarginine in the induction of degranulation, which may be attributed to the high content of Arg residues in these compounds and, in the case of histone, to its strong
The hydrophobic nature which may facilitate a more efficient interaction with the mast cell and basophil membrane.

High doses of morphine are routinely used as intravenous anaesthetics and frequently induce significant hypotension. Production of IgE antibodies has also been implicated in morphine-induced anaphylaxis [572]. In addition, intradermal injection of both morphine and codeine resulted in positive and immediate skin reactions [573], which, in the case of codeine, were 100-fold more potent in patients with chronic urticaria [574].

This study shows that the hypotensive effects of narcotic analgesics may, in part, be due to their direct histamine-releasing effect in human skin since morphine and codeine were specific histamine liberators from human skin mast cells, except for top concentrations of morphine, at which there was some activity from the uterine mast cells. This was in keeping with previous reports where morphine has been shown to degranulate isolated cutaneous mast cells [431,575] and both narcotics released histamine from skin slices [576,577]. Human basophil leukocytes [576] and isolated intestinal mucosal mast cells [431] were, however, refractory to the effects of this compound. The relatively high concentrations at which these agents were effective suggests that they induced release via a receptor-independent, non-specific interaction between their tertiary nitrogen atom (Appendix 1) and sites of negativity in the mast cell membrane. This is further supported by the finding that the effect of morphine on histamine release from human skin was unaffected by naloxone, an opioid antagonist [576].

Such a non-specific interaction may also account for the histamine-liberating properties of the H₂ antagonist, ranitidine, against the human skin mast cell which similarly possesses a tertiary nitrogen atom (Appendix 1). This compound was considerably less active against the uterine cell and has been reported to be an ineffective histamine liberator from isolated human lung mast cells [578], which suggests that fundamental differences in membrane structure exist between these cell types and the skin mast cell.

Reversal of heparin anticoagulation and retardation of insulin absorption constitute the two
current medical uses of protamine whilst d-tubocurarine is used surgically as a muscle relaxant. Although there has been an association between life-threatening reactions to these compounds and production of IgE and IgG antibodies [579,580], the present study has, however, demonstrated that histamine release results from a direct interaction between these compounds and human skin mast cells in accordance with a recent study [581]. Protamine has also been reported to release histamine following intradermal injection [582], but not with minced human lung [583].

The peptides, ACTH$_{1-24}$ and mastoparan, which are potent histamine releasers from the rat serosal mast cell (see chapter 3) also liberated histamine from the human skin mast cells whilst having no effect against the uterine and, in the case of ACTH$_{1-24}$, lung mast cells. The cationic residues, Arg and Lys, constitute roughly 29% of the structure of ACTH$_{1-24}$ and mastoparan contains approximately 21% Lys. The secretory activity of these compounds may result from an interaction between their basic residues and pockets of negative charge in the membrane. Recently, Lawrence et al. [584] conducted a detailed study into the structure-activity relationship of a number of bradykinin analogues and antagonists on isolated human skin mast cells. In general, addition of D-Arg to the N terminal of the peptide dramatically enhanced its properties as a secretagogue whereas addition of Lys was considerably less effective. Moreover, aromatic residue substitution also enhanced secretagogue activity, something which has also been reported for substance P [466,585]. Thus, the degree of hydrophobicity of the peptide is also of supreme importance for its effectiveness as a histamine liberator.

In the present study, the histamine releasing properties of the human anaphylatoxin, C3a, which is a potent histamine releaser from the human basophil leukocyte [586], and its analogues, C3a$_{\text{des Arg}}$ and the 21 residue carboxy terminal peptide, 21R, were tested on isolated human skin mast cells. C3a induced a substantial histamine release from the human skin mast cell, although this was somewhat lower than that obtained in a previous study [587] where longer incubation times were used. Interestingly, however, removal of the carboxy terminal Arg residue (by carboxypeptidase N), completely abolished the histamine-releasing properties of the peptide. The 21R peptide, on the other hand, which
retains this Arg and also contains five additional basic amino acids, was still active as a histamine releaser albeit to a lesser degree than the parent molecule, which comprises around 26% basic amino acids (Appendix 1), thus confirming the importance of clusters of positive charge for histamine release.

Previous studies have shown that C5a, which is also a strong human basophil degranulating agent [588], was more potent than histamine, Co. 48/80, C3a and morphine at producing a wheal and flare on injection into human skin [589]. This agent has also been shown to be a potent secretagogue for isolated human skin mast cells at low concentrations [431,590], although it was inactive against human pulmonary [590] and adenoidal [591] mast cells. As with C3a above, the C terminal Arg was essential for the actions of C5a in vivo [592] and in vitro [590].

It would appear from the use of the detergents, tween 20 and, even more strikingly, triton X-100, that the human skin mast cell membrane is more susceptible to lysis, when compared with its counterpart in the uterus. This may indeed reflect fundamental differences in membrane structure and/or fluidity between the skin cell and other mast cell types which might account for the greater reactivity of the former cell to a wide range of polycationic substances. Differences in membrane fluidity as a function of mast cell age have been reported in Wistar rats [593]. It has also been reported that histamine-releasing stimuli increase the fluidity and permeability of the lipid bilayer [568] and thus it could be postulated that the more intrinsically fluid the membrane the greater the potential histamine release. It may then be that the human skin mast cell membrane is more fluid, perhaps as a result of a higher phospholipid or a lower cholesterol content [594], compared to the lung or uterine cells and that this results in its increased reactivity to a wide variety of secretagogues. An increase in membrane fluidity is not, however, envisaged to occur as a function of age in these cells, as skin cells derived from adult tissue have been shown to be equally, if not more, responsive to polybasic secretagogues than their infant counterparts [430].

In summary, the human skin mast cell, despite signs of functional immaturity to
immunologically directed ligands and calcium ionophores, appeared to be unique compared with its counterparts in the lung and uterus in being highly responsive to the histamine-releasing properties of a wide variety of polybasic compounds. It has been postulated that the exquisite reactivity of the human skin mast cell compared to other human mast cells may be the result of a more fluid or more negatively charged membrane, which might facilitate the non-specific interaction of a diverse array of secretagogues with the cell to trigger histamine secretion.

The uterine mast cell, on the other hand, despite a similarly connective tissue origin, was both histochemically and functionally distinct from the skin mast cell and showed closer similarities to the lung mast cell. This was in contrast to the apparent sensitivity of the rat uterine mast cell to Co. 48/80 [31]. Determination of the tryptase and chymase content of the human uterine mast cell would be of great interest in the further elucidation of human mast cell heterogeneity and more detailed investigation, perhaps of the effect of EHRF on human uterine mast cell degranulation, is required before a clear role for the uterine mast cell in human physiology and pathology can emerge.
Fig. 5.1 Histamine release induced by anti-human IgE from isolated human mast cells (n=4-12).
Fig. 5.2  Histamine release induced by the calcium ionophores (a) A23187 and (b) ionomycin from isolated human mast cells (n=4-6).

(a) % histamine release

\[ \text{A23187 (\mu M)} \]

- skin
- uterus
- lung

(b) % histamine release

\[ \text{Ionomycin (\mu M)} \]

- skin
- uterus
- lung
Fig. 5.3  Histamine release induced by chlortetracycline from isolated human mast cells (n=3-6).

% histamine release

Chlortetracycline (μM) M

- skin  - lung
Histamine release induced by (a) compound 48/80 and (b) substance P from isolated human mast cells (n=4-14).

(a)

% histamine release

\[
\begin{array}{c}
0.1 & 1 & 10 & 100 \\
48/80 (\mu g/ml) & \hline \\
\text{skin} & \text{uterus} & \text{lung}
\end{array}
\]

(b)

% histamine release

\[
\begin{array}{c}
0.01 & 0.1 & 1 & 10 \\
\text{Substance P (\muM)} & \hline \\
\text{skin} & \text{uterus} & \text{lung}
\end{array}
\]
Fig. 5.5  The effect of preincubation with metabolic inhibitors on the response of human skin mast cells to (a) compound 48/80 and (b) substance P. Cells were preincubated for 20 minutes with the following buffers (i) + Glucose, (ii) - Glucose, (iii) 2 Deoxy-D-glucose (5 mM), (iv) Antimycin A (1 µM) and (v) 2 Deoxy-D-glucose plus Antimycin A (2Deoxy/AntiA), (n=3).

(a)  

% histamine release

48/80 (10 µg/ml)

+ Glucose  
- Glucose  
2Deoxyglucose  
Antimycin A  
2Deoxy/AntiA

(b)  

% histamine release

Substance P (50 µM)

+ Glucose  
- Glucose  
2Deoxyglucose  
Antimycin A  
2Deoxy/AntiA
Fig. 5.6  Histamine release induced by the polyamino acids (a) polyarginine and (b) polylysine from isolated human mast cells (n=4-12).

(a)

% histamine release

![Graph showing histamine release induced by polyarginine at various concentrations.]

Polyarginine (μg/ml)

- skin
- uterus
- lung

(b)

% histamine release

![Graph showing histamine release induced by polylysine at various concentrations.]

Polylysine (μg/ml)

- skin
- uterus
- lung
Fig. 5.7 Histamine release induced by polymixin B from isolated human mast cells (n=5-9).
Fig. 5.8  Histamine release induced by (a) protamine sulphate and (b) histone from isolated human mast cells (n=4-6).

(a)

% histamine release

\[
\begin{array}{c}
\text{Protamine sulphate (nM)} \\
0.1 & 1 & 10 & 100 & 1000 & 10000
\end{array}
\]

- skin
- uterus
- lung

(b)

% histamine release

\[
\begin{array}{c}
\text{Histone (μM)} \\
0.01 & 0.1 & 1 & 10 & 100
\end{array}
\]

- skin
- uterus
- lung
Fig. 5.9  Histamine release induced by (a) ACTH$_{1-24}$ and (b) mastoparan from isolated human mast cells (n=4-6).

(a) % histamine release

\begin{center}
\begin{tikzpicture}
\begin{axis}[
    width=\textwidth,
    height=0.45\textwidth,
    xlabel={ACTH (1-24) (\textmu M)},
    ylabel={% histamine release},
    xmode=log,
    ymode=log,
    xmin=0.01, xmax=10,
    ymin=0, ymax=14,
    legend style={at={(0.5,0.1)},anchor=north},
    legend pos=north west,
    ytick={0,2,4,6,8,10,12,14},
    xtick={0.01,0.1,1,10},
    grid=both,
]
\addplot coordinates {(0.01,2) (0.1,2) (1,2) (10,14)} node[right]{skin};
\addplot coordinates {(0.01,2) (0.1,2) (1,2) (10,14)} node[right]{uterus};
\addplot coordinates {(0.01,2) (0.1,2) (1,2) (10,14)} node[right]{lung};
\end{axis}
\end{tikzpicture}
\end{center}

(b) % histamine release

\begin{center}
\begin{tikzpicture}
\begin{axis}[
    width=\textwidth,
    height=0.45\textwidth,
    xlabel={Mastoparan (\textmu M)},
    ylabel={% histamine release},
    ymode=log,
    xmin=0.01, xmax=10,
    ymin=0, ymax=14,
    legend style={at={(0.5,0.1)},anchor=north},
    legend pos=north west,
    ytick={0,2,4,6,8,10,12,14},
    xtick={0.01,0.1,1,10},
    grid=both,
]
\addplot coordinates {(0.01,2) (0.1,2) (1,2) (10,14)} node[right]{skin};
\addplot coordinates {(0.01,2) (0.1,2) (1,2) (10,14)} node[right]{uterus};
\end{axis}
\end{tikzpicture}
\end{center}
Fig. 5.10  Histamine release induced by (a) d-tubocurarine and (b) ranitidine from isolated human mast cells (n=4-7).

(a)  

% histamine release

<table>
<thead>
<tr>
<th>d-Tubocurarine (mM)</th>
<th>skin</th>
<th>uterus</th>
<th>lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b)  

% histamine release

<table>
<thead>
<tr>
<th>Ranitidine (mM)</th>
<th>uterus</th>
<th>skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5.11  Histamine release induced by (a) morphine and (b) codeine from isolated human mast cells (n=4-5).

(a)

% histamine release

Morphine (mM)

- skin  - uterus  - lung

(b)

% histamine release

Codeine (mM)

- skin  - uterus  - lung
Fig. 5.12  Histamine release induced by (a) tween 20 and (b) triton X-100 from isolated human mast cells (n=2-3).

(a) % histamine release

Triton X-100 (μl/ml)

- skin  ○ uterus

(b) % histamine release

Tween 20 (μl/ml)

- skin  ○ uterus
**Fig. 5.13** Histamine release induced by the neuropeptide substance P and its analogue SP$_{1-4}$C$_{12}$ from isolated human skin mast cells (n=13-14).

![Graph showing histamine release induced by Sub P/SP(1-4)C12 (μM)]

**Fig. 5.14** Histamine release induced by the anaphylatoxin C3a and its analogues C3a$_{des \text{Arg}}$ and 21R from isolated human skin mast cells (n=4-6).

![Graph showing histamine release induced by C3a/C3adesArg/21R (μM)]
Fig. 5.15  Histamine release induced by compound 48/80 (10 μg/ml), substance P (50 μM), FMLP (1mM), polymixin (100 μg/ml), polylysine (100 μg/ml), polyarginine (100 μg/ml), histone (100 μM) and protamine sulphate (10 μM) from human basophil leukocytes (n=4-6).
Table 5.1 Some basic properties of mast cells isolated from human skin, lung and uterus (n=4-10).

<table>
<thead>
<tr>
<th></th>
<th>Skin</th>
<th>Lung</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast Cells (% total)</td>
<td>8.7±1.6</td>
<td>3.5±0.8</td>
<td>3.1±0.7</td>
</tr>
<tr>
<td>Histamine (pg/mast cell)</td>
<td>1.2±0.3</td>
<td>2.6±0.1</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>Viable Cells (% total)</td>
<td>90.2±12</td>
<td>90.5±1.1</td>
<td>83.2±3.8</td>
</tr>
<tr>
<td>Spontaneous histamine release (%)</td>
<td>6.7±1.5</td>
<td>3.9±0.5</td>
<td>5.0±1.2</td>
</tr>
<tr>
<td>Formaldehyde sensitive (%)</td>
<td>16.1±7.8</td>
<td>88.5±1.8</td>
<td>84.1±6.0</td>
</tr>
</tbody>
</table>
Table 5.2  Histamine release from human mast cells with the lectins Con A, PHA and WGA in the absence (a) and presence (b) of phosphatidylserine (15 μg/ml) (n=3-7). ND = not done.

(a)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Human Skin</th>
<th>Human Lung</th>
<th>Human Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>2.9±2.3</td>
<td>4.4±2.3</td>
<td>2.9±1.0</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>3.0±1.7</td>
<td>2.3±1.0</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>1.0±0.4</td>
<td>1.6±0.9</td>
<td>0.8±0.6</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>0.8±0.5</td>
<td>2.0±0.6</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>WGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>0.8±0.6</td>
<td>0.8±0.4</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>1.7±0.9</td>
<td>2.3±0.4</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Human Skin</th>
<th>Human Lung</th>
<th>Human Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>2.7±0.2</td>
<td>ND</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>1.0±0.3</td>
<td>ND</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>1.0±0.5</td>
<td>1.3±0.9</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>1.2±0.6</td>
<td>1.3±0.3</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>WGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>1.1±0.6</td>
<td>0.8±0.5</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>0.8±0.3</td>
<td>0.5±0.3</td>
<td>0.1±0.09</td>
</tr>
</tbody>
</table>
### Table 5.3 Histamine release from human mast cells with anti-human IgG (n=4-6).

<table>
<thead>
<tr>
<th>Concentration (dilution)</th>
<th>% Histamine release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human Skin</td>
</tr>
<tr>
<td>1/100</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>1/300</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>1/1000</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>1/3000</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>1/10000</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>
CHAPTER 6: THE EFFECT OF ANTI-ALLERGIC COMPOUNDS ON HISTAMINE RELEASE FROM HUMAN SKIN AND UTERINE MAST CELLS

6.1 Introduction

Although mast cells are widely distributed throughout the body, they are found most particularly in areas which come into contact with foreign substances, namely in association with blood vessels in the subepithelial connective tissue of the bronchi, conjunctiva, gut, ear, nose, throat and skin. This makes them ideally situated to participate in a range of allergic disorders and inflammatory processes and, as such, the mast cell has long been a target for drug therapy.

Mast cells elaborate a diverse array of mediators. The release of spasmogenic and vasoactive substances produces immediate clinical symptoms of allergy, including bronchoconstriction, oedema and peripheral vascular collapse. The liberation of chemotactic factors and hydrolases leads to a substantial, more prolonged chronic inflammatory response characterized by cellular infiltration and tissue damage. Mast cells from the rat have provided a useful model in which to study the mechanisms associated with mediator release and have yielded invaluable information for the design of mast cell stabilizing drugs.

However, the existence of interspecies heterogeneity, which is clearly observed with secretagogues [438,452,457], is also apparent in the response of mast cells to anti-allergic compounds. Salbutamol, which is one of the most effective bronchodilators currently available for relief of asthmatic symptoms, is, however, without effect in the supression of rat mast cell exocytosis. In addition, the pronounced tachyphylaxis observed in rat mast cells with disodium cromoglycate is in contrast to the long term effectiveness of this agent in the management of human asthma. Marked variation has also been reported in the effectiveness of cromoglycate in the treatment of other human allergic conditions in which the mast cell plays a central role [36-40].
It is the aim of the present study to evaluate the effect of a number of anti-inflammatory compounds in isolated mast cells from the human skin and uterus in order to investigate further the extent of mast cell heterogeneity in man.

6.2 Methods

A full account of the relevant methods for this section can be found in chapter 2.

6.3 Results

6.3.1 Inhibition of histamine release from human mast cells by some anti-asthmatic compounds and flavonoids

Disodium cromoglycate was a poor inhibitor of histamine release from both human skin and uterine mast cells except at a concentration of 1 mM where inhibition was 14.6±5.1 and 23.3±4.1 %, respectively (Fig. 6.1a). In addition, nedocromil sodium was ineffective against the human cutaneous mast cell, although it proved more active against the uterine cell, inhibiting release by as much as 29.5±6.2 % from this cell type at 100 μM (Fig. 6.1b).

The uterine mast cell was unusual in failing to display tachyphylaxis with 1 mM DSCG when this agent was preincubated with the cells for 10 min (Fig. 6.2a). The effect of this agent was, in contrast, dramatically reduced following its preincubation with the skin cells and both cell types showed some tachyphylaxis with nedocromil sodium (Fig. 6.2b).

The flavonoids, quercetin and chrysin, both inhibited histamine release from human uterine and cutaneous mast cells in a dose-related fashion (Fig. 6.3). Quercetin was most potent in this regard, inhibiting release by a maximum of 91.3±2.8 and 93.4±3.8 % at 100 μM in the uterine and skin cells, respectively, compared with an inhibition of only 60.1±6.3 and 61.2±6.6 % induced from these cells by 100 μM chrysin.
6.3.2 *Inhibition of histamine release from human mast cells by agents which affect cyclic AMP levels*

Theophylline, isobutylmethylxanthine (IBMX, Fig. 6.4) and dibutyryl cyclic AMP (Fig. 6.5) were all highly effective inhibitors of histamine release from both the human skin and uterine mast cells. IBMX was a more potent inhibitor on a molar basis than theophylline. The response of both mast cell types to the latter compound was indistinguishable whereas the skin mast cells were considerably less sensitive than their counterparts in the uterus to the effects of dibutyryl cyclic AMP over the entire concentration range and to a lesser extent also IBMX.

6.3.3 *Inhibition of histamine release from human mast cells by H₂ and β₂ receptor-directed agents*

Mast cells from both human uterus and skin were sensitive to inhibition of histamine release by the H₂ receptor antagonist, cimetidine (Fig. 6.6). This agent induced a potent and identical dose-dependent inhibition in both cell types with a maximum inhibition of 94.9±3.8 and 91.1±6.2 % in the uterine and skin cells at 10 μM, respectively.

Salbutamol and isoprenaline, both β₂ agonists, were also moderate inhibitors of histamine release from both human mast cell types (Fig. 6.7). However, the uterine cells were notably more sensitive than the skin cells to both agents over the entire concentration range. These cells exhibited a maximum release of 52.2±7.0 and 54.4±9.4 % at 10 μM of salbutamol and isoprenaline, respectively, compared to only 43.0±8.2 and 43.2±4.8 % for these compounds in the cutaneous cells.

6.4 **Discussion**

Disodium cromoglycate has been used extensively by physicians in the treatment of a number of allergic disorders including allergic rhinitis, food allergy, atopic eye disease and ulcerative colitis, but it has proven most effective in the management of asthma. The
activity of DSCG in man was discovered when Altounyan, himself an asthmatic, found that inhalation of this agent afforded significant protection against bronchoconstriction induced by antigen inhalation [595]. The efficacy of cromoglycate has been attributed mainly to its capacity to inhibit mast cell degranulation, although its exact mechanism of action in this regard is a topic of considerable uncertainty, as discussed in chapter 3. Reports have recently emerged to suggest that DSCG and its more potent congener, nedocromil sodium, also display significant inhibitory activity against other inflammatory cells such as the neutrophil, eosinophil and monocyte [596-598], which have become established as important effector cells in the complex aetiology of asthma [599-601]. This observation, coupled with the apparent weak activity of this agent against isolated lung parenchymal mast cells [602,603], would seem to suggest that the efficacy of DSCG in asthma may be due to these more generalised inhibitory actions.

In the present study, neither DSCG nor nedocromil sodium were effective inhibitors of histamine release from isolated human skin mast cells. This was in accordance with a previous study which demonstrated that DSCG was an ineffective inhibitor of histamine secretion from human skin slices [525,604]. Given that the activity of these anti-asthmatic compounds varies markedly and inversely with the magnitude of induced release, it is conceivable that these agents might be significantly more active at inhibiting more physiological levels of histamine release in vivo. However, this would appear not to be the case, at least in skin, since local application of DSCG failed to reduce antigen-induced wheal and flare [433] in man. The anti-asthmatic compounds therefore appear to be of little clinical utility in the management of allergic skin disorders.

It would appear that DSCG induces a variety of responses in human mast cells. In the present study, this compound was a weak inhibitor in the uterine cell, whereas nedocromil sodium proved more effective in this regard. DSCG has been reported to be more active in cells from BAL than dispersed human lung, and showed no tachyphylaxis against the former cell type where this was marked against the latter [434,605,606]. Similarly, nedocromil sodium showed no tachyphylaxis against BAL and proved more potent than DSCG against both cell types. The characteristics displayed by DSCG against BAL in
vitro, in particular the absence of tachyphylaxis with this cell type, resembled the clinical profile of this drug more closely. It would therefore seem more likely that the cells recovered by BAL, which lie superficially within the airway mucosa, are more important in modulation of the initial phases of the allergic asthmatic response than their counterparts in the deeper parenchymal layers. The functional response of the uterine cell to DSCG was similar to BAL, and also to cells of the colonic mucosa [551], in failing to exhibit tachyphylaxis. However, this cell did show tachyphylaxis to nedocromil unlike the other two cell types.

The skin cell, on the other hand, showed similarities to the human basophil leukocyte against which both DSCG and nedocromil were ineffective [434,452]. Thus, the differences in the profile of responsiveness of these agents is both subtle and difficult to interpret. In addition, the varied activity of these anti-asthmatic compounds in a number of human mast cells serves to emphasize that no single compound can be expected to act as a panacea for all allergic disorders. Distinct problems also exist in defining suitable test systems for the screening of new, potential anti-allergic agents because of interspecies heterogeneity in their activity; DSCG is clearly effective in the rat PCA model [497,498] but is completely without effect in the corresponding system in man [607].

Despite the existence of considerable structural similarities between the flavonoids quercetin and chrysin and the chromone DSCG, all of which possess a benzopyrone group, the results of the present study indicate that their modes of action must clearly differ. Histamine release from both the human skin and uterine mast cells was strongly inhibited by quercetin and, to a somewhat lesser, but nonetheless significant extent, chrysin, which is in stark contrast to the effect of cromoglycate in these cells. In addition, quercetin has been reported to inhibit histamine release from the human basophil [608,609] which is refractory to the effects of DSCG [452], and both flavonoids were more active against isolated human intestinal mast cells and those from the lung parenchyma, than DSCG [133,551,552]. The exact mechanism of action of these compounds is, however, uncertain. They are not thought to act by raising intracellular cAMP but display manifold inhibitory actions against a number of key enzymes involved
in the secretory process [514,610].

Elevation of intracellular cAMP is thought to provide an inhibitory signal to exocytosis. Although the β2 agonists, salbutamol and isoprenaline, which raise cAMP by activation of membrane bound adenylate cyclase, were ineffective inhibitors in rat mast cells (chapter 3), this was attributed to the receptors being in an uncoupled or modified form. Both β2 agonists were moderate inhibitors of release from isolated human skin and uterine mast cells. These agents had previously been shown to inhibit histamine release from fragments of human lung [603], isolated human parenchymal cells [611,612], BAL [605] and isolated human intestinal mucosal mast cells [551]. Weak activity was also reported for salbutamol in skin slices [525] and the difference in activity between these and isolated human skin cells can probably be accounted for by the existence of a diffusional barrier to penetration of the drug in the skin fragments.

In each case, however, the maximal inhibition with these agents was rarely in excess of 60 %, whereas β agonists have been shown to abrogate completely the elaboration of the eicosanoids, PGD2 and LTC4 by human lung mast cells over a similar concentration range [612]. A suitable explanation for this might be that a fraction of immunologically released histamine is uncoupled from modulation by β receptors or that these agents might induce some histamine release via β receptors which are linked to pools of cAMP associated with triggering rather than inhibition of degranulation and that this limits their ability to inhibit completely release. Apparent differences in the maximal inhibition achieved by these compounds between cells may then reflect variation in β receptor number or the proportion of receptors that are linked to secretory cAMP pools.

On the other hand, elevation of intracellular cAMP by agents which bypass receptor-coupling such as the methylxanthines, theophylline and IBMX, which act by preventing its catabolism, or directly, using analogues such as Bu2 cAMP, resulted in a more marked inhibition of histamine release in both isolated skin and uterine mast cells. This was in accordance with a previous study which found theophylline and Bu2 cAMP to supress effectively antigen-induced histamine release from human skin fragments in vivo [613].
The slightly weaker effect of both IBMX and Bu₂cAMP against the human skin cells compared with the uterine cells may reflect variation in the utilization of internal cAMP to modulate the secretory process in these cells.

In contrast to the histamine-liberating properties of its counterpart, ranitidine, in human mast cells (see chapter 5), the H₂ antagonist, cimetidine, was a highly potent inhibitor of human mast cell histamine release in the present study. Unlike ranitidine, it lacks a tertiary nitrogen atom (Appendix 1) and would hence fail to interact with the mast cell membrane in such a way as to trigger secretion. Its action is most likely by stabilization of the membrane via intercalation into the lipid bilayer [480,481], thereby preventing granule fusion and exocytosis.

In summary, the present study indicates that, despite the marked heterogeneity observed in response to a variety of secretagogues (chapter 5), human mast cells displayed similar sensitivity to a number of inhibitory and anti-inflammatory compounds. Where heterogeneity was apparent, in the response of human mast cells to the anti-asthmatic compounds, disodium cromoglycate and its more potent congener nedocromil sodium, this was subtle and less easily defined than in the rodent.
Fig. 6.1 Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the anti-asthmatic compounds (a) disodium cromoglycate (DSCG) and (b) nedocromil sodium (n=8-11) (no preincubation). A concentration of anti-human IgE was chosen which gave control release of 13.5±3.9% (uterus) and 16.3±3.1% (skin).

(a) % inhibition

![Graph showing inhibition of histamine release by DSCG](image)

(b) % inhibition

![Graph showing inhibition of histamine release by nedocromil sodium](image)
Fig. 6.2

The effect on inhibition of histamine release from human mast cells by (a) disodium cromoglycate (DSCG, 1 mM) and (b) nedocromil sodium (0.1 mM) of a 10 minute preincubation with the drug (n=5-11).

(a) DSCG (1 mM)

% inhibition

(b) Nedocromil sodium (0.1 mM)

% inhibition
Fig. 6.3  Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the flavonoids (a) quercetin and (b) chrysin (n=4-6) (10 min preincubation). A concentration of anti-human IgE was chosen which gave control release of 11.2±3.2 % (uterus) and 9.5±1.1 % (skin).

(a)

% inhibition

Quercetin (µM)

skin  uterus

(b)

% inhibition

Chrysin (µM)

uterus  skin
Fig. 6.4  Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the methylxanthines (a) theophylline and (b) isobutylmethylxanthine (n=5-6) (10 min preincubation). A concentration of anti-human IgE was chosen which gave control release of 15.4±4.4 % (uterus) and 9.9±1.3 % (skin).

(a)

(b)
Fig. 6.5  Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the cyclic AMP analogue, dibutyryl cyclic AMP (n=4-5) (10 min preincubation). Control release was 9.3±4.0 % (uterus) and 8.6±1.2 % (skin).

Fig. 6.6  Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the H₂ antagonist, cimetidine (n=4-6) (10 min preincubation). Control release was 16.0±5.5 % (uterus) and 9.4±1.3 % (skin).
Fig. 6.7 Inhibition of histamine release from isolated human cutaneous and uterine mast cells by the β₂ agonists (a) salbutamol and (b) isoprenaline (n=5-7) (10 min preincubation). A concentration of anti-human IgE was chosen which gave control release of 15.1±4.8 % (uterus) and 9.9±1.3 % (skin).

(a)

(b)
CHAPTER 7: CHARACTERISATION OF MAST CELLS ISOLATED FROM THE BLADDER MUCOSA AND SUBMUCOSA/MUSCLE OF NORMAL INDIVIDUALS AND ONE PATIENT WITH INTERSTITIAL CYSTITIS (IC)

7.1 Introduction

Interstitial cystitis (IC) is a chronic inflammatory disorder of the urinary bladder of unknown aetiology, which predominantly affects young and middle-aged females. A striking characteristic of the complaint is an increased number of mast cells in the detrusor muscle and elevated levels of urinary histamine [41,42,614,615], suggesting the involvement of this cell in the chronic inflammation associated with the disease. The exact role for this cell type is, however, uncertain and as the incidence of bladder removal in cases of IC is very low, detailed study of enzymically isolated mast cells from this tissue is limited.

We have managed, however, to obtain a whole bladder specimen from a 78 year-old female patient with chronic, advanced IC and the results of the functional and histochemical characterisation of isolated mast cells from the submucosa/muscle and mucosa of this tissue sample are described here. In addition, the characteristics of these cells were compared with mast cells isolated from the same locations in normal bladder specimens, removed in cases of bladder carcinoma. Preliminary findings are discussed in terms of human mast cell heterogeneity.

7.2 Methods

The methods pertaining to this section can be found in full in chapter 2.
7.3 Results

7.3.1 Some basic characteristics of mast cells isolated from normal human bladder mucosa and submucosa/muscle compared with those from a patient with IC

From histological studies (table 7.1) it has been shown that collagenase digestion of samples of both normal and chronically inflamed (IC) human bladder submucosa/muscle (sm) and mucosa (m) resulted in highly viable cellular suspensions, as judged by trypan blue dye exclusion and low basal histamine release (< 3.5 %).

Mast cells comprised 6.9 ± 0.7, 5.3, 9.2 and 5.0 % of the cellular suspensions obtained from submucosa/muscle and mucosa of normal and IC bladder specimens, respectively. The histamine content of all four cell types was comparable, if slightly higher in the submucosa/muscle cells in both normal and IC specimens. The staining of all mast cells with alcian blue was dramatically reduced by formalin fixation, regardless of location, and the cells were universally resistant to safranin counterstaining.

7.3.2 Histamine release from mast cells isolated from normal bladder induced by immunologically directed stimuli and calcium ionophores

Mast cells isolated from both the mucosa and submucosa/muscle of normal bladder specimens responded in a dose-dependent manner to challenge with anti-human IgE (Fig. 7.1) following routine passive sensitization of the cells with high titre atopic serum. The response of the mucosal cell was, however, considerably weaker than that of its counterpart in the submucosa/muscle, releasing only 17.4 % histamine compared with 45.5±7.5 % in the latter cell type with a top dilution of 1/100. In addition, the cells from the submucosal layer responded in a dose-related fashion to anti-human IgG, releasing up to 26.7 % histamine with top concentrations of this agent.

The submucosa/muscle bladder mast cells similarly responded vigorously to the lectin, concanavalin A, with 100 μg/ml of this compound inducing as much as 24.9±7.5 %
histamine release from this cell type (Fig. 7.2). This was in contrast to the comparatively weak response of the mucosal cells to this agent.

Similarly, the calcium ionophores, A23187 and ionomycin induced a pronounced concentration-dependent histamine release from mast cells of the submucosa/muscle (Fig. 7.3) whilst having a more moderate effect on the mucosal cell. Optimal release (ca 70%) occurred at 0.3 μM (A23187) and 1 μM (ionomycin) for the former cells, where maximum release was only 41.7% with 3 μM of A23187 from the mucosal cell.

7.3.3 Histamine release from isolated, normal bladder mast cells induced by polybasic compounds

Both the polyamine, Co. 48/80, and the neuropeptide, substance P, induced a strong, dose-dependent secretion from isolated bladder submucosa/muscle mast cells (Fig. 7.4), maximal release reaching 23% and 21.9±9.5% for top concentrations of these agents, respectively. Again, the response of the bladder mucosal cell was considerably weaker than its submucosal counterpart, but nonetheless significant (maximum ca 12% for each compound).

Such a profile of responsiveness was also observed for the polyamino acids, polyarginine and polylysine (Fig. 7.5). The bladder mucosal mast cells were essentially refractory to the effects of either compound while the submucosa/muscle cells exhibited a dose-dependent response to both agents. The effect of polyarginine on these cells was particularly marked, with release peaking at 10 μg/ml (16.2%), whereas the top concentration of polylysine only induced 6.9% release in the same cells.

7.3.4 Inhibition of histamine release from the normal bladder submucosa/muscle mast cells by anti-asthmatic compounds, the cAMP-directed agent, theophylline and the β2 agonist, isoprenaline

Histamine release induced in mast cells isolated from the bladder submucosa/muscle of
normal individuals was unaffected by the chromone, disodium cromoglycate (table 7.2a), with or without a 10 min preincubation. The anti-asthmatic agent, nedocromil sodium, on the other hand, was a moderately effective inhibitor of histamine release at a top concentration of 100 µM, although the response was reduced by almost half after a 10 min preincubation with the drug (table 7.2b).

In contrast, the methylxanthine, theophylline, induced a pronounced concentration-dependent inhibition of release from this cell type, with a maximum inhibition of 94.2% at 10 mM of the compound (Fig. 7.6a). The β2 agonist, isoprenaline, had a comparatively weaker inhibitory action, however, reducing release by a maximum of only 40% (Fig. 7.6b).

7.3.5 Histamine release induced by anti-human IgE, anti-human IgG, the lectin, Con A, and the calcium ionophores from mast cells isolated from the bladder submucosa/muscle and mucosa of a patient with IC

Mast cells from both the submucosa/muscle and mucosa released histamine in response to anti-human IgE prior to routine passive sensitization of these cells with high titre atopic serum (Fig. 7.7). This was especially marked for the submucosa/muscle cells which liberated 40.7% of their total cellular histamine with a 1/100 dilution of the agent which was increased to 56.1% on passive sensitization. These cells were also highly responsive to anti-human IgG (Fig. 7.7a). Similarly, mast cells from the bladder mucosa responded more vigorously to anti-human IgE following sensitization, liberating 44.6% histamine with a 1/100 dilution, compared with 17.4% at the same dilution prior to sensitization.

A strong response was also observed with the lectin Con A, which, like anti-IgE, was more marked in mast cells from the submucosa/muscle than the mucosa (Fig. 7.8). The ionophore, A23187, however, induced an identical dose-dependent histamine release in both cell types (Fig. 7.9). The response induced in the submucosa/muscle mast cells by ionomycin appeared to drop at high concentrations of the agent.
7.3.6  **Histamine release induced by a variety of polybasic agents from bladder mast cells isolated from a patient with IC**

The classic histamine liberator, Co. 48/80, and the polyamino acids, polyarginine and polylysine, induced a marked and dose-related histamine release in mast cells isolated from the submucosa/muscle (Fig. 7.10a). Co. 48/80 proved most potent in this regard, with polylysine inducing the weakest response. The response of the mucosal cells to polyarginine was comparable to that from the submucosa/muscle cells, although both Co. 48/80 and polylysine were less potent but nonetheless effective releasers in the former cell type (Fig. 7.10b).

The neuropeptide, substance P, and the wasp venom peptide, mastoparan, induced a pronounced concentration-dependent secretion from cells of the bladder submucosa/muscle, with maximal release of 23.6 % and 37.9 % at top concentrations of these agents, respectively (Fig. 7.11a). Histone was a marginally effective releaser in these cells only at the highest concentration. The response of the bladder mucosal cells to substance P was less vigorous than their counterparts in the submucosa/muscle but a maximal histamine release of 12 % was obtained at 50 µM of the compound (Fig. 7.11b).

7.3.7  **Inhibition of histamine release from submucosa/muscle and mucosal mast cells isolated from a patient with IC using a variety of inhibitory agents**

Neither of the two anti-asthmatic compounds, disodium cromoglycate nor nedocromil sodium, showed any inhibitory effect against isolated mast cells from the submucosa/muscle or mucosa of this chronically inflammed bladder specimen (table 7.3). In contrast, the flavonoid, quercetin, was a highly potent inhibitor from the submucosa/muscle mast cells (Fig. 7.12).

The inhibitory activity of the β₂ agonists, isoprenaline and salbutamol, was similar against mast cells isolated from both locations in the bladder (Fig. 7.12). In both the submucosa/muscle and mucosal cells isoprenaline appeared to be marginally more potent
than salbutamol, but neither agent induced maximal inhibition exceeding between 40-60%
. The methylxanthine, theophylline was also equiactive against both cell types, inducing
near total inhibition of secretion at top concentrations.

The H2 antagonists, cimetidine and ranitidine, displayed very different profiles of activity
against the submucosa/muscle cells (Fig. 7.13). Cimetidine was a highly effective
inhibitor (max=99.8 %) whereas histamine secretion from these cells was only weakly
suppressed with ranitidine (max=22.1 %). Dimaprit, which is an H2 agonist, also proved
to be an effective inhibitor of release from the submucosa/muscle cells.

7.4 Discussion

In an attempt to extend our present understanding of mast cell heterogeneity in man, two
novel mast cell types, isolated from human bladder submucosa/muscle and mucosa from
both normal individuals and a patient suffering IC, have been both histochemically and
functionally characterised.

In accordance with the theory that mast cells may be implicated in the aetiology of IC
there appeared to be a slight elevation in the number of mast cells present in the
submucosa/muscle of the IC bladder compared to that of normal controls, although with
such a small sample number the significance of this could not be ascertained. Mast cell
numbers in the IC mucosa were largely unchanged compared to the normal mucosa.
Previous studies, using biopsy tissue, have described increased mast cell numbers in both
the submucosa [616] and detrusor muscle [41,615,616] of patients with IC compared with
normal controls. In addition, a number of mast cells in the submucosa of IC bladder
appeared to have degranulated [616] and electron microscopic studies have indicated that
at least some of the mast cells within the detrusor muscle are also degranulating [41]
which suggests that they are actively participating in the disorder. It has been speculated
that the stimulus for mast cell degranulation in IC is of T cell origin since these cells have
been shown to infiltrate the bladder submucosa in this disease [617]. An equally
attractive proposal is that T cells may influence mast cell proliferation in this tissue by
elaboration of mast cell growth factors such as IL3.

In the present study, mast cells from the normal and IC human bladder submucosa/muscle and mucosa were predominantly formaldehyde sensitive and did not counterstain with safranin O. This is in conflict with a number of other studies in the literature. Aldenborg et al. [615] have reported that normal bladder mast cells and those from the submucosa/muscle in IC patients were predominantly formalin resistant and only those in the IC mucosa showed significant formalin sensitivity [615]. In addition, Christmas et al. [616] found that there were no discernible differences between long and short toluidine blue staining times in IC or normal mast cell preparations fixed with formalin.

The bladder mast cells were also tested for their functional reactivity to a variety of secretagogues and for their susceptibility to inhibition by a range of compounds.

Mast cells isolated by collagenase digestion from the bladder submucosa/muscle and mucosa were functionally intact as evidenced by their strong reactivity to immunological stimuli and calcium ionophores. Normally, isolated human mast cells require routine passive sensitization with high titre atopic serum in order to display a significant response to anti-human IgE. Mast cells isolated from the IC bladder, however, showed a high level of histamine release with anti-human IgE prior to passive sensitization, which was similar in magnitude to the response of mast cells from normal bladders after incubation with high titre IgE. This indicates a high degree of endogenous sensitization of the mast cells involved in IC and is suggestive of the existence of high local levels of IgE from which it might be inferred that IC has an allergic basis. Indeed, a possible immunological mechanism has been proposed for the initiation and promotion of bladder injury in Painful Bladder Syndrome, brought about by disruption of the integrity of the glycosaminoglycan (GAG) layer of the bladder epithelium [618]. The GAG layer acts in a protective manner, preventing access of bacteria to the bladder mucosa [619]. Damage to this layer may result in urine, and any initiating agent therein, gaining access to the underlying tissue which may set up an allergic response [620]. Such a mechanism may also result in initiation of the inflammatory processes observed in IC.
The very much higher response of mast cells from both the submucosa/muscle and mucosa of IC samples compared to control bladders after sensitization suggests that bladder mast cells in IC patients may have a greater number of FcεRI than their normal counterparts. This observation is also suggestive of high local IgE levels since, as discussed previously in chapter 5, there is a good correlation between FcεRI receptor number and serum IgE levels [543]. In addition, the response of both normal and IC submucosa/muscle mast cells to anti-human IgG is indicative of a generalised, local elevation in the production of anaphylactic antibodies. IgG may be present bound to the surface of the mast cell via IgG or IgE receptors or there may also be some cross-reactivity of anti-IgG with IgE on the mast cell membrane, as displayed in RPMC [621].

Cross-linkage of FcεRI receptors with the lectin concanavalin A resulted in a strong histamine liberation from submucosa/muscle mast cells in both normal and IC bladders and a lesser, but nonetheless significant release from IC mucosal cells. Those from the normal bladder mucosa were, however, largely unresponsive to this agent. The release profile with the lectin roughly mirrored that of anti-human IgE and probably reflects the relative FcεRI receptor number in these cells. The calcium ionophores induced a strong response from all mast cell types except the normal mucosal mast cell which is typical of the generalised lower responsivity of this cell type to a variety of agents, as seen later.

One of the most striking features of the bladder mast cells observed in the present study was their profound sensitivity to a variety of polybasic secretagogues. As yet, apart from rather limited and conflicting reports concerning the reactivity of the human synovial mast cell [622-624], only mast cells isolated from the human skin have been observed to be responsive to Co. 48/80 and the neuropeptide, substance P [344,430,433] whereas isolated mast cells from the human lung, intestinal and colonic mucosa and submucosa/muscle [133,551,552], uterus and the human basophil leukocyte (chapter 5) were quiescent on challenge with these agents. It now appears that bladder mast cells from the submucosa/muscle and mucosa of both normal and IC samples are responsive to Co. 48/80 and substance P.
Interestingly, several neuropeptides have been demonstrated in the lower urinary tract of man [625,626] and other mammals, including VIP, substance P, SOM, enkephalin and neuropeptide Y (NPY). In the human bladder NPY- and VIP-containing nerves have been found throughout the detrusor muscle, around blood vessels, and at the base of the mucosa which also contains a few CGRP, substance P and SOM immunoreactive nerves. Indeed, substance P is involved in the control of the tone and motility of the bladder. It may then be that mast cell degranulation, under the control of peptidergic neurones in the bladder, may be involved in the normal physiological functioning of this organ, such as control of local blood flow, or more likely, in manifestation of pathophysiology.

The polyamino acids, polylysine and polyarginine were highly effective releasers from normal and IC submucosa/muscle mast cells. In addition, IC mucosal mast cells also displayed a pronounced release with polyarginine. This confirms the potency of polyarginine, which was highly effective even in the human basophil (chapter 5), as a histamine releaser. Finally, the polybasic compound, mastoparan induced a pronounced release from IC submucosa/muscle mast cells. This level of release is unusual in human mast cells; skin cells only released a maximum of 9% with this compound. In contrast, the extent of release in the bladder mast cells with histone was somewhat lower than that observed with this compound in the human skin mast cell and basophil leukocyte (chapter 5). These differences may reflect further subtle variation in the bladder mast cell membrane morphology compared with that of other mast cells.

The reactivity of the submucosa/muscle mast cells, which was greater than their mucosal counterparts, is perhaps not surprising given that these cells are located in a similar connective tissue environment to the skin mast cell, although as evidenced by the uterine cell (chapter 5), this is not necessarily associated with responsivity to polybasic secretagogues. In addition, their reactivity was not expected from their predominantly formalin sensitive staining characteristics. However, the behaviour of the bladder mucosal mast cell was very much at odds with its location at a mucosal surface. It has been suggested, since mast cells are also found in the bladder epithelium and in bladder washings in IC patients, that these cells have a migratory capacity [615]. It may then be
that mast cells in the detrusor muscle of the bladder, which due to their connective tissue environment have a "CTMC" phenotype, are able to migrate into the mucosal layer and these, together with the "mucosal-like" mast cells already resident there, give mast cell suspensions isolated from the mucosa partial CTMC characteristics. Such a migratory theory has also been suggested as an explanation for seasonal rhinitis in the nasal mucosa \[627,628\]; that on first exposure to allergen, prior to development of symptoms, mast cells migrate from their normal habitat in the stroma to the epithelial lining of the nasal mucosa. Given the responsivity of bladder mucosal mast cells from control patients to Co. 48/80 and substance P, however, it would appear that this is a normal phenomenon, not associated in particular with IC.

It would appear from the results obtained with the bladder mast cells that neither the location nor formalin sensitivity of human mast cells can be employed as accurate predictive indicators of their functional reactivity. Hence, use of the terms CTMC and MMC and the inferences which are drawn from mast cell staining characteristics, commonly used to define mast cell subtypes in the rodent, should be treated with great caution in a human mast cell context, until such times as a more suitable nomenclature evolves. Currently, typing of human mast cells on the basis of their neutral protease content appears to match the functional characteristics of these cells most closely \[115\]. This method has not, however, been employed in typing human uterine, synovial or bladder mast cells and this is eagerly awaited.

The profound inability of the anti-asthmatic chromone, disodium cromoglycate, to inhibit histamine release from the submucosa/muscle from both normal and IC bladder specimens and IC mucosal mast cells was in direct contrast to a report claiming that DSCG had a protective effect in a rat model of chemical cystitis \[629\]. This again emphasizes the inappropriateness of rodent models for human systems. The apparent refractiveness shown by the bladder mast cells to DSCG is similar to the response of the human skin mast cell to this agent (chapter 5), which is further evidence to suggest that the two cell types are alike in their functional characteristics. In contrast to mast cells isolated from both locations in the IC bladder, those from the submucosa/muscle of normal bladders
were moderately susceptible to inhibition by nedocromil sodium and showed tachyphylaxis with this agent following preincubation. In general, however, the weak profile of activity of this compound indicates that it would be largely ineffective in the treatment of IC.

A range of inhibitors was tested on mast cells isolated from the IC bladder sample, whereas those from normal submucosa/muscle were only tested with theophylline and isoprenaline. There was no apparent variation between the different cell types in their responses to these agents. As with other isolated mast cell types discussed in chapter 5, the $\beta_2$ agonists, salbutamol and isoprenaline, inhibited release in a dose-dependent fashion in the bladder mast cells, but only up to a maximum of between 40-60%. This demonstrates the existence of functional $\beta$ receptors on the bladder cells but would also appear to indicate that a certain fraction of released histamine is shielded from $\beta$ adrenergic control.

The methylxanthine, theophylline, and the flavonoid, quercetin, were potent inhibitors of histamine release from both normal and IC bladder mast cells. This would suggest that, as in other human mast cells, cAMP provides an inhibitory signal to exocytosis and emphasizes that the mechanism of action of quercetin is quite distinct from that of DSCG.

Interestingly, where the anti-histamine, cimetidine, proved to be a potent inhibitor in the submucosa/muscle IC bladder mast cells, another $H_2$ antagonist, ranitidine, proved only mildly effective. This latter agent has been tested for its histamine liberating properties in other isolated human mast cells in the present study (chapter 5), not as an inhibitor of mast cell secretion. This agent was a strong releaser of histamine in the human skin mast cell and it is probable that the secretory characteristics of ranitidine have masked its inhibitory actions in the bladder mast cell, given both the functional similarities that exist between the bladder and skin mast cells, and the basic nature of ranitidine. Although the $H_2$ agonist, dimaprit, has been reported to be a strong secretagogue in the RPMC [578], it has been reported to act as an inhibitor in human mast cells [Liu W.L., PhD Thesis, University of London, 1990]. In accordance with this, dimaprit induced a strong dose-dependent inhibition of secretion in the bladder submucosa/muscle mast cells from an IC
patient. Despite their apparent effectiveness \textit{in vitro}, however, anti-histamines have been used in the treatment of IC with limited success [630].

Such inhibitor studies using isolated human bladder mast cells may result in the discovery of an effective treatment for IC. Indeed, amitriptyline, a tricyclic drug, which is an effective inhibitor of histamine release from RPMC [631] has also proved clinically beneficial in IC [632].

In summary, the preliminary findings, reported in the present study, indicate that mast cells isolated from the human bladder submucosa/muscle and mucosa are of great interest and value in the further assessment of mast cell heterogeneity in man. Their pronounced reactivity to a variety of polybasic compounds, which was neither predictable from their location, in the case of the bladder mucosal cell, nor their sensitivity to formalin fixation, once again emphasizes that caution should be exercised in categorising human mast cells on the basis of phenomena employed in the rat system.

No striking differences were apparent between normal and IC bladders histochemically except for a slight elevation in mast cell number in the submucosa of IC bladder. Functionally, the responses of submucosa/muscle cells in both normal and IC bladders were very similar although the IC mucosal cell appeared to be marginally more reactive than its counterpart from normal bladders, supporting an increased mast cell migration from the muscle to the mucosa in IC. The marked endogenous sensitization of mast cells from the IC bladder would suggest that this disorder is associated with high, local levels of IgE and hence may have an allergic basis. The reader is reminded, however, that in the present study only one bladder from an IC patient was used due to the difficulties inherent in obtaining such specimens. More extensive use of this cell type will be required for its full and statistically relevant evaluation and the elucidation of a role for the mast cell in IC.
Fig. 7.1  Histamine release induced by anti-human IgE and anti-human IgG from isolated human mast cells from normal bladder mucosa and submucosa/muscle (sm) (n=2-3).

Fig. 7.2  Histamine release induced by concanavalin A from isolated human mast cells from normal bladder mucosa and submucosa/muscle (n=1-3).
Fig. 7.3  Histamine release induced by the calcium ionophores A23187 and ionomycin from isolated human mast cells from normal bladder mucosa and submucosa/muscle (n=2-3).
Fig. 7.4 Histamine release induced by (a) compound 48/80 and (b) substance P from isolated human mast cells from normal bladder mucosa and submucosa/muscle (n=1-3).

(a) % histamine release

\[
\begin{align*}
\text{48/80 (µg/ml)} & \quad 0.1 & 1 & 10 & 100 \\
\text{bladder mucosa} & \quad \circ & \quad \circ & \quad \circ & \quad \circ \\
\text{bladder submucosa} & \quad \times & \quad \times & \quad \times & \quad \times \\
\end{align*}
\]

(b) % histamine release

\[
\begin{align*}
\text{Substance P (µM)} & \quad 0.1 & 1 & 10 \\
\text{bladder mucosa} & \quad \circ & \quad \circ & \quad \circ \\
\text{bladder submucosa} & \quad \times & \quad \times & \quad \times \\
\end{align*}
\]
Fig. 7.5 Histamine release induced by the polyamino acids (a) polyarginine and (b) polylysine from isolated human mast cells from normal bladder mucosa and submucosa/muscle (n=1-2).

(a)

% histamine release

Polyarginine (μg/ml)

--- bladder mucosa  --- bladder submucosa

(b)

% histamine release

Polylysine (μg/ml)

--- bladder mucosa  --- bladder submucosa
Fig. 7.6  Inhibition of histamine release by (a) theophylline and (b) isoprenaline from isolated human mast cells from normal bladder submucosa/muscle (n=1-2). A concentration of anti-human IgE was chosen which gave control release of 37.7 % and 46.1 %, respectively.
Fig. 7.7  Histamine release induced by anti-human IgE before (no sens) and after (+ sens) passive sensitization, from mast cells isolated from (a) the bladder submucosa/muscle and (b) the bladder mucosa of a patient with IC. The effect of anti-human IgG against the bladder submucosa/muscle cells is also shown (a) (n=1).

(a)  

% histamine release

Anti-IgE/G dilution

- Anti-IgE no sens  - Anti-IgE + sens  - Anti-IgG

(b)  

% histamine release

Anti-IgE dilution

- Anti-IgE no sens  - Anti-IgE + sens.
Fig. 7.8  Histamine release induced by the lectin, concanavalin A from mast cells isolated from the human bladder submucosa/muscle (sm) and mucosa (m) of a patient with IC (n=1).

Fig. 7.9  Histamine release induced by the calcium ionophores, A32187 and ionomycin from mast cells isolated from the human bladder submucosa/muscle (sm) and mucosa (m) of a patient with IC (n=1).
Histamine release induced by the polyamine, Co. 48/80, and the polyamino acids polyarginine and polylysine from mast cells isolated from (a) the bladder submucosa/muscle and (b) the bladder mucosa of a patient with IC (n=1).

(a)  

% histamine release

Concentration (µg/ml)

Co. 48/80  Polyarginine  Polylysine

(b)  

% histamine release

Concentration (µg/ml)

Co. 48/80  Polyarginine  Polylysine
Fig. 7.11  Histamine release induced by the peptides, substance P and mastoparan, and the large cationic polypeptide, histone, from mast cells isolated from (a) the bladder submucosa/muscle and (b) the bladder mucosa of a patient with IC (n=1).

(a)

\[ \text{% histamine release} \]

\[ \begin{array}{c}
\text{Concentration (\mu M)} \\
0.1 & 1 & 10 & 100
\end{array} \]

---

(b)

\[ \text{% histamine release} \]

\[ \begin{array}{c}
\text{Substance P (\mu M)} \\
0.01 & 0.1 & 1 & 10 & 100
\end{array} \]

---

- Substance P
- Histone
- Mastoparan

---

Substance P
Fig. 7.12 Inhibition of histamine release by the $\beta_2$ agonists, isoprenaline and salbutamol, the flavonoid, quercetin, and the methylxanthine, theophylline, from mast cells isolated from (a) the bladder submucosa/muscle and (b) the bladder mucosa of a patient with IC (n=1). A concentration of anti-human IgE was chosen which gave control release of 55.2 % (sm) and 36.8 % (m).

% inhibition vs Concentration (µM)

(a) $\beta_2$ agonists: Isoprenaline, Salbutamol, Quercetin, Theophylline

(b) $\beta_2$ agonists: Isoprenaline, Salbutamol, Theophylline

% Inhibition vs Concentration (µM)
Fig 7.13  Inhibition of histamine release by the H₂ antagonists, cimetidine and ranitidine, and the H₂ agonist, dimaprit, from isolated mast cells from the human bladder submucosa/muscle of a patient with IC (n=1). Control release was 55.2 %.
**Table 7.1** Some basic properties of mast cells isolated from the submucosa/muscle and mucosa of normal (n=2-3) and IC bladders (n=1).

<table>
<thead>
<tr>
<th></th>
<th>Normal Bladder</th>
<th></th>
<th>IC Bladder</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Submucosa</td>
<td>Mucosa</td>
<td>Submucosa</td>
<td>Mucosa</td>
</tr>
<tr>
<td>Mast Cells (% total)</td>
<td>6.9±0.7</td>
<td>5.3</td>
<td>9.2</td>
<td>5.0</td>
</tr>
<tr>
<td>Histamine (pg/mast cell)</td>
<td>3.4±0.26</td>
<td>2.8</td>
<td>3.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Viable Cells (% total)</td>
<td>93.4±1.8</td>
<td>90.7</td>
<td>96.3</td>
<td>93.8</td>
</tr>
<tr>
<td>Spontaneous histamine release (%)</td>
<td>3.1±0.5</td>
<td>3.5±0.5</td>
<td>2.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Formaldehyde sensitive (%)</td>
<td>100.00</td>
<td>92.5</td>
<td>88.6</td>
<td>89.9</td>
</tr>
</tbody>
</table>
Table 7.2 Inhibition of histamine release from isolated mast cells from the normal bladder submucosa/muscle by (a) disodium cromoglycate and (b) nedocromil sodium (n=2).

(a)

<table>
<thead>
<tr>
<th>Concentration of DSCG (μM)</th>
<th>Time of preincubation</th>
<th>0 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control release (% histamine release)</td>
<td></td>
<td>39.1</td>
<td>41.0</td>
</tr>
</tbody>
</table>

% inhibition

<table>
<thead>
<tr>
<th>Concentration of DSCG (μM)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>4.4</td>
</tr>
<tr>
<td>100</td>
<td>-2.1</td>
</tr>
<tr>
<td>10</td>
<td>-3.5</td>
</tr>
<tr>
<td>1</td>
<td>-5.6</td>
</tr>
<tr>
<td>0.1</td>
<td>-4.4</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Concentration of Nedocromil Sodium (μM)</th>
<th>Time of preincubation</th>
<th>0 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control release (% histamine release)</td>
<td></td>
<td>39.1</td>
<td>41.0</td>
</tr>
</tbody>
</table>

% inhibition

<table>
<thead>
<tr>
<th>Concentration of Nedocromil Sodium (μM)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>29.9</td>
</tr>
<tr>
<td>10</td>
<td>-5.6</td>
</tr>
<tr>
<td>1</td>
<td>-4.7</td>
</tr>
<tr>
<td>0.1</td>
<td>-10.3</td>
</tr>
<tr>
<td>0.01</td>
<td>-11.2</td>
</tr>
</tbody>
</table>
Table 7.3

Inhibition of histamine release from isolated mast cells from (a) bladder submucosa/muscle and (b) bladder mucosa from a patient with IC with disodium cromoglycate and nedocromil sodium (n=1).

(a)

<table>
<thead>
<tr>
<th>Time of preincubation</th>
<th>Concentration</th>
<th>0 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control release (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSCG 1 mM</td>
<td>2.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>-5.5</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Nedocromil 0.1 mM</td>
<td>-0.5</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>0.01 mM</td>
<td>2.0</td>
<td>5.7</td>
<td></td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Time of preincubation</th>
<th>Concentration</th>
<th>0 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control release (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSCG 1 mM</td>
<td>-1.1</td>
<td>-1.0</td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>3.8</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Nedocromil 0.1 mM</td>
<td>0.5</td>
<td>-0.4</td>
<td></td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.5</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 8: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In the present study enzymic dispersion techniques [340,343,437,438] have been extended to a wide variety of tissues from the rat, guinea pig and man. In each case these methods yielded mast cells which were both highly viable and functionally intact as assessed by immunological stimuli and calcium ionophores.

The existence of both histochemical and functional mast cell heterogeneity was established by Enerbäck from his work using the gastrointestinal tract of the rat [337-339]. The present study has sought to extend such histochemical and functional investigations using mast cells from four connective tissue locations in the rat; the peritoneum, mesentery, lung and skin. From the results of this study, it appears that the strict dichotomy of two distinct mast cell subtypes in the rat, CTMC and MMC, as proposed by Enerbäck, is somewhat of an oversimplification. Histochemically, mast cells from the four connective tissue sites displayed a range of phenotypes following staining with alcian blue/safranin O and formalin fixation; those from the peritoneum were all safranin positive and resistant to the effects of formaldehyde whereas mast cells isolated from the lung were predominantly safranin negative and mainly formaldehyde sensitive. Mesenteric and skin mast cells, which were largely resistant to formaldehyde, exhibited intermediate staining characteristics. These findings suggest the existence of both chondroitin sulphate and heparin proteoglycans in varying proportions in connective tissue mast cells of the rat.

The use of a variety of structurally diverse compounds which share a predominantly basic nature in functional studies has confirmed the existence of a range of phenotypes within these rat connective tissue mast cell populations. The RPMC was consistently the most reactive cell type whereas the lung and skin mast cells were comparatively refractory to most polycationic agents. The mesenteric mast cell exhibited an intermediate reactivity. In addition, only the responses of the peritoneal and mesenteric cells to plant lectins were potentiated by addition of phosphatidylserine. These results therefore highlight the now inappropriate usage of the terms CTMC and MMC which represent two phenotypic extremes, between which a gradation of phenotypes clearly exists.
Given the range of basic compounds which act as histamine liberators from RPMC, it is likely that release occurs via a non-specific, receptor-independent mechanism. Consideration of the structure of such histamine releasers has given rise to a hypothesis concerning their mechanism of action which has been described in some detail by Repke et al. [475]. In brief, this hypothesis proposes a mechanism whereby hydrophobic moieties present in the compound intercalate with and anchor in the mast cell membrane. This disturbs the membrane conformation in such a way as to allow the ionic interaction of cationic residues within the molecule with pockets of negative charge within the lipid bilayer. These sites of interaction have recently been proposed to be G proteins from studies using RPMC [487,570,571]. In addition, it has also been suggested that the initial binding of these polybasic agents to the membrane might be through interaction with membrane sialic acid residues [568,569], which is supported in the present study by the inhibitory effect of neuraminidase incubation on the response of RPMC to Co. 48/80, substance P and mastoparan. It has been proposed that the differences observed here between the responsivity of connective tissue mast cells in the rat to polycationic agents may be attributable to variations in membrane morphology and/or charge between mast cells which determine the extent of the interaction of such compounds with the mast cell membrane.

Histochemical techniques failed to distinguish between guinea pig skin and uterine mast cells which were both safranin negative and formaldehyde sensitive. However, despite such similarities, the guinea pig skin mast cell released histamine on challenge with a range of polybasic agents to which the uterine cell was refractory. It is evident from this that the assumption, based on the rat system, that mast cells which do not counterstain with safranin O and display marked sensitivity to formalin fixation must also be refractory to polycationic secretagogues, cannot be employed with ease in reference to guinea pig mast cells.

In accordance with their striking differences in response to a variety of secretagogues, rat skin and peritoneal mast cells also varied in their susceptibility to inhibition by the anti-allergic chromone disodium cromoglycate, the former cell being entirely refractory to its
effects. However, unlike the rat intestinal cell [434], both the rat skin and peritoneal cells were similarly inhibited by nedocromil sodium. This was also true for a variety of anti-allergic compounds such as $H_2$ and $\beta_2$ directed agents, compounds which elevate intracellular cAMP and the flavonoids, quercetin and chrysin. Thus, heterogeneity amongst rat mast cells from connective tissue locations was not readily apparent for the majority of inhibitors tested.

On the basis of both histochemical and functional investigations conducted in the present study, two distinct mast cell subtypes appeared to exist in human connective tissues. The human skin mast cell was almost totally resistant to formalin fixation whereas mast cells isolated from other human connective tissues such as the uterus and lung were predominantly formalin sensitive. Again, a range of structurally diverse polycationic agents were employed in functional studies with isolated human mast cells. Compounds as diverse as the antibiotic, chlortetracycline, Co. 48/80, peptides such as substance P, mastoparan and ACTH$_{1-24}$, the analgesics, morphine and codeine, the alkaloid d-tubocurarine, the large, cationic polypeptides, protamine and histone, and the $H_2$ antagonist ranitidine selectively induced non-immunological histamine secretion from the human skin mast cell. The fact that the skin mast cells, which were obtained mainly from infant foreskin samples, appeared to be less functionally mature than mast cells obtained from other locations in the adult, as indicated from their comparatively weak response to immunological stimuli and calcium ionophores, renders their unique releasability on challenge with polybasic compounds even more striking. In addition, the skin mast cells appeared to be more susceptible to cell lysis with the detergents triton X-100 and tween 20 compared to their uterine counterparts and this undoubtedly reflects differences in membrane morphology between the two cell types. Interestingly, the connective tissue origin of uterine mast cells did not endow them with characteristics of releasability with polybasic agents, as might have been expected, and further, in depth studies with this cell type are required for an accurate definition of its role in uterine physiology and dysfunction [31,37].

The sensitivity of the human skin mast cell response to polybasic agents following
treatment with neuraminidase and pertussis toxin would be of great interest in determining whether the mode of action of polycationic agents in human mast cells involves interaction with either sialic acid residues or G proteins, respectively, both of which have been postulated for the rat peritoneal mast cell [487,568-571]. In addition, measurement of the relative electric charge on human mast cells from different locations by whole cell electrophoresis or of the relative fluidity of these membranes by calculation of cholesterol/phospholipid ratios [594] might be of value in uncovering the fundamental differences between human mast cells which provide a basis for mast cell heterogeneity.

There were no marked differences in the susceptibility of human skin and uterine mast cells to inhibition with a variety of anti-allergic compounds, namely agents which elevate intracellular cAMP, \( \beta_2 \) agonists and flavonoids. The relative lipophilicity and basicity of the \( H_2 \) anti-histamine, ranitidine, which acted as a secretagogue against the skin mast cell, render it a considerably less useful drug than its counterpart, cimetidine, a strong inhibitor of histamine release from both cell types, in treatment of the symptoms of human allergy. In addition, neither cell type was affected by DSCG and nedocromil sodium proved only moderately effective against the uterine cell. The varied activity of these effective anti-asthmatic drugs in a number of human mast cell types [434,452,551,605,606] is evidence that no single compound can act as a panacea for all allergic disorders. In addition, there is the question of finding suitable animal test systems for screening new anti-allergic agents since clearly results using rat models are rarely predictive of the efficacy of a given compound in man [497,498,607].

The recent findings, described in the present study, concerning the histochemical and functional characteristics of two novel mast cell types, isolated from the human bladder submucosa/muscle and mucosa, have indicated that our knowledge of human mast cell heterogeneity is far from complete. Mast cells isolated from these locations in both normal specimens and one from a patient suffering IC were highly responsive to a range of polybasic agents which was neither predictable from their formalin sensitivity nor, in the case of the mucosa, their location. The similarity of these mast cells to the human skin mast cell was further evident from their complete refractiveness to DSCG and
nedocromil sodium, although they behaved in an identical fashion to other human mast cells in their response to a variety of inhibitory agents.

Although it was only possible to obtain one IC bladder sample, it was nonetheless apparent that there was an elevation in mast cell number in the submucosa/muscle of this sample compared to normal controls, which is consistent with the characterisation of the disease [41,42,614,615]. Functional differences between normal and IC bladder mast cells were not marked and indeed were only evident in the relative responses of these cells to anti-human IgE. The IC bladder submucosa/muscle and mucosal mast cells showed a pronounced histamine release with anti-human IgE prior to passive sensitization with high titre atopic serum and therefore appeared to possess a high level of endogenous sensitization with IgE. This is suggestive of elevated, local levels of IgE which implies IC may have an allergic basis. The reactivity of the IC mucosal mast cell may, in part, be attributed to the migration of mast cells from the underlying muscle and/or submucosa into the mucosal layer [615]. Such a migratory theory has also been suggested as an explanation for seasonal rhinitis in the nasal mucosa [627,628].

Taken as a whole, the results of the present study demonstrate interspecies heterogeneity and confirm the inappropriate use of the rat system as a model for predicting the behaviour of, for instance, corresponding human mast cells. For example, where the human skin mast cell was exquisitely responsive to the undecapeptide substance P, the corresponding cell in the rat was refractory to its effects. This was observed consistently with the range of secretory compounds employed in this investigation. Moreover, use of the terms CTMC and MMC, which can now be regarded as an oversimplification in the rat, where connective tissue mast cells of graded functional reactivity clearly exist, is highly unsuitable for the human system. The skin and submucosa/muscle bladder mast cells originate from connective tissue but the reactivity displayed by these cells to polybasic agents is not shared by the uterine cell which also has a similar connective tissue origin. Moreover, the bladder mucosal mast cell is similarly responsive to these compounds despite its location at a mucosal surface.
In addition, while the degree of formalin sensitivity and safranin positivity of rat mast cells may serve to predict adequately their functional characteristics, use of these criteria for such a purpose in a human mast cell context is deeply misguided. Firstly, safranin counterstaining is rarely observed (< 3 %) in even the most functionally reactive human mast cell. Secondly, apart from the human skin mast cell which is predominantly formalin insensitive, the bladder submucosa/muscle and mucosal mast cells, which display a high degree of functional reactivity, are indistinguishable from the more quiescent human uterine, lung, intestinal [415,416] or colonic mucosal cells [551] on the basis of their formalin sensitivity. Categorising human mast cells on the basis of their neutral protease content [115] appears to date to be the most effective method available. However, as yet, the tryptase and chymase content of uterine, bladder submucosa/muscle, bladder mucosa and synovial mast cells has not been investigated and this is eagerly awaited.

These observations also apply in full to the guinea pig where both the skin and uterine mast cells were from connective tissue locations and were completely sensitive to formalin fixation, yet were quite distinct from one another on the basis of their functional characteristics. Indeed, from the studies conducted to date, it would appear that the guinea pig system closely resembles that of man and may function as an appropriate human model. However, further use of guinea pig mast cells from a variety of locations will clearly be needed before such a similarity can be concluded unequivocally.

The present study has extended our current understanding of the concept of mast cell heterogeneity within man, the rat and the guinea pig using both histochemical and detailed functional studies, the latter employing a wide range of polycationic secretagogues and anti-allergic agents. There is extensive variation amongst mast cells within the same species and between mast cells from the same location in different species in their functional characteristics. However, the fundamental underlying mechanisms which are responsible for this are, as yet, undefined. Future studies into mast cell heterogeneity might consider the investigation of relative mast cell membrane morphology as differences here could conceivably constitute the cellular basis of functional heterogeneity.
Refinements of existing techniques for the purification of human mast cells [344,422] and the successful growth of large numbers of human mast cells in culture [376] will undoubtedly facilitate such detailed investigations.

With regard to the generation of multiple mast cell phenotypes in vivo, it certainly seems plausible that this is somehow under the control of the mast cell’s microenvironment [378-383, for a comprehensive review see Ref. 633]. From the findings of the present study it would appear that such regulation must be more subtle and complex than at first thought from the traditional concept of the "connective tissue" and "mucosal" environments, in order that such a range of phenotypes should arise. Hopefully, the fascinating and emergent area of cytokine research might hold the key to unravel this mystery. Mast cells may themselves regulate their own phenotype by elaboration of a myriad of cytokines, such as IL3, GM-CSF and/or IL4 [158,160,161], in response to local tissue regulatory factors. Indeed, an insight into how mast cells interact with their surroundings and other cells present therein, such as fibroblasts [384] and other inflammatory cells, is vital to our continued understanding of mast cell heterogeneity.
BIBLIOGRAPHY

Gastroenterology 97, 575-585.
478.


217

Bibliography

537-546.

<table>
<thead>
<tr>
<th>Number</th>
<th>Reference</th>
</tr>
</thead>
</table>
394. Dvorak A.M., Mihm M.C., Osage J.E., Kwan T.H., Austen K.F. and Wintroub


413. Eliakim R., Gilead L., Ligumsky M., Okon E., Rachmilewitz D. and Razin E.


145, 1214-1221.


Bibliography

533. Cocchiara R., di Trapani G., Azzolina A., Albeggiani G., Ciriminna R., Cefalu E.,
537. Foley M.E., Griffin B.D., Zuzel M., Aparicio S.R., Bradbury K., Bird C.C.,
med. J. 2, 322-324.
66, 16-22.
Invest. 62, 176-181.
Allergy 9, 119-123.
546. Scott M.G., Nahm M.H., Macke K., Nash G.S., Bertovich M.J., MacDermott R.P.
and Actions 30, 70-73.
2604-2610.


71, 489-494.


APPENDIX 1

STRUCTURES

ACTH_{1-24}


Mastoparan

Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_{2}

Substance P

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_{2}

SP_{1-4C_{12}}

Arg-Pro-Lys-Pro-(CH_{2})_{11}-CH_{3}

C3a (21R=57-77)

Protamine Sulphate: Gly (5.9%), Ala (1.7%), Val (4.5%), Ile (1.3%), Ser (8.9%), Pro (8.5%), Arg (69.2%)
average mwt. 7500

Histone: Gly (16.1%), Ala (1.7%), Val (7.6%), Leu (8.5%), Ile (5.5%), Ser (2.5%), Thr (6.8%), Met (1%), Pro (1.5%), His (2.3%), Lys (11%), Arg (13.2%), Asp (5.5%), Glu (6.8%), Phe (1.8%)
average mwt. 16000

Polymixin B (DABA = αγ-diaminobutyric acid)

\[
\begin{align*}
\text{DABA-DABA-DABA-D-Phe} \\
\text{L-Thr-DABA-L-Leu-DABA} \\
\text{6-methyloctanoic acid}
\end{align*}
\]

d-Tubocurarine

Morphine/Codeine

Ranitidine
Appendix 1

Cimetidine

Chlortetracycline

Compound 48/80
### APPENDIX 2

#### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>Wild type mice</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Ab-MuLV</td>
<td>Abelson murine leukemia virus</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AGEPC</td>
<td>Alkylglyceryletherphosphorylcholine</td>
</tr>
<tr>
<td>Anti A</td>
<td>Antimycin A</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BDTA</td>
<td>Benzyldimethyltetradecylammonium chloride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Bu&lt;sub&gt;2&lt;/sub&gt;cAMP</td>
<td>Dibutylryl cAMP</td>
</tr>
<tr>
<td>C3/C3a</td>
<td>Complement fragment 3/3a</td>
</tr>
<tr>
<td>c-kit</td>
<td>Gene encoding the tyrosine kinase receptor</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3’-5’-(cyclic) monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>Cromolyn binding protein</td>
</tr>
<tr>
<td>CEC</td>
<td>Critical electrolyte concentration</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>Co. 48/80</td>
<td>Compound 48/80</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue type mast cell</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDA</td>
<td>2’,5’-dideoxyadenosine</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DSCG</td>
<td>Disodium cromoglycate</td>
</tr>
<tr>
<td>ECF-A</td>
<td>Eosinophil chemotactic factor of anaphylaxis</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration causing 50 % histamine release</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
</tbody>
</table>
EGF  Epithelial growth factor
EHRF  Embryo-derived histamine-releasing factor
ETI   5,8,11-eicosatriynoic acid
ETYA  Eicosa-5,8,11,14-tetraynoic acid
Fab'  Papain digested antigen binding (ab’) immunoglobulin fragment (F)
Fc    Papain digested membrane binding (c) immunoglobulin fragment (F), c = crystallizable
FcεRI High affinity IgE receptor
FcεRII Low affinity IgE receptor
FHT   Full HEPES Tyrode’s
FMLP  F-methionine-leucine-phenylalanine, bacterial peptide
GAG   Glycosaminoglycan
GDW   Glass distilled water
GM-CSF Granulocyte/macrophage-colony stimulating factor
GppNHp Guanylyl-5’-[β,γ-imido]triphosphate
GTPγS Guanosine-5’-[γ-thio]triphosphate
GTP   Guanosine triphosphate
HEPES N-2-hydroxyethyl-piperazine-N’-2-ethane sulphonic acid
IBMX  Isobutylmethylxanthine
IC    Interstitial cystitis
IF-A  Inflammatory factor of anaphylaxis
IFNγ Interferon γ
IgE/G Immunoglobulin E/G
IL3/4 Interleukin 3/4
IP3   Inositol-3-phosphate
KiSV  Kirsten murine sarcoma virus
LDH   Lactate dehydrogenase
LHRH  Leutinising hormone releasing hormone
LPR   Late phase reaction
LT    Leukotriene
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>m/rBMMC</td>
<td>Mouse/rat bone marrow-derived mast cells</td>
</tr>
<tr>
<td>mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCCP</td>
<td>Mast cell committed progenitor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIPα/β</td>
<td>Macrophage inflammatory protein α/β</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal type mast cell</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NANA</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>NANC</td>
<td>Non-adrenergic, non-cholinergic nerves</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OPT</td>
<td>O-Phthaldialdehyde</td>
</tr>
<tr>
<td>p-BPB</td>
<td>p-Bromophenacyl bromide</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCA</td>
<td>Passive cutaneous anaphylaxis</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA₂/C</td>
<td>Phospholipase A₂/C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PtX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RBL 2H3 cell</td>
<td>Rat basophilic leukemia cell line 2H3</td>
</tr>
<tr>
<td>rIL</td>
<td>Recombinant interleukin</td>
</tr>
<tr>
<td>RMCPI/II</td>
<td>Rat mast cell protease I/II</td>
</tr>
<tr>
<td>RPMC</td>
<td>Rat peritoneal mast cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>S-AM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SI/SI&lt;sup&gt;d&lt;/sup&gt;</td>
<td>WCB6F&lt;sub&gt;1&lt;/sub&gt;-Sl/SI&lt;sup&gt;d&lt;/sup&gt; mice, with mutations at the steel (Sl) locus</td>
</tr>
<tr>
<td>SRS-A</td>
<td>Slow reacting substance of anaphylaxis</td>
</tr>
<tr>
<td>TC&lt;sub&gt;MC&lt;/sub&gt;</td>
<td>Human mast cells containing both tryptase and chymase</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt; cell</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;MC&lt;/sub&gt;</td>
<td>Human mast cells containing tryptase alone</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α or cachectin</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>W/W&lt;sup&gt;V&lt;/sup&gt;</td>
<td>WBB6F&lt;sub&gt;1&lt;/sub&gt;-W/W&lt;sup&gt;V&lt;/sup&gt; mice, with mutations at the white spotting (W) locus</td>
</tr>
<tr>
<td>WE/μl</td>
<td>Worm equivalent/μl</td>
</tr>
<tr>
<td>WEHI-3</td>
<td>Myelomonocytic leukemia mastocytoma (transformed cell line, rich in IL3)</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>3-DZA</td>
<td>3-Deazaadenosine</td>
</tr>
<tr>
<td>5HETE/5HPETE</td>
<td>5-Hydroxy-/5-hydroperoxy-eicosatetraenoic acid</td>
</tr>
<tr>
<td>5HT</td>
<td>5-Hydroxytryptamine or Serotonin</td>
</tr>
</tbody>
</table>
PUBLICATIONS


