The Role of Adenosine in Mast Cell Degranulation

by

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To

My Mother
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Abstract

We have used a range of selective agonists and antagonists to examine the effect of adenosine in different mast cell phenotypes and on smooth muscle preparations. The agonists included the adenosine A₁ receptor ligands R(-)N⁶-(2-phenylisopropyl)-adenosine (R-PIA) and N⁶-cyclopentyladenosine (CPA), the A₂a and A₂b compounds 2-p-(2-carboxyethyl)phenethylamino-5’-N-ethylcarboxamidoadenosine hydrochloride (CGS21680) and N-[(2-methylphenyl)methyl]-adenosine (metrifudil), the A₃ agents 1-deoxy-1-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuron-amide (IB-MECA) and N⁶-[2-(4-aminophenyl)ethyl]-adenosine (APNEA) and the non-selective agonist 5’-N-ethylcarboxamidoadenosine (NECA). The antagonists comprised the A₁ ligand 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), the A₂ compound 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazdin-5-amine (CGS15943), the A₁/A₂ ligand 8-phenyl-theophylline (8PT) and the A₃ compound (E)-3-(3-(1,2,3,6-tetrahydro-2,6-dioxo-1,3-dipropyl-9H-purin-8-yl)phenyl) acrylic acid (BW3911). The agonists potentiated (by up to 120 %) immunologic histamine release from rat peritoneal mast cells with a rank order of potency of IB-MECA>metrifudil=CPA=NECA>R-PIA>CGS21680. The dose-response curve for IB-MECA was shifted to the right by BW3911, but was less affected by 8PT, DPCPX and CGS15943. Mechanistic studies showed augmented histamine release was coupled with a parallel rise in IP₃ production, indicating the activation of A₃ receptors. The agonists both induced histamine release (by up to 50 %) and potentiated secretagogue induced release from mouse peritoneal mast cells with the first effect probably the result of A₃ receptor activation, and the second due to stimulation of either A₂b or A₃ receptors.

In contrast, adenosine and its analogues inhibited (by up to 90 %) immunologic histamine release from human lung mast cells and basophils. In the basophil, IB-MECA was the most potent inhibitor, NECA showed significant activity and the remaining compounds were ineffective. In the lung, all of the agonists, apart from CGS21680, showed comparable activity. These data suggest that inhibition is mediated through A₃ receptors in the basophil and A₂b receptors in the lung mast cell.

Adenosine was shown to have a dual effect on smooth muscle, inducing relaxation through A₂ receptors and contraction thorough activating either A₁ or A₃ receptors.
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1.1 Historical aspects

The first person to describe the mast cell was Paul Ehrlich in 1877 [1] who identified them based on their metachromatic staining properties with certain basic dyes such as methylene blue and toluidine blue. He named them from the german “mastzellen” meaning well-fed cells, since the cell’s cytoplasm appeared stuffed with granular material which he wrongly assumed the cell had ingested. He subsequently identified the circulating equivalent of the mast cell, the peripheral blood basophil leukocyte [2].

The first recognition of a possible link between the mast cell and a pathological condition was discovered by Unna in 1894 [3], investigating the dermatological complaint urticaria pigmentosa. He observed that the cutaneous lesions consisted almost exclusively of mast cells.

The role of the mast cell in immediate hypersensitivity reactions was first described by Portier and Richet in 1902 [4]. They noted that dogs injected with sea anemone toxin suffered anaphylaxis on a later administration of the same toxin, often with fatal consequences. This work was extended by Dale [5] and Shultz [6] who demonstrated that tissue sensitised to an antibody mimicked the characteristics of anaphylaxis. Histamine was first exposed as an important mediator of allergic reactions by Dale and Laidlaw [7] in 1919. They demonstrated that the amine produced the symptoms of anaphylaxis when injected directly into laboratory animals or used on isolated tissues. Later work by Webb [8] on rat peritoneal mast cells demonstrated that the cells were also capable of reacting to foreign substances without prior sensitisation.

Jorpes [9] and his co-workers, investigating the anticoagulant properties of heparin in the 1940s, discovered that there was a good correlation between the heparin content and mast cell number in a given tissue. These findings were confirmed by Rocha e Silva [10] and Scroggie and Jaques [11] who showed that in perfused dog liver, both heparin and histamine were released when stimulated by agents that in sensitised animals would cause anaphylactic shock. It was not until 1953, however, that Riley and West,
following extensive work in a variety of tissues, established that the storage site of histamine was the mast cell \[12-13\].

The presence of reaginic antibody in the serum of allergic individuals was first demonstrated by Prausnitz and Küstner in 1921 \[14\]. They found that serum taken from individuals sensitive to fish protein, when injected intradermally into normal subjects, could elicit an immediate hypersensitivity reaction following antigen challenge 24 hours later. This concept that hypersensitivity could be transferred was given further support by the Ishizakas \[15-16\] in the 1960s who identified the nature of this transferable factor as a novel immunoglobulin termed immunoglobulin E (IgE). They later showed that both mast cells and basophils expressed high affinity cell surface receptors for IgE \[17-18\].

1.2 The role of mast cells in health and disease

Mast cells are widely distributed throughout normal connective tissue, and are particularly abundant in sites that come into frequent contact with foreign substances, suggesting that they play an important role in the initial defence mechanisms of the host. They are, therefore, located predominantly in the skin, respiratory tract, lung, bladder and the digestive system \[19-20\]. They are also to be found in connective tissue particularly adjacent to blood and lymphatic vessels \[21\] and nerves \[22-24\].

It has been proposed that mast cells, IgE, complement and eosinophils interact in order to provide an effective mechanism against invasion by helminthic parasites \[25\]. Skin penetration by the parasite stimulates the production of specific immunoglobulin G (IgG) and activates both the classic and alternative complement cascades. The migration of the helminth through the skin triggers the tissue mast cells, primed by anti-helminth IgE, which adhere to the parasite by their membrane complement receptors, to release a number of pharmacological mediators including eosinophil chemotactic factor of anaphylaxis (ECF-A) and histamine. Eosinophils are thus recruited to the site of infection where they are stimulated to carry out parasite killing via IgG and complement mediated release of granular constituents.
Mast cells and basophils have been implicated in a variety of pathological disorders, in particular in immediate hypersensitivity and inflammatory reactions. Histologic and biochemical studies suggest that mast cells and their products play a role in conditions such as asthma \[26\], allergic rhinitis \[27-29\], conjunctivitis \[30\], rheumatoid arthritis \[31-32\], inflammatory bowel diseases \[33\], interstitial cystitis \[34-36\], sarcoidosis \[37-38\], pulmonary fibrosis \[39-40\] and coronary artery diseases \[41-43\].

In addition to their role in immediate hypersensitivity reactions, there is evidence that mast cells may also be involved in the early stages of delayed type hypersensitivity reactions \[44-47\], where release of vasoactive mediators from the mast cell is required for effector T cells to leave the intravascular space, enter the tissues and become activated by antigen.

There is also increasing evidence that mast cells may play a role in a number of non-allergic immune reactions. Mast cell numbers have been shown to increase in scar tissue, callus tissue, in osteoporosis-linked diseases and in various neuropathies \[48\].

### 1.3 The immune system

The mammalian immune response is believed to have evolved as a last line of defence against pathogenic invaders such as bacteria, viruses and fungi \[49\]. The immune system’s surveillance role protects the organism by searching out and destroying agents which cause infections and cancer \[50\]. This defence mechanism is therefore indispensable and vital for the survival of the individual.

Although the manifestations of the allergic reaction are so varied, the events that set them in motion are universal to all reactions \[51\]. The first step is called “sensitisation” and involves the action of lymphocytes of which there are several types, the granular, B and T-lymphocytes, killer (cytotoxic) cells and natural killer cells. The T-cells are involved in the regulation of many immune responses whilst B-cells are specific for antibody production.
During sensitisation, an allergen which has entered the body reaches the local lymph nodes where it is "presented" to the lymphocytes on the surface of either non-phagocytic cells termed "antigen-presenting cells" (APC), or by scavenger cells and macrophages which have engulfed the antigen, broken it into pieces and displayed the resulting fragments on the cell surface. APC release the soluble factor interleukin 1 (IL-1) which helps facilitate this recognition.

T lymphocytes that express the cluster of differentiation 4 (CD4) antigen can be divided into two discrete subsets, designated T helper (Th)1 and Th2 which along with mast cells and other cell types release small molecular weight proteins called cytokines. Both types of cells are activated by APC, but respond by producing different patterns of interleukins \[^{[52-53]}\]. Th1 cells secrete interleukin (IL)-2, IL-3 and interferon γ (IFNγ). They are also induced to express high affinity IL-2 receptors. Th2 cells produce IL-3, IL-4, IL-5 and IL-6 and are involved in the proliferation and terminal differentiation of B-lymphocytes into antigen producing plasma cells \[^{[54]}\]. During the late phase of allergic asthma, there is a significant increase in the number of activated T-helper cells and a switch from the Th1 to the Th2 phenotypes \[^{[53]}\].

A reciprocal relationship between IL-4 and IFNγ on IgE regulation has been clearly established \[^{[55-56]}\] with the former an essential mediator for IgE synthesis and the latter having a negative regulatory effect on the production of the immunoglobulin. IL-5 \[^{[57]}\] IL-6 \[^{[52]}\] and tumour necrosis factor α (TNFα) all enhance IL-4 induced IgE production whilst IFNα and transforming growth factor β (TGFβ) inhibit synthesis of the antibody.

The proliferation of B cells is limited by their interaction with suppressor T (Ts) cells which release soluble factors that are inhibitory to the events described above. It has been proposed that T cells from allergic patients have an enhanced ability to augment IgE production relative to those of normal subjects \[^{[58]}\], suggesting an imbalance in the numbers of Th and Ts cells.
1.4 Development and differentiation of mast cells

Studies by Kitamura and co-workers\(^{[59-62]}\) on mice with specific genetic characteristics helped demonstrate the origin of mast cells. Three particular types of animal model were used:

1. mice with Chediak-Higashi syndrome. These mice have mast cells which contain characteristically giant granules.
2. mice where a gene product on the mast cell precursor necessary for differentiation has been knocked out.
3. mice which have an absence of a gene product on the fibroblast which participates in mast cell development.

Transplantation of bone marrow from mice with Chediak-Higashi syndrome (C57BL/6-bg\(^{+/+}\)) or from respectively normal litters (WBB6\(^{+/+}\)) into genetically mast cell deficient mutants (WBB6\(^{-W/W}\) or WCB6\(^{-S1/S1}\)) led to the development of mast cells in the recipient with characteristics of donor origin, indicating that the pluripotential stem cells in the bone marrow were the precursors for mast cells and basophils.

Other cells derived from bone marrow (erythrocytes, eosinophils, neutrophils, platelets and basophils) complete their differentiation within the bone marrow, whilst the mast cell leaves the bone marrow undifferentiated\(^{[63]}\). This circulating mononuclear precursor resembles a lymphoid cell which on leaving the blood and entering the tissue differentiates into either a mucosal or connective tissue mast cell\(^{[64-66]}\).

T-lymphocytes together with IL-4 are required for the differentiation of mucosal mast cells (MMC). The differentiation of the connective tissue mast cells (CTMC), however, is dependent on contact of the mast cell with a molecule expressed on the surface of fibroblasts\(^{[67]}\).

The differentiation into the two distinct mast cell types, although following separate pathways, is not a static process. Phenotypic changes are observed when mast cells are removed and supplanted in a different environment\(^{[68-70]}\). For example, CTMC if grown
in suspension culture with IL-3 and IL-4 convert into MMC. If these MMC are then injected into the peritoneal cavity of mast cell deficient mice, they revert back to having properties characteristic of CTMC. Perhaps even more surprisingly, CTMC when injected into the stomach wall of mast cell deficient mice produce MMC in the mucosa and CTMC in the submucosa. These environment induced phenotypic changes between sub-populations of mast cells are possibly a result of the extensive proliferative potential of differentiated mast cells. Mast cells proliferating in an environment that is different from their original mileu have resulting progeny whose phenotype is determined by the tissue environment in which the second differentiation occurs.

This microenvironmental regulation of mast cell development and phenotype is thought to be linked to the cytokines present with IL-3\textsuperscript{[71]}, IL-4 \textsuperscript{[72]}, nerve growth factor (NGF) \textsuperscript{[73]} and stem cell factor (SCF) \textsuperscript{[74]} being particularly important in mast cell proliferation.

### 1.5 Heterogeneity

Studies have shown that mast cells from various tissues differ in their phenotypes, and that this heterogeneity tends to have either biological or clinical significance \textsuperscript{[62]}. They show marked heterogeneity in their histochemistry, ultrastructure and pharmacological properties \textsuperscript{[75]}. A summary of the differences between mast cells and basophils is given in Fig 1.1.

#### 1.5.1 Heterogeneity in rodents

Maximow et al \textsuperscript{[76]} were the first to observe that mast cells in the intestinal tract were different in structure to those located elsewhere. They proposed, therefore, that mast cells did not represent a homogenous population. Subsequent work by Enerbäck \textsuperscript{[77-78]} in the 1960s, on rat gastrointestinal mucosa, identified a different type of mast cell to that previously observed in connective tissue. The mucosal mast cells were smaller, varied more widely in shape, were more sparsely granulated and had a much lower histamine and 5-hydroxytryptamine content (5HT) \textsuperscript{[75]} than typical mast cells. In addition, their granules stained red rather than violet with toluidine blue. This led to the classification
of mast cells into two distinct phenotypes, MMC located in the mucosa, and CTMC found in the lower layer of the intestinal wall, connective tissue and serosal cavities.

Rat MMC stain blue with alcian blue, but do not counterstain with safranin, whilst CTMC stain red with safranin [78]. This difference in staining properties reflects the binding of the dye to the proteoglycan. MMC have a relatively soluble matrix containing the poorly sulphated glycosaminoglycan chondroitin. CTMC have a much less soluble proteoglycan matrix containing heparin. The result is an increased degree of sulphation of the matrix and hence increased affinity for safranin. CTMC can also be identified by staining with the cationic dye berberine [79-80]. The dye reacts with heparin to form a highly fluorescent complex which can be used for the quantitative cytofluorometric determination of the proteoglycan content of the cell. MMC do not react with berberine and hence cannot be visualised by this technique.

The enzymatic content of the granules, and the mediators released by the mast cell upon activation, are also phenotype dependent. Anaphylactic mediators are divided into two groups: preformed mediators and those synthesised “de novo”. The newly synthesised mediators are produced by the oxidative metabolism of arachidonic acid [81-82], and can derive either from the lipoxygenase pathway which leads to the production of leukotrienes, or the cyclooxygenase pathway which generates prostaglandins and thromboxanes. Both of these pathways are observed in the two rat mast cell types, but their relative importance is dependent on the cell phenotype. CTMC predominantly generate prostaglandin (PG)D2 by activation of the cyclooxygenase pathway [81][83]. Arachidonic acid is almost exclusively processed by this pathway so that little or no leukotriene production is observed. In contrast MMC synthesise mainly leukotrienes (LT)B4 and LTC4 [84-86].

The enzyme content of the two subpopulations also differs. Both cells contain a chymotrypsin-like neutral protease, denoted rat mast cell protease I (RMCPI) in CTMC, and rat mast cell protease II (RMCPII) in MMC [87]. These enzymes are very similar in action, and have substantial homology in their amino-acid sequences. They are, however, immunologically distinct.
Unsurprisingly, these phenotypic differences are related to the functional differences between the two mast cell types. Antigens, anti-IgE antibodies and concanavalin A (con A), which mediate their effects via the cross-linking of IgE molecules, and calcium ionophores, cause histamine release from both CTMC and MMC. Compound 48/80 and bee venom peptide (mast-cell degranulating peptide), however, although potent secretagogues for CTMC, fail to activate MMC \cite{88-89}. In addition, the anti-allergy compound disodium cromoglycate (DSCG), a potent inhibitor of anti-IgE induced histamine release in peritoneal and other connective tissue cells and extensively used in the treatment of human bronchial asthma, is essentially ineffective in MMC \cite{89}.

Although the differences between CTMC and MMC have been well defined, more recent studies have shown that the concept of the existence of two distinct mast cell phenotypes is an over simplification. Tanish and Peace \cite{90} studied the histochemical and functional properties of mast cells taken from the mesentery, lung, skin and peritoneum, all connective tissues. Interestingly, they saw a marked graduation in responsiveness to a wide range of compounds and on histological examination it became evident that cells from the lung, skin and mesentery contained both CTMC and MMC. They concluded from their investigation that the terms CTMC and MMC were inappropriately used, since they represented two phenotypic extremes between which a gradation of phenotypes existed, exhibiting widespread variation in functional reactivity.

1.5.2 Heterogeneity in humans

In human mast cells the concept of heterogeneity is more complex since the terms “connective tissue” and “mucosal” are no longer immediately relevant. In addition, ultrastructural analysis with antithrombin III gold has shown that all human mast cells contain heparin \cite{91}. Although heterogeneity does exist in human mast cells, it is not strictly dependent on location. Studies by Liu et al \cite{92-93} on gastric mucosal cells showed that they were functionally similar to lung and colonic mast cells whilst Pearce et al \cite{94} showed that cutaneous mast cells were both histochemically and functionally distinct from mast cells obtained from the colonic mucosa and muscle, gastric mucosa, lung and uterus. In addition, there are known to be two different types of mast cells present in the lung, with one predominating \cite{95}.
Human mast cells are, however, conveniently classified by their enzymatic content [96]. The neutral protease of human mast cell granules can be either a chymase or a tryptase. Some cells, e.g. human skin cells, contain both enzymes and are therefore classified as (TC+) cells. Lung and intestinal mucosal mast cells contain only tryptase and are therefore denoted (T+). No cells have been identified with chymase only. The TC+ and T+ cells can be distinguished by immuno-histochemical staining and show marked differences in their ultrastructures. TC+ cell granules are generally larger and more numerous than T+ granules, which tend to be more variable in shape. Only TC+ cells have granules with grating and lattice structures whilst T+ cells have granules that contain scrolls [97-98]. Brading et al [99] demonstrated that human mast cells were also functionally heterogeneous with respect to cytokine expression. IL-4 was distributed among both cell phenotypes but was preferentially expressed by TC+ cells. In contrast, IL-5 and IL-6 were almost exclusively confined to TC+ cells.

Patella et al [100] demonstrated that mast cells from the human heart, lung and skin released different preformed and do novo synthesised mediators upon cross-linking of the high-affinity IgE receptor (FcεRI). Human heart mast cells contained lower tryptase levels than skin cells but more than lung mast cells. Human skin mast cells synthesised more PGD₂ than heart mast cells but much less LTC₄, whilst recombinant human C5a anaphylatoxin and protamine both induced histamine release from skin and heart mast cells but not from lung.

TC+ cells purified from the skin are activated by compound 48/80, morphine and substance P. Other TC+ and T+ cells from lung, heart and intestinal mucosa are not. Histamine release from human lung mast cells, but not TC+ cells, is inhibited by DCSG [101]. Basophils behave in a similar manner to T+ mast cells in that they are not stimulated by compound 48/80 nor is histamine release inhibited by DSCG [102].

1.6 The high affinity IgE receptor (FcεRI)

There are two receptors which bind IgE. The FcεRI receptor has a high affinity for IgE and is found almost exclusively on mast cells and basophils [103], although it is also found on human epidermal Langerhan’s cells [104]. The numbers of FcεRI on human
basophils\textsuperscript{105}, rat mast cells\textsuperscript{106} and rat basophilic leukaemia cells (RBL 2H3 cells)\textsuperscript{107} have been estimated as being 30,000-100,000, 300,000 and 1,000,000 per cell, respectively. The IgE receptor found on platelets, eosinophils, lymphocytes and macrophages has an affinity at least two orders of magnitude lower than that on the mast cell and has thus been denoted Fc\(_e\)RII\textsuperscript{108}. These receptors have been cloned, and sequence analysis has shown that they are very different structurally\textsuperscript{109}. It should also be noted that although the Fc\(_e\)RI receptor is an IgE receptor, it also has low affinity for IgG\textsuperscript{110}. The significance of this is uncertain, although it is possible that IgG plays a role in mast cell activation.

Fc\(_e\)RI is a tetrameric structure consisting of one \(\alpha\), two \(\beta\) and two \(\gamma\) subunits\textsuperscript{111}. The \(\alpha\)-chain binds IgE, whilst the \(\beta\) and \(\gamma\)-chains insert the \(\alpha\)-chain into the membrane and are involved in signal transduction. Binding studies have shown that the receptor binds to the Fc region of the IgE molecule via the C\(_e\)3 domain\textsuperscript{112}. The trigger for mast cell degranulation occurs when multivalent antigens bridge and cross-link the cell-bound IgE molecules\textsuperscript{113}.

### 1.7 Activation of mast cells

Mast cells and basophils can be activated to release their chemical mediators by a number of immunological and non-immunological stimuli.

#### 1.7.1 Immunological stimulation

Sensitisation of the mast cell involves the binding of IgE antibodies to the cell surface. Activation occurs when the body encounters the allergen at a later stage, triggering the series of reactions commonly known as an anaphylactic response. This second stage takes place very rapidly. The antigen binds to the IgE antibodies on the surface of the mast cells, within seconds of coming into contact with the tissue. Bi- or polyvalent antigens are required for histamine secretion since the antigen activates the cell by cross-linking two neighbouring IgE molecules, thereby forming a bridge between them\textsuperscript{114}. This action enables receptor aggregation (cross-linking of the Fc\(_e\)RI) in the membrane, and hence mast cell degranulation.
Immunological activation can be achieved independently by any method which cross-links FcεRI receptors, i.e. by using (1) anti-IgE, an IgG antibody directed against determinants in the Fc region of the IgE molecule [115], (2) con A, a lectin which binds to the carbohydrate on the IgE molecule, or on the receptor itself [116]. It exists as a tetramer and is unable to activate mast cells if broken down into monomeric units, (3) chemically dimerized IgE [117], (4) anti-receptor antibody. This IgG antibody prepared against determinants in the receptor itself can activate the cells in the absence of IgE [113][115][117]. In fact, the anti-receptor antibody cannot bind if the IgE molecule occupies the receptor, since it blocks the sites to which the anti-receptor antibody would normally attach.

In the absence of any cross-linking ligand, a small amount of histamine release is still observed. This “spontaneous” release could be the result of random collisions between receptors in the membrane, i.e. the membrane is fluid, and the FcεRI receptors are free to move within it [115]. Although not proven, the evidence suggests that the spontaneous release is not just non-specific leakage of histamine and it would appear, therefore, that simple collisions are sufficient to cause limited mast cell degranulation.

1.7.2 Non-immunological stimulation

There are many substances which cause histamine release from mast cells and basophils with some having important and functional significance. These stimuli fall into two distinct groups. The first are cytotoxic agents, causing lysis of the cell and leaving it irreversibly damaged. This effect is non-selective and mediated by detergents such as Triton X-100 and Tween 20 [118-119]. The second group are non-cytotoxic liberators, selective in their action and inducing histamine release without the loss of cytoplasmic markers. The latter secretagogues include the following.

1. The polybasic liberators such as compound 48/80 [118], often described as the “classical mast cell degranulating agent” although it does not induce histamine release from all mast cell types, neuropeptides e.g. substance P [120], mastoparan (the classical guanine nucleotide-binding regulatory protein (G-protein) activating agent) [121], mast cell degranulating peptide (MCDP or peptide 401) [122] and polybasic amino acids e.g. polylysine [123].
High molecular weight dextrans. Given systemically, dextran is a potent histamine liberator in the rat, although not other animals. In vitro, it induces release from rat serosal mast cells, and to a much lesser extent mesenteric and cutaneous mast cells \(^{[124]}\).

Anaphylatoxins C3a, C4a and C5a \(^{[125-126]}\).

NGF, a protein required for growth and maintenance \(^{[127]}\).

Phosphatidylserine, which potentiates dextran, NGF and immunological release from rat serosal mast cells \(^{[128]}\).

Calcium ionophores A23187 \(^{[129]}\) and ionomycin \(^{[130]}\).

Drugs e.g. morphine \(^{[131]}\).

Enzymes e.g. phospholipase A\(_2\) (PLA\(_2\)) \(^{[132]}\).

Cytokines e.g. SCF \(^{[133]}\).

Adenosine-5'-trisphosphate (ATP) \(^{[134]}\).

The mechanisms of action of many of the above secretagogues are discussed in chapter 3, and have been described by Lagunoff et al \(^{[135]}\).

1.8 Ultrastructural changes following mast cell degranulation

Mast cell activation leads to exocytosis and the release of histamine and other chemical mediators into the extracellular fluid following granule-cell membrane fusion \(^{[136-137]}\).

In rat peritoneal mast cells, the initial ultrastructural changes are observed with the most peripherally located granules \(^{[137]}\). These granules swell, enabling fusion of the two membranes. Many intergranular fusions follow, resulting in the formation of extensive labyrinthic cavities which communicate with the extracellular milieu through multiple openings. Hence, some of the granules are expelled from the cell whilst others are just exposed to the external environment from within the confines of the cell.

In human mast cells, stimulation by anti-IgE results in degranulation but via a different mechanism to that observed in rat mast cells \(^{[138]}\). Initial swelling of the granules leads to the fusion of individual cytoplasmic granules, resulting in the formation of interconnecting chains of swollen granules. Opening of these channels to the
extracellular medium through narrow fusion points with the cell membrane initiates degranulation. Subsequent release of mediators follows, but no apparent expulsion of the granules occurs. The recovery of human mast cells is complex and involves the re-usage of the granular membrane following degranulation, to repackage newly condensed, crystalline granular contents [139-140].

1.9 Mast cell mediators

The chemical mediators of inflammation released from the mast cell fall into two broad classes; 1: those that are preformed and stored in granules, 2: those that are synthesised “de novo” following activation of the cell (Fig 1.2).

1.9.1 Preformed mediators

1.9.1.1 Histamine

Histamine is probably the best established mediator released from the mast cell. It is synthesised in a simple one-step process from the amino acid L-histidine by the enzyme L-histidine decarboxylase [141]. This enzyme displays a high substrate specificity for histidine and the only other amino acid it decarboxylates is the naturally occurring 3-methylhistidine. Catabolism occurs along two separate pathways. The main route for inactivation of histamine involves its metabolism to N-methylhistamine, a process which is catalysed by N-methyltransferase [142]. The second metabolic route involves direct oxidative deamination of histamine into imidazole acetic acid by diamine oxidase.

The amine comprises between 5 and 10 % of the dry weight of the mast cell granule [142] where it associates by ionic bonds with the acidic residues of the glycosaminoglycan side chains of proteoglycans in the granular matrix [143]. Histamine release occurs via a simple exchange process with extracellular ions [144].

Histamine elicits its effects by combining with $H_1$, $H_2$ and $H_3$ receptors [145]. Peripherally, $H_1$ receptors are linked to the phosphatidylinositol pathway and the mobilisation of intracellular calcium [145-146]. They are located in the ileum, uterus,
bronchi and bronchioles, where upon stimulation they cause contraction of the smooth muscle\cite{145}. Bronchiolar constriction by histamine can be severe and is believed to be one of the major factors that causes airflow reduction in bronchial asthma\cite{147}. H$_1$ receptors are also located in the cardiovascular system, where they mediate relaxation of the arteries (resulting in vasodilatation, flushing and a fall in blood pressure), contraction of the veins and increased vascular permeability (which leads to fluid escape and hence swelling). The swelling that results is aided by the endothelial cells becoming rounder in shape, thus leaving large gaps between them through which fluid can escape.

H$_2$ receptors are coupled to adenylate cyclase and once activated increase 3'-5'-cyclic adenosine monophosphate (cAMP) formation\cite{148}. They are located on the parietal cells in the stomach where they act to increase gastric acid secretion\cite{149-150}. They are also found in the heart where they regulate atrial flow\cite{147}.

H$_3$ receptors control histamine synthesis in the brain\cite{151}. They have also been observed on cholinergic nerves of guinea pig and human airways, predominantly presynaptically, where they are thought to be responsible for inhibiting cholinergic and non-adrenergic non-cholinergic (NANC) transmission\cite{145}. It has been suggested that the H$_3$ receptors might inhibit histamine release from airway mast cells\cite{151}. If so, H$_3$ agonists could prove to have therapeutic effects in the treatment of allergen-induced bronchoconstriction.

In addition to the effects histamine produces directly, it is thought to have an immunomodulatory role, inhibiting histamine release from basophils\cite{152}, regulating lymphocyte activity and controlling eosinophil and neutrophil chemokinesis\cite{153-154}.

1.9.1.2 Serotonin or 5HT

5HT is present in rodent but not human mast cells\cite{155}. Its actions are numerous, complex and species dependent i.e. it causes bronchoconstriction in rat\cite{156} but has little or no effect in human subjects\cite{142}. Its main effects are on blood vessels, where the larger vessels are usually constricted, although it can mediate relaxation. In the microcirculation, constriction of the venules and dilatation of the arteries results in fluid escape from the capillaries, and subsequent oedema. This is further aided by the direct
effect of 5HT on the capillaries, which makes them more permeable to proteins. The amine is also found in human platelets \[144\] and is a well-recognised neurotransmitter in the central nervous system \[157\].

### 1.9.1.3 Neutral proteases

Mast cell granules contain a group of proteolytic enzymes termed neutral proteases or serine esterases which make up approximately one third of the total protein content of the cell and unlike other mediators remain tightly bound to heparin following degranulation. These enzymes cleave peptide and ester bonds and have optimal activity at neutral pH \[144\].

As discussed previously, the distribution of these enzymes throughout rat and human mast cells is an important indicator of the heterogeneous nature of mast cells from different tissue sites. Rat mast cells contain either the chymotryptic enzyme RMCPI or RMCPII \[87\] whilst human mast cells all contain typtase, with cells from the skin, intestinal mucosa and to a lesser extent the lung also containing chymase \[96\].

Tryptase is tetrameric with catalytic sites on each subunit, although it is inactive as a monomer. It cleaves peptide substrates on the carboxyl side of lysine and arginine residues \[158\]. Two different isoforms of the enzyme have been separated by chromatography and have been shown to exist as tetramers of homologous units. These different isoforms differ in their specificity and rate of cleavage of various substrates.

Tryptase causes contraction of bronchial smooth muscle \[159\], providing a possible role for the enzyme in asthma and bronchial hyperresponsiveness. This action of the enzyme may be linked to the ability of human lung typtase to break down vasoactive intestinal peptide (VIP), the main bronchorelaxant neurotransmitter of NANC nerves in the lung \[160\].

The enzyme has been shown to be involved in complement activation, by cleaving C3 into its component parts, namely C3a the anaphylatoxin and direct spasmogen and C3b \[161\]. When heparin is present, however, tryptase further cleaves C3a rendering it inactive. Studies on rheumatoid synovium have implicated tryptase in the pathogenesis
of matrix degradation observed in the rheumatoid joint \[162-163\]. The enzyme activates matrix metallo-proteinase 3 (MMP-3) which in turn activates latent collagenase in an enzyme cascade. The protease also has a local anticoagulant action through the degradation of fibrinogen \[164\] and high molecular weight kininogen \[158\]. Tryptase stimulates deoxyribonucleic acid (DNA) synthesis in bronchial epithelial cells \[165\], indicating a possible role in epithelial repair, and is a potent mitogen for fibroblasts in vitro \[166\], indicating a possible role in fibrosis.

Chymase is a monomer and exists as different isoforms which show species variation in their activity. Human chymase catalyses the conversion of angiotensin I to angiotensin II \[167-168\] independently of the angiotensin converting enzyme (ACE) \[169\]. It inactivates bradykinin and kallidin, metabolises basement membrane components \[170\] and cleaves both VIP and substance P \[171\]. Rat chymase, like human tryptase, binds to and metabolises C3a \[172-173\].

Rat and human mast cells also contain carboxypeptidase. The rat peritoneal mast cell enzyme has characteristics similar to bovine carboxypeptidase A \[174\], whilst the enzyme isolated from human mast cells is functionally similar to bovine carboxypeptidase A, but has structural similarity to bovine carboxypeptidase B \[175\]. The human isoform is found solely in TC+ cells \[176\].

1.9.1.4 Other granule associated enzymes

A variety of other enzymes in addition to the neutral proteases have been localised to mast cell granules. These include arylsulphatases, β-glucuronidase, exoglycosidases and hexosaminidase \[177\]. The latter is often measured to determine the extent of mast cell degranulation.

1.9.1.5 Proteoglycans

The highly sulphated proteoglycans, heparin and chondroitin sulphate, are the major structural components of the mast cell granule and are responsible for the characteristic metachromatic staining seen with basic dyes \[144\][178-179]. They comprise a central protein
core attached to glycosaminoglycan (GAG) chains. The GAG components consist of hexosamine and either hexuronic acid or galactose units arranged in alternating unbranched sequence, with sulphate substituents in various positions \[180\]. The main proteoglycan expressed is tissue and species dependent \[144\].

The proteoglycan binds and packages the other preformed mediators so that they are essentially inactive whilst in the cell. When degranulation occurs, the neutral proteases chymase and carboxypeptidase A, complexed with heparin, remain at the surface of the activated mast cell whilst the other biochemical mediators diffuse away \[181\].

The main function of extracellular heparin is as a powerful anticoagulant \[182\], although it has been suggested that it acts as a natural anti-inflammatory agent \[183\]. Since heparin is highly anionic, it is able to neutralise cytotoxic mediators such as major basic protein, eosinophil cationic protein and eosinophil peroxidase, thereby limiting tissue damage. In addition, heparin has been shown to inhibit antigen-induced bronchoconstriction \[184\] and to block the increased vascular permeability induced by many mediators \[185\]. Heparin also inhibits lymphocyte activation \[186\] and delayed hypersensitivity reactions \[187\]. Further, the proteoglycan has been shown to inhibit chymase activity whilst potentiating the tryptase response \[144\], inhibit the complement cascade \[188\] and modulate growth factor activity \[189\].

1.9.1.6 Chemotactic factors

Mast cells also release substances which are chemotactic for eosinophils and neutrophils. The release of ECF-A has been observed following immunological activation of guinea pig \[190\] and human \[191\] lung and rat peritoneal mast cells \[192\]. Neutrophil chemotactic factor of anaphylaxis (NCF-A) is also released during immediate hypersensitivity reactions \[193\] and has been detected in increased concentrations in the serum following allergen bronchial challenge in asthmatic subjects \[194\]. The cellular infiltration characteristically observed in the latter stages of allergic and inflammatory conditions is almost certainly due at least in part to the release of these two mediators.
1.9.2 Newly generated mediators

This second group of highly potent inflammatory mediators is synthesised during cellular activation. Two major classes of mediator have been identified, the eicosanoids and cytokines.

1.9.2.1 Eicosanoids

These chemical mediators consist of the prostaglandins, thromboxanes and leukotrienes which are derived from arachidonic acid (5,8,11,14-eicosatetraenoic acid). Phospholipids in the cell membrane are the main source of arachidonic acid, and normally have the acid esterified at the 2'-acyl position. Cleavage of the fatty acid is the rate limiting step in eicosanoid biosynthesis and is normally catalysed by PLA$_2$\textsuperscript{[142]}. Arachidonic acid is metabolised by two distinct pathways. Firstly, the enzyme cyclooxygenase proceeds, via the intermediate 11-hydroperoxyeicosatetraenoic acid (11-HPETE), to produce the cyclic endoperoxides, prostaglandin PGG$_2$ and PGH$_2$. PGH$_2$ is a precursor for thromboxane synthesis (TXA$_2$ and TXB$_2$) and for the biologically active prostaglandins PGD$_2$, PGE$_2$, PGF$_{2\alpha}$ and PGI$_2$\textsuperscript{[195-196]}. The major cyclooxygenase product produced in human and rodent mast cells is PGD$_2$ although other prostaglandins and thromboxanes are detected in smaller quantities\textsuperscript{[83][197]}.

The second enzyme 5-lipoxygenase converts arachidonic acid into 5-monohydroxyeicosatetraenoic acid (5-HETE) or LTA$_4$ which is unstable. LTA$_4$ is converted to LTB$_4$ by the addition of water, or by the addition of the tripeptide glutathione to LTC$_4$, LTD$_4$ and LTE$_4$, which comprise the biological activity of the slow reacting substance of anaphylaxis (SRS-A)\textsuperscript{[198-201]}.

It is postulated that some, although not all, of the eicosanoids contribute significantly to the symptoms observed in inflammation. The major contributors appear to be PGE$_2$ and PGI$_2$, both of which relax vascular smooth muscle and are potent dilators of precapillary arterioles\textsuperscript{[142][202]}. This results in the characteristic redness seen with acute
inflammation. Increased blood flow to the inflamed tissue also enhances plasma loss induced by histamine and bradykinin.

PGD<sub>2</sub>, PGF<sub>2α</sub> and TXA<sub>2</sub> appear to play a significant role in asthma since they produce constriction of bronchial smooth muscle<sup>[203-205]</sup>. PGE<sub>1</sub> and PGE<sub>2</sub> have bronchodilator activity<sup>[203]</sup>. In addition, PGF<sub>2α</sub> enhances airway mucus production<sup>[206]</sup> whilst PGD<sub>2</sub> induces erythema and wheal formation<sup>[207]</sup>. The leukotrienes have relatively short half-lives, but potent biological activity. LTA<sub>4</sub> is very unstable and its main role, therefore, appears to be as a precursor for other leukotrienes. LTB<sub>4</sub> has potent chemotactic, chemokinetic and aggregatory actions on polymorphonuclear leukocytes, and may be the main cause of eosinophilia often observed in the lungs of asthmatics<sup>[208]</sup>. LTC<sub>4</sub> and LTD<sub>4</sub> stimulate the production of platelet-activating factor from human endothelial cells, leading to neutrophil adherence<sup>[209]</sup>. In addition, LTB<sub>4</sub> has been identified as an impotent inflammatory mediator in gout<sup>[210]</sup>. Pharmacological studies have shown that the concentrations of leukotrienes in the lavage fluid are significantly elevated in asthmatic patients relative to normal subjects<sup>[211-212]</sup>. They have, therefore, been implicated in many of the symptoms observed in asthmatics i.e. narrowing of the airways<sup>[213]</sup>, excess mucus secretion<sup>[212]</sup>, oedema of the bronchial mucosa and plugging of the airways<sup>[214]</sup>. In addition to their inflammatory actions, LTC<sub>4</sub> and LTD<sub>4</sub> are potent vasoconstrictors and could therefore be involved in ischaemic conditions such as angina<sup>[198]</sup>. The role that eicosanoids play in inflammation has been further confirmed by the anti-inflammatory properties of drugs which inhibit arachidonic acid metabolism. Aspirin, which inhibits prostaglandin synthesis, is an anti-inflammatory, analgesic and anti-pyretic agent<sup>[215]</sup>. Reducing leukotriene production in addition to suppressing cyclooxygenase activity also prevents leukocyte activation<sup>[198]</sup>, an important part of the inflammatory process.
1.9.2.2 Platelet activating factor (PAF)

In addition to the prostaglandins, leukotrienes and thromboxanes, platelet activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glyceryl-phosphorylcholine) is also generated and released upon stimulation of mast cells, neutrophil polymorphs, macrophages, eosinophils and basophils from several species although not from humans [142]. Perhaps surprisingly, a significant amount of the PAF generated is retained within the cell. The reason for this is uncertain, although it is possible that it acts as a second messenger.

PAF mediates its effects by binding to high affinity sites on many inflammatory cells including mast cells [216], basophils [217] and eosinophils [218]. The exact mechanism by which PAF induces mediator release is still uncertain although activation of human basophils leads to a rapid transient rise in intracellular calcium [217] and PAF activation of mast cells has been shown to be inhibited by cAMP active drugs and phosphodiesterase inhibitors [216]. The PAF response is also significantly diminished in the absence of neutrophils [217], suggesting that priming by other inflammatory cells may be necessary for a maximum response.

PAF produces a wide range of biological actions including local vasodilatation, increased vascular permeability [219], wheal formation [220], platelet aggregation [221], accumulation of eosinophils [222], bronchoconstriction and bronchial-hyperresponsiveness [223]. It is, therefore, considered to be an important mediator of inflammation and is known to be active at very low concentrations. The selective attraction of eosinophils by PAF may explain why the mediator produces such a prolonged hyper-responsiveness in asthmatic subjects, since eosinophils are thought to play a role in the damage and shredding of the epithelium. PAF antagonists could, therefore, prove to be very effective as anti-inflammatory agents.

1.9.2.3 Cytokines

Cytokines are produced by many different cells and appear to be synthesised "de novo" following cellular activation. They are normally extremely potent, producing effects at nanomolar concentrations. They act on many different cells to produce a huge variety of effects mediated in both an autocrine and paracrine manner. Mast cell populations have
been shown to release the interleukins IL-1 \[^{224}\] , IL-3 \[^{225}\] , IL-4 \[^{226-227}\] and IL-6 \[^{228}\] , tumour necrosis factors TNF\(_\alpha\) \[^{227}\][229]\) and TNF\(_\beta\) \[^{230}\] and IFN\(_\gamma\) \[^{224}\] once stimulated. They play an important role in up-regulating allergic inflammation and IL1 in particular is thought to be centrally involved in rheumatoid arthritis \[^{162}\] .

The actions of the cytokines are very similar, and it is difficult to determine whether they function separately or together in vivo. Their functions include switching B cell immunoglobulin synthesis from IgG to IgE \[^{54}\] , inducing mast cell and basophil development and differentiation \[^{71-74}\] , inducing endothelial-leukocyte adhesion \[^{231}\] and many more numerous actions of inflammation \[^{225}\] . In addition, recent studies have demonstrated an altered pattern of cytokine secretion in lungs from asthmatic subjects \[^{232}\] .

1.10 Signal transduction mechanisms

Activation of mast cells and basophils resulting in subsequent degranulation involves several inter-linking biochemical events. The mechanisms involved are still not clearly understood and much of the work has been conducted using RBL 2H3 cells, results from which do not always compare directly with human mast cells.

1.10.1 Calcium and phospholipid metabolism

One of the earliest observations of antigen induced histamine release from mast cells was that it was dependent upon calcium in the extracellular medium. The calcium ion gradient, extracellular (1 mM) / intracellular (0.1 \(\mu\)M) i.e. 10,000 : 1, is maintained by the active transport of calcium ions out of the cell and by the membrane’s limited permeability to calcium in resting cells \[^{233}\] . It is only following cellular activation that this homeostasis is disturbed.

Secretion in human basophils appears to be almost entirely dependent on the influx of external calcium. In mast cells, calcium from internal stores and calcium bound to regulatory sites in the membrane also play important roles \[^{234-235}\] .

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Entry of calcium into the cell via specific membrane channels can be blocked by using lanthanum, manganese or magnesium \[^{[233]}\]. Preventing this influx inhibits antigen induced histamine secretion \[^{[236]}\]. In addition, artificial elevation of calcium levels using ionophores such as A23187, which move calcium down the concentration gradient therefore elevating intracellular concentrations, induces secretion of histamine in both mast cells and basophils \[^{[237]}\]. In the absence of extracellular calcium, sub-optimal histamine release is mediated by the release of calcium from intracellular stores and by the mobilisation of membrane-bound calcium.

Hence, activation of the cell results in elevated calcium levels and subsequent histamine release. The calcium signal peaks rapidly and then falls to a lower, stable level, above resting calcium concentrations \[^{[238]}\]. The initial transient rise has been shown to be due to intracellular calcium mobilisation, whilst the sustained elevated level which follows is due to calcium influx. This initial rapid peak can in some cells occur more than once resulting in an oscillation of calcium spikes, the frequency of which is dependent on the agonist concentration \[^{[67][239]}\].

The mechanism for the production of elevated calcium levels is, however, unclear. Investigations by Fewtrell et al \[^{[240]}\] demonstrated that aggregation of the IgE receptors by antigen resulted in increased membrane permeability to calcium, suggesting that calcium influx occurs through receptor mediated calcium channels. Studies by Lindau et al \[^{[241]}\], however, found that blocking calcium activated channels inhibited the conductance change but did not affect degranulation. It has also subsequently been suggested that the whole mechanism of calcium release and uptake by the stores is far more complicated than previously thought, with the calcium which enters the cell being pumped directly into the intracellular stores \[^{[242]}\]. Only when a particular store is full, can it be activated to release the calcium into the cytosol.

The mobilised intracellular calcium mediates its effects by binding to intracellular calcium receptors (calcium binding proteins) with calmodulin being the most important \[^{[243-244]}\]. Calmodulin is present in all eukaryotic cells. It binds up to four calcium ions in a reversible manner inducing conformational changes in the protein. The activated calcium-calmodulin complex then interacts with a multitude of target proteins including cyclic nucleotide phosphodiesterase, adenylate and guanylate cyclase and various
phosphatases, ATPases and kinases involved in the phosphorylation of membrane proteins. Drugs that combine with the calcium-calmodulin complex (e.g. the phenothiazines) prevent its interaction with target proteins and inhibit histamine release from mast cells, giving further evidence of the importance of the protein in cellular reactions \[245\].

The release of calcium from internal stores is mediated through hydrolysis of membrane bound inositol phospholipids \[246\] (Fig 1.3). IgE receptor cross-linking activates a G-protein present in the membrane. This stimulates the formation of inositol 1,4,5 triphosphate (IP$_3$) in addition to various other inositol phosphates through phospholipase C (PLC) activation \[247\]. IP$_3$ acts on the ligand-gated intracellular stores in the endoplasmic reticulum, inducing calcium release into the cytosol \[248-249\]. It has also been proposed that IP$_3$ induces influx of external calcium through specific calcium permeable channels \[250\]. In the mast cell, this calcium plays a role in amplifying the signal by stimulating further IP$_3$ production \[246\].

IP$_3$ is metabolised by its rapid conversion to either inositol 1,4-bisphosphate (IP$_2$) or inositol-1,3,4,5-tetraphosphate (IP$_4$) \[251\]. Whereas IP$_2$ is essentially inactive, IP$_4$ has been shown to have some signal transduction activity and it has been suggested that it might be involved in opening membrane calcium channels \[246\].

In addition to IP$_3$ generation, hydrolysis of inositol phospholipids by the calcium dependent PLC produces diacyglycerol (DAG) which remains in the membrane, where it interacts with protein kinase C (PKC) \[252\]. PKC is normally present in the cytosol, but associates with the membrane upon DAG generation. The activation of PKC provides a signal which is mechanistically similar to that generated by the calcium ionophores, and hence the two mechanisms may have some overlap \[253\]. The precise role of PKC in histamine secretion is, however, still unclear.

1.10.2 Cyclic nucleotides

cAMP and 3'5'-cyclic guanosine monophosphate (cGMP) are important second messengers in many different cell types, although their precise role in mast cells and basophils is still unclear since many studies have proved either contradictory or
inconclusive. Immunological stimulation leads to activation of adenylate cyclase via a Gs type of G protein resulting in ATP dephosphorylation and cAMP generation. Studies in rat peritoneal mast cells have shown that challenge with anti-IgE leads to a rapid rise in intracellular cAMP levels prior to histamine secretion \[254\] with levels returning to baseline following the onset of degranulation \[255\]. Lewis et al \[256\] reported that immunological activation of rat serosal cells gave an early and a late monophasic rise in both cAMP and cGMP. Low levels of indomethacin (the cyclooxygenase inhibitor) abolished both monophasic rises in cGMP and the second rise in cAMP without affecting histamine release. This indicates that only the initial elevation in cAMP is involved in coupling the activation-secretion response.

Leung et al \[257\], however, reported that the direct addition of cAMP analogues, β-adrenoreceptor agonists and phosphodiesterase inhibitors all blocked to various extents histamine release from rat, mouse and hamster peritoneal mast cells. These findings were confirmed by Sydom and Fredholm \[258\] who found that theophylline, whilst having no effect on spontaneous release, significantly inhibited antigen, compound 48/80 and phosphatidylserine induced mast cell degranulation. Berridge \[259\] postulated that cAMP was involved in the regulation of calcium homeostasis and attempted to rationalise these effects by subdividing them into two categories, monodirectional and bidirectional. In monodirectional systems, cAMP enhances calcium induced secretion whilst in bidirectional systems cAMP inhibits the calcium response by activating calcium ATPases, thereby promoting the removal of cytosolic calcium either into intracellular stores or into the extracellular environment.

Other results indicate that cAMP plays no part in non-immunological stimulation of the mast cell, and the nucleotide is, therefore, probably only involved in the early stages of transduction which are by-passed by pharmacological agonists \[255\][260\].

In human lung mast cells and basophil leukocytes, there is contradictory evidence as to whether immunological stimulation augments or inhibits cAMP levels following cell activation. Ishizaka et al \[261\] and Hughes et al \[262\] reported an augmentation in levels of the nucleotide following cross-linking of IgE receptors in both cell types. In contrast, Peachell et al \[263\] found that anti-IgE failed to elevate intracellular cAMP levels in
either cell whilst Lichtenstein et al. [264] found that anti-IgE challenge of human basophils led to a significant fall in cAMP levels prior to the onset of histamine release.

As with rat mast cells, agents which act to elevate levels of cAMP, such as β-adrenergic agents and methylxanthines, inhibit antigen induced degranulation of human lung mast cells [265], basophils [262] and leukocytes [266]. The data suggests, therefore, that cAMP is in general inhibitory to histamine release.

The role of cAMP in mast cell activation has, therefore, never been truly clarified. The effects of cAMP are mediated by activating cytoplasmic cAMP-dependent protein kinases which can phosphorylate distinct serine and threonine residues [267]. In rat mast cells, two protein kinase isoenzymes termed Type I and Type II have been identified. Studies have shown that the phosphodiesterase inhibitor theophylline activates both enzymes resulting in subsequent inhibition of immunologic histamine release [268].

1.10.3 G-proteins

Interaction of agonists with specific receptors on the cell surface represents the initial step in cellular activation. Receptor stimulation often results in activation of effector proteins which trigger a cascade of events involving the mobilisation of various second messengers. A family of heterotrimeric G-proteins act to convey this receptor generated signal across the cell membrane [269].

The three subunits of the protein are denoted α, β and γ. In its resting state the α subunit is bound to guanosine 5’-diphosphate (GDP) and is associated with the β and γ subunits. Following receptor stimulation, the GDP dissociates from the protein allowing guanosine trisphosphate (GTP) to bind. This causes the dissociation of the trimer into α-GTP and βγ subunits [270]. It is the α-GTP subunit that is the active member of the trimer and the role of the βγ-subunit is unknown, although it has been proposed that it acts to attenuate the action of the α subunit.

The α subunit acts on a variety of effector proteins including adenylate cyclase, enzymes and ion channels [271]. Gomperts [272] demonstrated that the introduction of
non-hydrolysable GTP analogues into the cytosol in the presence of extracellular calcium was sufficient to induce mast cell exocytosis even in the absence of a secretagogue.

Deactivation of the protein is caused by hydrolysis of GTP to GDP. This results in the rebinding of the $\alpha$ and $\beta \gamma$ subunits $^{[271]}$.

**1.10.4 Phosphorylation of cellular proteins**

An intracellular supply of ATP is required for mast cell degranulation. Synthesis comes from glycolytic and oxidative metabolism and either of these processes is sufficient to sustain histamine release $^{[273-274]}$.

ATP is required for protein phosphorylation which follows stimulation of the cell, and is an important part of the secretory mechanism. Phosphorylation and dephosphorylation of several proteins has been reported following stimulation with various secretagogues. These include the $\beta$ and $\gamma$ chains of the Fc$_e$RI, PLC-$\gamma$1 and proteins with molecular weights of 130, 110, 105, 91, 86, 77, 72, 60, 57, 55, 40 and 38 kDa which are phosphorylated on tyrosine residues $^{[275-277]}$. Sieghart et al $^{[278]}$ reported that stimulation of rat peritoneal mast cells by compound 48/80 led to phosphorylation of several proteins with molecular weights 42, 59, 68 and 72 kDa. The same effect was observed with calcium ionophore A23187 except that phosphorylation of the 78 kDa protein was diminished. The first three proteins are phosphorylated as histamine is released and are believed, therefore, to be part of the process which initiates secretion. This mechanism is calcium dependent. Phosphorylation of the 78,000 protein is much slower, and hence cannot be involved in the induction of secretion. It is possible, therefore, that it plays an inhibitory role and this hypothesis is supported by the action of anti-allergic drugs, e.g. DSCG, which induce rapid phosphorylation of this protein $^{[279-281]}$. 

38
1.10.5 Serine esterases

Studies by Emadi-Khiav and Pearce [282-284] have implicated the involvement of a chymotryptic protease in IgE mediated activation of rat mast cells. This enzyme is only involved in immunologic histamine release and plays, therefore, no role in mast cell degranulation induced by basic compounds such as 48/80, mastoparan and substance P. In addition, inhibitors of the enzyme are only effective if present at the time of cellular activation. It seems likely, therefore, that activation of this serine esterase represents one of the earliest steps in cellular activation since the basic secretagogues are thought to by-pass some of the initial steps of antigen induced release.

1.10.6 Phospholipase D

Receptor mediated hydrolysis of phospholipids is catalysed by various phospholipases including PLC, PLA\textsubscript{2} and PLD. The latter catalyses the conversion of phosphatidylcholine to phosphatidic acid (PA) and choline. PA is subsequently metabolised by PA phosphohydrolase (PPH) to form DAG [285].

Experiments by Lin et al [286] have shown that PLD is activated by the cross-linking of IgE receptors on the surface of RBL 2H3 cells and Kennerly [287] demonstrated that in rat serosal mast cells, approximately 75% of DAG was actually derived from phosphatidylcholine, rather than from the breakdown of phosphatidylinositol by PLC as had previously been thought. Blocking this formation of DAG has been shown to inhibit degranulation [288].

PLD activation appears to be carefully controlled through multiple mechanisms including protein kinase C activation, intracellular calcium concentrations and GTP-binding proteins [285][289].

1.10.7 Membrane ion channels

In excitable cells such as adrenal chromaffin cells and pancreatic β cells, calcium influx through voltage-activated Ca\textsuperscript{2+} channels is sufficient to induce secretion [290]. Mast cells
do not possess such channels and are, therefore, termed “non-excitable” secretory cells. Studies have indicated, however, that ion fluxes do occur in mast cells, but their relevance in stimulus-secretion coupling has yet to be clarified [291-292].

It has been postulated that mast cells possess calcium channels controlled by G-proteins and IP$_4$ [293]. In addition, Romanin et al [292] have shown that there are immunologically activated chloride channels involved in rat mast cell degranulation. These channels are activated by intracellular cAMP and calcium [291]. The chloride current hyperpolarises the cell, providing a driving force for calcium influx. Blocking these chloride channels inhibits immunological activation of mast cells, suggesting that this process is necessary for exocytosis [292]. In addition, DSCG has been shown to inhibit antigen induced chloride influx in parallel with serotonin release.

1.11 Adenosine

Adenosine is a naturally occurring purine nucleoside present in the extracellular fluid at a concentration of approximately 30 nM. Its main biosynthetic route is from the cleavage of adenosine 5’-monophosphate (AMP) by the enzyme 5’-nucleotidase, although other metabolic routes have been identified, including the demethylation of S-adenosyl methionine and de-novo synthesis. It has an autacoid function in many physiological systems and modulates inflammation by acting on a wide variety of cells, including mast cells, leucocytes, monocytes, eosinophils and basophils. It is believed to play an important role in bronchoconstriction. In addition, adenosine acts on several organ systems, where it elicits a large number of biological responses.

1.11.1 Adenosine receptor subtypes

The effects of adenosine are mediated through G-protein coupled receptors, of which currently four subtypes have been cloned: A1, A2a, A2b and A3. A1 receptors have classically been associated with the inhibition of adenylate cyclase via $G_i$ proteins [296-297], although it is now known that they can act through alternative effector systems including potassium channels, calcium channels, phospholipases $A_2$ and $C$, and guanylate cyclase [298]. $A_2$ receptors stimulate adenylate cyclase via binding to $G_s$.
proteins, and were originally subdivided into two classes, based on their agonist binding affinity and anatomical distribution [299]. The high affinity receptor subtypes located predominately in the striatum were later designated A2a, and the low-affinity type distributed throughout the brain, A2b [300].

The existence of a third A3 receptor was first proposed by Ribeiro and Sebastiao [301] who observed that in atrial tissue and at pre-junctional sites on neuronal tissue, the binding affinities of the adenosine agonists were not consistent with either A1 or A2 receptors. They also found that adenosine mediated its effects via an adenylate cyclase independent mechanism. This first putative A3 receptor has since been shown to be pharmacologically distinct from the cloned A3 receptor and much active scientific debate has taken place as to whether such a receptor actually exists.

Meyerhof et al [302] first reported the identification of a novel receptor clone from a rat testis cDNA library. They noted that the clone had the highest homology (> 40 %) with canine A1 and A2a adenosine receptors, but they could not identify a ligand for the receptor. Zhou et al [303] detected a clone designated R226 from rat striatum. R226 was found to encode for an adenosine receptor subtype, and on the basis of its pharmacological profile being distinct from that of either the A1 or A2 receptors, it was designated A3. Sheep [304], rabbit [305] and human [306] A3 adenosine receptors have subsequently been cloned and, in contrast to A1 and A2 receptors, display much lower homology among species at the amino acid level. In addition, pharmacological characterisation has shown that the A3 receptors from different species exhibit remarkable differences in their affinity for adenosine agonists and antagonists [307].

The second messenger pathway activated by the adenosine A3 receptor is still under debate. When expressed in CHO cells, homologues of the adenosine A3 receptor from all four species noted above have been shown to inhibit forskolin-stimulated cAMP accumulation through a pertussis toxin sensitive G-protein [303-306], suggesting coupling to G1 or Go proteins. In RBL-2H3 cells, however, receptor activation results in rises in intracellular calcium and inositol phosphate levels [308]. This stimulation is blocked by both cholera toxin and pertussis toxin, suggesting that activation of PLC by adenosine is via a different G-protein to that involved in agonist induced activation. The exact nature of the G-protein remains to be determined, but it has been postulated that it is G1 [309].
1.11.2 Role of adenosine in bronchoconstriction

Studies in subjects with asthma and chronic bronchitis have demonstrated that they have an increased amount of adenosine in the bronchial lavage fluid, relative to that of normal subjects [310]. This indicates that adenosine release may be increased in asthmatic lung and areas of inflammation. Once produced, adenosine induces bronchoconstriction in both allergic and non-allergic asthmatic subjects [311] but not in normal subjects. Guanosine, which is structurally similar to adenosine, is completely ineffective, suggesting a degree of selectivity for the adenine moiety. In addition, the inhalation of AMP and adenosine 5'-diphosphate (ADP) induce bronchoconstriction with identical characteristics to that of adenosine [312], suggesting that their effects are mediated by extracellular hydrolysis to the purine nucleoside. Theophylline, the adenosine receptor antagonist, was also shown to have a significant protective effect on adenosine and AMP induced bronchoconstriction, but not on histamine mediated contraction [313-314], giving further confirmation that the effects of adenosine are likely to be due to specific stimulation of airway purinoreceptors. Drugs that inhibit cellular uptake of adenosine would, therefore, be expected to augment the bronchoconstrictor response. Dipyridamole, a potent inhibitor of adenosine uptake, increased airway responsiveness to adenosine in asthmatic subjects and shifted the dose-response curve for the nucleoside to the left [315].

1.11.3 Mechanism of adenosine induced bronchoconstriction

The exact mechanism of adenosine induced asthma is unknown, but is thought to involve the action of the nucleoside at two different sites (Fig 1.4) [316]. Firstly, activation of the mast cell by adenosine causes the release of histamine and other bronchoconstrictor mediators which act directly to contract the smooth muscle of the airways. Secondly, adenosine acts on sensory nerves, inducing the local release of neuropeptides and indirect mast cell activation.

Adenosine induced bronchoconstriction is inhibited by DSCG and nedocromil sodium. In atopic subjects, the concentration of AMP required to reduce the FEV₁ by 20 % was increased sevenfold by DSCG and twenty-seven fold by nedocromil sodium. These
drugs are thought to mediate their effects, at least in part, by preventing mast cell degranulation.

The above hypothesis is supported by Rafferty et al. [317] who showed that terfenadine, a potent H₁ receptor antagonist, inhibited AMP induced bronchoconstriction by approximately 80%. They proposed, therefore, that AMP mediated the majority of its effects by inducing histamine mobilisation. Crimi et al. [318], however, found that pretreatment with indomethacin (an inhibitor of cyclooxygenase) also decreased adenosine hyperresponsiveness, suggesting a role for arachidonic acid derivatives in adenosine mediated bronchoconstriction. These findings were confirmed by Polosa et al. [319] who found that the prompt reduction in airway calibre following endobronchial stimulation with AMP was paralleled by a significant rise in PGD₂, histamine and tryptase levels in the lavage fluid.

Hence, there is now significant evidence that the responses produced by adenosine are not merely due to non-specific airway hyperresponsiveness, but rather to selective interaction with inflammatory cells. The receptor responsible for mediating these effects, however, remains to be determined. In addition, there is growing support for the theory that adenosine may mediate some of its effects by activating cholinergic neural pathways [320-321].

1.11.4 Role of adenosine in rhinitis

Adenosine elicits nasal symptoms in subjects with allergic rhinitis [322], and this is accompanied by a significant elevation in histamine levels in the nasal lavage fluid [323]. Cetirizine, the specific H₁ receptor antagonist, completely abolishes the symptoms [324], if given prior to nasal challenge with AMP, indicating that in the human nose, adenosine may act to mimic rhinitis by inducing histamine release from mucosal mast cells.

1.11.5 Adenosine mediated hypotension

Fozard and Carruthers [325] showed that N⁶-2-(4-aminophenyl)ethyladenosine (APNEA), the adenosine A₃ receptor agonist, induced hypotension in the angiotensin II-
supported circulation of the pithed rat. This effect was not antagonised by the general adenosine receptor antagonist 8-(p-sulphophenyl)theophylline (8-SPT), although follow up studies demonstrated that the effect was substantially reduced by treatment with pertussis toxin \[^{[326]}\] and by the A\(_3\) antagonist 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-phenylxanthine (I-ABOPX) \[^{[327]}\]. It seems likely, therefore, that adenosine mediates hypotension via activation of the A\(_3\) receptor to induce mast cell degranulation. In agreement with this hypothesis, the fall in blood pressure and heart rate observed with APNEA is very similar to that observed with compound 48/80 \[^{[328]}\]. Plasma and serum histamine concentrations are also markedly increased in both APNEA and compound 48/80 treated animals.

### 1.11.6 Other effects of adenosine

It has been proposed that caffeine and other alkyl-xanthines that act as physiological stimulants mediate their effects by blocking adenosine receptors \[^{[329]}\]. Adenosine acts as a neuromodulator in the brain and is produced locally in response to stress or increased activity \[^{[330]}\], e.g. during cardiac hypoxia \[^{[331]}\] or central nervous system ischemia \[^{[332]}\]. It is thought to act as a neuroprotective agent by inhibiting platelet \[^{[333]}\] and neutrophil \[^{[334]}\] function, in addition to activating cellular antioxidant enzymes \[^{[335]}\].

Adenosine mediates most of its effects by acting on the A\(_1\) and A\(_2a\) receptor subtypes. Activation of the A\(_1\) receptor mediates a decreased energy demand, whilst A\(_2a\) activation results in an increased oxygen supply to the organs \[^{[330]}\].

Adenosine has been shown to be involved in pain \[^{[336]}\], cognition, anxiety and depression \[^{[329]}\]. Agonists of A\(_1\) receptors produce anti-arrhythmic, anti-diabetic \[^{[330]}\], anti-convulsant \[^{[337]}\] and neuroprotective effects. The latter is due in part to counteraction of the damage caused by excessive glutamate release \[^{[338]}\]. The A\(_2a\) receptor mediates anti-convulsive \[^{[339]}\], hypotensive \[^{[340]}\] and anti-psychotic \[^{[341]}\] effects. In addition, the positive interaction between adenosine A\(_2a\) antagonists and L-DOPA \[^{[342]}\] indicates that such antagonists may prove useful in the treatment of Parkinson’s disease \[^{[343]}\]. A\(_3\) agonists mediate prophylactic neuroprotection \[^{[344-345]}\] and hypotension \[^{[325-328]}\], in addition to regulating behaviour \[^{[346]}\].
1.12 Aims of the present study

Adenosine is known to modulate a number of physiological and pathological processes, including the release of histamine from mast cells. There have been many contradictory results published in the literature, however, as to the exact nature of its role in mast cell activation, and this may be due partly to the fact that its effects are thought to vary according to the mast cell phenotype [347].

The aim of this study was, therefore, to elucidate the role of adenosine in the release of inflammatory mediators from mast cells and basophils by studying the effects of the nucleoside on a number of different mast cell subtypes and using a range of agonists and antagonists in an attempt to define the receptors involved.

Additional studies were carried out to try to determine the second messenger system involved in the action of adenosine on mast cells, and we also investigated the effects of the nucleoside on smooth muscle preparations.
**Fig. 1.1. Comparison between mast cells and basophils**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mast Cells</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>Surface processes</td>
<td>Long and thin</td>
<td>Short and thick</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><strong>Surface receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgE receptor</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-3 receptor</td>
<td>Yes (Mouse)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No (Human)</td>
<td></td>
</tr>
<tr>
<td>SCF receptor (c-kit)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Natural history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>Hematopoietic stem cell</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>Site of maturation</td>
<td>Connective or mucosal tissue</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mature cells in the 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><strong>Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate hypersensitivity</td>
<td>Involved</td>
<td>Involved</td>
</tr>
<tr>
<td>Cutaneous basophil hypersensitivity</td>
<td>No</td>
<td>Involved</td>
</tr>
<tr>
<td>Augmentation of acute infection</td>
<td>Involved</td>
<td>No</td>
</tr>
<tr>
<td>Rejection of ticks</td>
<td>Involved</td>
<td>Involved</td>
</tr>
<tr>
<td>Rejection of intestinal parasites</td>
<td>Involved</td>
<td>?</td>
</tr>
</tbody>
</table>

Adapted from reference 68.
Fig. 1.2. Mast cell mediators

- **Lipid mediators**
  - PGD,
  - LTC,
  - LTB,
  - TXA,
  - PAF

- **Granule associated pre-formed mediators**
  - Proteoglycans
  - Histamine
  - Serotonin
  - Neutral proteases
  - Chemotactic factors

- **Cytokines**
  - GM-CSF
  - TNFα
  - IFNγ
  - IL-1α
  - IL-3
  - IL-4
  - IL-5
  - IL-6

Redrawn from reference 294.
The (1) binding of agonists to a surface receptor activates PLC through the intermediacy of a G protein (2). PLC catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to IP$_3$ and DAG (3). The water soluble IP$_3$ stimulates the release of Ca$^{2+}$ sequestered in the endoplasmic reticulum (4) which, in turn, activates numerous cellular processes through the intermediacy of calmodulin and its homologs (5). The nonpolar DAG remains associated with the membrane where it activates PKC to phosphorylate and thereby modulate the activities of a number of cellular proteins (6). This latter activation process also requires the presence of the membrane lipid phosphatidylinerine (PS) and Ca$^{2+}$.

Redrawn from reference 295.
Fig. 1.4. Possible mechanism of adenosine induced bronchoconstriction

Redrawn from reference 316.
Chapter 2

Materials and methods
Chapter 2

2.1 Animals

Sprague Dawley, Wistar and Hooded Lister rats (150-350 g), MF1 and TO mice (30-40 g) and Duncan Hartley guinea pigs (250-400 g) were used for this study. All animals were male and obtained either 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 supplied by surgeons from St. George’s Hospital, London, following resection for bronchial carcinoma. Human foreskins were provided from circumcisions by surgeons from the Chelsea and Westminster Hospital, London. Peripheral blood was obtained by venipuncture from normal healthy volunteers.

2.3 Physiological buffers

All buffers were made up in glass distilled water.

2.3.1 Buffers employed in the isolation of mast cells and in whole cell experiments

All experiments were carried out in a modified Tyrode’s solution buffered with N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES) as described below, unless otherwise stated. The pH of all solutions was adjusted to 7.2-7.4, by the addition of sodium hydroxide (4 M) and hydrochloric acid (3 M).

2.3.2 Full HEPES buffered Tyrode’s (FHT)

<table>
<thead>
<tr>
<th>Component</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>
</tbody>
</table>
2.3.3 Glucose-free-Tyrode’s (GFT)

The composition of GFT was the same as for FHT only omitting glucose.

2.3.4 BSA Tyrode’s (BSA-FHT)

Identical composition to that of FHT but supplemented with bovine serum albumin (BSA, 1 mg/ml).

2.3.5 10 x Calcium-free-Tyrode’s (10xCFT)

This buffer comprised all the reagents used in FHT at 10 times the concentration except for calcium chloride.

2.3.6 Krebs solution

Krebs solution of the following composition was used for smooth muscle preparations.

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>2.5 mM (368 mg/l)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>5.5 mM (0.99 g/l)</td>
</tr>
<tr>
<td>KCl</td>
<td>4.8 mM (358 mg/l)</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.2 mM (163 mg/l)</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1.2 mM (144 mg/l)</td>
</tr>
<tr>
<td>NaCl</td>
<td>118 mM (6.90 g/l)</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>2.5 mM (210 mg/l)</td>
</tr>
</tbody>
</table>
2.4 Isolation and purification of mast cells

2.4.1 Peritoneal mast cells (Isolation)

The animals were sacrificed through asphyxiation with a rising concentration of carbon dioxide, followed by cervical dislocation. The abdominal skin was removed and heparinized (50 units/ml) FHT (15-20 ml per rat, 7-8 ml per mouse) was injected into the peritoneal cavity through the exposed abdominal wall, taking care to avoid penetration of the gastro-intestinal tract. The abdomen was then gently massaged (2 min per rat, 10 sec per mouse) before an incision was made along the midline to expose the peritoneal cavity. The peritoneal lavage fluid was carefully recovered using a plastic liquipipette, and placed in polypropylene tubes. It was important to avoid the introduction of air bubbles into the sample, as this would have affected the cells. Any samples heavily contaminated with blood were discarded at this stage. The cells were kept on ice for up to 4 h, before centrifuging (100 g, 2.5 min, 4 °C). They were then washed twice with warm FHT (37 °C), before being either purified or resuspended in the required volume of warm buffer for use directly in functional studies. If necessary cells from more than one animal were pooled.

2.4.2 Peritoneal mast cells (Purification)

Mixed peritoneal mast cells (section 2.4.1) were purified using density gradient centrifugation in Percoll. The cells were resuspended in BSA-FHT (1 ml) and then mixed with a Percoll solution (4 ml) consisting of 9 parts Percoll and 1 part 10xCFT. BSA-FHT (1 ml) was then carefully layered over the Percoll/cell mixture, so that a clear interface was produced. The sample was then centrifuged (140 g, 25 min, 4 °C) before the supernatant was aspirated to leave a pure mast cell pellet at the bottom of the tube. The pellet was washed twice in BSA-FHT and twice in FHT before use in functional studies.
2.4.3 Human basophil leukocytes

Peripheral blood was collected from healthy volunteers and placed in polypropylene tubes. One part isotonic saline (10 ml) with dextran (6 %), glucose (30 mg/ml) and heparin (50 units/ml) was mixed with 4 parts blood and the solution left to stand for 90 min (RT). The top plasma layer, which was rich in platelets and leukocytes, was carefully aspirated with a liquipipette and centrifuged (150 g, 5 min, 4 °C) to sediment the basophils. The platelet rich supernatant was discarded and the cell pellet washed twice in FHT before use in functional studies.

2.4.4 Human cutaneous mast cells

Human foreskin tissue was dissected free of underlying fat, washed thoroughly with FHT and chopped into small pieces. The tissue fragments (<1 mm²) were then incubated (90 min, 37 °C) in BSA-FHT containing collagenase (160 units/ml) and hyaluronidase (500 units/ml) with constant stirring. At the end of the digestion period, the tissue was disrupted gently by expression through a syringe (20 ml) and the resulting solution filtered through moistened gauze to remove tissue debris. The cells were recovered by centrifugation (100 g, 5 min, 4 °C) and resuspended in BSA-FHT. The supernatant (containing the enzyme) was added to any undigested skin fragments and the procedure repeated. Finally, all the cell suspensions were pooled, centrifuged (100 g, 5 min, 4 °C) and resuspended in BSA-FHT. The cells were then washed twice in FHT before use in functional studies.

2.4.5 Human lung parenchymal mast cells

Macroscopically normal human lung tissue was recovered following surgery for bronchial carcinoma. The specimen was dissected free of major airways, pleural tissue and blood vessels. The tissue was chopped into small pieces (approx. 1 cm³) using scissors and washed thoroughly with FHT. The tissue was then chopped into much finer fragments (< 1 mm²) and washed again with FHT. The tissue fragments were then incubated (90 min, 37 °C) in BSA-FHT containing collagenase (160 units/ml) with constant stirring. At the end of the digestion period, the tissue was disrupted by expression through a syringe (50 ml) and the resulting solution filtered through
moistened gauze to remove tissue debris. The cells were recovered by centrifugation (100 g, 8 min, 4 °C) and resuspended in BSA-FHT. The supernatant (containing the enzyme) was added to any undigested lung fragments and the procedure repeated. Finally, all the cell suspensions were combined, centrifuged (100 g, 8 min, 4 °C) and resuspended in BSA-FHT. The cells were then washed twice in FHT before use in functional studies.

2.5 Mast cell number and purity

To determine mast cell number and purity, an aliquot of the cells (40 μl) was gently mixed with FHT (60 μl) and Kimura staining solution (100 μl) [348].

Kimura solution consisted of the following components.

(a) Toluidine blue solution (0.05 %)
   toluoidine blue (0.05 g), sodium hydroxide (1.8 %, 50 ml), ethanol (96 %, 22 ml), mixed together and made up to 100 ml with distilled water.
(b) Saturated solution of saponin in ethanol (50 %)
(c) Sodium dihydrogen phosphate (0.066 M, 1.03 g/100 ml distilled water)

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

2.6 Histamine release from isolated mast cells and basophils

Aliquots of isolated mast cells (200 μl) were added to disposable polypropylene tubes containing FHT (250 μl) and allowed to equilibrate in a water bath (5 min, 37 °C). A solution of the secretory stimulus (50 μl) was then added and the reaction allowed to proceed for 10 min (passively sensitised cells, 15 min; basophils, 20 min) before being terminated by the addition of ice cold buffer (1 ml). Tubes containing cells alone were set up in duplicate to determine spontaneous histamine release. Cells were pelleted by centrifugation (murine peritoneal mast cells 100 g, 2 min; basophils 150 g, 5 min; cutaneous mast cells 100 g, 5 min; lung mast cells 100 g, 3 min; 4 °C). The supernatant containing the secreted histamine was then decanted off into correspondingly labelled
tubes. The cell pellets were resuspended in FHT (1.5 ml). Rat peritoneal mast cell pellets were boiled for 10 minutes (manual assay). For the other cells, both supernatant and cell pellet tubes were treated with 70% v/v perchloric acid (final concentration 0.4 M, automated assay).

The histamine content of both supernatant and cell pellet tubes was measured, and the release expressed as a percentage of the total amine content;

\[ H_R = 100 \times \frac{H_S}{(H_S + H_C)} \]

Where

- \( H_R \) = Histamine Release (%)
- \( H_S \) = Histamine in Supernatant
- \( H_C \) = Histamine in Cell Pellet

The releases were then corrected for the spontaneous release produced in the absence of any stimulus.

### 2.6.1 Kinetic studies

To determine the kinetics of histamine release, cells were incubated with one concentration of the releasing agent ± the agonist for a series of pre-selected times and the reaction terminated as before.

### 2.6.2 The effect of metabolic inhibitors

To investigate the metabolic requirements for histamine release, aliquots of mast cells (200 µl, GFT) were incubated in the presence of 2-deoxy-D-glucose (5 mM, GFT) and antimycin A (1 µM, GFT). The samples were then challenged with the appropriate secretagogue (GFT). The reaction was terminated by addition of ice-cold FHT and histamine releases determined as above.
2.6.3 Potentiation effect of agonists in murine peritoneal mast cells

Polypropylene tubes containing FHT (125 µl), the test agonists (125 µl) and the secretory stimulus (50 µl) were allowed to equilibrate in a water bath (5 min, 37 °C) before addition of the cell suspension made up in the appropriate volume of warm buffer (200 µl). Tubes containing secretagogue alone were set up in duplicate to determine control release, which was maintained at about 20-30%. The effect of the test agonist was also monitored to check whether it elicited release in an additive or synergistic manner. Histamine releases were determined as above. Potentiation of histamine release was expressed in terms of the percentage of enhancement of the control release (i.e. release in the absence of the agonist)

\[
\text{Potentiation (\%) } = 100 \times \frac{R_P - R_C}{R_C}
\]

Where

\(R_C\) = Control release
\(R_P\) = Release in the presence of the potentiator

2.6.4 Inhibitory effect of antagonists in murine peritoneal mast cells

In antagonist studies, cells were incubated with antagonists (125 µl, 30 min, 37°C) instead of FHT prior to challenge. Cells were then stimulated either by the secretagogue and test agonist or by the secretagogue alone. Inhibition of histamine release was expressed in terms of the percentage inhibition of the control release (i.e. release in the absence of the inhibitor)

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

Where

\(R_I\) = Release in the presence of the inhibitor
\(R_C\) = Control release
2.6.5 Agonists in human mast cells

In human mast cell experiments, the test agonists were either pre-incubated with the cells (30, 15 or 0 min) prior to antigen challenge, or the agonists were added post-challenge (5 or 15 min).

2.6.6 Antagonists in human mast cells

In antagonist studies, cells were incubated with the antagonists (5 min, 37 °C) prior to addition of the agonist (basophil leukocytes, 30 min; lung parenchymal cells, 15 min) before challenge with antigen.

2.7 Measurement of mast cell mediators

2.7.1 Manual assay

The methodology describing the assay of histamine from rat peritoneal mast cells was first published by Shore et al [349]. Histamine is reacted with o-phthaldialdehyde (OPT) under alkaline conditions to generate a fluorescent adduct. This reaction is shown in Fig. 2.1. The fluorescence was then measured with a commercially available spectrometer, using excitation and emission wavelengths of 360 nm and 440 nm respectively.

Briefly, NaOH (1 M, 200 μl) was added to each sample, followed by OPT (1 % w/v in methanol, 75 μl). The tubes were allowed to incubate (4 min, RT) before quenching the reaction by the addition of hydrochloric acid (3 M, 100 μl). The tubes were vortexed following each addition to ensure adequate mixing. This generated fluorescence that was directly proportional to the histamine content in the cell. The sensitivity of the manual technique enabled measurement of histamine concentrations of 5-10 ng/ml. If immunoglobulin was used as the secretagogue, particularly at high concentrations, the addition of the acid often resulted in protein precipitation. To counteract this, samples were centrifuged (200 g, 10 min) before being assayed.
2.7.2 Automated assay

All other samples were assayed using a commercial autoanalyser (Technicon Autoanalyser II). This involves selectively extracting the histamine from the sample, before the reaction with OPT and measurement of fluorescence\[350\]. Samples were prepared for analysis by acidification with perchloric acid (72 %, 50 µl) to liberate residual histamine and induce protein precipitation. The tubes were vortexed and then centrifuged (200 g; 10 min murine peritoneal mast cells, 20 min tissue mast cells and basophils; 4 °C). The samples were then decanted into sample cups and assayed in automated sequence.

The histamine in the samples was concentrated and purified by a sequence of reactions and washes. Initially, the samples were made alkaline and the histamine extracted with salt saturated butan-1-ol. The organic phase was then separated and retained, before being washed in a less alkaline medium. The solution was then made less polar by the addition of n-heptane, before the histamine was back-extracted using dilute HCl. The amine was then made alkaline again before being reacted with OPT. Subsequent acidification stabilised the adduct, the fluorescence of which was measured by a fluorophotometer attached to a chart recorder. This process allowed measurement of basal histamine concentrations of 1 – 10 ng/ml.

2.8 Measurement of cytosolic calcium

It is possible to monitor intracellular calcium levels by using fluorescent indicators with green excitation wavelengths. Such an indicator, fluo-3, can easily be loaded into cells by incubating with the pentaacetyoxymethyl (AM) ester \[351\]. The ester is hydrolysed intracellularly by endogenous enzymes to yield fluo-3, which can indicate changes in calcium levels induced by stimulation with agonists. The structure of fluo-3 is shown in Fig 2.2.

Aliquots of purified rat peritoneal mast cells (10^6 cells/ml, 900 µl) were incubated (30 min, 37 °C) with fluo-3 AM (4 µM, 100 µl) and p-[dipropylsulfamoyl] benzoic acid (probenecid, 0.25 mM) in BSA-FHT. Probenecid was used to prevent leakage of the dye from the cells and did not interfere with the action of the secretagogues or the agonists used in the present study. A parallel portion of the cells containing no dye was
also incubated and used to determine the autofluorescence. The cells were recovered by centrifugation (140 g, 3 min, 4 °C), washed twice and resuspended in FHT (1900 µl) containing probenecid (0.25 mM). The loaded cells were placed in a quartz cuvette, and maintained at 37 °C with constant stirring. The fluorescence was measured in a luminescence spectrophotometer (excitation wavelength 506 nm, slit size 2.5 nm; emission wavelength 440 nm, slit size 5nm).

The cells were stimulated by addition of the test agonist (100 µl), and the resulting change in fluorescence recorded. Digitonin (30 µM, 100 µl) was added finally to determine the total fluo-3 content of the cells.

Intracellular calcium levels were determined as below:

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

Where

- \( K_d \) = the equilibrium constant for the dissociation of the fluo-Ca\(^{2+}\) complex and has a value of 400 nM at vertebrate ionic strength (37 °C)
- \( F_{\text{min}} \) = Fluorescence with no dye (autofluorescence)
- \( F_{\text{max}} \) = Fluorescence of total fluo-3 in cells (found by adding digitonin)
- \( F \) = Fluorescence with test secretagogue

### 2.9 Extraction of IP\(_3\)

IP\(_3\) was extracted from purified rat peritoneal mast cells by mixing the cell suspension with perchloric acid (20 %, 0.2 v/w, 0 °C) and incubating on ice (20 min) before centrifuging (2000 g, 15 min, 4 °C). This sedimented any proteins in the sample. The supernatants were then decanted into glass tubes (to minimise loss of phosphorylated inositol species) and neutralised (pH 7.5) by titrating with NaOH (2 M) buffered with HEPES (60 mM). The NaClO\(_4\) was sedimented by centrifugation (2000 g, 15 min, 4 °C), and the supernatants decanted into pre-labelled tubes for the assay.
2.10 Measurement of IP₃

The IP₃ samples were assayed using a commercially available radioimmunoassay kit (Amersham). The number of cells in each sample was measured before the experiment. In the assay, unlabelled IP₃ competes with a fixed amount of [³H]-labelled IP₃ for a limited number of bovine adrenal IP₃ binding proteins. The radioactivity measured is, therefore, inversely proportional to the IP₃ content of each sample.

The samples from the experiment (performed in duplicate) were assayed with working standards so that an IP₃ standard curve could be produced. Briefly, assay buffer (100 µl), experimental samples and standards (100 µl), diluted tracer (100 µl) and binding protein (100 µl) were added to all tubes, vortexed and left to incubate on ice (15 min). In addition to these samples, a total counts tube (TC) (assay buffer 100 µl, deionized water 200 µl, diluted tracer 100 µl), a zero standard tube (B₀) (assay buffer 100 µl, deionized water 100 µl, diluted tracer 100 µl, binding protein 100 µl), and a non-specific binding tube (NSB) (assay buffer 100 µl, stock standard 100 µl, diluted tracer 100 µl, binding protein 100 µl) were also prepared in duplicate. All tubes except TC were then centrifuged (2000 g, 10 min, 4 °C). Following centrifugation, the tubes were placed in decantation racks and the supernatant discarded. The tubes were inverted, placed on absorbent tissue and allowed to drain (2 min). The inverted tubes were then firmly blotted, and any droplets of liquid removed from inside the tube by using suitable swabs. Care was taken not to disturb the pellet during this process. NaOH (0.15 M, 1 ml) was then added to all tubes, except TC, the mixture vortexed, ensuring full resuspension of each pellet and left to incubate (RT, 10 min). Acetic acid (10 %, 50 µl) was then added to each tube, except TC, the mixture vortexed again, and decanted immediately into scintillation vials. Scintillation fluid (Optiphase; LKB; 10 ml) was added, and the radioactivity measured by β-scintillation spectrometry (4 min/sample).

The results were expressed as below.

\[
\% \frac{B}{B₀} = 100 \times \frac{(B \text{ cpm} - \text{NSB cpm})}{(B₀ \text{ cpm} - \text{NSB cpm})}
\]

Where

B = Sample or standard reading
Using the IP₃ standards, it was possible to plot a standard curve, allowing the IP₃ content of each sample to be determined. Values were expressed as pmol per 10⁶ mast cells. Preliminary samples and dilutions were tested prior to performing the assay to ensure that the concentration of IP₃ in the unknown samples fell in the concentration range (0.19 to 25 pmol/tube). If necessary, dilutions were completed to verify that the sample IP₃ levels coincided with the standard curve.

2.11 Active sensitisation (Rats)

2.11.1 Sensitisation of rats with the nematode *Nippostrongylus brasiliensis*

Rats (150-200 g) were sensitised by subcutaneous injection of the third stage larvae (L₃, 2500) of *Nippostrongylus brasiliensis* in sterile physiological saline (SPS). The larvae were obtained either from faecal cultures of previously infected rats (as described below) or generously provided by Mr. S. Pickersgill, Department of Applied Biology, University of Leeds. The rats were ready for experimentation 21 days post injection and remained sensitised for a further 3-4 weeks.

2.11.2 Preparation of L₃ of *Nippostrongylus brasiliensis*

Faeces from the rats were collected on days 5-8 following injection of the 2500 L₃ larvae. They were washed with tap water and then gently ground with an equal weight of activated granular charcoal until homogenous. The mixture was then transferred to moistened filter paper contained in covered petri dishes and incubated in the dark (2 weeks, 27 °C). The mixture was checked every two to three days to ensure that it remained moist. The larvae were isolated by filtering the mixture through two layers of cotton gauze interleaved with a layer of lens tissue, which had been fitted into a glass funnel containing warm tap water (37 °C). The larvae were allowed to sediment (60-90 min) into graduated test tubes. They were then washed three to four times in warm
physiological saline to remove any residual charcoal, before being resuspended in an appropriate volume of saline to give a concentration of 10,000 worms / ml.

2.12 Active sensitisation (Guinea pigs)

Guinea pigs were sensitised by subcutaneous injection of pertussis toxin (20,000 / ml, 0.5 ml) containing ovalbumin (5 mg/ml). A booster injection was given the following day. The animals were ready for experimentation 21 days post injection and remained sensitised for a further 3-4 weeks.

2.13 Passive sensitisation

Cell suspensions were washed twice and resuspended in warm BSA-FHT (2 rats/ml). Cells were incubated (1-2 h, 37 °C) with mouse monoclonal anti-dinitrophenol antibody (anti-DNP, 1 μg/ml). Mouse anti-DNP is an IgE antibody previously shown to bind to rat mast cells and to facilitate mast cell degranulation upon antigen challenge [352]. Following sensitisation the cells were centrifuged (100 g, 5 min, 4 °C), washed twice and resuspended in warm FHT. Cell aliquots were then incubated with test agonists and challenged simultaneously with human serum albumin dinitrophenol (HSA-DNP). Fifteen minutes after challenge, cells were sedimented by centrifugation (100 g, 2.5 min, 4 °C).

2.14 Smooth muscle preparations

2.14.1 Rat duodenum

Rats were killed by cervical dislocation and the duodenum dissected out by cutting at the base of the pylorus and at 1.5 cm from that point. Any connective tissue was then removed and the duodenum washed thoroughly in Krebs solution. The tissues were mounted in organ baths (5 ml), aerated with 95 % O₂/ 5 % CO₂ and maintained at 35 °C [353].
A resting tension of 1 g was applied to the tissues, and isometric responses recorded with a transducer attached to a chart recorder. Tissues were equilibrated (45-60 min) before addition of drugs.

The inhibitory responses to the test agonists were quantified by pre-contracting the tissue to carbachol (0.5 µM) before challenging with the agonist. The relaxation was expressed as the percentage inhibition of the carbachol-induced contraction. The test agonists were added 15 min before challenge with carbachol. For the antagonist studies, the tissues were incubated with the antagonist (15 min) before challenge with the agonist (15 min) prior to addition of carbachol.

2.14.2 Guinea Pig trachea

The animals were sacrificed by cervical dislocation and the trachea removed and placed in Krebs solution. Any extraneous tissue was then removed and the trachea cut into segments of 3-4 mm. These segments were suspended in organ baths (25 ml) aerated with 95 % O₂/ 5 % CO₂ and maintained at 35 °C. A resting tension of 1 g was applied to the tissues, and isometric responses recorded with a transducer attached to a chart recorder. Tissues were equilibrated (60 min) before addition of drugs.

To ascertain tissue viability, the trachea was first contracted to histamine (cumulatively) until a maximum was reached. The tissue was then washed, and following recovery of basal tone, increasing concentrations of the adenosine agonists were added cumulatively to the bath. The results are expressed as contractile response (g).

2.15 Materials

2.15.1 Secretagogues

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human IgE</td>
<td>Dako Pharmaceuticals</td>
</tr>
<tr>
<td>Anti-rat IgE</td>
<td>ICN Biochemicals</td>
</tr>
<tr>
<td>Calcium ionophore A23187</td>
<td>Sigma</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>Sigma</td>
</tr>
<tr>
<td>Con A</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dextran 70 (110,000 MW)</td>
<td>Pharmacia Fine Chemicals</td>
</tr>
</tbody>
</table>
HSA-DNP Sigma
Lyso-phosphatidylserine Sigma
Mastoparan Neosystems Ltd
NGF Sigma
Substance P Neosystems Ltd

2.15.2 Inhibitors

2-deoxy-D-glucose Sigma
Antimycin A Sigma
DSCG Fisons

2.15.3 Adenosine agonists

Adenosine Sigma
APNEA Glaxo Wellcome
2-\(p\)-(2-\(\text{Carboxyethyl}\))phenethylamino-5'-\(\text{N}\)-ethylcarboxamidoadenosine hydrochloride (CGS21680) Research Biochemicals Inc.
\(N^6\)-Cyclopentyladenosine (CPA) Sigma
\(N\)-[\(2\)-Methylphenyl]methyl]-adenosine (Metrifudil) Glaxo Wellcome
5'-\(\text{N}\)-Ethylcarboxamidoadenosine (NECA) Sigma
\(R(-)N^6\)-(2-\(\text{Phenylisopropyl}\))adenosine (R-PIA) Sigma

2.15.4 Adenosine antagonists

8-\(\text{Phenyltheophylline (8PT)}\) Research Biochemicals Inc.
\((E)-3-(1,2,3,6\)-tetrahydro-2,6-dioxo-1,3-dipropyl-9H-purin-8-yl)phenyl) acrylic acid (BW3911) Glaxo Wellcome
9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazin-5-amine (CGS15943) Glaxo Wellcome
8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) Sigma
2.15.5 Materials for buffers

Calcium chloride 2-hydrate
D- (+)-glucose
Heparin
HEPES
Hydrochloric acid (analar)
Magnesium sulphate
pH standards
Potassium chloride
Potassium dihydrogen orthophosphate
Sodium chloride
Sodium dihydrogen orthophosphate
Sodium hydrogen carbonate
Sodium hydroxide (Analar)

2.15.6 Solvents

Butan-1-ol
Chloroform
Dimethylsulphoxide (DMSO)
n-Heptane
Methanol

2.15.7 Miscellaneous compounds

Absorbent gauze
Activated charcoal (particle size 0.85 -.170 mm)
Anti-DNP
Bovine serum albumin (BSA)
Collagenase (Type 1A)
Dextran (6 %) 70 intravenous infusion BP in 0.9 % NaCl
Digitonin
Fluo-3-AM
Hyaluronidase (Type 1-S) Sigma
IP$_3$ [${}^3$H] assay kit Amersham p.l.c.
Isotonic saline (0.9 % NaCl) Baxter Healthcare Ltd.
Lens tissue Whatman Ltd.
Nytex gauze R. Cadisch & Sons.
OPT Sigma
Ovalbumin Sigma
Perchloric acid (Analar) BDH Laboratory Supplies
Percoll Pharmacia Fine Chemicals.
Phosphate buffered saline (PBS) Sigma
Probenecid Sigma
Toluidine blue BDH Laboratory Supplies
Triton-X-403 BDH Laboratory Supplies

2.16 Stock solutions of drugs used

2.16.1 Secretagogues

Anti-rat IgE was obtained in lyophilised form. It was dissolved in distilled water (2 ml) and the resultant solution was then aliquoted into microcentrifuge tubes and stored (-20 °C) until required. Anti-human IgE was obtained in liquid form and diluted when needed. Compound 48/80 was dissolved in distilled water (1 mg/ml) and kept in the refrigerator for up to one week. Con A (1 mg/ml FHT) and dextran (200 mg/ml FHT) were made when needed. Stock solutions of mastoparan, substance P (5 mM) and DNP-HSA (10 mg/ml) were made by dissolution in distilled water. Anti-DNP (0.2 mg, w/v) was diluted ten fold in PBS, calcium ionophore stock (10 mM) was prepared by dissolving in DMSO, NGF (10 μg/ml) in FHT and lyso-phosphatidylinerine (10 mg/ml) in 95 % chloroform, 5 % methanol. All these drugs, once aliquoted, were stored (-20 °C) until required.
2.16.2 Adenosine compounds

Adenosine stock (2 mM) was made when required by dissolving in HCl (1 M, 50 µl), neutralising with NaOH (1 M, 50 µl) and then diluting in warm FHT.

NECA and R-PIA (0.2 M) were prepared by dissolving in DMSO, APNEA (0.2 M), IBMECa (0.02 M), Metrifudil (0.02 M) and CPA (0.02 M) in warm HCl (1 M, 1 M, 0.1 M and 0.01 M respectively), and CGS21680 (0.02 M) in warm NaOH (0.1 M).

BW3911 and 8PT (0.2 M) were dissolved in warm NaOH (1 M) and CGS15943 (0.02 M) in DMSO. DPCPX (0.2 M) was dissolved in DMSO (100 µl) and heated under the hot tap, before adding NaOH (1 M, 50 µl). The solution was then heated again before diluting in distilled water (850 µl).

All these drugs (except adenosine), once aliquoted, were frozen (-20 °C) until needed. It was ensured that the final concentration of DMSO, HCl and NaOH in each sample was less than 0.1 % as concentrations greater than this may have affected histamine release.

2.17 Numerical analysis

All values given are mean ± standard error of that mean (SEM) for the number (n) of experiments undertaken. Hence the points on the graph represent the mean, and the vertical error bars show the SEM. All values have been corrected for the spontaneous release. Statistical analysis of results was carried out using the student’s t-test for the difference of two independent means. Where appropriate a paired t-test was performed. Significant differences of p < 0.05, p < 0.01 and p < 0.001 are denoted by *, ** and ***, respectively.
Fig. 2.1. The reaction of histamine with OPT

\[
\text{OPT} + \text{histamine} \rightarrow \text{Condensation product}
\]
Fig. 2.2. Structure of fluo-3
Chapter 3

Mast cell heterogeneity in the rat
Marquardt et al. [355] were the first to show that adenosine had the ability to enhance mediator release induced by various secretagogues from isolated rat mast cells. They reported that adenosine (1 nM – 0.1 mM) had no effect on the spontaneous release, but significantly potentiated release induced by anti-IgE, con A, 48/80 and calcium ionophore A23187. Interestingly, they found that adenosine did not alter the activation period, i.e. the time between application and onset of mediator release, but did affect the rate of release. They were unable to determine the biochemical basis for the potentiation but postulated that it involved changes in cAMP metabolism, a theory later disputed by Leoutsakos et al. [254].

Further studies by Nishibori et al. [356] showed that both adenosine and its R-site analogue, N^methyladenosine, had a bi-phasic effect on anti-IgE induced histamine release. They observed inhibition at low concentrations (20 %, 10 nM adenosine) and potentiation at high concentrations (30 %, 10 μM adenosine). Surprisingly, when looking at mediator release induced by compound 48/80 and α-chymotrypsin, the potentiatory arm of the response was abolished. Instead adenosine produced a dose-dependent inhibition of secretion, the effect being significant at concentrations of 33 μM and above. Typical levels of inhibition (10 mM adenosine) were 40 % and 30 % for the two secretagogues respectively, at control histamine releases of 40 %. Furthermore, adenosine (100 nM – 1mM) was found to have no effect on substance P and neurotensin induced histamine release.

Hence, the effects of adenosine on rat mast cell degranulation have been the source of some contradictory results. Marone et al. [347] reported that in human mast cells and basophils, the effects of adenosine varied according to the mast cell phenotype. Since, as discussed in chapter 1, heterogeneity between different cell types tends to have either biological or clinical significance [75][357], it was the aim of this study to try to clarify the role of adenosine in mast cell activation by studying the effects of the nucleoside and its
more potent, non-selective agonist NECA on a wide variety of different secretagogues, using mast cells from three different rat strains.

3.2 Materials and methods

All the materials and methods employed in this study are described in chapter 2. The protocol for potentiation studies, and the equation used to determine potentiation is given in section 2.6.3.

3.3 Results

Rat mast cells from all three strains were incubated in the presence of adenosine (0.01 – 1000 μM) and NECA (0.001 – 100 μM) for 10, 15 and 20 min at 37 °C. No effect on the spontaneous release was observed at these concentrations (i.e. the release with the agonists was equal to the spontaneous release ± 2 %). It was not possible to use 1000 μM NECA since at this concentration the agonist produced significant histamine release (approximately 60 %).

3.3.1 The effects of adenosine and NECA on anti-IgE induced histamine release from rat peritoneal mast cells (RPMC)

PMC from rats which had previously been sensitised to the nematode *Nippostrongylus brasiliensis* were challenged simultaneously with anti-IgE and either adenosine or NECA.

In the Sprague-Dawley rats, adenosine inhibited (< 20 %) the anti-IgE mediated response at relatively low concentrations (0.01 – 1 μM) and potentiated at 100 μM (< 20 %, Fig. 3.1). These results were not significant except with the lowest concentrations of adenosine and were consistent for both dilutions of anti-IgE.

NECA also had a dual effect on anti-IgE induced histamine release, but the potentiation observed was far greater (Fig. 3.2). A slight inhibition (< 10 %) was observed with low concentrations (0.001 – 0.1 μM) and potentiation at high concentrations (1– 100 μM), with the maximum enhancement at 10 μM. The percentage potentiation was dependent
on the control release, with a greater potentiation being seen at lower levels of control release. Maximum potentiations were $49.9 \pm 12.4\%$, $30.8 \pm 7.3\%$ and $29.9 \pm 10.4\%$ at control releases of $21.0 \pm 2.3\%$, $28.3 \pm 4.0\%$ and $35.5 \pm 4.7\%$, respectively.

When the experiments were repeated using Wistar rats, (Figs 3.3 and 3.4), neither agonist produced inhibition of the anti-IgE response at low concentrations. Both compounds dose-dependently potentiated the release induced by the secretagogue, with maximum enhancement observed with 100 μM adenosine ($76.3 \pm 18.1\%$, control release 16.9 ± 3.2 %) and 10 μM NECA ($270.3 \pm 48.6\%$, control release 8.5 ± 0.7 %).

The response observed with adenosine in Hooded Lister rats was different from either of the other two rat strains (Fig. 3.5). Adenosine had a slightly greater effect than with the Sprague-Dawley rats, but far less than with the Wistars. Inhibition was again observed with low adenosine concentrations (0.01 – 0.1 μM) and potentiation at high concentrations (10 – 100 μM). Maximum potentiation was $26.6 \pm 7.4\%$, control release 13.7 ± 1.4 %. Surprisingly, with an anti-IgE dilution of 1/100, inhibition was observed with all adenosine concentrations. The results with NECA (Fig. 3.6) were similar to those seen in the Sprague-Dawley rats. Maximum potentiation with NECA (100 μM) was $52.8 \pm 7.5\%$, control release 13.2 ±1.4 %.

### 3.3.2 The effect of adenosine on calcium ionophore A23187 induced histamine release from RPMC

Fig. 3.7 shows that adenosine also had a dual effect on calcium ionophore A23187 induced histamine release from PMC of Sprague-Dawley rats, with inhibition at low concentrations (0.01 and 0.1 μM) and potentiation at high concentrations (10 – 1000 μM). Maximum potentiation with adenosine (100 μM) was $80.9 \pm 15.9\%$, control release $18.3 \pm 8.1\%$.

These preliminary experiments showed that the potentiation induced by adenosine and NECA at high concentrations of the agonists was the most significant part of the response. We decided therefore to investigate the effects of adenosine (10 – 1000 μM) and NECA (1 – 100 μM) on a wide range of secretagogues, in all three rat strains. Control releases were maintained at about 30 %, to enable direct comparisons to be
made. The main aim of the study was to determine whether the effects of adenosine and NECA were universal to all stimuli.

3.3.3 The effect of adenosine and NECA on 48/80, mastoparan and substance P induced histamine release from RPMC

Neither adenosine nor NECA were strikingly effective at potentiating histamine release induced by the basic secretagogues from Sprague-Dawley rats (Figs 3.8 and 3.9). Typical levels of potentiation with adenosine (1000 μM) were 48/80 (10.4 ± 3.3 %), mastoparan (19.2 ± 2.2 %) and substance P (25.4 ± 4.7 %). With NECA (10 μM), potentiation for the secretagogues was 48/80 (23.0 ± 6.3 %), mastoparan (7.8 ± 3.8 %) and substance P (1.9 ± 3.5%).

When the experiment was repeated for Wistar rats (Figs. 3.10 and 3.11), the potentiation was still small (relative to the enhancement with anti-IgE) but greater than that observed in the Sprague-Dawley rats. Maximum potentiation levels observed with the agonists were 48/80 (100 μM adenosine, 33.8 ± 4.9 %), mastoparan (10 μM NECA, 17.9 ± 4.9 %) and substance P (1000 μM adenosine, 42.9 ± 5.8 %).

No consistent potentiation was achieved with either agonist in Hooded Lister rats (Figs 3.12 and 3.13).

3.3.4 The effect of adenosine and NECA on dextran, NGF and calcium ionophore A23187 induced histamine release from RPMC

Figs 3.14 and 3.15 show the effects of adenosine and NECA on dextran, NGF and A23187 induced histamine release in the Sprague-Dawley rats. All three stimuli were significantly potentiated by both agonists. Typical levels of potentiation (100 μM adenosine) were dextran (59.4 ± 17.5 %), NGF (40.9 ± 10.0 %) and A23187 (56.4 ± 16.1 %). The enhancement produced by NECA (10 μM) was similar, dextran (81.1 ± 18.9 %), NGF (58.8 ± 15.2 %) and A23187 (49.9 ± 16.4 %).

Figs 3.16 and 3.17 show the results for the same experiment in Wistar rats. Again, both adenosine and NECA potentiated histamine release induced by all three secretagogues,
but this time the potentiation was greater. Potentiation with adenosine (100 μM) and NECA (10 μM) was dextran (82.2 ± 11.6 %, 99.2 ± 14.5 %), NGF (50.9 ± 9.1 %, 56.3 ± 10.2 %) and A23187 (88.8 ± 8.7 %, 98.3 ± 12.2 %) respectively.

With Hooded Lister rats (Figs 3.18 and 3.19), adenosine and NECA potentiated histamine release induced by dextran and A23187, but had only a small effect on NGF induced release. Potentiation with adenosine (100 μM) and NECA (10 μM) was dextran (102.8 ± 6.2 %, 128.8 ± 10.1 %), NGF (30.0 ± 7.5 %, 29.8 ± 10.8 %) and A23187 (68.2 ± 10.1 %, 96.9 ± 17.2 %) respectively.

3.3.5 The effect of adenosine and NECA on con A induced histamine release from RPMC

The con A response in the Sprague-Dawley rats was potentiated more that 120 % by both agonists (Figs 3.20 and 3.21). In the Wistar rats, maximum potentiation with adenosine (1000 μM) and NECA (100 μM) of histamine release induced by con A was 84.6 ± 15.3 % and 93.4 ± 18.4 % respectively.

The effects of adenosine and NECA on anti-IgE induced histamine release in both Sprague-Dawley and Wistar rats were included for direction comparison, and are in agreement with the findings discussed in section 3.3.1.

The problem of sufficiently sensitising the Hooded Lister rats prevented the study of con A induced histamine release in this rat strain, since it was not possible to obtain control releases in the order of 20 – 30 %.

3.4 Discussion

The results from this study provide conclusive evidence that extracellular adenosine can significantly regulate mast cell activation at physiological concentrations. In keeping with all previous reports, we found that adenosine failed to produce mast cell degranulation in the absence of a secretagogue, but could significantly modulate induced histamine release.
The initial experiments on the effects of adenosine and NECA on anti-IgE induced histamine release from the three rat strains indicate yet another important example of mast cell heterogeneity. Whilst both agonists had a bi-phasic effect on anti-IgE induced release from Sprague-Dawley rats (i.e. inhibition at low concentrations and potentiation at high), in the Wistar rats only dose-dependent potentiation was observed. In peritoneal mast cells taken from Hooded Lister rats, the results were similar to those seen with the Sprague-Dawleys, except when an anti-IgE dilution of 1/100 was used. At this concentration, inhibition of the secretagogue induced release was observed with all adenosine concentrations. Since Hooded Lister rats are capable of producing extremely high levels of IgE, it is possible that at this dilution of anti-IgE, histamine release is already at a maximum. Any other agent will therefore have no effect or only succeed in antagonising release.

Since the inhibition observed at low agonist concentrations was small (20 – 25 % of the control release), we decided to concentrate solely on the potentiatory arm of the response. We therefore investigated the effect of the top three concentrations of the two agonists on mast cell degranulation induced by eight different secretagogues.

The main area of dispute over the effects of adenosine and its analogues has been on mediator release induced by the basic secretagogues. Marquardt et al [355] and Leoutsakos et al [254] reported that adenosine augmented compound 48/80 induced histamine release, whilst Nishibori et al [356] observed an inhibitory effect. In contrast Sydbom et al [258], whilst detecting a substantial enhancement of antigen induced release by adenosine, reported that 48/80 release was completely unaffected by exogenous concentrations of the nucleoside as high as 100 μM. Perhaps most surprising is that all these experiments were performed on Sprague-Dawley rats, and reasons for these discrepancies therefore remain to be determined.

Our results indicate that the effects of adenosine and NECA on histamine release induced by the basic secretagogues in all three rat strains are small. In the Sprague-Dawleys, maximum levels of potentiation by the agonists were between 20 and 25 %. In Wistar rats, the maximum enhancement observed was slightly higher (30 - 40 %). In
Hooded Listers, the agonists fail to produce any consistent potentiation. In contrast, both adenosine and NECA inhibited the release induced by mastoparan.

In keeping with previous reports, adenosine and NECA significantly potentiated histamine release induced by the calcium ionophore A23187, from all three rat strains. Marquardt et al \([355]\) had shown that release induced by the ionophore from Sprague-Dawley rats was enhanced considerably by adenosine, but that adenosine did not decrease the minimum concentration required to induce degranulation. Lohse et al \([358]\) noted the same effect in Wistar rats and demonstrated that the enhancing capability of adenosine and NECA was extremely variable, being very dependent on the control release in the absence of the nucleoside. Burt et al \([359]\) investigated this effect further and found no elevation of cAMP or evidence for the activation of adenylate cyclase in adenosine's mechanism of action. Forsythe et al, on investigating the effect of adenosine on A23187 induced release from Hooded Lister rats \([360]\) reported that the potentiation observed was dependent on the external calcium concentration and the time of adenosine addition. They postulated, therefore, that adenosine mediated its effects by elevating intracellular calcium levels.

Expanding the study to look at both dextran and NGF induced release, we found that adenosine and NECA also significantly potentiated (by up to 120 %) release induced by both secretagogues in all three rat strains, except in the Hooded Listers where the effect of NGF was only potentiated by 20 - 30 %.

Previous studies have shown that adenosine potentiated release induced by con A \([361,362]\), anti-IgE \([363]\) and antigen \([364]\). The ability to reverse this enhancement by the addition of theophylline was disputed by Gilfillan et al \([365]\) who found that the stimulatory effects of adenosine agonists on both histamine and leukotriene release were reversed by pre-treatment of the cells with pertussis toxin, but were unaffected by purinoreceptor antagonists and uptake blockers. Our results show that the effects of adenosine on IgE-directed stimuli again appear to be dependent on the rat strain, since although adenosine and NECA enhance con A induced release from both Sprague-Dawley and Wistar rats, they only potentiate anti-IgE induced release in Wistars.
Hence this study shows that the effects of adenosine are not only species dependent, but that there is also considerable variation within a given species. The degree of potentiation observed has been shown to be dependent on the rat strain, the stimulus used and the magnitude of the control release in the presence of the secretagogue alone. These differences probably account for at least some of the confusing and conflicting evidence observed in previous studies. Since adenosine was a universal potentiator only in Wistar rats, and since the greatest degree of enhancement was observed in this rat strain, all subsequent work was performed with PMC from Wistars.

The mechanisms of action of the secretagogues used in this study are very varied. The ability of compound 48/80, mastoparan and substance P to induce histamine release is due to their exposed positive charge linked to a hydrophobic moiety. The wide diversity in their structures means that they are unlikely to act via a classical receptor. Instead, it is postulated that the basic secretagogues initially bind to sialic acid residues on the surface of the mast cells and then interact with a pertussis toxin sensitive G-protein to cause activation of PLC \cite{121}. Calcium ionophore A23187 is an ion-carrier molecule that specifically binds calcium. It dissolves in the cell membrane and allows the transportation of calcium ions from the extracellular fluid into the cytosol, elevating the levels of intracellular calcium independently of any other secretory or gating mechanism \cite{129}. High molecular weight dextran is thought to initiate degranulation via cross-linking of glucoreceptors on the cell surface \cite{366}. NGF is postulated to induce histamine release by binding to a novel receptor on the mast cell membrane \cite{127}. Anti-IgE binds to the IgE anti-bodies on the surface of the mast cell, inducing cross-linking and hence receptor aggregation \cite{367}. Con A is a lectin that binds to the carbohydrate on the IgE molecule, inducing cross-linking and subsequent degranulation \cite{116}. Adenosine’s ability to potentiate both immunologically and non-immunologically induced mast cell degranulation implies that its effects are mediated by interference at a relatively late stage in cellular activation as both the basic secretagogues and A23187 are thought to by-pass some of the initial steps of antigen induced release.

In total, these results indicate that adenosine may be an important modulator of mast cell function. The receptor and mechanism involved is explored in subsequent chapters.
Fig. 3.1 Potentiation by adenosine of histamine release from PMC of Sprague-Dawley rats stimulated with anti-IgE (n=5, Control releases; 1/250 = 10.0 ± 0.9%, 1/100 = 12.9 ± 2.3%)

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

Fig. 3.2 Potentiation by NECA of histamine release from PMC of Sprague-Dawley rats stimulated with anti-IgE (n=4, Control releases; 1/1000 = 21.0 ± 2.3%, 1/500 = 28.3 ± 4.0%, 1/250 = 35.5 ± 4.7%)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.3 Potentiation by adenosine of histamine release from PMC of Wistar rats stimulated with anti-IgE (n=5, Control releases; 1/1000 = 16.9 ± 3.2 %, 1/500 = 24.8 ± 4.1 %, 1/250 = 30.5 ± 4.1 %)

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

Fig. 3.4 Potentiation by NECA of histamine release from PMC of Wistar rats stimulated with anti-IgE (n=5-7, Control releases; 1/2000 = 8.5 ± 0.7 %, 1/1000 = 18.1 ± 1.0 %, 1/500 = 30.1 ± 1.8 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.5 Potentiation by adenosine of histamine release from PMC of Hooded Lister rats stimulated with anti-IgE (n=4-5, Control releases; 1/500 = 13.7 ± 1.4 %, 1/250 = 19.7 ± 1.9 %, 1/100 = 17.7 ± 2.6 %)

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

Fig. 3.6 Potentiation by NECA of histamine release from PMC of Hooded Lister rats stimulated with anti-IgE (n=4-6, Control releases; 1/1000 = 13.2 ± 1.4 %, 1/500 = 21.6 ± 1.8 %, 1/250 = 33.9 ± 3.0 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.7 Potentiation by adenosine of histamine release from PMC of Sprague-Dawley rats stimulated with calcium ionophore A23187 (n=5-8, Control releases; 0.08 μM = 18.3 ± 8.1 %, 0.09 μM = 12.9 ± 3.5 %, 0.1 μM = 28.6 ± 5.4 %, 0.2 μM = 64.1 ± 7.9 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of adenosine. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.8 Potentiation by adenosine of histamine release from PMC of Sprague-Dawley rats stimulated with various basic agents (n=6, Control releases; 48/80 = 26.8 ± 1.7 %, M astoparan = 25.0 ± 2.7 %, Substance P = 27.3 ± 3.3 %)

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

Fig. 3.9 Potentiation by NECA of histamine release from PMC of Sprague-Dawley rats stimulated with various basic agents (n=6, Control releases; 48/80 = 26.8 ± 1.7 %, Mastoparan = 25.0 ± 2.7 %, Substance P = 27.3 ± 3.3 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p< 0.01, ***p<0.001)
Fig. 3.10 Potentiation by adenosine of histamine release from PMC of Wistar rats stimulated with various basic agents (n=6, Control releases; 48/80 = 29.1 ± 0.8 %, Mastoparan = 32.6 ± 3.2 %, Substance P = 25.5 ± 2.9 %)

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

Fig. 3.11 Potentiation by NECA of histamine release from PMC of Wistar rats stimulated with various basic agents (n=6, Control releases; 48/80 = 29.1 ± 0.8 %, Mastoparan = 32.6 ± 3.2 %, Substance P = 25.5 ± 2.9 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.12 Potentiation by adenosine of histamine release from PMC of Hooded Lister rats stimulated with various basic agents (n=5-6, Control releases; 48/80 = 31.4 ± 3.0 %, Mastoparan = 30.3 ± 2.8 %, Substance P = 29.4 ± 3.2 %)

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

Fig. 3.13 Potentiation by NECA of histamine release from PMC of Hooded Lister rats stimulated with various basic agents (n=5-6, Control releases; 48/80 = 31.4 ± 3.0 %, Mastoparan = 30.3 ± 2.8 %, Substance P = 29.4 ± 3.2 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.14 Potentiation by adenosine of histamine release from PMC of Sprague-Dawley rats stimulated with various agents (n=6, Control releases; A23187 = 27.7 ± 7.7 %, Dextran = 31.2 ± 3.5 %, NGF = 29.1 ± 4.2 %)

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

![Graph showing potentiation by adenosine](image)

Fig. 3.15 Potentiation by NECA of histamine release from PMC of Sprague-Dawley rats stimulated with various agents (n=6, Control releases; A23187 = 27.7 ± 7.7 %, Dextran = 31.2 ± 3.5 %, NGF = 29.1 ± 4.2 %)

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

![Graph showing potentiation by NECA](image)
Fig. 3.16 Potentiation by adenosine of histamine release from PMC of Wistar rats stimulated with various agents (n=6, Control releases; A23187 = 25.8 ± 2.8 %, Dextran = 33.9 ± 4.0 %, NGF = 30.0 ± 2.0 %)

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

Fig. 3.17 Potentiation by NECA of histamine release from PMC of Wistar rats stimulated with various agents (n=6, Control releases; A23187 = 25.8 ± 2.8 %, Dextran = 33.9 ± 4.0 %, NGF = 30.0 ± 2.0 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.18 Potentiation by adenosine of histamine release from PMC of Hooded Lister rats stimulated with various agents (n=6, Control releases; A23187 = 27.1 ± 3.7 %, Dextran = 26.9 ± 1.4 %, NGF = 30.1 ± 2.0 %)

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

Fig. 3.19 Potentiation by NECA of histamine release from PMC of Hooded Lister rats stimulated with various agents (n=6, Control releases; A23187 = 27.1 ± 3.7 %, Dextran = 26.9 ± 1.4 %, NGF = 30.1 ± 2.0 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.20 Potentiation by adenosine of histamine release from PMC of Sprague-Dawley rats stimulated with IgE-directed stimuli (n=3-6, Control releases; Con A = 21.9 ± 4.4 %, anti-IgE = 27.1 ± 3.3 %)

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

Fig. 3.21 Potentiation by NECA of histamine release from PMC of Sprague-Dawley rats stimulated with IgE-directed stimuli (n=3-6, Control releases; Con A = 21.9 ± 4.4 %, anti-IgE = 27.1 ± 3.3 %)

(Asterisks denote those releases that were significantly different from those obtained in the absence of NECA. * p<0.05, **p<0.01, ***p<0.001)
Fig. 3.22 Potentiation by adenosine of histamine release from PMC of Wistar rats stimulated with IgE-directed stimuli (n=6, Control releases; Con A = 24.6 ± 3.1 %, anti-IgE = 24.7 ± 2.6 %)

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

![Graph showing potentiation by adenosine](image)

Stimuli

Con A (5 μg/ml) | anti-IgE (1/500)

--- | ---

10 μM adenosine | 100 μM adenosine | 1000 μM adenosine

Fig. 3.23 Potentiation by NECA of histamine release from PMC of Wistar rats stimulated with IgE-directed stimuli (n=6, Control releases; Con A = 24.6 ± 3.1 %, anti-IgE = 24.7 ± 2.6 %)

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

![Graph showing potentiation by NECA](image)

Stimuli

Con A (5 μg/ml) | anti-IgE (1/500)

--- | ---

1 μM NECA | 10 μM NECA | 100 μM NECA

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Chapter 4

The adenosine receptor in the rat
4.1 Introduction

Historically, elevated levels of cAMP in the cytosol have been associated with the inhibition of mediator secretion from mast cells \[368\]. More recent studies, however, have shown that immunological activation produces a transient rise in intracellular cAMP levels prior to the onset of histamine release \[369\]. This effect is thought to be due to the coupling of IgE-Fc receptors to adenylate cyclase in the mast cell membrane.

As discussed in chapter 1, adenosine A_1 receptors inhibit adenylate cyclase, by interacting with the G_i GTP regulatory protein \[296-297\], whilst A_2 receptors activate adenylate cyclase by binding to the G_s-protein \[299-300\]. In addition to the effects that adenosine produces externally by combining with membrane bound receptors, it also binds to an internal P-site \[370\] on the inner surface of the membrane which is stimulated by agents such as 2',5'-dideoxyadenosine (DDA) \[371-372\] and antagonised by agents which prevent the uptake of adenosine into cells (e.g. dipyridamole) \[373\]. Surprisingly, stimulation by DDA suppresses both the augmentation of the transient rise in cAMP and the increased histamine release \[363\].

It seems likely, therefore, that adenosine mediates its effects by binding to a receptor on the cell surface. Enhancing or suppressing the transient elevation in cAMP with appropriate adenosine analogues produces a parallel change in histamine secretion \[254\]. Studies with theophylline, however, showed that the purinoreceptor antagonist prevented the elevation of cAMP levels seen with adenosine, NECA and PIA, both non-selective adenosine agonists, but did not affect the potentiation of histamine release. Church and Hughes had reported the same effect using 8PT \[374\]. Vardey and Skidmore \[375\] reported that not only were theophylline, 8PT and 1,3 diethyl-8-phenylxanthine all ineffective at blocking the effects of adenosine, but that NECA, L-PIA and D-PIA, all known to have differential effects on the A_1 and A_2 adenosine receptors, were all essentially equieffective at potentiating mediator release.
It seems possible, therefore, to be able to separate the effects of adenosine and its analogues on histamine release from the changes observed in cAMP levels. These results imply that the mast cell must contain at least two functional cell surface receptors, one which couples to adenylate cyclase, and one which does not.

These findings were confirmed by Marquardt and Walker \(^{[376]}\) who showed that adenosine activated adenylate cyclase and PLC in murine mast cells in addition to potentiating mediator release. Using a specific inhibitor of cAMP dependent protein kinase (KT5720), they inhibited antigen-induced release of beta-hexosaminidase and LTC\(_4\). Adenosine, however, even in the presence of KT5720, was able to potentiate beta-hexosaminidase release in both antigen and A23187 stimulated cells. This provided convincing evidence that the ability of adenosine to augment mast cell degranulation was independent of cAMP.

Marquardt and Walker \(^{[377]}\) carried out experiments on mouse bone marrow derived mast cells, and made two important discoveries. Firstly, adenosine induced the translocation of PKC from the cytosol to the membrane. In the absence of a secretagogue, this did not alter mast cell secretion. Secondly, agents which activated PKC enhanced A23187 induced beta-hexosaminidase release but induced mast cell hyporesponsiveness to adenosine. Inhibition of beta-hexosaminidase release by agents that block PKC activity also blocked the response to adenosine. They concluded that activation of PKC by itself did not necessarily induce degranulation and that in all probability an additional signal was required for mast cell exocytosis. Also, the enhancement of histamine release seen with adenosine requires an intact pathway to PKC activation. Agents which either enhance or inhibit PKC activity suppress the effects of adenosine.

Ali et al \(^{[309]}\) doing work on RBL-2H3 cells (which are believed to contain an adenosine receptor distinct from \(A_1\) and \(A_2\)) \(^{[378]}\), found that NECA caused the release of inositol phosphates and substantially increased levels of calcium in the cytosol. This transient rise in calcium was insufficient by itself to activate the secretory response, but markedly enhanced the response to antigen. They concluded that, in RBL cells, adenosine was coupled via a receptor to PLC. IgE receptors also couple to PLC but via a different G-protein. The G-protein which couples to the IgE receptor is not blocked by either
cholera or pertussis toxin, whilst the adenosine receptor coupled G-protein is inhibited by both. This is in keeping with previous reports which have shown that PLC can be activated by different stimuli acting through different G-proteins \cite{378}.

Ramkumar et al \cite{308} found conclusive evidence that the $A_3$ receptor is the only receptor present in RBL cells by using the selective $A_3$ receptor agonist APNEA. This produced little activation of the mast cell by itself, but enhanced antigen induced responses 2-2.5 fold. Northern blotting studies showed that the RNA from RBL-2H3 cells hybridised to $A_3$ adenosine receptor cDNA but not that of the $A_1$ or $A_2$ adenosine receptor cDNA probes.

It is thought that adenosine analogues cause a transient but substantial release of inositol phosphates but importantly produce no sustained influx of calcium \cite{378}, and hence are unable to induce secretion in the absence of a secretagogue. They therefore have no effect on spontaneous release, but are able markedly to potentiate sub-optimal antigen induced exocytosis.

Recent experiments by Ali et al \cite{379} have demonstrated that NECA has the ability to enhance the secretory responses to calcium ionophore and to antigen for up to 20 min after stimulation. PLC activation is transient and therefore only associated with a brief release of mediators in the early stages of activation. In addition, NECA dose-dependently activates PLD as indicated by the formation of PA. This activation was coupled to an increase in diglyceride production and PKC activity. Hence, it has been postulated that PLD responses may persist after the PLC response has subsided, and it is this, in combination with PKC activation, that best correlates with the ability of adenosine and its analogues to enhance histamine release from mast cells.

Hence, most evidence implies that it is the adenosine $A_3$ receptor which is responsible for causing degranulation in RBL-2H3 cells. It is probable, therefore, that it is also the $A_3$ receptor responsible for mediating the effects of adenosine in rat mast cells, although it is possible that more than one mast cell adenosine receptor may contribute to the complex chain of biochemical events necessary for enhancement of mediator release. The lack of specific adenosine receptor agonists and antagonists has, however, prevented full characterisation of the adenosine receptors on rat mast cells in the past.
The aim of this study was hence to identify the adenosine receptor responsible for enhancing antigen-induced mediator release from isolated rat mast cells, and to determine the second messenger system involved.

4.2 Materials and methods

All the materials and methods employed in this study are described in chapter 2.

4.3 Results

4.3.1 The effect of different adenosine receptor agonists on antigen and anti-IgE induced histamine release from PMC of Wistar rats

PMC from male Wistar rats were either sensitised to the nematode *Nippostrongylus brasiliensis* or were passively sensitised with anti-DNP. They were then challenged with anti-IgE or HSA-DNP, respectively, simultaneously with the adenosine receptor agonist. None of the agonists tested produced any effect on histamine release in the absence of a secretagogue.

For passive sensitisation, the cells were incubated for either 1 or 2 h (37 °C), in the presence of calcium (Fig 4.1). Antigen stimulation produced a concentration-dependent histamine release, reaching a maximum (20 ng/ml) of 32.1 ± 4.3 % and 39.4 ± 7.8 % at 1 and 2 h respectively. In order to achieve control releases in the order of 30 % cells were, therefore, incubated for 1 h (8 ng/ml) in all subsequent experiments.

The non-selective adenosine receptor agonist, NECA (0.001 – 100 µM), produced a concentration-dependent increase in histamine release induced by HSA-DNP (maximum enhancement 102.6 ± 9.3 %, 10 µM, Fig 4.2). The same effect was observed with each of the other agonists, with a rank order of potency of IB-MECA>metrifudil>R-PIA>NECA>CPA>CGS21680. IB-MECA was not only the most potent agonist tested, it also produced a greater potentiation of immunologic release than any of the other compounds (maximum enhancement 126.2 ± 10.7 %, 0.5 µM).
If the cells were left incubating in the bath (37 °C) for any significant period of time (> 15 min) prior to challenge with HSA-DNP and IB-MECA, then the enhancement produced by the adenosine receptor agonist was significantly reduced (Fig 4.3). Maximum enhancements were 126.2 ± 10.7 %, 0.5 μM, 66.5 ± 12.9 %, 0.1 μM and 55.5 ± 7.5 %, 1 μM at 0, 15 and 30 min pre-incubation periods respectively.

When the cells were challenged with anti-IgE and the adenosine receptor agonists (Fig 4.4), similar results were observed to those obtained with antigen. NECA (0.001 – 100 μM) produced a concentration-dependent increase in the anti-IgE response (maximum enhancement 79.5 ± 19.3 %, 10 μM). The same effect was observed with each of the other agonists, with a rank order of potency of IB-MECA > APNEA > R-PIA = CPA > NECA > CGS21680. Surprisingly, the greatest enhancement was observed with APNEA (146.3 ± 24.8 %, 1 μM). Lack of availability of the A2b agonist metrifudil, prevented its use in this experiment.

Since IB-MECA was by far the most potent compound tested, all subsequent experiments concentrated on the enhancement produced by this agonist. Purification of the mast cells (Fig 4.5) did not significantly reduce this potentiation.

4.3.2 The effect of different adenosine receptor antagonists on antigen and anti-IgE induced histamine release from PMC of Wistar rats

The adenosine receptor antagonists had no effect on the spontaneous histamine release, but BW3911 (A3) and CGS15943 (A2) dose-dependently inhibited both the antigen and anti-IgE responses (Figs 4.6 and 4.7). Maximum inhibition of HSA-DNP induced release was 69.1 ± 8.5 %, 50 μM and 60.0 ± 9.0 %, 5 μM for BW3911 and CGS15943 respectively. Lack of solubility prevented the use of concentrations greater than 5 μM for CGS15943. In comparison, 8PT (A1/A2) and DPCPX (A1) were much less effective (maximum inhibition of antigen response was 20.5 ± 8.0 %, 50 μM and 36.1 ± 8.2 %, 50 μM, respectively). Similar results were observed with the antagonists on anti-IgE induced histamine release but, in this experiment, both 8PT and DPCPX were essentially ineffective (maximum inhibitions of release were 99.0 ± 3.9 %, 50 μM, 60.8 ± 5.0 %, 5 μM, 3.7 ± 3.8 %, 50 μM and 2.5 ± 4.2 %, 50 μM, for BW3911, CGS15943, DPCPX and 8PT respectively).
4.3.3 The effect of different adenosine receptor antagonists on 48/80 induced histamine release from PMC of Sprague-Dawley and Wistar rats

Figs 4.8 and 4.9 show the effect of the antagonists on 48/80 induced histamine release from PMC of Sprague-Dawley and Wistar rats. Neither DPCPX nor CGS15943 produced any appreciable inhibition of the 48/80 response in either rat strain (Maximum inhibition = 9.5 ± 5.3 %, 50 μM and 4.9 ± 8.2 %, 5 μM for the two antagonists, respectively, in Sprague-Dawley rats and 20.7 ± 5.2, 50 μM and 18.2 ± 5.2 %, 5 μM in Wistars). BW3911 and 8PT, however, significantly inhibited the release induced by compound 48/80 in both rat strains (Maximum inhibition = 100.8 ± 2.0 %, 50 μM and 61.4 ± 2.4 %, 50 μM respectively in Sprague-Dawley rats and 105.0 ± 3.1 %, 50 μM and 51.1 ± 2.7 %, 50 μM in Wistars).

4.3.4 The effect of different adenosine receptor antagonists on the enhancement by IB-MECA of anti-IgE induced histamine release from PMC of Wistar rats

We investigated the effect of the antagonists on IB-MECA’s enhancement of anti-IgE rather than antigen induced histamine release, since the compounds were producing less non-specific antagonism in this system. BW3911, 8PT, DPCPX (10 μM) and CGS15943 (1 μM) were incubated with the cells (30 min) prior to challenge with anti-IgE and IB-MECA. At these concentrations, antagonism of the anti-IgE response by BW3911 and CGS15943 was kept to a maximum of 30 – 40 %.

8PT had no effect on the enhancement of histamine release induced by the adenosine receptor agonist (Fig 4.10). DPCPX produced a slight rightward shift of the concentration-effect curve to IB-MECA whilst CGS15943 and BW3911 produced significant shifts to the right of the dose-response curve, with the effect produced by BW3911 being the greatest (Enhancement of anti-IgE induced release was 123.8 ± 14.8 % in the presence of IB-MECA alone, 0.05 μM, and 55.2 ± 8.5 %, 70.8 ± 6.8 %, 103.7 ± 13.0 % and 105.9 ± 7.4 % with BW3911, CGS15943, DPCPX and 8PT respectively).
4.3.5 The effect of disodium cromoglycate on the enhancement by IB-MECA of calcium ionophore A23187 induced histamine release from PMC of Wistar rats

DSCG was incubated with the cells simultaneously with calcium ionophore A23187 and IB-MECA. A23187 was chosen as the secretory stimulus rather than anti-IgE since DSCG is known to dose-dependently inhibit the anti-IgE response (inhibition being significant at 10 µM and above) but to be much less active against the ionophore. Cells were challenged simultaneously with DSCG, A23187 and IB-MECA since pre-incubation of the cells with the inhibitor prior to antigen challenge irreversibly blocks the effect of DSCG.

DSCG (10 µM) completely abolished the potentiation by IB-MECA but had no effect on A23187 induced histamine release (Fig 4.11). DSCG (1 µM) reduced the potentiation of the ionophore response induced by IBMECA by approximately 50%, an effect that was unsurmountable i.e. it could not be reversed by increasing the adenosine receptor agonist concentration (Maximum enhancements with IB-MECA were 238.1 ± 25.8 %, 1 µM and 121.9 ± 11.8 %, 5 µM in the absence and presence of DSCG respectively, Fig 4.12). DSCG (0.1 µM) was ineffective at inhibiting the IB-MECA response (Fig 4.13).

4.3.6 The measurement of intracellular calcium levels in RPMC following stimulation with anti-IgE ± IB-MECA

RPMC loaded with fluo-3 dye showed an immediate and pronounced increase in fluorescence (calcium concentration) following stimulation with anti-IgE (1/200 dilution), from a base line of 0.21 ± 0.02 µM to 0.42 ± 0.03 µM, (Fig 4.14). This rise in cytosolic calcium was not enhanced by stimulating the cells in the presence of IB-MECA (0.5 µM), where the increase was from 0.24 ± 0.05 µM to 0.44 ± 0.07 µM, (Fig 4.15). In both systems, lysis of the cells with digitonin (30 µM) caused a further large increase in fluorescence.
4.3.7 The effect of IB-MECA on the kinetics of anti-IgE induced histamine release from purified PMC from Wistar rats

IB-MECA increased the rate of histamine release as well as enhancing the amount of release in anti-IgE stimulated mast cells (Fig 4.17). The release induced by anti-IgE alone (1/200) was 31.3 ± 4.2 %, reaching a maximum after 180 s. When cells were stimulated with anti-IgE (1/200) and IB-MECA (0.5 μM), maximum levels of secretion of 53.9 ± 4.0 % were achieved after 40 s.

4.3.8 The effect of IB-MECA on the kinetics and level of IP₃ production in purified PMC from Wistar rats

Since IB-MECA significantly increased the rate of histamine release, we decided to investigate its effects on the IP₃ production induced by anti-IgE. We postulated that IB-MECA was acting by augmenting calcium levels, but that the effect was too rapid and transient to be detected by the fluo-3 system. Activation of purified RPMC with anti-IgE (1/200) produced a gradual increase in IP₃ production, reaching a maximum after 20 s (baseline 85 ± 109 pmol/10⁶ cells, peak value after stimulation 438 ± 146 pmol/10⁶ cells). Cells stimulated with anti-IgE and IB-MECA showed a rapid rise in IP₃ production, reaching a maximum after 5 s before returning after 10 s to the levels observed with anti-IgE alone (baseline 149 ± 56 pmol/10⁶ cells, levels after 5 s and 10 s were 820 ± 168 pmol/10⁶ cells and 420 ± 38 pmol/10⁶ cells respectively).

4.4 Discussion

The results from this study provide conclusive evidence that adenosine agonists enhance both antigen and anti-IgE stimulated histamine release from RPMC. The majority of the experiments were performed on crude preparations of cells, which contain predominately macrophages with only a small percentage of mast cells (approximately 5 %). However, since mast cells were the only cells in the preparation that contained histamine and since purification did not significantly alter the enhancement produced by the adenosine receptor agonist IB-MECA, it is reasonable to conclude that the effects seen were mast cell mediated.
In antigen stimulated cells, the selective adenosine A$_3$ receptor agonist, IB-MECA $^{[380]}$, was approximately 10 times more potent that the non-selective agonist NECA. There was no significant difference between the potencies of the modestly selective A$_{2b}$ agonist metrifudil $^{[381]}$, NECA, and the adenosine A$_1$ receptor agonists CPA and R-PIA $^{[381]}$. The A$_{2a}$ agonist CGS21680 $^{[381]}$, however, was by far the weakest compound tested.

In anti-IgE stimulated cells, the A$_3$ agonists IB-MECA and APNEA $^{[382]}$ were approximately 100 and 50 times more potent than NECA, respectively. R-PIA and CPA were about 10 times more potent than NECA, and CGS21680 was about 10 times less potent. This rank order of potency is inconsistent with the activation of A$_1$, A$_{2a}$ or A$_{2b}$ receptors $^{[381]}$.

Surprisingly, incubating the cells in the water-bath (37 °C), prior to challenge with antigen and IB-MECA, significantly reduced the enhancement produced by the adenosine receptor agonist. In contrast, the response to antigen was unaffected. This implies either a reduction in adenosine receptor number, or a desensitisation of the receptor to adenosine.

The phenomenon of desensitisation or tachyphylaxis is known to occur following chronic stimulation of receptors by their agonists $^{[383]}$. The reverse, up-regulation of response, is often observed with long-term treatment with antagonists $^{[384-385]}$.

Longabaugh et al $^{[386]}$ found that the A$_1$ receptor adenylate cyclase system in rat adipocytes underwent heterologous desensitisation following chronic in vivo exposure to R-PIA. Following treatment for 6 days with the adenosine receptor agonist, they observed a reduction in the total number of receptors as well as uncoupling of the receptor from its G protein. Mundell and Kelly $^{[387]}$, studying the responses of different adenosine receptor ligands in NG108-15 cells, found that NECA induced desensitisation of both the A$_{2a}$ and the A$_{2b}$ receptor responses, the half-life of the desensitisation being approximately 20 min. Marquardt and Walker $^{[388]}$ observed a similar effect in mouse bone marrow derived mast cells where NECA induced a homologous desensitisation of the adenosine receptors.

Studies of chronic administration of caffeine, on the number and function of central adenosine receptors, have shown an up-regulation of central A$_1$ receptors without any
effect on the adenosine $A_1$ receptor mRNA $^{[389]}$. In contrast, $A_{2a}$ receptor binding and mRNA expression appear to be completely unaffected.

Ramkumar et al $^{[390]}$, working with RBL cells, found that dexamethasone, whilst suppressing the antigen response, augmented the cellular response to NECA by increasing IP$_3$ production and increasing degranulation. They showed that this effect was due to the upregulation of both $A_3$AR and their associated G-proteins, an effect that was optimal following 16 h of treatment.

Hence, adenosine receptors are obviously susceptible to both up and down regulation. It is possible, therefore, that cells incubated in the absence of a secretaglogue undergo receptor desensitisation due to the level of endogenous adenosine in the mast cell preparation. We observed a 50 % reduction in the response to IB-MECA following pre-incubation of the cells for 15 min. Incubating the cells for 30 min, resulted in a further reduction in the response. These results are in keeping with the findings by Mundell and Kelly $^{[387]}$. However, these authors used very high concentrations of NECA (10 μM and 100 μM) for studying desensitisation of the $A_{2a}$ and the $A_{2b}$ receptors, respectively. It is possible, therefore, that the adenosine $A_3$ receptor undergoes desensitisation at much lower agonist concentrations than either of the $A_2$ receptors.

The non-selective inhibition by the antagonists means that interpretation of these data has to be carried out with a certain degree of caution. The reason for this effect is unknown, but it is unlikely to be due to blocking the effects of endogenous adenosine since 8PT at micromolar concentrations significantly inhibited 48/80 induced histamine release in Sprague-Dawley rats, a system that is relatively insensitive to the actions of adenosine (section 3.3.3). The considerable inhibition of 48/80 induced histamine release by BW3911 is most likely to be due to simple chemical antagonism i.e. BW3911 is a negatively charged molecule, whilst 48/80 is highly positively charged. The $A_3$ antagonist produces maximum inhibition at concentrations of 5 μM and above. At these concentrations, the molar ratio of BW3911 to 48/80 in the extracellular fluid will be approximately 10:1.

Theophylline and other alkylxanthines block histamine release from RPMC by inhibition of phosphodiesterase activity resulting in augmented levels of cAMP in the cytosol, in addition to antagonism at adenosine receptors $^{[391]}$. The phosphodiesterase-inhibiting capacity of these compounds varies considerably depending on their structure. DPCPX is only a very weak phosphodiesterase inhibitor and requires
concentrations 10,000 fold higher for inhibition of phosphodiesterase activity than those required for antagonism at the adenosine $A_1$ receptor. 8PT, however, has been shown to be a much more potent phosphodiesterase inhibitor and this may account for the inhibition of 48/80 induced release by this agent in both Sprague-Dawley and Wistar rats.

In antigen and anti-IgE stimulated rat peritoneal mast cells, BW3911 and CGS15943, produced significant inhibition, with CGS15943 being approximately ten times more potent than BW3911. Ali et al. [392], working on RBL-2H3 cells, found that certain xanthines, whilst being unable to antagonise the binding of adenosine to the $A_3$ receptor, did interfere with the antigen-induced generation of IP$_3$ and increased Ca$^{2+}$ levels. In addition, the same compounds inhibited the binding of the antigen to cell attached DNP-specific IgE. It is possible, therefore, that BW3911 could be acting via a similar mechanism. The reason for the inhibition by CGS15943, however, remains to be determined since it is a non-xanthine adenosine antagonist with no known phosphodiesterase activity [393].

Hence, although the mechanism of the inhibition of antigen and anti-IgE induced histamine release is still unknown, the antagonist data support the hypothesis that the enhancement of histamine release induced by the adenosine receptor agonists is not due to activation of either $A_1$ or $A_2$ receptors. 8PT at concentrations that block both $A_1$ and $A_2$ receptors [394] failed to diminish the IB-MECA response. DPCPX, the $A_1$ receptor antagonist [394], also failed significantly to inhibit the potentiation produced by the $A_3$ adenosine receptor agonist. However, the $A_3$ antagonist BW3911 produced a significant shift to the right of the concentration effect curve. The enhancement of anti-IgE induced release by IB-MECA was also significantly blocked by CGS15943, and the reason for this remains unclear. Reeves et al. [395] observed the same effect on 5HT release from pleural cells, but reported that CGS15943 had no inhibitory effect on the responses to IB-MECA in vivo.

These data, taken together with the agonist results, suggest that the potentiation of antigen and anti-IgE induced histamine release from RPMCs is via activation of the adenosine $A_3$ receptor.
DSCG has been widely used for the treatment of bronchial asthma, since its introduction in 1968, although its exact mechanism of action is still unknown. It has been shown to inhibit both immunological and non-immunological release from rat mast cells[^396-397] and blocks antigen induced degranulation in human lung mast cells[^398]. Cross linking of IgE receptors leads to the rapid phosphorylation of several proteins which may be involved in the induction of histamine release[^399-400]. Subsequent later phosphorylation of a 78,000 protein is thought to terminate the secretory process and to prevent further degranulation[^278]. It is thought that DSCG acts by phosphorylation of this 78,000 protein[^279][^281], i.e. it acts by utilising the cell’s natural switch-off mechanism. This would explain why DSCG has no known side effects.

In addition to blocking immunologic mediator release, DSCG also inhibits adenosine induced bronchoconstriction[^316]. Cushley et al[^401] found that inhaled DSCG had no effect on airway responsiveness to histamine but did protect against adenosine induced bronchoconstriction in four of the seven subjects studied. They postulated that DSCG was inhibiting adenosine induced bronchoconstriction via a mechanism other than mast cell stabilisation. Our results show that DSCG blocks the enhancement by IB-MECA of the calcium ionophore response at concentrations too low to affect either immunological or non-immunological release. This effect is, however, unsurmountable, and is therefore not due to simple competitive antagonism at the adenosine A3 receptor.

Having studied the nature of adenosine’s effect on mast cell degranulation, we then went on to try to determine the second messenger system involved.

IgE activation initiates a series of biochemical events that result in mast cell degranulation. Cross linking of the receptor by the appropriate ligand induces activation of a G-protein, which then stimulates PLC to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP2) to form IP3 and DAG[^402-403]. IP3 then liberates calcium ions from intracellular stores[^248][^403], whilst DAG activates PKC[^252][^404]. Calcium mediates its effects by activating specific calcium binding proteins, of which calmodulin is the most important[^405]. All eukaryotic cells contain calmodulin, which can activate numerous protein kinases, phosphatases, cyclic nucleotide phosphodiesterases and ATPases[^243-244].
The studies with fluo-3 show that anti-IgE stimulation of mast cells leads to a transient increase in intracellular calcium levels and subsequent mast cell degranulation. Although there was an increase in histamine release when the experiment was repeated with anti-IgE and IB-MECA, no augmentation of calcium levels was observed. This was in contrast to Tinton et al [406] who had noted that adenosine in Fura-2-loaded hepatocytes increased intracellular calcium levels, both through activating release from internal stores and via calcium entry from the extracellular medium.

Studies on the kinetics of histamine release showed that addition of IB-MECA rapidly increased the rate of degranulation. We postulated, therefore, that intracellular calcium levels were augmented in the presence of IB-MECA, but that the effect was too rapid and transient to be detected by the fluo-3 system.

Measurement of IP$_3$ levels confirmed this. Anti-IgE produced a temporary increase in intracellular IP$_3$, reaching a maximum after 20 s. When cells were stimulated with anti-IgE and IB-MECA simultaneously, a rapid rise in IP$_3$ levels resulted that peaked after 5 s, dropping to the levels observed with anti-IgE alone after 10 s. It would appear, therefore, that this large increase in IP$_3$ production, although transient, is sufficient to enhance mediator release.

In keeping with all previous reports, we found that the receptor agonists by themselves were unable to induce mast cell degranulation. Assem et al [407] working on individual RBL-2H3 cells, found that antigen stimulation produced asynchronous Ca$^{2+}$ oscillations or irregular spikes, an effect that was dependent on the presence of extracellular calcium. NECA produced a transient rise in intracellular calcium, but could not produce a maintained oscillatory response.

In conclusion, the present study indicates that adenosine receptor agonists induce histamine release from rat peritoneal mast cells via stimulation of adenosine A$_3$ receptors, leading to an increase in IP$_3$ production and probably a transient augmentation in intracellular calcium levels. This is consistent with reports that stimulation of adenosine A$_3$ receptors enhances allergic mediator release from RBL-2H3 cells [308] and indirectly produces hypotension [408] and bronchoconstriction [409] in the rat via mast cell degranulation.
Fig. 4.1 Histamine release from PMC of Wistar rats following passive sensitisation with anti-DNP and then stimulation with HSA-DNP (n=3)

![Histamine release graph](image1)

Fig. 4.2 The effect of different adenosine receptor agonists on antigen induced histamine release from passively sensitised PMC of Wistar rats (n=6, Control release = 27.2 ± 1.4 %)

![Agonist effect graph](image2)
Fig. 4.3 The effect of incubation at 37 °C, for different time periods on PMC of Wistar rats, prior to challenge with IB-MECA and HSA-DNP (n=5-6, Control release = 25 – 30 %)
Fig. 4.4 The effect of different adenosine receptor agonists on anti-IgE induced histamine release from PMC of Wistar rats (n=6, Control release = 24.6 ± 1.4 %)

![Graph showing the effect of different adenosine receptor agonists on histamine release.](image)

Fig. 4.5 The effect of purification on IB-MECA's enhancement of anti-IgE induced histamine release from PMC of Wistar rats (n=6, Control releases; non-purified = 24.6 ± 1.4 %, purified = 23.2 ± 5.6 %)

(Purity = 97.1 ± 0.6 %)

(The releases from non-purified and purified cells were not significantly different)

![Graph showing the effect of purification on IB-MECA's enhancement of histamine release.](image)
Fig. 4.6 The effect of different adenosine receptor antagonists on HSA-DNP induced histamine release from PMC of Wistar rats (n=6-7, Control release = 21.9 ± 2.3 %)

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

Fig. 4.7 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from PMC of Wistar rats (n=6, Control release = 29.9 ± 2.3 %)

![Graph showing inhibition of histamine release](image)
Fig. 4.8 The effect of different adenosine receptor antagonists on 48/80 induced histamine release from PMC of Sprague-Dawley rats (n=5, Control release = 35.4 ± 3.0 %)

Fig. 4.9 The effect of different adenosine receptor antagonists on 48/80 induced histamine release from PMC of Wistar rats (n=6, Control release = 37.7 ± 6.0 %)
Fig. 4.10 The effect of different adenosine receptor antagonists on the enhancement by IB-MECA of anti-IgE induced histamine release from PMC of Wistar rats (n=6, Control releases = 18 - 23 %)

![Graph showing the effect of different adenosine receptor antagonists on IB-MECA enhanced histamine release from PMC of Wistar rats.](image)

Fig. 4.11 The effect of disodium cromoglycate (10 μM) on the enhancement by IB-MECA of A23187 induced histamine release from PMC of Wistar rats (n=8-12, Control release = 16.4 ± 1.5 %)

![Graph showing the effect of DSCG on IB-MECA enhanced histamine release.](image)
Fig. 4.12 The effect of disodium cromoglycate (1 μM) on the enhancement by IB-MECA of A23187 induced histamine release from PMC of Wistar rats (n=8–9, Control releases; A23187 only = 16.8 ± 1.6 %, + DSCG = 17.1 ± 1.7 %).

Fig. 4.13 The effect of disodium cromoglycate (0.1 μM) on the enhancement by IB-MECA of A23187 induced histamine release from PMC of Wistar rats (n=9, Control releases; A23187 only = 15.1 ± 5.6 %, + DSCG = 14.8 ± 1.7 %).
Fig. 4.14 Measurement of intracellular calcium in RPMC containing fluo-3 treated with anti-IgE (1/200 dilution)

(Traces are representative of at least 3 similar recordings)

Fig. 4.15 Measurement of intracellular calcium in RPMC containing fluo-3 treated with anti-IgE (1/200 dilution) + IB-MECA (0.5 μM)

(Traces are representative of at least 3 similar recordings)
Fig. 4.16 The changes in intracellular calcium levels induced by anti-IgE ± IB-MECA

(The changes in intracellular calcium levels induced by anti-IgE and IB-MECA together were not statistically different from the changes induced by anti-IgE alone)

<table>
<thead>
<tr>
<th></th>
<th>anti-IgE (1/200)</th>
<th>anti-IgE (1/200) + IB-MECA (0.5 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca] Resting</td>
<td>0.21 ± 0.02 μM</td>
<td>0.24 ± 0.05 μM</td>
</tr>
<tr>
<td>[Ca] Activated</td>
<td>0.42 ± 0.01 μM</td>
<td>0.44 ± 0.07 μM</td>
</tr>
<tr>
<td>Change in [Ca]</td>
<td>0.21 ± 0.03 μM</td>
<td>0.20 ± 0.04 μM</td>
</tr>
</tbody>
</table>

Fig. 4.17 The effect of IB-MECA on the kinetics of anti-IgE induced histamine release from purified PMC of Wistar rats (n=3, Maximum releases; anti-IgE = 31.3 ± 4.2 %, 180 s, anti-IgE + IB-MECA = 53.9 ± 4.0 %, 40 s)

(Purity = 93.3 ± 0.7 %)
Fig 4.18 IP₃ Standard Curve
Fig. 4.19 The effect of IB-MECA on the kinetics and level of IP₃ production in purified PMC from Wistar rats (n=3) (Purity = 93.3 ± 0.7 %)

(Asterisks denote those reaction times where the IP₃ accumulation produced by stimulation with anti-IgE was significantly different in the presence of IB-MECA, to that obtained with anti-IgE alone.

* p<0.05)
Chapter 5

The adenosine receptor in the mouse
Chapter 5

5.1 Introduction

Marquardt et al. [410], working on mouse bone marrow-derived mast cells, found that the simultaneous addition of adenosine, NECA or L-PIA along with a secretagogue significantly augmented beta-hexosaminidase release. Similar results were observed when adenosine was pre-incubated with the cells for 10 min prior to challenge with antigen or the calcium ionophore A23187. The release of newly generated arachidonic acid metabolites was not, however, significantly increased, suggesting that the nucleoside had a differential effect on the release of granule-associated mediators. Adenosine transiently elevated cAMP concentrations in resting cells. Maximum levels were achieved within 15 sec and remained above baseline for at least 60 sec, although no effect on the spontaneous release of mediators was observed. Binding studies showed that the cells possessed high-affinity adenosine receptors of the $A_2$ subtype that were up-regulated on exposure to aminophylline, an adenosine receptor antagonist.

Since adenosine is known to be released from the lung under hypoxic conditions [411] and antigen challenge [412] at concentrations that are sufficient to effect mediator release, studies were performed to investigate production and release of adenosine from stimulated mouse bone marrow-derived mast cells [413]. Significantly elevated levels of adenosine were observed in the extracellular fluid upon challenge with A23187, although the enhancement was much lower than that observed in rat serosal cells receiving equal stimuli.

Using cDNA probes, Marquardt et al. [414] revealed the presence of $A_{2a}$ and $A_{2b}$ but not $A_1$ adenosine receptor subtypes in mouse bone marrow-derived mast cells. CGS21680, the $A_{2a}$ agonist, failed to potentiate stimulated beta-hexosaminidase release, suggesting that it was either the $A_{2b}$ and/or another receptor subtype that was responsible for mediating the effects of adenosine observed in previous studies. The $A_{2a}$ receptor has since been studied more fully in $A_{2a}R$-knockout mice [340] and has been shown to mediate many physiological processes. Knockout mice are less exploratory, less
responsive to pain, more anxious and aggressive and suffer from much higher blood pressure than normal mice.

Studies by McWhinney et al [415] on murine J774.1 macrophages, found that they predominately express adenosine A\textsubscript{3} relative to A\textsubscript{1} and A\textsubscript{2} receptor RNA. Hon et al [416], investigating the effect of adenosine and its agonists on nitric oxide synthase (NOS) activity and nitrite production induced by lipopolysaccharide in RAW 264.7 murine macrophages, found that the nucleoside and its agonists enhanced both NOS activity and nitrite production by up to 2.5 times. The effect was dose-dependent and was neither A\textsubscript{1} nor A\textsubscript{2} receptor mediated. Hence, it is possible that the cellular adenosine released during inflammatory conditions and trauma augments production of nitric oxide (NO) in macrophages via activation of the adenosine A\textsubscript{3} receptor.

It has been postulated, therefore, that murine mast cells, like murine macrophages and rat mast cells, express an adenosine A\textsubscript{3} receptor in addition to the A\textsubscript{2a} and A\textsubscript{2b} receptor subtypes. The aim of this study was to try to clarify the effects of adenosine in the mouse and to attempt to define the receptor(s) involved by using a range of adenosine receptor agonists and antagonists.

### 5.2 Materials and methods

All the materials and methods employed in this study are described in chapter 2.

### 5.3 Results

#### 5.3.1 Histamine release from PMC of MF1 and TO mice following stimulation with anti-IgE, con A and A23187

Two different mouse strains were used for this study, MF1s and TOs. Cells from MF1 mice were much more responsive than cells from TO mice. This was demonstrated by their reactivity in the absence of a secretagogue. In total, 53 experiments were performed on MF1 mice (mean spontaneous 12.9 ± 0.6 %). Only 38 experiments were done with cells from TO mice (mean spontaneous 8.2 ± 0.3 %), since it was possible to
do more samples per mouse with this strain. (Average number of samples per mouse were 5 and 8 for the MF1s and TOs respectively). Sample numbers had to be restricted for the MF1 mice to prevent a large increase in the spontaneous histamine release.

The animals had not previously been sensitised to the nematode *Nippostrongylus brasiliensis* and anti-rat IgE, therefore, failed to produce significant histamine release from either strain (< 3 %, Fig. 5.1). Con A, however, although having only a limited effect on TO mice (maximum release 11.8 ± 5.4 %, 5 µg/ml, Fig 5.2), dose-dependently (0.1 - 10 µg/ml) induced histamine release from MF1 mice (maximum release 52.3 ± 2.5 %, 10 µg/ml). The calcium ionophore A23187 (0.01 - 1 µM) induced histamine release from mast cells of both mouse strains in an identical fashion (maximum releases were 80.9 ± 1.3 % and 75.9 ± 3.2 %, 0.5 µM for MF1 and TO mice respectively, Fig 5.3).

**5.3.2 Histamine release induced by adenosine receptor agonists from PMC of MF1 and TO mice**

The non-selective adenosine receptor agonist, NECA (0.001 – 100 µM) produced a concentration-dependent release of histamine from PMC of MF1 mice (maximum release 34.1 ± 3.6 %, 10 µM, Fig. 5.4) in the absence of any other secretagogue. The same effect was observed with each of the other agonists, with a rank order of potency of IB-MECA>R-PIA>APNEA>metrifudil>NECA>adenosine>CGS21680. IB-MECA was not only the most potent agonist tested, it also produced a greater degree of release than any of the other compounds (maximum release 51.2 ± 2.5 %, 0.1 µM). All the other agonists produced maximum releases of 26 – 34 %, except metrifudil and CGS21680, whose maximum histamine releases were 18.5 ± 1.5 %, 1 µM and 21.6 ± 2.0 %, 100 µM, respectively.

When PMC of TO mice were challenged with the adenosine receptor agonists (Fig. 5.5), similar results were observed to those with MF1 mice, except that the maximum releases induced by the agonists were now much smaller. NECA (0.001 – 100 µM) produced a concentration-dependent histamine release from the PMC (maximum release 14.3 ± 2.8 %, 10 µM). The same effect was observed with IB-MECA and adenosine
(maximum releases = 13.7 ± 2.1, 0.1 μM and 13.5 ± 2.4, 1000 μM respectively). The other agonists were all much less effective (maximum releases < 7 %)

5.3.3 The effect of metabolic inhibitors on adenosine, NECA and IB-MECA induced histamine release from MF1 mice

Histamine release induced by adenosine (100 and 1000 μM), NECA (10 and 100 μM) and IB-MECA (0.1 – 1 μM) from PMC of MF1 mice was partially reduced by the omission of glucose from the incubation medium (Figs. 5.6 and 5.7) and was virtually abolished in the presence of the metabolic inhibitors 2-deoxy-D-glucose (2-DOG) and antimycin A. The same effect was observed when the cells were incubated with antimycin A alone. 2-DOG, however, caused significant mast cell degranulation when incubated with the cells in the absence of antimycin A (histamine release 39.4 ± 3.3 %, Fig 5.8). In the presence of the adenosine agonists, the 2-DOG response increased slightly (total releases < 56 %) except with adenosine (1000 μM), where the nucleoside slightly inhibited the release induced by the sugar (release 29.8 ± 2.4 %). Con A was included in all experiments as a control and produced the expected release of histamine.

5.3.4 The effect of adenosine and NECA on con A induced histamine release from PMC of MF1 mice

Having shown that the adenosine receptor agonists induced histamine release from both mouse strains in the absence of a secretagogue, we decided to investigate whether they could also enhance release induced by various agents. Neither adenosine nor NECA significantly potentiated the histamine release induced by the lectin, con A, from PMC of MF1 mice (Figs 5.9 and 5.10).

5.3.5 The effect of adenosine and NECA on calcium ionophore A23187 induced histamine release from PMC of MF1 mice

Figs 5.11 and 5.12 show the effect of adenosine and NECA on histamine release induced by the calcium ionophore A23187 from mast cells of MF1 mice. The release
induced by A23187 was potentiated slightly by the adenosine agonists, the effect being significant for adenosine (1-1000 μM) and NECA (0.1 and 1 μM).

The histamine releases induced by the adenosine receptor agonists alone, however, were so large that it was difficult to observe a true synergistic effect. We decided, therefore, to concentrate our potentiation studies on TO mice, where the adenosine receptor agonists were much less effective at inducing degranulation in the absence of a secretagogue.

5.3.6 The effect of adenosine receptor agonists on calcium ionophore A23187 induced histamine release from PMC of TO mice

We did not investigate the effects of the compounds on con A induced histamine release in TO mice, since the lectin response was so small (<12 %). The nature of the experiments meant that it was impossible to study more than one adenosine receptor agonist at a time. Control releases were, therefore, maintained between 22 and 24 %, to enable direct comparisons to be made. Figs 5.13-5.19 show the effect of the adenosine receptor agonists, adenosine, NECA, R-PIA, CGS21680, metrifudil, IB-MECA and APNEA on A23187 induced histamine release, respectively.

Adenosine (1-1000 μM) and NECA (0.1 -1 100 μM) dose-dependently increased the A23187 response in a synergistic manner. The same effect was observed with each of the other agonists. When the two effects produced by the adenosine receptor agonists (induction of release and potentiation of the ionophore response) were added together (Fig 5.20), the rank order of potency of the compounds was; IB-MECA>metrifudil≥R-PIA=NECA≥APNEA> adenosine=CGS21680.

5.3.7 The effect of different adenosine receptor antagonists on agonist induced histamine release from MF1 and TO mice

In agreement with the experiments in rat mast cells, the adenosine receptor antagonists produced no effect on the spontaneous histamine release. All four compounds, however, dose-dependently inhibited the IB-MECA (0.01 μM) response in MF1 mice (Figs 5.21). CGS51943 and BW3911 completely abolished the histamine release induced by the adenosine receptor agonist at concentrations of 10 and 100 μM respectively. DPCPX
and 8PT were less effective, producing maximum inhibitions of 85.5 ± 2.4 % and 36.9 ± 7.5 %, 100 μM respectively.

Similar results were observed with the antagonists on R-PIA (1 μM) induced histamine release in MF1 mice and IB-MECA (0.1 μM) induced histamine release in TO mice (Figs 5.22 and 5.23), where the rank order of potency of the antagonists was CGS15943>BW3911>DPCPX>8PT.

5.4 Discussion

The results from this study provide conclusive evidence that adenosine is an important modulator of murine mast cell function. Due to the heterogeneous nature of adenosine mediated effects in the rat, we decided to investigate the effects of the nucleoside and its analogues in two different mouse strains. The initial studies showed that neither strain responded to stimulation by anti-rat IgE. This was as predicted since the animals had not previously been sensitised to the nematode *Nippostrongylus brasiliensis*, and the anti-IgE used was specific for rat rather than mouse IgE anti-bodies. Both strains responded in an identical fashion to stimulation by A23187. Surprisingly, however, con A dose-dependently induced histamine release from PMC from MF1 mice, whilst producing only a small response in cells from TO mice. Barrett and Pearce [417] had reported that con A induced significant mast cell degranulation in mast cells from Porton mice, without prior experimental sensitisation of the animals. This implies that MF1 and Porton, but not TO mice, exhibit some degree of natural sensitisation towards environmental allergens.

In contrast to findings by Marquardt et al [410], we found that the adenosine receptor agonists dose-dependently induced histamine release from both mouse strains in the absence of a secretagogue. The reason for this discrepancy may be due to the nature of the mast cells studied. Marquardt et al performed all of their experiments on mouse bone marrow-derived mast cells. Studies on the ultrastructural, biochemical and functional characteristics of these cells [418] lead to them being tentatively identified as mucosal mast cells. However, further studies [419] showed that, in common with mast cells from the gastrointestinal mucosa, mouse bone marrow-derived mast cells were
heterogeneous in their expression of proteinases, and a proportion of them resembled serosal mast cells in their protease content. Our studies were all performed on primary cells from the peritoneum (classic connective tissue mast cells\textsuperscript{420}). Since the adenosine response has been shown to vary considerably, depending on the model studied, it is perhaps unsurprising that cells from different sites exhibit marked variations in their response to the nucleoside.

In keeping with our results in the rat, we discovered that the mast cells from the two strains differed in their responsiveness to adenosine and its analogues, with NECA inducing 2.5 times the level of histamine release from MF1 compared to TO mice. Previously, we showed that mast cells from Wistar rats were much more responsive to the nucleoside than cells from either Sprague-Dawley or Hooded Lister rats (chapter 4). They were also more sensitive to antigen, anti-IgE and con A challenge. Since, the same effect has now been observed in the mouse, there appears to be a good correlation between the sensitivity of mast cells to adenosine and to the levels of histamine release induced by IgE directed stimuli. As discussed in chapter 1, studies on patients suffering from asthma and chronic bronchitis have shown that they have an increased amount of adenosine in the bronchial lavage fluid, relative to that of normal subjects\textsuperscript{310}, and that adenosine induces bronchoconstriction in asthmatic\textsuperscript{311} but not in normal subjects. Since, MF1 mice also have a higher spontaneous release of histamine than TO mice, it appears possible to equate the differences in sensitivity of mast cells to adenosine to the difference in sensitivity to the nucleoside in asthmatic and non-asthmatic subjects.

In addition to NECA, all the test agonists induced histamine release in cells from MF1 mice (Fig. 5.4), although only IB-MECA and adenosine induced significant mast cell degranulation in cells from TO mice (Fig. 5.5). Despite the difference in levels of histamine release triggered by the agonists in the two strains, IB-MECA was by far the most potent compound tested in both experiments. None of the other agonists produced any significant release at concentrations below 0.1 µM. The rank order of potency of the agonists in MF1 mice was IB-MECA\textsuperscript{2}R-IA\geq\text{APNEA=metrifudil=NECA=adenosine\geq CGS21680}. This rank order of potency of the agonists is not consistent with the activation of adenosine $A_1$, $A_2A$, or $A_2B$ receptors. The sensitivity of the cells to R-PIA is surprising, since Marquardt et al\textsuperscript{414} showed that bone marrow-derived mast cells have
no A\textsubscript{1} receptors. Linden \cite{421}, however, reported that agonists such as R-PIA, NECA and CGS21680 all activate A\textsubscript{3} receptors in the range 0.1 –1 \(\mu\)M and at these concentrations it is possible that the effect observed is the result of A\textsubscript{3} receptor activation.

We decided to investigate whether the histamine release induced by the adenosine receptor agonists from MF1 mice was cytotoxic, by using the metabolic inhibitors 2-DOG and antimycin A. For active secretion to occur, the utilisation of glucose and the production of ATP must be involved. The two inhibitors block glycolysis and oxidative phosphorylation respectively \cite{273-274}. The histamine release induced by all the compounds tested (adenosine, NECA and IB-MECA) was virtually abolished in the presence of antimycin A or antimycin A and 2-DOG. This indicates that these are non-cytotoxic processes. It was not possible, however, to study the effect of 2-DOG alone on the release induced by the adenosine agonists, since 2-DOG caused significant histamine release from the mouse cells, even in the absence of a secretagogue. The reason for this is unclear, but it could be just another example of the reactivity of MF1 murine peritoneal mast cells.

Since the releases induced by the adenosine receptor agonists alone in MF1 mice were so considerable, it was difficult to determine whether the compounds significantly augmented mediator release induced by other secretagogues, or whether the effects were purely additive. The low reactivity of cells from TO mice to con A meant that the best model for studying potentiation was in cells stimulated with concentrations of A23187 designed to give control releases in the order of 20 %.

All the agonists significantly potentiated the ionophore response. When the results of both effects induced by the agonists were added together, IB-MECA and metrifudil were shown to be the most potent compounds tested, with adenosine and CGS21680 being the weakest. This is in keeping with previous studies \cite{414} which suggested that the potentiating effect of adenosine in the mouse is not the result of A\textsubscript{1} or A\textsubscript{2a} receptor stimulation.

The studies with the adenosine receptor antagonists concentrated on trying to block the release induced by the agonists alone. The nature of the experiment meant that it was too problematic to try to reverse the potentiation of the ionophore response.
None of the antagonists were effective at concentrations lower than 0.1 \( \mu \text{M} \). At concentrations of 1 \( \mu \text{M} \) and above, the rank order of potency of the antagonists was \( \text{CGS15943} > \text{BW3911} > \text{DPCPX} > \text{8PT} \). However, at these concentrations, \( \text{BW3911} \) and \( \text{CGS15943} \), in particular, are known to produce non-selective inhibition in the rat (chapter 4). Also, data provided on the binding affinities of the four antagonists in human studies \( ^{[422]} \) show that at micromolar concentrations all four antagonists are capable of binding to all four adenosine receptors. Indeed, the order of potency of the antagonists at these concentrations is consistent with binding at the human \( A_3 \) receptor. The lack of data on the binding affinities of the antagonists in mouse mast cells, and the high concentrations required to reverse the agonist response, means it is difficult to draw any further conclusions from these results.

Studies by Marquardt and Walker \( ^{[423]} \) demonstrated that adenosine’s ability to potentiate beta-hexosaminidase release and augment cAMP levels in mouse mast cells was attenuated by pre-treatment with pertussis toxin. This implies that the nucleoside mediates its effects by activating a G-protein coupled receptor on the cell surface. Mast cell desensitisation to IgE failed to induce a parallel adenosine receptor desensitisation \( ^{[424]} \), indicating that the signal transduction events stimulated upon antigen challenge are not sufficiently linked to adenosine’s signalling pathway to induce similar unresponsiveness. Activation of PKC directly, however, by culturing the cells with the phorbol ester PMA \( ^{[425]} \) was sufficient to block the effects of adenosine. This is in agreement with findings that adenosine activates PKC by inducing translocation of the protein from the cytosol to the membrane \( ^{[377]} \). Since the potentiation of mediator release has been shown to be independent of cAMP \( ^{[376]} \), it is possible that adenosine mediates its effects in the mouse via a similar mechanism to that observed in the rat.

In conclusion, therefore, the present study indicates that adenosine receptor agonists both induce histamine release and potentiate secretagogue induced release from mouse PMC. The first effect is probably the result of \( A_3 \) receptor activation, and the second due to stimulation of either \( A_{2b} \) or \( A_3 \) receptors. The use of more selective antagonists effective at much lower concentrations in the mouse is, however, required to verify the nature of the receptor.
The reactivity of mast cells from MF1 mice has shown them to be an ideal model for studying the effects of the agonists in vitro. They might prove, therefore, to be a useful animal model for studying the effects of adenosine and its analogues on bronchospasm in vivo.
Fig. 5.1 Histamine release from PMC of MF1 and TO mice following stimulation with anti-rat IgE (n=3-4) 

(The releases induced by anti-IgE were not significant from either mouse strain)

Fig. 5.2 Histamine release from PMC of MF1 and TO mice following stimulation with concanavalin A (n=3-4)
Fig. 5.3 Histamine release from PMC of MF1 and TO mice following stimulation with calcium ionophore A23187 (n=3)
Fig. 5.4 Histamine release induced by adenosine receptor agonists from PMC of MF1 mice (n=3-7)

Fig. 5.5 Histamine release induced by different adenosine receptor agonists from PMC of TO mice (n=5-7)
Fig. 5.6 The effect of metabolic inhibitors on adenosine and NECA induced histamine release from PMC of MF1 mice (n=4-5)

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

![Graph showing the effect of metabolic inhibitors on adenosine and NECA induced histamine release from PMC of MF1 mice.]

Fig. 5.7 The effect of metabolic inhibitors on IB-MECA induced histamine release from PMC of MF1 mice (n=4)

(Asterisks denote those releases that were significantly different from those obtained when using FHT.
*p<0.05, **p<0.01, ***p<0.001)
Fig. 5.8 The effect of the metabolic inhibitor 2-deoxy-D-glucose on histamine release from PMC of MF1 mice stimulated with various agents (n=4-5)

(Releases corrected for spontaneous release in FHT)
Fig. 5.9 The effect of adenosine on concanavalin A (3 μg/ml) induced histamine release from PMC of MF1 mice (n=3)

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

Fig. 5.10 The effect of NECA on concanavalin A (3 μg/ml) induced histamine release from PMC of MF1 mice (n=3)

(Asterisks denote the releases induced by concanavalin A and NECA together that were significantly different from the sum of the releases obtained by concanavalin A and NECA separately. *p<0.05, **p<0.01, ***p<0.001)
Fig. 5.11 The effect of adenosine on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of MF1 mice (n=4)

(Asterisks denote the releases induced by A23187 and adenosine together that were significantly different from the sum of the releases obtained by A23187 and adenosine separately. *p<0.05, **p<0.01, ***p<0.001)

![Graph showing the effect of adenosine on histamine release](image)

Fig. 5.12 The effect of NECA on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of MF1 mice (n=4)

(Asterisks denote the releases induced by A23187 and NECA together that were significantly different from the sum of the releases obtained by A23187 and NECA separately. *p<0.05, **p<0.01, ***p<0.001)

![Graph showing the effect of NECA on histamine release](image)
Fig. 5.13 The effect of adenosine on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and adenosine together that were significantly different from the sum of the releases obtained by A23187 and adenosine separately. *p<0.05, **p<0.01, ***p<0.001)

Fig. 5.14 The effect of NECA on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and NECA together that were significantly different from the sum of the releases obtained by A23187 and NECA separately. *p<0.05, **p<0.01, ***p<0.001)
Fig. 5.15 The effect of R-PIA on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and R-PIA together that were significantly different from the sum of the releases obtained by A23187 and R-PIA separately. *p<0.05, **p<0.01, ***p<0.001)

Fig. 5.16 The effect of CGS21680 on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and CGS21680 together that were significantly different from the sum of the releases obtained by A23187 and CGS21680 separately. *p<0.05, **p<0.01, ***p<0.001)
Fig. 5.17 The effect of metrifudil on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and metrifudil together that were significantly different from the sum of the releases obtained by A23187 and metrifudil separately. *p<0.05, **p<0.01, ***p<0.001)

![Graph showing histamine release (%) vs. metrifudil concentration (μM).](image)

Fig. 5.18 The effect of IB-MECA on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and IB-MECA together that were significantly different from the sum of the releases obtained by A23187 and IB-MECA separately. *p<0.05, **p<0.01, ***p<0.001)

![Graph showing histamine release (%) vs. IB-MECA concentration (μM).](image)
Fig. 5.19 The effect of APNEA on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4)

(Asterisks denote the releases induced by A23187 and APNEA together that were significantly different from the sum of the releases obtained by A23187 and APNEA separately. *p<0.05, **p<0.01, ***p<0.001)

Fig. 5.20 The effect of different adenosine receptor agonists on calcium ionophore A23187 (0.1 μM) induced histamine release from PMC of TO mice (n=4, Control releases = 20-25 %)

(Releases corrected for spontaneous release only)
Fig. 5.21 The effect of different adenosine receptor antagonists on IB-MECA (0.01 μM) induced histamine release from PMC of MF1 mice (n=4, Control releases = 32.7 ± 3.8 %)

Fig. 5.22 The effect of different adenosine receptor antagonists on R-PIA (1 μM) induced histamine release from PMC of MF1 mice (n=4, Control releases = 29.5 ± 5.5 %)
Fig. 5.23 The effect of different adenosine receptor antagonists on IB-MECA (0.1 \( \mu \text{M} \)) induced histamine release from PMC of TO mice 
\( (n=4, \text{Control releases} = 19.9 \pm 0.9 \%) \)
Chapter 6

The adenosine receptor in humans
6.1 Introduction

As discussed in chapter 1, adenosine has been shown to play an important role in asthma and allergic rhinitis. The increase in plasma histamine concentrations following challenge of the airways with AMP implies that the bronchoconstriction is mediated, at least in part, by the activation of airway mast cells \[426\]. Björck et al \[427\] found that bronchi isolated from asthmatic subjects contracted in response to adenosine in vitro. This effect was blocked by a combination of histamine and leukotriene antagonists in addition to non-selective adenosine receptor antagonists.

Walker et al \[428\], studying lung and airway tissue, identified A\(_3\) receptors on eosinophils but not on mast cells. They found that the A\(_3\) agonist N\(^6\)-(4-amino-3-iodobenzyl)adenosine dose-dependently inhibited platelet activating factor-induced eosinophil chemotaxis and postulated, therefore, that the adenosine A\(_3\) receptor may play an important role in eosinophil dependent diseases. Further studies by Kohno et al \[429\] showed that the eosinophil adenosine receptors, once stimulated, induced the release of calcium from intracellular stores followed by subsequent calcium influx. They suggested, therefore, that the adenosine A\(_3\) receptors expressed on human eosinophils, like the A\(_3\) receptors on rat mast cells, couple to PLC.

Adenosine receptors have also been shown to be present in platelets where their activation results in accumulation of cAMP and inhibition of aggregation. Feoktistov and Biaggioni \[430\], testing a range of adenosine receptor agonists and antagonists in platelets and human erythroleukemia cells (often used as a model for platelets), proposed that they possess adenosine A\(_2\) receptors. The striking differences in the relative order of potencies of the compounds in the two cell types, however, led to the conclusion that they possess different adenosine receptor subtypes. Platelets appear predominately to express the A\(_{2a}\) receptor, whilst the biochemical characteristics of the receptor on erythroleukemia cells corresponds more to that of the A\(_{2b}\) subtype.
Studies on the effects of adenosine on the human mast cell line, HMC-1 [431], have shown that the cells express $A_{2a}$ and $A_{2b}$ receptors which couple to adenylate cyclase. In addition, the $A_{2b}$ receptor also couples to PLC and evokes IL-8 release. This latter effect is blocked by the adenosine receptor antagonists theophylline and enprofylline.

Suzuki et al [432], working on human cultured mast cells, found that adenosine inhibited FcεRI-mediated tryptase release in a dose dependent manner, and that this effect was $A_{2a}$ mediated.

Studies on canine BR mastocytoma cells, and HEK-293 cells stably transfected with recombinant human adenosine $A_{2b}$ receptors [433], showed that the receptors activated PLC and mobilized calcium by signalling through the $G_q$-protein. Since canine adenosine receptors have been shown to be pharmacologically more similar to human receptors than those of the rat, they postulated that it was solely the $A_{2b}$ receptor that serves to facilitate degranulation from human mast cells.

Marone et al [347] demonstrated that the adenosine receptor agonists NECA, R-PIA, 2-chloroadenosine (CADO) and adenosine inhibited anti-IgE-induced histamine and LTC$_4$ release from human basophils in a concentration dependent manner. In human lung parenchymal cells, they showed that micromolar concentrations of adenosine, NECA and R-PIA potentiated anti-IgE induced mediator release, whilst in human skin mast cells challenged with anti-IgE, submillimolar concentrations of the adenosine agonists inhibited release of both histamine and PGD$_2$.

Peters et al [434] reported that in dispersed human lung mast cells, adenosine enhanced release at low concentrations and inhibited release at high concentrations. Hughes et al [435], however, reported that the mechanisms of action of adenosine were much more complex and that both inhibition and potentiation of the anti-IgE response was observed, depending on whether the nucleoside was added before or after immunological challenge. The effects were much more pronounced with low levels of histamine release and subsequent agonist and antagonist studies showed that both effects appeared to be mediated by an $A_2$ cell surface adenosine receptor.
Forsythe et al. working on human bronchoalveolar lavage (BAL) mast cells found that adenosine induced histamine release in the absence of other secretagogues. This effect was mimicked by the adenosine receptor agonists R-PIA, NECA and CGS21680, whilst pre-incubation of the cells with the adenosine receptor antagonist xanthine amine congener significantly inhibited the nucleoside response. They also noted an inverse correlation between endogenous adenosine levels in the lavage fluid and the maximal responses to in vitro adenosine challenge of the lavage cells.

Hence the nature of the adenosine response in human mast cells is dependent on the model studied. This heterogeneity meant that it was necessary to study the effects of adenosine in several different phenotypes in an attempt to clarify the role of adenosine in mast cell degranulation. We decided therefore to investigate the effects of the nucleoside on human basophils, lung and skin mast cells.

6.2 Materials and methods

All the materials and methods employed in this study are described in chapter 2.

6.3 Results

None of the agonists or antagonists tested produced any histamine release themselves in the absence of a secretagogue.

6.3.1 The effect of adenosine and NECA on anti-IgE induced histamine release from human basophils

Dose response curves to anti-IgE were obtained for all the blood donors prior to commencing the experiments with adenosine and its related analogues, to ensure that all control releases were maintained at approximately 20%. This was important since the sensitivity of the basophils from the different donors varied considerably, with maximum histamine releases (anti-IgE 1/1000) between 20 and 80%.
Adenosine (0.01 – 1000 μM, Fig. 6.1) dose-dependently inhibited immunologic histamine release from human basophils if the cells were pre-incubated with the nucleoside prior to challenge with anti-IgE (maximum inhibition at 15 min pre-incubation, 51.8 ± 6.8 %, 1000 μM) or if the cells were challenged simultaneously with the nucleoside and the secretory stimulus (maximum inhibition 31.6 ± 4.0 %, 1000 μM). This effect disappeared if adenosine was added post challenge.

Similar results were obtained when the experiment was repeated with the non-selective agonist NECA (0.001 – 100 μM, Fig. 6.2). Maximum inhibitions of 58.1 ± 6.9 %, 52.8 ± 5.8% and 32.0 ± 3.2 %, 100 μM were obtained at 30 min, 15 min and 0 min pre-incubation periods respectively. No inhibition of the anti-IgE response was observed when the cells were post-challenged with the adenosine receptor agonist.

The importance of maintaining control releases in the order of 20 %, was demonstrated by measuring the degree of inhibition of the anti-IgE response induced by NECA (15 min pre-incubation, 100 μM, Fig. 6.3) for control releases between 5 and 65 %. An inverse correlation between control release and percentage inhibition was observed, with a correlation coefficient of –0.779.

6.3.2 The effect of different adenosine receptor agonists on anti-IgE induced histamine release from human basophils

We investigated the effects of the selective adenosine receptor agonists (0.01 – 100 μM, Fig. 6.4) at 30 min pre-incubation, since this was when maximum inhibition of the anti-IgE response was observed with NECA. NECA and the A3 agonists IB-MECA and APNEA dose-dependently inhibited anti-IgE induced histamine release with a rank order of potency of IB-MECA>NECA>APNEA (maximum inhibitions of 40.7 ± 4.1, 10 μM, 39.9 ± 4.7 %, 100 μM, and 37.6 ± 7.3 %, 100 μM respectively). Lack of solubility of the adenosine receptor agonist IB-MECA prevented using concentrations higher than 10 μM. The other agonists were all much less effective (maximum inhibitions < 8 %)
6.3.3 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human basophils

The adenosine receptor antagonists dose-dependently inhibited anti-IgE induced histamine release at both 30 min and 0 min pre-incubation periods (Figs 6.5 and 6.6). CGS15943 was the most potent compound tested, producing maximum inhibitions of 67.8 ± 5.2 % and 54.7 ± 6.1 %, 5 μM at 30 and 0 min pre-incubation periods respectively. In comparison BW3911, 8PT and DPCPX were all much less effective, with the compounds only weakly antagonising the anti-IgE response at concentrations below 5 μM (maximum inhibitions < 22 %). High antagonist concentrations (50 μM), however, produced inhibitions of 73.9 ± 7.1 %, 38.9 ± 8.4 % and 29.0 ± 6.9 % for BW3911, 8PT and DPCPX respectively at 30 min, and 65.8 ± 11.0 %, 42.1 ± 4.4 % and 10.0 ± 4.6 % respectively at 0 min. Lack of solubility prevented the use of concentrations greater than 5 μM for CGS15943.

6.3.4 The effect of different adenosine receptor antagonists on the inhibition by NECA of anti-IgE induced histamine release from human basophils

We investigated the effect of the antagonists on the inhibition produced by NECA at 30 min pre-incubation, since this was when maximum inhibition of the anti-IgE response was observed, and the non-selective inhibition induced by the antagonists varied little between the 30 min and 0 min pre-incubation periods (Fig. 6.7). BW3911, 8PT, DPCPX (1 μM) and CGS15943 (0.1 μM) were incubated with the cells (5 min) prior to addition of NECA (30 min) before challenge with anti-IgE (20 min). At these concentrations, inhibition of the anti-IgE response by the antagonists was kept to a maximum of 10 %.

All four antagonists produced a rightward shift of the concentration-effect curve to NECA (i.e. they reversed the inhibition of the anti-IgE response induced by the adenosine receptor agonist) with a rank order of potency of CGS15943>BW3911>DPCPX=8PT. Inhibition of anti-IgE induced release was 32.3 ± 3.5 % in the presence of NECA alone, 10 μM, and 10.0 ± 7.4 %, 17.3 ± 6.0 %, 21.4 ± 6.7 % and 23.6 ± 2.6 % with CGS15943, BW3911, DPCPX and 8PT respectively.
6.3.5 The effect of adenosine and NECA on anti-IgE induced histamine release from human lung mast cells

When human lung mast cells were challenged with anti-IgE and adenosine (Fig. 6.8), similar results were obtained to those with human basophils, except that this time the inhibition produced by the nucleoside was greater. Adenosine (0.01 – 1000 μM) dose-dependently inhibited immunologic histamine release from human lung mast cells if the cells were pre-incubated with the nucleoside prior to challenge with anti-IgE (maximum inhibition at 15 min pre-incubation 90.4 ± 3.8 %, 1000 μM) or if the cells were challenged simultaneously with the nucleoside and the secretory stimulus (maximum inhibition 87.6 ± 4.6 %, 1000 μM). This effect disappeared if adenosine was added post anti-IgE challenge.

Similar results were observed when the experiment was repeated with the non-selective agonist NECA (0.001 – 100 μM, Fig. 6.9). Maximum inhibitions of 67.1 ± 7.6 % and 47.1 ± 8.1 %, 100 μM were obtained at 15 min and 0 min pre-incubation periods respectively. Again, no inhibition of the anti-IgE response was observed if the adenosine agonist was added post-challenge. Indeed, this time a slight potentiation was seen at 15 min post-stimulation (maximum enhancement 11.5 ± 5.1 %, 100 μM).

N.B. As with human basophils, all control releases were maintained at approximately 20 %.

6.3.6 The effect of different adenosine receptor agonists on anti-IgE induced histamine release from human lung mast cells

When the experiment was repeated with the selective adenosine receptor agonists (0.001 – 100 μM, 15 min pre-incubation, Fig. 6.10), NECA was shown to be the most potent compound tested (maximum inhibition 57.2 ± 6.1 %, 100 μM). The same effect was observed with each of the other agonists, with a rank order of potency of NECA>metrifudil=IB-MECA>APNEA>R-PIA>CGS21680.
6.3.7 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human lung mast cells

BW3911 and CGS15943 dose-dependently inhibited anti-IgE induced histamine release at both 15 and 0 min pre-incubation periods (Figs 6.11 and 6.12). Maximum inhibition of anti-IgE induced release was $43.7 \pm 5.4\%$, $5 \mu M$ and $86.5 \pm 5.6\%$, $50 \mu M$ for CGS15943 and BW3911 respectively at 15 min pre-incubation and $49.1 \pm 10.1$, $5 \mu M$ and $75.0 \pm 8.0\%$, $50 \mu M$, for the two compounds respectively at 0 min pre-incubation. In comparison, DPCPX was much less effective (maximum inhibition of anti-IgE response was $21.1 \pm 9.1\%$, and $20.4 \pm 4.9\%$, $50 \mu M$ at 15 min and 0 min pre-incubation periods respectively). 8PT was essentially ineffective at reversing anti-IgE induced histamine release.

6.3.8 The effect of different adenosine receptor antagonists on the inhibition by NECA of anti-IgE induced histamine release from human lung mast cells

With human lung mast cells, we studied the effect of the antagonists on the inhibition by NECA at 15 min pre-incubation (Fig. 6.13). BW3911 was essentially ineffective whilst the other three antagonists all produced a slight rightward shift of the concentration-effect curve to NECA. The rank order of potency was $8PT > CGS15943 > DPCPX$. Inhibition of anti-IgE induced release was $26.3 \pm 9.5\%$ in the presence of NECA alone, $1 \mu M$, and $-8.3 \pm 2.2\%$, $1 \mu M$, $0.9 \pm 3.5\%$, $0.1 \mu M$ and $11.6 \pm 1.6\%$, $1 \mu M$ with 8PT, CGS15943 and DPCPX respectively.

6.3.9 The effect of adenosine on anti-IgE and calcium ionophore A23187 induced histamine release from human skin mast cells

Adenosine ($0.1 - 1000 \mu M$, 0 min pre-incubation, Fig. 6.14) had no effect on calcium ionophore induced histamine release from human skin mast cells but dose-dependently inhibited anti-IgE induced release (maximum inhibition $78.1 \pm 6.8\%$, $1000 \mu M$). When the experiment was repeated at the different time periods (Fig 6.15), the cells were first pre-incubated with recombinant SCF (15 min) prior to challenge with anti-IgE and the nucleoside in order to augment the control release $^{437}$. As with basophils and lung mast cells, adenosine ($1 - 1000 \mu M$) dose-dependently inhibited immunologic histamine
release if the cells were pre-incubated with the nucleoside prior to challenge with anti-IgE (maximum inhibition at 15 min pre-incubation 70.7 ± 5.7 %, 1000 μM) or if the cells were challenged simultaneously with the nucleoside and the secretory stimulus (maximum inhibition 80.8 ± 5.9 %, 1000 μM). This effect disappeared if adenosine was added post anti-IgE challenge, and again a slight potentiation was observed at 15 min post-stimulation (maximum enhancement 12.4 ± 5.4 %, 1000 μM).

Lack of tissue samples prevented studying the effects of the selective agonists and antagonists on human skin mast cells.

6.4 Discussion

The results from this study provide conclusive evidence that extracellular adenosine can significantly regulate human mast cell and basophil function at physiological concentrations. Previous studies on the affects of adenosine in human basophils have produced conflicting results. Church et al [438] found that pre-incubating the cells with the nucleoside prior to anti-IgE challenge inhibited histamine release, whilst addition after challenge potentiated release, with peak responses for both effects occurring 15 min before and after challenge respectively. The capacity of adenosine to mediate both effects was shown to be inversely related to the strength of the stimulus. Theophylline, the non-selective adenosine receptor antagonist, antagonised both inhibition and potentiation of the IgE dependent response at concentrations that did not modify basophil histamine release in the absence of the nucleoside. In addition, the purinergic transport inhibitor dipyridamole, enhanced both effects of the nucleoside slightly, indicating that adenosine activates a cell surface coupled receptor. The transient rise in cAMP that followed receptor stimulation, and the order of agonist potency, led to them designating the receptor A₂.

Subsequent studies, however, failed to detect the potentiatory arm of the response [439-440]. Peachell et al [440] showed that adenosine inhibited anti-IgE induced histamine and LTC₄ release in addition to elevating cAMP levels. The order of potency for substituted adenosine analogues was NECA>CADO>R-PIA, which implied that the receptor being activated was of the A₂ type. The adenosine receptor antagonist 8PT antagonised both the elevated cAMP levels and the inhibition of mediator release seen with NECA.
Dipyridamole, however, reversed the adenosine induced inhibition of histamine release but not that of the newly synthesised mediator LTC₄. This indicated that the biochemical processes involved in arachidonic metabolism were more effectively inhibited by adenosine than the release of the preformed mediators. Dipyridamole also failed to eliminate adenosine induced augmentation of cAMP levels. It was concluded, therefore, that adenosine mediated its effects in the main by binding to an A₂ receptor on the cell membrane which couples to adenylate cyclase, causing an increase in cAMP levels upon activation. In addition, adenosine which enters the cell interacts with an intracellular, cAMP independent site, involved solely in regulating release of preformed mediators.

In keeping with these experiments, we observed that adenosine and NECA dose-dependently inhibited anti-IgE induced histamine release from human basophils if the cells were pre-incubated with the agonists prior to challenge with anti-IgE or if the cells were challenged simultaneously with the agonists and the secretory stimulus. We did not, however, observe a potentiation of the immunological response when the agonists were added post-challenge. In the study conducted by Church et al [438], addition of adenosine to a final concentration of 1 μM, 15 min after challenge, prolonged the rapid phase of release. At 45 min, histamine release was, therefore, potentiated 12.8 ± 1.1 % by the presence of the nucleoside. The limited extent of the potentiation compared to the inhibition, and the length of time taken for the effect to develop, might explain why it has not been detected in subsequent studies.

We also observed a negative correlation between the control histamine release induced by anti-IgE and the subsequent inhibition by NECA.

Previous studies have only investigated a limited number of adenosine receptor agonists when attempting to define the receptor responsible for mediating adenosine induced inhibition in the basophil. Marone et al [439] found that NECA>CADO>adenosine>R-PIA>S-PIA whilst Peachell et al [440] showed that PIA was essentially ineffective at inhibiting histamine release. These agonist profiles are inconsistent with the activation of A₁ receptors. They do not, however, distinguish between A₂ and A₃ receptors.
The aim of this study was, therefore, to use a range of adenosine receptor agonists and antagonists in an attempt to define the receptor(s) involved. Only the non-selective agonist NECA and the A\textsubscript{3} agonists IB-MECA and APNEA were effective at inhibiting the anti-IgE response. This indicates that in the basophil, it is solely the A\textsubscript{3} receptor that is responsible for inhibiting mast cell degranulation, since IB-MECA is A\textsubscript{3} selective in all species [441]. The rank order of potency of the antagonists is also consistent with adenosine A\textsubscript{3} receptor activation [422], since CGS15943 binds to human A\textsubscript{3} receptors with a far higher affinity than it binds to rat A\textsubscript{3} receptors [442].

Cloning and characterisation of the human A\textsubscript{3} adenosine receptor [306][443], however, has shown that it is probably coupled to a G\textsubscript{i}-protein inhibiting adenylate cyclase. All previous studies in human basophils have shown that adenosine augments cAMP levels in addition to inhibiting histamine release. It is possible, therefore, that in human basophils the adenosine A\textsubscript{3} receptor has an alternative effector system, independent of adenylate cyclase, and that augmentation of cAMP levels is mediated via a different receptor, possibly A\textsubscript{2}. This is consistent with a report by Hughes et al [444] who studied the effect of several secretagogues on basophil-rich human leukocytes and found there was an inconsistent quantitative and kinetic relationship between histamine release and cAMP production. They concluded, therefore, that cAMP did not play a key role in the biochemical events leading to the secretion of mediators from human basophil leukocytes.

Studies on the effects of adenosine and its analogues on human lung mast cells have produced many contradictory reports. Hillyard et al [445] showed that adenosine inhibited both histamine and SRS-A release from human lung fragments, whilst Peachell et al [446] reported that adenosine potentiated immunologic and calcium ionophore induced release. Further studies [440] claimed that adenosine had a bi-phasic effect on the anti-IgE response. At low concentrations, adenosine potentiated release of histamine and LTC\textsubscript{4} whereas, at high concentrations, release of mediators was inhibited. NECA and R-PIA were also shown to modulate release in a bi-phasic manner, implying that this action was surface receptor mediated. As with basophils they found that NECA was more potent than R-PIA for both parts of the bi-phasic response. Low concentrations of adenosine produced only slight increases in cAMP levels, whilst higher concentrations produced much larger elevations of cAMP. Both the bi-phasic, modulatory effects of
NECA on mediator release and its elevation of cAMP levels were antagonised by 8PT. From these results it was concluded that, in human lung mast cells, the inhibitory arm of the response was mediated via an adenylate cyclase coupled $A_2$ receptor. Explaining the pre-secretory response, however, proved more problematic, and the authors postulated that it involved either the same receptor coupled to a different effector system or a distinct new receptor that had yet to be characterised.

Studies by Hughes et al \cite{435} on human lung mast cells also demonstrated a bi-phasic response, but one which was not dependent on adenosine concentrations, but instead on whether the cells were pre-stimulated with adenosine prior to challenge with anti-IgE, or whether the cells and anti-IgE were incubated together and then post-challenged with adenosine. They saw that pre-incubation of the cells with adenosine inhibited histamine release, whilst post-challenge with the nucleoside potentiated release. They also found that both theophylline and 8PT competitively antagonised the effects of adenosine, indicating the activation of a surface $A_2$ purinoreceptor.

Additional studies by Ott et al \cite{447} showed that the potentiating effects of adenosine were blocked by nucleoside transport inhibitors, indicating that adenosine mediates some of its effects by binding to an internal site which may regulate the slight potentiating seen with adenosine in post-stimulation experiments.

Our studies show that adenosine and NECA produce a similar effect in human lung mast cells to that observed in basophils. Hence, pre-incubation of the cells with the adenosine receptor agonists prior to immunological challenge, or simultaneously challenging the cells with the agonists and the secretory stimulus, inhibited histamine release. Post-stimulation of the cells with adenosine or NECA, however, abolished this inhibition. Indeed NECA produced a slight potentiation of the anti-IgE response ($11.5 \pm 5.1 \%$, $100 \mu M$). When we investigated the effects of the selective adenosine receptor agonists in human lung mast cells, we noted that unlike in basophils, all the agonists displayed some activity, although NECA, metrifudil and IB-MECA were the most potent. This is consistent with activation of adenosine $A_{2b}$ receptors since, in human mast cells, NECA remains the most potent $A_{2b}$ agonist \cite{448}. 

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The studies with the adenosine receptor antagonists also indicated an A₂ mediated effect since 8PT and CGS15943 were the most potent compounds tested. Data provided on the binding affinities of the antagonists in human studies \cite{372,422} show that CGS15943 binds to the A₂ receptors at much lower concentrations than 8PT. Our results, however, show that 8PT was more effective than CGS15943 at blocking the NECA response. This discrepancy can probably be attributed to the fact that the concentration of CGS15943 used was ten fold lower than for any of the other antagonists. It was not possible to use higher concentrations of the triazoloquinazoline due to the non-selective nature of the inhibition at higher concentrations. The profiles of neither the agonists nor the antagonists are consistent with the activation of A₁ or A₃ receptors, and the inability of CGS21680 to produce any appreciable inhibition of the anti-IgE response is inconsistent with activation of A₂a receptors. It is probable, therefore, that in human lung mast cells adenosine mediates its effects via activation of the A₂b receptor \cite{449}.

The inhibitory effects of adenosine in human lung mast cells in vitro are in direct contrast to the effects of the nucleoside when inhaled by asthmatic subjects (chapter 1). This discrepancy was explained by Forsythe and Ennis \cite{450-451}, who have done extensive work with BAL cells. They postulated that two distinct lung mast cell phenotypes are involved in the asthmatic response. The first population is situated beneath the basement membrane, close to blood vessels and the fibrous stroma and comprises what are commonly termed human lung mast cells. The second population is located between the basement membrane and the epithelium and comprises BAL cells. Adenosine induces histamine release from BAL cells \cite{436} even in the absence of a secretagogue and it is possible that these cells play an important role in the initial stages of the asthmatic response. In contrast, human lung mast cells may be implicated in the long-term effects of adenosine in inflammatory diseases.

Our preliminary studies on human skin mast cells have shown that adenosine has no effect on calcium ionophore induced histamine release but that it inhibits the anti-IgE response in an identical manner to that in basophils and lung mast cells. This is in keeping with work by Mita et al \cite{452} who observed that adenosine only exerted inhibitory effects on histamine release from human foreskin mast cells. Due to lack of availability of human tissue, we were, however, unable to characterise this response further.
In conclusion, the present study indicates that adenosine is an important modulator of human basophil and mast cell function. The results indicate that the nucleoside inhibits immunological histamine release in all three of the cell types studied, and that this effect is probably mediated through the A3 receptor in basophils and the A2b receptor in human lung mast cells.
Fig. 6.1 The effect of adenosine on anti-IgE induced histamine release from human basophils (n=4-5, Control releases = 19-24 %)

Fig. 6.2 The effect of NECA on anti-IgE induced histamine release from human basophils (n=6-8, Control releases = 15-27 %)
Fig. 6.3 Variation of percentage inhibition with control release (human basophils, 15 min pre-incubation, 100 μM NECA)

![Graph](image)

$r = -0.779$

Fig. 6.4 The effect of different adenosine receptor agonists on anti-IgE induced histamine release from human basophils (n=5-7, Control release = 20.8 ± 2.4 %, 30 min pre-incubation)

![Graph](image)
Fig. 6.5 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human basophils (n=5, Control release = 26.9 ± 5.0 %, 30 min pre-incubation)

Fig. 6.6 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human basophils (n=5, Control release = 25.8 ± 4.5 %, No pre-incubation)
Fig. 6.7 The effect of different adenosine receptor antagonists on the inhibition by NECA of anti-IgE induced histamine release from human basophils (n=5-6, Control releases = 25–28 %, 30 min pre-incubation)
Fig. 6.8 The effect of adenosine on anti-IgE induced histamine release from human lung mast cells (n=5, Control releases = 19–24 %)

Fig. 6.9 The effect of NECA on anti-IgE induced histamine release from human lung mast cells (n=4, Control releases = 19–26 %)
Fig. 6.10 The effect of different adenosine receptor agonists on anti-IgE induced histamine release from human lung mast cells (n=4, Control release = 23.9 ± 4.8 %, 15 min pre-incubation)
Fig. 6.11 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human lung mast cells (n=4, Control release = 21.4 ± 8.3 %, 15 min pre-incubation)

Fig. 6.12 The effect of different adenosine receptor antagonists on anti-IgE induced histamine release from human lung mast cells (n=4, Control release = 22.6 ± 7.8 %, No pre-incubation)
Fig. 6.13 The effect of different adenosine receptor antagonists on the inhibition by NECA of anti-IgE induced histamine release from human lung mast cells (n=3, Control releases = 18-24 %, 15 min pre-incubation)
Fig. 6.14 The effect of adenosine on anti-IgE and calcium ionophore A23187 induced histamine release from human skin mast cells (n=3-4, Control releases; anti-IgE = 7.1 ± 2.1 %, A23187 = 31.1 ± 2.9 %, No pre-incubation)

![Graph showing inhibition (%) of adenosine at different concentrations.]

Fig. 6.15 The effect of adenosine on anti-IgE + stem cell factor induced histamine release from human skin mast cells (n=4-5, Control releases = 11-15%)

![Graph showing inhibition (%) of adenosine at different concentrations with and without pre-incubation.]
Chapter 7

The effects of adenosine and its analogues on smooth muscle contraction
Chapter 7

7.1 Introduction

Purines nucleotides and nucleosides have been shown to have pharmacological actions on a variety of smooth muscle preparations by activating either P₁ or P₂ purinoreceptors [453]. P₁ receptors are stimulated by adenosine and competitively antagonised by the methylxanthines. They are sub-divided into A₁, A₂a, A₂b and A₃ receptors as discussed in previous chapters. P₂ receptors preferentially bind ATP and have been subdivided into P₂X and P₂Y based on the different structure-activity relationships of ATP analogues at these receptor subtypes [454,455]. P₂X receptors usually mediate contraction whilst P₂Y receptors, once activated, induce relaxation of smooth muscle [456].

Following the observation that adenosine induces bronchoconstriction in allergic and non-allergic asthmatics (chapter 1), several experiments have been carried out in animal models in an attempt to define the mechanism involved. Initial experiments, however, produced conflicting results. Krzanowski et al [457], Ito and Takeda [458] and Brown and Collis [459] found that adenosine relaxed the airway smooth muscle of the dog, cat and guinea pig respectively. Holroyde [460] discovered that low concentrations of adenosine induced contraction of guinea pig trachea denuded of epithelium, although these findings were not confirmed by others [461]. Finney et al [462] found that both adenosine and ATP contracted human isolated bronchiolar smooth muscle and Pauwels and Van der Straeten [463] observed similar effects with adenosine in anaesthetised rats.

It was necessary, therefore, to find a suitable animal model for studying airway responsiveness induced by adenosine. Martin [464] reported that the guinea pig and the Basenji-greyhound were the best animal models for demonstrating airway responsiveness since challenge of the airways with a bronchoconstrictive agent induced transient vascular leakage in addition to bronchoconstriction. This is similar to the effects observed in asthmatic human subjects. In addition, Thorne and Broadley [465] demonstrated that guinea pigs sensitised to antigen or made hyporesponsive to spasmogens were bronchoconstrictive to adenosine.
Advenier et al [466] demonstrated that the effects of adenosine and ATP in guinea pig isolated trachea were dependent upon the initial tone of the organ. Both compounds were equipotent at relaxing trachea that had previously been contracted to acetylcholine, whilst they moderately contracted trachea under resting tone. The contractile effects of the agonists were antagonised by inhibitors of cyclooxygenase and thromboxane synthetase, indicating that the compounds act via release of arachidonic acid metabolites.

Caparrotta et al [467] showed that the adenosine receptor agonist PIA had a bi-phasic effect on guinea pig trachea, inducing contraction at low concentrations and relaxation at higher doses. Theophylline and 8PT competitively antagonised the inhibitory arm of the response, but had no effect on the contraction. Inhibitors of the arachidonic acid cascade blocked the contractile effects of PIA whilst potentiating the relaxation, suggesting that the effects of adenosine on tracheal tone may be regulated by prostaglandin turnover. Farmer et al [354] demonstrated that R-PIA>N6-cyclohexyladenosine(CHA)>CADO>S-PIA at inducing contraction, whilst NECA>CADO>R-PIA>S-PIA at mediating relaxation. They concluded, therefore, that the relaxation was the result of A2 receptor activation, whilst the contraction was A1 mediated. Their antagonist data was less conclusive, however, due to the non-selective relaxation induced by the compounds.

In contrast, Manzini and Ballati [468] found that a transient vagally mediated bronchospasm could be induced by stimulation of A2 receptors in anaesthetised guinea pigs.

In the rat duodenum and urinary bladder, Nicholls et al [353] noted that the responses to both adenosine and ATP were present as early as postnatal day 2, which was the earliest day studied. In the bladder, adenosine mediated relaxation, whilst the ATP-analogues induced contraction. The rank order of potency of the agonists suggested that the latter was the result of P2X receptor activation. In the duodenum, adenosine also mediated relaxation, whilst ATP induced relaxation at low concentrations and contraction at higher concentrations. Both effects of ATP appeared to be mediated by the activation of P2Y receptors. They concluded, therefore, that the urinary bladder and the duodenum were ideal models for studying P2X and P2Y receptors, respectively.
Gaion et al \[469\], demonstrated that the relaxation induced by adenosine in rat duodenum was associated with a marked decrease in the amplitude of the spontaneous contractions. The effects of adenosine were blocked by both theophylline and 8PT in a dose-dependent manner. Increasing concentrations of the two antagonists progressively shifted the concentration-response curve to adenosine to the right, with no effect on the maximum relaxation. The inhibition by the methylxanthines was also specific to adenosine, since neither compound antagonised the relaxations induced by noradrenaline or isoprenaline.

Studies by Nicholls et al \[470\] on rat duodenum and urinary bladder using selective adenosine receptor agonists and antagonists suggested that the rat duodenum contained a mixture of A₁ and A₂ receptors whilst the bladder contained only the latter. The limited potency of the selective A₂a agonist CGS21680 in both tissues suggested that the A₂ receptors were of the low affinity A₂b subclass. Further studies showed that when the two layers of the duodenum that contract in the longitudinal plane (the longitudinal muscle layer and the muscularis mucosae) were separated, the longitudinal muscle layer possessed both A₁ and A₂b receptors which induced relaxation, whilst the muscularis mucosae contained solely A₂b receptors, which induced contraction \[471-472\].

Peachey et al \[473\] discovered that the A₁ receptors in the rat duodenum were functional from day 20 and their density increased with age up to that found in the adult. In contrast, the A₂b receptors responded to NECA from day 5.

Mulè et al \[474\], investigating the relaxation induced by adenosine in isolated duodenal segments, reported that the differences in the morphology of relaxation induced by adenosine and electrical field stimulation, in addition to their sensitivity to theophylline, ruled out the possibility that adenosine was acting as a neurotransmitter of the NANC system. These findings were confirmed by Serio et al \[475\] and Postorino et al \[476\].

The aim of this study was to try to clarify the effects of adenosine on smooth muscle preparations, and to identify the receptors responsible for mediating contraction and relaxation, by using a range of adenosine receptor agonists and antagonists on guinea pig trachea (an ideal model for studying contraction) and rat duodenum (for relaxation). It was not possible to investigate the effects of adenosine on rat trachea, since it failed to
respond to stimulation by adenosine. Histamine also failed to produce a contractile effect in this tissue [477].

7.2 Materials and methods

The materials and methods employed in this study are described in chapter 2. All the rats used were Wistars and had previously been sensitised to the nematode *Nippostrongylus brasiliensis*. The guinea pigs were Duncan Hartleys and had been sensitised by subcutaneous injection of pertussis toxin (20 000 / ml, 0.5 ml) containing ovalbumin (5 mg/ml). It was important to use tissue from sensitised animals since adenosine induces bronchoconstriction in both allergic and non-allergic asthmatic subjects [311] but has no effect in normal subjects. In addition, studies by Matera et al [478] found that adenosine induced contraction of sensitised guinea pig trachea, but caused relaxation of trachea from non-sensitised animals.

7.3 Results

7.3.1 The effect of different agonists on guinea pig isolated trachea under basal tone

Ovalbumin (0.001 – 1 µg/ml) produced a concentration-dependent contraction of the guinea pig trachea (Fig 7.1) with a maximum increase in tension of 762.5 ± 81.8 mg, 0.3 µg/ml. Similar results were observed with histamine (0.01 – 10 µg/ml), except that the amine was less efficaceous than antigen at concentrations below 1 µg/ml, but induced far greater contraction at higher concentrations with a maximum increase in tension of 1939 ± 273 mg, 10 µg/ml.

Due to significant variation in the sensitivity of the guinea pig trachea to the adenosine receptor agonists, these compounds were only examined in tissues that responded well to histamine (the maximum response to the agonists was approximately 15-20 % of the maximum contraction induced by histamine). In addition, direct comparison of the potencies of the agonists was only made when the compounds were tested on the same preparation.
Adenosine induced contraction of the guinea pig trachea at low concentrations (0.1 – 10 μM). At concentrations above 10 μM, the contraction decreased, to be replaced by a slight relaxation at 1000 μM, Fig 7.2. The maximum tension increase was 187.5 ± 17.5 mg, 10 μM, which is much smaller than the maximum contractions to ovalbumin and histamine. Similar results were obtained with the adenosine A₃ receptor agonist IB-MECA and the A₁ agonist R-PIA, although these compounds were significantly more potent and efficacious than adenosine. Maximum tension increases of 377.5 ± 76.2 mg and 330 ± 89.6 mg, 1 μM were observed with IB-MECA and R-PIA respectively. This was approximately 50% of the maximum response to antigen. At concentrations above 1 μM, the contractile response to both compounds was reduced, with the response to R-PIA being replaced by a relaxation at 100 μM (reduction in tension of 132.5 ± 90.6 mg).

When the adenosine receptor agonists IB-MECA, R-PIA, APNEA and CPA were compared directly in the same study, the contraction induced by all the test compounds increased in the range 0.001 to 1 μM, and then decreased over the range 3 – 100 μM, with R-PIA inducing relaxation at 100 μM, Fig 7.3. The rank order of potency of the agonists was IB-MECA>R-PIA>APNEA= CPA.

Many experiments were carried out that compared three or four agonists in a single study. It was impossible to study all the different agonists on a single preparation due to the nature of the experiments. Various combinations of the nine agonists were, therefore, compared at any one time. Throughout the studies IB-MECA, R-PIA, APNEA and CPA were consistently the most potent compounds tested at inducing contraction. Fig 7.4 shows the results if all the agonist data are incorporated into the same graph. NECA, adenosine and inosine all induced contraction at low concentrations with this response decreasing at higher concentrations, although these compounds were much weaker than R-PIA, CPA, IB-MECA and APNEA. The CGS21680 response had no contractile component. At concentrations above 0.3 μM, the A₂a agonist weakly relaxed the tissue with a maximum reduction in tension of 103.3 ± 8.8 mg, 3 μM. Metrifudil, the A₂b agonist, had a very small contractile effect at low concentrations (maximum increase in tension of 60 ± 10 mg, 0.03 μM). It did, however, significantly relax the tissue at higher concentrations (maximum reduction in tension of 543.3 ± 133.5 mg, 10 μM) and was the most potent compound tested at inducing relaxation.
7.3.2 The effect of the BW3911 and mepyramine on the increase in tension induced by IB-MECA in guinea pig isolated trachea under basal tone

Preliminary studies carried out by Dr Assem, University College London, involving BW3911, have shown that the A₃ antagonist has no effect on trachea under resting tone at concentrations lower than 1 μM. At higher concentrations, a slight relaxation of the tissue is observed (change in tension < 50 mg). BW3911 and the H₁ antagonist mepyramine, however, significantly reduced the contraction induced by the adenosine receptor agonists. Mepyramine reduced the maximum response to IB-MECA by about 60%, whilst BW3911 completely reversed the contraction, so that significant relaxation of the tissue was observed, Fig 7.5. The increase in tension was 300 ± 150 mg in the presence of IB-MECA alone (1 μM), and 113.8 ± 36.3 mg and −418.8 ± 68.8 mg with mepyramine (2 μM) and BW3911 (0.2 μM) respectively. Similar effects were observed with both antagonists on APNEA induced contraction of the trachea. BW3911, however, whilst blocking the contraction, failed to relax tissue pre-contracted to either R-PIA or CPA (data not shown).

7.3.3 The effect of the different adenosine receptor agonists on carbachol induced contraction of the Wistar rat duodenum

The inhibitory responses to the adenosine receptor agonists were quantified by precontracting the tissue to carbachol (0.5 μM) before challenge with the agonist. It is usual to study relaxant actions of drugs on smooth muscle by raising the tone with an excitatory agonist. The response to carbachol was consistent and reproducible with a mean increase in tension of 3.8 ± 0.1 g (n=5). The relaxation was expressed, therefore, as the percentage inhibition of the carbachol induced contraction. The agonists were shown to have little or no effect on the tissue under basal resting tone.

The adenosine receptor agonist NECA (0.02 – 20 μM) dose-dependently relaxed the carbachol contracted rat duodenum with a maximum reduction of 42.3 ± 4.4 %, 20 μM, Fig 7.6. CPA, metrifudil and IB-MECA all produced similar responses with a rank order of potency of metrifudil > IB-MECA > NECA > APNEA. The A₁ agonist CPA, was essentially ineffective at concentrations below 2 μM. We also investigated the effects of
CGS21680 and discovered that the $A_{2a}$ agonist had no effect on the carbachol induced contractions.

7.3.4 The effect of BW3911 on IB-MECA's and APNEA’s reduction of carbachol induced contraction of the Wistar rat duodenum

The $A_3$ receptor antagonist BW3911, relaxed the carbachol-contracted rat duodenum by itself, in the absence of the adenosine receptor agonists, Fig 7.7 and 7.8. The maximum reduction was $20.5 \pm 7.7\%$, 0.2 $\mu$M. It failed, therefore, to reverse the relaxation induced by either APNEA or IB-MECA. The reductions in contraction induced by the agonists and the antagonist separately were not significantly different from those when the agonist and antagonists were added simultaneously.

7.3.5 The effect of CGS15943 on metrifudil's reduction of carbachol induced contraction of the Wistar rat duodenum

Figs 7.9-7.11 show the effects of the $A_2$ receptor antagonist CGS15943 on the relaxation in carbachol induced contraction produced by metrifudil at concentrations of 0.2, 2 and 20 $\mu$M respectively. CGS15943 significantly reversed the relaxant effects of the $A_{2b}$ agonist at all concentrations studied. For example, the reductions in carbachol induced contractions were $35.3 \pm 6.8\%$ in the presence of metrifudil alone (20 $\mu$M) and $24.9 \pm 3.9\%$ and $13.1 \pm 3.5\%$ in the presence of CGS15943 at concentrations of 2 and 20 $\mu$M, respectively.

7.4 Discussion

The results from this study provide conclusive evidence that extracellular adenosine can significantly regulate smooth muscle contraction at physiological concentrations. In keeping with findings by Caparrotta et al [467] and Farmer et al [354], we found that R-PIA had a bi-phasic effect on trachea smooth muscle, inducing contraction at low concentrations and relaxation at higher doses. The failure of others to observe this initial contractile effect has been attributed to the weak response of the trachea to the purine analogues relative to that of other agonists.
Ghai et al. [479], studying a range of adenosine receptor agonists and antagonists on guinea pig trachea, found conclusive evidence for the existence of two types of adenosine receptor. They noted that in tissue pre-contracted to histamine, carbachol, KCl or field stimulation, only concentration-related relaxation responses were observed. This indicates that the relaxing effects of the adenosine agonists are not specific for a single spasmogen. The rank order of potency of the agonists was indicative of A2 receptor activation. This is in keeping with findings by Advenier et al. [466] who showed that in tissues where the tone has been raised by an excitatory agonist, the contractile phase of the purine response was completely abolished. We noted that this effect was, however, short-lived since tissues that responded well to histamine also responded well to the adenosine analogues if given time to return to basal tone.

In tissue under resting tone, the above authors observed a concentration-related contraction with a rank order of agonist potency of R-PIA>CADO=CPA=CHA [479], indicating that the receptor being activated was of the A1 subtype. This was confirmed by the ability of the A1 antagonist 1,3 dipropyl-8-(2 amino-4-chlorophenyl) xanthine (PACPX), but not theophylline, to attenuate the response. This effect was not mediated by stimulation of cholinergic, adrenergic or histaminergic systems since pretreatment with atropine, propranolol, phentolamine and pyrilamine did not modify the response. It was, however, shown to diminish after repeated administration of R-PIA. In contrast, the relaxation response was not tachyphylactic.

We noted that the limited response of the trachea to the adenosine receptor agonists meant that it was impossible to compare the relative potencies of the compounds unless they were studied on the same tissue preparation. Our data shows that the A1 agonists R-PIA and CPA and the A3 agonists IB-MECA and APNEA all significantly contracted the trachea at low concentrations with this effect decreasing at higher doses. Similar results were obtained with NECA, adenosine and inosine, although these agonists were less potent and efficacious. Xiaowei et al. [480] recently reported that inosine binds to recombinant rat A3 adenosine receptors in addition to A3 receptors in guinea pig lung, and that it stimulates degranulation of RBL-2H3 cells. It is possible, therefore, that in the trachea it also acts as a weak A3 adenosine receptor agonist. The rank order of potency of the agonists for the contractile response was IB-MECA≥R-PIA>APNEA=CPA. It is not possible from the agonist data to define the receptor
responsible for mediating contraction since IB-MECA and R-PIA were significantly
more potent than any of the other compounds tested in all experiments.

Preliminary studies with the A\textsubscript{3} adenosine receptor antagonist BW3911 have shown that
not only does it reverse the contraction induced by APNEA, IB-MECA and CPA at
micromolar concentrations, but that it induces significant relaxation in tissue pre­
contracted to the A\textsubscript{3} agonists. The H\textsubscript{1} antagonist mepyramine, also partly blocks (by
approximately 50 - 60 \%) the agonist response. It is possible, therefore, that IB-MECA
has several effects on guinea pig trachea. Firstly, the A\textsubscript{3} agonist acts directly on the
smooth muscle. This effect has both a contractile and relaxant component, with
contraction observed at low agonist concentrations and relaxation at higher
concentrations. BW3911 blocks the contractile part of the agonist response so that only
relaxation is observed. In addition, IB-MECA mediates some of its effects by activating
mast cells to induce degranulation. This would explain why the H\textsubscript{1} antagonist
mepyramine partly blocks the contractile component of the IB-MECA response. This
theory, however, can only be confirmed by the completion of the antagonist data since
the effects of the selective A\textsubscript{1} receptor antagonist DPCPX have yet to be confirmed.

These results are in direct contrast to findings by El-Hashim et al \cite{481} who could find no
evidence for the role of the A\textsubscript{3} receptor in adenosine induced bronchoconstriction in
allergic rabbits. They observed that aerosol challenge with CPA but not APNEA elicited
a fall in dynamic compliance and an increase in airway resistance. The reason for this
discrepancy is unclear although in our studies on mast cells we have found that APNEA
was not a potent A\textsubscript{3} agonist. It would have been more conclusive, therefore, to have
looked at IB-MECA since this agonist has been shown to be A\textsubscript{3} selective in all species
\cite{441}.

Matera et al \cite{478}, found that the dose-response curve to R-PIA in trachea from non­
sensitised guinea pigs, was shifted to the right by the 5HT\textsubscript{2} antagonist ketanserin in
addition to PACPX. Diphenydramine, the H\textsubscript{1} receptor antagonist, and indomethacin
which blocks prostaglandin synthesis were, however, ineffective. It was concluded,
therefore, that the airway response to adenosine was probably mediated via activation of
5-HT\textsubscript{1} and/or 5-HT\textsubscript{2} receptors, potential sources of 5-HT within the pulmonary system
being platelets, nerve-terminals and mast cells.
Ali et al. [482] demonstrated that the increase in tension of the airway smooth muscle in bronchial rings from allergic rabbits, induced by the adenosine receptor agonist CPA, was accompanied by a quantitative increase in intracellular calcium. In calcium free medium, CPA was shown to be only 15 to 20% as effective at inducing contractions as in calcium containing medium. The CPA induced contraction and the changes in intracellular calcium were blocked by CGS15943 and theophylline in calcium containing medium, but only by CGS15943 in calcium free medium. The A1 antagonist, DPCPX also failed to block the response. These experiments indicate that calcium plays an important role in adenosine induced contraction of airway smooth muscle, and that the calcium independent part of the response is xanthine insensitive.

In addition to the effects induced by the A1 and A3 receptor agonists, our studies show that the A2 agonists CGS21680 and metrifudil induce significant responses in guinea pig trachea under resting tone. Both compounds induce relaxation at sub-micromolar concentrations with little or no contraction observed. Since metrifudil is significantly more potent than CGS21680, these data suggest that the receptor mediating relaxation is of the A2b subtype.

Losinski and Alexander [483], studying adenosine receptor mediated relaxation of guinea pig pre-contracted isolated trachea, found that the agonist profile was consistent with the activation of adenosine A2b receptors i.e. NECA=CADO>2-(2-aminoethy lamino)-carbonylethylphenylethlamino-5'-N-ethylcarboxamido adenosine (APEC)>CGS21680>adenosine. APEC, like CGS21680, is an A2a selective agonist. The rank order of potency of the antagonists was, however, more consistent with the activation of A2a receptors. They concluded, therefore, that the tracheal adenosine receptor was a new type of adenosine A2 receptor, distinct from the two currently identified.

In the duodenum, all the agonists tested induced relaxation. The rank order of potency of the compounds was consistent with the activation of either A2b or A3 receptors since NECA, metrifudil, APNEA and IB-MECA all showed similar potency. The A1 agonist CPA and the A2a agonist CGS21680 were essentially inactive. The failure of BW3911 to block the effects of APNEA and IB-MECA indicated that the response was not A3 mediated. The potent A2 antagonist CGS15943 effectively reversed the relaxation of the carbachol response induced by metrifudil at all concentrations tested. Since CGS21680
was not active in this system, this confirms the findings of Nicholls et al. that in the rat duodenum it is the A$_{2b}$ subclass of A$_2$ receptor that is responsible for mediating relaxation. We failed, however, to detect a noticeable A$_1$ mediated effect and the reason for this discrepancy is unclear.

Hargreaves et al. found that the agonist and antagonist profiles for adenosine mediated relaxation in dog lateral saphenous vein and in guinea pig aorta, were also consistent with the activation of A$_{2b}$ receptors.

Fredholm et al. investigated the mechanism by which theophylline relaxes tracheal smooth muscle, observed that it acts partly by antagonising endogenous adenosine and partly by inhibiting cAMP phosphodiesterase. cAMP has been indicated as the mediator responsible for inducing relaxation in many different smooth muscle types, and since adenosine A$_2$ receptors appear to couple exclusively to adenylate cyclase via binding to G$_s$ proteins, it seems probable that adenosine relaxes smooth muscle by A$_2$ receptor activation resulting in subsequent cAMP elevation in the cytosol.

Murthy et al. confirmed these findings by examining adenosine receptors and their signalling pathways in dispersed intestinal muscle cells. Adenosine, by itself, was shown to induce contraction in addition to augmenting intracellular calcium and cAMP levels. CPA mimicked the contraction, but DPCPX and pertussis toxin abolished both the contraction and the augmentation in calcium levels whilst increasing intracellular cAMP. Rp-cAMP[S], the cAMP kinase inhibitor, and CGS15943, the A$_2$ antagonist, both augmented contraction and the increase in intracellular calcium, whilst abolishing the rise in cAMP. The A$_{2a}$ agonist CGS21680 was again shown to be only a weak relaxant of smooth muscle.

In conclusion, the present study indicates that adenosine is an important modulator of smooth muscle function. The results indicate that in the guinea pig trachea there are at least two subtypes of receptor, one mediating contraction and the other relaxation. Where resting muscle tone is sufficient, an initial increase in tone is observed which is probably the result of A$_3$ receptor activation, although it is impossible to eliminate the involvement of A$_1$ receptors without further antagonist studies. At higher agonist concentrations, there is a progressive relaxation of tone which is likely to be due to the
activation of $A_2b$ receptors. In the rat duodenum, where pre-contraction with carbachol means that adenosine-induced contraction is masked and only relaxation is observed, the potency of the agonists and the antagonists indicates that the relaxation is probably $A_2b$ mediated. It seems likely, therefore, that regardless of the origin of the tissue there are at least two different types of adenosine receptor, one that either augments calcium levels or inhibits cAMP, resulting in contraction of the smooth muscle, and a second that augments cAMP levels to induce relaxation.
Fig. 7.1 The contractile effects of ovalbumin and histamine on guinea pig isolated trachea under basal tone (n=6-11)

Fig. 7.2 The effect of IB-MECA, R-PIA and adenosine on guinea pig isolated trachea under basal tone (n=4)
Fig. 7.3 The effect of IB-MECA, R-PIA, APNEA and CPA on guinea pig isolated trachea under basal tone (n=4)

![Graph showing the effect of different adenosine receptor agonists on guinea pig isolated trachea under basal tone.](image)

Fig. 7.4 The effect of different adenosine receptor agonists on guinea pig isolated trachea under basal tone (n=2-8)

![Graph showing the effect of different adenosine receptor agonists on guinea pig isolated trachea under basal tone.](image)
Fig 7.5 The effect of BW3911 and mepyramine on the increase in tension induced by IB-MECA in guinea pig isolated trachea under basal tone (n=3)
Fig. 7.6 The effect of different adenosine receptor agonists on carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=3-8)

Fig. 7.7 The effect of BW3911 on IB-MECA’s reduction of carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=3-5)

(Asterisks denote the reductions in carbachol induced contraction induced by IB-MECA and BW3911 together that were significantly different from the reductions obtained by IB-MECA and BW3911 separately. *p<0.05)
Fig. 7.8 The effect of BW3911 on APNEA’s reduction of carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=4)

(The reductions in carbachol induced contraction induced by APNEA and BW3911 together were not significantly different from the sum of the reductions obtained by APNEA and BW3911 separately.)

![Graph showing the effect of BW3911 on APNEA's reduction of carbachol induced contraction.]

Fig. 7.9 The effect of CGS15943 on Metrifudil’s (0.2 μM) reduction of carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=3)

(Asterisks denote the reductions in carbachol induced contraction induced by metrifudil that were significantly reversed by pre-incubating with CGS15943. * p<0.05, **p<0.01, ***p<0.001)

![Graph showing the effect of CGS15943 on Metrifudil's reduction of carbachol induced contraction.]
Fig. 7.10 The effect of CGS15943 on Metrifudil’s (2 μM) reduction of carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=3)

(Asterisks denote the reductions in carbachol induced contraction induced by metrifudil that were significantly reversed by pre-incubating with CGS15943. * p<0.05, **p<0.01, ***p<0.001)

Fig. 7.11 The effect of CGS15943 on Metrifudil’s (20 μM) reduction of carbachol (0.5 μM) induced contraction of the Wistar rat duodenum (n=3)

(Asterisks denote the reductions in carbachol induced contraction induced by metrifudil that were significantly reversed by pre-incubating with CGS15943. * p<0.05, **p<0.01, ***p<0.001)
Chapter 8

General synopsis and overview
Chapter 8

Adenosine is a naturally occurring purine nucleoside present in nanomolar concentrations in the extracellular fluid. It is cleaved from AMP by the enzyme 5'-nucleotidase and has a regulatory role in many physiological systems, where it modulates inflammation by acting on a wide variety of cells and is believed to have an important role in bronchoconstriction. Its effects are mediated by four G-protein coupled receptors; A\textsubscript{1}, A\textsubscript{2a}, A\textsubscript{2b} and A\textsubscript{3}, which bind to either adenylate cyclase or PLC. The effect of adenosine on mast cells and basophils is species dependent and determined by which receptors are activated.

The inhalation of adenosine or AMP causes rapid and reproducible bronchoconstriction in both atopic and non-atopic asthmatics. Since AMP is known to be the precursor for adenosine, and since the characteristics of the bronchoconstriction induced by the two substances are identical, it seems likely that the AMP induced bronchoconstriction is the result of extracellular hydrolysis of AMP to adenosine. The mechanism of adenosine induced asthma is unknown, but is thought to involve the action of the nucleoside at two different sites. Firstly, adenosine activates mast cells, inducing release of histamine and other bronchoconstrictor mediators which act to contract airway smooth muscle. Secondly, the nucleoside acts on sensory nerves to induce a local release of neuropeptides and indirect mast cell activation.

In light of this, it was the aim of the present study to elucidate further the role of adenosine in mast cell degranulation by examining the effects of adenosine on different mast cell phenotypes and using a range of agonists and antagonists in an attempt to define the receptors involved.

Marquardt et al showed that adenosine had the ability to enhance anti-IgE, con A, 48/80 and calcium ionophore induced histamine release, at concentrations as low as 1 nM, without affecting the spontaneous release of the amine. The potentiation by adenosine of both immunologic and non-immunologic mast cell degranulation implied that its effects were mediated by interference at a relatively late stage in cellular activation. The named authors were unable to determine the biochemical basis for the
potentiation but postulated that it involved changes in cAMP metabolism since the phosphodiesterase inhibitor theophylline blocked the potentiating effects of adenosine without otherwise affecting mast cell function. Further studies by Nishibori et al \[^{356}\], however, showed that adenosine had a bi-phasic effect on anti-IgE induced histamine release, causing inhibition at low concentrations and potentiation at high concentrations.

Our preliminary experiments in RPMC showed that the nature of the adenosine response was dependent upon the mast cell phenotype, with cells from three different rat strains exhibiting marked differences in their responses to adenosine. The nucleoside enhanced anti-IgE induced histamine release from PMC of Wistar rats, whilst in Sprague-Dawley and Hooded Lister rats, both adenosine and NECA had a bi-phasic effect on histamine release.

The main contradictory results in the literature were over the effects of adenosine and its analogues on mediator release induced by the basic secretagogues, with different authors reporting that the nucleoside potentiated \[^{254}\][\[^{355}\]], inhibited \[^{356}\] or had no effect \[^{258}\] on the response to 48/80. Our results indicate that the potentiation by adenosine of histamine release induced by 48/80, mastoparan and substance P was substantially less than for all the other secretagogues tested.

In keeping with reports by Lohse et al \[^{358}\], we found that adenosine potentiated calcium ionophore A23187 induced release, the magnitude of the response being dependent upon the magnitude of the control release. The same effect was observed with NGF, dextran, anti-IgE and con A, with the degree of potentiation observed being also dependent upon the rat strain and the stimulus used.

Hence our results show conclusively that, in addition to the effects of adenosine being dependent on the strain of the rat, there is also considerable variation within a given strain according to the nature of the stimulus. This probably accounts for previous conflicting results reported in the literature. Only in the Wistar rat was adenosine a universal potentiator, and since the greatest degree of potentiation was observed with this strain, all subsequent experiments involving agonist potency and second messenger systems were performed with PMC from this source.
Levels of cAMP are known to be elevated in the mast cell following immunological activation. Whether this rise has an excitatory or inhibitory effect is still under debate, but it appears to be an important part of the secretory process \cite{368-369}. Leoutsakos and Pearce \cite{254} found that enhancing or suppressing that transient elevation of cAMP by appropriate adenosine analogues produced a parallel change in histamine secretion. Studies with theophylline, however, showed that the adenosine receptor antagonist prevented the elevation of cAMP but did not affect the potentiation of mediator release.

Subsequent experiments by Marquardt and Walker \cite{376-377} indicated that the effects of adenosine might be mediated by PLC and PKC activation. Ali et al \cite{309} and Ramkumar et al \cite{308} found conclusive evidence that RBL-2H3 cells contained solely A3 receptors and that adenosine and its analogues augmented histamine release by releasing inositol phosphates and substantially elevating calcium levels in the cytosol.

Our results with the adenosine receptor agonists showed that they potentiated histamine release from both passively and actively sensitised mast cells from Wistar rats with a rank order of potency of IB-MECA > metrifudil > R-PIA = CPA > NECA > CGS21680. The concentration-response curve was shifted to the right by BW3911 and, to a lesser extent, by CGS15943, but was unaffected by 8-PT and DPCPX. These results are in agreement with an earlier report \cite{395} and indicate that, like in RBL-2H3 cells, the enhancement of IgE-dependent histamine release from RPMC is via activation of the adenosine A3 receptor.

To determine whether adenosine potentiated histamine release by augmenting intracellular calcium levels, cytosolic concentrations of the cation were measured using the fluorescent indicator fluo-3. Stimulation of mast cells with anti-IgE led to a transient elevation in intracellular calcium prior to histamine release. This elevation was not significantly increased in the presence of the agonist IB-MECA.

Kinetic studies showed that the addition of IB-MECA significantly reduced the time taken for maximum release of histamine to occur. In the absence of IB-MECA, histamine release with anti-IgE reached a maximum after 180 s. When the A3 agonist was added, this time was reduced to 40 s. Since the fluo-3 system only detects overall changes in calcium levels, we decided to investigate the correlation between histamine...
release and IP₃ production. Cells stimulated with anti-IgE alone demonstrated an increase in intracellular IP₃ which reached a maximum after 20 s. Cells stimulated simultaneously with anti-IgE and IB-MECA produced a rapid rise in IP₃ levels, which peaked after 5 s before falling to the levels observed with anti-IgE alone after 10 s. It would appear, therefore, that this transient elevation in IP₃ is probably sufficient to potentiate histamine release. Adenosine and NECA by themselves have been shown to release inositol phosphates and increase calcium levels in the cytosol. They do not, however, stimulate a sustained uptake of calcium from the external environment. The failure of adenosine to affect the spontaneous release of histamine has been attributed to its inability to induce calcium influx in the absence of a secretagogue [309].

Studies on mouse bone marrow derived mast cells showed that the simultaneous addition of adenosine and its analogues with the secretory stimulus significantly potentiated beta-hexosaminidase release but had no effect on the spontaneous release of the enzyme [410]. In resting cells, adenosine transiently elevated cAMP levels and binding studies showed that the cells possessed A₂ but not A₁ receptors. The inability of CGS21680 to potentiate stimulated beta-hexosaminidase release suggested that it was the A₂b receptor responsible for modulating degranulation. Subsequent studies by McWhinney et al [415], however, found that murine J774.1 macrophages predominantly expressed adenosine A₃ receptors.

In contrast to previous reports, we found that the adenosine receptor agonists directly induced histamine release from mouse PMC. The magnitude of the effect was strain dependent, with cells from MF1 mice being much more responsive than cells from TO mice. In keeping with our findings in the rat, we found that cells from MF1 mice were also more sensitive to immunological challenge. It appears, therefore, that there is a good correlation between sensitivity to antigen and responsiveness to adenosine. This is in keeping with reports that showed adenosine induces bronchoconstriction in asthmatic but not in normal subjects [311].

All the test agonists induced histamine release from MF1 mice although only IB-MECA and adenosine induced significant mast cell degranulation in cells from TO mice. Despite the significant differences in the extent of histamine release induced by the agonists from the two different strains, IB-MECA was the most potent compound tested.
in both studies. This release was abolished in the presence of the metabolic inhibitors antimycin A and 2-DOG, indicating that the release was not cytotoxic. In addition, all the agonists significantly potentiated the ionophore response in TO mice, with IB-MECA and metrifudil being the most potent compounds tested.

The release induced by IB-MECA was reversed by pre-incubation with adenosine receptor antagonists. The order of potency was CGS15943>BW3911>DPCPX>8PT. The concentrations of the antagonists required to produce significant inhibition were, however, very high and BW3911 and CGS15943, in particular, are known to produce non-selective inhibition under these conditions. The rank order of potency of the antagonists is, however, consistent with binding at the human A

_3_ receptor.

It would appear, therefore, that in mouse PMC the adenosine agonists both induce and potentiate secretagogue induced release. The first effect is probably mediated via activation of adenosine A

_3_ receptors and the latter via either A

_2b_ or A

_3_.

In contrast to murine PMC, but in keeping with previous reports [438-440], adenosine inhibited immunologic histamine release from human basophils and lung mast cells. There was a negative correlation between the control release induced by anti-IgE and the subsequent inhibition by NECA. The effect was most apparent if the cells were pre-incubated with the nucleoside but disappeared if the compound was added after the secretory stimulus.

Peachell et al [440] showed that, in the basophil, the inhibition by adenosine of both histamine and LTC

_4_ release was accompanied by an elevation in cAMP. The order of agonist potency indicated that the effect was A

_2_ mediated. They found that 8PT antagonised both the elevated cAMP levels and the inhibition of mediator release but not that of LTC

_4_. Our results show that only the non-selective agonist NECA and the A

_3_ agonists IB-MECA and APNEA effectively inhibited anti-IgE induced histamine release. The remaining agonists were all essentially ineffective. NECA’s inhibition was reversed to some extent by all the adenosine receptor antagonists tested, although the rank order of potency is most consistent with binding at the A

_3_ receptor [422] since CGS15943 binds to this receptor in the human with a far higher affinity than in the rat [442].
In human lung mast cells, previous studies have produced many conflicting results, with reports that adenosine inhibits \cite{445}, potentiates \cite{446} and has a bi-phasic effect \cite{440} on the anti-IgE response, with low concentrations of the nucleoside potentiating histamine release and higher concentrations inhibiting. Hughes et al \cite{435} also demonstrated a bi-phasic response but one that was dependent on whether the cells were pre-stimulated with adenosine prior to anti-IgE challenge or whether the cells and anti-IgE were incubated together and post-challenged with the nucleoside. We observed a similar response in human lung mast cells to that observed in basophils, with pre-incubation with the nucleoside inducing inhibition, and this effect disappearing if the compound was added after the secretory stimulus. All the agonists apart from CGS21680 showed broadly comparable activity although NECA, metrifudil and IB-MECA were the most potent. The antagonist data also indicated an A\(_{2b}\) mediated effect since 8PT and CGS15943 were the most potent compounds tested.

In keeping with work by Mita et al \cite{452}, adenosine also inhibited anti-IgE induced histamine release from human skin mast cells.

In order to determine the effects of adenosine on smooth muscle function, it was necessary to find animal models ideal for studying both the contractile and relaxant components of the nucleoside response. We studied, therefore, a range of adenosine receptor agonists and antagonists on guinea pig trachea \cite{464-465} and rat duodenum \cite{353}, respectively.

In keeping with findings by Caparrotta et al \cite{467}, we observed that the \(\text{A}_1\) and \(\text{A}_3\) agonists induced contraction of the guinea pig trachea at low concentrations with this response being reversed at higher concentrations. NECA, adenosine and inosine, although weaker, exhibited similar responses. The rank order of agonist potency for the contractile component was IB-MECA \(>\) R-PIA \(>\) APNEA \(=\) CPA. Neither CGS21680 nor metrifudil produced a substantial contraction of the tissue. Indeed, metrifudil significantly relaxed the trachea at concentrations above 0.1 \(\mu\text{M}\). Preliminary antagonist results have shown that the contractile component of the response produced by APNEA, CPA and IB-MECA is completely abolished in the presence of BW3911. The \(\text{A}_3\) antagonist reverses the IB-MECA and APNEA responses so that a relaxation of the tissue is observed. The \(\text{H}_1\) antagonist, mepyramine, also partly blocks the agonist
response indicating that adenosine mediates some of its effects on smooth muscle by
inducing the release of mediators from mast cells. Ghai et al \cite{479} and Caparrotta et al
\cite{467} proposed that this effect was $A_1$ mediated, but they failed to test any agonists that
were selective for the $A_3$ receptor. However, they did show that the $A_1$ antagonist
PACPX, but not theophylline, attenuated the contractile response. Whether the
contraction is $A_1$ or $A_3$ mediated will only be confirmed by the completion of the
antagonist studies and it is possible that the trachea contains both receptor subtypes. Our
results thus far, however, indicate that the guinea pig trachea probably contains $A_3$
receptors that mediate contraction and $A_{2b}$ receptors that mediate relaxation.

Studies by Nicholls et al \cite{470} on rat duodenum and urinary bladder suggested that the
duodenum contained a mixture of $A_1$ and $A_2$ receptors, whilst the bladder contained
only $A_2$. Further studies, however, showed that if the two layers of the duodenum that
contract in the longitudinal plane were separated, the longitudinal muscle layer
possessed both $A_1$ and $A_{2b}$ receptors which induced relaxation and the muscularis
mucosae contained solely $A_{2b}$ receptors which mediated contraction.

Our results show that all the agonists tested induced relaxation of the rat duodenum, the
rank order of potency being consistent with the activation of either $A_{2b}$ or $A_3$ receptors.
The $A_1$ agonist CPA and the $A_{2a}$ agonist CGS21680 were ineffective. BW3911 failed to
reverse the relaxation induced by APNEA or IB-MECA whilst CGS15943 effectively
blocked the effects of metrifudil at all concentrations tested, indicating that the receptor
being activated is of the $A_{2b}$ subclass. This is in keeping with findings by Hargreaves et
al \cite{464}, who found that the agonist and antagonist profiles for relaxation of the dog
lateral saphenous vein and the guinea pig aorta were consistent with the activation of
adenosine $A_{2b}$ receptors.

In summary, in keeping with previous reports, adenosine was shown to potentiate
histamine release from RPMC. The magnitude of the effect varied with the
secretagogue and the strain of animal. The effects of selective agonists and antagonists
indicated that the enhancement was probably mediated through $A_3$ receptors. Adenosine
and its analogues both potentiated induced histamine release and themselves evoked
secretion from mouse PMC. Both effects appear to be mediated through $A_3$ receptors.
Further studies on the mechanism of release indicated that, in the rat, adenosine
mediates its effects by augmenting IP₃ production. In contrast to murine mast cells, adenosine and its analogues inhibited histamine release from human basophils and lung mast cells, with the rank order of potency of the agonists and antagonists suggesting the involvement of A₃ and A₂b receptors, respectively. The functional experiments demonstrated that adenosine is an important modulator of smooth muscle function. The nucleoside probably mediates contraction of the muscle by activating A₃ receptors resulting in an augmentation of calcium levels and relaxation by activating A₂b receptors resulting in a rise in intracellular cAMP levels.

In conclusion, the present study has indicated a fundamental role for adenosine in mast cell activation and smooth muscle contraction, the control of which may be therapeutically invaluable in the treatment of inflammatory disease.
References

1 Ehrlich P., Doctoral Thesis, University of Leipzig, Leipzig, East Germany, 1878
3 Unna P.G., Miliaria Syphilide, Leopold Voss, Hamburg, 1894
6 Schultz W.H., J. Pharmacol. Exp. Ther., 1910, 1, 549
7 Dale H.H., Laidlaw P.P., J. Physiol., 1919, 52, 355
8 Webb R.L., Am. J. Anat., 1931, 49, 283
12 Riley J.F., West G.B., J. Physiol., 1953, 120, 528
14 Prausnitz C., Küstner H., Z. Bakt., 1921, 86, 160

193
25 Kay A.B., J. Allergy Clin. Immunol., 1979, 64, 90
32 Godfrey H.P., Ilardi C., Engber W., Granziano F.M., Arth. Rheum., 1984, 27, 852
36 Enerbäck L., Fall M., Aldenborg F., Agents Actions, 1989, 27, 113

194
<table>
<thead>
<tr>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 Lichtenstein L.M., Scientific Am., 1993, Special Issue, 85</td>
</tr>
<tr>
<td>52 Miezel S.B., FASEB. J., 1989, 3, 2379</td>
</tr>
<tr>
<td>53 Mygind N., Allergy, 1993, 48, 476</td>
</tr>
<tr>
<td>58 Buckley R.H., FASEB. J., 1981, 40, 2159</td>
</tr>
<tr>
<td>60 Kitamura Y., Go S., Hatanaka K., Blood, 1978, 52, 447</td>
</tr>
<tr>
<td>61 Kitamura Y., Go S., Blood, 1979, 53, 492</td>
</tr>
<tr>
<td>64 Hatanaka K., Kitamura Y., Nishimune Y., Blood, 1979, 53, 142</td>
</tr>
<tr>
<td>65 Hayashi C., Sonada T., Nakano T., Nakayama H., Kitamura Y., Dev. Biol., 1985, 109, 234</td>
</tr>
<tr>
<td>66 Kitamura Y., Fujita J., Bio-Essays, 1989, 10, 193</td>
</tr>
<tr>
<td>69 Kitamura Y., Kanakura Y., Sonada S., Asai H., Nakano T., Int. Arch. Allergy Appl. Immunol., 1987, 82, 244</td>
</tr>
<tr>
<td>70 Galli S.J., Lab. Invest., 1990, 62, 5</td>
</tr>
</tbody>
</table>
75 Pearce F.L., Pharmacol., 1986, 32, 61
79 Enerbäck L., Histochem., 1974, 42, 301
87 Gibson S., Miller H.R.P., Immunol., 1986, 58, 101

196
97 Craig S.S., Schechter N.M., Schwartz L.B., Lab. Invest., 1988, 58, 682
98 Craig S.S., Schwartz L.B., Lab. Invest., 1990, 63, 581
102 Bienenstock J., Befus A.D., Denburg J., Goodacre R., Pearce F.L., Shanahan F., Monogr. Allergy, 1983, 18, 124
110 Moodley I., Mongar J.L., Agents Actions, 1981, 11, 77
113 Ishizaka T., Ishizaka K., J. Immunol., 1978, 120, 800
114 Siraganian R.P., Hook W.A., Levine B.B., Immunochem., 1975, 12, 149
115 Kazimierczak W., Diamant B., Prog. Allergy, 1978, 24, 295

197
120 Krumins S.A., Broomfield C.A., Neuropeptides, 1992, 21, 65
122 Ennis M., Atkinson G., Pearce F.L., Agents Actions, 1980, 10, 222

198
144 Schwartz L.B., Austen K.F., Prog. Allergy, 1984, 34, 271
147 Pearce F.L., Agents Actions, 1991, 33, 4
150 Black J.W., Shankley N.P., TiPS, 1987, 8, 486

199
174 Everitt M.T., Neurath H., FEBS. Letts., 1980, 110, 292
179 Thompson H.L., Schulman E.S., Metcalfe D.D., J. Immunol., 1988, 140, 2708
189 Ruoslahti E., Yamaguchi Y., Cell, 1991, 64, 867
194 Howarth P.H., Durham S.R., Lee T.H., Kay A.B., Church M.K., Holgate S.T.,
197 Ennis M., Barrow S.E., Blair I.A., Agents Actions, 1984, 14, 397
199 Piper P.J., TiPS, 1983, 4, 75
202 Holgate S.T., Robinson C., Church M.K., In: Allergy, Principles and Practice,
(Ed. Middleton E., Reed C.E., Ellis E.F., Adkinson N.F., Yunginger J.W.), The
C.V. Mosby Co., 1988, 135
204 Hardy C.C., Robinson C., Tattersfield A.E., Holgate S.T., N. Eng. J. Med., 1984,
311, 209
Acad. Sci. U.S.A., 1984, 81, 2191
1986, 83, 2204
Immunol., 1988, 81, 711
Immunol., 1989, 84, 19
213 Barnes N.C., Piper P.J., Costello J.F., Thorax, 1984, 39, 500
Prostaglandins, 1983, 25, 155

201
215 Holgate S.T., Kay A.B., Clin. Allergy, 1985, 15, 221
216 Mustafa S.B., Pearce F.L., Agents Actions, 1992, 37, C265
236 Mohr F.C., Fewtrell, J. Biol. Chem., 1987, 262, 10638

202


240 Fewtrell C., Sherman E., Biochem., 1987, 26, 6995


244 Hidaka H., Ishikawa T., Cell Calcium, 1992, 13, 465

245 Peachell P.T., Pearce F.L., Br. J. Pharmacol., 1989, 97, 547

246 Rana R.S., Hokin L.E., Physiol. Rev., 1990, 70, 115


252 Nishizuka Y., Nature, 1984, 308, 693


257 Leung K.P.B., Barrett K.E., Pearce F.L., Agents Actions, 1984, 14, 461


262 Hughes P.J., Benyon R.C., Church M.K., J. Pharmacol. Exp. Ther., 1987, 242, 1064

203
266 Lichtenstein L.M., Margolis S., Science, 1968, 161, 902
267 Cohen P., TiBS, 1992, 17, 408
269 Hepler J.R., Gilman A.G., TiBS, 1992, 17, 383
279 Theoharides T.C., Sieghart W., Greengard P., Douglas W.W., Science, 1980, 207, 80
281 Wells E., Mann J., Biochem. Pharmacol., 1983, 32, 837
282 Emadi-Khiav B., Pearce F.L., Agents Actions, 1992, 37, C272
283 Emadi-Khiav B., Pearce F.L., Agents Actions, 1994, 41, C37
287 Kennerly D.A., J. Immunol., 1990, 144, 3912
290 Penner R., Neher E., TiNS, 1989, 12, 159
292 Romanin C., Reinsprecht M., Pecht I., Schindler H., EMBO. J., 1991, 10, 3603
1988, 143, 259
296 Van Calker D., Müller M., Hamprecht B., J. Neurochem., 1979, 33, 999
302 Meyerhof W., Müller-Brechlin R., Richter D., FEBS. Letts., 1991, 284, 155
305 Hill R.J., Oleynek J.J., Hoth C.F., Kiron M.A.R., Weng W., Wester R.T., Tracey
1997, 280, 122
1994, 33, 51

205
315 Crimi. N., Palermo F., Oliveri R., Maccarrone C., Palermo B., Vancheri C., Polosa R., Mistretta A., Allergy, 1988, 43, 179
316 Church M.K., Holgate S.T., J. Allergy Clin. Immunol., 1993, 92, 190
330 Collis M.G., Hourani S.M.O., TiPS, 1993, 14, 360

206
343 Richardson P.J., Kase H., Jenner P.G., TiPS, 1997, 18, 338
Nicholls J., Hounani S.M.O., Kitchen I., Br. J. Pharmacol., 1990, 100, 874
Nishibori M., Shimamura K., Yokoyama H., Tsutsumi K., Saeki K.,
Arch. Int. Pharmacodyn., 1983, 265, 17
1392
Hughes P.J., Church M.K., Agents Actions, 1986, 18, 81
6800
Fredholm B.B., Sydbom A., Agents Actions, 1980, 10, 145
467
Ishizaka K., Ishizaka T., J. Immunol., 1969, 103, 588
Church M.K., Hughes P.J., Br. J. Pharmacol., 1985, 85, 3
Vardey C.J., Skidmore I.F., In: Purines, Pharmacology and Physiological Roles,
Marquardt D.L., Walker L.L., Agents Actions, 1994, 43, 7
Beaven M.A., Gonzaga H.M.S., In: The Biology and Medicine of Signal

208
385 Shi D., Nikodijevic O., Jacobson K.A., Daly J.W., Arch. Int. Pharmacodyn., 1994, 328, 261
391 Ukena D., Schudt C., Sybrecht G.W., Biochem. Pharmacol., 1993, 45, 847
Sagi-Eisenberg R., Foreman J.C., Raval P.J., Cockcroft S., Immunol., 1987, 61, 203
Kurosawa M., Parker C.W., Biochem. Pharmacol., 1987, 36, 131
Assem E.S.K., Personal Communication, EHRS, Lodz, 1998
Hon W-M., Moochhala S., Khoo H-E., Life Sci., 1997, 60, 1327
Linden J., TiPS, 1994, 15, 298
430 Feoktistov I., Biaggioni I., Mol. Pharmacol., 1993, 43, 909
435 Hughes P.J., Holgate S.T., Church M.K., Biochem. Pharmacol., 1984, 33, 3847
438 Church M.K., Holgate S.T., Hughes P.J., Br. J. Pharmacol., 1983, 80, 719
444 Hughes P.J., Holgate S.T., Roath S., Church M.K., Biochem. Pharmacol., 1983, 32, 2557


Feoktistov I., Biaggioni I., Biochem. Pharmacol., 1998, 55, 627


Mita H., Ishii T., Yamada T., Akiyama K., Shida T., Life Sci., 53, 775


Kennedy C., Arch. Int. Pharmacodyn. Ther., 1990, 303, 30


Brown C.M., Collis M.G., Br. J. Pharmacol., 1982, 76, 381

Holroyde M.C., Br. J. Pharmacol., 1986, 87, 501


Martin J.G., Rev. Mal. Resp., 1994, 11, 93


Manzini S., Ballati L., Br. J. Pharmacol., 1990, 100, 251


212
479  Ghai G., Zimmerman M.B., Hopkins M.F., Life Sci., 1987, 41, 1215
Appendix 1

Structures
General Compounds

2-Deoxy-D-glucose

Antimycin A
Calcium Ionophore A23187

Compound 48/80
Dextran

Disodium Cromoglycate
Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH₂

**Mastoparan**

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

**Substance P**

**Adenosine Agonists**

![Adenosine structure](image)

**Adenosine**
Metrifudil

NECA
Adenosine Antagonists
DPCPX
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-DOG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>5-HETE</td>
<td>5-Monohydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>5HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>8PT</td>
<td>8-Phenyltheophylline</td>
</tr>
<tr>
<td>8-SPT</td>
<td>8-(p-Sulphophenyl)theophylline</td>
</tr>
<tr>
<td>10xCFT</td>
<td>10xC Calcium-free Tyrode’s</td>
</tr>
<tr>
<td>11-HPETE</td>
<td>11-Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>Anti-DNP</td>
<td>Mouse monoclonal anti-dinitrophenol antibody</td>
</tr>
<tr>
<td>Anti-human IgE</td>
<td>Rabbit antiserum to human anti-IgE</td>
</tr>
<tr>
<td>Anti-rat IgE</td>
<td>Sheep anti-serum to rat IgE</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>APEC</td>
<td>2-(2-Aminoethylamino)carbonyl ethylphenylethylamino-5’-N-ethylcarboxamidoadenosine</td>
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<tr>
<td>APNEA</td>
<td>N^6-[2-(4-Aminophenyl)ethyl]-adenosine</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5’-trisphosphate</td>
</tr>
<tr>
<td>B0</td>
<td>Zero standard tube</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BW3911</td>
<td>(E)-3-(3,1,2,3,6-Tetrahydro-2,6-dioxo-1,3-dipropyl-9H-purin-8-yl)phenyl acrylic acid</td>
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<td>C3a/C4a/C5a</td>
<td>Complement fragments</td>
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<tr>
<td>CADO</td>
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<tr>
<td>cAMP</td>
<td>3’-5’-Cyclic adenosine monophosphate</td>
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<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
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<td>cGMP</td>
<td>3’5’-Cyclic guanosine monophosphate</td>
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<td>9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazin-5-amine</td>
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<td>CGS21680</td>
<td>2-p-(2-Carboxyethyl)phenethylamino-5’-N-ethylcarboxamido adenosine hydrochloride</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>CHA</td>
<td>N°-Cyclohexyladenosine</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CPA</td>
<td>N°-Cyclopentyladenosine</td>
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<tr>
<td>CTMC</td>
<td>Connective tissue mast cells</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DDA</td>
<td>2',5'-Dideoxyadenosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPCPX</td>
<td>8-Cyclopentyl-1,3-dipropylxanthine</td>
</tr>
<tr>
<td>DSCG</td>
<td>Disodium cromoglycate</td>
</tr>
<tr>
<td>ECF-A</td>
<td>Eosinophil chemotactic factor of anaphylaxis</td>
</tr>
<tr>
<td>FcERI</td>
<td>High affinity receptor for IgE</td>
</tr>
<tr>
<td>FcERII</td>
<td>Low affinity receptor for IgE</td>
</tr>
<tr>
<td>FHT</td>
<td>Full HEPES buffered Tyrode’s buffer</td>
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<tr>
<td>Fluo-3-AM</td>
<td>Pentaacetoxymethyl ester of fluo-3</td>
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<tr>
<td>G-protein</td>
<td>Guanine nucleotide-binding regulatory protein</td>
</tr>
<tr>
<td>GAG</td>
<td>Glysocaminoglycan</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GFT</td>
<td>Glucose-free-Tyrode’s</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-trisphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N°-2-Hydroxyethylpiperazine-N°-2-ethanesulphonic acid</td>
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<td>HETE</td>
<td>Monohydroxyeicosatetraenoic acid</td>
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<td>HSA-DNP</td>
<td>Human serum albumin dinitrophenol</td>
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<td>I-ABOPX</td>
<td>1-Propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-phenylxanthine</td>
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<td>1-Deoxy-1-[[6-[[3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>Inositol-1,4-bisphosphate</td>
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<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol-1,4,5-trisphosphate</td>
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<tr>
<td>IP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Inositol-1,3,4,5-tetraphosphate</td>
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<td>L&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Third stage larvae of <em>Nippostrongylus brasiliensis</em></td>
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<td>LT</td>
<td>Leukotriene</td>
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<tr>
<td>Lyso-PC</td>
<td>Lysophosphatidylcholine</td>
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<td>MCDP</td>
<td>Mast cell degranulating peptide</td>
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<tr>
<td>Metrifudil</td>
<td>N-[(2-Methylphenyl)methyl]-adenosine</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<td>MMC</td>
<td>Mucosal mast cells</td>
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<td>Matrix metallo-proteinase 3</td>
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<td>NANC</td>
<td>Non-adrenergic non-cholinergic</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>NCF-A</td>
<td>Neutrophil chemotactic factor of anaphylaxis</td>
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<tr>
<td>NECA</td>
<td>5’-N-Ethylcarboxamidoadenosine</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>NSB</td>
<td>Non-specific binding tube</td>
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<tr>
<td>OPT</td>
<td>o-Phthalaldehyde</td>
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<td>PA</td>
<td>Phosphatidic acid</td>
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<td>PACPX</td>
<td>1,3 Dipropyl-8-(2 amino-4-chlorophenyl) xanthine</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<td>Phosphate buffered saline</td>
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<td>Prostaglandin</td>
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<td>Protein kinase C</td>
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<td>Phospholipase C</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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<td>PMC</td>
<td>Peritoneal mast cells</td>
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</table>
Probenecid p-[Dipropylsulfamoyl] benzoic acid
RBL Rat basophilic leukaemia
RMCP II Rat mast cell protease I
RMCP II Rat mast cell protease II
R-PIA R(-)N^6-(2-Phenylisopropyl)adenosine
RPMC Rat peritoneal mast cells
RT Room temperature
SCF Stem cell factor
sec Second
SEM Standard error of the mean
SPS Sterile physiological saline
SRS-A Slow-reacting substance of anaphylaxis
T+ Tryptase only
TC Total counts tube
TC+ Tryptase and chymase
TGF Transforming growth factor
T_H T-helper
TNF Tumour necrosis factor
T_S T-suppresser
TX Thromboxane
VIP Vasoactive intestinal peptide
ACh Acetylcholine
FEV\_1 Forced expiratory volume in one second
NP Neuropeptides