SOME STUDIES ON THE EFFECTS OF BRADYKININ AND OTHER BIOACTIVE PEPTIDES ON MAST CELLS

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ABSTRACT

The effect of a number of serum-derived bioactive peptides on different histaminocytes was examined. Bradykinin induced a dose-dependent release of histamine and prostaglandin D$_2$ from rat peritoneal mast cells. The release was rapid, non-cytotoxic, independent of extracellular calcium and inhibited by extremes of pH and temperature. A comparable release was produced by peptide analogues with both agonist and antagonist activity at conventional bradykinin receptors and by a range of other polybasic compounds. These data suggest that bradykinin may act through the putative mast cell polyamine receptor. Consistently, the release was inhibited by benzyltrimethyltetradecylammonium chloride, was not accompanied by any change in intracellular cAMP levels and exhibited cross-tachyphylaxis with other polycations. Bradykinin also produced a synergistic enhancement of IgE-mediated histamine release from rat mast cells. The effects of bradykinin were highly species and site specific. Serosal mast cells of the rat and hamster were the most responsive, tissue mast cells of the rat and skin mast cells from man were weakly reactive, but other tissue mast cells and basophil leucocytes from man, together with mesenteric mast cells from the guinea pig, were refractory to the peptide.

The anaphylatoxin C3a, des-arg-C3a and the analogue peptide 2IR also released histamine from rat peritoneal cells but had no effect on human lung or guinea pig mesenteric mast cells or, contrary to report in the literature, on human basophils.

Thrombin produced a limited release of histamine from peritoneal cells of the rat but was without effect on these cells from the mouse, pulmonary and intestinal cells of the rat and human, basophils of man and bone marrow-derived mast cells of the mouse.

In total, the present data show that a number of serum-derived peptides are able to induce histamine release from different mast cells. The effects are, however, very species and site specific and generally resemble those produced by other polyamines. On this basis, the possible role of these agents in allergic inflammation is discussed.
ACKNOWLEDGEMENTS

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To my parents
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Anti-IgE</td>
<td>Antibody to IgE</td>
</tr>
<tr>
<td>BAC</td>
<td>Benzalkonium chloride</td>
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<tr>
<td>BDTA</td>
<td>Benzyltetradecylylammonium chloride</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow derived-mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CBP</td>
<td>Carboxypeptidase</td>
</tr>
<tr>
<td>CMF</td>
<td>Calcium and magnesium free</td>
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<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
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<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DDA</td>
<td>2',5'-Dideoxyadenosine</td>
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<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-Dinitrophenyl</td>
</tr>
<tr>
<td>DSCG</td>
<td>Disodium cromoglycate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECF-A</td>
<td>Eosinophil chemotactic factor of anaphylaxis</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diaminetetraacetic acid</td>
</tr>
<tr>
<td>ETYA</td>
<td>5, 8, 11, 14-eicosatetraynoic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HMWK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidyl inositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>IP₂</td>
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LDH  Lactate dehydrogenase
LMWK Low molecular weight kininogen
LT  Leukotriene
lyso-PS Lyso-phosphatidyl serine
MMC Mucosal mast cell
NCF Neutrophil chemotactic factor
NGF Nerve growth factor
NSAID Non-steroidal anti-inflammatory drug
NT Neurotensin
OAG 1-Oleoyl-2-acetyl-glycerol
OPT o-Phthaldialdehyde
PC Phosphatidyl choline
PG Prostaglandin
PGD$_2$ Prostaglandin D$_2$
PKC Protein kinase C
PLA$_2$ Phospholipase A$_2$
PLC Phospholipase C
PS Phosphatidyl serine
RBL Rat basophilic leukaemia
RMCP Rat mast cell protease
RPMC Rat peritoneal mast cell
RT Room temperature
SP Substance P
SPF Specific pathogen free
SRS-A Slow reacting substance of anaphylaxis
Som Somatostatin
TCA Trichloroacetic acid
TPA 12-O-tetradecanoylphorbol-13-acetate
VIP Vasoactive intestinal peptide
CHAPTER 1

Introduction

1.1 The mast cell

The mast cell has been studied extensively, yet its precise function and physiological role remain obscure. Upon appropriate stimulation, an impressive array of pro-inflammatory mediators is liberated. It is located at sites which form mechanical barriers to infection, such as the connective tissue of the skin, gastrointestinal, respiratory and genito-urinary tracts, sclera of the eye etc. [1-2]. Since these sites are particularly exposed to environmental antigens, this suggests a role for mast cells in host defence and this proposition is supported by the pro-inflammatory nature of the cell mediators.

Mast cells are linked to the immune system through immunoglobulin E (IgE), which forms one of the activation mechanisms of the cell. As with all antibodies, IgE is produced by lymphocytes, integral components of the immune system. Upon contact with antigenic material, B lymphocytes differentiate into antibody-secreting cells and memory cells, specific to the antigen. The production of antibody is controlled by T lymphocyte helper and suppressor cells [3,4]. However, certain individuals lose the ability to discriminate between harmful and harmless agents. Antibodies, specifically IgE, may be generated in response to innocuous substances such as pollen [4] and it is this dysfunction that is proposed as the basis of allergic disease.

Specific receptors with high affinity for IgE exist on the mast cell membrane [5]. Adjacent antibody molecules can be cross-linked by the same antigen that provoked their production, leading to discharge of granules and associated mediators into the extracellular environment [6-10]. In allergic individuals, IgE is produced in response to certain agents and the subject is said to be sensitized to that agent, or allergen. On subsequent exposure, mast cell activation occurs and results in the release of potentially harmful mediators.

Mast cells are also involved in the elimination of helminths [11-12]. Evidence includes reports of elevated serum IgE, mast cell hyperplasia and eosinophilia coincident with such infestations. The identification of normally benign substances as allergens may be a malfunction in the immune system and a possible consequence of the purported anti-helminthic role of the mast cell. This supports the view that the allergic response is a breakdown in the immune recognition system, where T cells are unable to discriminate between harmful or harmless agents. This is reflected by the implication of mast cells in the
aetiology of a variety of allergic and auto-immune disorders such as asthma [13], allergic rhinitis [14-16], conjunctivitis [17], inflammatory bowel disease [18], interstitial cystitis [19-21], rheumatoid arthritis [22,23] and skin disorders like urticaria, dermatitis and mastocytosis [24].

1.2 Mast cell heterogeneity

Mast cells derived from different species and tissues within the same species may vary substantially in their responses to histamine liberators [205,207] and in their histochemical, ultrastructural and cytochemical properties [207,211].

Two distinct subclasses of histaminocytes have been identified in the rat. The serosal cavities and connective tissues produce heparin-containing mast cells, which are non-replicating. Mucosal tissue supports proteoglycan-containing cells, which can proliferate upon appropriate stimulation [212]. Mucosal mast cells (MMCs) increase in number after helminthic infection for example, but this probably does not involve the division of end cells, rather the multiplication and/or the recruitment of undifferentiated precursors. Connective tissue mast cells (CTMCs) are located in the lower layers of the gastrointestinal wall and MMCs are found in the lamina propria [212]. Morphologically, MMCs are smaller than CTMCs, have fewer granules of variable size and contain less histamine [206,210]. MMCs contain RMCP II, while CTMCs contain RMCP I. CTMCs are susceptible to the inhibitory effects of anti-allergic compounds such as disodium cromoglycate (DSCG), nedocromil sodium and theophylline, to which MMCs are resistant [232]. Histochemically, MMCs stain metachromatically with toluidine blue, but lack appreciable affinity for the dyes berberine and safranin [213,214]. In addition, the activation of these distinct subtypes may tend to produce different products of arachidonic acid metabolism. Thus, MMCs generate mainly leukotrienes (LT) and CTMCs predominantly prostaglandins (PG) [215-217]. Within the lung and intestine of man, at least two types of mast cell have been described which bear some histochemical similarities to the CTMC and MMC of the rat. However, the distinction between the two human cell types is less clearly defined [218], particularly as cells resembling MMCs are not confined to the intestinal mucosa, but are also to be found in the submucosa and muscle. In addition, this cell type is widely distributed throughout the parenchyma of human lung outnumbering the CTMC populations [219,220]. It is clear that the histochemical criteria for distinguishing between subpopulations of rat mast cells are probably restricted to this species [229]. Therefore, the terms mucosal and connective tissue mast cells should not be extrapolated to other species without caution and the usefulness of these definitions should be questioned.
It is appreciated that histaminocytes from different tissues and species display wide variety in their pharmacological characteristics [205-207,221]. Mast cells universally respond to anaphylactic and related stimuli and the heterogeneity of mastocyte response is best illustrated in the reaction to peptide and chemical stimuli. In the rat, only CTMCs are responsive to peptides, although there have been reports of a weak response in the MMCs to substance P and somatostatin. Recent studies have shown that even in adult rats, the peritoneal mast cells are heterogenous and can be distinguished by a number of functional properties, including their responsiveness to compound 48/80 [222]. In summary, it would seem that the ability of an agent to elicit or inhibit mediator secretion only bears real significance if the studies are conducted on the specific mast cell system with which the agent is thought, or intended, to interact.

The polybasic compounds possess a typical profile of tissue and species specificity. In general, the compounds are most active on mast cells such as those of the rat peritoneum, pleural cavity, mesentery and skin and the hamster peritoneum. Typically, the basic agonists do not release histamine from cells of the rat lung and intestine; guinea pig mesentery and lung; human lung and intestine and from human blood basophils [82]. Indeed, susceptibility to secretion induced by these compounds may be used as a criterion in the characterization of mast cells.

1.3 Mast cell derived mediators
Mast cell activation culminates in exocytosis and the release of preformed and/or newly generated mediators. The granular constituents may differ according to the species and tissue of origin [10, 25-27] and preformed mediators are ionically attached to a protein matrix. Although widespread differences also exist in the mechanism of anaphylactic degranulation [28], histamine and other mediators are solubilized and released from the matrix through a ubiquitous ion exchange reaction between the mediators and the cations that enter the cell when it is stimulated [29,30].

1.3.1 Preformed mediators
These are stored within the granule, which consists of a protein matrix to which the mediators are ionically attached. The granule matrix consists of proteoglycan. It is this matrix that allows mast cells to be visualized in light microscopy - heparin, for instance, can shift the colour absorption and emission spectrum of certain basic dyes such as toluidine blue, giving it a characteristic purple stain (metachromasia). Mast cells are the sole source of extracellular heparin, liberated on degranulation, which has a wide variety of biological
properties: it is an anticoagulant and an inhibitor of complement activation and eosinophil cytotoxicity [31,32].

1.3.1.1 Histamine

Histamine is the major preformed mediator stored in mast cell and basophil granules 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 in the granules. Extracellularly, histamine is rapidly metabolized by methylation (N-methyl transferase) and, to a lesser extent, oxidation (diamine oxidase or histaminase) and excreted in the urine. The amine possesses both pro- and anti-inflammatory activity in addition to several chemotactic properties and the effect on a particular tissue is defined by the nature of the local receptor subtype present. Pro-inflammatory effects are mediated via $H_1$-receptors and include smooth muscle contraction, increased vascular permeability, bronchospasm, increased mucus production and enhanced neutrophil and eosinophil chemotaxis [25,26]. $H_2$-mediated effects include the stimulation of T suppressor cells, IFN$\gamma$ production, bronchodilatation, stimulation of airway mucus production, mediation of acid secretion [33] and inhibition of IgE-mediated basophil [34] and skin mast cell histamine release [35]. The most recently discovered receptor, $H_3$, has been located in rat cerebral cortical slices and a presynaptic autoreceptor role has been described in both the CNS and the periphery [36]. In addition, $H_3$ 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 [37].

1.3.1.2 Chemotactic factors

Other granule associated mediators include eosinophil, neutrophil and lymphocyte chemotactic factors, of which eosinophil chemotactic factor of anaphylaxis (ECF-A) and neutrophil chemotactic factor (NCF) are the most prominent [25-27]. These factors recruit inflammatory cells towards the sites of inflammation where these chemotaxins are liberated. They enhance the surface expression of various receptors (eg. C3b and IgG), reducing the threshold at which these cells will respond to other stimuli. These factors may be involved in the second wave of inflammation which occurs 3-6 hours after the immediate hypersensitivity reaction and which is characterized by the influx of neutrophils and eosinophils. Such reactions dominate conditions such as asthma, eczema and urticaria.
1.3.1.3 Cytokines

The cytokines, the most recently investigated mediators, 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. Tumour necrosis factor-α (TNF-α) was shown to be produced by certain cultured murine mast cell lines [38,39], mast cell/basophils from human bone marrow cultures [40] and mouse peritoneal mast cells [41].

1.3.1.4 Granular enzymes

A number of enzymes are also available preformed within mast cell granules. They form the structural basis of lysosomal granules and, in mast cell granules, act as storage matrices for other preformed mediators such as histamine. On stimulation, some are secreted in parallel with histamine while others remain complexed to the heparin of the granule matrix and dissociate more slowly [11,42]. These enzyme–heparin complexes may result in the delay or suppression of any anti-coagulant effect of heparin [305]. The three main classes of enzyme are the neutral proteases, the acid hydrolases and the oxidative enzymes.

The acid hydrolases are enzymes which act optimally at acid pH: β-hexosaminidase, β-glucuronidase and β-galactosidase degrade glycoproteins and proteoglycans, and aryl sulphatase hydrolyses aromatic sulphate esters. All of these hydrolases have been found in rat and human mast cells in various isomeric forms and β-hexosaminidase is commonly used as a marker of mast cell degranulation in vitro. Oxidative enzymes include superoxide dismutase and peroxidase. These remove superoxide anions and peroxidase inactivates dihydroxy and sulphidopeptide leukotrienes.

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) or aromatic (chymase), or terminal aromatic (carboxypeptidase) amino acids. Mast cells from different locations contain different enzymes: in the rat, connective tissue mast cells contain a chymotryptic serine esterase, chymase or rat mast cell protease I (RMCP I) [43], whose substrates include fibrinogen and components of the basement membrane, fibronectin and type IV collagen; those from mucosal sites contain a more soluble form (RMCP II) [44]. In rodent granules, chymase is the major neutral protease [45]. Carboxypeptidase A is found in association with RMCP in rodent mast cells, while in human cells, carboxypeptidase B is found with tryptase. These enzymes complement the actions of the other neutral proteases in protein degradation. These neutral proteases include dipeptidase, kininogenase, kallikrein and plasminogen activator.
In man, 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, the lung [46]. A very small amount of the enzyme (< 1%) is found in basophils [47]. The catalytic form of tryptase is tetrameric and it is inactive as a monomer.

Chymase and tryptase have been isolated from RPMCs and chymase was found to induce histamine release, elevate cAMP levels and evoke PGD$_2$ production [84,85]. The enzyme is reported to remain associated with the mast cell surface, together with heparin and some undefined protein, after immunologic or A23187 activation [86]. Tryptase from the granules is liberated along with histamine, in a fixed ratio to histamine, following anti-IgE treatment of RPMCs and human pulmonary cells [84,87]. These observations suggest that tryptase acts in the extracellular environment and chymase on the cell surface, subsequent to stimulation. The enzymes are released as complexes with the appropriate associated inhibitor, heparin or trypstatin, indicating that a modulatory role for these inhibitors may exist [85].

There is also the distinct possibility that the involvement of mast cells in allergic processes may involve either the generation or degradation of these bioactive peptides by serine proteases released upon mast cell degranulation. Rat mast cell chymase has been shown to generate C3a from human C3 [289] and inactivate bradykinin [252], while human tryptase has been shown to be involved in both the generation [240] and degradation [241] of kinins.

In addition, there have been reports of IgE-mediated release of a kallikrein-like enzyme from human basophils, thought to be an arginine esterase [253]. Human mast cell chymase catalyses the conversion of angiotensin I to angiotensin II [50], inactivates bradykinin and lys-bradykinin, degrades basement membrane components and cleaves VIP and substance P. Tryptase from human lung mast cells has also been shown to catalyze the destruction of fibrinogen [286,288,290] and high molecular weight kininogen (HMWK) [288], activate collagenase [288] and to generate C3a from human C3 [48,288]. The enzyme may also play a role in bronchial hyperresponsiveness in asthma through its ability selectively to degrade and inactivate vasoactive intestinal peptide (VIP), the main bronchodilator neurotransmitter of non-adrenergic, non-cholinergic (NANC) nerves in the lung [49]. These enzymes may be released complexed to heparin [86] or other proteoglycans which has the effect of stabilizing the proteins.

Thus, serine proteases may also have a role in allergic inflammation through possible action on components of the inflammatory system such as the kinin, complement and fibrinogen systems. The selective presence of these enzymes in mast cells allows its use as a marker for mast cell degranulation [307]. Tryptase levels in BAL fluid are higher in smokers [308] and atopic asthmatic subjects [309] than in the corresponding controls. It is known that tryptase [305] and chymase [86] are released from the mast cell complexed to heparin, which
has a stabilizing effect on these enzymes and prolongs their immunoreactivity. Thus, the release of these substances through mast cell degranulation may have subtle effects on the long term pathogenesis of allergic disease [288,289].

1.3.2 Newly synthesized mediators

Mast cells are able to generate a variety of metabolites upon suitable activation. These include chemotactic factors, platelet activating factor (PAF), cytokines and the products of arachidonic acid metabolism such as the prostaglandins and leukotrienes [51,53]. These are all mediators of great potency.

A slow-acting, spasmogenic agent, traditionally termed the slow reacting substance of anaphylaxis (SRS-A), is now recognised to be a mixture of LTC₄, LTD₄, and LTE₄. These leukotrienes can induce smooth muscle contraction and increased vascular permeability. In human bronchi, they are some 1000-fold more potent than histamine. The prostaglandins are also powerful spasmogens, in addition to increasing vascular permeability [54,56]. Elevated levels of PGD₂ metabolites have been observed in the plasma and urine of patients during attacks of mastocytosis [223] and some evidence also suggests a role for newly formed mediators in the generation of the late phase reaction (LPR) in man [57]. These two classes of mediators can also produce synergistic pro-inflammatory effects, illustrating an interaction distinct from that with other mediators. This may serve to intensify the overall development of inflammatory events [51].

Platelet activating factor, PAF, or alkylglyceryl ether phosphocholine (AGEPC), was thus named because of its ability to aggregate and degranulate rabbit platelets. When the cell is stimulated, PAF is synthesized from 1-0-alkyl-2-acyl-glyceryl-3-phosphocholine, which is hydrolysed by phospholipase A₂ (PLA₂) to yield lyso-AGEPC, the precursor of PAF. PAF is produced by a wide variety of inflammatory cells, including some murine mast cell lines [58,59] and purified preparations of isolated human lung mast cells [60], following challenge of these cells by anti-IgE and the calcium ionophore A23187. PAF also induces chemotaxis and the activation of eosinophils and neutrophils. In the lungs, it causes pulmonary oedema, vasoconstriction and bronchoconstriction and systemically produces hypotension. When injected into human skin, PAF produces a wheal and flare reaction and is 100-1000 times more potent than histamine on a molar basis.

1.4 Cell activation

Non-cytotoxic mast cell agonists can be broadly classified as immunologic and non-immunologic stimuli [2]. Examples of the former include secretagogues such as
anti-IgE, antigens and concanavalin A (con A). Common non-immunological agents are compound 48/80, basic peptides such as substance P, somatostatin, bradykinin, neurotensin, peptide 401 from bee venom, polymyxin, polyarginine and polylysine, and permeabilizing agents such as ATP and ionophores.

1.4.1 Immunologically-induced histamine release

1.4.1.1 The IgE receptor

Basophils and mast cells exhibit high affinity binding to IgE [5,61-63] but the association with IgG and IgA is thought to be much weaker [64]. The IgE receptors on the surface of mast cells and basophils belong to a family of molecules named Fc-receptors. These receptors are not confined to the mast cell or basophil, but have been characterised on lymphocytes, macrophages, eosinophils and platelets [65-67]. However, the nature and full significance of such binding is unknown.

The interaction between cell-bound IgE and antigen induces granule discharge [61-63] through the aggregation of the IgE receptors. It is this receptor aggregation that actually initiates the biochemical events culminating in the release of mediators [68,69]. IgE binds to the Fc-receptor through its Fc portion [70], but the precise location of the binding site within this fragment remains unclear. Suggestions have been made that sites within the C3 and C5 domains are involved [71]. More recently, a single interaction with the C3 domain has been favoured [69,72]. An indirect mechanism for Fc-receptor aggregation is effected by the subsequent cross-linking of Fab regions of adjacent, cell-bound IgE with multivalent antigen [73,74].

Experiments with fluorescently labelled, monomeric IgE have shown that the Fc-receptor is univalent, laterally mobile and diffusely distributed on the mast cell membrane [75,76]. On the assumption that the maximum number of bound IgE molecules is equivalent to the number of Fc-receptors, studies have shown that each rat mast cell carries 2.5 x 10^5 molecules of receptor, while the range for rat basophilic leukaemia (RBL) cells and human basophils was found to be 0.3-1.0 x 10^6 molecules per cell [29].

The IgE receptor on the RBL cell is a glycoprotein with a molecular weight of about 87 kDa. The protein is divided into subunits (α: 45 kDa, β: 33 kDa, γ: 9 kDa). The α, β and γ subunits are all further subdivided into α1, α2, β1, β2: the two γ chains are identical. The α subunit is directed to the surface of the cell and binds IgE, while the β and γ subunits cannot be labelled from the cell surface and are presumed buried in the cell membrane. The functions of these subunits are, at present, unknown [30,224-228]. The research into the IgE receptor
has centered around receptors of the RBL cell. However, similarities between IgE receptors in the rat and other species is likely since human and rodent IgE may interact with the same receptor [77,78].

1.4.1.2 Membrane receptor activation

IgE bound to the surface of the mast cell is essential for the immunological stimulation of the mast cell. Actual activation of the cell is achieved when specific antigen binds to two or more of these cell-bound antibodies, bringing about the aggregation of two or more adjacent antibody receptors. It is this aggregation that brings about cell activation. Thus, monovalent antigens are ineffective and any substances that can achieve receptor aggregation will induce degranulation. Bi- or polyvalent antigens cross-link adjacent IgE molecules by combining with the Fab regions, indirectly causing Fc-receptor aggregation. Anti-IgE (IgG antibody directed against the Fc heavy chains of IgE) cross-links IgE, at a site different to that of antigen. The lectin concanavalin A binds to IgE-associated carbohydrate and also cross-links adjacent antibody. Anti-Fc-receptor antibodies will also induce degranulation in the rat peritoneal mast cell (RPMC) [75].

Nonetheless, observed data do not unreservedly support the concept that histamine release is directly proportional to the dimeric cross-linking of receptors. Basophils from certain subjects fail to release histamine, despite the certainty that cross-links are formed. This may represent the uncoupling of Fc-receptors from the signal transduction mechanisms which connect membrane events to the biochemical processes leading to mediator secretion. Also, both qualitative and quantitative characteristics of histamine release vary according to the type of cross-linking ligand. The number of receptors cross-linked in an aggregate may also influence release levels [2].

1.4.2 Non-immunological histamine release

Non-immunologic agents do not appear to rely upon the presence of antibody in order to activate the mast cell [229]. Certain agents are thought to circumvent membrane events by directly introducing calcium into the cytosol. ATP permeabilizes the membrane, and so allows an influx of extracellular calcium into the cell. Ionophores, on the other hand, act as “carrier” molecules to transport calcium into the cytosol. In addition, chymase and tryptase from RPMC granules have been found to induce histamine release, elevate cAMP levels and cause PGD$_2$ production [84,85] (see section 1.3.1.4). However, the most widely investigated group of non-immunologic mast cell stimuli are the basic peptide and polycationic stimuli.
1.4.2.1 Basic peptide secretagogues

The differences between non-immunological and polycationic stimuli have been thoroughly investigated [229,230,82]. Peptides such as substance P (SP) [302], somatostatin [81], neurotensin (NT) [322], bradykinin (BK) [95], calcitonin gene-related peptide (CGRP) [81], vasoactive intestinal peptide (VIP) [320] and several endorphins [321] have been shown to release histamine from RPMCs. The structures of some of these peptides are shown in fig. 1.1.

In brief, certain characteristics distinguish polybasic agents from immunological stimuli. Histamine releases induced from RPMCs by different polybasic stimuli possess features in common. Polycationic and basic peptide secretagogues do not require the presence of antibody on the cellular surface and are unaffected by the application of exogenous phosphatidyl serine. Secretion is rapid and independent of the presence of extracellular calcium. Also, the agents are inhibited by BDTA (benzyldimethyltetradecylammonium chloride) and not desensitized by primary challenge at 4 °C [82,231]. Inhibition by BDTA appears to be specific to polybasic and basic peptide stimuli [82]. These pharmacological agents do not appear to release histamine from human mast cells, save those of the skin [82,323]. By contrast, immunologically-induced release is slow (approx 15 min), enhanced by the presence of phosphatidyl serine, dependent on external calcium, desensitized by primary challenge at 4 °C and not inhibited by BDTA [82].

In the RPMC, the activity of these basic agents has been attributed to clusters of positively charged groups in the molecule. Extensive structure-activity studies with substance P and related peptides have indicated that the N-terminal positively charged amino acids, arginine and lysine, are involved in histamine-releasing activity of these peptides [256,324]. It was shown that the presence of a cluster of basic amino acids, usually lysine or arginine, at the N-terminal end is essential for activity, together with a blocked carboxyl group at the C-terminal. In addition, many of these active peptides have hydrophobic residues, such as tryptophan and phenylalanine, at the C-terminal portion of the molecule. These features are noted in substance P, somatostatin and other peptides.

Nonetheless, the C-terminal portion is also required for secretion since there is a progressive reduction in the histamine-releasing activity with progressive shortening of the C-terminal sequence of substance P [302,303]. Moreover, the N-terminal tetrapeptide, SP$_{1-4}$, which contains two positively charged amino acids, has only 0.015 times the activity of substance P and the C-terminal octapeptide SP$_{4-11}$ is practically inactive [302,303,321]. In addition, secretory potency is increased after certain amino acid substitutions in the C-terminal part of substance P, as with undecapeptide substance P antagonists [D-Trp$^{7,9,10}$]SP$_{1-11}$ and
Some sensory neuropeptides, for instance, substance P and somatostatin, the bronchodilatory peptide, VIP, and bradykinin, a pro-inflammatory peptide, are capable of reproducing some of the pathologic features of asthma, including contraction of airway smooth muscle, oedema, plasma extravasation and mucus hypersecretion [80]. In addition, substance P, somatostatin, CGRP and VIP induce a wheal and flare reaction when injected into human skin [81,338]. These observations have led to theories that these basic peptides might act through the release of histamine from the relevant mast cells [339].

Nevertheless, in vitro experiments do not universally support this view. A number of basic peptides, such as the examples above, evoke histamine release from RPMCs. Such secretion may be induced by a range of polycationic compounds, varying widely in their chemical structure. Thus, this raises the question of whether the action of the peptides is specific or a manifestation of this general effect.

Basic secretagogues are thought to act through an ill-defined polyamine receptor on the surface of the mast cell. Release induced in this fashion is selectively antagonised by benzalkonium chlorides such as BDTA [231]. As outlined above, there are characteristic features which distinguish secretion induced by polybasic compounds from that produced by other ligands and these compounds have a typical profile of reactivity across mast cells from different species and tissues. In particular, since the peptides are unable to evoke a response from human basophil leucocytes and pulmonary mast cells, it is unlikely that they have a role in histamine release in asthma. It is thus not possible to sustain a generalised model of neurogenic inflammation involving peptide-mast cell interaction in man, except possibly in the skin [82,83,323].

1.5 Biochemical events involved in mediator secretion

1.5.1 Calcium

In 1961, Douglas and Rubin coined the term stimulus-secretion coupling to denote the concept of calcium linking the receptor-mediated events at the plasma membrane with exocytosis [88]. Since then, much effort has been devoted to elucidating the mechanisms involved in the coupling process in the context of the mast cell. However, in view of the heterogeneity of mast cells, it is important to mention that the main body of work has been
1.5.1.1 The role of calcium as a second messenger

The response of the mast cell to stimulation requires a system for transducing cell surface signals across the plasma membrane. This is commonly achieved by activation of biochemical second messenger cascades within the cell. Calcium, for example, is a second messenger ubiquitous throughout many systems [89]. Mongar and Schild [90] used fragments of guinea pig lung to provide the initial evidence that anaphylactic secretion of histamine from mast cells was dependent on the presence of calcium ions in the extracellular medium. Secretion was also found to be calcium-dependent in the human basophil [91], rabbit leucocytes [92], human cutaneous mast cells [93] and cells from the guinea pig mesentery [94]. In most cases, reduction in extracellular calcium resulted in significantly depressed histamine release.

Studies on RPMCs have shown that histamine secretion is severely impaired after immunological challenge in the absence of calcium. However, alkaline earth cations such as strontium (10 mM) and barium (16 mM) could substitute for calcium (1 mM), but only at higher concentrations [95].

Uptake of extracellular calcium by mast cells following antigen challenge was subsequently demonstrated by Foreman et al. [96]. They investigated cation fluxes following cellular activation by using radioisotopic $^{45}\text{Ca}^{2+}$. These studies indicated that the time course, magnitude and pH effects on calcium influx correlated to the degree of histamine liberation. Also, addition of phosphatidyl serine (PS) to the incubation medium enhanced $^{45}\text{Ca}$ uptake and histamine release. The increase in calcium uptake under the effects of PS was proportional to the increased level of secretion. It was thus concluded that entry of this cation from the extracellular milieu induced exocytosis.

Related work by Ishizaka et al. [97] showed an increase in membrane permeability on cell activation, as a consequence of Fc-receptor cross-linking. In addition, calcium entry from the extracellular milieu induced exocytosis. The increased permeability of the membrane to calcium declines quite rapidly after initial challenge [96] and, if mast cells are presented with antigen in the absence of extracellular calcium and $^{45}\text{Ca}$ reintroduced at discrete intervals after challenge, both $^{45}\text{Ca}$ uptake and histamine secretion decreases as the time interval between calcium reintroduction and challenge is increased. Despite this, reservations have been expressed on the validity of relating $^{45}\text{Ca}$ uptake with a discrete functional role for the cation in the release process [2,98-100]. The argument centres on the
possibility that increased $^{45}$Ca uptake may reflect non-specific binding to sites that are exposed as a consequence of exocytosis. More importantly, there is no distinction between increased influx and decreased efflux of the cation, nor between a net increase in intracellular calcium and exchange of the labelled for unlabelled cation across the cell membrane [2,101]. Furthermore, the $^{45}$Ca uptake studies indicate an increase in cytosolic calcium concentration from a resting level of approximately $10^{-8}$ M into the millimolar range after activation. Fluxes of this magnitude are incompatible with a messenger or signalling function of calcium since the cells possess a greater sensitivity to elevations in the levels of calcium. Also, increases into the micromolar range are thought to be operative in stimulus-secretion coupling [100]. Nevertheless, these arguments cannot detract from the fact that optimal anaphylactic release is promoted in the presence of extracellular calcium. Instead, they merely question the validity of the $^{45}$Ca studies as a means of examining the functional significance of calcium in the release process.

Ionophores are compounds that can specifically elevate cytosolic calcium concentrations [340]. This is achieved by carrier-assisted, passive diffusion and so circumventing biochemical events at the cell membrane level. Thus, experiments involving ionophores can be used directly to investigate the effects of increased levels of intracellular calcium.

After prolonged incubation of mast cells in calcium-free media, exposure to high concentrations of calcium (16-110 mM) provoked histamine release. Calcium deprivation was considered to render the plasma membrane more permeable to the cation, such that reintroduction to high concentrations provoked a secretory response. This was not mimicked by magnesium [106].

The ion La$^{3+}$ has an ionic radius similar to that of calcium and strontium and its trivalent state should allow binding to any calcium sites on mast cell membranes with greater affinity. La$^{3+}$ and other lanthanide ions have been shown to inhibit histamine release stimulated by basic inducers and La$^{3+}$ has been shown competitively to antagonise the adherence of calcium to a superficial site [109,110]. Basic compounds are thought to mobilize internal calcium stores in circumstances of calcium deprivation. Another study confirmed these data, but also proved La$^{3+}$ to be the least effective of the lanthanide ions in inhibiting histamine release evoked by these agents. Lanthanum-induced inhibition obtained in the presence of calcium was immediate and did not require preincubation. In the absence of calcium, the abrogatory effect of the ion was increased by preincubation. This suggests that penetration into less accessible sites in the membrane by lanthanum could lead to a displacement of bound calcium that would usually be at the disposal of the secretagogue. An alternative explanation could be that lanthanide ions may exert a general stabilizing effect on the cell membrane, so
preventing the mobilization of sequestered stores [2,108]. Thus, La$^{3+}$ can be employed as a specific Ca$^{2+}$ antagonist.

Direct evidence for the involvement of calcium in mediator secretion arrived from the experiments of Kanno and coworkers who succeeded in inducing rapid degranulation of rat mesenteric mast cells by microinjection of the cation into these cells [109]. Such degranulation was specific for Ca$^{2+}$ and could not be reproduced by injection of magnesium or potassium or by direct mechanical insult.

1.5.1.2 Extracellular calcium

Studies have established cytosolic calcium as a critical factor in degranulation [110, 111]. In these experiments, the fluorescent Ca$^{2+}$ indicator quin-2 is used to monitor fluctuations in intracellular calcium, becoming fluorescent when it binds to free calcium in the cytosol. The sensitivity of the compound makes it possible to monitor discrete fluctuations in intracellular calcium as an increase in fluorescence corresponds to a rise in calcium levels.

In the RBL 2H3 cell, the resting intracellular calcium level was $10^{-7}$ M and rose into the micromolar range upon stimulation with antigen [111]. La$^{3+}$ blocked both the calcium signal and histamine release associated with cellular activation by antigen. This effect is consistent with a mechanism of action for La$^{3+}$ as an antagonist of calcium entry into the cell. Moreover, cells challenged with antigen in the absence of extracellular calcium failed to release histamine and did not generate a calcium signal. Similar results were obtained when these experiments were conducted on RPMCs [112].

The physiological concentration of calcium in the extracellular space is $10^{-3}$ M, at least four times greater than the intracellular concentration. This creates a large concentration gradient, augmented by an electrochemical gradient, which promotes an inward flow of calcium into the cell. When the cell is under resting conditions, cellular homeostatic control mechanisms arrest calcium entry. Recent experiments have shown that extrusion of calcium ion is facilitated through the operation of a sodium-calcium antiporter [113]. The mechanism was unaffected by metabolic inhibitors but wholly dependent on extracellular sodium ions. This impermeability of the mast cell membrane is essential for maintaining its integrity.

1.5.1.3 Intracellular calcium

While mast cells respond to non-immunological stimuli in the absence of extracellular calcium, immunologic stimuli provide only a token response [2,114]. The elevation of
cytosolic calcium levels is usually attributed to the utilization of intracellularly bound stores of the ion. Thus, non-immunologic secretagogues appear more adept at mobilizing these stores than immunologic ligands [115].

Depletion of intracellular stores of calcium is brought about by prolonged incubation in a calcium-free medium (1-3 hours) with agents such as EDTA, a calcium chelator, and calcium ionophores such as A23187. Following such treatment, the mast cell becomes unresponsive to stimulation [116]. The most effective way of depleting cells of their more firmly bound calcium stores appears to be a combination of these two agents [117]. The cells regain responsivity when restored to a calcium-containing medium, and this secretion is comparable to that obtained with control cells [116-119].

The effects of intracellular calcium can also be studied through ionophores and cell permeabilization. Ionophores A23187 and ionomycin possess some specificity for calcium and produce non-cytotoxic, dose-dependent release of histamine from mast cells [102]. Permeabilization of cells by ATP [103], Sendai virus [104] or liposomes loaded with calcium [105] can also raise cytosolic levels of calcium. Degranulation ensues, and visual monitoring by microscopy revealed that degranulation was localized to discrete areas of the mast cell, probably corresponding to points of fusional contact puncturing the membrane with concomitant cation entry.

1.5.1.4 Membrane bound calcium

In many cell types, the two most common sites associated with calcium sequestration are the mitochondrial apparatus and the cell membrane. Lanthanum ions are progressively inhibitory in the absence of added calcium [107] and the cations are thought not to traverse the membrane into the cytosol. Therefore, calcium stores are likely to be deeply buried within, or on, the cytosolic surface of the cell membrane in the absence of added calcium. Brief preincubation with EDTA (5 minutes) enhances the secretion produced by most secretagogues in the absence of extracellular calcium. By contrast, supraoptimal concentrations of calcium inhibit histamine release by apparent membrane stabilization [2]. Regulatory sites may exist on the cell membrane which, when saturated, would reduce cellular responsivity to ligand challenge. Hence, removing calcium from these sites through a brief preincubation with calcium chelator may destabilize the membrane, providing an explanation for the enhanced secretion observed in these circumstances.

Thus, three major calcium pools have been identified in the mast cell. A superficial membrane site on the cell surface, an intracellular store of the cation and a regulatory site
on the cell membrane. Occupation of this site stabilizes the membrane, while its depletion renders the cell more responsive.

1.5.1.5 Calcium channels in the mast cell

Early experiments indicated that there were no voltage-operated calcium channels in the mast cell membrane [120]. However, recent experiments involving patch clamping have revealed the existence of a channel which was sensitive to elevated levels of cytosolic calcium [121]. When RPMCs were stimulated with either compound 48/80 or substance P, the studies showed the existence of a sustained plateau of elevated calcium, which was dependent on external calcium. The plateau followed the initial, large, transient increase in internal calcium. The calcium plateau, in contrast to the early rise in internal calcium, was ideally timed to influence the secretory response.

Three ionic mechanisms were identified which explained this calcium influx. A chloride channel, activated by internal adenosine 3',5'-cyclic monophosphate (cAMP), provides the electrical driving force necessary for the uptake of calcium. This facilitates the opening of calcium-specific channels in the membrane, which are also sensitive to inositol-(1,4,5)-trisphosphate (IP₃). Finally, there is a non-specific channel through which divalent cations can permeate. These channels are not voltage-gated, but are potential-dependent in as far as the calcium influx depends on an electrical driving force. Thus, it appears that calcium influx in the mast cell is regulated by membrane channels sensitive to the two intracellular messengers IP₃ and cAMP. Where depolarization in excitable cells results in the opening of voltage-gated calcium channels, hyperpolarization has often been associated with calcium influx in non-excitable cells.

1.5.1.6 Calmodulin

The mechanism by which calcium elicits secretion is thought to be by interaction with the polypeptide, calmodulin (CaM), which is generally accepted as being the universal intracellular receptor for the cation [122-124]. The protein is ubiquitously distributed in eukaryotic cells and its structure has been found to be highly conserved [2,122,125]. It is an extremely acidic molecule with a molecular weight of 17 000 and binds a maximum of four calcium ions with high affinity. Binding calcium confers stability upon the protein, and the ensuing conformational changes reveal active sites on the surface of the molecule, thereby allowing interaction with and activation of apoenzymes or effector proteins.

Neuroleptic drugs, principally those of the phenothiazine family, are able to combine with the calcium-CaM complex. Consistently, these compounds inhibited histamine release from
mast cells and basophils [126,127]. However, inhibition was not specific for calmodulin, as they displayed binding to membranes, cell surface receptors and protein kinase C.

A number of key enzymes involved in important cellular processes such as secretion and proliferation are regulated by calmodulin. These include adenylate cyclase, guanylate cyclase, methyl transferase, phospholipase A₂, the ATPase and calcium pump of the plasma membrane and sarcoplasmic reticulum, and a number of kinases. Mast cell activation initiates phosphorylation of three proteins of molecular weight 46, 55 and 58 kDa in calcium-dependent fashion ([28]. In addition, the phosphorylation of a 78 kDa protein is coincident with its involvement in the termination of secretion. Since this phosphorylation may be important for the induction and termination of histamine release, calmodulin may thus play a role in these processes, possibly through protein kinase C.

Calmodulin is known to promote the disassembly of microtubules (129) and, thus, activation of calmodulin may be involved in the termination of secretion. Calmodulin activation may also have profound effects on the regulation of cyclic nucleotides and calcium homeostasis and it has been implicated in mediation of the characteristic transient increase in cAMP following immunological stimulation of the mast cell [130].

1.5.2 Phospholipids

1.5.2.1 The role of the phosphoinositide cycle

The initial studies into phospholipid hydrolysis were performed by Hokin and Hokin and extended by Michell [131], who proposed that breakdown of membrane phosphatidyl inositol (PI) is an essential step in the formation and control of calcium channels. This has been extended to the mast cell and it is now thought that inositol phosphate (IP) metabolism plays a critical role in stimulus-secretion coupling.

Phospholipid hydrolysis is investigated by incubating intact tissues and cells with radiolabelled precursors such as [³²P] phosphate or D-myo [H³] inositol in the presence of Li⁺, which allows the accumulation of labelled breakdown products [132]. The pathways for this process are highly complex [133-135]. PI, the parent compound, is rapidly converted by sequential addition of two phosphate groups by the action of ATP-dependent kinases to phosphatidyl inositol-4,5-bisphosphate (PIP₂). Phosphomonoesterases then convert PIP₂ back to PI, thus constantly cycling these molecules in the cell membrane. When a receptor is occupied by its agonist, PIP₂ is converted by phospholipase C to inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ is converted to either
inositol-4,5-bisphosphate (IP$_2$) or inositol-1,3,4,5-tetrakisphosphate (IP$_4$). The dephosphorylation of IP$_4$ generates inositol-1,3,4-trisphosphate [136,137], an isomer of IP$_3$. Evidence indicates that IP$_3$ and IP$_4$ are involved in the mobilization of calcium following receptor activation. Physiologically relevant concentrations of IP$_3$ (0.2 - 0.5 \mu M) are able to produce half maximal release of calcium from non-mitochondrial intracellular sites, using permeabilized cells. These effects were specific for IP$_3$ [138,139]. IP$_3$ and IP$_4$, however, have been recently implicated in the regulation of specific calcium channels in the plasma membrane, thus amplifying the calcium signal [341].

Influx of extracellular calcium is thought to occur in order to replenish intracellular stores. This persists for the duration of IP$_3$ production and once production ceases and stores of calcium are replenished, calcium entry ceases [140]. This theory is supported by studies of RBL 2H3 cells [141,142], where the phosphoinositide response of the cells was not affected by blockade of calcium influx by lanthanum or induced by direct mobilization of calcium by ionophore A23187. However, these results contradicted earlier studies, where PI hydrolysis in RBL cells, stimulated by antigen, was shown to be dependent on the presence of calcium in the extracellular fluid. Since IP$_3$ is thought to be involved in the release of intracellular stores of calcium, its generation ought not to be calcium-dependent. The discrepancy is now attributed to the slow kinetics of receptor aggregation particular to the antigen used in the earlier studies. Investigations using antigen of higher binding affinity have confirmed this, showing that IP$_3$ generation in the RBL cell precedes the onset of the calcium signal [143].

DAG is the other product of PIP$_2$ hydrolysis and is retained in the membrane. It serves as a second messenger to activate protein kinase C (PKC), which phosphorylates certain proteins. PKC is a calcium and phospholipid-dependent enzyme, detected in a variety of tissues and mast cells [144,145]. 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 calcium. A small amount of DAG then dramatically increases the calcium affinity of PKC, thus activating the enzyme without any change in calcium levels. Activation of the enzyme induces the translocation of the protein from the cytosol to the membrane.

Mast cells have been shown to produce DAG [146], and research has found that membrane-associated PKC activity begins on stimulation, preceding histamine release and rising to a maximum after 30 s [115,147]. In addition, staurosporine and K-252a, potent PKC inhibitors, strongly inhibited the release of histamine and PGD$_2$ from rat serosal mast cells [148,149], and LTC$_4$ from mouse bone marrow-derived mast cells (BMMC) when
stimulated with anti-IgE. The effects of compound 48/80 were only partially affected, suggesting that the mechanism by which compound 48/80 induces mastocyte responses is not linked to activation of this enzyme. This is borne out by work with exogenously applied PKC activators.

There are numerous reports of the use of structural analogues of DAG, such as the phorbol ester PMA, 1-oleoyl-2-acetyl-glycerol (OAG) and 12-O-tetradecanoylphorbol-13-acetate (TPA), in studies of this effect. They have varied effects on the mast cell and both synergistic and inhibitory effects have been demonstrated, depending on experimental conditions. This indicates a complex and possibly multifunctional role for the PKC system [150-152]. When TPA was applied to cells together with antigen, both at concentrations which do not individually stimulate secretion, they synergised to produce an enhanced level of histamine release. When OAG and TPA are applied, they phosphorylate four cytosolic proteins and induce PI hydrolysis and exocytosis, suggesting that PKC has a role in the activation of mast cells [153]. Phosphorylation of these proteins does not occur with a rise in intracellular calcium. TPA-induced histamine release is calcium independent and the ester synergistically enhances release due to agonists that require the presence of external calcium for optimal effect. Exogenous PS [153] or lyso-PS [154], together with TPA, enhanced release evoked by calcium-dependent agents, and taken together, evidence indicates that the calcium-dependent pathway, promoted by lipids or PKC activation by TPA alone, can induce exocytosis.

The inhibitory effect of PKC is demonstrated using RBL cells, where low concentrations of PMA caused a rapid abrogation of antigen-induced PI hydrolysis and the calcium signal [141,145,155,156]. Higher concentrations completely inhibited these effects. Nonetheless, secretion was only moderately affected and, thus, the exact nature of this contribution is unclear.

1.5.2.2 Phosphatidyl serine

PS enhances anaphylactic histamine release from RPMCs but that evoked by compound 48/80 is unaffected [157,158]. The extent of the enhancement varies between mast cells from different locations, while being totally inactive against human basophils. While dextran-induced secretion from RPMCs is potentiated by the addition of PS, the lipid is not required for dextran-induced histamine release from rat skin or mesentery mast cells. This potentiation of anaphylactic release was not produced by ethanolamine, choline or inositol phospholipids [159,160].
In RPMCs, PS acts almost immediately to increase the magnitude of histamine release. Calcium is necessary for the enhanced release induced by dextran or anaphylactic stimuli [95]. Thus, PS is supposed to potentiate secretion by facilitating the uptake of calcium during anaphylactic stimulation [50].

Acidic phospholipids such as PS and PI, are essential components of cell membranes. They have been shown to interact with calcium and possibly other divalent ions, while the neutral phospholipids, such as phosphatidyl ethanolamine (PE), do not. Studies have shown that the carboxyl group of PS is important in the biological activity of PS and calcium binding is a function of the degree of ionization of phosphate and carboxyl groups [161,162]. This may be the reason for the inactivity of PE, the decarboxylated derivative of PS.

1.5.2.3 Phospholipid methylation

The methylation of some membrane phospholipids appears to be linked to Fc-receptor cross-linking. This cross-linking is thought to result in the decarboxylation of PS to PE [163-165]. Membranes appear to contain two methyl transferases (MT) requiring S-adenosyl-L-methionine as a donor, located in the inner section of the membrane bilayer and the outer section. The first enzyme (MT I) methylates PE to yield phosphatidyl-N-monomethylethanolamine. This is then converted by the second enzyme (MT II), to phosphatidyl choline (PC). Thus, it appears that PS from the inner layer of the membrane is converted to PC in the outer layer. PC may be a substrate for phospholipase A₂, which converts it to lyso-PC and arachidonic acid (AA) [165,166].

The methylation reactions have been shown to precede histamine secretion [167,168] and are related to calcium influx into the mast cell [50,74]. While MT inhibitors prevent histamine secretion and calcium influx, dose-response curves for these inhibitors do not exclude the possibility that inhibition of MT activity is unrelated to calcium movement and histamine secretion [169,170]. However, the PC is produced in the outer membrane leaflet where the phospholipid already forms the bulk of the membrane. Thus, it is difficult to determine how this system generates a signal to the cell, though it is said that the process can increase membrane fluidity [342,343].

1.5.3 Arachidonic acid activation

The metabolism of membrane phospholipids by enzymes such as phospholipase A₂ (PLA₂), phospholipase C (PLC) and diacylglycerol lipase produces free AA which is utilized by the cyclooxygenase and lipoxygenase pathways, generating a variety of prostaglandins,
leukotrienes and thromboxanes. These compounds are thought to have a role in the modulation of the release process.

Highly purified PLA₂ induced non-cytotoxic histamine release from RPMCs and human basophils [171-174] and p-bromophenacyl bromide (p-BPB) [173-175] and mepacrine [172,176] suppressed antigen- and A23187-induced release from these cells. However, non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin [177,178] were, in contrast, ineffective at inhibiting secretion.

Nonetheless, 5-hydroperoxy- and 5-hydroxy-eicosatetraenoic acid (5HPETE, 5HETE) enhance antigen-induced secretion. These substances are produced by the lipoxygenase pathway. The highly specific 5-lipoxygenase inhibitor, L651-392 (4-bromo-2,7-dimethoxy-3-H-phenothiazine-3-one), was found not to affect histamine release from human pulmonary mast cells and blood basophils, despite inhibiting the release of LTC₄ and 5-HETE.

1.6 Regulatory components of signal transduction mechanisms

1.6.1 GTP regulatory proteins (G-proteins)

G-proteins are a large homologous family of trimeric proteins and consist of an α subunit that binds guanine nucleotides and β and γ subunits, which are tightly associated and link the α subunit to the receptor [179]. A guanine nucleotide-dependent regulatory protein, designated Gp, is an essential component in the transduction mechanism which, in the case of PI turnover, links receptor occupancy with PLC activation [180]. The G-protein is cycled between an active GTP-bound form and an inactive GDP-bound conformation which results from an intrinsic GTPase activity of the protein itself. This reaction is catalysed by receptor occupancy. The activated receptor has a high affinity for the G-protein conformation where the single guanine nucleotide binding site is empty and the α and βγ subunits are associated. When the activated receptor binds to the G-protein, it induces GTP binding to the α subunit, resulting in dissociation of Gp into its component α-GTP and βγ complexes. The former complex activates the effector system through a largely unknown mechanism.

G-proteins are of importance in mast cell activation [181]. This is particularly illustrated by experiments using mast cells permeabilized by ATP⁺. Permeabilization is a technique whereby the entry of substances into the cell cytosol can be precisely controlled since, following permeabilization, cells can be resealed by the addition of exogenous magnesium, so trapping exogenously applied compounds within the cell. Introduction of non-hydrolysable analogues of GTP, GTP₇S or GppNHp
(guanylyl-5’-[βγ-imido]triphosphate) into the cells induced calcium-dependent histamine release. Pertussis toxin, which inactivates the inhibitory G-protein (Gi) of the adenylate cyclase transduction mechanism by ADP-ribosylation of the α subunit, suppressed GTP-induced exocytosis from permeabilized RPMCs and secretion induced by compound 48/80 from intact cells [182,183]. The toxin was ineffective when applied against IgE-mediated secretagogues, suggesting that pertussis-sensitive G-proteins are not involved in transduction of the signal produced upon the coupling of IgE receptors [183,184]. GTPγS does not affect Gp, as shown by studies using streptolysin-O permeabilized cells, where the analogue induced secretion in the presence of calcium, even in the presence of low concentrations of neomycin, an inhibitor of the metabolism of inositol phospholipids.

Two G-proteins are implicated in secretion: one at the receptor level, linked to PLC, and a second directly activated G-protein, Ge, which exists distal to the calcium signal [185,186]. Patch-clamp experiments, taking membrane capacitance as a measure of exocytosis, showed that even with high concentrations of EGTA, GTPγS evoked histamine release. This work strongly suggests that G-protein activation was taking place independently of the calcium signal. Further evidence for the involvement of Ge is provided by H-rasval[12], a constitutively activated G-protein, which is thought to activate the cell by mimicking Ge. The oncogenic transformant can effect degranulation on microinjection of calcium into the cell cytosol [311].

1.6.2 Serine esterases

The regulation of methyl transferase enzymes [187] involves activation of proteolytic enzymes. Diisopropylfluorophosphate (DFP), a potent and irreversible serine esterase inhibitor, blocked IgE-dependent secretion from rat serosal mast cells [188,189] and fragments of guinea pig and human lung [187,190]. DFP inhibited a number of biochemical events such as phospholipid methylation, 45Ca2+ uptake and the transient cAMP rise [191] when presented together with the secretagogue, suggesting the involvement of a serine esterase following initiation of the secretion following receptor bridging. Further evidence arises from results showing that α-chymotrypsin and rat mast cell chymase induced secretion in RPMCs [192], and substrates and inhibitors of these enzymes also inhibited phospholipid methylation and the cAMP spike produced on immunologic stimulation of mast cells.

1.6.3.3 The role of cAMP

Adenosine 3’,5’-cyclic monophosphate (cAMP) is an important second messenger in many cell types, but its role in the mast cell is unclear [195,196]. cAMP is produced by the action
of adenylate cyclase on adenosine 5'-triphosphate (ATP), and metabolized by phosphodiesterase. Adenylate cyclase is associated with the membrane and may be stimulated via a receptor. Berridge attempted to classify possible control mechanisms as monodirectional and bidirectional [233]. In the former, cAMP acts in concert with cGMP (guanosine 3',5'-cyclic monophosphate) to enhance calcium-induced secretion, while in bidirectional systems, the nucleotides antagonize each other and cAMP opposes the effect of calcium.

Traditionally, cAMP was considered to be inhibitory to histamine secretion [196,197]. Phosphodiesterase inhibitors, thought to elevate intracellular levels of the nucleotide, suppressed secretion from human basophils and chopped human lung. The observed decrease in cAMP levels in cells exposed to 48/80 seemed to confirm this. However, there is evidence that this is not so. Concentrations of drugs required for the suppression of cAMP are far above those needed to inhibit histamine release [198,199].

On immunological stimulation, there is an initial sharp rise in cAMP levels, which takes place within the first minute, followed by a second, smaller rise 30 s later. The initial rise in nucleotide levels is thought to be specific to immunological stimuli, as only the later smaller increase is observed when cells are stimulated with pharmacological agonists such as compound 48/80 [200]. When indomethacin was applied, at concentrations which did not affect histamine release, prostaglandin production was suppressed and the late phase cAMP response was totally abolished [201]. Thus, it may be inferred that the late rise in cAMP levels is probably related to arachidonic acid metabolism [202].

Potentiation of histamine release by adenosine and its analogues produces a correspondingly greater rise in the level of cAMP, suggesting a causal role for the nucleotide in secretion. However, pharmacological stimuli, also susceptible to potentiation by adenosine, do not produce this early rise in cAMP, showing that it is not obligatory for exocytosis. Furthermore, during adenosine-induced potentiation of secretion, the elevation of cAMP levels may be suppressed by concentrations of theophylline that do not affect histamine release or the potentiation induced by the nucleotide [203,204]. Nevertheless, this is a real event that could either be part of the immunological release mechanism, that is bypassed by other forms of activation, or merely an epiphenomenon. Further research is necessary to clarify the role of this nucleotide.
Aims of this thesis

The kinin, complement and coagulation systems are linked through the inflammatory system. All three events are part of the innate vascular response to inflammation and have been linked in an investigation on human anaphylaxis [306]. The study reported that the two subjects with the most severe shock showed evidence of intravascular coagulation, characterized by a diminution of coagulation factors V and VIII, fibrinogen and high molecular weight kininogen (HMWK) as well as changes in the complement system. Such depletion of critical coagulation factors could account for the defective coagulation seen after clinical and experimental anaphylaxis. In addition, kinins have been implicated in asthma and rhinitis [235,248].

Elements of these systems have been shown to release histamine from mast cells: bradykinin, a product of the kinin cascade, and the anaphylatoxin peptides of the complement system release histamine from RPMCs [260] and thrombin, an enzyme essential to clotting, has been shown to release histamine from bone marrow-derived mast cells (BMMC) [281-284]. However, the exact role of these peptides in allergic disease and their relation to mast cells remains to be elucidated. Accordingly, we have now examined their effect on mast cells from a number of different sources and have, in particular, compared their action with other polycationic histamine liberators.
Fig. 1.1 Primary structure of some basic peptides.

Bradykinin arg-pro-pro-gly-phe-ser-pro-phe-arg

Lys-bradykinin lys-arg-pro-pro-gly-phe-ser-pro-phe-arg

Substance P arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met

Somatostatin ala-gly-cys-lys-asn-phe-phe-trp-lys-thr-phe-thr-ser-cys

Neurotensin pGlu-leu-tyr-glu-asn-lys-pro-arg-arg-pro-tyr-ile-leu
CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 TISSUE AND CELL SOURCES

2.1.1.1 Animal cells and tissue
Closed, random-bred colonies of Sprague-Dawley rats (200-300 g), Porton mice (60-100 g), Syrian hamsters (130-180 g) and Dunkin-Hartley guinea pigs (250-350 g) were obtained from the Joint Animal House, University College London. Specific pathogen free (SPF) Sprague-Dawley rats (250 g) were procured from Charles River, Kent. Cultured mouse bone marrow cells (BMMC, Wehi-con A) were provided by Dr. T. Lomas.

2.1.1.2 Human tissue
Whole venous blood was obtained from healthy, adult volunteers and tissue was supplied by co-workers from the Middlesex and University College hospitals. All tissue, except infant foreskin, was obtained from carcinoma operations and judged as macroscopically normal.

2.1.2 BUFFERS

2.1.2.1 Full Tyrode’s buffer
Full Tyrode’s-based buffer (pH 7.3) was the standard experimental medium used in these studies:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137.0 mM</td>
</tr>
<tr>
<td>glucose</td>
<td>5.6 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>CaCl2</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

The weak acid HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane sulphonic acid) buffered the solution in the correct range, and the pH was adjusted with either NaOH (4 M) or HCl (3 M). The solution was modified where required.
2.2.1.2 Modifications to full Tyrode's buffer

(a) *Heparinized Tyrode's*: Full Tyrode's with heparin (50 I.U./ml).

(b) *BSA Tyrode's*: Full Tyrode's with bovine serum albumin (BSA, 1% w/v).

(c) *Experiments involving calcium*: Calcium free Tyrode's (CMF-Tyrode's) was used in preparation of buffers with twice the final concentration of calcium, or EDTA (2 x EDTA-Tyrode's), or strontium where appropriate.

(d) *pH experiments*: Twice the usual amount of HEPES was used and pH was adjusted using either NaOH (4 M) or HCl (3 M).

(e) *D₂O-Tyrode's*: Buffer was prepared using D₂O instead of H₂O.

2.1.2 SECRETAGOGUES AND INHIBITORS OF HISTAMINE RELEASE

2.1.2.1 Secretagogues

(a) *Bradykinin and peptide analogues*:

- bradykinin (BK)
- lys-BK
- [lys₁⁻BK
- met-lys-BK
- ile-ser-BK
- des-arg⁹-BK

(b) *Bradykinin receptor antagonists*:

- ([D-Phe⁷]-BK)
- [Thi⁵,6-D-Phe⁷]-BK
- [Hyp²-Thi⁵,8-D-Phe⁷]-BK
- [Hyp³-Thi⁵,8-D-Phe⁷]-BK

(c) *Anaphylatoxin peptides*:

- C3a
- des-arg-C3a
- peptide 21R

(d) *Polyaminic and peptide secretagogues*:

- compound 48/80 (MW of trimer = 520)
- polylysine, MW 70 000
polyarginine, MW 70 000
somatostatin
neurotensin
F-met-leu-phe
substance P Peninsula

(e) Other secretagogues:
bovine thrombin Sigma
human thrombin ..
dextran, MW 70 000 Pharmacia

(f) Immunological secretagogues:
sheep anti-rat IgE Miles
sheep anti-rat IgG ..
goat anti-guinea pig IgG Nordic Immunology
sheep anti-human IgE Dako Ltd.
concanavalin A Sigma

2.1.2.2 Inhibitors
(a) Metabolic inhibitors:
antimycin A Sigma
2-deoxyglucose ..

(b) Other inhibitors:
theophylline Sigma
disodium cromoglycate gift from Fisons plc
indomethacin Sigma
polyglucoses: MWs 1600, 2100 Dr. K. Granath, Pharmacia
BDTA (benzyldimethyltetradecyl Fluka
ammonium chloride)

2.1.2.3 Phospholipids
phosphatidyl serine (PS) Lipid Products
lyso-phosphatidyl serine (lyso-PS) Sigma
2.1.2.4 Radioimmunoassay materials

- [I\(^{125}\)]cAMP radioimmunoassay kit
- [H\(^{3}\)]PGD\(_2\) radioimmunoassay kit

2.1.2.5 Other materials

(a) Dispersion and purification:

- collagenase, type 1A
  Sigma
- hyaluronidase
  ..
- bovine serum albumin (BSA)
  ..
- foetal calf serum (FCS)
  Gibco
- Percoll
  Pharmacia
- trypan blue
  George T. Gurr Ltd.
- toluidine blue
  Sigma

(b) Others:

- deuterium oxide (D\(_2\)O)
  Sigma
- o-phthalaldialdehyde (OPT)
  Sigma
- heparin
  Weddell Pharmaceuticals

(c) Disposable polystyrene equipment:

- centrifuge tubes, 10 ml
  Sarstedt
- pipettes, 1 and 1.5 ml
  Sterilin
- universal tubes, 20 and 50 ml
  ..
- syringes, 10 and 20 ml
  ..

Plastic and siliconized glass equipment was used throughout all handling of cells and tissues.
2.2 METHODS

2.2.1 Isolation of histaminocytes

2.2.1.1 Isolation of peritoneal and pleural mast cells

(a) Peritoneal mast cells
Rats and mice were sacrificed by an excess of nitrous oxide. The abdomen was exposed and warm heparinized buffer (10 ml, 37 °C) was injected into the peritoneal cavity. The abdomen was gently massaged (2 min) then opened down the mid-line, and the fluid within extracted with a disposable polystyrene pipette and placed in Sarstedt tubes. In experiments where a greater efficiency of recovery of mast cells was needed, the cavity was washed twice, with a greater quantity of buffer. The cells were recovered by centrifuging (100 g, 2 min, RT). The recovered cells were resuspended in an appropriate volume of buffer (usually 3 ml), centrifuged as above, the supernatant discarded, and the cells resuspended in a final volume suitable to the experiment.

Sacrifice by anaesthetic overdose was unsuitable for hamsters and guinea pigs. These were more quickly dispatched by light asphyxiation with carbon dioxide, followed by decapitation and exanguination. Each rat yielded sufficient cells for 30-50 samples, mice 6-10 and hamsters up to 30. Cells from SPF rats were isolated as quickly as possible after arrival to minimize antibody formation.

(b) Pleural mast cells
Warm heparinized buffer (6 ml, 37 °C) was injected into both sides of the pleural cavity through the diaphragm. The thorax was gently massaged (2 min), the rib cage cut away, and the cell suspension removed with a disposable pipette. The cells were then treated as for peritoneal cells. One rat yielded enough for about 20 samples.

2.2.1.2 Animal tissue mast cells

(a) Lung and mesenteric mast cells
These cells were isolated by enzymic dispersion. Lung was dissected free of pleura and major airways, and mesentery of lymph nodes. The tissue was washed and chopped roughly (= 2 min) and then finely chopped using a McIlwain tissue chopper. The tissue was then incubated in a dispersion medium of heparinized BSA-Tyrode’s with collagenase (type 1A, 160 units/ml), in a shaking water bath (60-90 min, 37 °C). After dispersion, the suspension was drawn several times through a syringe (10 ml) to disrupt tissue clumps, and filtered.
through a double layer of gauze moistened with BSA-Tyrode’s. Cells were recovered by centrifuging (150 g, 5 min, 4 °C), washed twice in heparinized BSA-buffer (4 °C) and resuspended in the appropriate medium.

*Guinea pig mesenteric mast cells:* The same procedure as above was followed except that, during enzymic digestion, the tissue was oxygenated with an O₂-CO₂ mixture (95% and 5% respectively).

(b) Rat intestinal mast cells

The small intestine was removed and flushed free of faecal matter with full Tyrode’s. Any attached mesentery and Peyer’s patches were removed from the bowel, which was then cut into longitudinal strips (= 2mm wide) and rinsed thrice with full Tyrode’s buffer (37 °C, 10 min each, stirring) to rid the tissue of residual mucus.

The digestion involved 2 separate incubations in 20% FCS-Tyrode’s (100 ml, 60 min, 37 °C) containing collagenase (40 units/ml). Following the first incubation, the buffer now containing freed cells, was poured off and replaced with fresh medium for a second spell. The buffer from the first digestion was filtered through a double layer of gauze, the cells recovered by centrifugation (150 g, 3 min, RT) and kept in BSA-Tyrode’s (37 °C) until use. After the final digestion, the tissue was disrupted through a syringe before filtration and recovery. The recovered cells were pooled and washed twice in experimental buffer and thus used.

### 2.2.1.3 Human histaminocytes

(a) Human colonic and lung parenchymal mast cells

Lung tissue was dissected free of major airways and blood vessels, while colonic tissue was divided into mucosal and submucosal layers and treated separately. The method of dispersion was a slight variation from that of rat intestinal mast cells (section 2.2.1.2b). The tissue was rinsed twice (37 °C, 10 min each, stirring), cut up and passed through the tissue chopper. This was followed by digesting twice (37 °C, 60 min each) in BSA-Tyrode’s (25 ml/g of tissue) with collagenase (120 U/ml). The cells were also recovered by centrifugation (150 g, 3 min), between as well as after digests, following the protocol above.

(b) Cutaneous mast cells

Infant foreskin or adult breast skin was cut up and chopped as above. The tissue was digested thrice in BSA-Tyrode’s (37 °C, 120 min each) containing collagenase (160 units/ml) and
hyaluronidase (500 units/ml). Freed cells were recovered between and after dispersions as above (100 g, 3 min) and stored in heparinized BSA-Tyrode’s (4 °C) until required.

(c) Basophil leucocytes

Venous blood (20 ml) was extracted from healthy, adult volunteers and placed in sterile universal tubes (20 ml) containing glucose (150 mg), heparin (50 000 I.U./ml, 20 µl) and dextran (MW 70 000, 5 ml). The blood was left to stand at room temperature while the erythrocytes sedimented (approx. 1 h) and the leucocyte suspension was drawn off progressively and stored in clean Sarstedt tubes (37 °C). When this process was complete, the suspension was centrifuged (150 g, 3 min, RT), the supernatant discarded and the cell pellet, containing basophils and other leucocytes, was washed twice in normal buffer (37 °C) and resuspended in the appropriate experimental medium. The basophils could be stored for up to an hour (0 °C).

*Basophils for use in experiments with complement peptides:* The leucocyte suspension was stored at room temperature until use, and washed in buffer, also at room temperature. The basophils were used immediately and were not brought up to 37 °C at any time before use. Immediately prior to the experiment, the cells were allowed 5 min to equilibrate to the experimental temperature, 37 °C.

2.2.1.4 Purification of rat peritoneal mast cells

Cells obtained by peritoneal lavage contain 2-5% mast cells, the remainder comprising peritoneal macrophages, neutrophils, eosinophils and erythrocytes. Purified preparations of mast cells are required for biochemical determinations such as the measurement of eicosanoids and cyclic nucleotides. Percoll (polyvinyl pyrrolidone-coated silica particles, PVP) is of low osmolarity and viscosity and produces mast cell suspensions of high purity and yield, with good preservation of morphological and biological integrity.

The Percoll solution was prepared by mixing 9 parts Percoll with 1 part of 10-fold concentrated CMF-Tyrode’s buffer (calcium was added in a minimum volume after mixing the two). Peritoneal cells were suspended in 1 ml BSA-Tyrode’s (5% w/v) containing heparin (250 I.U./ml) and mixed with 4 ml of Percoll solution. 1 ml of normal buffer was layered on top and the suspension was centrifuged (200 g, 25 min, 4 °C). Best results were obtained when cells from no more than two rats were allowed for each tube. Finally, the purified cells were washed twice in BSA-heparin-Tyrode’s (10 ml, 4 °C) and resuspended in buffer appropriate to the experiment. Prior to use, cells were checked for viability, using the Trypan blue exclusion test (see section 2.2.1.5).
2.2.1.5 Staining of mast cells

(a) Metachromatic staining with toluidine blue

Purified mast cells were mixed, one part to nine parts, with toluidine blue solution (0.1% w/v in 0.9% saline) and incubated at 37 °C for 5 min. The stained preparation was examined microscopically using a haemocytometer (Neubauer improved). Mast cells were readily identified by the metachromatic staining (violet) of the granules, in contrast to the blue stain of other cells. Thus, the purity of the sample was found to be greater than 95%.

The cell count was determined in the following way:

\[
\text{No. of cells/ml} = \frac{\text{total counts in 9 squares} \times \text{dilution factor}}{\text{total volume} \times 1000}
\]

where:
- depth of haemocytometer = 0.2 mm
- total area = 9.0 mm\(^2\)
- total volume = 1.8 mm\(^3\)
- dilution factor = 110/100

(b) Determination of cell viability - Trypan blue exclusion test

Purified cells were mixed, one part to three parts, with trypan blue (0.1% w/v in 0.9% saline) and incubated for 5 min at 37 °C. The cells were loaded into a haemocytometer and counted as above. Viable cells excluded the dye and were seen as clear cells surrounded by a dark ring.

2.2.2 Preparation of reaction agents

2.2.2.1 Water-soluble agents

Most agents used were water-soluble and could be prepared in experimental buffer. All were freshly prepared except compound 48/80 which could be prepared, concentrated, in glass-distilled water and stored for up to a week at 4 °C. Care was taken with substance P which could hydrolyse in 30 min. Complement peptides were dissolved in full Tyrode’s to give stock solutions that were aliquoted and stored (-20 °C). Where necessary the molecular weight of the polymer, compound 48/80, was taken from the trimer (MW 520) as it is its most abundant form. The molecular weight of polylysine was taken as 70 000, according to the suppliers recommendation.

Anti-rat and anti-guinea pig antibodies and thrombin were dissolved to give stock solutions according to recommendations and stored at -20°C in aliquots until use. Anti-human IgE was stored at 4 °C.
2.2.2.2 Non-water-soluble agents

(a) Dimethyl sulphoxide (DMSO)

DMSO was used to dissolve antimycin A at 1000 times the final concentration.

(b) Ethanol

Ethanol was used as a solvent for BDTA. 30 mg of BDTA was dissolved in 1666 μl ethanol and 20 μl of this was made up to 12 ml with full Tyrode’s buffer to produce a 30 mg/ml stock. Subsequent dilutions were made from this, such that the final concentration of ethanol was less than 0.16 μg/ml.

(c) Phospholipids

Phosphatidyl serine (PS) was supplied in chloroform/methanol (ratio 3:1, 20 mg/ml). Lyso-PS came in solid form and was also dissolved in chloroform/methanol in the same proportions as for PS. Stock solutions of the phospholipids were then obtained by placing 25 μl of the organic solution in a glass tube and evaporating the solvent over a stream of pure, dry nitrogen, rotating the tube so as to obtain a thin even film of solid over the bottom. The solid was suspended in CMF Tyrode’s (500 μl) to give a stock solution (1 mg/ml). The final concentrations required of PS and lyso-PS were 15.0 and 0.5 μg/ml, respectively.

2.2.3 Histamine release experiments

The experimental reaction volume used was 200 μl. Typically, cells (100 μl) were added, in timed fashion, to Sarstedt tubes containing the prepared experimental agents. Thus, the total volume of all agents included in the experiment was 100 μl, and initial concentrations adjusted to fit. When preincubation of cells with an agent, or equilibration with a particular medium was required (CMF- or EDTA-Tyrode’s, inhibitor, pH, temperature), the subsequent agent was added in a minimum volume (max. 20 μl).

Histamine release was allowed to proceed for ten minutes (37 °C, shaking) irrespective of preincubation time. The reaction was terminated by the addition of ice-cold buffer (4 °C, 2 ml) which arrested further secretion by lowering the temperature and diluting the agonist to relatively ineffective concentrations. The cells were sedimented (4 °C), and the supernatants decanted into fresh, labelled tubes. The cell pellets were resuspended to the same volume as the supernatant tubes (2 ml) and heated in a water bath to release residual histamine. Hence, the amount of histamine release could be determined from the relative concentrations of amine in the cell pellet and supernatant tubes.
Where cells from enzymic dispersion and basophils were involved, the reaction was quenched with 800 μl of buffer in readiness for automated assay (section 2.2.4.3). In addition, basophils were allowed a 30 min incubation owing to a slower response to stimulation.

A sample tube of cells was included in all experiments to determine the spontaneous level of histamine release. This was achieved by exposing all cells to identical handling in the absence of any reagents. Spontaneous release was consistently 2 - 5% with 10% taken as an experimental limit to the validity of the experiment. Higher values for the rat pleural mast cell (mean 10%) and purified RPMCs (5 - 10%) were unavoidable.

(a) Temperature-dependence experiments

The cell pool was divided equally and each portion was equilibrated to 0, 25, 37 and 45 °C before the start of the experiment (10 min). The experiment was then performed in the usual way at the specified temperatures.

(b) Experiments concerning the significance of extracellular calcium

RPMCs were isolated in standard Tyrode's buffer, washed in CMF-Tyrode’s, then added to test tubes, where they were incubated for five minutes in either the absence or presence of calcium (CMF- or normal Tyrode’s), or with the calcium chelator EDTA (EDTA-Tyrode’s), where the concentrations of calcium were 1.0 and 0.1 mM respectively and the concentration of EDTA, 0.1 mM. These cells were then used in the experiment.

2.2.4 Histamine assay

2.2.4.1 Principles of assay

Secretion was assessed by measuring the relative concentrations of amine in supernatant and cell pellet tubes. The assay, developed in 1959 [310], relies upon the formation of a fluorescent adduct, where the fluorescence generated by the sample is directly related to the concentration of histamine.

Under basic conditions, histamine is in free amine form, and condenses with o-phthaldialdehyde (OPT) to give a fluorescent adduct. The adduct is stabilised by acidification. The level of fluorescence is determined by exciting the sample at 360 nm and assessing the emitted fluorescence at 440 nm. The fluorescence reading of the sample is directly proportional to the histamine concentration and the relation between the supernatant and cell pellet concentrations is used in calculation of the results.
2.2.4.2 Manual assay

The sample (2 ml) was made alkaline by the addition of NaOH (1 M, 300 µl), and OPT (1% w/v in methanol, 100 µl) was added with vortexing. The reaction was allowed to proceed for 4 min, and then arrested by application of HCl (3 M, 150 µl). The samples were transferred to plastic cuvettes and assessed on a Perkin-Elmer spectrophotometer LF-5B.

The samples were analysed as soon as possible, as the fluorescence generated by the assay decayed quickly, especially on exposure to U.V. light. Nonetheless, it was possible to maintain the viability of the samples by storing them either in the dark at room temperature for up to 6 h, or overnight at -20 °C.

2.2.4.3 Automated assay

Automated assay of an experiment (Technicon Autoanalyser II) was performed when the samples held an excess of debris and protein, or contained substances that interfered with the assay. The process exploits amine chemistry, where histamine takes the form of the free base under alkaline conditions, and is converted to the salt by acidification. In accord with these properties, histamine has preferential solubility in organic and aqueous phases. The Autoanalyser performs sequential solvent extractions under the appropriate conditions, retaining histamine and leaving the sample relatively free of impurities, and ready for OPT assay.

The samples (1 ml) were treated with perchloric acid (72% w/v, 30 µl) and mixed well to precipitate excess protein that could otherwise block the fine-bore tubing of the analyser. Blank sample tubes were included as controls. The precipitate was removed by centrifugation (200 g, 4 °C, 20 min), and the samples loaded onto the Autoanalyser for analysis. Results were expressed as peaks rising from a base line, where the height of the peaks indicated fluorescence and was taken to represent the concentration of histamine. The Autoanalyser was regularly calibrated using histamine standards and maintained as part of the standard laboratory routine.

2.2.4.4 Calculation of results

Determinations of absolute concentrations of histamine were not needed and the degree of histamine liberation was calculated from the relative amounts of amine in supernatant and cell pellet tubes, where:

\[
\text{% histamine release} = \frac{\text{amount of supernatant histamine}}{\text{(cell pellet + supernatant) histamine}} \times 100\%
\]
The actual level of histamine release was determined by subtracting the value of spontaneous histamine release from the experimentally determined level of secretion calculated above. Inhibition and potentiation were calculated from the difference in histamine release in the presence (% release) and absence (% control release) of the agent in question where:

\[
\text{% inhibition} = \frac{\% \text{ control release} - \% \text{ release}}{\% \text{ control release}} \times 100\% \\
\text{% potentiation} = \frac{\% \text{ release} - \% \text{ control release}}{\% \text{ control release}} \times 100\%
\]

2.2.5 Biochemical investigations

2.2.5.1 Prostaglandin D\(_2\) (PGD\(_2\))

Purified RPMCs (50 μl) were placed in Sarstedt tubes containing the stimulus, to a final reaction volume of 1 ml, and incubated for 10 min (37 °C). The reaction was terminated by centrifuging (150 g, 2 min, RT) and 500 μl of the supernatant was aliquoted into Eppendorf tubes and snap-frozen in liquid nitrogen, in preparation for prostanoid analysis. The remainder was assayed for histamine release, and correlated to prostaglandin release.

PGD\(_2\) levels were evaluated by radioimmunoassay using a commercially available kit. The method employs competition between the experimentally generated PGD\(_2\) and [H\(^3\)]-PGD\(_2\) for binding to a specific antibody (overnight). Any unbound prostaglandin is subsequently removed by adsorption onto activated charcoal (10 min, 4 °C). The supernatants were decanted and mixed with scintillant (5 ml) and radioactivity measured on a β-scintillation counter (Packard Model 3255 Tris-carb liquid scintillation spectrometer, 2 min). PGD\(_2\) levels were determined from a standard curve, and results expressed as ng PGD\(_2\)/10⁶ mast cells.

2.2.5.2 Adenosine 3',5'-cyclic-monophosphate (cAMP)

Purified cells (100 μl) were added to the secretagogue (100 μl) in Eppendorf tubes (= 10 000 cells/tube) and incubated at 37 °C. The reaction was allowed to proceed for the required length of time and terminated by the addition of ice-cold absolute ethanol (200 μl), with vortexing. The fluid was then evaporated (air, 37 °C, 24 h) and the residue dissolved in assay buffer (RIA kit) and stored until assay (−20 °C).

The assay employed a commercially available RIA kit and involved competition between cAMP and [\(^{125}\)I]-cAMP for a specific antibody. Separation of unbound antibody was
achieved by precipitating bound antiserum with an antiserum-antibody. Counts were made on a gamma-counter (Nuclear Enterprises 1600) and levels of cAMP determined through a standard curve and expressed as pmol cAMP/10^6 mast cells.

2.2.6. Statistical treatment

A minimum of three, though usually four, experiments were conducted for each study and the data were treated statistically. This involved calculation, with the aid of a programmable calculator, of the standard error of the numerical mean (SEM), which expresses the variation of individual results within the mean. Hence, results are expressed in terms of the mean ± SEM, together with the number of experiments, where appropriate. An effect was deemed valid if the statistical significance, \( p \), was calculated such that \( p < 0.05 \).
CHAPTER 3

The Effects of Bradykinin on the Rat Peritoneal Mast Cell

3.1 INTRODUCTION

Bradykinin is a nonapeptide featuring an arginine residue at each terminal.

\[ \text{arg - pro - pro - gly - phe - ser - pro - phe - arg} \]

The peptide, together with its analogues lys-BK and met-lys-BK, have been found in man and implicated in inflammation and allergic disease [250,251]. Bradykinin has also been shown to release histamine from RPMCs [237,314] and this study extends and confirms previous findings.

3.1.1 The kinins

Bradykinin and lys-BK are kinins, potent polypeptides with many biological properties such as the contraction or relaxation of smooth muscle, the promotion of fluid movement through the vascular space and the production of pain [250,251]. These properties make kinins ideal mediators of inflammation and, consequently, the peptides may also be involved in allergic inflammation, in conditions such as asthma and rhinitis [316].

The kinins are implicated in the response of organisms to injury as part of the kallikrein-kinin system. Nonetheless, while the peptides produce the classical features of inflammation, it is not yet possible to demonstrate directly whether kinins are important mediators of inflammation. This ignorance stems largely from the lack of a specific kinin receptor antagonist or inhibitor of the kinin generating enzymes, although antagonists are being currently developed. It is also impracticable to measure directly the rate and magnitude of kinin metabolism at areas of inflammation since bradykinin is degraded very quickly, \textit{in vivo}, by several common proteases. Kinin generation may also result in an amplification of inflammatory processes, giving rise to a proliferation of inflammatory mediators, oxygen free radicals, fibrin split products, proteolytic enzymes and other materials. Thus, tracing a specific mediator is particularly difficult. However, while kinins are largely involved in inflammation, the peptides do have other effects such as the stimulation of the proliferation of a wide variety of cells, \textit{in vivo} and \textit{in vitro}, including thymocytes.

Pharmacological characteristics of the kinins include the ability to increase vascular permeability, vasodilation, the relaxation or contraction of smooth muscle of different
origins and nociceptive properties. These events appear to be effected largely through the release of arachidonic acid metabolites and catecholamines and a direct activation of nociceptors and nerve reflexes which regulate heart rate and vessel tone.

3.1.2 The production of kinins

The kinins are found in the blood, and are present as precursors called the high and low molecular weight kininogens (HMWK, LMWK). The kinin sequence is contained within the kininogen molecule and proteolytic cleavage of these precursors by specific enzymes, the plasma and tissue kallikreins, results in the liberation of bradykinin and lys-BK respectively [250]. Lys-BK may then be converted to bradykinin by aminopeptidase.

The kininogen molecules are encoded by the same gene and consist of a heavy and light peptide chain, separated by the kinin sequence. Both kininogens may serve as substrates for tissue kallikrein, but the activity of plasma kallikrein is restricted to HMWK. This is the result of variations in the structure of the two molecules. Whilst the heavy chains of both kininogens are similar, the light chain of HMWK bears binding sites for plasma prekallikrein and Factor XI (zymogens of plasma kallikrein and the coagulation cascade, respectively) and a region that binds to negatively charged surfaces. These light chain binding sites are essential for the liberation of kinin by plasma kallikrein and allow for correct orientation of the participating proteins during the process of kinin generation. The LMWK light chain is much smaller and lacks these binding sites and, as a result, LMWK cannot be proteolysed by plasma kallikrein.

The tissue kallikreins, or kininogenases, are found in the kidney, pancreas, skin, salivary gland and bronchopulmonary tissue. The kininogenases are antigenically related, save that of the skin, while plasma kallikrein derives from a separate source. Tissue kallikreins are synthesized on demand in exocrine cells and release kinin from circulating kininogenase. The liberated kinins are rapidly proteolysed by the abundance of kininase in the blood and lung, ensuring that the peptide acts only in situ. The excess of active kallikrein is deactivated by the plasma-borne inhibitors, C1 inhibitor (C1 INH) and α-macroglobulin. Thus, the location of kallikrein in these tissues indicates that kinin production is part of the physiological regulation of local events. Indeed, the peptide appears to be implicated in the control of water and electrolyte movement and in the regulation of local blood flow. Studies have also shown that kallikrein in the exocrine cells of these tissues may cleave prohormones such as prorenin and proinsulin. These findings illustrate a potential regulatory role for kinins.
The effects of tissue kallikrein are limited to its site of synthesis. The kinin thus generated may fulfill a homeostatic purpose and is swiftly deactivated by circulating proteases. However, bradykinin is produced at sites of inflammation throughout the body. This is enabled by plasma kallikrein, which is borne in plasma and extracellular spaces in the form of its zymogen, plasma prekallikrein. A large proportion of prekallikrein is bound to its eventual substrate, HWMK, and the prekallikrein-HMWK complexes circulate until activated by contact with Hageman factor, in the presence of a negatively charged surface. Such a surface would be exposed in instances of injury and infection and presented by substances such as bacterial lipopolysaccharides, glycosaminoglycans, heparins, glomerular basement membrane, collagens, elastins and sodium urate crystals.

The process of kinin liberation involves HMWK as both substrate and co-factor. The binding of the kininogen to a negatively charged surface and the prekallikrein-HMWK complex positions the zymogens for reaction, resulting in mutual activation of prekallikrein and Hageman factor and the release of bradykinin. Consequently, the production of bradykinin occurs with the initiation of coagulation and fibrinolytic cascades as activated Hageman factor is a key enzyme in these pathways. This system of bradykinin release is alternatively known as the intrinsic coagulation-kinin pathway or contact activation. In addition, activated Hageman factor may also trigger the complement cascade.

Factor XI, the second zymogen of the intrinsic coagulation cascade, may also form complexes with HMWK. These complexes are activated in a similar way to the prekallikrein-HMWK complexes, again resulting in bradykinin production and the co-activation of Factor XI and Hageman factor. Activated Factor XI is one of the enzymes in the intrinsic coagulation cascade, so that release of bradykinin by Factor XI further aids the clotting pathways. In this way, the contact system links the kinin and intrinsic clotting systems by their activation and can amplify the response to noxious stimuli.

The kinins are degraded by kininases which are also non-specific peptidases that act on a whole variety of peptides and proteins. Kininase I, or carboxypeptidase N (CBP N), is found largely in plasma while kininase II, or angiotensin converting enzyme (ACE), is found in tissues, mainly in the lung. CBP N is a zinc metalloenzyme that cleaves C-terminal arginine or lysine residues from several biologically active peptides including anaphylatoxins and fibrinopeptides. It is probably the major inactivator of anaphylatoxins in plasma while inactivating kinins slightly less rapidly. ACE converts angiotensin I to angiotensin II and the enzyme removes the two C-terminal residues from these peptides. The action of CBP on bradykinin produces des-arg-BK, which can then be degraded by ACE. These peptide degradation mechanisms are extremely efficient and the half-life of bradykinin in plasma is less than thirty seconds.
3.1.3 Bradykinin and histamine release

Bradykinin is a pro-inflammatory peptide, and has been connected with allergy and associated inflammation [250,316]. Evidence of contact system activation has been found in asthma and rhinitis [234,235] and the peptide induces bronchoconstriction in asthmatic subjects [236]. Bradykinin also liberates histamine from RPMCs [237,303], suggesting a possible mechanism for the bronchoconstrictive action of bradykinin and providing a role for the peptide in allergy. However, RPMCs are quite dissimilar to most human histaminocytes in structure and function [82] and, particularly, in the responsiveness of this cell type to polybasic compounds and basic peptide secretagogues. These non-immunologic stimuli induce histamine release in a different way to immunologic ligands - they appear to bind directly to the cell membrane, bypassing the immunologic route of activation. In addition, the release characteristics of these immunologic and non-immunologic agonists are distinctively different. Experimental evidence suggests that polybasic agonism is due to the presence of positively charged moieties on the peptide or compound [256]. More specifically in basic peptides, secretory ability is thought to rely on basic amino acids at the N-terminal portion and hydrophobic residues at the C-terminus. The requirement for basicity is paramount and is the only common factor in agonists of this type, which otherwise vary enormously in structure and function [82,256,304].

Bradykinin is a basic molecule and is likely to act in a similar way to other basic peptides and polyaminic compounds. This study outlines the release profile for bradykinin, comparing it to other mast cell stimuli, including compound 48/80 and anti-IgE. Similarities between bradykinin and other basic compounds would indicate that bradykinin releases histamine from RPMCs as a direct result of its basicity.
3.2 RESULTS

3.2.1 Basic aspects of bradykinin-induced histamine release

3.2.1.1 Histamine release by bradykinin and basic agonists
Bradykinin liberated histamine from RPMCs at concentrations greater than $10^{-5}$ M. The peptide was less potent than compound 48/80, polylysine, substance P and somatostatin, but more active than neurotensin (fig. 3.1).

3.2.1.2 The effect of metabolic inhibitors
Metabolic inhibitors were tested in the absence of glucose. Cells were preincubated with the appropriate inhibitor in glucose-free Tyrode’s for 20 min prior to challenge with bradykinin. The histamine release induced by bradykinin ($10^{-4}$ M) was virtually identical in both the absence and presence of glucose. Antimycin A (1 μM) completely abolished secretion when used alone, or with 2-deoxyglucose (5 mM), but the sole use of 2-deoxyglucose proved ineffective (fig. 3.2).

3.2.1.3 Temperature dependence
RPMCs were equilibrated to 0, 25, 37 and 45 °C before the start of the experiment. Histamine liberation induced by bradykinin ($10^{-4}$ M) was virtually abolished at 0 and 45 °C, while optimal release was attained at 37 °C (fig. 3.3).

3.2.1.4 Kinetics
Secretion effected by bradykinin ($3 \times 10^{-4}$ M) was rapid, being essentially complete in 20 s, and analogous to that of compound 48/80 (1 μg/ml) where histamine was released within 10 s. In contrast, anti-IgE (1/300 dilution) released histamine slowly and gradually, and the process was still ongoing after 10 min (fig. 3.4).

3.2.1.5 The effect of pH on histamine release
In all pH experiments, cells were washed in full Tyrode’s, then added in a minimum volume to buffer adjusted to the required pH, and then allowed to equilibrate (10 min). Secretion was terminated with standard buffer (pH 7.3, 4 °C). Bradykinin ($3 \times 10^{-5}$ M) was surprisingly sensitive to slight variations in pH, and histamine release was virtually eliminated at pH 8.0.
Figure 3.1  Dose response curves for histamine release induced by bradykinin (●), compound 48/80 (△), polylysine (□), substance P (■), somatostatin (▲) and neurotensin (○) from RPMCs. Values are means ± SEM, n=4.
Figure 3.2 The effect of metabolic inhibitors antimycin A (1 μM) and 2-deoxyglucose (5 mM) on histamine release induced by bradykinin (10^{-4} M) from RPMCs. Values are means ± SEM, n=4.

Figure 3.3 Histamine secretion from RPMCs induced by bradykinin from RPMCs (10^{-4} M) at 0, 25, 37 and 45 °C. Values are means ± SEM, n=4.
Figure 3.4 The kinetics of histamine release induced by bradykinin (●; 3 x 10^{-4} M), compound 48/80 (■; 1 µg/ml) and anti-IgE (▲; 1/300 dilution). Cells used: RPMCS. Values are means ± SEM, n=4.
Figure 3.5 The effect of pH on histamine secretion induced by bradykinin (●, 3 x 10^{-4} M), compound 48/80 (▲, 0.6 μg/ml) and anti-IgE (■, 1/300 dilution). Cells used: RPMCs. Values are means ± SEM, n=6.
Compound 48/80 (0.6 \mu g/ml) and anti-IgE (1/300 dilution) were much less affected by the changes in pH (fig 3.5).

3.2.1.6 The effect of phosphatidyl serine on histamine release
Phosphatidyl serine (PS, 15 \mu g/ml) was added to the cells 15 s before challenge with the agonist. There was no effect on the action of bradykinin (10^{-6} - 10^{-4} M) and compound 48/80 (0.01 - 1 \mu g/ml). On the other hand, the effects of the ligand-directed secretagogues anti-IgE (1/30 000 - 1/300 dilution) and concanavalin A (1 - 100 \mu g/ml) were greatly enhanced by the phospholipid (figs 3.6 a, b, c).

3.2.1.7 The significance of extracellular calcium
RPMCs were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca^{2+}, 1 mM). Following this, the agonist (dissolved in CMF-Tyrode’s), was added to the cells and the experiment allowed to proceed as usual. Bradykinin-mediated histamine release was greater in the absence of calcium and after the brief pretreatment with EDTA than in the presence of the cation. Compound 48/80 displayed a similar profile, although secretion evoked by the polyamine was less affected by the different media. However, in both cases, at the highest concentration of secretagogue used, the secretion evoked in the presence of calcium was comparable to or greater than that evoked in CMF- and EDTA-Tyrode’s. In contrast, secretion effected by anti-IgE (1/300 dilution) was greater in the presence than in the absence of the cation (figs 3.7 a,b,c).

3.2.1.8 The effects of extracellular calcium
RPMCs were incubated in a range of calcium and strontium concentrations (0.01 - 20 mM) for 20 min prior to challenge with bradykinin (6 x 10^{-5} M), compound 48/80 (0.2 \mu g/ml) and anti-IgE (1/300 dilution). High concentrations of calcium suppressed secretion by bradykinin and compound 48/80, while strontium proved to be less inhibitory than calcium. Anti-IgE-induced histamine release was significantly enhanced by supraoptimal concentrations of strontium (10, 20 mM; \( p < 0.05 \)) (figs. 3.8 a, b, c).
Figure 3.6 (a, b) Histamine release induced by bradykinin (○, ●) and compound 48/80 (△, ▲) in the presence (open symbols) and absence (closed symbols) of PS (15 μg/ml). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.6 (c, d) Histamine release induced by anti-IgE (□, ■) and concanavalin A (▼, ▼) in the presence (open symbols) and absence (closed symbols) of phosphatidyl serine (15 μg/ml). Cells: RPMCs. Values are means ± SEM, n=4.
Figure 3.7 (a) Cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with bradykinin. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.7 (b) Cells were preincubated (5 min, 37 °C) in CMF- (△, no calcium), EDTA- (□, 0.1 mM) or calcium-containing (○, 1 mM) buffer, then challenged with compound 48/80. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.7 (c) Cells were preincubated (5 min, 37 °C) in CMF- (⊥, no calcium), EDTA- (®, 0.1 mM) or calcium-containing (□, 1 mM) buffer, then challenged with anti-IgE. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.8 (a) The effect of calcium (●) and strontium (○) on histamine release induced by bradykinin (6 x 10^{-3} M). RPMCs were incubated in the appropriate calcium and strontium concentrations for 20 min prior to challenge. Values are means ± SEM, n=4.
Figure 3.8 (b) The effect of calcium (▼) and strontium (▼) on histamine release induced by compound 48/80 (0.2 µg/ml). RPMCs were incubated in the appropriate calcium and strontium concentrations for 20 min prior to challenge. Values are means ± SEM, n=4.
Figure 3.8 (c) The effect of calcium (■) and strontium (○) on histamine release induced by anti-IgE (1/300 dilution). RPMCs were incubated in the appropriate calcium and strontium concentrations for 20 min prior to challenge. Cells used: RPMCs. Values are means ± SEM, n=4.
3.2.2 Inhibition of bradykinin-induced histamine release

3.2.2.1 Theophylline

RPMCs were simultaneously challenged with agonist and the inhibitor, theophylline (0.1 - 20 mM). The agonists were bradykinin (6 × 10⁻⁵ M, control histamine release: 50.0 ± 5.9%), compound 48/80 (0.6 µg/ml, 51.5 ± 4.9%) and anti-IgE (1/300 dilution, 42.8 ± 7.0%). These compounds were all similarly inhibited by theophylline (fig. 3.9).

3.2.2.2 Disodium cromoglycate

Bradykinin (5 × 10⁻⁵ M, 57.1 ± 3.5%), compound 48/80 (0.6 µg/ml, 53.3 ± 2.4%), concanavalin A (100 µg/ml, control histamine release: 52.5 ± 1.3%) and anti-IgE (1/300 dilution, 30.3 ± 3.2%) were all inhibited by disodium cromoglycate upon simultaneous challenge (fig. 3.10).

Inhibition of bradykinin- and compound 48/80-induced secretion plateaued at 55% and 30% respectively, rising to maximums of 75% and 60%. The maximum inhibition observed for concanavalin A was approximately 98%, while that of anti-IgE was 70%. The IC₅₀ of bradykinin, compound 48/80, concanavalin A and anti-IgE are 8.3 µM, 650 µM, 7.5 µM, 2.2 µM respectively.

3.2.2.3 Indomethacin

Bradykinin (3 × 10⁻⁵ M, control histamine release: 22.9 ± 4.9%), compound 48.80 (0.05 µg/ml, 29.3 ± 5.8%), substance P (10⁻⁵ M, 29.3 ± 4.3%), somatostatin (3 × 10⁻⁶ M, 31.1 ± 4.6%), concanavalin A (30 µg/ml, 20.8 ± 7.2%) and anti-IgE (1/300 dilution, 36.3 ± 3.9%) were added to RPMCs together with indomethacin. Histamine release was inhibited at and above 10⁻⁴ M indomethacin, but at concentrations below this, the compound was observed to potentiate secretion. (fig. 3.11).
3.2.3 Biochemical aspects of bradykinin-induced histamine release

3.2.3.1 PGD$_2$ release induced by bradykinin

Bradykinin ($10^{-4}$-$10^{-6}$ M) was observed to evoke the release of PGD$_2$, along with histamine, from purified RPMCs in dose-dependent fashion. The cells were confirmed viable and secreted histamine normally in response to anti-IgE (dilution 1/3000: 10.7 ± 5.2%, 1/1000: 20.4 ± 6.8%) (fig. 3.12).

3.2.3.2 Investigating cAMP levels during bradykinin-induced secretion

The purified cells obtained for the experiments were viable, and liberated histamine in response to anti-IgE and bradykinin (data not shown). Bradykinin ($3 \times 10^{-4}$ M) had no effect on cAMP production, while anti-IgE (1/300 dilution) induced an early rise in nucleotide levels. The average basal level of cAMP was 1.42 pmol/10$^6$ cells (fig. 3.13). The standard curve used in calculation of the results is given in fig. 3.14.
Figure 3.9 Theophylline-induced inhibition of secretion induced by bradykinin (●, $6 \times 10^{-5}$ M, 50.0 ± 5.9%), compound 48/80 (▲, 0.6 µg/ml, 51.5 ± 4.9%) and anti-IgE (■, 1/300 dilution, 42.8 ± 7.0%). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.10  Inhibition by disodium cromoglycate of histamine release induced by bradykinin (●, $5 \times 10^{-5}$ M, $57.1 \pm 3.5\%$), concanavalin A (□, 100 μg/ml, $52.5 \pm 1.3\%$), anti-IgE (🍋, 1/300 dilution, $30.3 \pm 3.2\%$) and compound 48/80 (▲, 0.6 μg/ml, $53.3 \pm 2.4\%$). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 3.11 Effect of indomethacin on histamine release evoked by bradykinin (●, 3 x 10^{-5} M, 22.9 ± 4.9%), compound 48.80 (▲, 0.05 μg/ml, 29.3 ± 5.8%), substance P (○, 10^{-5} M, 29.3 ± 4.3%), somatostatin (▲, 3 x 10^{-6} M, 31.1 ± 4.6%), concanavalin A (□, 30 μg/ml, 20.8 ± 7.2%) and anti-IgE (■, 1/300 dilution, 36.3 ± 3.9%). Cells used: RPMCs. Values are means ± SEM, n = 5.
Figure 3.12  PGD<sub>2</sub> (●) and histamine (○) release induced by bradykinin from RPMCs. Values are means ± SEM, n=4.
Figure 3.13  cAMP levels in RPMCs induced by bradykinin (●, 3 x 10^{-4} M) and anti-IgE (□, 1/300 dilution). Values are means, n=2, vertical bars indicate the range.
Figure 3.14 Typical standard curve for radioimmunoassay of cAMP.
3.3 DISCUSSION

The data presented confirm and extend previous findings that bradykinin is an effective histamine liberator from rat peritoneal mast cells [237,314]. Results indicate that bradykinin behaves in a similar fashion to the classic mast cell degranulator, compound 48/80. On a molar basis, bradykinin is less potent than compound 48/80, polylysine, substance P and somatostatin, though it is more active than neurotensin.

This similarity was evident at the earliest stages of the investigation. A characteristic of basic inducers such as compound 48/80 and polylysine is an extremely rapid rate of histamine release, essentially complete within 10 s [82]. By contrast, immunological stimuli tend to initiate comparatively slower rates of secretion. Thus, the rapid rate of bradykinin-induced secretion is reminiscent of compound 48/80-induced histamine release. It was also determined that secretion was an active, non-cytotoxic process as it was arrested by extremes of temperature and the action of metabolic inhibitors. However, the reactivity of bradykinin was affected by changes in pH, whereas the actions of other basic secretagogues were only slightly impaired. This result is further investigated in the following chapter.

PS selectively potentiated exocytosis induced by anti-IgE, but had absolutely no effect on bradykinin- and compound 48/80-induced secretion. The ability of the lipid to discriminate between immunological and non-immunological stimuli is attributed to its known ability to increase membrane fluidity and thus aid and maintain the formation of ‘calcium channels’.

The response to PS may be related to the differing calcium requirements of the two classes of agonist. Anti-IgE-induced secretion relies on an influx of calcium from the external medium, while polybasic agonists are thought able to meet the calcium requirement by liberating intracellular stores of the cation. Hence, polybasics are at least equivalently active liberators of histamine in the absence of external calcium. The magnitude of this effect varies according to the secretagogue, and ligands such as compound 48/80 [194], peptide 401 [194] and polylysine [118] exhibit near maximal release under these conditions. As a basic peptide, bradykinin would be expected to behave in the same way. The results of the present study support this contention and show that degranulation mediated by bradykinin and compound 48/80 is independent of the presence of external calcium, while that induced by anti-IgE was calcium-dependent.

A calcium regulatory site is thought to exist on the surface of the mast cell and its occupancy favours stabilization of the mast cell membrane. Brief pretreatment of the cell with EDTA or preincubation in CMF-Tyrode’s may abstract calcium from these sites. The consequent destabilization may assist the liberation of calcium from intracellular stores on the cytosolic face of the membrane, enhancing the cellular response. Correspondingly, the release
induced by bradykinin and compound 48/80 in EDTA-Tyrode’s was enhanced above the value obtained with these ligands in a calcium-free medium.

The proposed regulatory sites may also be involved in the inhibition of secretion that is observed upon incubation with supramaximal concentration of extracellular calcium. High concentrations of calcium may lead to oversaturation of these sites, perhaps encouraging stabilization of the membrane and leading to depression of exocytosis. In contrast, IgE-directed stimulation of the cell is not negatively affected by high levels of external calcium. Activation by IgE is thought to rely on an influx of extracellular calcium and an increase in the amount of external calcium would increase the influx of calcium. This may be sufficient to compensate for the stabilizing effects described above. Alternatively, high concentrations of calcium may selectively compete with polyamines for negatively charged binding sites on the membrane.

Strontium ions supported histamine release induced by bradykinin and compound 48/80. Calcium was inhibitory at high concentrations, while strontium had a lesser effect. With anti-IgE-induced release, strontium was only able to substitute for calcium at higher concentrations of this cation. Similar results have been reported for compound 48/80 and peptide 401, the mast cell degranulating (MCD) peptide from bee venom [194]. Mast cells pretreated with EDTA still released histamine in response to the polyamines, provided that calcium, strontium or barium, but not magnesium, were added with the inducer. Thus, it follows that these cations are able to promote histamine release in some way, perhaps by displacing bound calcium which is inaccessible to the action of EDTA and basic releasers. The findings of Foreman and Mongar [321] indicate that calcium has a greater affinity, but lesser efficacy, for ion binding sites than strontium. This might explain the smaller inhibitory effect of high strontium concentrations, when compared with calcium, when these ions displace bound calcium. Thus, the behaviour of bradykinin, in this respect, is again typical of polybasic releasers.

The anti-allergic drugs, theophylline and disodium cromoglycate, inhibited the release induced by all stimuli. There was no real difference in theophylline-mediated inhibition of non-immunologic stimuli and anti-IgE-induced secretion. However, cromoglycate was found to strongly inhibit concanavalin A-induced secretion while having a lesser effect on bradykinin- and compound 48/80-induced secretion. Past work has concluded that disodium cromoglycate inhibits secretion induced by IgE-directed ligands more effectively than that induced by pharmacological agents but, for reasons not obvious, inhibition of anti-IgE-mediated secretion was consistently not greater than that of bradykinin.
Bradykinin stimulates arachidonic acid metabolism, and particularly, the production of PGE\textsubscript{2} and PGI\textsubscript{2} and many of the effects of bradykinin are attributed to this process [251,335]. These effects may be blocked by suitable agents such as indomethacin and thus, its effect on bradykinin-induced histamine secretion was investigated, while taking into account the variable and imprecise effects of this compound on histamine release induced by other compounds [291]. The results obtained showed that the effect of indomethacin on bradykinin-induced secretion did not differ from that on other secretagogues: it inhibited secretion only at concentrations above 10\textsuperscript{-4} M and, as previously found, there was no distinction between immunological and non-immunological stimuli.

Bradykinin stimulated PGD\textsubscript{2} production in parallel with the release of histamine. This is the only prostaglandin produced by the rat mast cell and is generated upon stimulation of arachidonic acid metabolism by diverse agents [291]. Thus, the peptide resembles other immunologic and non-immunologic mast cell secretagogues that have been examined for PGD\textsubscript{2} production [317].

The intracellular level of cAMP is unaffected by the application of bradykinin and duplicates the response of the rat mast cell to polyanionic and basic peptides. This is quite unlike the response elicited by IgE-directed ligands. The latter produce a rapid, transient rise in intracellular cAMP within ten seconds of stimulation and this is followed, five minutes later, by a more gradual rise and fall [202]. This second rise is blocked by indomethacin and is associated with arachidonic acid metabolism and the release of PGD\textsubscript{2}. The initial rise in nucleotide levels is a general phenomenon with IgE-directed ligands and dextran, but is not seen with basic peptide and polyanionic stimuli, while the second increase has been observed irrespective of stimuli [203]. As such, it helps discriminate between the two classes of mast cell stimuli and the results obtained locate bradykinin along with other polybasic secretagogues.

Bradykinin possesses a basic amino acid at the N-terminus, which is deemed a necessary requirement for the induction of secretion by basic peptides. However, a further requirement has been reported to be a hydrophobic C-terminal portion and in bradykinin, a second basic arginine moiety is situated at this end of the molecule [302-304]. Nevertheless, bradykinin has a reaction profile similar to that of other basic inducers. For instance, release induced by bradykinin is independent of extracellular calcium, rapid and is unaffected by the application of PS. The response to the peptide thus appears typical of polybasic stimuli despite its reaction to slight increases in pH. It is clear that further investigation is needed to extend the similarity between bradykinin and the basic peptide and polycationic stimuli. Thus, the following chapters attempt to determine if the histamine-releasing ability of bradykinin is a function of its basicity or an expression of its kinin nature.
CHAPTER 4

Aspects of bradykinin-induced histamine release

4.1 INTRODUCTION

The histamine release profile in the RPMC of bradykinin closely mimics that of other basic peptide and polycationic mast cell stimuli. This chapter seeks to extend these parallels by looking more closely at the nature of the site of interaction between the peptide and the RPMC. Since bradykinin resembles basic mast cell agonists, it is possible that the peptide activates the cell through the putative polyamine receptor. Nonetheless, despite the probability that the secretory ability of bradykinin is distinct from its kinin function, a parallel study was conducted to examine the possible existence of specific bradykinin receptors on the RPMC.

4.1.1 Bradykinin receptors

Kinins are released in response to tissue injury and activate sensory pain fibres, contract venous smooth muscle and stimulate the synthesis of both prostacyclin (PGI₂) and endothelium-derived relaxing factor (EDRF). As a result, there is an increase in vascular permeability and blood flow to the damaged region and a full inflammatory response is obtained [206].

Bradykinin receptors have been classified into B₁ and B₂ subtypes. They are defined by the rank order of potency of bradykinin analogues [206]:

\[
\begin{align*}
B_1 & : \text{met-lys-BK} > \text{des-arg}^9\text{-BK} > \text{lys-BK} > \text{bradykinin} \\
B_2 & : \text{bradykinin} > \text{lys-BK} > \text{met-lys-BK} \gg \text{des-arg}^9\text{-BK}
\end{align*}
\]

B₁-receptor mediated responses are unique, as they only arise when allowed to develop slowly over several hours in isolated tissue preparations [336]. The primary agonist for the B₁-receptor is des-arg⁹-BK, the result of in vivo metabolism of bradykinin by peptidases, and activation of this receptor is antagonized by leu⁸-des-arg⁹-BK. It is conceivable that these receptors develop as a response to tissue injury, though evidence is scanty and the significance of these receptors remains to be established. This effect may have significance in the pathogenesis of human asthma (see Chapter 5).

The B₂-receptor is present in the majority of systems affected by kinins and antagonists have been developed with a view to their potential in therapy. Recent work with these suggests
that the $B_2$-receptor spans several distinct subtypes in different cells and tissues responsive to kinins [206,245]. Kinins affect numerous responses which have been shown to result from activation of different transduction systems in the various tissues. Bradykinin receptors are known to be linked to intracellular events including PI hydrolysis, the formation of cGMP and the opening of ion channels. Depending on the cell type, the peptide also activates the phospholipases C and A$_2$ (PLC, PLA$_2$) Activation of PLC causes generation of inositol phosphates and diacylglycerols from membrane phospholipids. These messengers cause the release of intracellular calcium and the stimulation of protein kinase C, respectively. Stimulation of PLA$_2$ results in the formation of arachidonic acid and eicosanoids such as PGE$_2$ and PGI$_2$. Bradykinin enhances the effects of these prostaglandins, producing a state of hyperalgesia. Responses to the peptide may often be blocked by the application of drugs that inhibit arachidonic acid metabolism [250].

Mediation of responses in diverse systems by subtypes of receptor is not unexpected. The potential exists for distinct bradykinin receptors to be coupled by different G-proteins to various second messenger pathways, leading to arachidonic acid production. The present system of classification is inexplicit and elucidation of these events could contribute towards a more precise definition of receptor sub-types.

### 4.1.2 $B_2$-receptor antagonists

The essential modification required to generate a $B_2$-receptor antagonist is the replacement of the pro$^7$ residue of bradykinin by an aromatic D-amino acid such as D-phe [315]. [D-phe$^7$]-BK is a partial antagonist of the $B_2$-receptor and further modifications produce antagonists such as [thi$^{5,8}$D-phe$^7$]-BK, [hyp$^2$thi$^{5,8}$D-phe$^7$]-BK and [hyp$^3$thi$^{5,8}$D-phe$^7$]-BK. The latter three analogues antagonize both B$_1$- and B$_2$-receptor types.

Recent work has reported an agonist action at ‘classical’ B$_2$-receptor systems by [thi$^{5,8}$D-phe$^7$]-BK: it potentiates the twitch tension (neurogenic) response in the presynaptic kinin receptor of the vas deferens and increases PI turnover in the neuronal cell line N1E-115. In the rat vas deferens postjunctional receptor site, the analogue is also antagonistic. Further studies are required to confirm the existence of a distinct, novel receptor in neuronal systems where kinins are known to influence neurotransmitter release. This receptor may not be present in all neuronal cell types [315].

### 4.1.3 The polyamine receptor

Basic agonists are all thought to act at a common site on the RPMC, the putative polyamine receptor. This receptor appears to be non-specific and accepts a wide range of basic
substances, conforming to broad structure-activity requirements. These agonists include basic neuropeptides such as substance P and somatostatin and polycations such as compound 48/80, the mast cell degranulating peptide from bee venom (MCD peptide) and polylysine. These substances are unrelated save in their ability to release histamine from RPMCs.

Extensive structure-activity studies have revealed several features of peptide molecules that determine their potency as RPMC agonists. These studies have largely concentrated on substance P, as this peptide is found in the skin and is possibly involved in neurogenic transmission of wheal and flare. The presence of basic amino acids, usually arginine and lysine residues, at the N-terminal end was found to be essential for activity, together with a blocked carboxyl group at the C-terminal. Also, active peptides such as substance P and somatostatin have hydrophobic residues, including phenylalanine and tryptophan, in the C-terminal region. This is thought to increase the affinity of the peptide for the binding site and, consequently, to enhance release. This was demonstrated by studies involving a hybrid molecule, formed from the N-terminal sequence of substance P and dodecylamine [304]. Results indicate that the properties of substance P which relate to histamine release are the specific structure of the N-terminal region, in which basic amino acids are essential, and a non-specific hydrophobic property of the C-terminal sequence. In summary, the peptide is thought to anchor to the RPMC membrane via its hydrophobic C-terminal, freeing the basic amino acids at the N-terminus for cell activation.

A variety of basic, sensory neuropeptides induce secretion from RPMCs, as do a wide range of polycationic agents. Histamine release induced by these agents has some characteristic features in common - the process is rapid, essentially independent of added calcium or phospholipids, not mediated through cell-fixed antibody, inhibited by antagonists of the putative polyamine receptor, and is species and tissue specific. Bradykinin fulfills the first two conditions and has a similar pattern of species- and tissue-selectivity; this study seeks to extend these parallels. The pH effects observed in the third chapter were studied and the possibility of synergistic action between bradykinin and anti-IgE was also examined. Enhancement of IgE-mediated secretion by bradykinin would be of clinical interest.
4.2 RESULTS

4.2.1 The involvement of bradykinin receptors

4.2.1.1 An investigation of bradykinin analogues

The rank order of potency of bradykinin analogues was ascertained in an attempt to examine the possibility of a bradykinin receptor as the site of interaction between the peptide and the mast cell. The peptides were found to induce histamine release, with the following rank order of potency:

met-lys-BK > lys-BK > [lys\(^{-}\)]-BK > bradykinin > ile-ser-BK > des-arg\(^{9}\)-BK

The order of potency runs roughly like that of a B\(_{2}\)-receptor and accordingly, some B\(_{2}\)-receptor antagonists were investigated (fig. 4.1).

4.2.2 An investigation of B\(_{2}\)-receptor antagonists

[D-phe\(^{7}\)]-BK, [thi\(^{5,8}\)D-phe\(^{7}\)]-BK, [hyp\(^{2}\)thi\(^{5,8}\)D-phe\(^{7}\)]-BK and [hyp\(^{3}\)thi\(^{5,8}\)D-phe\(^{7}\)]-BK are B\(_{2}\)-receptor antagonists. They freely liberated histamine in the rank order of potency:

[D-phe\(^{7}\)]-BK = [thi\(^{5,8}\)D-phe\(^{7}\)]-BK

> [hyp\(^{2}\)thi\(^{5,8}\)D-phe\(^{7}\)]-BK = bradykinin

> [hyp\(^{3}\)thi\(^{5,8}\)D-phe\(^{7}\)]-BK

[D-phe\(^{7}\)]-BK is a partial B\(_{2}\)-receptor antagonist, while the others are reputed to be complete antagonists. The secretory potential is thus obviously unrelated to their antagonistic activity (fig 4.2).

4.2.2.3 The effect of extracellular calcium on secretion induced by bradykinin analogues and receptor antagonists.

RPMCs were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca\(^{2+}\), 1 mM). Following this, RPMCs were challenged with lys-BK, des-arg\(^{9}\)-BK, [D-phe\(^{7}\)]-BK and bradykinin and the experiment allowed to proceed as usual. Secretion induced by all these peptides was not dependent upon calcium in the external environment (figs. 4.3 a, b, c, d).
Figure 4.1  Histamine release induced by bradykinin (●) and its analogues, lys-BK (▲), des-arg²-BK (▼), met-lys-BK (■), [lys¹]-BK (○), ile-ser-BK (□) from RPMCs. Values are means ± SEM, n=4.
Figure 4.2  Histamine release induced by bradykinin (●) and B\textsubscript{2}-receptor antagonists, [D-phe\textsuperscript{7}]-BK (■), [thi\textsuperscript{5,8}D-phe\textsuperscript{7}]-BK (▲), [hyp\textsuperscript{2}thi\textsuperscript{5,8}D-phe\textsuperscript{7}]-BK (△) and [hyp\textsuperscript{3}thi\textsuperscript{5,8}D-phe\textsuperscript{7}]-BK (▼). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.3 (a) Cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with lys-BK. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.3 (b) Cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with des-arg⁹-BK. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.3 (c) Cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with [D-phe²]-BK. Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.3 (d) Cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with bradykinin. Cells used: RPMCs. Values are means ± SEM, n=4.
4.2.2 Nature of the binding site

4.2.2.1 The significance of cell bound antibody

Sprague-Dawley rats, bred in a sterile environment (SPF, 250 g) to ensure a minimum of antibody production, were used in this experiment. The animals were sacrificed as soon as possible after their arrival of the animals, and peritoneal cells were isolated and challenged with the usual range of concentrations of anti-IgE, anti-IgG and bradykinin. A low level of surface bound antibody was confirmed by the negligible response of the cells to the two immunological ligands. In contrast, the response to bradykinin was unimpaired (fig 4.4).

4.2.2.2 The effect of the polyamine inhibitor, BDTA

The polyamine inhibitor, benzyltrimethyltetradecylammonium chloride (BDTA), is a compound that antagonizes the secretion induced by polyamines and basic peptides when presented simultaneously to RPMCs [82,318]. The compound dose-dependently inhibited the actions of bradykinin (3 x 10^{-5} M, 28.3 ± 2.8%) and compound 48/80 (0.25 μg/ml, 44.6 ± 3.1%), but potentiated that of anti-IgE (1/300 dilution, 17.7% ± 1.6%) (fig 4.5).

4.2.2.3 The effect of polyglucoses (MWs 1000, 2600)

This experiment compares the action of bradykinin to that of dextran. Dextran (MW 70 000) is thought to activate the cell through glucoreceptors [345], since preincubation with high concentrations of polyglucoses inhibits dextran-mediated secretion. The RPMCs were preincubated (5 min) with the appropriate polyglucose concentrations before challenge with either bradykinin or dextran. Polyglucoses of MWs 2100 and 1600 dose-dependently inhibited the effect of dextran (30 μg/ml, + PS 15 μg/ml, 30.2 ± 4.7%), but potentiated that of bradykinin (0.25 x 10^{-5} M, 20.0 ± 1.5%) (fig 4.6 a,b).

4.2.2.4 The effects of cold on the activated state

Peritoneal cells are unresponsive at 4 °C, and this feature was employed in inspecting the activated state of the cells stimulated with bradykinin (3 x 10^{-5} M, 31.2 ± 3.0%), compound 48/80 (0.1 μg/ml, 29.2 ± 3.6%) and anti-IgE (1/300 dilution, 25.6 ± 4.3%). The cells were incubated with the secretagogue at 0 °C (10 min). After this period, they were transferred to 37 °C and the reaction between cells and agonist allowed to proceed for 10 min. A portion of cells was also incubated at 0 °C without the agonist and was used as a control, to see if
Figure 4.4  Histamine secretion from specific-pathogen free rats by bradykinin (●), anti-IgE (▼) and anti-IgG (▲). Values are means ± SEM, n=4.
Figure 4.5  The effect of BDTA on histamine release from RPMCs evoked by bradykinin (●, 3 x 10⁻⁵ M, 28.3 ± 2.8%) and compound 48/80 (▲, 0.25 µg/ml, 44.6 ± 3.1%) and anti-IgE (■, 1/300 dilution, 17.7 ± 1.6%). Values are means ± SEM, n=4.
Figure 4.6 (a) The effect of polyglucose MWs 1000 (▼) and 2600 (○) on histamine release induced by bradykinin (2.5 x 10^{-5} M, 20.0 ± 1.5%). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.6 (b) The effect of polyglucose MWs 1000 (▼) and 2600 (○) on histamine release induced by dextran (30 μg/ml, + PS 15 μg/ml, 30.2 ± 4.7%). Cells used: RPMCs. Values are means ± SEM, n=4.
Figure 4.7  RPMCs were preincubated (10 min) at 0 °C in both the presence and absence of the agonists, bradykinin (3 x 10^{-5} M, 31.2 ± 3.0%), compound 48/80 (0.1 μg/ml, 29.2% ± 3.6) and anti-IgE (1/300 dilution, 25.6 ± 4.3%). After 10 min, they were warmed to 37 °C and the reaction allowed to proceed as usual. Control levels of histamine release, shown in brackets, were obtained by challenging with the agonist at 37 °C without preincubation at 0 °C. Values are means ± SEM, n=4; * denotes value significantly less than control value, p < 0.05.
incubation at 0 °C affected the subsequent cellular response to the secretagogue. These cells responded normally to the agonist when challenged upon transfer to 37 °C. Control responses were derived from cells held at 37 °C throughout. It was found that only the response of mast cells incubated with anti-IgE was significantly diminished by cold, but the reaction of the cells to bradykinin and compound 48/80 was unaffected (fig 4.7).

4.2.2.5 Cross-desensitization of polybasic compounds to bradykinin

The response of RPMCs to basic agonists has been shown to be desensitized by incubation (37 °C, 10 min) with the agent in the presence of theophylline (10 mM). Initial experiments established that the inhibitory effect of theophylline was reversible - cells treated in this way regained full responsiveness after thorough washing.

RPMCs were incubated with bradykinin (5 x 10^{-5} M) in the presence of theophylline (10 mM). Cells were washed thrice (3 ml, 37 °C) and incubated for 10 min in between washes (37 °C). This treatment was aimed towards ridding the cells of theophylline. They were then re-challenged with the agonist, and the reaction allowed to proceed under the usual conditions (37 °C, 10 min). Control releases were established from cells treated with theophylline alone. Bradykinin (5 x 10^{-5} M, 51.2±5.1%), neurotensin (10^{-4} M, 13.6±4.2%), somatostatin (5 x 10^{-5} M, 40.7±2.5%), substance P (3 x 10^{-5} M, 66.5±2.3%) and compound 48/80 (0.6 μg/ml, 57.5±3.3%) were all significantly desensitized to some extent (p < 0.05), while responses to anti-IgE (1/300 dilution, 21.8±5.6%) and concanavalin A (100 μg/ml, 23.8±4.1%) were slightly, but not significantly, potentiated (fig 4.8).

4.2.2.6 Cross-desensitization of bradykinin analogues to bradykinin

Initial tests were carried out as in the previous experiment. RPMCs were incubated with bradykinin (5 x 10^{-5} M) in the presence of theophylline (10 mM). Cells were washed thrice (3 ml, 37 °C) and incubated for 10 min in between washes (37 °C). They were then re-challenged with bradykinin and the reaction allowed to proceed under the usual conditions (37 °C, 10 min). Control releases were established from cells treated with theophylline alone. The bradykinin analogues lys^1-BK (5 x 10^{-5} M, 65.1±3.1%), lys-BK (2 x 10^{-5} M, 40.9±2.6%), met-lys-BK (3 x 10^{-5} M, 74.9±6.2%), ile-ser-BK (5 x 10^{-5} M, 52.2±3.9%) and [D-phe^7]-BK (10^{-5} M, 56.0±3.1%) were all successfully cross-desensitized to bradykinin in the presence of theophylline (fig 4.9).
Figure 4.8 RPMCs were preincubated in the presence of the agonist and theophylline for 10 min. The cells were then washed thrice and rechallenged with the agonist. Control releases for the agonists, established from cells preincubated with theophylline alone, were: bradykinin (5 x 10^{-5} M, 51.2 ± 5.1%), neurotensin (10^{-4} M, 13.6 ± 4.2%), somatostatin (5 x 10^{-5} M, 40.7 ± 2.5%), substance P (3 x 10^{-5} M, 66.5 ± 2.3%) compound 48/80 (0.6 μg/ml, 57.5 ± 3.3%), anti-IgE (1/300 dilution, 21.8 ± 5.6%) and concanavalin A (100 μg/ml, 23.8 ± 4.1%). Values are means ± SEM, n = 5; * denotes values significantly less than control value, p < 0.05.
Figure 4.9  RPMCs were preincubated in the presence of bradykinin (5 x 10^{-5} M) and theophylline (10 mM) for 10 min. The cells were then washed thrice and rechallenged with bradykinin, lys^1-BK (5 x 10^{-5} M, 65.1 \pm 3.1\%), lys-BK (2 x 10^{-5} M, 40.9 \pm 2.6\%), met-lys-BK (3 x 10^{-5} M, 74.9 \pm 6.2\%), ile-ser-BK (5 x 10^{-5} M, 52.2 \pm 3.9\%) and [D-phe^7]-BK (10^{-5} M, 56.0 \pm 3.1\%). Control releases were established from cells preincubated with theophylline alone. Values are means \pm SEM, n = 5; * denotes values significantly less than control value, p < 0.05.
4.2.3 An investigation of pH effects

4.2.3.1 The effect of pH on bradykinin and some analogues

Peritoneal mast cells were challenged with varying concentrations of bradykinin, lys-BK, [D-phe^7]-BK and compound 48/80 at pH 7.0 and 8.0. The response at pH 7.0 was slightly greater than at physiological pH (7.3), as previously shown (fig 3.8). At pH 8.0, cells were completely unresponsive to bradykinin throughout the concentration range. Lys-BK and [D-phe^7]-BK were also ineffective except at the highest concentration (10^-4 M), where secretion levels were, respectively, 35.2% and 82.0% of the corresponding release at pH 7.0. Compound 48/80 was used as a control, and histamine secretion produced by this agent was unaffected by the difference in pH (fig 4.10 a,b,c,d).

4.2.3.2 Comparison with other basic mast cell agonists

Histamine secretion evoked by basic mast cell agonists, bradykinin (2 x 10^-5 M), neurotensin (10^-4 M), somatostatin (20 μg/ml), compound 48/80 (0.5 μg/ml), polylysine and polyarginine (both 10 μg/ml) was compared at pH 7.0 and pH 8.0. Of these, only bradykinin-induced secretion was significantly suppressed at pH 8.0 (fig 4.11).

4.2.3.3 The persistence of the inhibitory effect of pH 8.0

RPMCs were incubated (10 min) at pH 8.0 in the presence of bradykinin (10^-4 M), then washed once and resuspended at pH 7.0. The cells were then rechallenged with bradykinin and compound 48/80 (10^-6 and 10^-4 M, 0.1 - 1.0 μg/ml respectively). In both cases, histamine release was undiminished by the pretreatment. Control releases were established from cells held at pH 7.0 throughout (fig. 4.12).

4.2.3.4 The effect of pH on RPMCs

This experiment was an attempt to elucidate the effect of pH on the cells alone. Mast cells were preincubated at pH 8.0 for 10 min, restored to pH 7.3 as above and challenged. The responses to bradykinin (10^-6-10^-4 M), compound 48/80 (0.01-0.1 μg/ml) were undiminished, indicating that the cells were not irreversibly affected by incubation at this pH (fig. 4.13).
Figure 4.10 (a) RPMCs were challenged with a range of bradykinin concentrations at pH 7.0 (○) and pH 8.0 (▼). Values are means ± SEM, n=4.
Figure 4.10 (b) RPMCs were challenged with a range of lys-BK concentrations at pH 7.0 (○) and pH 8.0 (▼). Values are means ± SEM, n=4.
Figure 4.10 (c) RPMCs were challenged with a range of [D-phe\(^7\)]-BK concentrations at pH 7.0 (○) and pH 8.0 (▼). Values are means ± SEM, n=4.
Figure 4.10 (d) RPMCs were challenged with a range of compound 48/80 concentrations at pH 7.0 (○) and pH 8.0 (△). Values are means ± SEM, n=4.
Figure 4.11  Histamine secretion from RPMCs evoked by basic mast cells agonists, bradykinin (BK, 2 x 10^{-5} M) neurotensin (NT, 10^{-4} M), somatostatin (Som, 20 μg/ml), compound 48/80 (0.5 μg/ml), polylysine and polyarginine (both 10 μg/ml) was compared at pH 7.0 and pH 8.0. Values are means ± SEM, n=4;  * denotes value significantly different to value at pH 7.0, p < 0.05.
Figure 4.12  RPMCs were incubated (10 min) at pH 8.0 in the presence of bradykinin (10^{-4} M), washed once and resuspended at pH 7.0. The cells were then rechallenged with (a) bradykinin and (b) compound 48/80. Values are means ± SEM, n=4.
RPiMCs were preincubated at pH 8.0 for 10 min, restored to pH 7.3 as above and challenged with bradykinin and compound 48/80. Values are means ± SEM, n=4.
4.2.3.5 The effect of pH on the bradykinin peptide

To investigate the effect of pH on the bradykinin peptide, bradykinin and compound 48/80 were prepared at pH 8.0, in stock solutions of $10^{-3}$ M and 10 µg/ml respectively. The compounds were left to stand thus for 15 min. The secretagogues were then diluted for the experiment in buffer (pH 7.0), immediately prior to use, such that the final pH of the reaction mixture was pH 7.3 (the physiological pH used in these experiments). The activity of the compounds preincubated at pH 8.0 was equivalent to the activity of those preincubated at pH 7.0 (fig. 4.14 a,b).

4.2.4 Synergy

4.2.4.1 Synergy between bradykinin and anti-IgE

This experiment investigated the possible effects of bradykinin on anti-IgE induced secretion. Bradykinin ($10^{-5}$, $2 \times 10^{-5}$ M) or compound 48/80 (0.03 µg/ml) were simultaneously presented to RPMCs with anti-IgE (1/3000, 1/1000 dilution). The theoretically predicted levels of secretion were calculated by adding histamine release levels obtained by challenging cells with each agonist alone.

The levels of secretion induced by the agents alone were:

- bradykinin $10^{-5}$ M: 3.5 ± 1.2%, $2 \times 10^{-5}$ M: 16.3 ± 2.4%
- compound 48/80 0.03 µg/ml: 4.9 ± 0.7%
- anti-IgE dilutions 1/3000: 10.7 ± 5.2%, 1/1000, 20.4 ± 6.8%

The combination of bradykinin and anti-IgE gave a significantly enhanced level of release ($p < 0.05$). This effect was greater for lower levels of predicted release. Combinations of compound 48/80 and anti-IgE, and peptide and polyamine merely released the predicted levels of histamine (fig. 4.15 a, b).

4.2.4.2 Synergy between anti-IgE and other peptides

The peptides bradykinin ($2 \times 10^{-5}$ M), somatostatin (3 µg/ml), substance P ($10^{-6}$ M), neurotensin ($3 \times 10^{-5}$ M) and compound 48/80 (0.03 µg/ml) were individually presented in combination with anti-IgE (dilutions 1/3000, 1/1000). Of these, only bradykinin displayed significant synergy with anti-IgE (fig. 4.16 a, b).
Predicted levels of histamine release to be obtained from the combination of agonists were derived as in the above experiment (section 4.2.4.1). The levels of secretion induced by the agents alone were:

- **bradykinin** 2 x 10^{-5} M: 7.0 ± 2.2%
- **somatostatin** 3 μg/ml: 10.2 ± 4.4%
- **substance P** 10^{-6} M: 6.9 ± 2.8%
- **neurotensin** 3 x 10^{-5} M: 22.6 ± 9.5%
- **compound 48/80** 0.03 μg/ml: 27.4 ± 3.3%
- **anti-IgE** dilutions 1/3000: 8.4 ± 5.4%, 1/1000: 20.6 ± 9.5%

### 4.2.4.3 Synergy between anti-IgE and bradykinin in the release of PGD2

RPMCs were simultaneously challenged with anti-IgE (dilutions 1/1000, 1/3000) and bradykinin (2 x 10^{-5} M, 3 x 10^{-5} M).

Levels of PGD2 release obtained from the agents alone were:

- **bradykinin** 2 x 10^{-5} M: 13.9 ng PGD2/10^6 cells (21.6, 6.2 ng PGD2/10^6 cells)
- 3 x 10^{-5} M: 14.4 ng PGD2/10^6 cells (21.2, 7.6 ng PGD2/10^6 cells)
- **anti-IgE** diln. 1/1000: 13.1 ng PGD2/10^6 cells (18.5, 7.7 ng PGD2/10^6 cells)
- diln. 1/3000: 5.8 ng PGD2/10^6 cells (5.1, 6.5 ng PGD2/10^6 cells)

Levels of histamine secretion induced by the agents alone were:

- **bradykinin** 2 x 10^{-5} M: 3.5% (2.3%, 4.7%)
- 3 x 10^{-5} M: 16.3% (13.9%, 18.7%)
- **anti-IgE** diln. 1/3000: 10.7% (7.0%, 14.2%)
- diln. 1/1000: 20.4% (18.2%, 22.8%)

No significant enhancement of prostaglandin release was observed (fig. 4.17).
Figure 4.14 (a, b) Histamine release induced by bradykinin (○, •) and compound 48/80 (□, ■). Compounds were preincubated either at pH 7.0 (open symbols) or at pH 8.0 (closed symbols). The difference between values at pH 7.0 and 8.0 were not significant. Cells used: RPMCs. Values are means ± SEM, n=4.
(a) Simultaneous challenge with bradykinin & anti-IgE

Figure 4.15 (a) RPMCs were simultaneously challenged with bradykinin \( (10^{-5}, 2 \times 10^{-5} \text{ M}) \) and anti-IgE \( (1/3000, 1/1000 \text{ dilution}) \). Theoretically predicted levels of secretion were calculated by adding histamine release levels obtained by challenge of cells with each agonist alone. The levels of secretion induced by the agents alone were: bradykinin \( 10^{-5} \text{ M}: 3.5 \pm 1.2\% \), \( 2 \times 10^{-5} \text{ M}: 16.3 \pm 2.4\% \); anti-IgE dilutions \( 1/3000: 10.7 \pm 5.2\% \), \( 1/1000: 20.4 \pm 6.8\% \). * Denotes values significantly different to predicted values, \( p < 0.05 \). Values are means ± SEM, \( n=4 \).
Figure 4.15 (b) RPMCs were simultaneously challenged with bradykinin (10^{-5}, 2 \times 10^{-5} \text{ M}) and compound 48/80 (0.03 \mu g/ml). Theoretically predicted levels of secretion were calculated by adding histamine release levels obtained by challenge of cells with each agonist alone. The levels of secretion induced by the agents alone were: Bradykinin 10^{-5} \text{ M}: 3.5 \pm 1.2\%, 2 \times 10^{-5} \text{ M}: 16.3 \pm 2.4\%; compound 48/80 0.03 \mu g/ml: 4.9 \pm 0.7\%. The differences between the experimental and predicted levels were all not found to be statistically significant. Values are means \pm SEM, n=4.
(a) Simultaneous challenge with peptide & anti-IgE, 1/3000 dilution

Figure 4.16 (a) RPMCs were challenged simultaneously with anti-IgE (fig.(a): dilution 1/3000, fig.(b): dilution 1/1000) and each of the peptides bradykinin (2 x 10^{-5} M), somatostatin (3 μg/ml), substance P (10^{-6} M), neurotensin (3 x 10^{-5} M) and compound 48/80 (0.03 μg/ml). Levels of histamine release obtained from the compounds alone were: bradykinin 2 x 10^{-5} M: 17.0 ± 2.2%; somatostatin 3 μg/ml: 10.2 ± 4.4%; substance P 10^{-6} M: 6.9 ± 2.8%; neurotensin 3 x 10^{-5} M: 22.6 ± 9.5%; compound 48/80 0.03 μg/ml: 27.4 ± 3.3%; anti-IgE dilutions 1/3000: 8.4 ± 5.4%, 1/1000 : 20.6 ± 9.5%. The difference between experimental and actual levels of secretion was statistically significant only in the case of bradykinin for both concentrations of anti-IgE (*, p < 0.5). Values are means ± SEM, n=4.
(b) Simultaneous challenge with peptide & anti-IgE, 1/1000 dilution

Figure 4.16 (b) see previous page for comments.
Figure 4.17 RPMCs were simultaneously challenged with anti-IgE (1/3000, 1/1000 dilution) and bradykinin (2 x 10^{-5}, 3 x 10^{-5} M). Predicted levels of prostaglandin secretion were calculated by adding prostaglandin release levels obtained by challenge of cells with each agonist alone. Levels of PGD\(_2\) release obtained from the agents alone were: bradykinin 2 x 10^{-5} M: 13.9 ng PGD\(_2\)/10^6 cells, 3 x 10^{-5} M: 14.4 ng PGD\(_2\)/10^6 cells; anti-IgE diln. 1/1000: 13.1 ng PGD\(_2\)/10^6 cells, diln. 1/3000: 5.8 ng PGD\(_2\)/10^6 cells. Levels of histamine secretion induced by the agents alone were: bradykinin 2 x 10^{-5} M: 3.5%, 3 x 10^{-5} M: 16.3%; anti-IgE diln. 1/3000: 10.7%, 1/1000: 20.4%. Anti-IgE did not synergise with bradykinin to produce significantly enhanced levels of release of PGD\(_2\). Values are means, n=2, vertical bars indicate the range.
4.3 DISCUSSION

Similarities between bradykinin and the polyamine compound 48/80 were observed in the third chapter. In the light of the nature of these polybasic stimuli [82], it is probable that bradykinin is of this class of mast cell secretagogue.

The possibility that bradykinin may act through cell-fixed antibodies was investigated. Peritoneal cells of normally housed rats possess significant quantities of surface bound IgE and IgG and challenge with ligands directed against these proteins causes exocytosis. These cells responded normally to both bradykinin and anti-IgE. By contrast, cells obtained from SPF animals were relatively free of cell bound antibody and these cells were almost totally refractory to anti-IgE and slightly hyperresponsive to bradykinin.

A token presence of immunoglobulin on the mast cell membrane would account for the nominal response to anti-IgE and anti-IgG observed with SPF cells. IgE, in particular, may be generated in response to innocuous substances, despite the absence of pathogens. The full response on stimulation with bradykinin is indicative of a IgE-independent release mechanism. The apparent hyperresponsivity of cells from SPF animals to challenge with bradykinin may be a steric effect, where the absence of bulky immunoglobulin molecules may facilitate the approach of bradykinin to the mast cell membrane.

Continuing this line of investigation, experiments were conducted with BDTA, a tridecyl derivative of benzalkonium chloride (BAC). The BACs are positively charged quaternary ammonium compounds which are thought selectively to inhibit polyamine-induced histamine secretion. Antagonism is not extended to other types of stimuli, such as the immunologic, cytotoxic and ionophore releasers [82]. BDTA was shown to be the most potent of the BAC series [318] and their use is thought to provide evidence for the existence of a polyamine receptor. Simultaneous presentation of BDTA and polyamine is essential for experimental validity [318] and, under these conditions, the interaction is surmountable and competitive. Bradykinin was antagonized in a similar fashion to other polyamines, indicating that the peptide may bind to the mast cell membrane.

Experiments were carried out to exclude the possibility of a glucoreceptor-mediated interaction between bradykinin and the RPMC. The mode of action of dextran differs from that of the polybasic agents. It is assumed to activate the mast cell by cross-linking specific glucoreceptors on the cell membrane and high concentrations of low molecular weight glucose polymers specifically inhibit histamine release induced by dextran [319]. The response of the mast cell to bradykinin was unaffected by treatment with polyglucoses, indicating that a glucoreceptor-mediated mode of action is unlikely. Moreover, release
evoked by bradykinin is faster than that of dextran, and strontium was able to substitute for 
calcium, a feature not noted with dextran [319].

The decay of the mast cell response to antigen, induced by incubation in the cold, is said to 
be related to the activated state of the cells. When cells are stimulated with antigen, there 
is a transient increase in the permeability of the membrane to calcium. In the cold, this state 
decays rapidly and permanently. Upon restoration to physiological temperatures, the cell 
releases sub-maximal levels of histamine due to reduced uptake of external calcium. As 
antigen-induced secretion is dependent on extracellular calcium, it is susceptible to such 
treatment. In contrast, polybasic stimuli are resistant to cold treatment as they may act 
independently of external calcium, through their ability to mobilize internal stores of the 
cation. Bradykinin was not affected by preincubation in the cold and therefore, the peptide 
probably meets the calcium requirement for secretion by mobilizing internal stores. Thus, 
bradykinin demonstrates another similarity to compound 48/80.

Desensitization to IgE-directed ligands may be demonstrated by incubating RPMCs with the 
ligand in the absence of calcium. On restoration to optimum conditions and re-challenge 
with the agonist, a sub-maximal response is observed. This is thought to be due to activation 
or depletion of an element that may be part of the transduction pathway between receptor 
stimulation and histamine release. Accordingly, as antigen and anti-IgE work through the 
same receptor, it is possible to cross-desensitize the cell with these stimuli. In line with this 
reasoning, these stimuli do not cross-desensitize to polybasics.

Cross-desensitization has also been demonstrated between the basic peptide and polyaminic 
agonists. Bradykinin has been proposed as a member of this class of stimuli and should 
therefore cross-desensitize to these secretagogues. However, the calcium independence of 
polycation-induced degranulation precludes the use of calcium-free media to expedite 
desensitisation. To overcome this problem, polycation-induced secretion was inhibited with 
theophylline. Control experiments showed the cells to be desensitised to bradykinin under 
these conditions but fully responsive after theophylline treatment alone and subsequent 
removal of the inhibitor by washing. Cross-desensitisation between bradykinin and the other 
ligands was established but no desensitisation occurred between bradykinin and the 
immunological stimuli. Thus the peptide may be inferred to occupy the same activation site 
as that utilized by other polybasic stimuli.

The desensitization experiment agrees with existing evidence for two, distinct mechanisms 
of stimulation for IgE-directed ligands and basic agonists. Moreover, polyamine agonists 
collectively act at a non-specific receptor or binding site. The structural requirements for
activation of this binding site are extremely liberal, in biological terms. Hence, bradykinin and the other polybasics cross-desensitize as shown.

The above series of experiments have drawn a parallel between bradykinin and polyamines, indicating that the mechanistic pathway for bradykinin-induced histamine release is distinct from that of dextran and IgE-directed ligands. The exact mechanism has yet to be determined, but the evidence indicates that bradykinin probably acts through the polyamine receptor.

The possibility that bradykinin could act through defined receptors was investigated through the use of specific analogues and antagonists. The rank order of potency of bradykinin agonists appeared to be similar to that of a B<sub>2</sub>-receptor, in that lys-BK and bradykinin were more active than des-arg<sup>9</sup>-BK. This latter analogue was about half as potent as bradykinin on a molar basis. However, met-lys-BK was a better secretagogue than either bradykinin or lys-BK, whilst being a weaker B<sub>2</sub>-receptor agonist. Moreover, the B<sub>2</sub>-receptor antagonists [D-phe<sup>7</sup>]-BK, [thi<sup>3,8</sup>D-phe<sup>7</sup>]-BK, [hyp<sup>3</sup>thi<sup>5,8</sup>D-phe<sup>7</sup>]-BK and [hyp<sup>2</sup>thi<sup>5,8</sup>D-phe<sup>7</sup>]-BK were also potent histamine releasers. Thus, it is unlikely that bradykinin acts through bradykinin receptors. Instead, these findings are in agreement with the concept of bradykinin as a basic peptide secretagogue. This is further supported by the calcium independence of secretion induced by the analogues lys-BK, des-arg<sup>9</sup>-BK and the antagonist [D-phe<sup>7</sup>]-BK, and by the general characteristics of bradykinin-induced histamine release from RPMCs (see chapter 3). These results are in also agreement with those of Devillier et al. [300,326], whose studies concluded that the receptor site involved in kinin-induced degranulation was not a B<sub>2</sub>-receptor.

Bradykinin is unusual among polycationic histamine-releasing peptides as it possesses a basic arginine residue at the C-terminal. However, this residue obviously contributes to the secretory activity of bradykinin as des-arg<sup>9</sup>-BK released histamine with only half the potency of the native molecule. Similar findings were reported by Devillier et al. [303]. Nonetheless, since N-acetylation of the N-terminal arginine apparently leads to a totally inactive compound, Ac-BK [300], it appears that the N-terminal arginine may be of major importance for the secretory activity of bradykinin.

Along with the requirement for basic groups at the N-terminal, it is necessary for these groups to be coupled to an hydrophobic structure, such as the C-terminal sequence of substance P or the 12 carbon chain of dodecylamine [304]. In this study, the bradykinin antagonists tested were noticeably more active than bradykinin. These results are in agreement with other workers [326,328]. Thus, the increase in activity could be due to the increased hydrophobicity of the antagonists by virtue of substitution of the central amino acids by
aromatic residues. This is partially consistent with the findings of Foreman et al. [328] where increasing the hydrophobicity of the C-terminal portion of substance P enhanced its activity so that [D-trp\(^{7,9}\)]SP is 12-fold more potent than its parent peptide [81]. This was interpreted as a requirement for a hydrophobic C-terminal. However, the activity of des-arg\(^{9}\)-BK, as shown in this study and others [303] may indicate that the hydrophobic portion need not necessarily be situated at this end of the molecule.

In substance P, the peptide appears to act non-specifically, anchoring the molecule to the cell surface at the C-terminal, thus allowing the basic residues at the N-terminal to interact with the receptor site involved in triggering the secretory mechanism [302-304]. However, in bradykinin, the C-terminal position is occupied by a basic amino acid, arginine. Since the increased potency of the antagonist could be attributed to the increased aromaticity of the central residues, and the C-terminal arginine is necessary for full activity, it is feasible that bradykinin binds to the cell membrane through its central hydrophobic portion and activates the cell through its terminal arginines. In addition, this mode of interaction would account for the lesser potency of bradykinin when compared with substance P and somatostatin. Bradykinin is a short peptide, and the folding of the peptide molecule as it binds to the cell through its hydrophobic portion could impose steric constraints on the activation of the cell by the basic residues at each terminal.

Secretion of histamine induced by bradykinin was unexpectedly sensitive to changes in pH. The level of histamine release induced by the peptide fell rapidly when the pH was increased from 7.0 to 8.0. Other basic agonists tested also responded maximally at this point, with diminished activity on either side of this value, but none was as severely depressed as bradykinin (see Chapter 3). At pH 8.0, secretion evoked by bradykinin (10\(^{-6} \text{ to } 10^{-4} \text{ M}\)) was completely suppressed and lys-BK and [D-phe\(^{7}\)]-BK (both 10\(^{-6} \text{ to } 10^{-4} \text{ M}\)) were also distinctly inhibited by pH 8.0, although the more potent agonist, [D-phe\(^{7}\)]-BK, was less affected. Bradykinin does not contain amino acid residues that should be markedly affected, in terms of ionisation, within the pH range selected, indicating that the pH effect is extremely subtle. Cells preincubated at pH 8.0, then restored to pH 7.0, regained full responsiveness to bradykinin, anti-IgE and compound 48/80. Similarly, bradykinin dissolved in pH 8.0 buffer prior to dilution at pH 7.0 buffer was as active as the peptide maintained at pH 7.0 throughout. The effect is then not due to an irreversible modification of cell function or of the peptide itself.

In this study, the suppression of secretion on preincubation of cells with the peptide at pH 8.0 was employed to see if cells could be desensitized to bradykinin or compound 48/80 in this manner. This was not accomplished, inferring that receptor occupation had failed to take place. It is unlikely that the receptor site was affected by pH, as the secretion induced by other
basic peptides tested was only slightly affected by pH 8.0 and, thus, any effect was slight and reversible. Therefore, it is more likely that the suppression of secretion at pH 8.0 may be a result of pH sensitivity of the interaction between the peptide and the cell.

A surprising discovery was that bradykinin synergised with anti-IgE to produce enhanced levels of histamine secretion. Of the peptides tested, this property was exclusive to bradykinin. PGD$_2$ levels were also investigated but no enhancement was observed and the differences between the experimental and theoretically predicted levels of prostaglandin release were not statistically significant. Further study is needed fully to define the nature of this effect and a range of IgE-directed ligands and the effect of external calcium on the process should be investigated. Augmentation by bradykinin of immunological histamine release could clearly be of clinical importance.

In summary, these findings confirm and extend those of previous investigators who surmised that bradykinin is unlikely to operate on the RPMC via bradykinin receptors. Its mode of operation closely mimics that of compound 48/80, following in the tradition of the neuropeptides substance P, somatostatin and neurotensin. The peptide responds slightly differently from these other basic peptides, in that it is extremely sensitive to changes in pH and synergises with anti-IgE.

The variations between bradykinin and other basic bioactive peptides may derive from the binding of the peptide, where bradykinin is bound to the plasma membrane through its central portion and activation of the cell is performed by the terminal residues. This mode of attachment may provide less affinity to the receptor due to steric constraints, resulting in the relatively weaker activity of bradykinin. In accordance with this view, the increased aromaticity of the bradykinin antagonists may serve to strengthen the binding between the cell and the peptide, leading to an increase in secretory activity. Indeed, the activity of the antagonists [D-phe$^7$]-BK and [thi$^5$D-phe$^7$]-BK was comparable to that of substance P and somatostatin. Nonetheless, the evidence for this proposed *modus operandi* of bradykinin is circumstantial and further investigation is required. Indeed, the sensitivity of bradykinin and its analogues to pH may then be a result of an effect of pH on the proposed mode of anchoring by bradykinin, where a small rise in pH may be sufficient reversibly to affect this interaction. Thus, since lys-BK and [D-phe$^7$]-BK may be less affected by pH than bradykinin since the analogues probably interact more strongly with the cell, and are thus more able to overcome the effects of higher pH.

The ability of bradykinin receptor antagonists to liberate histamine from RPMCs shows clearly that this activity is unlikely to be mediated through the bradykinin receptor. Instead, the activity of these antagonists strengthens the proposition that bradykinin-induced
secretion is mediated through the putative polyamine receptor [82]. This site of action is considered non-specific as agonists of this class are peptides or polyamines that otherwise differ in structure and function, and the only unifying factor appears to be the presence of positively charged moieties.

Recent reports indicate that peptides and polyamine compounds may act through G-proteins. Degranulation induced by compound 48/80 and somatostatin is markedly inhibited by prior exposure to a small amount of islet-activating protein, pertussis toxin, which is a specific modifier of G-proteins [327-329]. Moreover, the wasp venom toxin, mastoparan, a tetrapeptide containing three lysine residues, has been reported to induce histamine release in rat mast cells by directly activating a G-protein [330]. Recently BAC has been reported competitively to antagonize the effect of mastoparan on a G-protein, potently stimulating nucleotide exchange [330]. Since BAC is the only reliable antagonist of compound 48/80-induced histamine release, this research may provide strong evidence for non-specific activation of G-proteins as a mode of action for compound 48/80 and other basic peptides. These findings may also explain the increased potency observed by increasing aromaticity of the central residues in bradykinin receptor antagonists and the C-terminal of substance P. This feature could increase the binding of peptide to cell, sinking the molecule further into the cell membrane and thus bringing the basic residues and G-proteins into closer proximity. This would result in a greater degree of cell activation through activation of a larger number of G-proteins.

In the following chapter, the response of mastocytes from different tissues and species to bradykinin is examined. This would extend the similarities between the peptide and other basic stimuli. Bradykinin has also been implicated in allergic disease, therefore it is important to clarify the role of the peptide with respect to histamine release from human mast cell.
CHAPTER 5

The effect of bradykinin on various histaminocytes

5.1 INTRODUCTION

5.1.1 Mast cell heterogeneity

Mast cells release histamine on anaphylactic or pharmacological stimulation and the heterogeneity of mast cell responses is well illustrated by peptide and chemical stimuli. While anaphylactic stimulation is implicit with mast cell function, not all mastocytes liberate histamine when treated with polyamines and basic peptides. These agents are strikingly similar in their tissue and species selectivity and are almost exclusively active on murine and hamster serosal-type mast cells. Among human histaminocytes, only the skin mast cell will release histamine in response to polybasic agonists [301], and compound 48/80, polyllysine, substance P and somatostatin are essentially ineffective against human basophils, lung and intestinal mast cells [82,313].

Bradykinin releases histamine from RPMCs and the characteristics of the response are very similar to those of other basic peptide and polyaminic stimuli [82] and so it is probable that the peptide activates this cell through the putative polyamine receptor. In this study, the effect of the peptide on various histaminocytes is examined and the analogues des-arg^9-BK and [D-phe^7]BK are also tested, in order to verify their similarity to other basic peptides and polyamines.

5.1.1 Bradykinin in inflammation and allergy

Kinins are capable of producing the classic signs of inflammation, but evidence of their involvement in such processes is circumstantial. The paucity of solid evidence is largely due to the lack of a specific kinin-receptor antagonist or inhibitor of the kinin-generating enzymes, although putative antagonists have now been described [257]. It is also difficult to measure the rate and amount of activation and inactivation of kinins at the target area of inflammation. The inflammatory event is extremely complex and involves a large number of mediator systems and cellular responses, making it particularly difficult to assess the contribution of a given mediator. For instance, besides the production of kinins, activated Hageman factor can also initiate the coagulation enzyme cascade and complement system [332,333]. In disease, the contact system is thought to play a role in hereditary angioedema,
gout and intravascular coagulation in septic shock and typhoid fever [250,251]. Activated Hageman factor may also be involved in hypotensive episodes in certain inflammatory diseases such as acute pancreatitis [250,251].

In human anaphylaxis, levels of HMWK are reduced [297,306]. In addition, bradykinin itself has been implicated in the pathogenesis of various allergic disorders including asthma and rhinitis. Elevated levels of immunoreactive kinin are found in the bronchial lavage fluid of asthmatic subjects [235] and bradykinin produces bronchoconstriction in asthmatic, but not normal, individuals [236]. This is in contrast to nasal challenge with the peptide, where symptoms of rhinitis are induced irrespective of atopic status [248]. Kinins are generated in nasal secretions during the immediate and late-phase responses to allergen challenge [234] where the release of kinins and histamine are highly correlated, occurring almost simultaneously [234,244]. The exact role of kinins in allergic inflammation is not clear. They could act directly on the smooth muscle and microvasculature or indirectly by the local activation of tissue mast cells [237]. To define more clearly the role of bradykinin in clinical allergy, we have now examined the effect of the peptide on isolated human mast cells.
5.2 RESULTS

5.2.1 The effect of bradykinin on various histaminocytes
Bradykinin (10^-4-10^-6 M) was applied to a range of histaminocytes from several species (for the method of isolation of these cells, see chapter 2). The peptide was active on rat peritoneal, pleural and mesenteric mast cells and hamster peritoneal mast cells and weakly active on the human cutaneous and mouse peritoneal mast cell. Rat lung and intestinal mast cells, guinea-pig mesenteric mast cells, human pulmonary and intestinal mast cells and human basophils were completely unresponsive (fig 5.1).

5.2.2 The calcium dependence of bradykinin-induced secretion from various histaminocytes
RPMCs, rat pleural, mesenteric and hamster peritoneal mast cells were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca^2+, 1 mM). Following this, the agonist (dissolved in CMF-Tyrode’s), was added to the cells and the experiment allowed to proceed as usual. In each case, histamine liberation in the absence of extracellular calcium was greater than that evoked in the presence of the cation, except at the highest concentration of bradykinin (figs. 5.2 a, b, c, d).

5.2.3 The action of des-arg^9-BK and [D-phe^7]-BK on various mastocytes
The species-selectivities of the bradykinin analogue, des-arg^9-BK, and BK-receptor antagonist, [D-phe^7]-BK, were found to be virtually identical to bradykinin. Both compounds induced secretion of histamine from rat pleural, peritoneal and mesenteric mast cells and hamster peritoneal mast cells. Rat lung and intestinal mast cells, guinea-pig mesenteric mast cells, human basophils and human pulmonary and intestinal mast cells were completely unresponsive. Mouse peritoneal cells were unresponsive to des-arg^9-BK, but [D-phe^7]-BK induced slight histamine release (10.2 ± 1.2%) at the highest concentration tested (10^-4 M) (figs 5.3 a,b,).

5.2.4 The calcium dependence of des-arg^9-BK-induced secretion from various histaminocytes
Rat mesenteric and hamster peritoneal mast cells were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca^2+, 1 mM). Following this, the agonist (dissolved in CMF-Tyrode’s), was added to the cells and the experiment allowed to proceed as usual. Histamine release in the absence of extracellular...
Figure 5.1  Bradykinin-induced histamine release from rat peritoneal (○) pleural (●) and mesenteric (□), hamster peritoneal (▼) and human cutaneous (◆) mast cells. Human basophils and mast cells from human lung, rat lung and intestine, and guinea-pig mesentery were totally unresponsive (●). The mouse peritoneal mast cell (△) was very slightly responsive. Values are means ± SEM, n=4.
Figure 5.2 (a) RPMCs were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with bradykinin. Values are means ± SEM, n=4.
Figure 5.2 (b) Rat pleural mast cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with bradykinin. Values are means ± SEM, n=4.
Figure 5.2 (c) Rat mesenteric mast cells were preincubated (5 min, 37 °C) in CMF- (Δ, no calcium), EDTA- (□, 0.1 mM) or calcium-containing (○, 1 mM) buffer, then challenged with bradykinin. Values are means ± SEM, n=4.
Figure 5.2 (d) Hamster peritoneal mast cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with bradykinin. Values are means ± SEM, n=4.
Figure 5.3 (a) Histamine release induced by des-arg⁹-BK from rat peritoneal (○) and mesenteric (□), and hamster peritoneal (▼) mast cells. Human basophils and mast cells from human lung, rat lung and intestine, the mouse peritoneum and guinea-pig mesentery were totally unresponsive (●). Values are means ± SEM, n=4.
Figure 5.3 (b) Histamine release induced by [D-phe$^7$]-BK from rat peritoneal (○) and mesenteric (□), hamster peritoneal (▼) and mouse peritoneal (△) mast cells. Human basophils and mast cells from human lung, rat lung and intestine, and guinea-pig mesentery were totally unresponsive (●). Values are means ± SEM, n=4.
calcium was greater than that evoked in the presence of the cation, except at the highest concentration of the analogue (figs. 5.4 a, b).

5.2.5 The calcium dependence of [D-phe$^7$]-BK-induced secretion from various histaminocytes

Rat mesenteric and hamster peritoneal mast cells were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca$^{2+}$, 1 mM). Following this, the agonist (dissolved in CMF-Tyrode’s), was added to the cells and the experiment allowed to proceed as usual. Secretion in the absence of extracellular calcium was greater than that evoked in the presence of the cation, except at the highest concentration (figs. 5.5 a, b).

5.2.6 Synergy between bradykinin and anti-IgE

While bradykinin did not release histamine from human lung and intestinal mast cells, the possibility of synergistic effects between the peptide and anti-IgE were investigated. Bradykinin (10$^{-4}$ - 10$^{-6}$ M) and anti-IgE (dilutions 1/1000, 1/3000) were presented simultaneously to human lung and intestinal mast cells. Human skin mast cells were not studied as there were insufficient cells. The levels of histamine release obtained were identical to those of the control cells stimulated with anti-IgE alone (fig 5.6 a,b).
Figure 5.4 (a) Rat mesenteric mast cells were preincubated (5 min, 37 °C) in CMF- (△, no calcium), EDTA- (□, 0.1 mM) or calcium-containing (○, 1 mM) buffer, then challenged with des-arg⁹-BK. Values are means ± SEM, n=4.
Figure 5.4 (b) Hamster peritoneal mast cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (●, 1 mM) buffer, then challenged with des-arg⁹-BK. Values are means ± SEM, n=4.
Figure 5.5 (a) Rat mesenteric mast cells were preincubated (5 min, 37 °C) in CMF- (△, no calcium), EDTA- (■, 0.1 mM) or calcium-containing (⊗, 1 mM) buffer, then challenged with [D-phe⁷]-BK. Values are means ± SEM, n=4.
Figure 5.5 (b) Hamster peritoneal mast cells were preincubated (5 min, 37 °C) in CMF- (▲, no calcium), EDTA- ( ●, 0.1 mM) or calcium-containing ( ○, 1 mM) buffer, then challenged with [D-phe$^7$]-bradykinin. Values are means ± SEM, n=4.
Figure 5.6 (a) Human lung mast cells were challenged simultaneously with anti-IgE (dilutions 1/1000, 1/3000) and bradykinin (10^{-6}-10^{-4} M). Alone, the peptide evoked 0% histamine release for all concentrations of the peptide. Values are means ± S.E.M., n = 4.
Figure 5.6 (b) Human intestinal mast cells were challenged simultaneously with anti-IgE (dilutions 1/1000, 1/3000) and bradykinin (10^-6-10^-4 M). Alone, the peptide evoked 0% histamine release for all concentrations of the peptide. Values are means ± S.E.M., n=3.
5.3 DISCUSSION

The release profile of bradykinin and its analogues through a range of mastocytes largely conforms with the species-specificity exhibited by other polyamines and basic peptides, such as compound 48/80, substance P, polylsine and somatostatin. Bradykinin was completely without effect on human pulmonary and intestinal mast cells and blood basophils, but was slightly active on human skin mast cells. These results are in accord with those of previous findings [239]. The analogues, des-arg^{9}-BK and [D-phe^{7}]-BK (a BK-receptor antagonist), also had a similar pattern of species-specificity to bradykinin. This pattern, and the calcium dependence of bradykinin and its analogues, is characteristic of basic peptide and polycationic stimuli [82,256]. Together with the evidence from the previous chapters, bradykinin is most likely to be of this class of agents.

In the previous chapter, bradykinin exhibited synergy when presented simultaneously with anti-IgE in RPMCs. This feature was observed to be unique to the peptide and was not shared by other polybasic compounds. However, such synergy was not observed in experiments with human lung and intestinal mast cells and, therefore, does not support a role for bradykinin in histamine release in these systems. Thus, while kinins evidently participate in the allergic process, there is no evidence that kinin-induced histamine release is involved.

Sound evidence implicates the kinin system in the pathogenesis of asthma and rhinitis. Elevated levels of tissue kallikrein and immunoreactive kinin are found in the bronchial lavage fluid of asthmatic subjects [235] and kinins are generated in nasal secretions during the immediate and late-phase responses to allergen challenge [234]. Bradykinin produces bronchoconstriction in asthmatic, but not normal, individuals [236] while nasal challenge with the peptide induces symptoms of rhinitis irrespective of atopic status [248]. The peptide has little effect on human airways in vitro [236], suggesting that the bronchoconstrictor action is indirect and that human airway smooth muscle does not have a significant population of bradykinin receptors.

In the lungs, the peptide may cause local vasodilation of the airways and stimulation of unmyelinated sensory nerve endings, perhaps inducing the release of sensory neuropeptides such as substance P, CGRP and somatostatin, which have long been implicated in asthma. To some extent, bradykinin-induced bronchoconstriction is diminished by anticholinergic drugs, implicating a vagal cholinergic reflex [236]. There is evidence that bradykinin may activate unmyelinated C-fibre afferent nerve endings in the bronchi [242], so that the effect of the peptide may be due to cholinergic reflex bronchoconstriction. Since exposure of nerve endings can occur as a result of the desquamation of the surface respiratory epithelium, C-fibre activation could explain the ability of bradykinin to induce bronchoconstriction in
asthmatic, but not normal subjects. In addition, disodium cromoglycate (DSCG) inhibited bradykinin-induced bronchoconstriction [236]. The mechanism of action of DSCG is still unknown, but its effects have been postulated to arise from an ability to suppress reflex responses caused by the stimulation of C-fibres or related sensory receptors in the human lung [344]. Should this theory prove correct, C-fibre activation could provide a partial explanation of the selective bronchconstrictor action of bradykinin.

The induction of bronchospasm by bradykinin in asthmatics could be a result of the hyperreactivity of asthmatic airways [236]. This hyperreactivity, which is an enhanced responsiveness of the airways to non-specific stimulation, is a fundamental characteristic of asthmatic airways. While a component of this hyperresponsiveness may be inherited, compelling evidence suggests that much of it is acquired through interaction of the airways with environmental stimuli [204,246], resulting in chronic inflammation of the lung. These inflammatory changes in asthmatic airways can result in extensive tissue damage of the bronchial lumen and thus provide the ideal surface for the activation of Hageman factor and the subsequent generation of kinins. As discussed, damage to the bronchial epithelium may also expose afferent nerve endings and generate local axon reflexes to amplify and spread the inflammatory response [245]. Thus, while normal airways are resistant to the inflammatory effects of bradykinin, asthmatic airways probably lack the usual defenses, such as an intact epithelium, which would mechanically protect nerve endings and provide appropriate chemical defence, such as ACE produced by endothelial cells in the lung [245]. Thus, the presence of kinins in the lavage fluid of asthmatics probably reflects the inflamed state of asthmatic airways and bradykinin would then induce bronchospasm through its inflammatory effects rather than through histamine release. Bradykinin also releases prostaglandins in several species, suggesting that its effects might be mediated via bronchoconstrictor prostaglandins. However, aspirin has no effect on bradykinin-induced bronchoconstriction [247] although released prostaglandins could again contribute to the underlying inflammatory response.

Cutaneous late-phase reactions in the skin are caused by a prior IgE-mediated reaction in which there is rapid activation of skin mast cells and secretion of a variety of proinflammatory substances, resulting in a wheal and flare reaction. Hageman factor-dependent pathways are among the mediator systems activated during the late phase; this appears to be due to plasma leakage at the site of the skin lesion and local activation of mast cells [249]. The mechanism for such activation is unknown but could include dilution (which lessens inhibitor effects), heparin released from mast cells and other negatively charged surfaces presented by exposed tissue or enzymes which cleave Hageman factor. This is perhaps a common mechanism by which kinin formation can occur as a late event.
in an inflammatory mechanism. Kinin formation might thus have a role in perpetuating the inflammatory response, or lead to chronicity and it is likely that the kinin system is one of the factors contributing to the late-phase allergic reaction. However, while human skin mast cells released a small quantity of histamine, the high concentrations of bradykinin required for histamine release from human skin mast cells indicate that there is no physiological significance in this result. This conclusion is in agreement with that obtained by Lawrence et al [239].

Nonetheless, although bradykinin does not liberate histamine from human mast cells, it is possible that the activation of mast cells in an allergic reaction has a role in kinin production, since mast cell granule enzymes may interact with elements of the kinin system [258]. The mast cell contains many proteases and it is an attractive possibility that these may be involved in kinin generation. The serine proteases of the mast cell have been shown to hydrolyse various plasma proteins. Rat chymase has been shown to destroy type IV collagen [288] and inactivate bradykinin [252], while human tryptase has been shown to hydrolyse HMWK and fibrinogen [241, 288, 305]. These reports suggest that mast cell proteases may have a role in the degradation of kinins and kininogens. However, there have also been reports of IgE-mediated release of a kallikrein-like enzyme from human basophils [253] and human lung mast cells [240, 299]. While the kallikrein from basophils is thought to be an arginine esterase [253], the kininogenase of the human lung mast cell has been identified as tryptase [299]. Thus, the degranulation of the human lung mast cell could conceivably lead to the exacerbation of allergic inflammation by kinins, through the effects of these released proteases. Alternatively, the mast cell could have a role in the degradation of kinins. These reports obviously require further clarification.

Another possible role for mast cells in the production of kinins could be in the provision of a negatively charged surface for activation of the Hageman factor system. The mast cell granule matrix is an ideal surface and both heparin and chondroitin sulphate E have been shown to activate the contact system [254]. Thus, the mast cell could play a role in amplifying allergic inflammation by promoting the production of kinins. This could help account for the presence of kinins on nasal and bronchial challenge with allergen [234, 235]. Thus, mast cells could mediate the production of kinins in these conditions, and the bronchoconstriction observed upon challenge with the peptide can be explained in a number of ways. Kininogenase is present in asthmatic BAL fluid and thus, it is possible that the degranulation of mast cells and the resultant release of proteolytic enzymes would then generate kinins as part of the asthmatic process.

Thus, these experiments provide further support to the view that bradykinin is a typical basic peptide mast cell agonist. It is also apparent that bradykinin-induced histamine release is not
likely to be a factor in human allergic disease. In view of current knowledge, bradykinin is more likely to be present as part of the inflammation induced by the allergic reaction and may serve to amplify the release and/or production of other putative mediators in asthma. The role of the mast cell is unclear, but it is probable that a connection exists between the mast cell and the kinin system. This study confirms and extends previous reports that polycationic compounds induce histamine release as part of a general effect and that the action of such peptides is non-specific. The significance of these interactions is uncertain, and largely confined to particular murine mast cells, but there exists the distinct possibility that mast cells may partake in the generation or degradation of these peptides.
CHAPTER 6

The action of anaphylatoxin peptides on mast cells

6.1 INTRODUCTION

6.1.1 The complement system

The complement system constitutes a complex series of events involving a cascade of serum proteins. The action of these peptides protects against infection through their ability to opsonize and lyse a wide variety of micro-organisms. Some peptides of the system can activate and are chemotactic for inflammatory cells and also stimulate smooth muscle cells, lymphocytes and endothelial cells, and can evoke the production of inflammatory mediators. In essence, the complement system is the principal humoral effector system in inflammation. The complement proteins are designated C1q, C1r, C1s, C2, C3, C4 . . . . C9, including the fragments C3a, C4a and C5a among others. These peptide fragments are also known as the anaphylatoxins and are generated from C3, C4 and C5, respectively. These cationic peptides are potent inflammatory agents and increase vascular permeability, induce contraction of smooth muscle and attract and activate granulocytes. Inhalation of nebulized C5a induces airway hyperresponsiveness to histamine in the rabbit.

The complement cascade can be initiated classically, through the antigen-antibody reaction, or through the alternative pathway, which may be stimulated by substances such as bacterial lipopolysaccharide, endotoxin and zymosan among others. The general form of reactions within the cascades is depicted in figure 6(i). The classical pathway is triggered by the combination of the first component of the complement system, C1q, and antigen-antibody complexes formed by the opsonization of a micro-organism by IgM or IgG, where the antigenic moiety is usually a membrane constituent of these pathogens. C1q then adsorbs C1r and C1s, resulting in an activated enzymic complex, C1 (qrs), or the initiation unit. This unit acts on C2 and C4 to produce C4b2a, a C3 convertase, releasing the peptide C4a in the process. C3 convertase is considered the most important enzyme of the system. Its substrate, C3, is the most plentiful complement protein in plasma and the action of C3 convertase produces an amplification of the original stimulus. C3 convertase activates C3, forming C5 convertase (C3b4b2a) and several fragments, including C3a. C5 convertase activates C5 to produce C5a and C5b, where the latter forms part of the membrane attack unit. This unit is a powerful cluster of hydrophobic proteins that achieve membrane lysis and the death of the pathogens. Other products of the cascade aid the fight against infection by inducing,
directly or indirectly, changes in vascular flow, opsonization of micro-organisms and the accumulation and/or activation of neutrophils and monocytes. Receptors for complement have now been identified on most circulating cells, including lymphocytes and erythrocytes. The anaphylatoxin fragments C3a and C5a have also been shown to release histamine from blood basophils.

The complement cascade hinges on the ability to convert C3. Unlike the classical pathway of activation, the alternative pathway generates C3 convertase without the need for antibody, C1, C2 or C4. This activation route is faster and more effective than the classical pathway and centres round the formation of a complex between C3 and Factor B, a humoral enzymatic component. The complex is activated by Factor D, a member of the cascade, which cleaves a fragment from B, leaving a C3 convertase, C3b.Bb. Adequate supplies of B guarantee the formation of more convertase. Following this, C5 convertase is formed by the addition of one or more molecules of C3 to C3b.Bb. Two inhibitors H and I both bind to C3 and cleave it to C3bi, thus limiting the supply of convertase. A decay process also operates spontaneously, whereby the complex simply dissociates if left to itself. Besides the activating substances mentioned above, initiation of the alternative pathway can be achieved by any enzyme with the ability to act as C3 convertase, such as trypsin, thrombin, plasmin, leucocyte cathepsin, cobra venom, activated Hageman factor and neutral proteases from neutrophils and macrophages.

6.1.2 Histamine release induced by anaphylatoxins

The extent to which mast cells are involved in responses to complement peptides has been controversial and varies according to the species. Among humans, basophil leucocytes have long been known to release histamine when challenged with purified anaphylatoxins, and recent studies have shown that isolated cutaneous, but not pulmonary, mast cells from humans are also susceptible to very low concentrations of purified C5a.

There have been numerous reports on histamine release from human basophils. Earlier work relied upon crude, unfractionated human and porcine anaphylatoxin, although later workers were able to obtain more purified material. Despite this, the results obtained by the various workers were reasonably consistent [264-276]. Human basophils will release up to 70% of their histamine content when challenged with C5a, while C3a is less effective and produced histamine secretion of up to 40% [265]. Where experiments were performed on unfractionated leucocytes, the action of the peptides was not thought to be affected by other cell types and levels of release attained in purified and non-purified cells were equivalent.
In addition, the rapidity with which histamine release occurred (under 2 min) [269] argues against the involvement of other cell types.

Anaphylatoxin-induced secretion from human basophils was determined to be an active process, with no release of LDH, and complete within 2 min [269,272]. Secretion was calcium dependent [272]; the optimal concentration of the cation was shown to be 0.5 mM and the presence of EDTA (2 mM) abolished any response. There was no evidence of 2-stage activation, nor was there any demonstrable synergism when the peptide was applied together with anti-IgE. Desensitization of basophils to antigen does not affect their response to the peptides. The optimum temperature for anaphylatoxin-induced histamine release has been variously reported at 25 °C [269] and 37 °C [272]. Histamine release was markedly affected by pH, and secretion at pH 6.6 (37 °C) was 50% of that attained at pH 7.4 (37 °C) [269]. C5a was shown to induce degranulation without concomitant release of SRS-A, and indomethacin was observed to augment C5a-induced secretion. C5a-induced histamine release was also inhibited by disodium cromoglycate [276]. This result was unusual as the drug is usually inactive against human basophils. Treatment of basophils with deuterium oxide and cytochalasin B enhances complement-induced secretion of histamine by human basophils, suggesting that microtubules and/or microfilaments are involved in this process [268]. Cross-desensitization was not observed between complement and pollen allergens [272].

Basophils were desensitized to anaphylatoxins by preincubating the cells with the peptides either in the presence of EDTA or at 0 °C. It was also observed by different laboratories that, upon isolation, the responsivity of the basophils to C3a and C5a was markedly reduced if the leucocytes were held at 37 °C [269]. To overcome this, the basophils were handled and stored at room temperature until commencement of the experiment.

Mast cells of the guinea pig lung appear to be sensitive to C5a [269], while histaminocytes of the dog and rabbit appear to be resistant [263,278]. However, it is not clear if RPMCs release histamine in response to these peptides as reports in the literature are contradictory [260,261,274]. Isolated human cutaneous mast cells have also been shown to be responsive to purified anaphylatoxins while human pulmonary mast cells did not release histamine when challenged with C5a [275]. The supernatants from these experiments remained fully active and induced secretion from basophil leucocytes, thus excluding the possibility of inactivation of the peptide by carboxypeptidase as an explanation for the unresponsiveness of the pulmonary cells. Thus, the species-specificity of these peptides is yet another demonstration of the heterogeneity of mastocyte responses.
The experiments in the present study were performed with anaphylatoxin produced and supplied by Dr. T. Hugli. The anaphylatoxin peptides supplied were C3a, des-arg-C3a and peptide 2IR, a synthetic analogue of C3a, with a molecular weight of 2462.
Figure 6 (i) Outline of the general form of the reactions in the complement cascade.

Ag-Ab

\[ C_1(q + r + s) \rightarrow C_1(qrs) \]

\[ C_4 + C_2 \rightarrow C_4b.2a \]

\[ C_5 \rightarrow C_5b \]

Figure 6 (ii) Diagram of the initiation and activation stages of the classical pathway. A bar over a complex indicates enzymic activity.
Figure 6 (iii) A comparison of the alternative and classical pathways of activation.
6.2 RESULTS

6.2.1 Histamine release from human histaminocytes

6.2.1.1 Histamine release from human pulmonary mast cells

C3a, des-arg-C3a and peptide 2IR produced absolutely no histamine secretion from pulmonary mast cells (n=2). The cells were tested for viability by challenge with anti-IgE (1/1000 diln: 18.4%, 26.8%; 1/3000 diln: 5.0% 12.8%). (fig.6.1).

6.2.1.2 Histamine release from human cutaneous mast cells

The peptides C3a, des-arg-C3a and peptide 2IR induced some histamine release (<10%) from cutaneous mast cells (young male foreskin, n=1). The cells responded weakly, but positively, to anti-human-IgE (1/100 diln: 4.3%) and substance P (5x10^-6 M: 8.6%). (fig. 6.2).

6.2.1.3 Histamine release from human basophil leucocytes

The histamine release induced by C3a, des-arg-C3a and peptide 2IR from basophils was negligible. These basophils responded normally to anti-IgE and FMLP peptide and the spontaneous histamine release from these cells was 0.3 ± 0.3%. These results clearly contradict previous reports on anaphylatoxin-induced histamine release from basophil leucocytes. (figs 6.3 a,b).

6.2.1.4 Investigating the lack of effect of the complement peptides on human basophils

The results obtained above (section 6.2.1.1) differed from published literature, where there were no reports of any failure of the anaphylatoxins, both purified and unpurified, to induce secretion from human basophils. The isolation of basophils followed the previous recommendations [268-270], where the cells were held at room temperature until the start of the experiment. This factor was apparently crucial to the responsivity of basophils to anaphylatoxin peptides.

The method of preparation of the peptides was also standard: peptides were dissolved in full Tyrode’s buffer at a high concentration, portioned into Eppendorf tubes and stored immediately (-20 °C). Freshly prepared complement peptides were used in the initial histamine release experiment with the basophils. At all other times, the peptides were taken from storage just before the start of the experiment and reconstituted for immediate use.
Figure 6.1 Human pulmonary mast cells were challenged with C3a (○), des-arg-C3a (□) and peptide 2IR (▲). The cells responded normally to anti-human-IgE (1/1000 diln: 18.4%, 26.8%; 1/3000 diln: 5.0% 12.8%) Values are means, n=2, vertical bars indicate the range.

Figure 6.2 Human cutaneous mast cells were challenged with C3a (○), des-arg-C3a (□) and peptide 2IR (▲). The cells responded weakly but positively to anti-human-IgE (1/100 diln: 4.3%) and substance P (5x10^{-4} M: 8.6%). n=1.
Figure 6.3 (a) Human basophil leucocytes were challenged with C3a (○), des-arg-C3a (□) and peptide 2IR (△). The cells responded normally to anti-human-IgE (1/1000 diln: 32.4 ± 5.1%; 1/3000 diln: 25.2 ± 3.2%) and FMLP peptide (see fig. 6.3b). Values are means ± SEM, n=4.

Figure 6.3 (b) Human basophil leucocytes were challenged with FMLP (▲). The cells responded normally to anti-human-IgE (1/1000 diln: 32.4 ± 5.1%; 1/3000 diln: 25.2 ± 3.2%). Values are means ± SEM, n=4.
Due to the need to preserve limited peptide stocks, investigations into the unresponsiveness of basophil leucocytes were confined to the following three experiments.

6.2.1.4 (a) Length of basophil response

The standard reaction time allowed for histamine release from basophils was 30 min. In this experiment, the reaction time was increased to 45 min (n=1). All cells used were tested for a positive response to FMLP peptide \(10^{-6}, 10^{-7}\) M and anti-human-IgE \(1/1000, 1/3000\) diln. Spontaneous release of histamine was consistently below 1%. The histamine release induced by the complement peptides after 45 min was again negligible (fig. 6.4).

6.2.1.4 (b) Use of anticoagulant

Basophils were isolated in the usual way. Twice the usual amount of blood \((2 \times 20\) ml) was drawn from the donor and portioned into two Universal tubes. The first contained the usual mixture of glucose, dextran and heparin (see Chapter 2), while the second contained 0.01 M EDTA instead of heparin. Both samples were equally treated but neither sample released histamine (fig. 6.5).

6.2.1.4 (c) Possible enhancement of complement-mediated secretion by \(D_2O\)

Deuterium oxide, a microtubule stabilizing agent, has been reported to enhance C5a-mediated secretion from basophils [268]. An experiment was thus performed by using \(D_2O\) buffer but no enhancement of histamine release by the complement peptides was observed. Secretion induced by anti-IgE (dilns. 1/3000, 1/1000) was strikingly increased by this treatment, while FMLP \((10^{-6}, 10^{-7}\) M) was only marginally affected (table 1).

6.2.1.4 (d) Comparison with an independent operator

An experiment was performed together with an independent operator (Dr. H.Y.A. Lau), with the cell pool divided into two after sedimentation of the erythrocytes by the independent operator. Thereafter, each investigator performed independent experiments and assays, using the same drug stock. The leucocytes handled by the independent operator released up to 5.2% of total cellular histamine, but no obvious dose-dependency was observed. Those handled by the author were unresponsive (0% at 3 \(\mu\)M) (fig. 6.6).
Figure 6.4 Human basophil leucocytes were challenged with C3a (⊗), des-arg-C3a (■) and peptide 2IR (▲) for (a) 30 min and (b) 45 min. The cells responded normally to anti-human-IgE (1/1000 diln: 18.1%; 1/3000 diln: 15.3%) and FMLP peptide (10^{-7} M: 21.7%, 10^{-6} M: 28.3%). n=1.
Figure 6.5  Human basophil leucocytes were challenged with C3a (☉), des-arg-C3a (●) and peptide 2IR (▲). The cells responded normally to anti-human-IgE (1/1000 diln: 20.2%; 1/3000 diln: 12.2%) and FMLP peptide (10⁻⁷ M: 47.8%, 10⁻⁶ M: 68.0%). n=1.
Table 1. Histamine release induced by C3a, des-arg\(^9\)-C3a and peptide 2IR (all 1.0, 2.0 μM), and anti-IgE (dilutions 1/3000, 1/1000) and FMLP (10\(^{-7}\), 10\(^{-6}\) M) in normal and D\(_2\)O-Tyrode’s. n=1.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>% Histamine release in normal Tyrode’s</th>
<th>% Histamine release in D(_2)O-Tyrode’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3a</td>
<td>1 μM</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Des-arg(^9)-C3a</td>
<td>1 μM</td>
<td>2.2</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Peptide 2IR</td>
<td>1 μM</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Anti-IgE</td>
<td>1/3000 diln</td>
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<td>71.6</td>
</tr>
<tr>
<td></td>
<td>1/1000 diln</td>
<td>47.8</td>
<td>74.2</td>
</tr>
<tr>
<td>FMLP</td>
<td>0.1 μM</td>
<td>19.2</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>22.4</td>
<td>24.6</td>
</tr>
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</table>
Figure 6.6 Human basophil leucocytes were challenged with C3a (●), des-arg-C3a (□) and peptide 2IR (▲). The cells responded normally to anti-human-IgE (1/1000 diln: 32.1%; 1/3000 diln: 15.7%) and FMLP peptide (10⁻⁷ M: 53.8%, 10⁻⁶ M: 56.8%). n=1.
6.2.2 Complement-induced secretion from animal mast cells

6.2.2.1. The effect of anaphylatoxin on RPMCs

C3a, des-arg-C3a and peptide 2IR induced significant histamine release from RPMCs in dose-dependent fashion. All the peptides released histamine from RPMCs. C3a was the most active peptide (fig. 6.7).

6.2.2.2. The effect of anaphylatoxin on guinea pig mesenteric mast cells

The anaphylatoxin peptides have been reported to induce secretion from these cells [269]. Despite this, C3a, des-arg-C3a and peptide 2IR failed to elicit any histamine release in the present experiments (fig. 6.8).
Figure 6.7  Histamine release from rat peritoneal mast cells induced by C3a (●), des-arg-C3a (■) and peptide 2IR (▼). Values are means ± SEM, n=3.

Figure 6.8  Histamine release from guinea-pig mesenteric mast cells by C3a (○), des-arg-C3a (□) and peptide 2IR (▲). Values are means, n=2, vertical bars indicate the range.
6.3 DISCUSSION

It is not known why the results obtained in the present study were different to those reported by previous authors. The basophils did not release significant amounts of histamine in response to any of the complement peptides while, at the maximum concentrations used, releases of up to 25% would have been expected [264]. However, since the supply of peptide was extremely limited, it was necessary to restrict the investigation of this anomalous result.

The method of preparation of the peptides was standard: the peptides were dissolved in full Tyrode's buffer at a high concentration, portioned into Eppendorf tubes and stored immediately (-20 °C). Freshly prepared complement peptides were used in the initial histamine release experiment with the basophils. At all other times, the peptides were taken from storage just before the start of the experiment and reconstituted for immediate use.

Experiments using a longer reaction time and a different anticoagulant showed no difference in the responsivity of the basophils. In the experiment conducted with an independent operator, neither worker achieved any release in excess of 5%. An experiment was also performed in D₂O buffer, which was reported to enhance C5α-mediated histamine release [268], but this also proved unsuccessful. Throughout, the functionality of the cells was monitored in terms of their responses to anti-IgE and FMLP.

In contrast to the above, C3α (3 μM) produced a pronounced release of histamine from RPMCs (22.1 ± 4.2%) and, in a pilot experiment, some release (7.4%) from human skin mast cells. These values are very similar to those reported in the literature for this cell type [260]. At first sight, these results appear to confirm that the peptide is functionally intact. However, des-arg-C3α was also active in these systems and showed comparable activity to peptide 2IR, although both molecules were rather less active than native C3α. In most systems, human des-arg-C3α is totally inactive while peptide 2IR is nearly as active as the parent molecule.

Under these conditions, the interaction of the anaphylatoxins with rat peritoneal and human skin mast cells may be relatively non-specific and simply reflect the unique sensitivity of these particular histaminocytes to polybasic compounds [277]. Similar reservations concerning the specificity of the interaction of anaphylatoxins with mast cells have been expressed by Hugli [264]. Consistent with this view, human basophils and pulmonary mast cells, and guinea pig mast cells, are essentially unresponsive to polyamines [82,263].

The reason for the lack of effect of C3α on human basophils in our hands remains obscure. It would have been relevant to have tested higher concentrations of the peptide, if sufficient supplies had been available, although the maximum concentrations used by us were comparable to other workers and should have induced a marked secretion (≤ 25%). It is possible that our materials were denatured or inactivated in some other way, although this...
seems highly unlikely especially in the case of the synthetic peptide 2IR. Alternative biological testing of the peptides, for example of their spasmogenic activity, might have shed further light on this consideration. In addition, any potential proteolysis of the peptides in the incubation media might have been prevented by the use of suitable inhibitors, although this has not been reported necessary in other systems.

The samples of C3a and des-arg-C3a were extremely highly purified, while peptide 2IR was chemically synthesized. Thus, it is also conceivable, albeit improbable, that the observations of earlier workers, many of whom used very crude preparations, were due to contaminants in their samples. Clearly, additional studies on the effect on mast cells and basophils of C3a, des-arg-C3a and synthetic derivatives, given further supplies of these relatively inaccessible materials, would be of great interest.

The concentrations of anaphylatoxin claimed to induce histamine release from basophils are high and it must be questionable whether anaphylatoxin-induced histamine release is a significant contributor in allergic reactions. The only human mastocytes reported to undergo histamine release in response to anaphylatoxins are the basophil and the cutaneous mast cell. All other human mast cells were found not to respond to these peptides. The sensitivity of skin mast cells to these peptides is expected and the reactivity of these cells to peptides has been well documented. The response of the basophil, on the other hand, is slightly unusual as this cell type does not usually respond to basic peptides [82]. On closer examination, the reported characteristics of anaphylatoxin-induced histamine secretion differ from the usual in a number of respects. Firstly, basophils held at 37 °C slowly cease to respond to the anaphylatoxins and secondly, anaphylatoxin-induced degranulation is complete in under 2 min [269]. This is abnormal for basophil histamine release, where the time taken for complete histamine release is generally 15 - 20 min.

An alternative interaction between mast cells and the complement system could involve the mast cell proteases. Human lung mast cell tryptase has been shown to generate C3a from C3 [48]. The presence of heparin inhibited this reaction and, instead, purified C3a was rapidly degraded by this mixture. Parallel results were also obtained with RPMC chymase [289]. Since these proteases are secreted in a complex with heparin, it is possible that after the degranulation of mast cells [86], C3a is rapidly degraded by released protease in the presence of heparin proteoglycan. Thus, the mast cell may be important in deactivating the anaphylatoxin peptides.
CHAPTER 7

The action of thrombin on mast cells

7.1 INTRODUCTION

7.1.1 Thrombin

Thrombin is a serine protease that is one of the major enzymes in the coagulation cascade. The coagulation system consists of proteins, mostly pre-enzymes, which cascade upon activation of the first pre-enzyme, Hageman factor or Factor XII. This results in the rapid formation of effective concentrations of thrombin. There are two pathways of clotting - the intrinsic, which arises upon contact with an abnormal surface, and the extrinsic, which is initiated through tissue trauma. Both pathways result in the production of Factor X, which then acts on prothrombin to produce thrombin. Coagulation ensues when thrombin acts on fibrinogen, forming fibrin. The enzyme also aids this process by stimulating platelet aggregation, essential to clotting. The first factor in the coagulation cascade, activated Hageman factor, can also act as a C3 convertase in the complement system, initiate the fibrinolytic cascade and is a key factor in the production of kinins. These pathways are interacting, where the formation of plasmin tends to increase kinin formation and decrease the coagulation cascade [333,337].

Activated clotting factors such as thrombin have a short lifetime: the enzymes may be deactivated by proteases or specific inhibitors, or removed by the liver. Antithrombin III inactivates thrombin by forming an irreversible complex and also inhibits Factors XI, X and IX of the coagulation cycle. Antithrombin III is potentiated by heparin, which acts by increasing the rate of reaction between the enzyme and its inhibitor.

7.1.2 Thrombin and histamine release

Serine proteases in mast cell granules, such as chymase, atypical chymase and tryptase, which are major proteins in the granules, may play an important role in the process of IgE-mediated degranulation and in pathobiological alterations in tissues [258]. Indeed, inhibitors of chymase, substrate analogues and antichymase F(ab)_2, but not inhibitors of tryptase, markedly inhibited histamine release induced by IgE-receptor bridging but not that induced by calcium ionophore.
RPMCs release histamine in response to rat proteases chymase or α-chymotrypsin [279,280] and the degranulation is inhibited by the enzyme inhibitors, DFP (diisopropyl fluorophosphate) and lima bean trypsin inhibitor [279]. In the study by Schick et al [85], chymase from the RPMC granules induced the release of β-hexosaminidase and exogenous 5HT. It was noted that the active site of chymase was required. Degranulation was blocked by heparin and also by pretreatment of RPMCs with trypsin inhibitor. A sharp rise in intracellular cAMP was also observed 30 s after initiation of the reaction. This rise in concentration was similar to that observed when the cell is stimulated with anti-IgE. The authors suggested that chymase could mimic natural proteolytic steps following the activation of the mast cell, and/or represent a secondary amplification mechanism on prolonged presentation of the granule complex to the extracellular environment [86].

Although RPMCs have been found to respond to other serine proteases, thrombin is reported not to activate this cell type [282]. However, the enzyme induced a marked, dose-dependent release of histamine from BMMCs (mouse bone marrow-derived mast cells, Wehi-E), a chondroitin sulphate E-containing mast cell. [281-284]. Degranulation induced by thrombin was found to be inhibited by DFP [282], antithrombin III [281] and hirudin [281], an enzyme inhibitor derived from leeches. There was no release of LTC₄, indicating that the 5-lipoxygenase pathway was not activated [281]. A cAMP spike was also observed 15 s after cell activation [282]. Thrombin-induced degranulation was complete within 2 min [281] and activation of BMMCs by the enzyme was found to be calcium dependent. However, ⁴⁵Ca uptake upon stimulation with thrombin was complete at 90 s, while the uptake induced by anti-IgE lasted for 5 min [283]. An increase in IP₁, IP₂ and IP₃ was detected 20 s after cell activation [284]. In addition, it was observed that depleting the cells of calcium resulted in an 80% reduction in β-hexosaminidase release upon stimulation with thrombin [284]. In view of the reported selectivity of thrombin, it was decided in the present study to examine the effect of the enzyme on histaminocytes from different sources.
Fig. 7. (i) The interrelationship between plasma-derived mediators of inflammation. Activated Hageman factor can initiate the coagulation, fibrinolytic, kinin and coagulation systems. (Adapted from *Textbook of Immunopharmacology*, chapter 1, 2nd edn, eds M. Dale, J.C. Foreman; Blackwell Scientific Publications, 1989.)
7.2 RESULTS

7.2.1 Histamine release induced from various histaminocytes of different species.

7.2.1.1 Thrombin-induced histamine release from BMMCs
BMMCs were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.1).

7.2.1.2 Thrombin-induced histamine release from rat intestinal mast cells
Rat intestinal mast cells were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.2).

7.2.1.3 Thrombin-induced histamine release from rat pulmonary mast cells
Rat pulmonary mast cells were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.3).

7.2.1.4 Thrombin-induced histamine release from human basophil leucocytes
Human basophil leucocytes were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.4).

7.2.1.5 Thrombin-induced histamine release from human intestinal mast cells
Human intestinal mast cells were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.5).

7.2.1.6 Thrombin-induced histamine release from human pulmonary mast cells
Human pulmonary mast cells were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells did not release histamine in response to either enzyme. (fig. 7.6).

7.2.1.7 Thrombin-induced histamine release from RPMCs
RPMCs were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells released histamine in dose-dependent fashion. (fig. 7.7).
Figure 7.1  BMMCs were challenged with bovine (△) and human (○) thrombin. The cells responded normally to ionophore Λ23187 (10^{-8} M: 21.3 ± 4.2%; 10^{-9} M: 13.2 ± 3.1%) Values are means ± SEM, n=4.

Figure 7.2  Rat intestinal mast cells were challenged with bovine (△) and human (○) thrombin. The cells responded normally to ionophore (10^{-8} M: 13.5 ± 3.1%; 10^{-9} M: 11.2 ± 3.0%) Values are means ± SEM, n=4.
Figure 7.3  Rat pulmonary mast cells were challenged with bovine (▲) and human (●) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 24.2 ± 6.2%; 1/1000: 15.6 ± 3.1%) Values are means ± SEM, n=3.

Figure 7.4  Human basophil leucocytes were challenged with bovine (▲) and human (●) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 53.4 ± 7.1%; 1/1000: 32.6 ± 5.2%) Values are means ± SEM, n=4.
Figure 7.5 Human intestinal mast cells were challenged with bovine (△) and human (○) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 21.7 ± 6.2%; 1/1000: 12.9 ± 3.1%) Values are means ± SEM, n=3.

Figure 7.6 Human pulmonary mast cells were challenged with bovine (△) and human (○) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 29.3 ± 2.9%; 1/1000: 16.6 ± 3.8%) Values are means ± SEM, n=4.
7.2.1.8 Thrombin-induced histamine release from mouse peritoneal mast cells

Mouse peritoneal mast cells were challenged with human and bovine thrombin (0.01 - 10 units/10^6 cells). The cells were completely unresponsive to the enzyme. (fig. 7.8).

7.2.2 Histamine secretion induced by thrombin from RPMCs

7.2.2.1 The effect of metabolic inhibitors

Metabolic inhibitors were tested in the absence of glucose. The histamine release induced by human and bovine thrombin (10 U/ml) was virtually identical in both the absence and presence of glucose. In both cases, antimycin A (1 μM) completely abolished secretion when used alone, or with 2-deoxyglucose (5 mM). 2-deoxyglucose proved ineffective when used alone (fig. 7.9 a, b).

7.2.2.2 The effect of phospholipids

PS (final conc. 15 μg/ml) or lyso-PS (final conc. 0.5 μg/ml) was added to the cells 15 s before challenge with the agonist. The action of human thrombin was slightly enhanced by PS, while lyso-PS slightly potentiated secretion evoked by bovine thrombin. However, neither effect was significant (p > 0.05) (fig. 7.10 a, b).

7.2.2.3 The effect of extracellular calcium

RPMCs were preincubated for 5 min in either CMF-Tyrode’s, EDTA-Tyrode’s (EDTA, 0.1 mM) or normal Tyrode’s (Ca^{2+}, 1 mM). Following this, the bovine or human thrombin (dissolved in CMF-Tyrode’s), was added to the cells and the experiment allowed to proceed as usual. Histamine release was greater in the presence than absence of calcium in both cases (fig. 7.11 a, b).
Figure 7.7 RPMCs were challenged with bovine (△) and human (○) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 35.6 ± 6.1%; 1/1000: 27.5 ± 4.5%). Values are means ± SEM, n=4.

Figure 7.8 Mouse peritoneal mast cells were challenged with bovine (△) and human (○) thrombin. The cells responded normally to anti-IgE (diln. 1/300: 31.9 ± 5.2%; 1/1000: 25.6 ± 4.5%). Values are means ± SEM, n=4.
Figure 7.9 The effect of metabolic inhibitors antimycin A (1 μM) and 2-deoxyglucose (5 mM) on histamine release induced from RPMCs by thrombin (10 U/ml). Values are means ± SEM, n=3.
Figure 7.10 (a, b) Histamine release induced by human and bovine thrombin in the absence (open symbols, □) and presence of PS (15 μg/ml, closed symbols, □) or lyso-PS (0.5 μg/ml, closed symbols, ■). Values are means ± SEM, n=4.
Figure 7.11 (a, b) Cells were preincubated (5 min, 37 °C) in CMF- (□, no calcium), EDTA- (▲, 0.1 mM) or calcium-containing (■, 1 mM) buffer, then challenged with human and bovine thrombin. Values are means ± SEM, n=4.
7.3 DISCUSSION

Contrary to expectations, neither bovine nor human thrombin released histamine from BMMCs. However, RPMCs were found to be responsive to both forms of the enzyme. The BMMCs used in this study were of the Wehi-conA line, while those used by Razin in his studies were of the Wehi-E line [281-284]. It is possible that the dissimilarity in these cell lines may explain the discrepancy in the response of the cells. To examine this phenomenon further, we examined a range of other cell types, namely mouse peritoneal, rat peritoneal, intestinal and pulmonary, human intestinal and pulmonary mast cells and human basophil leucocytes. Of these, the rat intestinal mast cell is considered closely to resemble the BMMC, both morphologically and functionally, and the two cells each contain chondroitin sulphate E as the major glycosaminoglycan. However, this cell type was unresponsive, as were all cell types tested except, surprisingly, the RPMC.

Histamine release induced from RPMCs by thrombin, both human and bovine, was non-cytotoxic and dependent upon external calcium. RPMCs are connective tissue type mast cells, and were found by Razin not to respond to thrombin [282]. Our findings are to the contrary; however, mouse peritoneal mast cells (also connective tissue type mast cells) were not activated by thrombin.

The reasons for our disparate results are not clear. It is possible that our observed histamine release from RPMCs is due to a contaminant in the enzyme preparation, but this is extremely unlikely as we employed two quite separate sources of the protein (bovine and human). Clearly, further collaborative study involving different laboratories is required to address this problem. In any event, the present data provide further evidence for the phenomenon of mast cell heterogeneity.

In this study, human mast cells did not respond to thrombin. However, it is possible that a relationship exists between mast cell proteases and the generation of thrombin. Tryptase from rat mast cells has been shown to convert bovine prothrombin to thrombin [290]. This may have clinical significance. In insect sting anaphylaxis, levels of histamine are elevated for up to ninety minutes after the attack [306], suggesting that mast cells and/or basophils may be releasing histamine during this period. Changes in the kinin and complement systems and levels of thrombin have also been noted during the inflammatory period [297,306] and mast cell proteases may be involved in the production of thrombin as discussed above.
CONCLUSIONS

The present study has examined the effects of the serum-derived bioactive peptides bradykinin, C3a and its analogues, and thrombin on a range of different histaminocytes. These peptides have long been implicated in the aetiology of allergic disease. Changes in the kinin, complement and coagulation systems have been noted during wasp sting anaphylaxis [297,306] and implicated in conditions such as asthma. However, these observations do not necessarily indicate that the peptides produce their effects by means of histamine release [297,306]. While the agents examined in this study release histamine from the RPMC, they did not activate most human mastocytes. With the exception of some weak responses from skin mast cells, these findings are in accord with other data in the literature. Thus, it is apparent that histamine secretion induced by these peptides is unlikely to have a general role in human allergic and inflammatory disease, except possibly in the skin. However, this does not preclude alternative interactions between mast cells and peptides and these cell types, and it is possible that mast cell proteases may be involved either in the generation or degradation of these molecules. Rat tryptase has been shown to generate C3a from C3 [241], while human tryptase has been shown to be involved in both the generation [240] and degradation [241] of kinins. Mast cell chymase inactivates bradykinin [252] and there have been reports of IgE-mediated release of a kallikrein-like enzyme from human basophils, thought to be an arginine esterase [253]. Human lung mast cell tryptase has been shown to generate C3a from human C3 [48], while RPMC chymase has been demonstrated to degrade human C3a in the presence of heparin. Tryptase from human lung mast cells has also been shown to have fibrinogenolytic activity [286], which complements other mast cell anticoagulant mediators such as heparin and suggests a significant prevention of coagulation by activated mast cells. In rat anaphylaxis, the slowing down of coagulation is one of the characteristics of anaphylaxis [289] and these factors indicate that the contribution of mast cells in allergy is more complex than mere histamine release.

The examples given above and throughout this thesis have shown that there is a definite possibility that mast cell proteases have a distinct role in inflammation. Thus, the interaction between mast cells and peptides may not be one involving histamine release, but rather, the action of granule proteases upon cascades of the inflammatory system, such as the kinin, complement and coagulatory systems. Mast cell proteases may then significantly enhance the severity of anaphylactic shock and allergic conditions through this mechanism.
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