The role of phospholipase A<sub>2</sub> in mast cell activation

by Maheshkumar Premji Varsani

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To

Bapa

without whose divine blessings and constant encouragement, education for me is an impossibility
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Abstract

The mast cell has a pre-eminent role in the immunopathology and pathogenesis of inflammatory conditions, including asthma. However, the precise mechanisms involved in the signal-transduction process in this cell remain unclear.

The enzyme phospholipase A\(_2\) (PLA\(_2\)) cleaves fatty acid moieties from the sn-2 position of the glycerol phosphate backbone to yield the lyso-phospholipid and free arachidonic acid. The latter may be further metabolised to generate leukotrienes and prostaglandins. In addition, PLA\(_2\) may also be intrinsically involved in stimulus-secretion coupling in the mast cell. Thus some reports, but not others, have claimed that purified preparations of the enzyme may evoke mast cell degranulation. In light of recent phylogenetic classification of the enzyme into different subtypes, its role in mast cell activation has been re-examined in the present study.

Purified PLA\(_2\) from bee and cobra (Naja naja) venom produced a dose-dependent release of histamine from different mast cell phenotypes. In some cases, the release was non-cytotoxic whilst, in others, components of the process were cytolytic. The non-cytotoxic release of histamine induced by PLA\(_2\) may be due to the generation of lyso-phosphatidic acid, which has a signalling function in other systems. In contrast, the lytic release of the amine may be caused by the production of lyso-phosphatidylcholine, which is a known surfactant. Suboptimal amounts of the enzyme also potentiated immunological histamine release from rat mast cells, possibly through the generation of lyso-phosphatidylserine. The present study has indicated that the differential effects of PLA\(_2\) on various histaminocytes reflect variations in overall membrane composition leading to the production of one or other lipid metabolite. PLA\(_2\) may also act through the production of arachidonic acid and its further metabolism to 5-hydroperoxyeicosatetraenoic acid, which is thought to modulate secretion. Thus compounds which block the metabolism of arachidonic acid were effective in inhibiting the release of histamine from mast cells.

Immunological and non-immunological activation of rat mast cells increased the activity of endogenous PLA\(_2\). Compounds which block the function of PLA\(_2\) also inhibited histamine release from a variety of mast cell phenotypes. Hence, the activation of endogenous PLA\(_2\) may be an essential step in signal transduction in the mast cell. Inhibition of this enzyme may then provide a novel target in the therapeutic modulation of this cell type.
# Contents

<table>
<thead>
<tr>
<th>Title page</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Contents</td>
<td>5-13</td>
</tr>
<tr>
<td><strong>Chapter 1</strong></td>
<td><strong>Introduction</strong></td>
</tr>
<tr>
<td>1.1</td>
<td>Prelude</td>
</tr>
<tr>
<td>1.2</td>
<td>Discovery, origin and development</td>
</tr>
<tr>
<td>1.3</td>
<td>Biological significance</td>
</tr>
<tr>
<td>1.4</td>
<td>Mast cell heterogeneity</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Mast cell heterogeneity in the rodent</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Mast cell heterogeneity in the human</td>
</tr>
<tr>
<td>1.5</td>
<td>Activation of mast cells</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Introduction to the immune system</td>
</tr>
<tr>
<td>1.5.2</td>
<td>The high affinity receptor for IgE</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Immunological activation</td>
</tr>
<tr>
<td>1.5.4</td>
<td>Non-immunological activation</td>
</tr>
<tr>
<td>1.6</td>
<td>Mast cell mediators</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Preformed mediators</td>
</tr>
<tr>
<td>1.6.1.1</td>
<td>Histamine</td>
</tr>
<tr>
<td>1.6.1.2</td>
<td>Serotonin</td>
</tr>
</tbody>
</table>
1.6.1.3 Neutral proteases
1.6.1.4 Acid hydrolases
1.6.1.5 Chemotactic factors
1.6.1.6 Proteoglycans
1.6.2 Newly generated mediators
1.6.2.1 Eicosanoids
1.6.2.2 Platelet activating factor
1.6.2.3 Cytokines
1.7 Transduction pathways which control the activation of mast cells
1.7.1 Elevation of intracellular calcium
1.7.2 Guanosine trisphosphate (GTP) binding proteins (G-proteins)
1.7.3 Cyclic nucleotides
1.7.4 Protein tyrosine phosphorylation
1.7.5 Serine esterase
1.7.6 Phospholipase D
1.7.7 Membrane ion channels
1.8 Introduction to PLA₂
1.8.1 PLA₂ and disease
1.8.2 Subclasses of PLA₂
1.9 Aims of the present study
<table>
<thead>
<tr>
<th>Chapter 2</th>
<th>Methods and materials</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Animals</td>
<td>52</td>
</tr>
<tr>
<td>2.2</td>
<td>Human subjects</td>
<td>52</td>
</tr>
<tr>
<td>2.3</td>
<td>Rat basophilic leukaemia cells (RBL-2H3)</td>
<td>52</td>
</tr>
<tr>
<td>2.4</td>
<td>Physiological buffers</td>
<td>52</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Buffers used in the isolation of mast cells and in standard whole cell experiments</td>
<td>53</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Full HEPES buffered Tyrode's (FHT)</td>
<td>53</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Calcium and magnesium free-Tyrode's (CMF)</td>
<td>53</td>
</tr>
<tr>
<td>2.4.4</td>
<td>EDTA-Tyrode's (EDTA)</td>
<td>53</td>
</tr>
<tr>
<td>2.4.5</td>
<td>Glucose-free-Tyrode's (GFT)</td>
<td>53</td>
</tr>
<tr>
<td>2.4.6</td>
<td>BSA Tyrode's (BSA-FHT)</td>
<td>54</td>
</tr>
<tr>
<td>2.4.7</td>
<td>10 x calcium free Tyrode's (10xCFT)</td>
<td>54</td>
</tr>
<tr>
<td>2.4.8</td>
<td>Magnesium buffer for permeabilisation (Mg-T)</td>
<td>54</td>
</tr>
<tr>
<td>2.4.9</td>
<td>High salt potassium buffer for extracting enzymes (HSP)</td>
<td>54</td>
</tr>
<tr>
<td>2.5</td>
<td>Isolation and purification of mast cells</td>
<td>54</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Peritoneal mast cells</td>
<td>54</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Purification of RPMC</td>
<td>55</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Rat and guinea pig cutaneous, mesenteric and lung cells</td>
<td>55</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Human lung parenchymal mast cells</td>
<td>56</td>
</tr>
<tr>
<td>2.5.5</td>
<td>Human basophil leukocytes</td>
<td>57</td>
</tr>
<tr>
<td>2.6</td>
<td>Mast cell number and purity</td>
<td>57</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Alcian blue stain</td>
<td>57</td>
</tr>
</tbody>
</table>
2.6.2 Kimura stain

2.7 Mediator release from isolated mast cells and RBL-2H3 cells

2.8 Inhibition of histamine release from mast cells

2.8.1 Inhibition of histamine release using metabolic blockers

2.9 Potentiation of histamine release

2.10 Desensitisation of RPMC

2.11 Permeabilisation of RPMC

2.12 Culturing of RBL-2H3 cells

2.13 Extraction of PLA₂ enzyme

2.14 Extraction of inositol 1, 4, 5-trisphosphate (IP₃)

2.15 Measurement of mast cell mediators

2.15.1 Manual assay of histamine

2.15.2 Automated assay of histamine

2.15.3 PGD₂ assay

2.16 IP₃ assay

2.17 PLA₂ assay

2.18 Measurement of cytosolic calcium

2.19 Determination of membrane phospholipid composition

2.20 Active sensitisation

2.20.1 Sensitisation of rats with the nematode *Nippostrongylus brasiliensis* (NB)

2.20.2 Preparation of L₃ of NB
### Chapter 2

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.21</td>
<td>Materials</td>
</tr>
<tr>
<td>2.21.1</td>
<td>Secretagogues used</td>
</tr>
<tr>
<td>2.21.2</td>
<td>Inhibitors used</td>
</tr>
<tr>
<td>2.21.3</td>
<td>Solvents used</td>
</tr>
<tr>
<td>2.21.4</td>
<td>Buffer reagents</td>
</tr>
<tr>
<td>2.21.5</td>
<td>Culture reagents</td>
</tr>
<tr>
<td>2.21.6</td>
<td>Miscellaneous</td>
</tr>
<tr>
<td>2.22</td>
<td>Stock solutions of drugs</td>
</tr>
<tr>
<td>2.23</td>
<td>Numerical analysis</td>
</tr>
</tbody>
</table>

### Chapter 3

**Effect of different PLA<sub>2</sub> isozymes on various mast cell phenotypes**

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods and materials</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Comparison of the histamine release from RPMC treated with PLA&lt;sub&gt;2&lt;/sub&gt; isolated from various sources</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Calcium ion dependency of PLA&lt;sub&gt;2&lt;/sub&gt; induced histamine release from RPMC</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Kinetics of histamine release from RPMC treated with PLA&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Cytotoxicity of histamine release from RPMC treated with PLA&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Histamine release from non-purified and purified RPMC treated with PLA&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Effects of various inhibitors on PLA\textsubscript{2} induced histamine release from RPMC</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Effect of pertussis toxin on PLA\textsubscript{2} induced histamine release from RPMC</td>
</tr>
<tr>
<td>3.3.8</td>
<td>Desensitisation of RPMC to various secretagogues</td>
</tr>
<tr>
<td>3.3.9</td>
<td>Effects of PLA\textsubscript{2} on different mast cell phenotypes</td>
</tr>
<tr>
<td>3.3.10</td>
<td>Cytotoxicity of histamine release induced by PLA\textsubscript{2} from different mast cell phenotypes</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
</tr>
</tbody>
</table>

**Chapter 4**  
Effect of PLA\textsubscript{2} on histamine release induced by different secretagogues: a comparison with PS and lyso-PS  

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>119</td>
</tr>
<tr>
<td>4.2</td>
<td>Methods and materials</td>
<td>121</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>122</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Effects of PLA\textsubscript{2} in combination with various ligands</td>
<td>122</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Effects of PS and lyso-PS on histamine release induced by various ligands</td>
<td>122</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Cytotoxicity of the potentiation of histamine release</td>
<td>123</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Potentiation by <em>Naja naja</em>-PLA\textsubscript{2} of histamine release from purified RPMC</td>
<td>123</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>123</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Effect of specific PLA&lt;sub&gt;2&lt;/sub&gt; inhibitors on histamine release from various mast cell phenotypes</td>
<td>151-184</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>152</td>
</tr>
<tr>
<td>5.2</td>
<td>Methods and materials</td>
<td>153</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>153</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Effects of various PLA&lt;sub&gt;2&lt;/sub&gt; inhibitors on compound 48/80 and anti-IgE induced histamine release from RPMC</td>
<td>153</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Effects of various PLA&lt;sub&gt;2&lt;/sub&gt; inhibitors on anti-IgE induced histamine release from human lung mast cells</td>
<td>155</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Effects of various PLA&lt;sub&gt;2&lt;/sub&gt; inhibitors on anti-IgE induced histamine release from human basophil leukocytes</td>
<td>155</td>
</tr>
<tr>
<td>5.3.4</td>
<td>Further studies into the inhibition by OEPC from RPMC induced by non-immunological stimuli</td>
<td>156</td>
</tr>
<tr>
<td>5.3.5</td>
<td>Effects of mannose-BSA on histamine release from RPMC</td>
<td>158</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>159</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 6</th>
<th>Effect of various phospholipids and lipoxygenase/ cyclooxygenase inhibitors on histamine release from different mast cell phenotypes</th>
<th>185-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>186</td>
</tr>
<tr>
<td>6.2</td>
<td>Methods and materials</td>
<td>188</td>
</tr>
<tr>
<td>6.3</td>
<td>Results</td>
<td>188</td>
</tr>
</tbody>
</table>
6.3.1 Effects of L-α-phospholipids and their corresponding lyso-derivatives on histamine release from different mast cell phenotypes

6.3.2 Cytotoxicity of the histamine release induced by lyso-PA and lyso-PC from different mast cell phenotypes

6.3.3 Potentiation of histamine release from RPMC treated with anti-IgE in combination with L-α-phospholipids and their corresponding lyso-derivatives

6.3.4 Further studies into the histamine release induced by lyso-PA from RPMC

6.3.5 PGD$_2$ release from RPMC treated with PLA$_2$ from Naja naja and bee venom

6.3.6 Effects of dual lipoxygenase and cyclooxygenase inhibitors on the histamine release induced by different ligands from RPMC

6.3.7 Effects of cyclooxygenase inhibitors on the histamine release induced by different ligands from RPMC

6.3.8 Effects of lipoxygenase inhibitors on the histamine release induced by different ligands from RPMC

6.4 Discussion

Chapter 7 Further studies into the role of PLA$_2$ in mast cell activation

7.1 Introduction

7.2 Methods and materials

7.3 Results
7.3.1 Effects of PLAP on histamine release from RPMC stimulated with anti-IgE and Naja naja-PLA₂

7.3.2 Effects of anti-IgE and compound 48/80 treatment on PLA₂ activity in RPMC

7.3.3 Effects of Naja naja-PLA₂, anti-IgE and compound 48/80 on IP₃ production and intracellular calcium ion concentration in RPMC

7.3.4 Changes in membrane composition between resting and stimulated RPMC and RBL-2H3 cells treated with Naja naja-PLA₂

7.4 Discussion

Chapter 8 General synopsis and overview

References

Appendix 1 Structures of selected general drugs

Appendix 2 List of abbreviations
Chapter 1

"Felix qui potuit rerum cognoscere causas."
Lucky is he who has been able to understand the cause of things.

Virgil 70-19 BC: Georgics
Mast cells are found in virtually all the organs and tissues of the body, mostly perivascularly and often close to neurons\(^{(1)}\). It has been estimated that if all the mast cells in a human body were assembled together into a single organ, their mass would be the same as the spleen\(^{(2)}\). Mast cells have a pre-eminent function in the immunopathology and pathogenesis of hypersensitivity and inflammatory reactions\(^{(3)}\). In addition to allergic and anaphylactic reactions, mast cells can participate in neurogenic inflammation through vasodilatation and leukocyte infiltration\(^{(1)}\). Other biological responses in which mast cells participate include host responses to parasites and neoplasms, blood vessel formation, fibrotic conditions, tissue remodelling and wound healing\(^{(3)}\). A recent review has stated that there is no disease, biological condition, or animal model yet identified that exhibits an absolute lack of mast cells from which or in which their biological role might be inferred\(^{(4)}\). Activation of the cells, by many different stimuli, leads to the release of a variety of vasoactive, nociceptive and proinflammatory, preformed and newly synthesised mediators, which then act on effector cells to produce diverse biological symptoms\(^{(5)}\).

**1.2: Discovery, origin and development**

It is Friedrich von Recklinghause to whom the discovery of mast cells in the year 1863 is accredited\(^{(6)}\). However, it was not until 1879 that Paul Ehrlich described what he called "mastung" (i.e., overfed) cells with cytoplasmic basophilic granules in the connective tissue of humans\(^{(7)}\). Hence the name
mast cell was given from this German derivation. Ehrlich noticed that the cells were stuffed full of granular material which he assumed had been ingested. This is now known to be not the case\(^8\). Mast cells do possess some phagocytic ability although the significance of this is unclear\(^8\). The granules within these cells contain stored material for release upon activation. Basic dyes such as methylene blue and toluidine blue showed a strong purple-red staining of the monomorphic cytoplasmic granules within the cells. This phenomenon is now called metachromasia. Mast cells are oval in shape and are between 10 and 30 μm in length. The dense cytoplasmic granules which they contain are between 0.2 and 0.4 μm in diameter and approximately 1 x 10^3 granules are contained in each mast cell\(^9\). Ehrlich subsequently also identified the circulating equivalent of the mast cell, the peripheral blood basophil\(^10\) which also shows metachromasia.

The role of the mast cell in immediate hypersensitivity, sometimes referred to as anaphylaxis, was first demonstrated by Portier and Richet\(^{11}\) in 1903. In these experiments, dogs were injected with sea anemone toxin. Upon first injection, some dogs did not die but they reacted dramatically towards a second. Histamine (β-imidazolyethylamine) was first isolated from tissues by Windaus and Vogt in 1907\(^{12}\). However, it was not until 1910 when Dale and Laidlow made a link between histamine and the phenomenon of anaphylaxis\(^{13}\). Histamine injected into animals and tissues mimicked the characteristics of anaphylaxis\(^{13, 14}\). Dale\(^{15}\) and Schultz\(^{16}\) also demonstrated that tissues that had been sensitised with an antibody could also elicit an anaphylactic reaction. These studies prompted the ideas that are taken for granted today that histamine is an important mediator of anaphylaxis and the antigen-antibody reaction is its initiating stimulus. Much later, in the 1950’s, Riley and West correlated tissue histamine contents and mast cell numbers and proposed that mast cells were the source of the tissue histamine\(^{17, 18}\). These ideas were given further credence in the 1960’s when Ishizaka et al identified a novel serum antibody, immunoglobulin E (IgE)\(^{19, 20}\). They also showed that mast cells expressed surface receptors for IgE\(^{21, 22}\). Thus the
mast cell clearly has a central role in the pathogenesis of hypersensitivity reactions.

For a long time, it was uncertain where the tissue mast cells originated from. There were suggestions that they had been derived from T-cells, fibroblasts, or macrophages\(^{23-25}\). Subsequent studies have shown that mast cells are derived from pluripotent hematopoietic stem cells in the bone marrow\(^{26}\). In these studies, bone marrow was transported from a mouse mutant with cytoplasmic large granules (C57BL/6 bg'/bg'), or from respectively normal littermates (WBB6F1-+/+), to genetically mast cell deficient mouse mutants (WBB6F1-W/W'). The mast cell deficient mouse developed either mast cells that carried large granules as a phenotypic marker or normal mast cells, respectively. Using cells from colony forming units which had been injected into mouse skin, it was demonstrated that mast cells originate from cells less differentiated than those precursors committed to either the neutrophil-macrophage or erythroid lineage\(^{26}\). However, unlike the other derivatives of the pluripotent stem cells, such as the erythrocytes, neutrophils, basophils or eosinophils, which do not leave the hematopoietic tissue until their differentiation is complete, mast cells leave as morphologically unidentifiable mast cell progenitors. Differentiation into identifiable mast cells only occurs when they enter their final connective or mucosal tissue location\(^{27-29}\). This may be an explanation why mast cells from different species and indeed mast cells from different tissues from the same species have marked variations in their biochemical, histochemical and functional properties, a phenomenon known as mast cell heterogeneity. Hence, the properties of mast cells are only fully established at their final location. Specific mast cell growth factors, including interleukin-3 (IL-3), IL-4, stem cell factor (SCF), IL-9 and IL-10 have been identified which give rise to mast cell proliferation from their precursors \textit{in vitro}\(^{30-32}\).
Chapter 1  Introduction

1.3: Biological significance

Mast cells are found abundantly at any site which can come into contact with the environment, such as the skin, respiratory tract, lung, bladder and the digestive system\(^{(33, 34)}\). This selective accumulation at the sites where foreign material could invade the host suggests that the initial defence mechanisms involve activation of the mast cell. Mast cells are also present in connective tissue particularly surrounding blood vessels, nerves and glandular ducts\(^{(35-37)}\). It is well established that the mast cell is a major effector cell in the pathogenesis of immediate type hypersensitivity reactions such as bronchial asthma, allergic rhinitis and urticaria. However, mast cells also have a role in a number of non-allergic immune reactions. An increase in mast cell numbers has been shown in scar tissue, callus tissue, in osteoporosis-linked diseases and in various neuropathies\(^{(3)}\). Mast cells have also been shown to participate in various inflammatory reactions, such as interstitial pneumonia, ulcerative colitis, intestinal helminthosis and ectodermal parasitosis\(^{(3)}\). The elimination of parasites such as the schistosomes and helminths is thought to be the original function of mast cells and the function for which they have evolved and been conserved throughout evolution\(^{(38)}\). Mast cells are also involved in such diverse skin conditions as psoriasis, atopic dermatitis, bullous pemphigoid, lichen planus, wound healing and cancer\(^{(39-44)}\). Activation of mast cells can be fatal, as in the case of anaphylaxis or, in contrast, can play a significant role in a life-saving host response to bacterial reactions\(^{(45, 46)}\). Mast cells are not only involved in these pathological conditions, but also have a role in normal physiological processes. For example, the mast cell mediators histamine and heparin enhance vascularisation and endothelial cell proliferation\(^{(47, 48)}\). The two-way communication between mast cells and sensory nerve cells may provide a homeostatic function such as the controlling of cutaneous blood flow\(^{(49)}\). The release of a broad spectrum of multifunctional cytokines from activated mast cells may also influence other cell types and contribute to some of the above pathological conditions\(^{(50)}\).
Chapter 1

1.4: Mast cell heterogeneity

Even as early as 1906, Maximow and other workers saw that the mast cells in the intestinal tract of several species were smaller and less granulated than those elsewhere\(^{(51)}\). They thus suggested that mast cells did not represent a homogeneous cell population. Pioneering work on heterogeneity was probably carried out by Enerbäck who used staining techniques to investigate the mast cells in the rat gastrointestinal tract\(^{(52, 53)}\). Mast cells that were located in the lower layer of the intestinal wall resembled those in other connective tissues and the serosal cavities, now called connective tissue mast cells (CTMC). The cells in the mucosa, called mucosal mast cells (MMC), displayed different properties. MMCs are smaller in size, more variable in shape, possess fewer granules and have a lower histamine and 5-hydroxytryptamine content\(^{(54)}\). These initial studies have stimulated extensive investigations into the heterogeneity of mast cells in rodents and in man.

Mast cell sub-populations differ not only in their histochemical and biochemical properties, but they may also be distinguished by their functional characteristics. Mast cells from different locations, mast cell phenotypes, exhibit functional heterogeneity in their responses to various secretagogues and a variety of drugs\(^{(4)}\). This is potentially very interesting as it may lead to the development of more potent drugs that are efficacious in only one phenotype\(^{(3)}\). Electron microscopy studies have shown that mast cells stimulated with IgE-dependent stimuli show a rapid swelling and fusion of mast cell granules and an abrupt mediator release. However, in certain disease states, a rather more piecemeal degranulation occurs with a gradual release of the chemical mediators. This again could reflect the heterogeneity with attendant different responses to IgE-mediated stimuli\(^{(55)}\).

Both mast cells and basophils contain the dense cytoplasmic granules which contain the bodies major sources of histamine\(^{(56)}\). They both also express the high affinity plasma membrane receptor for IgE\(^{(22)}\). However, there are clear
differences between the two, the most obvious one being the location of their maturation. (A summary of the comparison between mast cells and basophil leukocytes is given in Fig. 1.1) This has then prompted discussion about the origin of mast cell heterogeneity. Some workers think that the differing mast cell phenotypes represent the different stages on a single mast cell line\(^{(57)}\). However, others believe that the different phenotypes stem from different precursors\(^{(56, 59)}\). Kitamura et al. have suggested that the maturation into the different phenotypes is dependent on the anatomical microenvironment where the cells finally differentiate. Their studies have shown that mucosal type mast cells can differentiate into connective tissue type mast cells and vice versa\(^{(60, 61)}\), indicating that the surrounding tissue may actually dictate the phenotype produced. Mast cell heterogeneity can be traced back to certain cytokine patterns that are present in different microenvironments\(^{(61, 62)}\), including the presence of IL-3\(^{(63)}\) and SCF\(^{(64)}\).

1.4.1: Mast cell heterogeneity in the rodent

Two major rodent mast cell subtypes have been distinguished. The first are the atypical, T-cell-dependent MMCs which are found in the mucosa of the gastrointestinal tract and in the lamina of the respiratory tract. The proliferation of these cells is mediated by the cytokines IL-3, IL-4, IL-9 and IL-10\(^{(65)}\). The T-cell independent CTMCs occur mostly in the submucosa of the gastrointestinal tract, in the skin and in the peritoneal cavity. The development of this subtype is mediated by fibroblast-derived factors such as SCF\(^{(65)}\). A summary of the major characteristics of the two types is given in Fig. 1.2.

MMC's contain granules consisting of a relatively soluble matrix containing chondroitin sulphate and little histamine. Staining of these cells is blocked by common, formaldehyde-based fixatives\(^{(66)}\). CTMCs contain large amounts of histamine and heparin which produces the metachromatic staining pattern in response to dyes such as toluidine blue\(^{(66)}\).
The two types of mast cell also differ in the neutral protease content of their granules. The CTMCs contain a chymotrypsin-like neutral protease called rat mast cell protease-I\(^{(67)}\) and carboxypeptidase A, which is an enzyme which can hydrolyse the carboxy-terminal peptide bond of polypeptide chains\(^{(68)}\). MMCs, however, contain an immunologically distinct chymotryptic neutral protease called rat mast cell protease-I\(^{(67)}\).

In addition to release of their granular contents, mast cells synthesise and release a number of other potent mediators. Immunological activation of mast cells leads to the liberation of arachidonic acid (AA) from cellular membranes which can be further metabolised via the cyclooxygenase and lipoxygenase pathways to produce prostaglandins (PG) and thromboxanes (TX), or leukotrienes (LT), respectively. CTMCs isolated from the peritoneal cavity metabolise AA through the cyclooxygenase pathway to yield PG\(_D^2\) but little or no LT\(^{(69)}\). In contrast, MMCs isolated from the intestine synthesise comparable amounts of PG\(_D^2\) and LTB\(_4\) and LTC\(_4\)\(^{(70)}\).

The final example given here to demonstrate the differences between the two phenotypes is their respective responses to non-immunological stimuli. The “classical mast cell degranulating agent” compound 48/80 is highly specific in its actions. It induces a significant non-cytotoxic release of histamine from CTMCs but is ineffective against mucosal type cells\(^{(71)}\). The anti-asthma drug disodium cromoglycate (DSCG) is a potent inhibitor of immunologically induced histamine release from CTMCs but is totally inactive against its mucosal type counterpart\(^{(72)}\).
1.4.2: Mast cell heterogeneity in the human

Unlike in the rat, it is not possible strictly to classify the mast cells into mucosal and connective tissue types in humans. In the rat, the presence or absence of heparin proteoglycan results in formalin resistance and hence differing staining properties. A method of ultrastructural analysis using antithrombin III gold revealed that all human mast cells contain heparin\(^{(73)}\). However, a technique has been developed which uses staining and fixation properties to define mast cell types\(^{(74)}\). As in the rat, the main structural components of the secretory granules are neutral proteinases. These are enzymes that can catalyse the cleavage of peptide bonds. As depicted by their classification, they perform best at neutral pH\(^{(75)}\). Extensive investigations by Irani et al have shown that human mast cells can be distinguished and classified according to whether they contain only tryptase (MC\(_T\)) or both tryptase and chymase (MC\(_{TC}\))\(^{(76)}\). Most anatomical sites contain a mixture of both subtypes, however MC\(_{TC}\) is dominant in the skin and the small intestinal submucosa, whilst in the lung and intestinal mucosa, MC\(_T\) predominates\(^{(77)}\).

Immunoelectronmicroscopic studies have also differentiated the two subtypes according to their ultrastructures. MC\(_{TC}\) has secretory granules with grating and lattice substructures, whereas MC\(_T\) have granules containing scrolls\(^{(78)}\). However, scrolls have been sometimes detected in the peripheral regions of MC\(_{TC}\) granules\(^{(79)}\). The two types also generate and release different cytokines\(^{(80)}\). IL-4 is distributed among both phenotypes, however it is preferentially expressed by MC\(_{TC}\) (overall: 85 %, MC\(_{TC}\); 15 %, MC\(_T\)). In contrast, IL-5 and IL-6 was localised only to the MC\(_T\) subset. A summary of the characteristics of the two sub-populations of human mast cell types is given in Fig. 1.3.

There is also strong evidence of functional human mast cell heterogeneity. The majority of mast cells in the human heart are of the MC\(_{TC}\) phenotype.
These cells exhibit surface membrane antigens and functional properties similar to those of the lung and uterine mast cells\(^\text{61}\). Studies by Patella et al have indicated that immunological activation of the cells caused the release of preformed (histamine and tryptase) and \textit{de novo} synthesised (LTC\(_4\) and PGD\(_2\)) mediators\(^\text{62}\). However, the tryptase content of heart mast cells was much lower than in skin but much higher than in lung mast cells. Also, maximal stimulation with anti-IgE gave rise to the release of LTC\(_4\) and PGD\(_2\), whereas with skin, PGD\(_2\) was released much more abundantly with negligible amounts of LTC\(_4\). Recombinant human C5a anaphylatoxin and protamine induced histamine release from heart and skin mast cells but not from lung mast cells. Substance P and morphine induced degranulation from skin mast cells, but not from heart or lung mast cells. Compound 48/80 showed a release of histamine from skin and heart mast cells but not from their human lung counterpart\(^\text{62}\). DSCG has a moderate inhibitory action on histamine release from immunologically activated human colon, lung, uterine and bronchoalveolar lavage mast cells\(^\text{83}\) but has no effect on skin mast cells\(^\text{84}\).

Basophils only enter tissues under specific pathological conditions such as cutaneous basophil hypersensitivity\(^\text{85}\). Like the MC\(_T\) phenotype, human basophils do not respond to compound 48/80. Immunological activation of basophils is also unaffected by the application of DSCG\(^\text{85}\).

1.5: Activation of mast cells

1.5.1: Introduction to the immune system

The environment in which we exist is filled with a large variety of infectious agents, including bacteria, viruses, parasites and fungi. To some individuals, normally harmless agents, such as pollen and house dust mite may be regarded as being infectious. Vertebrates depend on physical barriers such as the protective barrier of the skin, the mucous membrane in the nasal passages and enzymes such as lysozyme in tears, as a first line defence
against these potentially pathogenic invaders. Pathogens that breach these physical barriers can be destroyed and eliminated by phagocytes, which are white blood cells that can engulf foreign agents. However, these are not sufficient to ward off the onslaught of various pathogenic agents and, in consequence, vertebrates have developed a specific and adaptable mechanism to aid protection from these agents - the immune system\(^{(66)}\).

The first role of the immune system is to recognise the invading pathogen. This is achieved by the lymphocytes, specialised white blood cells of which there are several types, the granular-, B- and T- lymphocytes, killer(cytotoxic) cells and natural killer cells. All of these are important elements in the recognition of pathogens in the immune system\(^{(67)}\). Once activated, the B-lymphocytes synthesise an antibody against the antigen\(^{(68)}\). Memory cells are also produced which, on subsequent exposure to the same antigen, are prompted readily to produce the correct antibodies\(^{(68)}\).

The process of B-cell expansion and maturation is controlled by the release of small molecular weight proteins, cytokines, from T-lymphocytes and many other cell types including mast cells\(^{(69)}\). T-cells expressing the cluster of differentiation 4 (CD4) antigen, broadly named T-helper cells (T\(_H\)), clonally expand around the activated B-cell. This is driven by IL-2. A range of other cytokines which have B-cell activating abilities are also released from T-cells. IL-1, -4 and -6 prime this expansion\(^{(90)}\). T-lymphocytes which express the CD8 antigen (T suppressor, T\(_S\)) release soluble factors which are inhibitory to this cascade of events. An abnormality in the T\(_H\) and T\(_S\) balance in allergic individuals has been detected and this may provide an explanation as to why some individuals react allergically to normally innocuous foreign substances\(^{(91-93)}\). A clear role has been shown for IL-4 and interferon \(\gamma\) (IFN\(_\gamma\)) in the up-regulation and down-regulation of IgE synthesis, respectively\(^{(94-97)}\). IL-5, -6 and tumour necrosis factor \(\alpha\) (TNF\(_\alpha\)) all enhance IL-4 induced IgE synthesis whilst IFN\(_\alpha\) and transforming growth factor \(\beta\) (TGF\(_\beta\)) inhibit synthesis of the antibody\(^{(94)}\).
Chapter 1  Introduction

Under certain circumstances, this protective system can react strongly to a normally inoffensive foreign substance and as a result of the immune system misdirecting or inappropriately deploying its effects on innocuous substances, the consequences can be deleterious rather than beneficial\(^{(86)}\). This is termed an allergic or hypersensitivity reaction\(^{(87)}\).

1.5.2: The high affinity receptor for IgE

The primary event in the initiation of an allergic response involves the binding of IgE to high affinity receptors (Fc\(_e\)RI) which are found on the surface of mast cells and basophils. Like the other antibody/immunoglobulin classes (IgA, IgD, IgG and IgM) IgE has two identical heavy chains and two identical light chains that are folded together into a globular domain. Each light and heavy chain has a variable and a constant region and its stability is maintained by the formation of disulphide bonds between the chains. The IgE serum content is merely a fraction of the total antibody present, typically being 50 - 300 ng/ml, compared to an IgG serum level of 10 mg/ml\(^{(98)}\). The difference between IgE and the other antibodies is the sequence of its constant heavy chain region (\(\varepsilon\) region). This region consists of four domains (Ce1-4), one more than IgA, IgD, or IgG, and it is the Ce3 domain which contains the Fc\(_e\)RI binding site\(^{(99, 100)}\). Each mast cell has approximately 1 - 5 \(\times\) 10\(^6\) Fc\(_e\)RI receptors studded on their surfaces\(^{(101)}\). IgE labelled with \(^{125}\)I has been shown to bind with high affinity almost exclusively to mast cells and basophils. Their affinity for IgE is approximately 10\(^9\) l/mol, whereas in the case of platelets, eosinophils and macrophages, it is several orders of magnitude less\(^{(102)}\). This low affinity IgE receptor is called Fc\(_e\)RII. They are also found free in the plasma\(^{(103)}\). Both high and low affinity receptors have been cloned and there seems to be little homology between the two\(^{(103)}\).
Binding of IgE to its receptor is not sufficient to elicit an activation of the mast cell. Cross-linking of adjacent cell fixed IgE molecules by an appropriate antigen is required for activation to occur\(^{104-106}\).

1.5.3: Immunological activation

Mast cells onto which IgE molecules have been affixed are termed “sensitised”. Much work has shown that cross-linking of the IgE molecules, and thus clustering of the Fc\(\varepsilon\)RI receptors, is the trigger required for mast cell activation\(^{106, 107}\). Recent work using mast cells that had been reacted with glass surfaces carrying densities of both antigen and bound IgE has confirmed the earlier work\(^{108}\). This has established the essential role of Fc\(\varepsilon\)RI immobilisation for initiating its signal transduction cascade. This report gives support to the notion that as few as two Fc\(\varepsilon\)RIls immobilised at van der Waals contact constitute an “elementary stimulatory unit” leading to a mast cell secretory response. In vivo, cross-linking by a polyvalent allergen activates the cell.

A range of common allergens, of apparently diverse nature, exists. These include pollens, animal danders, food components and certain drugs\(^{109}\). A short list of these is given in Fig. 1.4.

The plant lectin concanavalin A (Con A) binds to carbohydrate moieties on the immunoglobulin. In this way, cross-linking of adjacently bound IgE molecules can occur to activate the cells\(^{110}\). Divalent IgG antibodies raised against IgE (anti-IgE) are also used experimentally to accomplish in vitro cross-linking and thus degranulation.
Chapter 1

Introduction

1.5.4: Non-immunological activation

A large variety of substances cause activation of mast cells and basophils apart from the immunological IgE receptor cross-linking mechanism discussed previously. Some stimuli may cause histamine secretion in a non-selective or cytotoxic fashion by disruption of the cell membrane. Such agents include Triton X-100 and Tween-20\(^{(11)}\). These agents have detergent type properties.

Non-cytotoxic/active secretion requires an intact cell metabolism for the process to occur and intracellular markers such as lactate dehydrogenase are not lost. The effects of these agents are tissue and species dependent\(^{(12)}\). A summary of these stimuli is given in Fig. 1.5.

Compound 48/80 is a simple basic amine polymer which induces a non-cytolytic secretion of histamine from some mast cell phenotypes. Other agents include the neuropeptide substance P, the high molecular weight sugar dextran, the calcium ionophore A23187, the basic peptide from wasp venom mastoparan and enzymes such as exogenous phospholipase A\(_2\) (PLA\(_2\)\(^{(13)}\)), which is the subject of interest in this study. The mechanisms by which these and other agents induce secretion is outlined in chapter 4 and have been reviewed by Lagunoff et al\(^{(13)}\).

1.6: Mast cell mediators

Upon activation, mast cells secrete a wide range of chemical agents which go on to produce the diverse effects of inflammation. These mediators are either pre-formed and contained in the mast cell granules, or are synthesised de novo. A summary is given in Fig. 1.6.
1.6.1: Pre-formed mediators

1.6.1.1: Histamine

Histamine comprises between 5 and 15 % of the dry weight of the mast cell granules. The amine is formed in the Golgi apparatus of mast cells by the decarboxylation of histidine and metabolised almost completely by the enzymatic actions of N-methyl transferase and diamine oxidase which are found in the liver, kidney and small intestine. Once formed, histamine ionically associates with the acidic residues of the glycosaminoglycan side chains of proteoglycans. Dissociation of histamine during degranulation occurs by a cationic exchange with extracellular sodium ions. Mast cells and basophils are the major source of histamine in the body, although histaminergic nerves in the central nervous system and the periphery also exist. Further, certain enterochromaffin cells contain histamine and these may serve in normal gastric functioning.

The diverse biological effects of histamine are mediated by its binding to specific receptors, H₁, H₂ and H₃. H₁ receptors mediate bronchial, ileal, and uterine smooth muscle contraction and are coupled to inositol phosphate turnover. They also stimulate microvascular leakage through the contraction of epithelial cells in the post-capillary venules. H₂ receptors are coupled to adenylate cyclase and are involved in the secretion of gastric acid from parietal cells, stimulation of airway mucus secretion and bronchodilatation of human airways. H₃ receptors have primarily been found in the central nervous system where they have a presynaptic role in modulating neurotransmitter release.

Histamine also has some immunomodulatory activities. Via H₂ receptors, histamine can inhibit immunologically induced histamine release from basophils. Histamine is weakly chemotactic for eosinophils and neutrophils. It can also affect lymphocyte activity by suppressing B-cell
antibody production, lymphocyte proliferation, T-cell cytokine production and greatly enhance natural killer cell cytotoxicity\(^{(130, 131)}\). Thus, histamine has spasmogenic and pro-inflammatory activities and is also able to modulate other immunological processes\(^{(132)}\).

### 1.6.1.2: Serotonin

Serotonin (5-hydroxytryptamine) is contained within the granules of rodent mast cells with histamine in the granular proteoglycan matrix\(^{(133)}\). However, in humans, it is not stored in mast cells but is found instead in platelets\(^{(114)}\). Serotonin is generated by the decarboxylation of tryptophan and can produce bronchoconstriction, vasoconstriction and microvascular leakage in the periphery and can act as a neurotransmitter in the central nervous system\(^{(119)}\).

### 1.6.1.3: Neutral proteases

The large quantities of tryptase and chymase that are synthesised in mast cells suggest and emphasise the significance of these neutral proteases in mast cell function\(^{(134)}\). They are the major protein constituents of the granular matrix. Their abundance and specificity to mast cells makes them ideal quantitative markers of mast cell degranulation\(^{(135)}\). As stated earlier, the distribution of these enzymes is used as a tool to classify mast cell phenotypes.

Tryptase is stored almost exclusively in the granules as a fully active tetrameric serine endoprotease and has catalytic sites on each of the subunits\(^{(136)}\). Chymase is also a serine endoprotease with a specificity for chymotrypsin-like substrates and exists as various isoforms which exhibit interspecies variation.

Little and Johnson\(^{(137)}\) have demonstrated that human mast cell tryptase exists as two different isoforms. The forms differed in their specificity and rate...
Chapter 1

Introduction

of cleavage of various substrates. Stimulation of bronchial muscle with tryptase causes contraction\(^{138}\). The synthesis of deoxyribonucleic acid (DNA) in bronchial epithelial cells is stimulated upon treatment with tryptase, hence it can be regarded as a potent mitogen\(^{139}\). In the epidermis, tryptase releases and cleaves fibronectin as well as collagenase type IV and thus may have a role in tissue remodelling and matrix degradation\(^{140, 141}\). In blood coagulation, tryptase seems to play a role by way of fibrinogen cleavage and inactivation\(^{142}\). Tryptase can cleave the complement component C3 to form the anaphylatoxin and direct spasmogen C3a and thus reinforce mast cell degranulation\(^{143}\). Under certain conditions, tryptase can act on kininogen to yield the potent pro-inflammatory mediator bradykinin\(^{144}\).

Mast cell chymases vary greatly in their surface charge and their binding to proteoglycans. Several of these enzymes, especially canine and rat chymase I, bind strongly to heparin\(^{145-147}\). Human chymase has been shown to convert angiotensin I to angiotensin II independent of the angiotensin-converting enzyme \emph{in vivo} and \emph{in vitro}\(^{148}\) and may thus play a role in blood pressure regulation\(^{149, 150}\). Chymase, like tryptase, increases bronchial muscle contractility. Unlike tryptase, however, it can also hydrolytically inactivate bronchoactive peptides such as bradykinin and kallidin\(^{151}\) as well as certain neuropeptides such as substance P and vasoactive intestinal peptide, enabling chymase to inactivate certain bronchoconstrictors, which can then possibly limit mast cell degranulation\(^{152, 153}\). Chymase has been proposed, but yet not demonstrated, to cause the blistering seen occasionally in children with cutaneous mastocytosis\(^{154}\). Mast cell neutral proteases can thus have both pro- and anti-inflammatory actions.

1.6.1.4: Acid hydrolases

Mast cells contain numerous other granule associated enzymes, including arylsulphatases, \(\beta\)-glucuronidase, exoglycosidases and hexosaminidase\(^{155}\). These all have optimal activity at acidic pH.
1.6.1.5: Chemotactic factors

In addition to the release of potent mediators, mast cells from both rodents and human can secrete factors which are chemotactic for eosinophils (eosinophil chemotactic factor of anaphylaxis, ECF-A) and neutrophils (neutrophil chemotactic factor of anaphylaxis, NCF-A)\(^{156-158}\). Thus the mast cell degranulation in the immediate phase allergic response can result in the activation and recruitment of polymorphonucleocytes to the inflammatory zone and thus induce the late phase response. It has been demonstrated that allergen induced bronchial provocation results in a sustained increase in plasma NCF-A concentration\(^{158}\).

1.6.1.6: Proteoglycans

Proteoglycans comprise the major constituents of mast cell granules and it is the presence of these agents that conveys the characteristic staining phenomena of mast cells and basophils, metachromasia. The two major classes of proteoglycans found in mast cells are heparin and chondroitin sulphate, and the proportion of each in a particular phenotype is variable\(^{159}\). Proteoglycans are macromolecules comprised of glycosaminoglycan chains, consisting of disaccharide repeating units of uronic acid and hexosamine moieties, covalently linked to a protein core\(^{160}\). The proteoglycans bind histamine, neutral proteases and acid hydrolases in the granules and may facilitate the uptake and storage of these mediators\(^{161}\). When degranulation occurs, histamine and the acid hydrolases rapidly diffuse away, whilst the neutral proteases remain associated with heparin at the cell surface\(^{162}\).

Heparin has a role in inhibition of blood coagulation\(^{163}\), regulation of tryptase activation\(^{162}\), modulation of growth factors\(^{164}\), inhibition of the complement cascade\(^{165}\), inhibition of activities of several eosinophil pro-inflammatory mediators\(^{166, 167}\), as well as having anti-allergic properties\(^{168}\). There is also a theory that mast cell-derived heparin may limit eosinophil recruitment and the
actions of cationic proteins in vivo and thus may have a role in the pathogenesis of asthma\(^{(160)}\).

1.6.2: Newly generated mediators

When mast cells are activated, a de novo synthesis and release of potent chemical mediators occurs. Two general categories of mediators exist, the eicosanoids and the cytokines. Eicosanoids are released within minutes of activation and synthesis may continue for up to 30 minutes. In contrast, cytokines may not be synthesised until hours after the activation occurs. There are however some cytokines which are pre-formed and these are released more rapidly.

1.6.2.1: Eicosanoids

These chemical mediators comprise of PG, LT and TX. The initiation of their synthesis involves the cleavage of membrane phospholipids by PLA\(_2\) to yield free AA. This is then metabolised via either the cyclooxygenase or lipoxygenase pathways\(^{(170)}\). This is detailed in chapter 6 and thus is not extensively elaborated on here.

The activity of the cyclooxygenase enzyme and hence production of PG's can be blocked by non-steroidal anti-inflammatory drugs (NSAIDs). This is thought to be the major mechanism by which aspirin and related drugs exert their pharmacological effects\(^{(171)}\).

The eicosanoids exert their diverse biological effects by acting on specific receptors\(^{(172)}\). PGD\(_2\) is the principle cyclooxygenase product following immunological or calcium ionophore induced activation of human and rat mast cells\(^{(173, 174)}\). However, substance P induces histamine release from rat peritoneal and human skin mast cells without release of PGD\(_2\)\(^{(175)}\). Similarly, compound 48/80 induces PGD\(_2\) release from rat peritoneal mast cells.
(RPMC) but not from human skin mast cells\textsuperscript{(175, 176)}. Clinically, PGD\textsubscript{2}, causes bronchoconstriction, peripheral vasodilatation, inhibition of platelet aggregation, production of a wheal and flare response in human skin, and augmentation of LTB\textsubscript{4}-induced accumulation of neutrophils in human skin\textsuperscript{(177-181)}. PGD\textsubscript{2} is 3 times more potent as a bronchoconstrictor than PGF\textsubscript{2\alpha} and 30 times more effective than histamine\textsuperscript{(182)}. TXA\textsubscript{2} also has constrictor activity in human airways\textsuperscript{(182-184)}. In contrast, PGE\textsubscript{1}, PGE\textsubscript{2} and PGI\textsubscript{2} tend to oppose the biological activities of the above prostanoids. In this way, PGs may modulate allergic processes in both a beneficial and detrimental manner depending on the principle product formed.

Products of the lipoxygenase pathway are in general more potent than those of the cyclooxygenase pathway. LTC\textsubscript{4} and LTD\textsubscript{4} are between 1000 and 5000 times more efficacious as constrictors of human bronchial smooth muscle than histamine\textsuperscript{(185, 186)}. They are also potent stimulants of mucus secretion in human airways\textsuperscript{(187)}. In vascular tissue, LTC\textsubscript{4} and LTD\textsubscript{4} are potent elevators of microvascular leakage and produce the wheal and flare response\textsuperscript{(188, 189)}. LTB\textsubscript{4} is a potent chemotactic factor for leukocytes and LTC\textsubscript{4} and LTB\textsubscript{4} can increase the adhesiveness of leukocytes to endothelial cells and hence increase diapedesis\textsuperscript{(190-193)}. Mast cell derived LTs have been detected in various biological fluids of asthmatic individuals\textsuperscript{(194, 195)}. Together with their spectrum of activity, they then have been given major importance in allergic inflammatory disease\textsuperscript{(196)}.

1.6.2.2: Platelet activating factor

Platelet activating factor (PAF) is generated through a two-step process. PLA\textsubscript{2} hydrolyses membrane phospholipids to produce lyso-PAF and then an acetyltransferase enzyme acetylates lyso-PAF generating PAF\textsuperscript{(197)}. The biological actions of PAF include the activation of human platelets, eosinophils and degranulation of human basophils and RPMC\textsuperscript{(198-201)}. PAF
may also have a role in allergic asthma\textsuperscript{(202)}. These effects are all described further in chapter 6.

1.6.2.3: Cytokines

Cytokines influence many physiological processes including cellular proliferation and differentiation, cellular phenotype expression, regulation of immune responses, control of host defences against viral and parasitic infections, tissue remodelling and bone formation\textsuperscript{(203, 204)}. Cytokines are multifunctional proteins and are produced by many cell types. Their effects are conveyed by their binding to specific receptors\textsuperscript{(205)}. Mast cells produce and secrete various cytokines including IL-1, IL-3, IL-4, IL-6, TNF-\textsubscript{\textalpha}, and TGF-\textbeta\textsuperscript{(206, 207)}. Recent studies have shown that asthmatics had an altered pattern of cytokine secretion\textsuperscript{(208)}.

1.7: Transduction pathways which control the activation of mast cells

The addition of an immunological stimulus and subsequent mediator release has been extensively studied in the laboratory. However the underlying mechanisms coupling the two processes are complex and are still the target of much research. Several overlapping mechanisms are thought to be fundamental in the activation process of mast cells, whilst others may more subtly act to regulate the degranulation\textsuperscript{(209)}. Certain of these processes are mentioned below.

1.7.1: Elevation of intracellular calcium

This will be described in detail in chapter 7, but a brief summary is given below.
Chapter 1

Introduction

The normal intracellular (ca. 0.1 μM) / extracellular (ca. 1 mM) calcium ion gradient is also present in mast cells. Active transport of calcium ions and the relative impermeability of the cell membrane to Ca$^{2+}$ ensures that this homeostasis is maintained\(^{(210)}\). Artificial elevation of intracellular calcium using ionophores can initiate degranulation\(^{(211, 212)}\). Ca$^{2+}$ may also be released from intracellular stores. The immunological activation of mast cells involves uptake of Ca$^{2+}$ into the cell cytosol. Removal of external calcium reduces, but does not abolish, histamine release from mast cells\(^{(213)}\). This demonstrates that internal calcium stores are being mobilised. Ca$^{2+}$ mediates its effects through the activation of a variety of calcium binding proteins. Calmodulin seems to be of most importance\(^{(214)}\). It is present in all eukaryotic cells and exerts its pleiotropic cellular responses through activation of various proteins including protein kinases and phosphatases, cyclic nucleotide phosphodiesterase and ATPases\(^{(215, 216)}\). Inhibitors of calmodulin have been shown to inhibit histamine release from mast cells and basophils\(^{(217)}\) indicating the importance of the protein. Evidence from other cell systems suggests that the whole process may be controlled by the turnover of membrane phospholipids.

The cross-linking of IgE receptors may generate the calcium signals through the hydrolysis of membrane inositol phospholipids. The cross-linking activates tyrosine kinase, which initiates the formation of inositol 1,4,5-trisphosphate (IP$_3$) and inositol 1,3,4,5-tetraphosphate (IP$_4$) by activating the membrane enzyme phospholipase C (PLC)\(^{(218, 219)}\). Briefly, IP$_3$ releases calcium from internal stores in the endoplasmic reticulum\(^{(220, 221)}\). In the mast cell, the calcium signal produced in this way stimulates further IP$_3$ formation and hence amplification of the signal occurs\(^{(102)}\). The PLC mediated hydrolysis of inositol phospholipids also generates diacylglycerol (DAG) which activates protein kinase C (PKC)\(^{(102)}\). This enzyme is contained in the cytosol of resting state cells but it associates with the membranes when DAG is formed and then interacts with the kinase\(^{(102)}\). Activation of PKC provides a
Chapter 1

Introduction

signal that interacts synergistically with a calcium signal generated by a calcium ionophore to produce secretion, hence the two mechanisms may have some overlap\textsuperscript{(222, 223)}. An 80 kDa substrate for PKC, named myristilated alanine rich C kinase substrate (MARCKS) has been shown to exist\textsuperscript{(224)}. This becomes phosphorylated by PKC and then dissociates from the cell membrane. It then becomes free to bind calmodulin\textsuperscript{(225)}. MARCKS may synergise with Ca\textsuperscript{2+} at the level of calmodulin.

1.7.2: Guanosine trisphosphate (GTP) binding proteins (G-proteins)

G-proteins are a heterogeneous family of trimeric proteins which share a common cyclical mechanism of activation\textsuperscript{(226)}. The three sub-units are denoted \( \alpha \), \( \beta \) and \( \gamma \). G-proteins have been shown to exist downstream in many cell systems, linking membrane receptors with a range of effector systems. In the inactive state, the \( \alpha \) sub-unit is bound to guanosine 5'-diphosphate (GDP) and is associated with the \( \beta \) and \( \gamma \) sub-units. When an appropriate receptor/ agonist interaction occurs, and the G-protein is activated, the GDP becomes dissociated. This then allows binding with the more prevalent GTP. GTP binding with the \( \alpha \) sub-unit promotes the dissociation of \( \alpha \)-GTP from the \( \beta \gamma \)-complex\textsuperscript{(227)}. In this dissociated state, the sub-units can modulate various effectors including adenylate cyclase, enzymes and ion channels\textsuperscript{(228)}. Slow hydrolysis of \( \alpha \)-bound GTP by a GTPase intrinsic to the \( \alpha \) sub-unit leads to reassembly and inactivation of the G-protein\textsuperscript{(228)}. Electrophysiological and permeabilisation experiments on RPMC have shown that G-protein activation is a sufficient stimulus for mast cell activation\textsuperscript{(229)}.

1.7.3: Cyclic nucleotides

The cyclic nucleotides, cyclic 3', 5'-adenosine monophosphate (cAMP) and cyclic 3', 5'- guanosine monophosphate (cGMP), regulate biological
Chapter 1

Introduction

responses in a number of cell types. cAMP and cGMP activate protein kinases which can phosphorylate serine and threonine residues\(^{(230, 231)}\). Guanylate cyclase transforms GTP to cGMP\(^{(232)}\). Intracellular concentrations of cyclic nucleotides are controlled by the action of phosphodiesterases (PDE) which can hydrolyse and hence inactivate both cAMP and cGMP\(^{(233)}\). Some studies have reported elevated levels of cAMP following immunological activation of RPMC and human lung mast cells\(^{(234, 235)}\). Phosphodiesterase inhibitors also suppress histamine release from mast cells\(^{(236)}\). However, some studies have produced conflicting evidence and the role of these nucleotides is therefore not entirely clear\(^{(237)}\).

1.7.4: Protein tyrosine phosphorylation

Inhibitors of protein tyrosine kinase (PTK) have been shown to inhibit immunologically induced histamine release from RBL-2H3 cells, basophils and human lung mast cells\(^{(238-240)}\). Using RBL-2H3 cells, it has been shown that a phosphorylation of protein tyrosine residues occurs following immunological activation\(^{(241)}\). Mast cell targets for PTK activity include aggregated Fc\(\varepsilon\)RI receptors, PLC and several other cytosolic proteins which have not been fully elucidated\(^{(242-244)}\).

1.7.5: Serine esterase

Recent work using specific inhibitors of serine esterases have shown that immunological stimulation may involve activation of a membrane serine esterase\(^{(245)}\). Exogenously applied \(\alpha\)-chymotrypsin induces degranulation of mast cells suggesting that the mast cell serine esterase has \(\alpha\)-chymotryptic properties.
1.7.6: Phospholipase D

A substantial phospholipase D (PLD) mediated hydrolysis of phosphatidylcholine (PC) to form phosphatidic acid (PA) has been shown to occur upon antigen stimulation of RPMC and RBL-2H3 cells\[^{246-248}\]. The subsequent dephosphorylation of PA by phosphatidic acid phosphohydrolase produces DAG. This DAG formation seems to be essential for antigen induced secretion\[^{249}\].

1.7.7: Membrane ion channels

Mast cells do not possess voltage operated ion channels and are thus termed "non-excitable" secretory cells\[^{250}\]. However, various studies have indicated that ion fluxes do occur, although the importance of this in the signal transduction process is unclear\[^{251, 252}\]. It has been postulated that G-proteins and IP\(_4\) have a role in controlling Ca\(^{2+}\) channels\[^{253}\]. It has also been shown that the activation of a chloride channel occurs upon treatment with both Ca\(^{2+}\) and cAMP\[^{251}\]. The resulting chloride flux may slightly hyperpolarise the mast cell membrane and so facilitate Ca\(^{2+}\) influx down its electrochemical gradient. Blockers of chloride channels have been shown to inhibit mast cell degranulation\[^{252}\].

1.8: Introduction to PLA\(_2\)

Phospholipases are a group of enzymes which hydrolyse membrane phospholipids with great specificity to yield free fatty acids and the corresponding lyso-phospholipids. PLA\(_2\) cleaves the ester bond at the \(sn-2\) position of the phospholipid molecule\[^{254}\]. This is depicted in Fig. 1.7.

PLA\(_2\) was the first phospholipase to be identified. In the early 1900's, it was noted that if PC was incubated with pancreatic juices or cobra venom, free
fatty acids were formed\(^{(255)}\). \(\text{PLA}_2\) is found not only in pancreatic secretions, but is also abundant in the plasma, lymph and alveolar secretions\(^{(256)}\). These are termed the secretory \(\text{PLA}_2\)’s. In fact, \(\text{PLA}_2\) is found in all tissues and cells and is located either in the cytosolic compartment associated with the plasma membrane or stored within organelles of the vacuolar system such as secretory granules and lysosomes\(^{(257)}\). Hence, these are classified as the cytosolic \(\text{PLA}_2\)’s.

\(\text{PLA}_2\) participates in such diverse and specialised functions as membrane remodelling, digestion of phagocytosed bacteria, metabolism of pulmonary surfactant phospholipids and intestinal digestion of dietary phospholipids\(^{(258-261)}\). The action of \(\text{PLA}_2\) is also the initiation and rate limiting step for the release of fatty acid precursors and inflammatory eicosanoids\(^{(262,263)}\).

### 1.8.1: \(\text{PLA}_2\) and disease

\(\text{PLA}_2\) is involved in the pathogenesis of many diseases. Local and circulating levels of the enzyme are elevated during infections, inflammatory diseases, tissue injury and brain dysfunction\(^{(256,264,265)}\) and correlate with the severity, magnitude and duration of these disorders\(^{(256,266-269)}\). Uncontrolled or excessive \(\text{PLA}_2\) activity may promote chronic inflammation, allergic reactions, tissue injury and pathophysiological complications\(^{(270)}\). \(\text{PLA}_2\) products, or lipid mediators derived thereof, have been implicated in numerous activities that play an integral part in effector cell activation, chemotaxis, adhesion, degranulation, phagocytosis, and aggregation\(^{(271,272)}\). It has been proposed that an altered \(\text{PLA}_2\) activity or defects in its control and regulation may be a predisposing factor to affective illnesses including depression\(^{(273)}\).

Various cells, including macrophages, neutrophils and platelets release \(\text{PLA}_2\) upon stimulation\(^{(274-276)}\). The cytokine IL-1, which is produced after infection, injury or antigenic challenge has been reported to induce cellular biosynthesis of \(\text{PLA}_2\)\(^{(277)}\). TNF also induces a similar synthesis and
intravascular secretion of PLA₂ from the appropriate target cells. The products of PLA₂ hydrolysis then go on to produce the diverse clinical effects of PLA₂. This is summarised in Fig. 1.8. Human PLA₂ mRNA has been detected in human tonsils, kidney and placenta as well as in cells derived from human rheumatoid arthritic synovial fluid, inflamed synovial tissue and peritoneal exudate.

1.8.2: Subclasses of PLA₂

The effects of PLA₂ on mast cell activation have been contradictory. Some studies have indicated that the cells secreted histamine whilst others have shown no effects of applying the enzyme. Recent phylogenetic studies, involving a comparative alignment of the PLA₂'s of reptiles, insects and human, have led to the subdivision of the enzymes by virtue of their disulphide bonding patterns and primary amino acid sequences. This may explain the earlier antithetical results of mast cell activation by PLA₂. Hence, PLA₂ is not a single enzyme but is really an expanding “superfamily” of enzymes (isoenzymes). Characterisation has focused mainly on the extracellular PLA₂ enzymes due to their ubiquitous nature and high concentrations found in pancreatic juices and snake venoms. Traditionally, these extracellular enzymes were divided into three main groups. The isoenzymes isolated from the “old world” snake venoms of Elapid and Hydrophid variety, including Naja naja, and mamalian pancreas are classed as Type 1. Type II includes those isolated from the venoms of the Viperid and Crotalidae species, such as those from Crotalus atox and Crotalus adamenteus, human synovial fluid, platelets, mast cells and inflammatory exudates. Type III includes the isoenzymes isolated from bee venom.

All these isoenzymes have been isolated as extracellular enzymes and share many common characteristics such as a homology in both their amino acid sequence and X-ray crystal structures. The enzymes have a
molecular mass of approximately 14 - 18 kDa and differ in their states of aggregation. They are optimally active in the neutral to alkaline pH range and require Ca$^{2+}$ in the mM range for both catalysis and substrate binding. Their stability is conveyed in part by their high (seven) disulphide bond content. The catalytic mechanism by which these isoenzymes hydrolyse the phospholipid substrate involves the positioning of His-48 and Asp-99 to form an active Asp-His couple\(^{(287, 288, 290)}\). This couple polarises a bound water molecule which then attacks the carbonyl group and the Ca$^{2+}$ ion stabilises this transition. The cytosolic PLA\(_2\) isoenzymes have a higher molecular mass (31 - 110 kDa).

In the past few years, a great deal of work has been carried out in this field and numerous PLA\(_2\) isoenzymes have been identified, all essentially having the same function, but which do not fit into the above traditional categorisation. In many cases, several of these isoenzymes are present in the same cell\(^{(291)}\). A Ca$^{2+}$ independent, 85 kDa cytosolic PLA\(_2\) has been purified from human monocytes\(^{(292)}\). Another group of calcium independent PLA\(_2\) isoenzymes has been shown to exist in the brush-border membranes of the small intestine, lysosomes and a large variety of animal tissues such as the lung, myocardium and alveolar macrophages\(^{(293)}\).

### 1.9: Aims of the present study

A role for PLA\(_2\) in the pathogenesis of disease has been well established. It has even been reported that PLA\(_2\) induces a non-specific airway hyperreactivity which is the hallmark of asthma\(^{(294)}\). The role of the enzyme in mast cell activation has been emphasised by the finding that AA is released in parallel with histamine from cells which have been immunologically activated. Moreover, inhibitors of PLA\(_2\) not only inhibit AA release but also histamine release. The aim of the present study was further to elucidate the role of PLA\(_2\) in mast cell activation. The isoenzymes isolated from *Naja naja* and bee venom were eventually used as a paradigm of PLA\(_2\) activation in mast cells.
In view of the considerable functional variations between mast cell phenotypes, the effects of the isoenzymes on different mast cell populations were compared. Further studies were carried out to determine the mechanisms by which PLA$_2$ induces mediator secretion from mast cells and the role of the endogenous enzyme in the release process.
Fig. 1.1: Comparison of mast cells and basophil leukocytes

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>MAST CELLS</th>
<th>BASOPHILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>6-12 µm</td>
<td>5-7 µm</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Round or oval</td>
<td>Segmented</td>
</tr>
<tr>
<td>Cytoplasmic granules</td>
<td>Many and small</td>
<td>Few and large</td>
</tr>
<tr>
<td>Cytoplasmic lipid bodies</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Surface receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fc,R1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C-kit</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Interleukin-3</td>
<td>Yes (mouse)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No (human)</td>
<td></td>
</tr>
<tr>
<td>Natural history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>Haematopoietic stem cells</td>
<td>Haematopoietic stem cells</td>
</tr>
<tr>
<td>Site of maturation</td>
<td>Peripheral tissue</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mature cells in circulation</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Proliferation potential</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Life span</td>
<td>Weeks to months</td>
<td>Days</td>
</tr>
<tr>
<td>Mature cells recruited into tissue from circulation</td>
<td>No</td>
<td>Yes (during immunological inflammatory responses)</td>
</tr>
<tr>
<td>Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate hypersensitivity</td>
<td>Involved</td>
<td>Involved</td>
</tr>
<tr>
<td>Rejection of intestinal parasites</td>
<td>Involved</td>
<td>?</td>
</tr>
<tr>
<td>Rejection of ticks</td>
<td>Involved</td>
<td>Involved</td>
</tr>
<tr>
<td>Augmentation of acute infection</td>
<td>Involved</td>
<td>No</td>
</tr>
<tr>
<td>Cutaneous basophil hypersensitivity</td>
<td>No</td>
<td>Involved</td>
</tr>
</tbody>
</table>

Adapted from reference 62.
### Fig. 1.2: Characteristics of rat mucosal and connective tissue mast cells

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>MMC</th>
<th>CTMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining with alcian blue/safranin sequence</td>
<td>Blue</td>
<td>Red or blue and red</td>
</tr>
<tr>
<td>Fixation</td>
<td>Critical, aldehyde blocking</td>
<td>No aldehyde blocking</td>
</tr>
<tr>
<td>Appearance</td>
<td>Small, variable shape, variable-size granules</td>
<td>Larger, uniform shape, uniform-size granules</td>
</tr>
<tr>
<td>Behaviour</td>
<td>Migratory</td>
<td>Non-migratory</td>
</tr>
<tr>
<td>Dependent on</td>
<td>T cells, fibroblasts</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>Lifespan</td>
<td>Short: half life &lt;40 days</td>
<td>Long: half-life &gt;6 months</td>
</tr>
<tr>
<td>Histamine content</td>
<td>Low: &lt;2 pg/cell</td>
<td>High: &lt;25 pg/cell</td>
</tr>
</tbody>
</table>

**Proteoglycans**

<table>
<thead>
<tr>
<th></th>
<th>MMC</th>
<th>CTMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chondroitin sulfate di-B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chondroitin sulfate A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chondroitin sulfate E</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Adapted from reference 3.
Fig. 1.3: Characteristics of human $\text{MC}_{\text{TC}}$ and $\text{MC}_T$ cells

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>$\text{MC}_{\text{TC}}$ (pg/cell)</th>
<th>$\text{MC}_T$ (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease</td>
<td>Tryptase (35)</td>
<td>Tryptase (10)</td>
</tr>
<tr>
<td></td>
<td>Chymase (4.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carboxypeptidase (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cathepsin G (+)</td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>Heparin</td>
<td>Heparin</td>
</tr>
<tr>
<td></td>
<td>Chondroitin sulfates A, E</td>
<td>Chondroitin sulfates A, E</td>
</tr>
<tr>
<td>Granule morphology</td>
<td>Grating / lattice</td>
<td>Complete scroll-rich</td>
</tr>
<tr>
<td></td>
<td>Complete scroll-poor</td>
<td></td>
</tr>
<tr>
<td>T lymphocyte-dependence</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cytokine content</td>
<td>IL-4</td>
<td>IL-4, IL-5, IL-6</td>
</tr>
</tbody>
</table>

Adapted from reference 3.
Chapter 1

Introduction

Fig. 1.4: Common allergenic agents

INHALANTS
- Pollens
- Fungi
- Animal Products
- Dusts
- Algae

Sources of pollens
- Trees
- Grasses
- Weeds

Mammalian orthopod
- Dander
- Cuticle
- Saliva
- Urine
- Faeces

INGESTANTS
- Foods

Egg, Milk, Wheat, Peanut,
- Soy, Corn, Beef, Shrimp

Drugs

CONTACTANTS
- Dyes
- Rubber compounds
- Fungicides
- Cosmetics
- Bleach
- Mercury
- Nickel
- Copper

INJECTANTS
- Drugs
- Venom
- Insect saliva

Insect, Penicillin,
- Tetanus toxoid, Furosemide,
- Measles, mumps and rubella
- Vaccines

Adapted from reference 295.
Most of these drugs release histamine only from certain mast cell types and even then they do so at concentrations which are high or which exceed those achieved therapeutically.

IL-1 produces little histamine release. IL-3 produces a little release by itself but will potentiate histamine release induced by antigen.

Adapted from reference 102.
## Fig. 1.6: Mast cell mediators

<table>
<thead>
<tr>
<th>MEDIATORS</th>
<th>POSSIBLE PATHOPHYSIOLOGIC EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Granule-stored (pre synthesised)</strong></td>
<td></td>
</tr>
<tr>
<td>Arylsulfatases</td>
<td>Lipid / proteoglycan hydrolysis</td>
</tr>
<tr>
<td>Chemotactic factors</td>
<td>Infiltration of leukocytes</td>
</tr>
<tr>
<td>Chymase</td>
<td>Tissue damage, pain, angiotensin I and kinin metabolism</td>
</tr>
<tr>
<td>Cytokines (IL-1, 2, 3, 4, 5 &amp; 6, GM-CSF, INF-γ, MIF, TNF-α)</td>
<td>Tissue damage, leukocyte chemotaxis, pain</td>
</tr>
<tr>
<td>Enkephalins</td>
<td>Pain control</td>
</tr>
<tr>
<td>Heparin</td>
<td>Angiogenesis, nerve growth factor stabilisation</td>
</tr>
<tr>
<td>Histamine</td>
<td>Vasodilatation, angiogenesis, mitogenesis, pain</td>
</tr>
<tr>
<td>5-Hydroxytryptamine (5HT, serotonin)</td>
<td>Vasoconstriction, pain</td>
</tr>
<tr>
<td>Kinins</td>
<td>Vasodilatation, pain</td>
</tr>
<tr>
<td>Kinogenases</td>
<td>Synthesis of vasodilatatory kinins, pain</td>
</tr>
<tr>
<td>Somatostatin (SRIF)</td>
<td>Regulation of mediator secretion</td>
</tr>
<tr>
<td>Tryptase</td>
<td>Tissue damage, pain, neuropeptide processing</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide (VIP)</td>
<td>Vasodilatation, pain</td>
</tr>
<tr>
<td><strong>Newly synthesised</strong></td>
<td></td>
</tr>
<tr>
<td>Leukotriene B₄ (LTB₄)</td>
<td>Leukocyte chemotaxis</td>
</tr>
<tr>
<td>Leukotriene C₄ (LTC₄)</td>
<td>Bronchoconstriction, pain</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>Vasodilatation</td>
</tr>
<tr>
<td>Platelet activating factor (PAF)</td>
<td>Platelet activation, bronchoconstriction</td>
</tr>
<tr>
<td>Prostaglandin D₂ (PGD₂)</td>
<td>Vasodilatation</td>
</tr>
</tbody>
</table>

Abbreviations used under cytokines:
- GM-CSF = granulocyte monocyte-colony stimulating factor
- INF = interferon
- MIF = macrophage inflammatory factor
- TNF = tumour necrosis factor

Adapted from reference 1.
Fig. 1.7: Specificity of phospholipases

Fig. 1.7: outlines the structure of phospholipids and site of action of phospholipases. The numbering of the carbon atoms in the backbone is given on the left. Unsaturated fatty acids, such as AA, are usually esterified to position 2. Thus, the diagram indicates how arachidonate can be released in a one-step process. $X =$ hydrogen or phosphate esterified to choline, ethanolamine, serine, or inositol.

Adapted from reference 171.
Fig. 1.8: Proposed effector pathway of cytokine-induced PLA₂ activation and release of arachidonic acid derived mediators leading to the symptoms of inflammation.

Adapted from reference 278.
Chapter 2

“On principle, it is quite wrong to try founding a theory on observable magnitudes alone. It is the theory which decides what we can observe.”

A. Einstein, from J. Bernstein,

“The secrets of the Old Ones II”, New Yorker, 1973
2.1: Animals

In the present study, male Sprague-Dawley rats (200-300 g), male Dunkin Hartley guinea pigs (300-800 g) and female MF1 mice (30-40 g) were used. All animals were obtained from closed, random bred colonies kept at the Biological Services Division of University College London, or from commercial breeders (Harlan UK/ Charles River).

2.2: Human subjects

Human lung tissue was obtained from the surgeons of St. George’s Hospital, London, following surgical resection for bronchial carcinoma. Peripheral blood was obtained from healthy volunteers by phlebotomy.

2.3: Rat basophilic leukaemia cells (RBL-2H3)

RBL-2H3 cells were kindly donated by Dr. G. A. Mackay, The Randall Institute, King’s College London and Dr. B. Y. Wan, Department of Pharmacology, University College London.

2.4: Physiological buffers

All buffers were made using glass distilled water.
2.4.1: Buffers used in the isolation of mast cells and in standard whole cell experiments

Unless otherwise stated, all experiments were carried out using a modified Tyrode’s solution which had been buffered with N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES) as described in the following section. The pHs of the buffer solutions were adjusted within the range 7.2-7.4 by the addition of sodium hydroxide (NaOH, 4 M) or hydrochloric acid (HCl, 2 M).

2.4.2: Full HEPES buffered Tyrode’s (FHT)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1.0 mM (147 mg/l)</td>
</tr>
<tr>
<td>D-glucose</td>
<td>5.6 mM (1.0 g/l)</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0 mM (2.86 g/l)</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM (201 mg/l)</td>
</tr>
<tr>
<td>NaCl</td>
<td>137 mM (8.0 g/l)</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>0.4 mM (62.5 mg/l)</td>
</tr>
</tbody>
</table>

2.4.3: Calcium and magnesium-free-Tyrode’s (CMF)

The composition of this buffer was identical to that of the FHT buffer except for the omission of calcium chloride.

2.4.4: EDTA-Tyrode’s (EDTA)

This buffer was identical to CMF, except for the addition of EDTA (0.2 mM, 75 mg/l).

2.4.5: Glucose-free-Tyrode’s (GFT)

This consisted of FHT from which glucose had been omitted.
2.4.6: BSA Tyrode's (BSA-FHT)

BSA-FHT contained FHT supplemented with bovine serum albumin (BSA, 1 mg/ml).

2.4.7: 10x Calcium free Tyrode's (10x CFT)

This comprised a 10 fold greater concentration of all the reagents in FHT, except for the omission of calcium chloride.

2.4.8: Magnesium buffer for permeabilisation (Mg-T)

This comprised CMF to which magnesium chloride (20 mM, 4.62 g/l) had been added. The pH of this buffer was then adjusted to 7.8.

2.4.9: High salt potassium buffer for extracting enzymes (HSP)

This buffer consisted of potassium chloride (KCl, 2 M, 148 g/l), disodium hydrogen phosphate (Na₂HPO₄, 0.06 M, 8.52 g/l) and sodium dihydrogen phosphate (NaH₂PO₄, 0.04 M, 6.24 g/l) made up in glass distilled water. The pH of this buffer was then adjusted to 7.0.

2.5: Isolation and purification of mast cells

2.5.1: Peritoneal mast cells

Animals were asphyxiated in an enclosed chamber with an increasing concentration of carbon dioxide, followed by cervical dislocation. Abdominal skin was cut away and then heparinised (50 units/ml) FHT (20 ml per rat, 5 ml per mouse) was injected into the peritoneal cavity through the exposed abdominal wall. Special attention was taken to ensure that penetration of the
gastro-intestinal tract did not occur. The abdomen was gently massaged (2 min/ rat, 10 sec/ mouse) in order to detach the cells from the cavity walls. An incision was made along the midline and the peritoneal fluid was removed using a plastic pipette. This was then collected in polypropylene centrifuge tubes (cells tend to adhere to glass). Care was taken to avoid introduction of air bubbles into the samples and any preparations that were heavily contaminated with blood were discarded at this stage. The cellular suspension was centrifuged (100 g; 2.5 min; 4 °C) and the resulting cell pellet washed twice in FHT before either being purified or used directly in functional studies.

2.5.2: Purification of RPMC

Mixed RPMC (section 2.5.1) were purified using density gradient centrifugation in Percoll. Cells were suspended in BSA-FHT (1 ml) and mixed with a Percoll solution (4 ml, 9 parts Percoll: 1 part 10x CFT). BSA-FHT (1 ml) was carefully layered on top of this preparation, so as to produce an interface, and the sample was then centrifuged (140 g; 25 min; 4 °C). The supernatant was carefully aspirated to leave a pure mast cell pellet. This was washed twice in BSA-FHT and twice in FHT before being used in functional studies.

2.5.3: Rat and guinea pig cutaneous, mesentric and lung cells

The animals were sacrificed with (rat) or without (guinea pig) asphyxiation followed by cervical dislocation. The abdomen skin was shaved free of any fur and an area of approximately 5 cm² was dissected free. Any underlying tissue and fat was removed. The abdomen was then opened along the midline and the mesentery removed from both the small intestine and any attached lymph nodes. The lungs were removed from the chest cavity and any major airways were dissected out. Each of the tissues were rinsed several times in BSA-FHT and then chopped finely (<1 mm²) using scissors.
The tissue was digested (37 °C; 60 min, 90 min skin; with constant stirring) in BSA-FHT supplemented with collagenase (Type 1A, 120 units/ml; 160 units/ml skin). In the case of skin, the enzyme concoction was supplemented with hyaluronidase (Type 1S, 500 units/ml). Following this enzymatic dispersion, the tissue was further disrupted by repeated (10 times) extrusion through a syringe (20 ml). The resulting suspension was filtered through moistened cotton gauze to remove any undigested tissue. The cells were recovered by centrifugation (100 g; 3 min; 4 °C), washed twice in FHT and then used in functional studies without further purification. If, after the first dispersion, a substantial amount of undigested matter remained, a second digestion of this tissue was carried out. The same procedures as in the first digestion were used.

2.5.4: Human lung parenchymal mast cells

Macroskopically normal human lung parenchyma was dissected free of pleural tissue, major airways and blood vessels. The tissue was crudely chopped into fragments (~1 cm³) with scissors and thoroughly washed with FHT. The tissue was then cut into finer fragments (<1 mm³) and washed thoroughly once more in FHT. It was then enzymatically digested (37 °C; 90 min) in BSA-FHT containing collagenase (160 units/ml) with constant stirring. The tissue was subsequently further disrupted by extrusion (10 times) through a syringe (50 ml) and then filtered through moistened Nytex gauze. The filtrate was centrifuged (150 g; 3 min; 4 °C), the cell pellet resuspended in BSA-FHT and the supernatant (containing the enzyme) added to any undigested lung fragments. The digestion procedure was repeated for the undigested tissue. Finally, all the cell suspensions were combined, centrifuged (150 g; 3 min; 4 °C), resuspended in BSA-FHT and washed twice in FHT before being used directly in functional studies.
2.5.5: Human basophil leukocytes

Blood (120 ml per donor) was obtained by venepuncture and placed in polypropylene centrifuge tubes (60 ml) containing isotonic saline (10 ml) with dextran (6%), glucose (30 mg/ml) and heparin (50 units/ml), in a ratio of 1:4 (solution:blood). The solution was inverted and left to stand for 90 minutes (RT) or until the erythrocytes had settled to the bottom sector of the tubes. This resulted in a supernatant which was rich in platelets and leukocytes. This top layer was carefully aspirated with a liquipipette and centrifuged (100 g; 5 min; 4°C). The platelet rich supernatant was discarded and the cell pellet washed twice in FHT before being used in functional studies.

2.6: Mast cell number and purity

Two different staining methods were used to ascertain the number of mast cells present or to assess the purity of a cell suspension. The total cell number was determined by counting using an improved Neubauer haemacytometer. Cell purity was determined by counting the number of stained cells with respect to the total number of cells present.

2.6.1: Alcian blue stain

An aliquot of the cells was incubated (15 min; 37 °C; with shaking) in a solution of alcian blue (1 % w/v alcian blue; 0.9 w/v NaCl; 0.5 M HCl; 0.1 % v/v Tween 20) in a ratio of 9:1.

2.6.2: Kimura stain\(^{(296)}\)

An aliquot of the cells (40 µl) was gently mixed with Kimura stain (100 µl) and FHT (60 µl). The Kimura stain comprised the following.

i) Toluidine blue (0.05 %, 25 mg/25 ml in NaCl (1.8 %)) + 11 ml ethanol, made up to 50 ml with H₂O.
Chapter 2 Methods and Materials

ii) Saturated saponin in ethanol (50 %).

iii) Sodium dihydrogen phosphate (0.066 M, 1.03 g/100 ml H$_2$O)

The three solutions were mixed in a ratio of 22:1:10, respectively, filtered and stored at 4 °C.

2.7: Mediator release from isolated mast cells, basophils and RBL-2H3 cells

Aliquots of the cell suspensions (200 μl) were added to disposable polypropylene centrifuge tubes (containing 250 μl FHT) and allowed to equilibrate in a water bath incubator (37 °C, 5 min). A solution of the releasing agent (50 μl, at 10x required final concentration) was then added and the reaction allowed to proceed for 10 min (20 min for *Naja naja*-PLA$_2$ and basophils) before being stopped by the addition of ice cold FHT (1 ml). Samples which contained cells, but no secretagogue, were also used (in duplicate) in order to determine any spontaneous mediator release from the cells. Any secreted mediator was separated from the cells by centrifugation (100 g; 2 min; 4 °C) and decanted into correspondingly labelled tubes. The cell pellets were then resuspended in FHT (1.5 ml) and either boiled for 10 minutes (manual assay) or treated with 70 % v/v perchloric acid (final concentration 0.4 M, automated assay). In PGD$_2$ studies, an aliquot (250 μl) of the supernatant was removed after centrifugation. This was snap frozen in liquid nitrogen and stored at -70 °C until required. This prevented both further synthesis and degradation of the prostanoid.

The histamine contents in both the supernatant and the cell pellets were determined and the release expressed as a percentage of the total amount of histamine present originally in the cells.
\[ \text{Histamine release (\%)} = \left( \frac{H_S}{H_S + H_C} \right) \times 100 \]

where:
- \( H_S \) = amount of histamine in supernatant
- \( H_C \) = amount of histamine in cell pellet

In all experiments, values were corrected for the spontaneous release occurring in the absence of any stimulus.

2.8: Inhibition of histamine release from mast cells

Isolated mast cells (200 µl) were incubated with an inhibitor solution (250 µl at 2x required concentration) for a defined period of time (0, 10 or 20 min) before the addition of a stimulus (50 µl at 10x required concentration). The reaction was allowed to proceed as normal before being quenched and the suspension centrifuged in the same manner as in the histamine release protocol.

Inhibition of histamine release was expressed in terms of the percentage of inhibition of the control release (the release in the absence of the inhibitor).

\[ \text{Inhibition (\%)} = \left( \frac{R_C - R_I}{R_C} \right) \times 100 \]

where
- \( R_C \) = Control release
- \( R_I \) = Release in the presence of inhibitor

2.8.1: Inhibition of histamine release using metabolic blockers

Aliquots of isolated mast cells (200 µl, resuspended in GFT) were incubated (37 °C, 10 min) with the metabolic inhibitors antimycin A (1 µM, dissolved in GFT) and 2-deoxy-D-glucose (5 mM, dissolved in GFT). The samples were then challenged with the secretagogue (50 µl, dissolved in GFT; 10 min) after...
which the reaction was terminated by addition of ice-cold FHT (1 ml) and treated as before.

2.9: Potentiation of histamine release

Cells were simultaneously treated with two stimuli. The reaction was then treated as in the usual histamine release experimental procedure. Using this method, it could be detected whether the histamine release elicited by the two secretagogues in combination was additive or synergistic.

2.10: Desensitisation of RPMC

RPMC may be desensitised to a particular secretagogue, or secretagogues which act through the same mechanism according to the method employed by Dianzani and Foreman[297]. Briefly, cells were incubated with a secretagogue (0 °C, 10 min). The tubes were then centrifuged and the supernatants discarded. The cell pellet was then resuspended in FHT and incubated for a second time with the same, or different, secretagogue (37 °C, 10 min). The same procedure as in the standard histamine release experiment was subsequently followed.

2.11: Permeabilisation of RPMC

Purified RPMC were reversibly permeabilised by treatment with ATP, according to the method of Bennett et al[298]. Briefly, ATP (50 μM, 50 μl, made stable and neutral by the addition of Trizma base (100 μM)) was incubated (37 °C, 5 min) with aliquots of the cells resuspended in CMF (100 μl), CMF (50 μl) and the agent which is desired to enter the cell (eg. inhibitor, 250 μl). The ATP is able to permeabilise the cell membrane and facilitate penetration of the compound into the cell. Mg-T buffer (50 μl) was then added and the reaction allowed to proceed for a further 3 minutes. The excess of
magnesium ions combines with the ATP and terminates its action. Hence, all the created pores become re-sealed, leaving the test compound trapped in the cell cytosol. CMF (2 ml) was then added and the cells were washed twice in CMF and twice in FHT, before being stimulated with different secretagogues and then treated as before.

2.12: Culturing of RBL-2H3 cells

Cells were grown in adherent culture flasks (T-162) in DMEM medium (50 ml) supplemented with L-glutamate (2 mM) and foetal calf serum (FCS, 15 %). Cells seeded at 1×10^5 cells/ml doubled in number every 18 hours and reached confluency (4-6×10^6 cells/ml; 3-4×10^7 cells/flask) after incubation for 3-4 days (37 °C, 5 % CO_2).

Cells were passaged at confluency (twice weekly) by dissociating them from the flask using trypsin and EDTA. Briefly, the medium was removed and the cells rinsed with 3 ml of trypsin (0.05 %)/EDTA (0.02 %), incubated (37 °C, 5 min), centrifuged (100 g; 5 min; 4 °C), washed with, and resuspended in, growth medium and reseeded as above.

When required for functional studies cells at, or near, confluency were recovered with non-trypsin dissociating fluid as follows.

The medium was first removed and dissociating fluid (5 ml, trypsin (0.05 %)/EDTA (0.02 %)) added. The preparations were incubated (37 °C, 5 min), centrifuged (100 g; 5 min; 4 °C) and the cells washed with FHT and resuspended in this buffer at a density of 1×10^7 cells/ml. Each flask yielded 3-4×10^7 cells. They were then treated in the same way as peritoneal mast cells, except that the assay procedure was performed using the autoanalyser.
2.13: Extraction of PLA₂ enzyme

Total enzyme extraction from RPMC was achieved using a two step method developed by Fräki and Hopsu-Hava\(^{299}\).

Briefly, purified RPMC were snap frozen using liquid nitrogen. They were then allowed to thaw slowly at room temperature and HSP buffer (25 µl) was added. The cells were again frozen and allowed to thaw as before. This cycle of adding HSP followed by freeze-thawing was performed a total of 5 times. The cell extract was then allowed to stir overnight (4 °C). The following day, the extract was clarified by centrifugation (15000 g; 15 min; 4 °C), and immediately subjected to the enzyme assay procedure.

2.14: Extraction of inositol -1,4,5-trisphosphate (IP₃)

IP₃ from purified RPMC was extracted by the addition of perchloric acid (20 %, 0.2 v/w, 0 °C). The samples were immediately placed in ice (20 min) and centrifuged (2000 g; 15 min; 4 °C). The supernatants were decanted and neutralised (pH 7.5) using NaOH (4 M, 33 µl). The resulting KClO₄ was sedimented by centrifugation (2000 g; 15 min; 4 °C) and the supernatant transferred into pre-labelled test tubes for assaying.

2.15: Measurement of mast cell mediators

2.15.1: Manual assay of histamine

RPMC were assayed for histamine according to a method first described by Shore \textit{et al}\(^{300}\). This method measures the fluorescence generated by the alkaline condensation product of histamine with o-phthaldialdehyde (OPT). The scheme of this reaction is given in Fig. 2.1. The fluorescent adduct was measured using a commercially available spectrophotometer (Perkin Elmer
LS 5B) using an excitation wavelength of 360 nm and an emission wavelength of 440 nm. The fluorescence generated is proportional to the histamine content present in the sample. Briefly, the sample (1.5 ml) was made alkaline by addition of NaOH (1 M, 200 μl) and vortex mixed thoroughly. OPT (1% w/v in methanol, 75 μl) was added and the solution again vortex mixed. The reaction was allowed to proceed for precisely 4 minutes before being quenched by the addition of HCl (3 M, 100 μl). In this manner, histamine concentrations of 5-10 ng/ml could be measured.

If immunoglobulin was used as the secretagogue, especially if in high concentrations, protein precipitation often resulted on the addition of the acid. To counteract this, samples were centrifuged (200 g; 10 min; 4 °C) before being assayed.

2.15.2: Automated assay of histamine

Tissue mast cell preparations, mouse peritoneal mast cells, human basophils, RBL-2H3 cells or RPMC samples which contained drugs which interfered with the fluorescence obtained in the manual assay, were assayed using a commercial autoanalyser (Technicon Autoanalyser II). The final assay reaction is essentially the same as the manual method described above, but the automated process includes chemical extraction steps to purify the histamine samples prior to the reaction with OPT.

To prepare samples for analysis, perchloric acid (0.4 M final) was added to free any residual histamine from the cell pellet and to precipitate any protein from the samples. The latter were then vortex mixed, centrifuged (200 g; 10 min RPMC and RBL-2H3 cells, 20 min tissues and basophils; 4 °C) and decanted into sample cups. The sample was introduced into the autoanalyser and first made alkaline. The histamine was then extracted into the salt saturated butan-1-ol. The organic phase was separated and retained, washed once in a less alkaline medium, made less polar by the addition of n-
heptane and the histamine back extracted into dilute HCl. The sample was then made alkaline before being allowed to react with OPT. The fluorescent adduct was stabilised by acidification and quantified using a fluorophotometer and chart recorder. Using this assay method, histamine concentrations of 1-10 ng/ml could be measured.

2.15.3: PGD\textsubscript{2} assay

Amounts of PGD\textsubscript{2} generated and released from a reaction were measured using a commercially available radioimmunoassay (RIA) kit (Amersham). Purified RPMC preparations were used and the number of cells present in each sample (>1x10\textsuperscript{5} cells) was determined prior to performing the experiment. The assay is based on the competition between unlabelled PGD\textsubscript{2} and \textsuperscript{3}H-labelled PGD\textsubscript{2} for binding to a limited quantity of an antibody raised with a high specificity to PGD\textsubscript{2}. Thus the PGD\textsubscript{2} content of a sample would be inversely proportional to the radioactivity measured.

Briefly, a set of PGD\textsubscript{2} standards provided in the kit and experimental samples (100 µl) were pipetted into labelled polypropylene tubes and then \textsuperscript{3}H-PGD\textsubscript{2} tracer (100 µl), PGD\textsubscript{2} specific antiserum (100 µl) and assay buffer (100 µl) were added. In addition to these samples, a total counts tube (TC)(100 µl tracer; 100 µl antiserum; 200 µl buffer), a zero standard tube (B\textsubscript{0})(100 µl tracer; 100 µl antiserum; 200 µl buffer) and a non-specific binding (NSB) tube (100 µl tracer; 300 µl buffer) were prepared. They were all vortex mixed and incubated overnight (4 °C).

The following day, a dextran coated charcoal suspension (500 µl) was added to each tube (except TC tube, into which 500 µl assay buffer was added). The samples were immediately mixed, left to stand in an ice bath (15 min) and centrifuged (250 g; 4 °C; 15 min). All supernatants were decanted into scintillation vials and scintillant (Optiphase; LKB; 10 ml) was added. The
radioactivity in each sample was measured by β-scintillation spectrometry (4 min/sample). The results were expressed in terms of the quantity:

\[
100 \times \frac{(B - \text{NSB})}{B_0 - \text{NSB}}
\]

where 
- B is the sample reading
- \(B_0\) is the zero standard
- NSB is the non-specific binding

By using the PGD\(_2\) standards provided, a calibration curve was plotted and the PGD\(_2\) content of each sample determined. Prostanoid values were expressed as ng per \(10^6\) mast cells. To ensure that the unknown samples fell within the concentration range of the RIA kit (5-200 pg/ml), preliminary samples and dilutions of these were tested prior to assaying all the samples in full. If required, the samples were diluted as necessary to ensure that their PGD\(_2\) levels lay in the range of the standard curve.

**2.16: IP\(_3\) assay**

The second messenger IP\(_3\) which mobilises intracellular calcium was assayed using a commercially available radioimmunoassay kit (Amersham). Purified RPMC preparations were used and the number of cells in each sample was determined prior to the experiment. The assay is based on the competition between unlabelled IP\(_3\) and \(^3\)H labelled IP\(_3\) for binding to a limited quantity of an antibody raised with a high specificity to IP\(_3\). The IP\(_3\) content of a sample is then inversely proportional to the radioactivity measured.

Briefly, a set of IP\(_3\) standards provided in the kit and experimental samples (100 µl) were pipetted into labelled polypropylene tubes and then \(^3\)H-IP\(_3\) tracer (100 µl), IP\(_3\) binding protein (100 µl) and assay buffer (100 µl) were added. In addition to these samples, a total counts tube (TC)(100 µl tracer,
200 µl H₂O, 100 µl assay buffer), a zero standard tube (B₀)(100 µl tracer, 100 µl binding protein, 100 µl H₂O, 100 µl assay buffer) and a non-specific binding tube (NSB)(100 µl tracer, 100 µl binding protein, 100 µl stock standard, 100 µl assay buffer) were prepared. All tubes (except TC tubes) were centrifuged (2000 g; 10 min; 4 °C) and the supernatants discarded. Keeping the tubes inverted, they were placed on absorbent tissues and allowed to drain completely for 2 minutes. The tubes were firmly blotted and any droplets were wiped away using suitable swabs. NaOH (0.15 M, 1 ml) was then added to each tube (except TC tubes). The latter were vortex mixed and incubated for 10 minutes at room temperature. The solutions were then vortex mixed again and immediately decanted into scintillation vials. Scintillant fluid (Optiphase; LKB; 10 ml) was added and the radioactivity in each sample measured by β-scintillation spectrometry (4 min/ sample). The results were calculated as with the PGD₂ assay.

2.17: PLA₂ assay

Secretory PLA₂ was assayed using a commercially available colourimetric kit (Cayman Chemical Company). Purified RPMC preparations were used and the number of cells in each sample was determined prior to the experiment. The assay used the 1,2-dithio analogue of diheptanoyl phosphatidylcholine which serves as a substrate for most PLA₂'s (e.g. bee and cobra venoms, pancreatic, etc.) with the exception of cytosolic PLA₂. Upon hydrolysis of the thio ester bond at the sn-2 position by PLA₂, free thiols are detected using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) which is colourless at this wavelength. The resulting 5-thio-2-nitrobenzoic acid has a λ_max of 412 nm and an molar absorption (extinction) coefficient (ε) of 13600 M⁻¹cm⁻¹ and it is the generation of this compound which is being measured. The scheme of this reaction is given in Fig. 2.2. The detection range of this assay is from 0.021 to 0.211 µmol/min of sPLA₂ activity, which is equivalent to an absorbance increase of 0.01 to 0.1 units per min.
Chapter 2  Methods and Materials

Briefly, the positive control (bee venom PLA₂) provided in the kit and experimental samples (10 µl) were pipetted into standard plate reader wells. To each well was also added DTNB (10 µl) and assay buffer (5 µl). In addition, a blank (non-enzymatic control) well was also prepared (DTNB, 10 µl; assay buffer, 15 µl). All the reactions were initiated by the addition of substrate solution (200 µl), as quickly as possible. The microtiter plate was shaken gently and the absorbance read over a period of time (12-15 min) at 405 nm using a standard plate reader (Multiskan Plus Version 2.03).

The results were calculated as follows.

i) The change in absorbance (ΔA₄₀₅) per min was determined by plotting the absorbance values as a function of time and measuring the slope of the linear portion of the curve.

ii) The value of ΔA₄₀₅/min for the non-enzymatic control was also calculated and this was subtracted from the other samples.

iii) The sPLA₂ activity was calculated using the following formula.

\[ \text{sPLA}_2 \text{ activity (µmol/min)(per ml of sample)} = \frac{\Delta A_{405}/\text{min}}{10 \text{ mM}^{-1} x 0.225 \text{ ml}/0.01 \text{ ml}} \]

The figure 10 mM⁻¹ is the calculated molar absorption coefficient per cm for DTNB at 405 nm corrected for the actual pathlength of the solution in the well (0.784 cm). The molar absorption coefficient at 405 nm is 12800 M⁻¹cm⁻¹. Thus, 12800 M⁻¹cm⁻¹ x 0.784 cm = 10000 M⁻¹ = 10 mM⁻¹. The volume of solution in the well is 0.225 ml and the volume of added enzyme solution is 0.01 ml. One unit of enzyme activity is then defined as that amount of substance which hydrolyses one µmol of substrate per min at 25 °C.
2.18: Measurement of cytosolic calcium

It has been shown that fluo-3 can be loaded into specific cell types by incubation with the pentaacetoxymethylester (fluo-3 AM) of the dye and that the ester is hydrolysed intracellularly to yield genuine fluo-3 capable of indicating changes in intracellular calcium induced by agonist stimulation. The structure of fluo-3 is given in Fig. 2.3.

Aliquots of purified RPMC (10^6 cell/ml) were incubated (30 min, 37 °C) with fluo-3 AM (4 μM) in BSA-FHT. In all experiments, a control sample of the cell suspension was incubated without the dye. This was then used to determine the autofluorescence. Cells and entrapped dye were recovered by centrifugation (140 g; 3 min; 4 °C), washed and resuspended in FHT. Loaded cells (2.5x10^5 cell/ml) were maintained at 37 °C with continuous stirring in a quartz cuvette and the fluorescence was measured in a luminescence spectrophotometer (Perkin Elmer LS-5B). The cells were stimulated with a secretagogue and the resulting change in fluorescence was recorded after equilibration. The fluorescence of the total fluo-3 content in the cell was then determined by addition of the detergent digitonin (30 μM final). The excitation wavelength was 506 nm with a slit width of 2.5 nm. The emission wavelength was 526 nm with a slit width of 5 nm.

Intracellular calcium was calculated using the following formula.

\[
[Ca^{2+}] = \frac{K_d \cdot (F - F_{\text{min}})}{F_{\text{max}} - F}
\]

where

\( K_d \) is 400 nM at vertebrate ionic strengths (determined for fluo-3 at 37 °C).

\( F_{\text{min}} \) is fluorescence with no dye (autofluorescence).

\( F_{\text{max}} \) is fluorescence of total fluo-3 present in cells (achieved by using digitonin).

\( F \) is the fluorescence with a particular secretagogue.
2.19: Determination of membrane phospholipid composition

The phospholipid composition of purified RPMC and RBL-2H3 cells was determined by using a high performance liquid chromatography (HPLC) technique developed by Kurumi et al.\(^{(306)}\). Firstly, the total lipid content of the cells was extracted and then the HPLC analysis was performed.

The lipid extraction was performed according to the method of Blight and Dyer\(^{(306)}\). Briefly, extracting solution (2 ml) consisting of chloroform/ methanol/ 10 M HCl/ fluorescein (1 mg/ml in methanol) in a ratio of 100:200:2:1, was added to the cell suspension (100 μl) and mixed well. KCl (2 M, 600 μl) and chloroform (600 μl) were added to the solution and mixed for 30 seconds. The solution was then allowed to stand on ice (5 min) and centrifuged (1700 g; 10 min; 4 °C). Precisely 1 ml of the lower organic lower layer was then removed and dried with nitrogen gas. The sample was then immediately subjected to the HPLC procedure.

The equipment used for the HPLC analysis consisted of an Alphasil silica gel column (5 micron particle size; 250 mm length x 4.6 mm ID, HPLC Technology Ltd. UK) and an HPLC system comprising of a Gilson 305 pump, a Rheodyne 712S Injector, a Shimadzu 10A u.v. detector and a Water's 740 integrator. Elution was performed with a solution of acetonitrile/ methanol/ 85 % phosphoric acid (130:5:1.7, v/v/v) at a flow rate of 1 ml/ min at room temperature. Absorption was determined using a variable wavelength uv detector set at 203 nm. Lipid extracts or phospholipid standards were dissolved in methylene chloride (50 μl) and then 3 μl of the solution were injected into the HPLC system. By using phospholipid standards, it was possible to identify the retention times at which particular phospholipids were eluted and the peak area which corresponded to a particular phospholipid concentration. From these preliminary experiments, it was possible to identify and quantify the phospholipid composition of RPMC and RBL-2H3 cells.
2.20: Active sensitisation

2.20.1: Sensitisation of rats with the nematode *Nippostrongylus brasiliensis* (NB)

Rats (~200 g) were subcutaneously (hind leg) injected with third stage larvae (L₃; 2500) of NB suspended in sterile physiological saline. The larvae were obtained either from faecal cultures of previously injected rats (described below) or from Mr. S. Pickersgill, Department of Applied Biology, University of Leeds. This process induced an immunological sensitisation of the animals. They became ready for experimentation 14-21 days post injection and remained sensitised for a further 3-4 weeks.

2.20.2: Preparation of L₃ of *Nippostrongylus brasiliensis*

Faeces from injected rats were collected on the 5ᵗʰ, 6ᵗʰ and 7ᵗʰ days post injection. They were washed and gently ground with an equal mass of activated charcoal until an homogeneous mixture was obtained. This was then transferred to moistened filter paper and kept in petri dishes (15-20 g per dish; 25 °C; 14 days; dark). During this period, the cultures were regularly moistened with tap water. The larvae were then isolated by pouring the cultures onto two layers of cotton gauze interleaved with one layer of lens tissue which had been fitted into a glass funnel and filled with warm tap water. The larvae sedimented (60-90 min) into a graduated glass test tube which had been attached to the bottom of the apparatus. They were then washed in sterile physiological saline (3-4 times), counted and resuspended in an appropriate volume of saline (10 x 10³ worm/ ml), ready for injection.
2.21: Materials

All chemicals were purchased from Sigma, London except those stated in the following sections.

2.21.1: Secretagogues used

- Dextran 70 (110000 MW) (Pharmacia Fine Chemicals)
- Mastoparan (Neosystems Ltd.)
- PLA₂ activating peptide (PLAP) (ICN Pharmaceuticals)
- Rabbit antiserum to guinea pig IgG (anti-guinea pig IgG) (ICN Pharmaceuticals)
- Rabbit antiserum to human IgE (anti-human IgE) (Dako Pharmaceuticals)
- Sheep antiserum to rat IgE (anti-rat IgE) (ICN Pharmaceuticals)
- Substance P (Neosystems Ltd.)

2.21.2: Inhibitors used

- Arachidonyl trifluoromethyl ketone (AACOCF₃) (Calbiochem)
- (p-Amylcinnamoyl)anthranilic acid (ACA) (Calbiochem)
- L739010 and L746530 (Gift from Merck Frosst Canada Inc. Centre for Therapeutic Research)
- Mannose-BSA (E.Y. Laboratories, USA)
- Manoalide (ICN Pharmaceuticals)
- Oleoyloxyethyl phosphocholine (OEPC) (Calbiochem)
- Pertussis toxin (ICN Pharmaceuticals)
2.21.3: Solvents used

- Acetonitrile (HPLC grade) | BDH Laboratory Supplies
- Butan-1-ol | Fisons Ltd.
- Chloroform | BDH Laboratory Supplies
- Ethanol (analar and HPLC grade) | BDH Laboratory Supplies
- n-Heptane | BDH Laboratory Supplies
- Methanol (analar and HPLC grade) | BDH Laboratory Supplies
- Methylene chloride (HPLC grade) | BDH Laboratory Supplies
- Phosphoric acid (HPLC grade) | BDH Laboratory Supplies

2.21.4: Buffer reagents

- Calcium chloride 2-hydrate | BDH Laboratory Supplies
- D-(+)-glucose | BDH Laboratory Supplies
- Disodium hydrogen phosphate | BDH Laboratory Supplies
- Ethylenediaminetetraacetic acid (EDTA) | BDH Laboratory Supplies
- Heparin | C. P. Pharmaceuticals
- Hydrochloric acid (Analar) | BDH Laboratory Supplies
- Magnesium chloride | Fisons Ltd.
- pH standards | BDH Laboratory Supplies
- Potassium chloride | BDH Laboratory Supplies
- Sodium chloride | BDH Laboratory Supplies
- Sodium dihydrogen orthophosphate | Fisons Ltd.
- Sodium hydroxide (Analar) | BDH Laboratory Supplies
### Chapter 2

#### Methods and Materials

#### 2.21.5: Culture reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>DMEM Medium (supplemented with L-glutamine)</td>
<td>Gibco Life Technologies Ltd.</td>
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<tr>
<td>Foetal calf serum (FCS)</td>
<td>Gibco Life Technologies Ltd.</td>
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<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Gibco Life Technologies Ltd.</td>
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#### 2.21.6: Miscellaneous

<table>
<thead>
<tr>
<th>Item</th>
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<tbody>
<tr>
<td>Absorbant gauze</td>
<td>Smith Nephew</td>
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<tr>
<td>Activated charcoal (particle size 0.85 - 1.70 mm)</td>
<td>BDH Laboratory Supplies</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>BDH Laboratory Supplies</td>
</tr>
<tr>
<td>Dextran (6%) 70 intravenous infusion BP in 0.9% NaCl</td>
<td>Baxter Healthcare Ltd.</td>
</tr>
<tr>
<td>IP$_3$ [³H] assay kit</td>
<td>Amersham p.l.c.</td>
</tr>
<tr>
<td>Isotonic saline (0.9% NaCl)</td>
<td>Baxter Healthcare Ltd.</td>
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<tr>
<td>Lens tissue</td>
<td>Whatman Ltd.</td>
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<td>Nytex gauze</td>
<td>R. Cadisch &amp; Sons.</td>
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<td>Perchloric acid (Analar)</td>
<td>BDH Laboratory Supplies</td>
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<td>Percoll</td>
<td>Pharmacia Fine Chemicals</td>
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<td>PGD$_2$ [³H] assay kit</td>
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<td>sPLA$_2$ assay kit</td>
<td>Cayman Chemical Company, USA</td>
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<tr>
<td>Toluidine blue</td>
<td>BDH Laboratory Supplies</td>
</tr>
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</table>
Chapter 2 Methods and Materials

2.22: Stock solutions of drugs

Anti-rat IgE and anti-guinea pig IgG were obtained in lyophilised form. They were solubilised in distilled water (2 ml) and the resultant stock solution aliquoted into microcentrifuge tubes and stored (-20 °C) until required.

Anti-human IgE was obtained in liquid form. Subsequent dilutions were prepared freshly, as required.

Compound 48/80 (1 mg/ml; distilled water) was prepared each week and refrigerated until needed.

Con A, mastoparan, pertussis toxin and substance P were dissolved in distilled water, aliquoted into microcentrifuge tubes and stored (-20 °C) until required.

All the PLA\(_2\) isoenzymes, mannose-BSA, nerve growth factor and PLAP were dissolved in FHT, aliquoted and frozen (-20 °C) until needed. The PLA\(_2\) isoenzymes are commercially available in standardised units defined as the amount of enzyme which will hydrolyse 1.0 μmol of soybean L-α-PC to L-α-lyso-PC and a fatty acid per min at pH 8.9 at 25 °C.

The cyclooxygenase inhibitors, lipoxygenase inhibitors, dual lipoxygenase/cyclooxygenase inhibitors, and IBMX were dissolved in neat DMSO and used immediately. A small volume of this stock solution (20 μl) was added to a large volume (10 ml) of warm FHT in one step. By this method, the compounds were made to dissolve in the buffer solution. It was ensured that the final concentration of DMSO in each sample did not exceed 0.01 % as concentrations greater than this may cause inhibition of histamine release.
Chapter 2 Methods and Materials

All the PLA$_2$ inhibitors, A23187, fluo-3 AM, isatoic anhydride and manoalide were dissolved in neat DMSO, aliquoted and frozen (-20 °C) until required. Again, final DMSO concentrations did not exceed 0.01%.

All the phospholipids and lyso-phospholipids for functional studies were dissolved in chloroform/ methanol (95:5), aliquoted and stored (-20 °C) until required. To prepare an aqueous suspension for experimental use, an aliquot (20 µl) was pipetted into a glass test tube and chloroform (1 ml) added. The solvents were evaporated under a stream of dry, oxygen-free nitrogen. At the same time, the glass tube was gently rotated to obtain an even coating of the lipid on its surface. Warm saline (1 ml) was added and the suspension thoroughly Vortex mixed. This suspension was used directly, or further diluted in FHT, as required.

All other compounds were dissolved in FHT and prepared freshly on the day of use.

2.23: Numerical analysis

All values given are means ± standard error of the mean (SEM) for the number (n) of experiments performed. The points on the graphs are the means from the number (n) of experiments and the vertical bars represent the SEM. All the values have been corrected for the spontaneous secretion of histamine.

Statistical analysis of differences between groups of data was performed using the Student’s $t$-test. Where appropriate, a paired $t$-test for related samples was employed. Significant difference values of $p< 0.05$, $p< 0.01$ and $p< 0.001$ are denoted by *, ** and ***, respectively.
Fig. 2.1: Scheme of reaction for the OPT assay of histamine$^{(300)}$
Fig. 2.2: Scheme of reaction for the PLA$_2$ assay$^{(302, 303)}$
Fig. 2.3: Structure of fluo-3$^{304}$
Chapter 3

"Au fond de l'inconnu pour trouver du nouveau!"
Through the unknown, we'll find the new.

Charles Baudelaire 1821-67: Les fleurs du mal (1857)
Translated by Robert Lowell.
CHAPTER 3

Effect of different PLA₂ isoenzymes on various mast cell phenotypes

3.1: Introduction

As discussed in chapter 1, the direct effects of exogenously added PLA₂ on mast cell activation have been the source of contradictory results, with some studies indicating that PLA₂ caused histamine release from RPMC and others disputing these findings. These seemingly disparate conclusions may now be explained by recent phylogenetic studies which have professed the existence of several forms of the enzyme, or isoenzymes. A recent review has described PLA₂ as a superfamily of signal transduction enzymes, with the isoenzymes differing in their calcium dependency, their molecular mass and the number of disulfide bonds found in their structures. Hence, certain PLA₂ isoenzymes may cause mast cell degranulation, whilst others may have no effect.

In light of these findings, it was the initial aim of the present study to clarify and further elucidate some of the characteristics of the PLA₂ isoenzymes which induce mast cell activation. The phenomenon of mast cell heterogeneity has made it essential that these studies are also extended to a variety of mast cell phenotypes.

3.2: Methods and Materials

All the materials and methods that were used in these studies are described in chapter 2.
3.3: Results

3.3.1: Comparison of the histamine release from RPMC treated with PLA$_2$ isolated from various sources

Purified preparations of PLA$_2$ isolated from *Naja naja* (cobra) venom, and bee venom (0.3-10 units/ml) caused a significant dose-dependent release of histamine from RPMC, with maximum secretions of 72.5 ± 2.6 % and 19.0 ± 0.9 %, respectively. These proteins were then used in the rest of the study and the effects of these two isoenzymes on mast cells were further characterised. Over the same concentration range, the PLA$_2$ isolated from *Crotalus adamanteus* and *Crotalus atrox*, the eastern and western diamondback rattlesnakes respectively, and from the bovine pancreas were essentially ineffective or very weakly active in causing mast cell degranulation (maximum release <10 %) (Fig. 3.1).

3.3.2: Calcium ion dependency of PLA$_2$ induced histamine release from RPMC

The histamine release induced by *Naja naja*-PLA$_2$ (0.3-10 units/ml) was greatly reduced (from a maximum release of 72.1 ± 4.0 % in normal FHT buffer) when the cells were incubated in a calcium free medium (maximum release 21.8 ± 3.4 %), or in a medium containing the chelating agent EDTA (maximum release 16.7 ± 2.8 %) (Fig. 3.2). When stimulated with bee venom-PLA$_2$, the reduction in histamine release in calcium free or EDTA containing media was less pronounced (maximum secretion 12.3 ± 1.8 % and 14.4 ± 1.5 %, respectively, compared to 20.3 ± 1.6 % in the presence of calcium) (Fig. 3.3). As a control, this experiment was also performed with compound 48/80 (0.025-1 μg/ml). With this secretagogue, the maximum releases in the full calcium containing buffer, the calcium ion deficient medium and the EDTA medium were 67.5 ± 3.2 %, 10.5 ± 3.7 % and 43.7 ± 5.8 %, respectively (Fig. 81).
3.4. At low concentrations of secretagogue (≤ 0.1 μg/ml) the release in the presence of EDTA was greater than in the other two media.

3.3.3: **Kinetics of histamine release from RPMC treated with PLA₂**

The rate of histamine release induced by *Naja naja*-PLA₂ was slow (t₁/₂ = 180 sec, maximum release obtained after approximately 10 min) (Fig. 3.5). The release induced by bee venom-PLA₂ was much faster (t₁/₂ = 15 sec, maximum release obtained after approximately 60 sec)(Fig. 3.6). The kinetics of histamine release induced by bee venom-PLA₂ are comparable to those seen with compound 48/80 (t₁/₂ = 10 sec, maximum release obtained after approximately 30 sec)(Fig. 3.7). The kinetics of anti-IgE induced histamine release were slow (t₁/₂ = 30 sec, maximum release obtained after approximately 5 min)(Fig. 3.8). These were similar to the kinetics of *Naja naja*-PLA₂ induced release.

3.3.4: **Cytotoxicity of histamine release from RPMC treated with PLA₂**

The histamine release induced by *Naja naja*-PLA₂ (1-10 units/ml) was optimal when an incubation temperature of 37 °C was used. This was drastically reduced when the reactions were performed at 0 °C and at 45 °C. A slight reduction was seen at an incubation temperature of 22 °C (Fig. 3.9). This pattern was similar to that found when the cells were stimulated with compound 48/80 (Fig. 3.10). There was a less pronounced reduction of histamine release at the extreme temperatures with bee venom-PLA₂ (Fig. 3.11).

In the presence of the combined metabolic inhibitors 2-deoxy-D-glucose and antimycin A, the histamine release induced by *Naja naja*-PLA₂ (1-10 units/ml) was virtually abolished (Fig. 3.12). This pattern of reduced secretion by metabolic
blockade was also seen with compound 48/80 (1 μg/ml)(Fig. 3.13). The histamine release induced by bee venom-PLA₂ in the presence of the inhibitors was reduced, but still significant, when compared to the releases obtained in their absence (Fig. 3.14).

3.3.5: Histamine release from non-purified and purified RPMC treated with PLA₂

A dose-dependent release of histamine was induced by the *Naja naja*-PLA₂ and bee venom isoenzymes from purified preparations of RPMC (54.3 ± 1.9 % and 14.2 ± 3.4 %, with 10 units/ml). The corresponding releases from non-purified RPMC preparations were 74.3 ± 4.6 % and 20.2 ± 4.7 %, respectively (Fig. 3.15).

3.3.6: Effects of various inhibitors on PLA₂ induced histamine release from RPMC

Disodium cromoglycate (DSCG) (0.1 μM - 1 mM, no preincubation) produced a dose-dependent inhibition of histamine release (maximum inhibition 75.4 ± 4.6 %) when the cells were stimulated with *Naja naja*-PLA₂. A rather more pronounced inhibition was seen when the cells were stimulated with anti-IgE (maximum inhibition 87.3 ± 4.6 %) (Fig. 3.16).

The serine esterase inhibitor isatoic anhydride (100 μM) produced a maximum inhibition of 20.8 ± 3.1 % when the cells were pre-incubated with the compound for 10 min and subsequently stimulated with *Naja naja*-PLA₂. Under the same conditions, a maximal inhibition of 45.9 ± 4.6 % was obtained with anti-IgE as the secretagogue (Fig. 3.17).

Nedocromil sodium (0.01 - 100 μM, no preincubation), dibutryl cyclic AMP (dibutryl-cAMP, 0.1 - 10 mM, 30 min preincubation), and the
phosphodiesterase (PDE) inhibitors isobutylmethylxanthine (IBMX, 0.01 - 1 mM, 10 min preincubation) and theophylline (0.1 - 10 mM, 10 min preincubation) all produced a dose-dependent inhibition of histamine release from RPMC treated with *Naja naja*-PLA₂ and anti-IgE but were generally more effective against the latter ligand (Fig. 3.18 - 3.21). The maximum inhibitions in each case were nedocromil sodium 43.0 ± 4.3 and 91.2 ± 6.7 %, dibutryl cyclic AMP 34.7 ± 4.2 and 76.2 ± 5.6 %, IBMX 25.6 ± 3.9 and 55.0 ± 5.7, and theophylline 36.2 ± 2.7 and 81.2 ± 5.2 %.

Comparable unblocked releases of between 25 and 35 % were used in all these studies. The concentrations of the secretagogues were *Naja naja*-PLA₂ (2.5 units/ml) and anti-IgE (300 fold dilution).

### 3.3.7: Effect of pertussis toxin on PLA₂ induced histamine release from RPMC

Pertussis toxin (1-100 ng ml⁻¹, 2 hour incubation) dose-dependently inhibited the histamine release induced by compound 48/80 (maximum inhibition 82.7 ± 5.7 %). However, neither anti-IgE nor *Naja naja*-PLA₂ were affected by the presence of this G-protein inhibitor (maximum inhibition 8.8 ± 1.7 % and 5.3 ± 5.8 %, respectively)(Fig. 3.22).
3.3.8: Desensitisation of RPMC to various secretagogues

Primary stimulation with a given secretagogue under conditions in which the cells cannot release histamine may render the cells unresponsive to a second challenge with the same ligand. Such cells are said to be desensitised. Cross-desensitisation to a second secretagogue may indicate a common mode of action for the two agents.

RPMC were desensitised with compound 48/80 (0.5 μg/ml), *Naja naja*-PLA$_2$ (2 units/ml), anti-IgE (1/300 dilution), or substance P (5 μM) as described in section 2.10. Subsequent cross-stimulation with the four secretagogues yielded the following results.

Cells that were subsequently stimulated with compound 48/80 were significantly less responsive if they had first been desensitised by the same ligand. Cells desensitised to substance P were also somewhat less responsive, although these differences were not statistically significant. In contrast, the releases induced by compound 48/80 were unaffected by prior desensitisation with both *Naja naja*-PLA$_2$ and anti-IgE (Fig. 3.23).

Cells treated with *Naja naja*-PLA$_2$ showed essentially unaltered reactivity if they were first desensitised with compound 48/80, anti-IgE or substance P. A statistically significant reduction was only seen with *Naja naja*-PLA$_2$ desensitised cells (Fig. 3.24).

Cells stimulated with anti-IgE were desensitised only by prior treatment with the same ligand and no cross desensitisation to compound 48/80, *Naja naja*-PLA$_2$, or substance P was seen (Fig. 3.25). In fact, a slight but significant increase in histamine release was seen with cells desensitised to *Naja naja*-PLA$_2$ and subsequently treated with maximal concentration of anti-IgE. The origin of this effect is obscure.
Cells stimulated with substance P showed a reduced secretion of histamine if they had been first desensitised by compound 48/80 and, to a lesser extent, by the peptide itself. However, prior treatment with *Naja naja*-PLA$_2$ and anti-IgE did not affect the secretion of histamine (Fig. 3.26).

### 3.3.9: Effects of PLA$_2$ on different mast cell phenotypes

PLA$_2$ from *Naja naja* and bee venom (0.01-10 units/ml) induced a dose-dependent release of histamine from mast cells isolated from rat skin (maximum release with *Naja naja*-PLA$_2$, 52.7 ± 3.2 %; with bee venom-PLA$_2$, 64.5 ± 1.7 %)(Fig. 3.27), rat mesentery (*Naja naja*-PLA$_2$, 33.5 ± 3.7 %; bee venom-PLA$_2$, 35.3 ± 3.6 %)(Fig. 3.28), rat lung (*Naja naja*-PLA$_2$, 30.5 ± 4.6 %; bee venom-PLA$_2$, 44.6 ± 4.5 %)(Fig. 3.29), guinea pig skin (*Naja naja*-PLA$_2$, 41.2 ± 3.2 %; bee venom-PLA$_2$, 47.1 ± 2.7 %)(Fig. 3.30), guinea pig mesentery (*Naja naja*-PLA$_2$, 12.7 ± 0.6 %; bee venom-PLA$_2$, 11.1 ± 0.9 %)(Fig. 3.31), guinea pig lung (*Naja naja*-PLA$_2$, 24.5 ± 1.6 %; bee venom-PLA$_2$, 34.2 ± 3.3 %)(Fig. 3.32), mouse peritoneum (*Naja naja*-PLA$_2$, 13.4 ± 3.4 %; bee venom-PLA$_2$, 11.7 ± 3.0 %)(Fig. 3.33) and human lung (*Naja naja*-PLA$_2$, 29.9 ± 9.2 %; bee venom-PLA$_2$, 31.6 ± 8.9 %)(Fig. 3.34) and from human basophils (*Naja naja*-PLA$_2$, 5.6 ± 1.7 %; bee venom-PLA$_2$, 3.5 ± 0.9 %)(Fig. 3.35) and RBL-2H3 cells (*Naja naja*-PLA$_2$, 18.7 ± 0.5 %; bee venom-PLA$_2$, 19.7 ± 0.8 %)(Fig. 3.36).

### 3.3.10: Cytotoxicity of histamine release induced by PLA$_2$ from different mast cell phenotypes

The different mast cell phenotypes were stimulated with *Naja naja*-PLA$_2$ and bee venom-PLA$_2$ in the presence of the metabolic inhibitors antimycin A (1 μM) and 2-deoxy-D-glucose (5 mM). Even in the presence of these inhibitors, there was still a significant secretion of histamine from mast cells isolated from the skin, mesentery and lung of the rat (Figs. 3.37 - 3.39) and guinea pig.
(Figs. 3.40 - 3.42) and from RBL-2H3 cells (Fig. 3.43). Statistically, no significant reductions in histamine release were observed in any of these test systems, except with guinea pig lung mast cells (\textit{Naja naja}-PLA$_2$, 1 and 0.1 units/ml). In contrast, the secretion from mouse peritoneal (Fig. 3.44) and human lung (Fig. 3.45) mast cells and human basophils (Fig. 3.46) was markedly attenuated by the inhibitors. All the reductions were statistically significant.

3.4: Discussion

The present study has shown that PLA$_2$ isolated from \textit{Naja naja} and bee venom was able to induce a significant secretion of histamine from RPMC. The isoenzymes from \textit{Crotalus adamanteus}, \textit{Crotalus atrox} and bovine pancreas were essentially inactive in this system. Having ascertained which of the isoenzymes were effective, it was important to characterise further the effect.

We have shown that the histamine release induced by \textit{Naja naja}-PLA$_2$ is a calcium dependent process. In the absence of calcium in the extracellular environment, the extent of secretion was greatly reduced. This reduction was also seen when the calcium chelating agent EDTA was included in the incubation medium. Compound 48/80 was used in this experiment as a control. In the absence of extracellular calcium, the percentage of histamine release was again reduced. This was also the case with the EDTA containing medium but, under these conditions, there was a marked increase in the histamine release from that seen without any calcium. This occurred because compound 48/80 may act to mobilise internal stores of calcium and this labilisation may be facilitated by EDTA\textsuperscript{308}. This marked utilisation of the internal stores was not seen with \textit{Naja naja}-PLA$_2$. The histamine release elicited by bee venom-PLA$_2$ showed a less pronounced reduction when the reaction was performed in a calcium depleted, or EDTA containing, medium.
Hence, the effects of the two venom isoenzymes differ in their dependency on external calcium ions.

The histamine release induced by *Naja naja*-PLA$_2$ from the RPMC was a slow process, much like that induced by anti-IgE. In contrast, the kinetics of histamine release induced by bee venom-PLA$_2$ were much faster, similar to those of the histamine release induced by compound 48/80.

Two different methods were used to determine the cytotoxicity of the histamine release induced by the two PLA$_2$ isoenzymes. The first method involved the use of different incubation temperatures. An incubation temperature of 37 °C is optimal for a true secretory process to occur. At temperatures of 45 °C and 0 °C, the internal processes required for active secretion are not able to function efficiently, and thus the active secretion of histamine is diminished. If release of histamine does occur at these extreme temperatures, then the process probably does not require an intact cell metabolism and is likely to be cytolytic. The histamine release induced by compound 48/80 was completely eliminated at the extreme temperatures of 0 °C and 45 °C, and reduced at 22 °C. This showed that the process involved active secretion. There was also a reduction of histamine release at the extreme temperatures when the cells were treated with *Naja naja*-PLA$_2$, indicating that this was again a non-cytotoxic process. However, with bee venom-PLA$_2$ treated cells, a comparatively large component of the release persisted at the extreme temperatures, indicating that a significant proportion of the response was cytotoxic.

The second method utilised to ascertain the cytotoxicity of the secretion involved the use of the metabolic inhibitors 2-deoxy-D-glucose and antimycin A. For active secretion to occur, the utilisation of glucose and the production of ATP must be involved. The two inhibitors block glycolosis and oxidative phosphorylation, respectively. The histamine release induced by compound 48/80 in the presence of these inhibitors was diminished, indicating that this
was a non-cytotoxic process. That induced by *Naja naja*-PLA$_2$ was considerably reduced in the presence of the inhibitors, indicating a significant non-cytotoxic component of the release. Again, in contrast, the release induced by bee venom-PLA$_2$ was not significantly reduced by metabolic blockade, indicating that there was a major cytotoxic component of the release. This was in agreement with the results seen with the temperature method used to determine cytotoxicity.

It was next important to determine whether the observed secretion was due to a direct effect of PLA$_2$ on mast cells, or whether it depended on other cells that are present in mixed peritoneal washings such as macrophages and lymphocytes. Percoll was used to purify the cells to a homogeneity in excess of 95%. The histamine release was slightly reduced when using purified mast cells, but this phenomenon has been shown to occur with other secretagogues$^{(310)}$. Hence, it can be deduced that the histamine secretion from the mixed cell population was indeed due to a direct effect on the mast cells and further studies with mixed cells could be performed with confidence.

Various mast cell inhibitors working through a variety of different mechanisms were used to characterise further the release process induced by *Naja naja*-PLA$_2$.

Clinically, DSCG has been shown to be of major importance in the treatment of allergic and inflammatory diseases. However, its mechanism of action still remains unclear$^{(311)}$. Studies by Foreman *et al*$^{(312)}$ revealed that DSCG was able to prevent the uptake of $^{45}$Ca$^{2+}$ by mast cells that had been immunologically stimulated. This theory evoked further support when a cromolyn binding protein (60 kDa) was isolated from RBL cells$^{(313)}$ and it was suggested that this species was a calcium transporting protein. Activation of this transport mechanism was thought to be essential for Ca$^{2+}$ influx and histamine secretion from RBL cells. DSCG was thought to bind to the protein, impede its function and hence inhibit mast cell secretion$^{(311)}$. 

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89
A second theory centres on protein phosphorylation. Four major proteins, of molecular masses 67-68 kDa, 56-59 kDa, 42 kDa, and 78 kDa were shown to be phosphorylated on stimulation of RPMC with compound 48/80 and anti-IgE\cite{314}. It was suggested that the phosphorylation of the 78 kDa protein was an endogenous mechanism for the termination of exocytosis, whilst the other three phosphorylations were involved in the initiation of secretion. The 78 kDa protein has recently been identified as the myristoylated alanine-rich C-kinase substrate (MARCKS)\cite{315}, which has been reported in a number of secretory cells including macrophages and neutrophils\cite{316}. MARCKS may have an important modulatory role in the process of secretion. Both DSCG and its more potent congener nedocromil sodium may act by inducing phosphorylation of the MARCKS protein and thus initiating the endogenous mechanism for the termination of secretion in mast cells and other inflammatory cell types.

It is generally accepted that elevated intracellular levels of cAMP may inhibit the activation of mast cells and basophil leukocytes\cite{317,318}. This elevation may be achieved by the addition of cell-permeant analogues of the nucleotide, such as dibutyryl-cAMP, or PDE inhibitors, such as IBMX or theophylline, which prevent the breakdown of the nucleotide.

It has been proposed that the activation of one or more serine proteases may represent one of the earliest biochemical steps that ultimately lead to mediator release from mast cells\cite{319}. Thus, inhibitors of these putative proteases, such as isatoic anhydride which is a suicide inhibitor of chymotryptic enzymes, effectively block immunologic histamine release from RPMC\cite{320}.

In the present study, DSCG, nedocromil sodium, isatoic anhydride, dibutyryl cyclic AMP, IBMX and theophylline were all effective inhibitors of histamine release induced by anti-IgE but were much less active, particularly in the
latter cases against secretion evoked by *Naja naja*-PLA$_2$. The detailed mechanisms by which the two ligands induce secretion are thus presumably distinct.

It has been reported that compound 48/80 induces activation of mast cells via direct stimulation of a G-protein$^{(321)}$. Pertussis toxin is a potent inhibitor of this G-protein and is hence able to attenuate the histamine secretion by all secretagogues which act through this signalling molecule. Consistently, in agreement with previous reports$^{(322)}$, pertussis toxin was able dose-dependently to inhibit the secretion induced by compound 48/80. However, the secretion induced by anti-IgE, which does not act via activation of a pertussis toxin sensitive G-protein, was unaffected. The toxin was also ineffective against *Naja naja*-PLA$_2$, indicating a similar lack of involvement of such a G-protein.

By incubating mast cells with a particular secretagogue, it may be possible to desensitise them to further stimulation by the same ligand or to other agents which act through a similar mechanism$^{(297)}$. In the present work, some degree of cross-desensitisation was observed between compound 48/80 and substance P, suggesting that they may share a common mode of action. However, neither homologous nor heterologous desensitisation was complete in these systems and the data were thus somewhat equivocal. More importantly, neither anti-IgE nor *Naja naja*-PLA$_2$ showed any degree of cross-desensitisation to any of the other ligands tested, suggesting that they have unique modes of action.

From the results of these several sets of experiments, it can thus be concluded that the detailed mechanism by which *Naja naja*-PLA$_2$ induces histamine release is distinct from that of anti-IgE and polyamines, such as compound 48/80 or substance P, and does not involve activation of a pertussis toxin sensitive G-protein.
As previously discussed, mast cells isolated from different sources are phenotypically distinct. It was thence important to investigate the effects of PLA$_2$ on different mast cell phenotypes. It was shown that both *Naja naja*-PLA$_2$ and bee venom-PLA$_2$ were able to induce histamine release from the whole range of mast cell subtypes studied. Indeed, these isoenzymes may be the most general mast cell stimulating agents that have so far been identified. The enzymes induced a non-cytotoxic release of histamine from murine peritoneal mast cells, human lung mast cells and human basophils. This release may be due to the production of AA and its further metabolism to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is thought to modulate secretion$^{(523)}$. However, the releases from tissue mast cells of the rat and guinea pig and from RBL-2H3 cells were essentially unaffected by metabolic blockade and were presumably cytotoxic. This may be due to generation of lyso-PC (lyso-lecithin), which is a known surfactant$^{(324)}$. These differential effects of the PLA$_2$ isoenzymes on the various histaminocytes may reflect the overall membrane composition of the cell types, leading to the production of one or other metabolite. These effects are further investigated and discussed in subsequent chapters.

In total, this study has shown that PLA$_2$ is a potent inducer of histamine from various mast cell phenotypes. Some of these processes may be secretory whilst others may have cytotoxic components. The release from RPMC induced by *Naja naja*-PLA$_2$ is a non-cytotoxic, calcium dependent and relatively slow process. In contrast, bee venom-PLA$_2$ has a considerable cytotoxic and calcium independent component and this release is a relatively fast process. The mechanism by which PLA$_2$ evokes degranulation from RPMC is peculiar to itself and is distinct from immunologic or polyamine activation of this cell type.
Chapter 3  Characterisation and Heterogeneity

Fig. 3.1: Histamine release from RPMC treated with different PLA2 isoenzymes (n=5)

Fig. 3.2: Calcium ion dependency of histamine release from RPMC treated with *Naja naja*-PLA2 (n=4)
Fig. 3.3: Calcium ion dependency of histamine release from RPMC treated with bee venom-PLA$_2$ (n=4)

Fig. 3.4: Calcium ion dependency of histamine release from RPMC treated with compound 48/80 (n=4)
Fig. 3.5: Kinetics of histamine release from RPMC treated with *Naja naja*-PLA$_2$ (10 units/ml) (n=6)

Fig. 3.6: Kinetics of histamine release from RPMC treated with bee venom-PLA$_2$ (10 units/ml) (n=6)
Fig. 3.7: Kinetics of histamine release from RPMC treated with compound 48/80 (0.25 μg/ml) (n=6)

Fig. 3.8: Kinetics of histamine release from RPMC treated with anti-IgE (1/300 dilution) (n=5)
Chapter 3  Characterisation and Heterogeneity

Fig. 3.9: Temperature dependency of histamine release from RPMC treated with *Naja naja*-PLA₂ (n=4)
(Asterisks denote those releases that were significantly different from that obtained at 37 °C. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing temperature dependency of histamine release from RPMC treated with Naja naja-PLA₂ (n=4)](image)

Fig. 3.10: Temperature dependency of histamine release from RPMC treated with compound 48/80 (n=4)
(Asterisks denote those releases that were significantly different from that obtained at 37 °C. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing temperature dependency of histamine release from RPMC treated with compound 48/80 (n=4)](image)
Fig. 3.11: Temperature dependency of histamine release from RPMC treated with bee venom-PLA\(_2\) (n=4)

(Asterisks denote those releases that were significantly different from that obtained at 37 °C. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Fig. 3.11: Temperature dependency of histamine release](chart)

Fig. 3.12: Effects of metabolic inhibitors on histamine release from RPMC treated with *Naja naja*-PLA\(_2\) (n=4)

(Asterisks denote those releases that were significantly different from that obtained when using FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Fig. 3.12: Effects of metabolic inhibitors on histamine release](chart)
Fig. 3.13: Effects of metabolic inhibitors on histamine release from RPMC treated with compound 48/80 (n=4)
(Asterisks denote those releases that were significantly different from that obtained when using FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar chart showing the effects of metabolic inhibitors on histamine release from RPMC treated with compound 48/80.](image)

Fig. 3.14: Effects of metabolic inhibitors on histamine release from RPMC treated with bee venom-PLA₂ (n=4)
(Asterisks denote those releases that were significantly different from that obtained when using FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar chart showing the effects of metabolic inhibitors on histamine release from RPMC treated with bee venom-PLA₂.](image)
Fig. 3.15: Comparison of the histamine release from purified (95.7 ± 2.3%) and non-purified RPMC treated with PLA$_2$ isoenzymes (n=4)

![Graph showing histamine release vs. PLA$_2$ units/ml for different conditions.]

Fig. 3.16: Inhibition by DSCG (0 min preincubation) of histamine release from RPMC (n=4)

![Graph showing inhibition of histamine release vs. DSCG concentration (µM).]
Fig. 3.17: Inhibition by isatoic anhydride (10 min preincubation) of histamine release from RPMC (n=4)

Fig. 3.18: Inhibition by nedocromil sodium (0 min preincubation) of histamine release from RPMC (n=4)
Fig. 3.19: Inhibition by dibutyryl cyclic-AMP (30 min preincubation) of histamine release from RPMC (n=4)

Fig. 3.20: Inhibition by isobutylmethylxanthine (10 min preincubation) of histamine release from RPMC (n=4)
Fig. 3.21: Inhibition by theophylline (10 min preincubation) of histamine release from RPMC (n=4)

Fig. 3.22: Effects of pertussis toxin (2 hours preincubation) on histamine release from purified RPMC (purity 93.6 ± 2.4%) (n=5)
Fig. 3.23: Effects of compound 48/80 on RPMC following prior desensitisation by different secretagogues (n=4)
(Asterisks denote those releases that were significantly different from that obtained from non-desensitised cells. * p < 0.05, ** p < 0.01, *** p < 0.001.)
Fig. 3.24: Effects of *Naja naja*-PLA$_2$ on RPMC following prior desensitisation by different secretagogues (n=4)

(Asterisks denote those releases that were significantly different from that obtained from non-desensitised cells. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 3.25: Effects of anti-IgE on histamine release from RPMC following prior desensitisation by various secretagogues (n=4)

(Asterisks denote those releases that were significantly different from that obtained from non-desensitised cells. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 3.26: Effects of substance P on histamine release from RPMC following prior desensitisation by different secretagogues (n=4)

(Asterisks denote those releases that were significantly different from that obtained from non-desensitised cells. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 3.27: Histamine release from rat skin mast cells treated with PLA₂ (n=4)
(Control: compound 48/80 (1 μg/ml) 5.6 ± 1.2%)

![Histamine release from rat skin mast cells treated with PLA₂ (n=4)](image)

Fig. 3.28: Histamine release from rat mesenteric mast cells treated with PLA₂ (n=4)
(Control: compound 48/80 (1 μg/ml) 29.9 ± 2.0%)

![Histamine release from rat mesenteric mast cells treated with PLA₂ (n=4)](image)
Fig. 3.29: Histamine release from rat lung mast cells treated with \( \text{PLA}_2 \) (n=4)

(Control: compound 48/80 (1 \( \mu \)g/ml) -0.2 ± 0.7%)

![Graph showing histamine release from rat lung mast cells treated with PLA2](image1)

Fig. 3.30: Histamine release from guinea pig skin mast cells treated with \( \text{PLA}_2 \) (n=4)

(Control: anti-IgG (1/200 dilution) 25.1 ± 5.0%)

![Graph showing histamine release from guinea pig skin mast cells treated with PLA2](image2)
Fig. 3.31: Histamine release from guinea pig mesenteric mast cells treated with PLA$_2$ (n=4)
(Control: anti-IgG (1/200 dilution) 6.4 ± 3.6%)

Fig. 3.32: Histamine release from guinea pig lung mast cells treated with PLA$_2$ (n=4)
(Control: anti-IgG (1/200 dilution) 3.7 ± 1.7%)
Fig. 3.33: Histamine release from mouse peritoneal mast cells treated with \( \text{PLA}_2 \) \((n=4)\)

(Control: Con A (100 \( \mu \text{g/ml} \)) 13.7 \( \pm \) 3.2%)

![Graph showing histamine release from mouse peritoneal mast cells treated with \( \text{PLA}_2 \) (n=4).

Fig. 3.34: Histamine release from human lung mast cells treated with \( \text{PLA}_2 \) \((n=7)\)

(Control: anti-IgE (1/250 dilution) 18.4 \( \pm \) 7.2%)

![Graph showing histamine release from human lung mast cells treated with \( \text{PLA}_2 \) (n=7).]
Fig. 3.35: Histamine release from human basophil leukocytes treated with PLA$_2$ (n=4)

(Control: anti-IgE (1/500 dilution) 25.6 ± 6.2%)

Fig. 3.36: Histamine release from RBL-2H3 cells treated with PLA$_2$ (n=3)

(Control: A23187 (1 μM) 31.7 ± 1.5%)
Fig. 3.37: Cytotoxicity of histamine release from rat skin mast cells treated with PLA$_2$ (n=4)
(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release with different concentrations of PLA$_2$.]

Fig. 3.38: Cytotoxicity of histamine release from rat mesenteric mast cells treated with PLA$_2$ (n=4)
(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release with different concentrations of PLA$_2$.]
Fig. 3.39: **Cytotoxicity of histamine release from rat lung mast cells treated with PLA$_2$ (n=4)**

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release vs PLA$_2$ concentration.](image)

Fig. 3.40: **Cytotoxicity of histamine release from guinea pig skin mast cells treated with PLA$_2$ (n=4)**

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release vs PLA$_2$ concentration.](image)
Fig. 3.41: Cytotoxicity of histamine release from guinea pig mesenteric mast cells treated with PLA$_2$ (n=4)
(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release from guinea pig mesenteric mast cells treated with PLA$_2$.]

Fig. 3.42: Cytotoxicity of histamine release from guinea pig lung mast cells treated with PLA$_2$ (n=4)
(Asterisks denote those releases that were significantly different from that obtained from cells in FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release from guinea pig lung mast cells treated with PLA$_2$.]
Fig. 3.43: **Cytotoxicity of histamine release from RBL-2H3 cells treated with PLA$_2$ (n=3)**

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Graph showing histamine release from RBL-2H3 cells treated with PLA$_2$](image)

Fig. 3.44: **Cytotoxicity of histamine release from mouse peritoneal mast cells treated with PLA$_2$ (n=4)**

(Asterisks denote those releases that were significantly different from that obtained from cells in FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release from mouse peritoneal mast cells treated with PLA$_2$](image)
Fig. 3.45: Cytotoxicity of histamine release from human lung mast cells treated with PLA$_2$ (n=5)

(Asterisks denote those releases that were significantly different from that obtained from cells in FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release from human lung mast cells treated with PLA$_2$]({"data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAACAAAAAgCAYAAABzenr0AAAABGd7u8AAAAAt0lEQVR42mP8/A0Aw/wC1Mx2tKgAAAAASUVORK5CYII=})

Fig. 3.46: Histamine release from human basophil leukocytes treated with PLA$_2$ (n=4)

(Asterisks denote those releases that were significantly different from that obtained from cells in FHT. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release from human basophil leukocytes treated with PLA$_2$]({"data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAACAAAAAgCAYAAABzenr0AAAABGd7u8AAAAAt0lEQVR42mP8/A0Aw/wC1Mx2tKgAAAAASUVORK5CYII=})
Science is built up of facts, as a house is built of stones; but an accumulation of facts is no more a science than a heap of stones is a house.

**Henry Poincaré** 1854-1912

*Science and Hypothesis*. 1905
CHAPTER 4

Effect of PLA$_2$ on histamine release induced by different secretagogues: a comparison with PS and lyso-PS

4.1: Introduction

PLA$_2$ may be an integral part of a larger network of pro-inflammatory cytokines, growth factors and lipid mediators$^{(325)}$. Previous studies have shown that exogenous secretory non-pancreatic PLA$_2$ is inactive in the rat paw oedema assay yet, if an on-going inflammatory condition is present, the enzyme is able to induce additional swelling$^{(326, 326)}$.

Lyso-phosphatidylserine (lyso-PS) has been shown to exist at the sites of tissue injury$^{(327)}$ and there have been suggestions that the increase in intracellular calcium concentration, phosphoinositide hydrolysis and subsequent histamine release from mast cells upon stimulation with PLA$_2$ may be the result of the generation of lyso-PS$^{(327, 328)}$.

PLA$_2$ has been shown to potentiate anti-IgE induced histamine release$^{(329)}$. Martin and Lagunoff$^{(330)}$ showed that phosphatidylserine (PS) and lyso-PS also enhanced the secretory response of the mast cell in a highly specific manner. In contrast, PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), PA, lyso-PC and lyso-PE failed to produce this effect. PS and lyso-PS selectively enhance the response of mast cells to IgE mediated (antigen, Con A) secretagogues without increasing the response of the cells to IgE independent agents such as polymyxin B, chymotrypsin, compound 48/80 and A23187. The concentration of lyso-PS required to give 50% of the maximal potentiation with Con A was 0.065 μM but, for PS, it was 3-5 μM. The above authors
proposed that the effects may be due to a head-group specificity in the action of lyso-PS which was similar to that of PS.

The mechanism through which this potentiation occurs is unclear. Martin and Lagunoff\(^\text{330}\) argued that the conversion of PS to lyso-PS by a mast cell PLA\(_2\) is the important step in the process. Contrary to this postulation, however, Tamori-Natori and Nojima\(^\text{331}\) suggest that the deacylation by PLA\(_2\) is not required but the existence of an ester bond at position 1 is important for PS to show the effect. Mast cells may thus recognise the ester bond strictly. Hirata \textit{et al}.\(^\text{332}\) also proposed that the metabolism of PS is important for it to show its potentiating activity. They emphasised the significance of a Ca\(^{2+}\)-dependent PLA\(_2\) in the exocytotic stage of histamine release. Subsequent studies by Martin and Lagunoff\(^\text{333}\) have revealed the existence of a PLA\(_2\) which is primarily active towards exogenous PS. The affinity of this PLA\(_2\) is 3-4 fold higher towards PS than PC. A rather more recent communication has also revealed the existence of a PLA\(_2\) which preferentially hydrolyses PS\(^\text{334}\). These studies confirmed and continued the earlier findings that PS, and much more potently lyso-PS, was able to potentiate histamine and now PGD\(_2\) release induced by antigen. This study suggested that lyso-PS may act on RPMC as a lipid chemical mediator which induces eicosanoid generation as well as degranulation. A receptor-like molecule on the surface of CTMCs may exist which can recognise the configuration of the serine moiety\(^\text{334}\). Horigome \textit{et al}.\(^\text{335}\) suggested that lyso-PS was able to incorporate into mast cells more readily than PS and only phospholipids incorporated into mast cell membranes were able to express the potentiating activity. They suggested that a minute incorporation was required and the accumulation of lyso-PS was of major importance for optimal activity. Inserted lyso-PS may diffuse laterally and have access to the receptor, or receptor-like structure, which recognises the serine-like head group. However, PS may interact with the cells in the form of intact liposomes and hence be adsorbed onto the cell surface rather than being inserted into the membrane. Recognition of the
serine moiety by the membrane structure is specific since phospholipids having different head groups showed no activity\(^{(335)}\).

It is generally considered that optimal IgE-dependent histamine release requires extracellular Ca\(^{2+}\). Enhancement of histamine release from rat mast cells by lyso-PS may be due to enhancement of Ca\(^{2+}\) influx and/or inhibition of Ca\(^{2+}\) efflux. Previous studies have shown that lyso-PS increases stimulus dependent Ca\(^{2+}\) influx in RPMC suggesting that this influx or some earlier process may be the lyso-PS dependent step\(^{(335)}\). Similar results were also seen in mouse peritoneal mast cells indicating that this cell phenotype may also possess a similar receptor-like structure\(^{(335)}\).

The potentiation has been shown to be very specific for murine peritoneal and pleural mast cells but is limited or negligible in peritoneal mast cells of the hamster, chopped mesentery of the rat and chopped lung of the rat, guinea pig and man\(^{(336)}\). Enzymatically dispersed mast cells from rat and guinea pig mesentery, rat skin and intestine, and human lung, and basophils from man and the rabbit are also unaffected by the presence of PS\(^{(336)}\). The striking specificity of PS and lyso-PS may thus reflect the differences in membrane lipid composition in the diverse mast cell phenotypes\(^{(336)}\).

In summary, PLA\(_2\) is able to augment the histamine release induced by IgE-mediated secretagogues from RPMC, possibly through the generation of PS or lyso-PS. It was thus the aim of the present study to investigate this proposal further.

4.2: Methods and Materials

All the materials and methods employed in these studies are described in chapter 2. In particular, the protocol for potentiation studies is given in section 2.9.
4.3: Results

4.3.1: Effects of PLA$_2$ in combination with various ligands

PLA$_2$ from *Naja naja* (Fig. a in each case) and bee venom (Fig. b in each case) induced a dose-dependent potentiation of histamine release from RPMC when they were concurrently stimulated with anti-IgE, Con A, dextran, nerve growth factor (NGF), and from mouse peritoneal mast cells stimulated with Con A (Fig. 4.1 - 4.5, respectively). In contrast, the release from RPMC induced by the PLA$_2$ isoenzymes in combination with compound 48/80, substance P, mastoparan, calcium ionophore A23187, and from human lung mast cells and human basophil leukocytes stimulated with anti-IgE, was simply additive (Fig. 4.6 - 4.11, respectively).

In each of the cases above, PLA$_2$ (0.01-1 units/ml) was tested with several concentrations (>5) of each of the ligands to give a complete picture. However, for the purposes of this dissertation, only representative data, using secretagogue concentrations which elicited a 20-30 % secretion of total histamine, are given. In the case of dextran, results using the maximum concentration of 10 mg/ml are depicted as in the absence of added lipid, dextran is only able to elicit a very small (<3 %) secretion of histamine.

4.3.2: Effects of PS and lyso-PS on histamine release induced by various ligands

PS (Fig. a in each case) and lyso-PS (Fig. b in each case) dose-dependently augmented the secretion of histamine from RPMC which were concurrently stimulated with anti-IgE, Con A, dextran, NGF and from mouse peritoneal mast cells treated with Con A (Fig. 4.12 - 4.17, respectively). In contrast, the release from RPMC induced by compound 48/80, substance P, mastoparan,
A23187 and from human lung mast cells and from human basophils (Fig. 4.18 - 4.22) was unaffected by the presence of these lipids.

4.3.3: Cytotoxicity of the potentiation of histamine release

The cytotoxicity of the augmentation phenomenon was examined using the metabolic blockers antimycin A and 2-deoxy-D-glucose. The histamine releases from RPMC treated with \textit{Naja naja}-PLA$_2$ in combination with anti-IgE (Fig. 4.23) and NGF (Fig. 4.24) were completely abrogated by the metabolic blockade.

4.3.4: Potentiation by \textit{Naja naja}-PLA$_2$ of histamine release from purified RPMC

Purified mast cells were treated with \textit{Naja naja}-PLA$_2$ together with anti-IgE (Fig. 4.25) and NGF (Fig. 4.26). In both cases, the potentiation observed was similar to that in the mixed cell peritoneal population.

4.4: Discussion

The present study has shown that PLA$_2$ from both \textit{Naja naja} and bee venom is able to potentiate the histamine release from murine peritoneal mast cells induced by various IgE-dependent secretagogues, but has no effect on those agonists which act through mechanisms independent of the immunoglobulin. Dextran and NGF also work through immunoglobulin independent mechanisms. However there are similarities between these agents and the immunoglobulin dependent secretagogues in that PS and lyso-PS can potentiate histamine release induced by the compounds.

Anti-IgE binds to the Fc region of two adjacent IgE immunoglobulin macromolecules leading to their cross-linking and aggregation of the Fc$_{	ext{RI}}$
receptors. This aggregation is generally regarded as being the key event which then triggers the transduction cascade which eventually leads to degranulation\(^{337}\). The lectin Con A binds to carbohydrate moieties in the IgE immunoglobulin molecules to achieve cross-linking and subsequent aggregation of the Fc\(_e\)RI receptors\(^{338}\). High molecular weight dextran is thought to bind to glucose receptors on the surface of mast cells which, when cross-linked, initiate histamine secretion\(^{339}\). NGF is thought to induce exocytosis by interacting with specific receptors on the plasma membranes of the mast cells\(^{340}\). Compound 48/80 is a polybasic histamine releasing agent which is thought to activate a G-protein which then activates PLC and induces the secretion\(^{341}\). The neuropeptide substance P and mastoparan also initiate secretion by activating the G-protein\(^{342}\). Calcium ionophore A23187 induces histamine release by raising the intracellular calcium concentration which can then act as the trigger mechanism to induce degranulation\(^{343}\).

It is interesting to note that the very same ligands which exhibited augmented histamine release with the PLA\(_2\) isoenzymes also showed potentiation with PS and lyso-PS. Those ligands that were unaffected by the presence of PLA\(_2\) were also unaffected by the presence of PS or lyso-PS. This is summarised in Table 1. This potentiating effect was also seen when purified RPMC were used, indicating that the effect was indeed due to the mast cells and was not a consequence of the other cell types present in a mixed peritoneal cell population. The potentiated histamine release was suppressed by the presence of metabolic inhibitors indicating that the process was genuinely secretory. The effect was shown to be specific for murine mast cells as previously reported\(^{336}\). Neither human lung mast cells or basophils showed a potentiation of histamine release with PS or lyso-PS, and similarly showed no augmentation with the PLA\(_2\) isoenzymes.

On the basis of these results, it is not possible to ascertain unequivocally a mechanism through which PLA\(_2\) potentiates the histamine release from IgE
mediated secretagogues. However, from the results of these parallel experiments, it can be postulated that the generation of lyso-PS is a very likely mode of action. This has been further examined using more direct techniques in a subsequent chapter. These have categorically demonstrated that this hypothesis is, in fact, correct.
Chapter 4

Potentiation of Histamine Release

Fig. 4.1a: Effects of *Naja naja*-PLA$_2$ on anti-IgE (1/250 dilution) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA$_2$ together that were significantly different from the sum of the releases obtained by the ligand and PLA$_2$ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.1b: Effects of bee venom-PLA$_2$ on anti-IgE (1/250 dilution) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA$_2$ together that were significantly different from the sum of the releases obtained by the ligand and PLA$_2$ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.2a: Effects of *Naja naja*-PLA<sub>2</sub> on Con A (1 μg/ml) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA<sub>2</sub> together that were significantly different from the sum of the releases obtained by the ligand and PLA<sub>2</sub> separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar graph showing histamine release](image)

Fig. 4.2b: Effects of bee venom-PLA<sub>2</sub> on Con A (1 μg/ml) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA<sub>2</sub> together that were significantly different from the sum of the releases obtained by the ligand and PLA<sub>2</sub> separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar graph showing histamine release](image)
Fig. 4.3a:  Effects of *Naja naja*-PLA$_2$ on dextran (10 mg/ml) 
included histamine secretion from RPMC (n=4)  
(Asterisks denote the releases induced by the ligand and PLA$_2$ 
together that were significantly different from the sum of the releases 
obtained by the ligand and PLA$_2$ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release](image)

Fig. 4.3b:  Effects of bee venom-PLA$_2$ on dextran (10 mg/ml) 
included histamine secretion from RPMC (n=4)  
(Asterisks denote the releases induced by the ligand and PLA$_2$ 
together that were significantly different from the sum of the releases 
obtained by the ligand and PLA$_2$ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release](image)
Fig. 4.4a: Effects of *Naja naja*-PLA₂ on NGF (0.1 μg/ml) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA₂ together that were significantly different from the sum of the releases obtained by the ligand and PLA₂ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.4b: Effects of bee venom-PLA₂ on NGF (0.1 μg/ml) induced histamine secretion from RPMC (n=4)

(Asterisks denote the releases induced by the ligand and PLA₂ together that were significantly different from the sum of the releases obtained by the ligand and PLA₂ separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.5a: Effects of *Naja naja*-PLA2 on Con A (100 μg/ml) induced histamine secretion from mouse peritoneal mast cells (n=4)

(Asterisks denote the releases induced by the ligand and PLA2 together that were significantly different from the sum of the releases obtained by the ligand and PLA2 separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar chart showing the effects of PLA2 on Con A-induced histamine release.](image)

Fig. 4.5b: Effects of bee venom-PLA2 on Con A (100 μg/ml) induced histamine secretion from mouse peritoneal mast cells (n=4)

(Asterisks denote the releases induced by the ligand and PLA2 together that were significantly different from the sum of the releases obtained by the ligand and PLA2 separately. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar chart showing the effects of PLA2 on Con A-induced histamine release.](image)
Fig. 4.6a: Effects of *Naja naja*-PLA$_2$ on compound 48/80 (0.05 μg/ml) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

Fig. 4.6b: Effects of bee venom-PLA$_2$ on compound 48/80 (0.05 μg/ml) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)
Fig. 4.7a: Effects of Naja naja-PLA₂ on substance P (0.25 μg/ml) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA₂ together statistically different from the sum of those evoked by the ligand and PLA₂ separately.)

Fig. 4.7b: Effects of bee venom-PLA₂ on substance P (0.25 μg/ml) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA₂ together statistically different from the sum of those evoked by the ligand and PLA₂ separately.)
Fig. 4.8a: Effects of \textit{Naja naja}-PLA$_2$ on mastoparan (3 \textmu M) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

![Graph](image)

Fig. 4.8b: Effects of bee venom-PLA$_2$ on mastoparan (3 \textmu M) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

![Graph](image)
Chapter 4  Potentiation of Histamine Release

Fig. 4.9a: Effects of Naja naja-PLA$_2$ on A23187 (0.1 µM) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

![Graph showing the effects of Naja naja-PLA$_2$ on A23187-induced histamine secretion from RPMC](image)

Fig. 4.9b: Effects of bee venom-PLA$_2$ on A23187 (0.1 µM) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

![Graph showing the effects of bee venom-PLA$_2$ on A23187-induced histamine secretion from RPMC](image)
Chapter 4  
Potentiation of Histamine Release

Fig. 4.10a: Effects of *Naja naja*-PLA$_2$ on anti-IgE (1/250 dilution) induced histamine secretion from human lung mast cells (n=5)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)

Fig. 4.10b: Effects of bee venom-PLA$_2$ on anti-IgE (1/250 dilution) induced histamine secretion from human lung mast cells (n=5)

(In no cases were the releases induced by the ligand and PLA$_2$ together statistically different from the sum of those evoked by the ligand and PLA$_2$ separately.)
Fig. 4.11a: Effects of *Naja naja*-PLA₂ on anti-IgE (1/10000 dilution) induced histamine secretion from human basophils (n=4)

(In no cases were the releases induced by the ligand and PLA₂ together statistically different from the sum of those evoked by the ligand and PLA₂ separately.)

![Graph](image1)

Fig. 4.11b: Effects of bee venom-PLA₂ on anti-IgE (1/10000 dilution) induced histamine secretion from human basophils (n=4)

(In no cases were the releases induced by the ligand and PLA₂ together statistically different from the sum of those evoked by the ligand and PLA₂ separately.)

![Graph](image2)
Fig. 4.12a: Effects of PS on anti-IgE induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.12b: Effects of lyso-PS on anti-IgE induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.13a: Effects of PS on Con A induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.13b: Effects of lyso-PS on Con A induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.14a: Effects of PS on dextran induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.14b: Effects of lyso-PS on dextran induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.15a: Effects of PS on NGF induced histamine secretion from RPMC (n=4)
(Asterisks denote those releases that were significantly different from that obtained in the absence of PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.15b: Effects of lyso-PS on NGF induced histamine secretion from RPMC (n=4)
(Asterisks denote those releases that were significantly different from that obtained in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Chapter 4  
Potentiation of Histamine Release

**Fig. 4.16a:** Effects of PS on Con A induced histamine secretion from mouse peritoneal mast cells (n=4)  
(Asterisks denote those releases that were significantly different from that obtained in the absence of PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing effects of PS on Con A induced histamine secretion.](image)

**Fig. 4.16b:** Effects of lyso-PS on Con A induced histamine secretion from mouse peritoneal mast cells (n=4)  
(Asterisks denote those releases that were significantly different from that obtained in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing effects of lyso-PS on Con A induced histamine secretion.](image)
Chapter 4  
Potentiation of Histamine Release

Fig. 4.17a: Effects of PS on compound 48/80 induced histamine secretion from RPMC (n=4)
(In no cases were the releases in the absence and presence of PS significantly different.)

Fig. 4.17b: Effects of lyso-PS on compound 48/80 induced histamine secretion from RPMC (n=4)
(In no cases were the releases in the absence and presence of lyso-PS significantly different.)
Fig. 4.18a: Effects of PS on substance P induced histamine secretion from RPMC (n=4)

(In no cases were the releases in the absence and presence of PS significantly different.)

Fig. 4.18b: Effects of lyso-PS on substance P induced histamine secretion from RPMC (n=4)

(In no cases were the releases in the absence and presence of lyso-PS significantly different.)
Fig. 4.19a: Effects of PS on mastoparan induced histamine secretion from RPMC (n=4)
(In no cases were the releases in the absence and presence of PS significantly different.)

Fig. 4.19b: Effects of lyso-PS on mastoparan induced histamine secretion from RPMC (n=4)
(In no cases were the releases in the absence and presence of lyso-PS significantly different.)
Chapter 4  

Potentiation of Histamine Release

Fig. 4.20a: Effects of PS on A23187 induced histamine secretion from RPMC (n=4)

(In no cases were the releases in the absence and presence of PS significantly different.)

![Bar graph showing the effects of PS on A23187 induced histamine secretion.](image)

Fig. 4.20b: Effects of lyso-PS on A23187 induced histamine secretion from RPMC (n=4)

(In no cases were the releases in the absence and presence of lyso-PS significantly different.)

![Bar graph showing the effects of lyso-PS on A23187 induced histamine secretion.](image)
Fig. 4.21a: Effects of PS on anti-IgE induced histamine secretion from human lung mast cells (n=6)

(In no cases were the releases in the absence and presence of PS significantly different.)

Fig. 4.21b: Effects of lyso-PS on anti-IgE induced histamine secretion from human lung mast cells (n=6)

(In no cases were the releases in the absence and presence of lyso-PS significantly different.)
Chapter 4  
Potentiation of Histamine Release

Fig. 4.22a: Effects of PS on anti-IgE induced histamine secretion from human basophils (n=4)  
(In no cases were the releases in the absence and presence of PS significantly different.)

Fig. 4.22b: Effects of lyso-PS on anti-IgE induced histamine secretion from human basophils (n=4)  
(In no cases were the releases in the absence and presence of lyso-PS significantly different.)
Chapter 4  
Potentiation of Histamine Release

Fig. 4.23: Effects of metabolic inhibitors on the potentiation of histamine release induced by *Naja naja*-PLA$_2$ in combination with anti-IgE (n=3)

(Asterisks denote those releases that were significantly different from that obtained in the absence of the metabolic inhibitors. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 4.24: Effects of metabolic inhibitors on the potentiation of histamine release induced by *Naja naja*-PLA$_2$ in combination with NGF (n=3)

(Asterisks denote those releases that were significantly different from that obtained in the absence of the metabolic inhibitors. * p< 0.05, ** p< 0.01, *** p< 0.001.)
Fig. 4.25: Effects of *Naja naja*-PLA₂ on anti-IgE induced histamine release from purified RPMC (n=5)  
(Purity: 94.3 ± 4.2 %)  
(The releases from non-purified and purified cells were not significantly different.)

Fig. 4.26: Effects of *Naja naja*-PLA₂ on NGF induced histamine release from purified RPMC (n=5)  
(Purity: 94.3 ± 4.2 %)  
(The releases from non-purified and purified cells were not significantly different.)
Table 1: Summary of the effects of PLA₂, PS and lyso-PS on the potentiation of histamine release from several mast cell phenotypes stimulated with different ligands

<table>
<thead>
<tr>
<th>Mast cell Phenotype</th>
<th>Secretagogue</th>
<th>Potentiation by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PLA₂</td>
</tr>
<tr>
<td>RPMC</td>
<td>Anti-IgE</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>+</td>
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<tr>
<td></td>
<td>NGF</td>
<td>+</td>
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<tr>
<td></td>
<td>Compound 48/80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Substance P</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mastoparan</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A23187</td>
<td>-</td>
</tr>
<tr>
<td>Mouse peritoneal</td>
<td>Con A</td>
<td>+</td>
</tr>
<tr>
<td>Human lung</td>
<td>Anti-IgE</td>
<td>-</td>
</tr>
<tr>
<td>Human basophils</td>
<td>Anti-IgE</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter 5

"Science moves, but slowly slowly, creeping on from point to point."

Alfred, Lord Tennyson. 1809-92:

Locksley Hall. 1842
CHAPTER 5

Effect of specific PLA₂ inhibitors on histamine release from various mast cell phenotypes

5.1: Introduction

PLA₂ has been implicated in the process of mediator release from mast cells. This is primarily by virtue of the findings that the release of AA and its metabolites is a prominent event accompanying histamine secretion\(^{(344)}\). In addition, inhibitors of PLA₂ have been shown to block not only AA release but also histamine secretion\(^{(345, 346)}\). PLA₂ activation may thus also be a fundamental step in the degranulation of rat mast cells, rabbit and human leukocytes and RBL-2H3 cells, regardless of the stimulus\(^{(347, 348)}\).

The precise site in the transduction cascade by which PLA₂ may contribute to the exocytotic process is unclear\(^{(349)}\). However, a theory has arisen which may explain its exact role. Exocytosis of mast cells involves the fusion and fission of membranes and it has been suggested that calcium may initiate the alteration of the membrane lipid structure by activating a calcium-dependent PLA₂\(^{(350)}\). The enzyme may thus be involved in the fusion between plasma membranes and perigranular membranes resulting in chemical mediator release\(^{(347, 351)}\). Drugs which are capable of preventing these membrane changes, for example compounds which inhibit the activation of PLA₂, may then block the calcium dependent initial step in the release of AA and the subsequent synthesis and release of chemical mediators. This may offer a major site for therapeutic intervention in inflammatory diseases of diverse aetiology\(^{(256)}\).
Various studies have tried to identify selective inhibitors of PLA$_2$ which may be efficacious in the treatment of inflammatory diseases in man. However, such drugs have been difficult to obtain$^{362}$. Many of the previous studies have employed the use of compounds such as p-bromophenacyl bromide (p-BPB) and mepacrine. More specific and potent PLA$_2$ inhibitors, working through differing mechanisms, have become available. It was thus the aim of the present study to examine the effect of these agents on histamine secretion from different mast cell phenotypes in an attempt to add further credence to the hypothesis that PLA$_2$ is an essential factor in stimulus-secretion coupling in the mast cell.

5.2: Methods and Materials

All the materials and methods that were used in these studies are described in chapter 2. All unblocked releases used were between 30 - 40 %.

5.3: Results

5.3.1: Effects of various PLA$_2$ inhibitors on compound 48/80 and anti-IgE induced histamine release from RPMC

RPMC were stimulated with compound 48/80 (Fig. a in each case) and anti-IgE (Fig. b in each case) in the presence of p-BPB (0.1 - 10 μM), ACA (1.0 - 100 μM), manoalide (0.1 - 10 μM), OEPC (1 - 50 μM) and AACOCF$_3$ (10 - 100 μM).

p-BPB produced a dose-dependent and incubation time dependent inhibition of histamine release from RPMC stimulated with compound 48/80 (Fig. 5.1a, maximum inhibition $103.7 \pm 3.8 \%$, 20 min preincubation) and anti-IgE (Fig. 5.1b, maximum inhibition $97.5 \pm 2.4 \%$, 20 min preincubation).
ACA dose-dependently attenuated the histamine release induced by compound 48/80 (Fig. 5.2a, maximum inhibition 94.7 ± 1.8 %, 20 min preincubation) and anti-IgE (Fig. 5.2b, maximum inhibition 86.9 ± 2.6 %, 20 min preincubation).

The histamine release induced by compound 48/80 and anti-IgE was attenuated by the presence of manoalide (Fig. 5.3a and Fig. 5.3b, maximum inhibition 107.9 ± 4.4 % and 105.6 ± 4.6 %, respectively, 20 min preincubation).

OEPC dose dependently inhibited the histamine release induced by compound 48/80 (Fig. 5.4a, maximum inhibition 99.2 ± 3.4 %, 20 min preincubation), but was unable to attenuate the release induced by anti-IgE (Fig. 5.4b, maximum inhibition 7.3 ± 2.7 %, 20 min preincubation). However, when the compound was introduced into the cells using the permeabilisation technique, the immunologic secretion was dose dependently inhibited (Fig. 5.5, maximum inhibition 54.3 ± 3.1 %).

AACOCF$_3$ was ineffective in inhibiting secretion of the amine from cells stimulated with compound 48/80 or anti-IgE (Fig. 5.6a and Fig. 5.6b, maximum inhibition 5.6 ± 2.7 % and 5.9 ± 1.3 %, respectively, 20 min preincubation). However, by permeabilising the cells and allowing the compound to enter the cell cytosol, the histamine release induced by compound 48/80 and anti-IgE was dose dependently attenuated (Fig. 5.7, maximum inhibition 74.4 ± 3.7 % and 87.6 ± 2.5, respectively).
5.3.2: Effects of various PLA₂ inhibitors on anti-IgE induced histamine release from human lung mast cells

ρ-BPB (0.5 - 10 μM) dose dependently inhibited the histamine release induced by anti-IgE (Fig 5.8, maximum inhibition 100.7 ± 3.8 %, 20 min preincubation).

The histamine release was dose dependently attenuated by ACA (1 - 25 μM) (Fig 5.9, maximum inhibition 85.2 ± 4.4 %, 20 min preincubation).

Manoalide (0.3 - 25 μM) dose dependently inhibited release of the amine (Fig 5.10, maximum inhibition 93.5 ± 0.7 %, 20 min preincubation).

Both OEPC (5 - 50 μM) and AACOCF₃ (10 - 100 μM) were ineffective in inhibiting the histamine release from this cell type (Fig. 5.11 and Fig. 5.12, maximum inhibition 3.9 ± 0.3 % and 3.8 ± 1.4 %, respectively, 20 min preincubation.)

5.3.3: Effects of various PLA₂ inhibitors on anti-IgE induced histamine release from human basophil leukocytes

ρ-BPB (1 - 100 μM) dose dependently inhibited the histamine release induced by anti-IgE (Fig. 5.13, maximum inhibition 100.0 ± 1.4 %, 20 min preincubation).

ACA (50 - 500 μM) dose dependently inhibited the histamine release induced by anti-IgE (Fig. 5.14, maximum inhibition 37.3 ± 1.4 %, 20 min preincubation).

Manoalide (0.3 - 10 μM) dose dependently inhibited release of the amine (Fig. 5.15, maximum inhibition 89.1 ± 5.2 %, 20 min preincubation).
OEPC (50 - 500 μM) dose dependently inhibited the histamine release from the basophil leukocytes (Fig. 5.16, maximum inhibition 58.7 ± 2.5 %, 20 min preincubation).

AACOCF₃ (50 - 500 μM) was less effective in inhibiting secretion of the amine (Fig. 5.17, maximum inhibition, 21.3 ± 2.5 %, 20 min preincubation).

5.3.4: Further studies into the inhibition by OEPC from RPMC induced by non-immunological stimuli

OEPC dose dependently inhibited release of histamine from RPMC stimulated with compound 48/80 (0.03 - 30 μg/ml). Using differing concentrations of the inhibitor (1 - 80 μM), the dose response curves of histamine release induced by compound 48/80 were shifted to the right and the effect was surmountable at higher concentrations of the secretagogue (Fig. 5.18a). The concentration required to give a 50 % release of histamine in the absence and presence of each of the OEPC concentrations was used to produce a Schild plot. Regression analysis of these data yielded a straight line of slope 0.9 (Fig. 5.18b).

OEPC was able dose dependently to inhibit the histamine release from RPMC stimulated with substance P (1 - 50 μM). Increasing concentrations of OEPC (1 - 50 μM) shifted the dose response curves of histamine release induced by substance P to the right and the effect was surmountable at higher concentrations of the secretagogue (Fig. 5.19a). The concentration required to give a 30 % release of histamine in the absence and presence of each of the OEPC concentrations was used to generate the Schild plot. Regression analysis of these data yielded a straight line of slope 0.6 (Fig. 5.19b).
OEPC dose dependently inhibited the histamine release from RPMC stimulated with mastoparan (1 - 10 μM). The dose response curves were shifted to the right by increasing concentrations of OEPC (1 - 30 μM), and some suppression of the maximum response was observed. (Fig 5.20a). The Schild plot based on the concentrations of mastoparan required to elicit a 25 % histamine release in the absence and presence of each of the OEPC concentrations generated a regression line of slope 0.75 (Fig. 5.20b).

Benzalkonium chloride (BAC) dose dependently inhibited the release of histamine from RPMC stimulated with compound 48/80 (0.05 - 30 μg/ml). Increasing concentrations of BAC (1 - 3 μg/ml) shifted the dose response curves to the right and the effect was surmountable at higher concentrations of the secretagogue (Fig. 5.21a). The concentrations of compound 48/80 required to produce a 30 % histamine release in the absence and presence of each of the BAC concentrations were used to generate a Schild plot. The line of regression from these data had a slope of 1.97 (Fig. 5.21b).

Compound 48/80 (0.2 μg/ml) induced a 37.4 ± 3.3 % secretion of histamine from RPMC. Aliquots of the cells were incubated (10 min) with BAC (2.5 μg/ml) or FHT, spun down, washed and then re-incubated with either BAC or FHT. They were subsequently stimulated with compound 48/80 to see whether the wash was able to remove the BAC from the cells. The presence of BAC in the final incubation medium produced a reduced histamine release compared to that of the untreated cells. However, cells that had been incubated with BAC, washed and resuspended with FHT did not show an altered secretion, demonstrating that BAC could be washed from the cells (Fig 5.22a).

Using this same methodology, OEPC could not be washed from cells subsequently stimulated with compound 48/80 or substance P (10 μM) (Fig. 5.22b and Fig. 5.22c, respectively).
OEPC (1 - 50 μM) inhibited the histamine release from RPMC stimulated with polylysine (maximum inhibition, 113.9 ± 3.7 %), compound 48/80 (maximum inhibition, 101.0 ± 2.7 %), substance P (maximum inhibition, 92.8 ± 1.9 %) and mastoparan (maximum inhibition, 74.7 ± 2.2 %). The compound was less effective in inhibiting secretion of the amine from RPMC stimulated with anti-IgE (maximum inhibition, -2.3 ± 3.7 %), Con A (maximum inhibition, 23.6 ± 3.2 %), dextran (maximum inhibition, 17.5 ± 2.7 %), NGF (maximum inhibition, 11.1 ± 2.4 %) and A23187 (maximum inhibition, 14.1 ± 4.6 %) (Fig. 5.23).

5.3.5: Effects of mannose-BSA on histamine release from RPMC

Mannose-BSA (albumin, β-aminophenyl α-D-mannopyranoside, 26 mol monosaccharide/ mol albumin, 0.1 - 100 μg/ml) was ineffective in inducing secretion of histamine from RPMC. However, the presence of lyso-PS (1 μM) significantly increased the secretion with the higher concentrations of mannose-BSA (Fig. 5.24).

Mannose-BSA (100 μg/ml, 10 min preincubation) inhibited the release of histamine from RPMC stimulated with Naja naja-PLA₂ (0.1 - 10 units/ml). When cells were stimulated with bee venom-PLA₂, mannose-BSA was essentially ineffective (Fig. 5.25).

Mannose-BSA (0.1 - 250 μg/ml) dose and time dependently inhibited the release of histamine from RPMC treated with Naja naja-PLA₂ (Fig. 5.26, maximum inhibition, 102.6 ± 4.3 %, 20 min preincubation).

Mannose-BSA (0.1 - 250 μg/ml) dose dependently inhibited the histamine release from RPMC stimulated with dextran (maximum inhibition, 108.2 ± 3.2 %), anti-IgE (maximum inhibition, 101.0 ± 4.0 %) and Con A (maximum inhibition, 103.4 ± 3.4 %). The compound was less effective in inhibiting
secretion of the amine from RPMC stimulated with A23187 (maximum inhibition, 44.7 ± 1.8 %), compound 48/80 (maximum inhibition, 37.2 ± 3.2 %) or substance P (maximum inhibition, 38.2 ± 4.9 %, Fig. 5.27).

5.4: Discussion

The evaluation of PLA₂ inhibitors has been difficult due to the unique characteristics of the enzyme\(^\text{353}\). The extrapolation from in-vitro cell-free assays to cellular and/or in-vivo models has proved to be extremely tenuous\(^\text{354}\).

The present study has shown that various specific PLA₂ inhibitors, of diverse structural type and working through differing mechanisms, were able to inhibit the release of histamine from RPMC, human lung mast cells and human basophils leukocytes.

The compound that has been extensively used as a PLA₂ inhibitor is p-BPB (structure given in Fig. 5.28). p-BPB is a potent irreversible inhibitor of extracellular PLA₂\(^\text{355}\). This reagent alkylates a catalytic histidine residue resulting in the complete loss of enzymatic activity for most extracellular venom PLA₂s. In the present study, p-BPB was able to inhibit the histamine release from all three cell systems studied. However, with the basophils, a higher dose of the compound was required to elicit a complete attenuation of the secretion.

The most favourable results were probably seen using the marine natural product manoalide\(^\text{356}\) (structure given in Fig. 5.29). This compound is an irreversible inhibitor of several different PLA₂ isoenzymes\(^\text{357, 358}\). This inhibition has been associated with the loss of lysine residues by studies involving amino acid analyses\(^\text{357, 359}\). Mechanistic studies of the inhibition of PLA₂ by manoalide have revealed a limited number of lysines modified on bee venom-PLA₂ suggesting a selective binding site on PLA₂ for
manoalide\(^{(356, 360)}\). In the present study, manoalide was able completely to inhibit secretion of the amine from RPMC, human lung mast cells and human basophils. The concentrations required to produce this effect were comparable in all three test systems.

ACA (structure given in Fig. 5.30)\(^{(361)}\) was able dose-dependently to inhibit the histamine release from each of the test systems. However, the concentrations required for comparative inhibitions differed, with human lung mast cells requiring a much lower concentration than RPMC, whilst basophils required a much higher concentration of the compound. In other studies, ACA was shown to have no toxic properties\(^{(362)}\), but the precise mechanism of its action is unclear\(^{(363)}\).

AACOCF\(_3\) is a trifluoromethyl ketone analogue of AA in which the COOH group has been replaced with COCF\(_3\) (structure given in Fig. 5.31). This compound has been reported to be a cell-permeable, tight and slow-binding inhibitor of cytosolic human PLA\(_2\)\(^{(364, 365)}\). AACOCF\(_3\) inhibits PLA\(_2\) by directly binding to the enzyme rather than by decreasing the fraction of enzyme bound to the substrate interface\(^{(364)}\). However, in the present study, the compound was completely ineffective in RPMC and human lung mast cells, and was only moderately effective in human basophils. However, if the compound was introduced into the RPMC by permeabilisation, a dose-dependent inhibition of histamine release was seen. This indicates the importance of effective incorporation of the compound into the cell cytosol.

OEPC is a site-specific PLA\(_2\) inhibitor (structure given in Fig. 5.32)\(^{(366)}\). It blocks the myogenic response in perfused renal arcuate arteries of the dog and this effect has been claimed to be due to PLA\(_2\) inactivation\(^{(367)}\). In the present study, OEPC showed some interesting properties. OEPC inhibited the histamine release from RPMC stimulated with compound 48/80 but not anti-IgE. When the compound was introduced into the cell by permeabilisation, and the latter subsequently stimulated with anti-IgE,
inhibition was seen. This indicated that the compound was not able to penetrate the cell membrane. There then remains a paradox because a non-permeable compound should also be ineffective with compound 48/80. A more complex paradigm clearly existed. OEPC was also completely ineffective as an inhibitor in human lung mast cells and required much increased concentrations to show any inhibition in human basophils stimulated with anti-IgE.

To elucidate possible mechanisms behind the disparate results seen in RPMC, OEPC was further tested. It is possible that the positively charged group in OEPC may compete with compound 48/80 for binding to a putative receptor on the mast cell membrane and thus act as a competitive antagonist for the polyamine.

By definition, the effect of a true competitive antagonist should be reversible and surmountable. Thus, increasing concentrations of the antagonist should produce a parallel displacement to the right, without depression of the maximum effect, of the dose-response curves for a given agonist. A Schild plot, of log (dose ratio - 1) against the concentration of agonist, should give a straight line of unit slope. The dose ratio is the ratio of the concentration of agonist required to produce a given effect in the presence and absence of the antagonist\(^{(368)}\).

The dose response curves of compound 48/80 with OEPC showed a surmountable shift to the right. The Schild plot of these data gave a regression line of slope 0.9. However, OEPC in this system could not be washed from the cells and hence its effect was irreversible. Substance P and mastoparan showed similar results with Schild plots of slope 0.6 and 0.75, respectively.

Benzalkonium chloride (BAC) has been reported to be a competitive antagonist of compound 48/80\(^{(369, 370)}\). BAC is able to inhibit polyamine
induced histamine release from RPMC but not antigen-antibody reactions. In agreement with these reports, the present study has shown that BAC produces a reversible inhibition and its effect is surmountable at higher concentrations of compound 48/80. However, the Schild plot from the data generated gave a line of slope 1.97, indicating that BAC is not a simple, competitive antagonist.

Similarly to BAC, OEPC inhibited histamine release induced by polyamines (compound 48/80, substance P, mastoparan and polylysine) but not that evoked by IgE mediated secretagogues (anti-IgE, Con A), dextran, NGF or A23187. In total, OEPC may be a specific antagonist for polyamine ligands in addition to its ability as a PLA2 inhibitor.

A high affinity and specific binding site for bee venom-PLA2 was found on the surface of J774E macrophages. This binding site was entirely different from the binding site for pancreatic and snake venom PLA2. The binding of bee venom-PLA2 was completely antagonised by mannose-BSA, indicating it is probably mediated by macrophage mannose receptors. It was suggested that mannose-BSA and bee venom-PLA2 may bind to the same site on the macrophage.

To elucidate whether the same was true in the mast cell, RPMC were treated with mannose-BSA. The present study showed that the mannose conjugate significantly inhibited Naja naja-PLA2 induced histamine release but was less effective with bee venom-PLA2. In addition, mannose-BSA inhibited the secretion induced by the IgE related ligands (anti-IgE, Con A, dextran) but was less effective with A23187, compound 48/80 and substance P. This suggests that a mannose receptor may be involved in Naja naja-PLA2 induced secretion from RPMC. The signal transduction cascade of IgE mediated ligands may also involve the activation of this receptor.
In total, the present data indicate that the activation of endogenous PLA₂ may be an essential step in signal transduction in the mast cell. Inhibition of this enzyme may then provide for a novel target for the therapeutic modulation of this cell type.
Fig. 5.1a: Inhibition by ρ-BPB of histamine release from RPMC stimulated with compound 48/80 (0.2 µg/ml) (n=4)

Inhibition of histamine release (%)

ρ-BPB (µM)

Fig. 5.1b: Inhibition by ρ-BPB of histamine release from RPMC stimulated with anti-IgE (1/250 dilution) (n=4)

Inhibition of histamine release (%)

ρ-BPB (µM)
Chapter 5

PLA₂ Inhibitors

Fig. 5.2a: Inhibition by ACA of histamine release from RPMC stimulated with compound 48/80 (0.2 µg/ml) (n=4)

![Graph showing inhibition of histamine release by ACA at different preincubation times.]

Fig. 5.2b: Inhibition by ACA of histamine release from RPMC stimulated with anti-IgE (1/250 dilution) (n=4)

![Graph showing inhibition of histamine release by ACA at different preincubation times.]
Fig. 5.3a: Inhibition by manoalide of histamine release from RPMC stimulated with compound 48/80 (0.2 µg/ml) (n=4)

![Graph showing inhibition by manoalide of histamine release from RPMC stimulated with compound 48/80.](image)

Fig. 5.3b: Inhibition by manoalide of histamine release from RPMC stimulated with anti-IgE (1/250 dilution) (n=4)

![Graph showing inhibition by manoalide of histamine release from RPMC stimulated with anti-IgE.](image)
Fig. 5.4a: Inhibition by OEPC of histamine release from RPMC stimulated with compound 48/80 (0.2 μg/ml) (n=4)

Fig. 5.4b: Inhibition by OEPC of histamine release from RPMC stimulated with anti-IgE (1/250 dilution) (n=4)
Fig. 5.5: Inhibition by OEPC of histamine release from non-permeabilised and permeabilised RPMC stimulated with anti-IgE (1/250 dilution) (n=3)

Fig. 5.6a: Inhibition by AACOCF$_3$ of histamine release from RPMC stimulated with compound 48/80 (0.2 $\mu$g/ml) (n=4)
Fig. 5.6b: Inhibition by AACOCF$_3$ of histamine release from RPMC stimulated with anti-lgE (1/250 dilution) (n=4)

![Graph showing inhibition by AACOCF$_3$ of histamine release from RPMC stimulated with anti-lgE and various preincubation times.](image)

Fig. 5.7: Inhibition by AACOCF$_3$ of histamine release from non-permeabilised and permeabilised RPMC stimulated with anti-lgE (1/250 dilution) and compound 48/80 (0.2 µg/ml) (n=3)

![Graph showing inhibition by AACOCF$_3$ of histamine release from non-permeabilised and permeabilised RPMC.](image)
Fig. 5.8: Inhibition by ρ-BPB of histamine release from human lung mast cells stimulated with anti-IgE (1/250 dilution) (n=6)

Fig. 5.9: Inhibition by ACA of histamine release from human lung mast cells stimulated with anti-IgE (1/250 dilution) (n=6)
Fig. 5.10: Inhibition by manoalide of histamine release from human lung mast cells stimulated with anti-IgE (1/250 dilution) (n=6)

Fig. 5.11: Inhibition by OEPC of histamine release from human lung mast cells stimulated with anti-IgE (1/250 dilution) (n=6)
Fig. 5.12: Inhibition by AACOCF$_3$ of histamine release from human lung mast cells stimulated with anti-IgE (1/250 dilution) (n=6)

Fig. 5.13: Inhibition by $\rho$-BPB of histamine release from human basophils stimulated with anti-IgE (n=4)
Fig. 5.14: Inhibition by ACA of histamine release from human basophils stimulated with anti-IgE (n=4)

Fig. 5.15: Inhibition by manoalide of histamine release from human basophils stimulated with anti-IgE (n=4)
Fig. 5.16: Inhibition by OEPC of histamine release from human basophils stimulated with anti-IgE (n=4)

Fig. 5.17: Inhibition by AACOCF₃ of histamine release from human basophils stimulated with anti-IgE (n=6)
**Fig. 5.18a**: Inhibition by OEPC of histamine release from RPMC stimulated with various concentrations of compound 48/80 (n=5)

![Graph showing inhibition by OEPC of histamine release from RPMC stimulated with various concentrations of compound 48/80](image)

**Fig. 5.18b**: Schild plot of OEPC versus compound 48/80

(Concentrations of compound 48/80 required to induce a 50% release of histamine in the absence and presence of OEPC were calculated from data in Fig. 5.18a and used to generate this Schild plot.)

![Schild plot of OEPC versus compound 48/80](image)
**Fig. 5.19a:** Inhibition by OEPC of histamine release from RPMC stimulated with various concentrations of substance P (n=5)

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

Substance P alone

+ OEPC (50 µM)

+ OEPC (40 µM)

+ OEPC (30 µM)

+ OEPC (20 µM)

+ OEPC (10 µM)

+ OEPC (5 µM)

+ OEPC (1 µM)

**Fig. 5.19b:** Schild plot of OEPC versus substance P

(Concentrations of substance P required to induce a 30% release of histamine in the absence and presence of OEPC were calculated from data in Fig. 5.19a and used to generate this Schild plot.)

![Schild plot graph](image)

\[ y = 0.6x - 0.8 \]
Chapter 5

**PLA₂ Inhibitors**

Fig. 5.20a: Inhibition by OEPC of histamine release from RPMC stimulated with various concentrations of mastoparan (n=5)

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

Fig. 5.20b: Schild plot of OEPC versus mastoparan

(Concentrations of mastoparan required to induce a 25% release of histamine in the absence and presence of OEPC were calculated from data in Fig. 5.20a and used to generate this Schild plot.)

![Schild plot graph](image)

\[
y = 0.75x - 0.9
\]
Fig. 5.21a: Inhibition by benzalkonium chloride of histamine release from RPMC stimulated with various concentrations of compound 48/80 (n=5)

Fig. 5.21b: Schild plot of benzalkonium chloride versus compound 48/80
(Concentrations of compound 48/80 required to induce a 30% release of histamine in the absence and presence of BAC were calculated from data in Fig. 5.21a and used to generate this Schild plot.)
Fig. 5.22a: Effects of washing out benzalkonium chloride (2.5 \( \mu g/ml \)) from RPMC subsequently stimulated with compound 48/80 (0.2 \( \mu g/ml \)) (n=4)

(Asterisks denote those releases that were significantly different from that obtained from cells that were untreated with BAC. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar graph showing histamine release percentages with medium and drug treatments.

Fig. 5.22b: Effects of washing out OEPC (60 \( \mu M \)) from RPMC subsequently stimulated with compound 48/80 (0.2 \( \mu g/ml \)) (n=4)

(Asterisks denote those releases that were significantly different from that obtained from cells that were untreated with OEPC. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Bar graph showing histamine release percentages with medium and drug treatments.]
Fig. 5.22c: Effects of washing out OEPC (60 μM) from RPMC subsequently stimulated with substance P (10 μM) (n=4)
(Asterisks denote those releases that were significantly different from that obtained from cells that were untreated with OEPC. * p< 0.05, ** p< 0.01, *** p< 0.001.)

Fig. 5.23: Inhibition by OEPC (10 min preincubation) of histamine release from RPMC stimulated with various ligands (n=4)
(All unblocked releases were between 30 - 40 %)
Fig. 5.24: Effects of lyso-PS (1 μM) on RPMC treated with mannose-BSA (n=3)
(Asterisks denote those releases that were significantly different from that obtained from cells in the absence of lyso-PS. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release](image)

Fig. 5.25: Inhibition by mannose-BSA (10 min preincubation) of histamine release from RPMC treated with PLA₂ (n=3)
(Asterisks denote those releases that were significantly different from that obtained from cells in the absence of mannose-BSA. * p< 0.05, ** p< 0.01, *** p< 0.001.)

![Graph showing histamine release](image)
Chapter 5  
PLA₂ Inhibitors

Fig. 5.26: Inhibition by mannose-BSA of histamine release from RPMC treated with *Naja naja*-PLA₂ (2 units/ml) (n=4)

![Graph showing inhibition of histamine release over time with mannose-BSA concentrations.]

Fig. 5.27: Inhibition by mannose-BSA (10 min preincubation) of histamine release from RPMC stimulated with various ligands (n=4)

(All unblocked releases were between 30 - 40 %)

![Graph showing inhibition of histamine release with various ligands and mannose-BSA concentrations.]

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182
Fig. 5.28: Structure of $\rho$-BPB

![Structure of $\rho$-BPB](image)

Fig. 5.29: Structure of manoalide

![Structure of manoalide](image)

Fig. 5.30: Structure of ACA

![Structure of ACA](image)
Fig. 5.31: Structure of AACOCF$_3$

\[
\begin{align*}
\text{AACOCF}_3
\end{align*}
\]

Fig. 5.32: Structure of OEPC

\[
\begin{align*}
\text{OEPC}
\end{align*}
\]
Chapter 6

“I have long felt that biology ought to seem as exciting as a mystery story, for a mystery story is exactly what biology is.”

CHAPTER 6

Effect of various phospholipids and lipoxygenase/cyclooxygenase inhibitors on histamine release from different mast cell phenotypes

6.1: Introduction

One of the simplest modes of translating the information that is presented at a cell membrane into cellular signals which can then be interpreted by extracellular and intracellular sensors is to break the cellular membrane components and use these as the signalling elements. The membrane metabolites may themselves cause the biological response (primary metabolites e.g. lyso-phospholipids) or may act as precursors for the formation of secondary metabolites such as the eicosanoids (e.g. PGs or LTs) or PAF^{372}.

Biological membranes are comprised of organised sheet-like assemblies consisting mainly of proteins and lipids. The specific proteins mediate the distinct functions of membranes, serving as pumps, gates, receptors, energy transducers and enzymes. Membrane proteins are embedded in lipid bilayers, which create suitable environments for their actions. Membrane lipids are relatively small molecules that have both a hydrophobic and hydrophilic moiety. These lipids spontaneously form asymmetric closed bimolecular sheets in aqueous media^{373}. Lipids have a variety of biological roles, serving as fuel molecules, highly concentrated energy stores, and signalling molecules as well as components of membranes. It is the third of these functions which is presently of primary concern. The three major kinds of membrane lipids are phospholipids, glycolipids and cholesterol. PLA_{2} exerts its enzymatic actions on phospholipids hence this is the class of lipid
that is of relevance in the present study. The fatty acid chains of phospholipids usually contain an even number of carbon atoms, typically between 14 and 24, with the most common ones being those with 16- and 18-carbon atoms. AA, containing 20-carbon atoms, accounts for 5-30% of the total fatty acids in inflammatory cells. In animals, the hydrocarbon chains in the fatty acid moieties are usually unbranched and may be saturated or unsaturated. The configuration of the double bonds in unsaturated fatty acids is always cis. The phosphate groups of phospholipids become esterified to the hydroxyl group of one of several alcohols, choline, ethanolamine, inositol and serine.

The enzymatic hydrolysis by PLA\textsubscript{2} of these plasma membrane phospholipids may mobilise or generate a lipid substance such as a free fatty acid, lyso-phospholipid or related metabolite which may be responsible for inducing histamine release from mast cells. If AA is generated, this may be further metabolised to produce LTs and PGs. The generation of a lipid substance may promote the fusion of the mast cell membrane with secretory granules during the process of exocytosis. For example, lyso-PC has been reported to cause membrane fusion. Martin and Lagunoff reported that lyso-PC, lyso-PE and lyso-PS all caused histamine release from RPMC. The present study (Chapter 4) has also confirmed previous work that PS and lyso-PS are able to potentiate histamine release from murine mast cells. Certain of the lipid metabolites may also act as signalling molecules. For example, lyso-PI is known to be involved in the stimulus-secretion coupling mechanism in mast cells. This lyso-PI may be rapidly acylated to form PI, and an active PLA\textsubscript{2} may hydrolyse PI to re-generate lyso-PI. This “turnover” may induce secretion of histamine. Lyso-PA is a further naturally occurring intracellular signalling molecule. The action of PLA\textsubscript{2} on PA may thus generate lyso-PA which may then signal secretion of the amine.
Chapter 6 Membrane Metabolites

Hence, several membrane phospholipids may be potential inducers of the histamine release seen with PLA2. These possibilities have been further examined in the present chapter.

6.2: Methods and Materials

All methods and materials used in these studies have been described in chapter 2. In inhibition studies, unblocked releases of between 30 - 40 % were used. All the inhibitors were incubated with the cells for 10 min before application of the stimuli.

6.3: Results

6.3.1: Effects of L-ω-phospholipids and their corresponding lyso-derivatives on histamine release from different mast cell phenotypes

The L-ω-phospholipid (10 μM - 1 mM) PA induced modest or minimal amounts of histamine secretion from RPMC, human lung mast cells and human basophils (maximum releases, 6.4 ± 1.0 %, 1.7 ± 0.3 %, 0.4 ± 0.3 %, respectively, Fig. 6.1a). Its lyso-derivative, lyso-PA, was more potent in all three test systems (maximum releases, 48.8 ± 2.1 %, 4.0 ± 0.4 %, 14.5 ± 2.4 %, respectively, Fig. 6.1b).

The release induced by PC (10 μM - 1 mM) was also low, with maximum responses of 5.5 ± 2.3 %, 0.1 ± 0.2 % and 0.1 ± 0.4 % from RPMC, human lung mast cells and human basophils, respectively (Fig. 6.2a). However, lyso-PC was a much more potent liberator of the amine (maximum releases, 80.8 ± 2.7 %, 81.9 ± 3.9 %, 69.8 ± 5.0 %, respectively, Fig. 6.2b).
PS (1 - 100 μM) was essentially unable to induce secretion of the amine from the three mast cell phenotypes tested (maximum releases, 2.6 ± 0.5 %, 0.3 ± 0.6 %, 0.2 ± 0.1 %, respectively, Fig. 6.3a). Lyso-PS was only minimally more effective (maximum releases, 9.9 ± 1.8 %, 1.2 ± 0.7 %, 0.9 ± 1.2 %, respectively, Fig. 6.3b).

PE, PI, and their corresponding lyso-derivatives were essentially ineffective in all cases (maximum releases < 2 %, Fig. 6.4 and 6.5, a & b, respectively).

6.3.2: Cytotoxicity of the histamine release induced by lyso-PA and lyso-PC from different mast cell phenotypes

The histamine release induced by lyso-PA (0.1 mM - 1 mM) from RPMC, human lung mast cells and human basophils was suppressed by metabolic blockade with 2-deoxy-D-glucose and antimycin A (Fig. 6.6 - 6.8, respectively). In contrast, that evoked by lyso-PC was unaffected by these agents (Fig. 6.9 - 6.11).

6.3.3: Potentiation of histamine release from RPMC treated with anti-IgE in combination with L-α-phospholipids and their corresponding lyso-derivatives

Lyso-PS (1 μM) augmented immunological secretion of histamine from RPMC. Thus, the releases induced by anti-IgE (300-fold dilution), lyso-PS alone, and the two factors together were 32.3 ± 3.7 %, 8.2 ± 2.8 % and 73.9 ± 4.5 %, respectively (Fig. 6.12). These results are in agreement with those quoted in chapter 4. Similar results were also seen with PS. Here, the releases were 32.3 ± 3.7 %, 2.4 ± 1.8 % and 57.0 ± 2.6 %, respectively (Fig. 6.13).

None of the other lipids had any such potentiating effect (Fig. 6.14).
6.3.4: Further studies into the histamine release induced by lyso-PA from RPMC

The rate of histamine release induced by lyso-PA (0.5 mM) from RPMC was slow (t_{1/2} approximately 5 min, maximum release obtained after 30 min, Fig. 6.15) compared to that induced by compound 48/80 (t_{1/2} = 10 sec, maximum release obtained after 30 sec, Fig. 3.7), anti-IgE (t_{1/2} = 30 sec, maximum release obtained after 5 min, Fig. 3.8), Naja naja-PLA₂ (t_{1/2} = 180 sec, maximum release obtained after 10 min, Fig. 3.5) and bee venom-PLA₂ (t_{1/2} = 15 sec, maximum release obtained after 60 sec, Fig. 3.6).

The histamine release induced by lyso-PA (0.5 mM) was greatly increased (from a maximum release of 29.6 ± 3.1 % in normal FHT buffer) when the cells were incubated in a calcium free medium (maximum release 73.9 ± 4.8 %), or in a medium containing EDTA (maximum release 74.7 ± 3.9 %, Fig. 6.16).

In the presence of the metabolic inhibitors, the histamine release induced by lyso-PA (0.5 mM) from cells in a FHT buffer was abolished (as shown in Fig. 6.6). However, the blockers were ineffective when the cells were incubated in a calcium free medium (maximum release 71.8 ± 2.4 %), or in an EDTA containing buffer (maximum release 76.9 ± 4.5 %, Fig. 6.17).

RPMC suspended in a calcium free medium were stimulated with lyso-PA (0.5 mM, maximum release 89.4 ± 5.8 %). Increasing the concentration of calcium (0 - 1.0 mM) dose dependently decreased the histamine release (1.0 mM, 28.0 ± 2.1 %). A similar dose dependent reduction in histamine release was also seen with increasing concentrations of magnesium (0 - 1.0 mM), but the release at higher concentrations of the cation was now virtually abolished (1.0 mM, 3.7 ± 3.0 %, Fig. 6.18).
6.3.5: PGD$_2$ release from RPMC treated with PLA$_2$ from *Naja naja* and bee venom

PLA$_2$ from both *Naja-naja* and bee venom (0.1 - 10 units/ml) induced a dose dependent release of PGD$_2$ from RPMC (maximum release, 25.8 ± 5.3 ng/10$^6$ cells and 22.1 ± 7.1 ng/10$^6$ cells, Fig. 6.19a and 6.20a, respectively). The corresponding histamine releases were 70.2 ± 0.6 % and 24.7 ± 5.4 %, respectively (Fig. 6.19b and 6.20b, respectively). Anti-IgE (200-fold dilution, Fig. 6.21) and compound 48/80 (0.2 µg/ml, Fig. 5.22) produced PGD$_2$ releases of 18.5 ± 4.9 ng/10$^6$ cells and 4.1 ± 2.8 ng/10$^6$ cells, respectively. The corresponding histamine releases were 29.7 ± 2.1 % and 35.8 ± 6.5 %, respectively.

6.3.6: Effects of dual lipoxygenase and cyclooxygenase inhibitors on the histamine release induced by different ligands from RPMC

The dual lipoxygenase/cyclooxygenase inhibitors curcumin (0.3 - 15 µM, Fig. 6.23), meclofenamate sodium (10 - 50 µM, Fig. 6.24) and 1-phenyl-3-pyrazolidinone (phenidone, 5 - 75 µM, Fig. 6.25) inhibited the release of histamine induced by *Naja naja*-PLA$_2$ (2 units/ml), anti-IgE (1/300 dilution) and compound 48/80 (0.2 µg/ml). With curcumin, maximum inhibition was 73.4 ± 3.9 %, 45.9 ± 4.0 % and 39.4 ± 4.8 %, respectively. With meclofenamate sodium, the maximum inhibition seen was 34.9 ± 5.2 %, 26.4 ± 2.9 % and 28.1 ± 2.1 %, respectively. Phenidone produced maximal inhibitions of 37.4 ± 4.7 %, 28.3 ± 2.3 % and 22.4 ± 2.5 %, respectively.
6.3.7: Effects of cyclooxygenase inhibitors on the histamine release induced by different ligands from RPMC

The cyclooxygenase inhibitors indomethacin (0.1 - 10 μM, Fig. 6.26), diclofenac (1 - 75 μM, Fig. 6.27), phenylbutazone (1 - 50 μM, Fig. 6.28) and flurbiprofen (1 - 50 μM, Fig. 6.29) only slightly inhibited the release of histamine induced by *Naja naja*-PLA₂, anti-IgE and compound 48/80. With indomethacin, maximum inhibition was 5.4 ± 2.6 %, 5.8 ± 0.3 % and 6.3 ± 1.8 %, respectively. At lower concentrations, a slight potentiation of histamine release was seen. Diclofenac produced maximal inhibitions of 16.4 ± 2.1 %, 18.2 ± 2.7 % and 14.2 ± 2.9 %, respectively. Again, at lower concentrations, a potentiation of histamine release was seen. With phenylbutazone, maximum inhibitions of 5.8 ± 2.6 %, 10.9 ± 2.9 % and 8.4 ± 3.1 % were produced, respectively. A much lesser potentiation of histamine release was seen with lower concentrations of this compound. With flurbiprofen, maximal inhibitions were 5.7 ± 2.8 %, 2.3 ± 2.0 % and 11.6 ± 2.2 %, respectively.

6.3.8: Effects of lipoxygenase inhibitors on the histamine release induced by different ligands from RPMC

The lipoxygenase inhibitors 6, 7-dihydroxycoumarin (esculetin, 10 - 100 μM, Fig. 6.30), nordihydroguaiaretic acid (NDGA, 0.5 - 10 μM, Fig. 6.31), AA-861 (0.5 - 10 μM, Fig. 6.32), caffeic acid (0.5 - 10 μM, Fig. 6.33), L-739,010 (5 - 100 μM, Fig. 6.34) and L-746,530 (5 - 100 μM, Fig. 6.35), apart from the latter two agents, inhibited the histamine release induced by *Naja naja*-PLA₂, anti-IgE and, compound 48/80.

With esculetin, maximum inhibitions seen were 19.7 ± 2.1 %, 23.5 ± 2.7 % and 20.2 ± 2.9 %, respectively. NDGA produced maximum inhibitions of 71.4 ± 4.9 %, 60.1 ± 3.6 % and 61.0 ± 2.7 %, respectively. AA-861 gave maximal inhibitions of 13.5 ± 2.8 %, 25.5 ± 2.0 % and 17.8 ± 2.7 %, respectively.
Caffeic acid was more potent, giving maximal inhibitions of 80.0 ± 4.5 %, 53.4 ± 2.9 % and 61.8 ± 2.9 %, respectively. The specific lipoxygenase inhibitor L-739,010 inhibited the histamine release induced by *Naja naja*-PLA₂ and anti-IgE (maximum inhibition, 34.6 ± 2.9 % and 38.8 ± 2.1 %, respectively), but was ineffective in attenuating the histamine release induced with compound 48/80 (maximum inhibition, -2.9 ± 1.2 %). Similar results were seen with L-746,530. The compound attenuated the histamine release induced by *Naja naja*-PLA₂ and anti-IgE (maximum inhibition, 49.6 ± 2.7 % and 41.9 ± 3.5 %, respectively) but the release induced by compound 48/80 was essentially unaffected (maximum inhibition, 4.2 ± 0.9 %).

6.4: Discussion

The hydrolysis of an L-α-phospholipid by PLA₂ generates the lyso-phospholipid and free AA, both of which could possibly be responsible for the histamine release induced by the enzyme. The present study has shown that PA (structure in Fig. 6.36a), PC (structure in Fig. 6.37a), PS (structure in Fig. 6.38a) and their corresponding lyso-derivatives (structures in Fig. 6.36b - 6.38b, respectively) induced varying degrees of histamine release from the various cells studied. In contrast, PE (structure in Fig. 6.39a), PI (Fig. 6.40a) and their corresponding lyso-derivatives (structures in Fig. 6.39b - 6.40b) were essentially ineffective in all cases. Lyso-PC produced a marked release of histamine from all three mast cell types studied. Lyso-PA was rather less active against RPMC, showed modest activity against the basophils and was ineffective against the human lung mast cells. The releases induced by lyso-PA were suppressed by metabolic blockade with 2-deoxy-D-glucose and antimycin A whereas those evoked by lyso-PC were unaffected by these agents and were thus presumably cytolytic.

Lyso-PC (lyso-lecithin) has been reported to cause membrane fusion and to induce histamine secretion in a cytolytic fashion by virtue of its surfactant properties. The present study has confirmed these previous results. The
lytic release of histamine induced by PLA_2 (chapter 3) may then be caused by the production of lyso-PC.

The naturally occurring intercellular molecule lyso-PA has signalling functions in other systems having a role in cell growth and motility\(^\text{377, 379, 380}\). Studies in human skin fibroblasts suggest that lyso-PA acts by stimulating PLC activity resulting in the formation of inositol 1,4,5-trisphosphate (IP\(_3\)) which is accompanied by a concentration dependent increase in intracellular calcium concentrations\(^\text{380}\). Both the activation of PLC and the production of IP\(_3\) were sensitive to pertussis toxin suggesting that lyso-PA signalling involves the activation of a G\(_i\)-protein-coupled receptor in this cell type\(^\text{380}\). The present study has shown that the addition of exogenous lyso-PA induces a non-cytotoxic release of histamine. The non-lytic release of histamine induced by PLA\(_2\) may be then due to the generation of lyso-PA. The histamine release due to lyso-PA is much slower than that evoked by PLA\(_2\). This may be due to lyso-PA requiring a longer time to incorporate into the membrane. However, in a calcium free medium, the release was cytolytic. By increasing the concentration of calcium ions in the medium, the release decreased. This decrease was also seen with increasing concentrations of magnesium but now the release was completely abolished at non-cytolytic concentrations because magnesium cannot support active secretion whereas calcium can. It could be possible that lyso-PA may be mimicking a calcium ionophore type of drug. By applying the lyso-lipid to the cells in a calcium deficient environment, the resulting rapid sequestering of internal calcium stores may render it cytolytic. This reasoning has previously been applied to the histamine release induced by lyso-PS\(^\text{381}\).

Previous studies by West\(^\text{382}\) have reported that PA induces a non-cytotoxic release of histamine from RPMC. More recently, there have been suggestions that PA induces the liberation of arachidonate from RPMC\(^\text{383}\). Contrary to these findings, the present study has shown that PA induces a very low secretion of histamine. The reason for this paradox is unclear.
Mast cell activation has been shown to induce the rapid remodelling of arachidonate from PC to PE\(^{(384)}\). However, the present study did not show that exogenously applied PE was sufficient to induce activation of the cell types studied. The PI - lyso-PI “turnover” has been associated with the biochemical sequence of events leading to histamine release\(^{(376, 385)}\). The de novo PI synthesis may be a part of the triggering events for calcium ion mobilisation and subsequent mediator release\(^{(386)}\). However, in the present study, application of both PI and lyso-PI was insufficient to induce degranulation of the mast cell phenotypes examined.

Lloret and Moreno\(^{(327)}\) reported that lyso-PS induced a pertussis toxin sensitive release of histamine from mast cells. Indeed, they suggested that the release of histamine induced by PLA\(_2\) may be due to the production of lyso-PS. Studies have shown that the serine amino acid head group in PS and lyso-PS is required for association with a possible receptor in the mast cell membrane\(^{(387)}\). The present study has shown that PS or lyso-PS are not able to induce secretion by themselves but, in agreement with previous studies\(^{(388)}\), are able to enhance the immunological activation of the cell type. This specific effect of PS and lyso-PS has been examined in detail in chapter 4 of the present study. As none of the other lipids had any such effect in any of the test systems, it can be postulated that the potentiating effects of PLA\(_2\) (discussed in chapter 4) in combination with IgE-directed ligands in rat mast cells may then be due to generation of lyso-PS.

In resting platelets, the outer layer of the plasma membrane predominantly consists of the phospholipids PC and sphingomyelin. The inner layer contains PE and PS\(^{(386)}\). It is possible that this same asymmetry exists in mast cells. Bevers et al.\(^{(386)}\) reported that in activated platelets, a translocation of the inner phospholipids PE and PS into the outer plasma membrane occurs. This may possibly also occur during activation of mast cells, enabling these phospholipids to be accessible to the exogenously applied PLA\(_2\).
The second metabolite that is generated upon PLA\(_2\) hydrolysis of a membrane phospholipid is AA. This can be metabolised via the lipoxygenase or cyclooxygenase pathways to form a number of potent inflammatory mediators, the eicosanoids, principally PGs, LTs and TXs\(^{390, 391}\). The liberation of AA by PLA\(_2\) is the rate limiting step in eicosanoid biosynthesis\(^{392}\). Previous studies have shown that drugs that specifically inhibit the lipoxygenase pathway also block histamine secretion and hence it has been proposed that this pathway can generate intermediates which are essential to the release process\(^{393-396}\). The intermediate products 5-hydroxyeicosatetraenoic acid (5-HETE) and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) induce a dose dependent release of histamine from RPMC\(^{397, 398}\). In agreement with these reports, the present study has indicated that drugs that block the two divergent pathways, but in particular those that inhibit the lipoxygenase pathway, can inhibit the release of histamine induced by an immunological stimulus (anti-IgE), a non-immunological stimulus (compound 48/80) and PLA\(_2\). The histamine release associated with the PLA\(_2\) action may possibly be due to production of one of the intermediates which modulate secretion\(^{396}\). It is unclear, however, why the lipoxygenase inhibitors L-739,010 and L-746,530 are ineffective against cell activation by compound 48/80. A possible explanation may be that the ligand and the inhibitor could be physically interacting. Further work is required to decipher the precise mechanism of this effect. Inhibitors of the cyclooxygenase pathway, which include the non-steroidal anti-inflammatory drugs, have no effect or even potentiate histamine release\(^{393, 399, 400}\). In the present study, the cyclooxygenase inhibitors showed limited inhibition of histamine release induced by all three ligands. With the lower inhibitor concentrations, where their effects are probably more specific, a slight potentiation of histamine release was also seen. Several contradictory results have been cited in the literature. Masini et al\(^{401}\) reported that cyclooxygenase inhibitors did attenuate histamine release from rat mast cells. In contrast, Nagai et al\(^{402}\) stated that neither cyclooxygenase nor lipoxygenase inhibitors
had any inhibitory effect on histamine secretion from RPMC stimulated with
PLA$_2$, and concluded that the effects of the enzyme are not mediated by an
arachidonate metabolite. Both of these findings are disputed by the present
study.

AA can also be generated through a separate pathway involving PLC.
However, Yamada et al.$^{[403]}$ have shown that the major pathway responsible
for the AA generation in rat mast cells is indeed via PLA$_2$.

The entire AA metabolism cascade has been summarised in Fig. 6.54. Briefly,
the enzyme cyclooxygenase oxidises and causes the ring closure of AA. This
forms the unstable cyclic endoperoxide PGG$_2$ which is then rapidly reduced
to form PGH$_2$. The PGH$_2$ is further rapidly reduced to the more stable and
biologically active PGF$_{2\alpha}$, PGE$_2$ and PGD$_2$.$^{[404]}$ Recently it has become clear
that cyclooxygenase exists in two separate isoforms, termed cyclooxygenase
I and II. These two isoforms differ in the number of amino acids (602 and 604
residues, respectively), their molecular masses (69,054 kD and 69,093 kD,
respectively) and their messenger RNA (2.7 kb and 4.8 kb, respectively). The
emerging picture is that cyclooxygenase I is responsible for maintaining
resting PG synthesis in the gastric mucosa, platelets and the kidney, whereas
cyclooxygenase II is responsible for PG production in inflamed tissues.$^{[405]}$

A second enzymatic pathway involving TX and prostacyclin synthase forms
TXA$_2$ and PGI$_2$, respectively. These are then rapidly transformed into the
more stable but inactive metabolites TXB$_2$ and 6-keto-PGF$_{1\alpha}$, respectively.$^{[406]}$

In contrast to the cyclooxygenase enzyme, 5-lipoxygenase is cytosolic and
does not require calcium for its activity.$^{[404]}$ When the cell is activated, it is
thought that the 5-lipoxygenase is translocated to the cellular membrane$^{[407]}$
where it becomes activated by 5-lipoxygenase activating protein$^{[408]}$. The
enzyme is then able to convert the AA into 5-HPETE. Peroxidases can then
transform this into 5-HETE, or 5-lipoxygenase can further transform 5-HPETE
to form the unstable LTA$_4$\(^{409}\). This metabolite can be hydrolysed to LTB$_4$ or can become conjugated with glutathione to form LTC$_4$. Sequential proteolysis of the glutathione moiety can then generate LTD$_4$, LTE$_4$\(^{406, 410}\) and LTF$_4$\(^{411}\). AA can also be converted by 12-lipoxygenase into 12-HPETE and subsequently 12-HETE.

Mediators arising from AA are rapidly synthesised and secreted. This process begins within a few minutes of activation and can last for up to 30 minutes. The eicosanoids exert their biological effects by acting on specific receptors\(^{412}\). PGD$_2$ is the principle cyclooxygenase product following immunological and calcium ionophore activation of human and rat mast cells\(^{413}\). The biological effects of PGD$_2$ include bronchoconstriction\(^{414}\), peripheral vasodilatation\(^{415}\), inhibition of platelet aggregation\(^{416}\) and production of a wheal and flare response in human skin\(^{417}\). Metabolism of AA via the lipoxygenase pathway generates LTB$_4$ and C$_4$ from immunologically activated rat MMCS\(^{418}\). Purified human lung mast cells predominantly release LTC$_4$ upon immunological challenge\(^{419}\) as well as small amounts of LTB$_4$\(^{420}\). Their biological effects include constriction of arterial, arteriolar, intestinal and bronchial smooth muscle, increased vasopermeability and enhanced bronchial mucus secretion\(^{421}\). They are over 1000 times more potent constrictors of human bronchial smooth muscle than histamine both \textit{in vivo} and \textit{in vitro}\(^{422, 423}\).

In addition to these eicosanoids, the enzymatic action of PLA$_2$ can also yield PAF. This occurs via a concerted two step process first yielding lyso-PAF, which is then acetylated by an acetyltransferase enzyme generating PAF\(^{424}\). The biological actions of PAF include the activation of human platelets\(^{425}\) and human eosinophils\(^{426}\). PAF also induces histamine secretion from human basophils\(^{427}\) and RPMC\(^{428}\). However, mast cells from human lung, skin and uterus fail to respond to PAF\(^{427, 429}\). The linking of PAF to PLA$_2$ stimulation of mast cells has not been addressed in the present study, and
this would provide an important route for further work concerning the role of this enzyme.

In total, this series of experiments has indicated that the generation of a membrane metabolite may be the crucial factor which induces histamine release from mast cells stimulated with PLA$_2$. The enzyme variously produces a non-cytotoxic or cytolytic release of the amine from different mast cell phenotypes and potentiates immunologic histamine secretion from RPMC. These effects could be due to generation of lyso-PA, lyso-PC and lyso-PS, respectively, and/or to the production of one or more AA metabolites.
Chapter 6

Membrane Metabolites

Fig. 6.1a: Histamine release from different mast cell phenotypes treated with PA (n=4)

Fig. 6.1b: Histamine release from different mast cell phenotypes treated with lyso-PA (n=4)
**Chapter 6**

**Membrane Metabolites**

**Fig. 6.2a:** Histamine release from different mast cell phenotypes treated with PC (n=4)

**Fig. 6.2b:** Histamine release from different mast cell phenotypes treated with lyso-PC (n=4)
**Fig. 6.3a:** Histamine release from different mast cell phenotypes treated with PS (n=4)

**Fig. 6.3b:** Histamine release from different mast cell phenotypes treated with lyso-PS (n=4)
Fig. 6.4a: Histamine release from different mast cell phenotypes treated with PE (n=4)

Fig. 6.4b: Histamine release from different mast cell phenotypes treated with lyso-PE (n=4)
Chapter 6  Membrane Metabolites

Fig. 6.5a: Histamine release from different mast cell phenotypes treated with PI (n=4)

Fig. 6.5b: Histamine release from different mast cell phenotypes treated with lyso-PI (n=4)
Fig. 6.6: **Effects of metabolic inhibitors on histamine release from RPMC treated with lyso-PA (n=5)**

(Asterisks denote those releases that were significantly different from that obtained in the absence of the metabolic inhibitors. *p< 0.05, **p< 0.01, ***p< 0.001.)

Fig. 6.7: **Effects of metabolic inhibitors on histamine release from human lung mast cells treated with lyso-PA (n=3)**

(Asterisks denote those releases that were significantly different from that obtained in the absence of the metabolic inhibitors. *p< 0.05, **p< 0.01, ***p< 0.001.)
Fig. 6.8: Effects of metabolic inhibitors on histamine release from human basophils treated with lyso-PA (n=4)

(Asterisks denote those releases that were significantly different from that obtained in the absence of the metabolic inhibitors. *p< 0.05, **p< 0.01, ***p< 0.001.)

Fig. 6.9: Effects of metabolic inhibitors on histamine release from RPMC treated with lyso-PC (n=5)

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)
Chapter 6

Membrane Metabolites

Fig. 6.10: Effects of metabolic inhibitors on histamine release from human lung mast cells treated with lyso-PC (n=3)

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Bar graph showing histamine release from human lung mast cells treated with lyso-PC (n=3).]

Fig. 6.11: Effects of metabolic inhibitors on histamine release from human basophils treated with lyso-PC (n=4)

(In no cases were the releases in the presence and absence of the inhibitors statistically different.)

![Bar graph showing histamine release from human basophils treated with lyso-PC (n=4).]
Fig. 6.12: Effects of lyso-PS (1 \( \mu \text{M} \)) on anti-IgE (1/300 dilution) induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from the sum of the responses evoked by the two ligands applied separately. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).)

![Graph showing the effects of lyso-PS on histamine secretion](image)

Fig. 6.13: Effects of PS (1 \( \mu \text{M} \)) on anti-IgE (1/300 dilution) induced histamine secretion from RPMC (n=4)

(Asterisks denote those releases that were significantly different from the sum of the responses evoked by the two ligands applied separately. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).)

![Graph showing the effects of PS on histamine secretion](image)
Chapter 6

Membrane Metabolites

Fig. 6.14: Effects of different lipids (1 µM) on anti-IgE (1/300 dilution) induced histamine secretion from RPMC (n=4)

(In no cases were the releases induced in the absence and presence of the lipids statistically different.)

Fig. 6.15: Kinetics of histamine release from RPMC treated with lyso-PA (0.5 mM) (n=5)
Fig. 6.16: Calcium ion dependency of histamine release from RPMC treated with lyso-PA (0.5 mM) (n=4)

Fig. 6.17: Effects of metabolic inhibitors on the histamine release from RPMC treated with lyso-PA (0.5 mM) in different media (n=3)

(Asterisks denote those releases that were significantly different from that obtained in the absence of metabolic inhibitors. *p < 0.05, **p < 0.01, ***p < 0.001.)
Fig. 6.18: Effects of increasing calcium and magnesium ions on histamine release from RPMC treated with lyso-PA (0.5 mM) (n=5)

Fig. 6.19a: PGD$_2$ release from RPMC treated with *Naja naja*-PLA$_2$ (n=3)
Fig. 6.19b: Corresponding histamine release from RPMC treated with *Naja naja*-PLA$_2$ (n=3)

![Graph showing histamine release](image)

Fig. 6.20a: PGD$_2$ release from RPMC treated with bee venom-PLA$_2$ (n=3)

![Graph showing PGD$_2$ release](image)
Chapter 6

Membrane Metabolites

Fig. 6.20b: Corresponding histamine release from RPMC treated with bee venom-PLA$_2$ (n=3)

Fig. 6.21: PGD$_2$ and histamine release from RPMC treated with anti-IgE (1/200 dilution) (n=3)
Fig. 6.22: PGD$_2$ and histamine release from RPMC treated with compound 48/80 (0.2 μg/ml) (n=3)

![Graph showing PGD$_2$ and histamine release](image)

Fig. 6.23: Inhibition by curcumin of histamine release from RPMC (n=4)

![Graph showing inhibition by curcumin](image)
**Fig. 6.24:** Inhibition by meclofenamate sodium of histamine release from RPMC (n=4)

![Graph showing inhibition by meclofenamate sodium of histamine release](image1)

**Fig. 6.25:** Inhibition by phenidone of histamine release from RPMC (n=4)

![Graph showing inhibition by phenidone of histamine release](image2)
**Chapter 6**

**Membrane Metabolites**

**Fig. 6.26:** Inhibition by indomethacin of histamine release from RPMC (n=4)

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

**Fig. 6.27:** Inhibition by diclofenac of histamine release from RPMC (n=4)

![Graph showing inhibition by diclofenac of histamine release](image)
Fig. 6.28: Inhibition by phenylbutazone of histamine release from RPMC (n=4)

Fig. 6.29: Inhibition by flurbiprofen of histamine release from RPMC (n=4)
Chapter 6

**Membrane Metabolites**

Fig. 6.30: Inhibition by esculetin of histamine release from RPMC (n=4)

![Graph showing inhibition by esculetin of histamine release from RPMC](image)

Fig. 6.31: Inhibition by NDGA of histamine release from RPMC (n=4)

![Graph showing inhibition by NDGA of histamine release from RPMC](image)

218
Chapter 6  Membrane Metabolites

Fig. 6.32: Inhibition by AA-861 of histamine release from RPMC (n=4)

![Graph showing inhibition by AA-861](image)

Fig. 6.33: Inhibition by caffeic acid of histamine release from RPMC (n=4)

![Graph showing inhibition by caffeic acid](image)
Fig. 6.34: Inhibition by L-739,010 of histamine release from RPMC (n=4)

Fig. 6.35: Inhibition by L-746,530 of histamine release from RPMC (n=4)
Fig. 6.36a: Structure of PA

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{R}_2 - \text{C} - \text{O} - \text{CH} \\
&\text{C} - \text{O} - \text{P} - \text{O} - \text{H} \\
&\text{O} - \\
\end{align*}
\]

Fig. 6.36b: Structure of lyso-PA

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{HO} - \text{CH} \\
&\text{C} - \text{O} - \text{P} - \text{O} - \text{H} \\
&\text{O} - \\
\end{align*}
\]

Fig. 6.37a: Structure of PC

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{R}_2 - \text{C} - \text{O} - \text{CH} \\
&\text{C} - \text{O} - \text{P} - \text{O} - \text{CH}_2\text{CH}_2\text{N}^+\text{(CH}_3\text{)}_3 \\
&\text{O} - \\
\end{align*}
\]
Fig. 6.37b: Structure of lyso-PC

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{HO} - \text{CH} \\
&\text{C} - \text{O} - \text{P} - \text{O} - \text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3
\end{align*}
\]

Fig. 6.38a: Structure of PS

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{R}_2 - \text{C} - \text{O} - \text{P} - \text{O} - \text{CH}_2\text{CH}(\text{NH}_3^+)\text{COO}^-
\end{align*}
\]

Fig. 6.38b: Structure of lyso-PS

\[
\begin{align*}
&\text{H}_2\text{C} - \text{O} - \text{C} - \text{R}_1 \\
&\text{HO} - \text{CH} \\
&\text{C} - \text{O} - \text{P} - \text{O} - \text{CH}_2\text{CH}(\text{NH}_3^+)\text{COO}^-
\end{align*}
\]
Chapter 6

Membrane Metabolites

Fig. 6.39a: Structure of PE

\[
\begin{align*}
\text{R}_2 & \text{C} - \text{O} - \text{C} & \text{R}_1 \\
\text{H}_2\text{C} & - \text{O} - \text{C} & \text{R}_1 \\
\text{C} & - \text{O} - \text{P} & - \text{O} - \text{CH}_2\text{CH}_2\text{NH}_3^+ \\
\text{HO} & - \text{CH} & \\
\text{C}_2 & - \text{O} - \text{P} & - \text{O} - \text{CH}_2\text{CH}_2\text{NH}_3^+ \\
\text{O} & - & \\
\end{align*}
\]

Fig. 6.39b: Structure of lyso-PE

\[
\begin{align*}
\text{H}_2\text{C} & - \text{O} - \text{C} & \text{R}_1 \\
\text{HO} & - \text{CH} & \\
\text{C}_2 & - \text{O} - \text{P} & - \text{O} - \text{CH}_2\text{CH}_2\text{NH}_3^+ \\
\text{O} & - & \\
\end{align*}
\]

Fig. 6.40a: Structure of PI

\[
\begin{align*}
\text{R}_2 & \text{C} - \text{O} - \text{C} & \text{R}_1 \\
\text{H}_2\text{C} & - \text{O} - \text{C} & \text{R}_1 \\
\text{C} & - \text{O} - \text{P} & - \text{O} - \text{H}_2\text{O} \\
\text{H}_2\text{O} & - \text{OH} & \\
\text{OH} & - \text{OH} & \\
\text{OH} & - \text{OH} & \\
\text{OH} & - \text{OH} & \\
\end{align*}
\]
Chapter 6

Membrane Metabolites

Fig. 6.40b: Structure of lyso-Pl

Fig. 6.41: Structure of curcumin$^{(430)}$
Fig. 6.42: Structure of meclofenamate sodium

Fig. 6.43: Structure of phenidone

Fig. 6.44: Structure of indomethacin
Fig. 6.45: Structure of diclofenac\(^{434}\)

\[
\text{COONa} \quad \text{NH} \quad \text{Cl} \quad \text{Cl}
\]

Fig. 6.46: Structure of phenylbutazone\(^{435}\)

\[
\text{O} \quad \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 \quad \text{N} \quad \text{N} \quad \text{O}
\]

Fig. 6.47: Structure of flurbiprofen\(^{436}\)

\[
\text{F} \quad \text{CH}_3 \quad \text{CHCOOH}
\]
Fig. 6.48: Structure of esculetin$^{(437)}$

Fig. 6.49: Structure of NDGA$^{(438)}$

Fig. 6.50: Structure of AA-861$^{(439)}$
Fig. 6.51: Structure of caffeic acid\textsuperscript{(440)}

![Structural formula of caffeic acid]

Fig. 6.52: Structure of L-739,010\textsuperscript{(441)}

![Structural formula of L-739,010]
Fig. 6.53: Structure of L-746,530$^{(441)}$
Fig. 6.54a: Summary of the arachidonic acid metabolism cascade - Part 1 (See text for abbreviations)

(Adapted from reference 411)
Fig. 6.54b: Summary of the arachidonic acid metabolism cascade - Part 2 (See text for abbreviations)

(Adapted from reference 411)
Chapter 7

"The outcome of any serious research can only be to make two questions grow where one question grew before."

Thornstein Veblen, 1857-1929:
University of California Chronicle (1908).
CHAPTER 7

Further studies into the role of PLA$_2$ in mast cell activation

7.1: Introduction

The activation of PLA$_2$ via specific receptors may be a critical early biochemical event in the pathogenesis of allergic reactions. Release of histamine by mast cells and basophils, triggered by anti-IgE, involves the activation of PLA$_2$ and generation of AA metabolites$^{442, 443, 403}$. Such receptor mediated histamine release may be mimicked by exposure of mast cells to exogenous PLA$_2$$^{444, 445}$. During acute myocardial ischemia, PLA$_2$ may be activated due to increased intracellular calcium levels$^{446}$. Subsequently, the accumulation of lyso-phospholipids$^{447}$ and free fatty acids$^{448}$ may promote damage to membranes leading to irreversible cell injury and ultimately cell death. Various other physiological stimuli (cytokines, angiotensin II, bradykinin, prolactin and thrombin) activate PLA$_2$ when added to responsive cells$^{449}$. It has been proposed that altered PLA$_2$ activity and defects in its control and regulation correlate with disease severity$^{450}$. PLA$_2$ has thus been used as a biochemical marker of disease activity$^{449, 450}$.

In endothelial cells, it is generally thought that PLA$_2$ activity is regulated by the intracellular calcium concentration$^{451}$. Activation of PLC produces two molecules with second messenger properties. 1, 2-Diacylglycerol stimulates protein kinase C and 1, 4, 5-inositol trisphosphate (IP$_3$) can elicit release of calcium ions from intracellular stores$^{452}$. Since IP$_3$ can induce AA formation in permeabilised platelets$^{452}$, it has been suggested that a possible mechanism for the activation of PLA$_2$ is the IP$_3$ stimulated rise in cytosolic calcium concentration.
Studies using endothelial cells treated with LTD₄ have indicated that a protein is synthesised soon after application of the LT. This protein is thought to enhance the activity of PLA₂⁴⁵³, hence it has been named PLA₂ activating peptide (PLAP). This protein acts either by enhancing the endogenous PLA₂ activity, or by preventing inhibition⁴⁵³.

Murakami et al reported that a change in the cellular phospholipid composition occurred when a mouse bone marrow derived mast cell (BMMC) clone (MC-MKM) was cocultured with fibroblasts⁴⁵⁴. It is known that BMMC acquire the characteristics of CTMCs during their coculture with fibroblasts. In MC-MKM cells, PC was the predominant phospholipid class followed by PE. During their coculture for two weeks, the total phospholipid content in the MC-MKM cells increased 2.5 fold. Among the major phospholipid classes, the percentage of PE increased dramatically accompanied by a decrease in that of PC while no appreciable change in composition of other phospholipids was observed. The phospholipid composition of cocultured cells was closer to that of RPMC than that of the starting MC-MKM cells. They concluded that BMMCs converted their phenotype, in terms of their phospholipid composition, into CTMC-like cells by the interaction with fibroblasts⁴⁵⁴. According to these postulations, a similar variation in overall membrane composition may explain the differential effects of PLA₂ on the various histaminocytes studied in chapter 3 of the present study.

These several ideas have been investigated to see whether similar processes also occur in mast cells.

7.2: Methods and Materials

All the methods and materials used in the present studies have been described in chapter 2. In particular, the extraction and assay of PLA₂ has been given in sections 2.13 and 2.17, respectively. The extraction and assay of IP₃ is stated in sections 2.14 and 2.16, respectively. The measurement of
Chapter 7 Further Studies

cytosolic calcium is described in section 2.18 and the determination of membrane phospholipid concentration in section 2.19.

7.3: Results

7.3.1: Effects of PLAP on histamine release from RPMC stimulated with anti-IgE and *Naja naja*-PLA₂

*Naja naja*-PLA₂ (0.1 - 5 units/ml) induced a dose dependent release of histamine from RPMC (maximum release 51.0 ± 2.0 %). Addition of PLAP (0.001 - 1 μg/ml) was not able to alter the release of histamine significantly (Fig. 7.1a). Similar results were obtained by stimulating RPMC with anti-IgE (1/300 dilution) in the absence and presence of PLAP (Fig. 7.1b).

Mastoparan (0.01 - 0.5 μM) induced a dose dependent secretion of the amine from RPMC (maximum release 30.2 ± 3.6 %). In agreement with results shown in chapter 4 (Fig. 4.8a), the addition of *Naja naja*-PLA₂ did not augment the release of histamine (Fig. 7.2).

7.3.2: Effects of anti-IgE and compound 48/80 treatment on PLA₂ activity in RPMC

The PLA₂ activity in purified (96.3 ± 2.3 % purity) RPMC was measured and compared to that seen in cells activated with anti-IgE (1/250 dilution) and compound 48/80 (0.2 μg/ml). Resting RPMC had an activity of 0.10 ± 0.006 units/10⁶ cells. Activation of the cells significantly increased this PLA₂ activity (anti-IgE, 0.15 ± 0.005 units/10⁶ cells; compound 48/80, 0.15 ± 0.005 units/10⁶ cells)(Fig. 7.3).
7.3.3: Effects of *Naja naja*-PLA₂, anti-IgE and compound 48/80 on IP₃ production and intracellular calcium ion concentration in RPMC

Activation of purified RPMC with *Naja naja*-PLA₂ (10 units/ml), anti-IgE (1/200 dilution) and compound 48/80 (0.25 μg/ml) produced temporally distinct changes in IP₃ production in RPMC (baseline 259 ± 23 pmol/10⁶ cells, peak values after stimulation: *Naja naja*-PLA₂, 985 ± 47 pmol/10⁶ cells, 2 sec post stimulation; anti-IgE, 850 ± 54 pmol/10⁶ cells, 20 sec post stimulation; compound 48/80, 689 ± 42 pmol/10⁶ cells, 10 sec post stimulation; Fig. 7.4a). Both *Naja naja*-PLA₂ and bee venom-PLA₂ (0.3 - 10 units/ml) induced a dose dependent production of IP₃ (maximum production 782 ± 42 pmol/10⁶ cells and 744 ± 61 pmol/10⁶ cells, respectively, 5 sec post stimulation, Fig. 7.4b). A rather longer incubation time was used in these experiments for experimental convenience.

RPMC loaded with fluo-3 dye showed an immediate and pronounced increase in fluorescence (calcium concentration) following stimulation with both anti-IgE (1/250 dilution, from a base value of 0.31 ± 0.01 μM to 0.82 ± 0.01 μM calcium, Fig. 7.5a) and compound 48/80 (0.2 μg/ml, from a base value of 0.22 ± 0.02 μM to 0.70 ± 0.01 μM calcium, Fig. 7.5b). In contrast, the application of *Naja naja*-PLA₂ (5 units/ml) produced a very slow rise in fluorescence (Fig. 7.5c). In all three systems, lysis of the cells with digitonin (30 μM) caused a further large increase in fluorescence.
7.3.4: Changes in membrane composition between resting and stimulated RPMC and RBL-2H3 cells treated with *Naja naja*-PLA₂

Phospholipid standards (3 μl, 2.5 μg) and fluorescein as an internal standard were dissolved in methylene chloride and injected into the HPLC system. Ten peaks representing PI, PS, lyso-PS, PE, fluorescein, lyso-PA, PC, PA, lyso-PC and sphingomyelin were eluted in this order. A chromatograph representing the results of at least 4 similar experiments is given in Fig. 7.6.

Known amounts of phospholipid standards were injected into the column to obtain a calibration curve for each compound. The peak area of each standard correlated linearly with the injected dose (Fig. 7.7a - b).

The phospholipid compositions of unstimulated and *Naja naja*-PLA₂ stimulated RPMC and RBL-2H3 cells are given in Fig. 7.8 and Fig. 7.9, respectively. Data are presented in terms of the percentage (w/w) of each phospholipid and the actual content (μg) per 10⁶ cells. The total amounts of phospholipid in RBL-2H3 cells and RPMC were 6.22 ± 0.31 μg/10⁶ cells and 13.15 ± 0.52 μg/10⁶ cells, respectively. In RBL-2H3 cells, PC was the predominant phospholipid (53.35 ± 1.20 %, 3.21 μg). In RPMC, this proportion was reduced to 32.46 ± 2.03 % (4.26 μg), and the cells also contained substantial amounts of PE (23.42 ± 2.15 %, 2.93 μg) and sphingomyelin (19.49 ± 3.16 %, 2.56 μg).

During the process of stimulation, the proportions of lyso-PA, lyso-PC and lyso-PS in RPMC rose significantly (lyso-PA, from 0.22 ± 0.13 % to 1.95 ± 0.16 %, 0.029 μg/10⁶ cells to 0.256 μg/10⁶ cells; lyso-PC, from 3.36 ± 0.63 % to 7.074 ± 0.84 %, 0.442 μg/10⁶ cells to 0.93 μg/10⁶ cells; lyso-PS, from 3.09 ± 0.17 % to 11.72 ± 1.25 %, 0.406 μg/10⁶ cells to 1.541 μg/10⁶ cells). Correspondingly, there was a decrease in PA, PC and PS (PA, from 4.21 ±
Further Studies

0.92 % to 1.24 ± 0.25 %, 0.554 μg/10^6 cells to 0.162 μg/10^6 cells; PC, from 32.47 ± 2.03 % to 24.37 ± 1.94 %, 4.26 μg/10^6 cells to 3.47 μg/10^6 cells; PS, from 8.67 ± 0.43 % to 2.14 ± 0.46 %, 1.14 μg/10^6 cells to 0.28 μg/10^6 cells).

On stimulating RBL-2H3 cells, the proportions of lyso-PC and lyso-PS increased significantly (lyso-PC, from 3.0 ± 0.4 % to 23.7 ± 1.0 %, 0.026 μg/10^6 cells to 1.422 μg/10^6 cells; lyso-PS, from 2.01 ± 0.1 % to 3.9 ± 0.2 %, 0.12 μg/10^6 cells to 0.234 μg/10^6 cells). There was a decrease in PC and PE (PC, from 53.3 ± 1.2 % to 32.9 ± 0.8 %, 3.2 μg/10^6 cells to 1.97 μg/10^6 cells; PE, from 11.2 ± 0.4 % to 8.9 ± 0.3 %, 0.67 μg/10^6 cells to 0.53 μg/10^6 cells), but the change in PS was not significant.

7.4: Discussion

It has been shown that venoms from wasps, bees and hornets contain peptides which stimulate the activity of PLA_2\(^{(455)}\). Melittin is a PLA_2 stimulatory peptide isolated from bee venom\(^{(456)}\) and mastoparan from wasp venom\(^{(457)}\). A mammalian equivalent was isolated which was antigenically and functionally related to melittin\(^{(455)}\). This protein, PLAP, when purified to near homogeneity, selectively stimulated PLA_2 activity when PC was used as a substrate but had no effect when PE was used instead. Hence, its activity is probably specific for a subset of PLA_2 isoenzymes. The stimulation of PLA_2 appeared to involve an increase in the apparent \(V_{\text{max}}\) of the enzyme\(^{(455)}\). There are a number of possible mechanisms for this effect. Thus, PLAP could facilitate the proteolytic activation of PLA_2, could compete for the same regulatory site on the enzyme as the inhibitory protein lipocortin, or could interact with the substrate and thus enhance PLA_2 activity\(^{(455)}\). Clark et al\(^{(455)}\) hypothesised that PLAP was important in PLA_2 regulation and might be involved in the inflammatory process. PLAP has been shown to have a PLA_2 mediated contractile action on isolated smooth muscle from the rabbit rectosigmoid\(^{(458)}\) and to produce an activation of mechanoreceptors from goat palatal mucosa\(^{(459)}\).
The present study showed that PLAP is not able to enhance the release of histamine induced by anti-IgE or *Naja naja*-IgE. This may be because this peptide is structurally different from that produced in mast cells. Also, Clark *et al.*\(^\text{455}\) described how PLAP was effective only against certain PLA\(_2\) isoenzymes, and the peptide may be inactive against those forms used or involved in the present study. Alternatively, there may merely be a problem of access, whereby the peptide is too large to enter the cells and thus cannot interact with and then possibly activate further the endogenous PLA\(_2\). This latter idea may be explored in the future by permeabilising and introducing the peptide into the cell. In confirmation of earlier work (Chapter 4), mastoparan did not enhance histamine release from RPMC. Again, as the two peptides are structurally related, the same arguments for PLAP would be applicable to mastoparan.

It has been well documented that inflammation is accompanied by an increase in PLA\(_2\) activity\(^\text{450}\). A significant rise in PLA\(_2\) activity has been shown to occur with both anti-IgE and compound 48/80 stimulation of RPMC. This adds further credence to the idea that endogenous PLA\(_2\) activation is a significant event in the stimulus-secretion coupling process of mast cells.

There are two primary mechanisms which control the triggering of mast cell degranulation. The first involves the translocation of extracellular calcium into the cell and the second results from the release of intracellular stores of the cation\(^\text{460}\). The latter process may be controlled by the turnover of membrane phospholipids. Berridge and Irvine\(^\text{461}\) reported that a link existed between PI hydrolysis, an elevation of cytosolic calcium and cellular activation. The precise mechanism is complex and is continually expanding. However, a brief summary of the principle events is described below.

When an interaction between a ligand and a receptor occurs, a membrane G-protein becomes activated. The active sub-unit of this G-protein stimulates a
phosphoinositidase, PLC, which goes on to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP₂) to form IP₃ and DAG\(^{461\text{-}463}\). It has been demonstrated that IP₃ has the function of liberating calcium ions from intracellular stores\(^{463, 464}\) whilst DAG activates PKC\(^{465}\). IP₃ releases calcium ions from the endoplasmic reticulum by interacting with a specific receptor which leads to the opening of a calcium channel\(^{466}\).

Calcium mediates its actions by the activation of calcium binding proteins of which calmodulin is of primary importance\(^{467}\). Calmodulin has been found in all eukaryotic cells and exerts pleiotropic cellular responses through activation of numerous proteins, including protein kinases and phosphatases, cyclic nucleotide phosphodiesterases and ATPases\(^{468, 469}\).

In the present study, PLA₂, compound 48/80 and anti-IgE all produced temporally distinct increases in intracellular IP₃. Levels of the phosphate reached maximum values after 2, 10 and 20 sec, respectively. However, these changes were not simply related to increases in intracellular concentrations of calcium ions, which rose abruptly and thereafter declined following stimulation with compound 48/80 and anti-IgE but which increased progressively and much less markedly following activation with PLA₂. These data show that there is clearly a complex interaction between IP₃, which mobilises internal calcium stores, and the overall cellular concentration of the cation and also between calcium ions and the final secretory response. Indeed, studies using the patch clamp technique have shown that neither elevated levels of calcium ions nor internally applied IP₃ are alone able to induce degranulation\(^{470}\). The exact roles played by IP₃ and calcium in the signal transduction process obviously merit further investigation.

The membrane compositions of RPMC and RBL-2H3 cells were noticeably different. PC was the predominant phospholipid in the latter cells, comprising more than 50 % of the total. In contrast, PC amounted to only ca 30 % of the total lipid in RPMC which contained proportionally greater amounts of PE and
sphingomyelin. The present data with RPMC are generally in agreement with other studies\(^{471, 472}\) but the membrane composition of RBL-2H3 has not been previously reported. However, Murakami \textit{et al.}\(^{454}\) have described differences between mouse BMMC, which are mucosal-like mast cells analogous to RBL-2H3 cells, and murine connective tissue type mast cells, which are typified by RPMC.

On activation of RBL-2H3 cells, there was a striking reduction in the amount of PC and a concomitant increase in lyso-PC which comprised nearly 25 % of the total under these conditions. In RPMC, these changes were much smaller and lyso-PC comprised less than 10 % of the membrane lipid in activated cells. In contrast, there was a striking increase in the level of lyso-PS, and to a lesser extent lyso-PA, and a corresponding reduction in the parent lipids.

RPMC and RBL-2H3 cells were chosen for these studies, since they are readily available in large numbers in a purified form and, more importantly, since PLA\(_2\) produces diverse effects in these two systems. The enzyme induces a cytotoxic release of histamine from the latter cells and a non-cytolytic response from RPMC. Low concentrations of PLA\(_2\) also potentiated immunologic histamine release from the rat cells. We previously suggested (Chapter 4) that these observations might reflect differences in membrane composition between the cell types and proposed that the cytotoxic release of histamine might be due to the production of the surfactant lyso-PC while the non-cytolytic and augmentory response might be due to the production of the signalling molecules lyso-PA and lyso-PS. The present data are entirely in accordance with this proposal and provide a convincing explanation for the differential effects of PLA\(_2\) on the mast cell phenotypes.
Fig. 7.1a: Effects of PLAP on *Naja naja*-PLA$_2$ induced histamine secretion from RPMC (n=3)

(In no cases were the releases in the absence and presence of PLAP significantly different)

![Graph showing the effects of PLAP on Naja naja-PLA$_2$ induced histamine secretion from RPMC.](image)

Fig. 7.1b: Effects of PLAP on anti-IgE induced histamine secretion from RPMC (n=3)

(In no cases were the releases in the absence and presence of PLAP significantly different)

![Graph showing the effects of PLAP on anti-IgE induced histamine secretion from RPMC.](image)
Fig. 7.2: Effects of mastoparan on *Naja naja*-PLA₂ induced histamine secretion from RPMC (n=3)

(In no cases were the combined releases induced by mastoparan and PLA₂ alone significantly different from those induced by the two ligands added together)

![Graph showing histamine release with mastoparan alone, PLA₂ alone, and mastoparan + PLA₂ at different concentrations.]

Fig. 7.3: Effects of treatment with anti-IgE (1/250 dilution) and compound 48/80 (0.2 μg/ml) on PLA₂ activity in RPMC (n=4)

(Asterisks denote those activities that were significantly different from that obtained in unstimulated cells. * p< 0.05, ** p< 0.01, *** p< 0.001)

![Graph showing PLA₂ activity with unstimulated cells, anti-IgE stimulated, and compound 48/80 stimulated.]

243
Chapter 7  Further Studies

Fig. 7.4a:  Time course of IP₃ accumulation in RPMC treated with anti-IgE, compound 48/80 and *Naja naja*-PLA₂ (n=4)

![Graph showing time course of IP₃ accumulation](image)

Fig. 7.4b:  Effects of *Naja naja*-PLA₂ and bee venom-PLA₂ on IP₃ accumulation in RPMC (n=2-3)

(5 sec post stimulation)

![Graph showing effects of PLA₂](image)
Fig. 7.5a: Measurement of intracellular calcium in RPMC containing fluo-3 treated with anti-IgE (1/250 dilution) (Traces are representative of at least 4 similar recordings)
Fig. 7.5b: Measurement of intracellular calcium in RPMC containing fluo-3 treated with compound 48/80 (0.2 μg/ml) (Traces are representative of at least 4 similar recordings)
Fig. 7.5c: Measurement of intracellular calcium in RPMC containing fluo-3 treated with *Naja naja*-PLA$_2$ (5 units/ml) (Traces are representative of at least 4 similar recordings)
Fig. 7.6: Chromatograph of phospholipid standards
(Traces are representative of at least 4 similar recordings)
Chapter 7

Further Studies

Fig. 7.7a: Calibration curve for phospholipid standards (n=5)

Fig. 7.7b: Calibration curve for phospholipid standards (n=5)
Fig. 7.8: Phospholipid composition of untreated RPMC and following treatment with *Naja naja*-PLA₂ (n=4)

(Asterisks denote the phospholipid concentrations in stimulated cells that were significantly different from those in unstimulated cells. * p< 0.05, ** p< 0.01, *** p< 0.001)
Fig. 7.9: Phospholipid composition of untreated RBL-2H3 cells and following treatment with *Naja naja*-PLA$_2$ (n=4)

(Asterisks denote the phospholipid concentrations in stimulated cells that were significantly different from those in unstimulated cells. * p< 0.05, ** p< 0.01, *** p< 0.001)
Chapter 8

"No one means all he says, and yet very few say all they mean, for words are slippery and thought is viscous."

Henry Brooks Adams. 1838-1918:
The Education of Henry Adams (1907).
CHAPTER 8

General synopsis and overview

The enzyme PLA₂ hydrolyses fatty acid moieties from the sn-2 position of the glycerol phosphate backbone to yield the lyso-phospholipid and AA$^{(351)}$. The latter may then be metabolised to generate LTs and PGs$^{(351)}$. In addition, PLA₂ may also be intrinsically involved in stimulus-secretion coupling in the mast cell. These suggestions have occurred by virtue of experiments using inhibitors of PLA₂. The compounds not only blocked production of arachidonate, but also attenuated histamine release from the cells$^{(280)}$. However, the precise mechanism(s) involved in the action of the enzyme has not been fully elucidated. PLA₂ is found in inflammatory fluids and in inflammatory tissue exudates. The enzyme is also rapidly released by platelets, neutrophils and macrophages, as well as mast cells, when activated$^{(473)}$. However, application of the enzyme exogenously to mast cells has produced conflicting data$^{(280)}$. These contradictory results may be explained by recent studies which have classified the enzyme into different subtypes. In light of this, it was the aim of the present study to elucidate further the role of this enzyme in the degranulation process and thus give greater credence to the notion that PLA₂ plays a vital role in the inflammation process. Further to this study, each of the traditional PLA₂ subclasses have been further divided and more and more isoenzymes of the protein are being elucidated. It would therefore be interesting to characterise separately the role of each of these newer classes, in mast cell activation.

In agreement with previous reports$^{(474)}$, the present study has shown that PLA₂ from Naja naja venom induced a non-cytotoxic release of histamine from RPMC. The kinetics of release were slow, again as previously reported. The present study also showed that the release was unaffected by pertussis
toxin and its mechanism of action was distinct from the signal transduction cascades induced by both compound 48/80 and anti-IgE. Bee venom-PLA$_2$ induced histamine release from RPMC but to a lesser degree. The release in this case had a significant cytotoxic component and the kinetics of release were much faster. PLA$_2$ isolated from several other sources were essentially ineffective in inducing histamine release from RPMC.

Removal of calcium from the extracellular medium reduced the ability of *Naja naja*-PLA$_2$ to induce mast cell degranulation. There are two possible explanations for this observation. Optimal activities of most PLA$_2$ isoenzymes require the presence of millimolar concentrations of external calcium$^{(254)}$ and, thus, the enzymatic activity of PLA$_2$ may be responsible for the histamine release. Alternatively, the release is a calcium-dependent secretory process and the enzyme might increase the permeability of the cell to the cation, either by hydrolysis of membrane phospholipids or by interaction with a specific membrane receptor. The resulting influx of extracellular calcium could then trigger the degranulation process.

The present study has indicated that specific PLA$_2$ inhibitors are able significantly to reduce or abolish the histamine release induced by anti-IgE and compound 48/80. Previous work has employed the use of several types of compounds as inhibitors of PLA$_2$$^{(475, 476)}$. Certain of these compete with the phospholipid substrate for binding to the active site of the enzyme. Others, such as p-BPB inactivate the enzyme by binding to specific residues in its catalytic binding site$^{(477)}$. Additional work using specific PLA$_2$ inhibitors for the enzymatic site and receptor binding site would further elucidate the mechanism by which the enzyme evokes histamine release.

Hydrolysis of a plasma membrane phospholipid may generate a lipid substance(s) such as a free fatty acid, a lyso-phospholipid or a related metabolite which may be responsible for the induced histamine release. This lipid substance may promote the fusion of the mast cell plasma membrane
with the secretory granules during the process of exocytosis. The present study has indicated that the non-cytotoxic histamine release may be as a result of the production of lyso-PA, which has been shown to be a signalling molecule in other systems\(^{(478)}\). The cytotoxic release of the amine may be as a result of the generation of lyso-PC, which has surfactant properties\(^{(324)}\). The present study has unequivocally shown that stimulation of RPMC with *Naja naja*-PLA\(_2\) (non-cytotoxic releases) coincides with an increase in lyso-PA levels in the membrane, whilst stimulation of RBL-2H3 cells with the same secretagogue (cytotoxic releases) causes an increase in levels of lyso-PC.

Metabolism of the free AA may also contribute to the histamine release. The two pathways of AA metabolism, the lipoxygenase and cyclooxygenase cascades were blocked using specific inhibitors. In agreement with previous reports\(^{(395)}\), blockers of the lipoxygenase pathway inhibited histamine release induced by compound 48/80, anti-IgE and also *Naja naja*-PLA\(_2\). Inhibitors of the cyclooxygenase pathway did not inhibit the release. Thus, the generation of a metabolite of the lipoxygenase pathway, possibly 5-HETE or 5-HPETE, may be involved in the release process.

PLA\(_2\) could potentially act through a specific binding site on the mast cell membrane. A specific high affinity receptor for snake venom PLA\(_2\) has been cloned\(^{(479, 480)}\) and has been found on a variety of mammalian cells. Various biological responses have been reported to be elicited by PLA\(_2\) via this receptor\(^{(481, 482)}\). For example, rat uterine stromal cells have been shown to express such a receptor. It has the function of internalising the bound PLA\(_2\) and thus has important implications in its growth control mechanisms\(^{(482)}\). In rat mesangial cells, the receptor binds PLA\(_2\) with a Kd value of between 1 and 20 nM, depending on the PLA\(_2\) isoenzyme\(^{(479)}\). A recent report\(^{(483)}\) indicated that PGE\(_2\) production in rat mesangial cells was mediated through such a binding site and did not require the enzymatic properties of the protein. This 1458 residue receptor has 10 extracellular domains, and a short single transmembrane region followed by a short cytoplasmic tail\(^{(480)}\). However,
previous studies have indicated that RPMC, platelets and macrophages did not have a specific binding site for porcine pancreas-PLA$_2$\textsuperscript{(484)}. This may suggest that mast cells generally do not possess a specific binding site for the enzyme. The argument is supported by the observations discussed above which indicate that the induction of histamine release is dependent on the catalytic activity of the protein. Contradictory to this, experiments in the present study using mannose-BSA, which is thought to bind to the same site as bee venom-PLA$_2$\textsuperscript{(371)} inhibited histamine release from RPMC stimulated with \textit{Naja naja}-PLA$_2$ and a variety of other ligands. This may indicate that a receptor mediated event may be occurring. However, from the basis of these results, further conclusions cannot be made and additional work in this area is required.

PGD$_2$ was released in addition to histamine from RPMC following treatment with \textit{Naja naja}-PLA$_2$. Interestingly, similar amounts of PGD$_2$ were produced following treatment with bee venom-PLA$_2$ even though the levels of histamine released by the two isoenzymes were very different. It would be interesting to see if any of the non-histamine releasing PLA$_2$ isoenzymes were able to induce PGD$_2$ release.

Treatment of RPMC simultaneously with anti-IgE and \textit{Naja naja}-PLA$_2$ significantly enhanced the induced histamine release. This is in agreement with previous reports\textsuperscript{(402)} which have indicated that non-releasing concentrations of \textit{Naja naja}-PLA$_2$ potentiated antigen induced histamine release from RPMC. A similar potentiation was seen with low concentrations of both \textit{Naja naja}- and bee venom- PLA$_2$ in combination with a wide range of ligands. However, only those ligands that potentiated histamine release when the cells were simultaneously treated with PS or lyso-PS, showed a similar potentiation with the PLA$_2$ isoenzymes. With those ligands where the histamine secretion induced by simultaneous treatment with PS or lyso-PS was simply additive, the same was also true with their combination with PLA$_2$. Furthermore, none of the other lipids studied showed any potentiating
properties. These results may then indicate that the potentiating effect may be by virtue of the generation of lyso-PS and/or the mobilisation of PS. *In vivo*, this may indicate that PLA₂ may have a role in priming the mast cell for degranulation.

The precise sequence of events following immunological activation of mast cells is not fully understood. However, it is known that aggregation of the IgE receptors leads to the production of IP₃ and an increase in intracellular calcium due to the release of the cation from intracellular stores. With compound 48/80, the initial intracellular event is the activation of a pertussis toxin sensitive G-protein. This leads to activation of PLC and IP₃ production. Although optimal histamine release requires the presence of extracellular calcium, compound 48/80 can induce release in its absence\(^{(485)}\). This indicates that opening of calcium channels is not a prerequisite for histamine release induced by this agent.

The ability of PLA₂ to potentiate the histamine release induced by the immunologically related ligands suggests that the enzyme may be facilitating or maintaining the opening of membrane calcium channels. In the present study, a significant and rapid production of IP₃ was seen. The rise in intracellular calcium was however less pronounced and slow. This influx of extracellular calcium could be mediated by the opening of a calcium channel on the plasma membrane. An alternative mode of action may be through enzymatic disruption of the membrane leading to the observed calcium influx.

The study has shown that PLA₂ activity is significantly increased upon activation of mast cells. If this activity is inhibited using specific inhibitors, the cellular activation is also reduced. In addition, the activation of other cell types under inflammatory conditions may cause secretion of PLA₂ from them. This enzyme may then target mast cells and instigate degranulation. In conclusion, the present study has indicated a fundamental role for PLA₂ in
Chapter 8  

General Synopsis

mast cell activation, control of which may be therapeutically invaluable in the control of inflammatory diseases.
"If I have seen further it is by standing on the shoulders of giants."

**Isaac Newton.** 1642-1727:
Letter to Robert Hooke, (1676).
References


References


References


References


References


References


References


References


269
References


References


References


References


References


References


References


References


References


References


References


References


References


References


Appendix 1

"I have always suspected that the reading is right, which requires many words to prove it wrong; and the emendation wrong, that cannot without so much labour appear to be right."

Appendix 1

Structures of selected general drugs

2-Deoxy-D-glucose

Antimycin A
Calcium ionophore A23187

Compound 48/80
Appendix 1

Structures

Dextran

Dibutyryl cyclic AMP
Appendix 1

Structures

Disodium chromoglycate

IBMX

Isatoic anhydride
Appendix 1  

**Structures**

Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH$_2$

**Mastoparan**

![Mastoparan Structure](image)

Glu-Ser-Pro-Leu-Ile-Ala-Lys-Val-Leu-Thr-Thr-Glu-Pro-Pro-Ile-Ile-Thr-Pro-Val-Arg-Arg

**Phospholipase A$_2$ activating peptide**

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$

**Substance P**
Theophylline
“Were I to await perfection, my book would never be finished.”

Tai T'ung.

The Six Scripts: Principles of Chinese Writing.
### Appendix 2

#### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HT</td>
<td>5 Hydroxytryptamine</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AACOCF₃</td>
<td>Arachidonyl trifluoromethyl ketone</td>
</tr>
<tr>
<td>ACA</td>
<td>p-((Amylcinnamoyl)anthranilic acid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Anti-IgE</td>
<td>Immunoglobulin G raised against IgE</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-trisphosphate</td>
</tr>
<tr>
<td>B₀</td>
<td>Zero standard tube</td>
</tr>
<tr>
<td>BAC</td>
<td>Benzalkonium chloride</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow derived mast cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C3a/ C5a</td>
<td>Complement fragments</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic 3',5'-adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic 3',5'-guanosine monophosphate</td>
</tr>
<tr>
<td>CMF</td>
<td>Calcium and magnesium free HEPES buffer</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
</tr>
<tr>
<td>ΔA</td>
<td>Change in absorbance</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DSCG</td>
<td>Disodium cromoglycate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>ε</td>
<td>Molar absorbance (extinction) coefficient</td>
</tr>
<tr>
<td>ECF-A</td>
<td>Eosinophil chemotactic factor of anaphylaxis</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fc_RI</td>
<td>High affinity receptor for IgE</td>
</tr>
<tr>
<td>Fc_RII</td>
<td>Low affinity receptor for IgE</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FHT</td>
<td>Full HEPES buffered Tyrode's buffer</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GFT</td>
<td>Glucose free HEPES buffer</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine trisphosphate</td>
</tr>
<tr>
<td>H_{1,3}</td>
<td>Histamine receptors 1-3</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl piperazine N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HSP</td>
<td>High salt potassium buffer</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP_4</td>
<td>Inositol 1,3,4,5-tetraphosphate</td>
</tr>
<tr>
<td>L_3</td>
<td>Third stage larvae of NB</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Myristoylated alanine rich C kinase substrate</td>
</tr>
<tr>
<td>MC-MKM</td>
<td>BMMC clone</td>
</tr>
<tr>
<td>MCDP</td>
<td>Mast cell degranulating peptide</td>
</tr>
<tr>
<td>MC_T</td>
<td>Tryptase only mast cell</td>
</tr>
<tr>
<td>MC_{TC}</td>
<td>Tryptase and chymase mast cell</td>
</tr>
<tr>
<td><strong>Appendix 2</strong></td>
<td><strong>Abbreviations</strong></td>
</tr>
<tr>
<td>---------------</td>
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</tr>
<tr>
<td>Mg-T</td>
<td>Magnesium buffer</td>
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<tr>
<td>MIF</td>
<td>Macrophage inflammatory factor</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal mast cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>n</td>
<td>Number of experiments</td>
</tr>
<tr>
<td>NB</td>
<td><em>Nippostrongylus brasiliensis</em></td>
</tr>
<tr>
<td>NCF-A</td>
<td>Neutrophil chemotactic factor of anaphylaxis</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaretic acid</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
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<tr>
<td>OEPC</td>
<td>Oleoyloxyethyl phosphocholine</td>
</tr>
<tr>
<td>OPT</td>
<td>o-Phthalaldehyde</td>
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<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>PDE</td>
<td>Phosphodiesterase</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4, 5-bisphosphate</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
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<td>PLAP</td>
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<td>PLC</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RBL</td>
<td>Rat basophilic leukaemia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>pBPB</td>
<td>p-Bromophenacyl bromide</td>
</tr>
<tr>
<td>RPMC</td>
<td>Rat peritoneal mast cells</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<td>sec</td>
<td>second</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SRIF</td>
<td>Somatostatin</td>
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<td>Tc</td>
<td>Total counts tube</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TH</td>
<td>T-Helper cells</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>T-Supresser cells</td>
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<tr>
<td>TX</td>
<td>Thromboxane</td>
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<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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