

**THE EFFECT OF PLATELET ACTIVATING FACTOR (PAF) ON MAST
CELLS.**

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partial fulfilment of the requirements for
the degree of Doctor of Philosophy
in the Faculty of Science.

by

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To My Mother and Father.

THE EFFECT OF PLATELET ACTIVATING FACTOR (PAF) ON MAST CELLS.

Shamimunisa B. Mustafa

ABSTRACT

In recent years, platelet activating factor (PAF) has emerged as a potent proinflammatory mediator implicated in a diverse range of human pathologies including allergy and asthma. PAF can also attract and stimulate inflammatory cells such as eosinophils and neutrophils.

The present study has attempted to investigate the action of PAF on isolated histaminocytes from different species.

PAF (1-100 μM) induced histamine and prostaglandin D_2 release in a characteristic dose-dependent manner from rat peritoneal mast cells. Histamine release was cytotoxic at high concentrations (10 μM) of PAF but selective and non-cytotoxic at lower concentrations (5 μM). Under the latter conditions, the process was depressed at extremes of temperature, complete within approx. 10 min and independent of pH and calcium. Analogues of PAF (C17 and C18) and lyso-PAF also induced histamine release from rat peritoneal mast cells in a dose-related manner.

The non-cytotoxic release evoked by PAF and lyso-PAF from rat peritoneal mast cells was inhibited by anti-asthmatic compounds. Release induced by PAF was also inhibited by cAMP analogues, a naturally occurring flavonoid and to variable degrees by selective PAF antagonists.

The cytotoxic effect of PAF was found not to be highly tissue or species specific and comparable release was induced from isolated mast cells from rat lung, mesentery and skin, guinea-pig lung and mesentery, and human intestine, lung, skin and basophils.

PAF was seen to potentiate anti-IgE and concanavalin A induced histamine release from rat peritoneal mast cells. In contrast, it did not potentiate anti-IgE induced histamine release from human basophils or human lung mast cells.

Thus PAF is thought to act on mast cell membranes rather than through a receptor mediated mechanism, to produce mediator release.

Towards the end of this project, the effect of PAF on human neutrophils was studied. PAF (0.001-10 μM) induced a dose-related, non-cytotoxic release of β -glucuronidase from human neutrophils. The release was inhibited by the specific PAF receptor antagonist BN50730 but not by the anti-asthmatic chromone disodium cromoglycate. The present study indicates that PAF exerts its effects on human neutrophils through specific PAF receptors.

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CHAPTER ONE

INTRODUCTION

1.1 HISTORICAL BACKGROUND.

In 1878 Paul Ehrlich, an eminent bacteriologist, identified a certain connective tissue cell which, when stained with basic dyes such as toluidine blue, changed the colour spectrum of the dye in a process now known as metachromasia [1]. He also reported that these particular cells possessed many granules, thus appearing "overfed". Hence, Ehrlich named the cells "Mastzelle" from the German word "mästen" which means to feed. He later identified a similar cell type found in the peripheral blood system (now known as basophils) [2]. The similarity of the two kinds of cells lies in their cellular content of water-soluble cytoplasmic granules, which have a strong affinity for basic dyes.

In 1902 Portier and Richet, after conducting a series of experiments which involved injecting dogs with a sea-anemone toxin, put forward the theory of a syndrome they named "anaphylaxis" [3]. The theory was based on their findings in which some of the dogs did not die after the initial injection of the toxin, but entered a state of "confusion" upon a subsequent injection of the same toxin. They described this state of "confusion" as anaphylactic shock. These were the first steps taken in what was to become a vastly studied subject now known as immediate hypersensitivity reactions.

Dale and Laidlaw, in 1910, studied the physiological actions of a biological amine (later named histamine), and discovered it to be a potent vasoactive chemical [4]. After a series of experiments, they put forward the idea that histamine may play a vital role in immediate hypersensitivity reactions. Their conclusion was based on the

fact that injecting histamine systemically into animals or isolated tissues mimicked the signs and symptoms of anaphylactic shock [5].

Further work on the concept of anaphylaxis was carried out by Wilander and co-workers in 1938. They reported that mast cells in the liver of a dog undergoing anaphylactic shock became grossly damaged, thus liberating their cellular contents into the surrounding tissue [6]. Later findings indicated that not only heparin but also histamine was derived from this organ.

In 1939, Jorpes was working on an anticoagulant substance named heparin, as it had been initially derived from dog liver cells [7]. On finding that heparin stained metachromatically with toluidine blue, Jorpes and co-workers showed that a good correlation existed between the mast cell count of a particular tissue and the amount of heparin that was extracted from it. Thus, heparin was the first constituent of mast cells to be identified. It is now known that heparin is a highly sulphated proteoglycan and that it reacts with appropriate dyes to induce metachromasia.

It was not until the early 1950's that Riley and West identified the tissue mast cell as being the major source of histamine [8]. This finding provided an important advance towards the understanding of the role of histamine and mast cells in physiology and pathology.

During the late 1960's Ishizaka and co-workers identified a serum antibody responsible for immediate hypersensitivity reactions as belonging to a novel class of immunoglobulins named immunoglobulin E (IgE) [10-11]. Ishizaka and co-workers also discovered that human IgE preferentially bound to basophils via specific receptors [12]. Subsequent work identified surface receptors with a high affinity for this particular antibody on tissue mast cells [13].

1.1.1 ANAPHYLAXIS.

Following the pioneering work of Portier and Richet, our understanding of the term "anaphylaxis", and what it consists of, has greatly increased. Anaphylaxis is a mast

\ cell mediated, life-threatening, multiorgan disorder. The major organs involved include the skin, cardiovascular system, gastrointestinal tract and the respiratory airways. Table 1.1 illustrates the symptoms and pathological features of anaphylaxis together with the proposed mediators thought to initiate such responses.

1.2 THE LOCATION AND STRUCTURE OF MAST CELLS AND BASOPHILS.

It is now assumed that allergic disorders consist of numerous inflammatory processes involving several different cell types. Of these, the mast cell has assumed a pre-eminent position owing to its ability to release spasmogenic, chemotactic and inflammatory mediators and also due to its unique location within the body. Mast cells are widely distributed throughout the human body and in the bodies of other vertebrates. Mast cells are located in areas which come into frequent contact with noxious agents (antigens). They are exclusively found in the loose connective tissues of the ear, nose, conjunctiva, respiratory airways, lung, skin, gastrointestinal tract and in many internal organs [14,15]. Human basophils, once matured, leave the bone marrow and circulate in the peripheral blood [16]. Circulating mast cells are rare and occur almost exclusively in neoplastic disorders such as severe systemic mastocytosis.

Mammalian mast cells are mononuclear and ovoidal in shape. They have a diameter of 10-30 μm [17,18]. The cell surface possesses regularly spaced short narrow folds. Human mast cell granules have complex substructural patterns [19]. The major types of granule patterns observed in the human mast cells are scrolls, crystal, particle and mixed. However, in some cases, isolated human mast cells are filled with homogeneous membrane-bound dense granules which lack substructural patterns [20,21].

Human basophils are small, rounded cells with polylobed nuclei, which differentiate in the bone marrow and circulate in the peripheral blood. The cell surface displays irregularly placed blunt processes [22,23].

Mast cells and basophils share a number of properties. These include receptors for

IgE antibodies, large intracellular metachromatic granules and a variety of preformed and newly-generated mediators. Those mediators which are preformed are associated with intracellular granules and the newly formed mediators are generally derived from a variety of chemical interactions originating in the plasma cell membrane.

1.3 THE ROLE OF THE MAST CELL IN HEALTH AND DISEASE.

Owing to their unique distribution throughout the mammalian body, mast cells have been linked to a possible role in the body's defence mechanisms upon parasitic infestations. One such example is in the rapid elimination of helminthic parasites. It has been shown that a correlation between mast cell hyperplasia, eosinophilia and increased IgE antibody levels exists after parasitic infestations [24].

Traditionally, the mast cell and its blood counterpart the basophil have been assigned central roles in immediate hypersensitivity reactions. Activation of these cells is followed by degranulation, resulting in mediator release, which causes the symptoms of allergy and anaphylaxis. Therefore mast cells have been implicated in the aetiology of a variety of allergic conditions, such as conjunctivitis [25], asthma [26], rhinitis [27] and urticaria [28]. Another factor indicating that mast cells are linked to the pathogenesis of the above allergic conditions is that the number of mast cells in allergic subjects increases compared to normal subjects. Mast cells have also been implicated in a variety of other physiological processes which are not allergic diseases, such as menstruation [29] and embryo implantation [30].

1.4 THE ALLERGIC RESPONSE.

An allergic response occurs when an individual is exposed to ubiquitous, harmless antigens thus resulting in an increased IgE antibody production. Such antigens include pollen, house dust, grass, animal danders and certain foods. The immune system of a normal individual would not react to such antigens. Such individuals who develop an allergic response due to defects in their immune system are said to be atopic.

Cells involved in allergic reactions primarily include mast cells [31] as stated previously, tissue macrophages [32], eosinophils, neutrophils and macrophages [33]. In brief, mast cells are activated by IgE antibodies, thus triggering off a series of chemical reactions within the cells, resulting in mediator release. The mechanism of mediator release will be discussed later in detail. Once released, these chemical mediators act on nearby tissues thus producing inflammation, the result of an allergic response.

1.4.1 ALLERGIC RESPONSES IN THE SKIN.

The result of allergic inflammation depends on the cellular structure of the tissue and the type of mediators the tissue comes into contact with. An allergic reaction in the skin results in the formation of a wheal and flare. A flare develops due to vasodilatation which is caused by the release of neurotransmitters from the sensory nerve endings supplying the skin [34,35]. A wheal develops due to oedema formation caused by an increase in arteriolar blood flow and fluid leakage from nearby capillaries. There then follows a migration of inflammatory cells such as granulocytes to the site of oedema formation [36]. Mediators released from such cells further augment oedema formation and an increase in arteriolar dilatation.

1.4.2 ALLERGIC RESPONSES IN THE RESPIRATORY AIRWAYS.

An allergic response in the respiratory airways is far more complex than that which occurs in the skin owing to the large number of tissue components involved. The initial response consists of bronchial smooth muscle contraction due to direct stimulation by chemical mediators. An increase in acetylcholine release occurs due to the abnormal neural tone [33]. The allergic response also causes an increase in mucus production. This is brought about by direct stimulation of goblet cells by the released mediators [37,38].

Further disorders which occur in the respiratory airways include reduced mucociliary clearance, mucosal oedema and cellular influx into the lumen of the airways. The

migration of inflammatory cells into the airway results from the release of chemotaxins from the stimulated inflammatory cells.

1.4.3 THE ROLE OF HISTAMINE IN ALLERGIC DISEASES.

Histamine which is primarily stored in mast cells and basophils, is one of the main contributors to the allergic response. Increased plasma and tissue histamine levels occur during anaphylaxis and allergic disorders of the skin, nose and respiratory airways [39]. The biological effects of histamine are mediated through distinct receptors, of which three subtypes (H_1 , H_2 and H_3) have now been identified [40]. Important histamine effects in allergic reactions are mediated through the H_1 receptor. These include smooth muscle contraction, increased vascular permeability, prostaglandin generation and activation of vagal reflexes. The H_2 mediated effects include gastric acid secretion, increased mucus secretion and oesophageal contraction. A third histamine receptor, thought to mediate a negative feedback action on histamine synthesis, has recently been described in neural tissues [41].

Although histamine is a primary mediator in the allergic reaction, effects known to be mediated through histamine can also be mimicked by other mediators released from mast cells upon degranulation. This will be discussed later in detail.

1.5 THE IMMEDIATE HYPERSENSITIVITY REACTION.

1.5.1 THE FORMATION OF IgE ANTIBODIES.

As stated previously, allergic responses are brought about due to the increased production of IgE antibody molecules by atopic individuals. Immunoglobulins are glycoproteins composed of 82-96% polypeptide and 4-18% carbohydrate. There are five classes of immunoglobulins, designated IgA, IgD, IgE, IgG and IgM [42]. All the above antibodies possess the same basic unit structure, each consisting of two heavy and two light chains linked by disulphide bridges. There are two types of light chains, kappa and lambda, and five types of heavy chains alpha, delta, epsilon, gamma and mu [43-45]. Fig.1.1 shows that immunoglobulin molecules have a Y-

shaped configuration. The Fab regions contain the antigen binding sites and each immunoglobulin is bivalent. The lower part of the molecule, designated the Fc portion, binds to specific receptors on various cell surfaces. Mast cells and basophils possess high affinity IgE receptors [46].

In brief, once antigens have penetrated into an individual, the cascade process of the immune system is triggered off. Firstly, the antigen is processed by antigen presenting cells (APC). This is now known as antigen recognition. In order for helper T-cells to recognise the specific antigen, APC further processes the attached antigens in association with class II major histocompatibility molecules (MHC) [47]. The first indication that the production of IgE antibodies was T-cell dependent was reported in the early 1970's [48]. Helper T-cells are the principal orchestrators of the immune response, as their presence is vital in the activation of other effector cells involved in the immune response, ie, cytotoxic T-cells and antibody producing B-cells.

The activation of T-helper cells occurs quite early on in the immune pathway. To become activated, T-helper cells require firstly to bind to the class II MHC-antigen complex via its antigen receptor and secondly the presence of interleukin-1 (IL-1). IL-1 is a soluble protein produced by the APC. Together these two factors give rise to the production of interleukin-2 (IL-2) which is important for stimulating B-cells. IL-2 also amplifies the response initiated by the contact of helper T-cells with APC.

Antibody production requires both the activation of B-cells and their differentiation into antibody-producing plasma cells. This is achieved during the activation of helper T-cells, after which the helper T-cells secrete two factors, B-cell growth factor (BCGF) and B-cell differentiation factor (BCDF). BCGF stimulates proliferation of B-cells. This is followed by the action of BCDF, which induces activated B-cells to differentiate into IgE antibody-secreting plasma cells and a population of B-memory cells. B-memory cells can respond to subsequent encounters with the relevant antigen [49-54].

1.5.2 IgE ANTIBODY RECEPTORS.

Circulating, free IgE antibody molecules bind specifically to high affinity receptors (Fc_εR1) found exclusively on the surface of mast cells and basophils [55-56]. The average number of receptors on human basophils has been estimated as lying between 40,000 and 100,000 [57] and on rat mast cells as being 300,000 [58]. Detailed studies on rat peritoneal mast cells have now revealed that the receptor is comprised of a glycoprotein, of which 13% of the molecule consists of carbohydrates [59,60]. The relative molecular mass of the receptor is approximately 87,000. It consists of three subunits (alpha, 45,000; beta, 33,000; gamma, 9,000). The three subunits are further subdivided into alpha₁, alpha₂, beta₁ and beta₂ polypeptides, with the two gamma chains being identical (Fig.1.2). The IgE receptor transverses the plasma cell membrane with the two alpha-subunits facing outwards.

1.5.3 IMMUNOLOGICAL ACTIVATION OF MAST CELLS.

Once the IgE molecule has bound to its receptor on the mast cell surface via its Fc portion, leaving the Fab part free for antigen binding, the cell is said to be sensitised. Initial sensitisation does not result in degranulation of the cell. However, subsequent exposure to the same antigen causes cross-linking of IgE molecules, thus triggering off a complicated series of biochemical reactions within the mast cell, ultimately leading to mediator release [61].

1.5.4 NON-IMMUNOLOGICAL ACTIVATION OF MAST CELLS.

There are several other agents which also cause mast cell degranulation besides that directed by IgE antibody molecules. Certain chemicals such as the detergents Triton X-100 and Tween-20 cause cytotoxic histamine release from mast cells by totally disrupting the cell plasma membrane. Such agents are known as non-selective liberators [62].

Conversely there also exists a group of chemicals which induce mast cell degranulation leading to mediator release, but do not irreversibly damage the cell

plasma membrane. Such agents are known as selective liberators and include the polybasic cations [63-66], anaphylatoxins [67-68], calcium ionophores [69,70] and the plasma substitute dextran [71], (Table 1.2).

1.6 STRUCTURAL CHANGES IN A MAST CELL UNDERGOING DEGRANULATION.

Activation of resting mast cells by immunological or non-immunological stimulation leads to the exocytosis of granular contents into the external media. A certain amount of cellular granule heterogeneity was found to exist in human mast cells. In general, skin mast cells were found to contain crystal granules; lung mast cells, scroll granules; and colonic mucosal mast cells, particle granules [72-74].

In human mast cells, one minute after stimulation, the granules swell and dissolution of their matrix material can be seen. By three to five minutes after stimulation, the surrounding swollen membrane granules fuse with each other. This leads to the formation of channels through the plasma membrane extending outwards. Thus, the solubilized granular contents are then finally extruded into the surrounding medium [75].

Having undergone degranulation, human mast cells enter a phase known as the recovery period. Early recovery from degranulation is stimulated by the sealing of degranulation channels. The channels decrease in length and width. Vesicle cytoplasmic particles and minute lipid bodies migrate into the channels and become portioned into individual granules. This process eventually results in the formation of new cytoplasmic granules [76].

1.7 THE BIOCHEMISTRY OF MEDIATOR RELEASE.

The high granular content of mast cells indicates that these cells are highly specialised for secretion. This is one of the reasons why mast cells have been extensively studied during the last twenty years in the investigation of the secretory process. It is now known that mast cell activation and subsequent mediator release

involves not one but many complex, biochemical pathways.

In 1969, Johnson and co-workers [77], reported that histamine release from stimulated mast cells was non-lytic. Their findings were based on the fact that the enzyme lactate dehydrogenase and potassium ions remained within the cell after degranulation. Thus, non-lytic histamine release involves the selective fusion of intracellular granules with the plasma membrane, resulting in mediator release without irreversibly damaging the cell.

1.7.1 ACTIVATION OF SERINE ESTERASES.

Once the IgE antibody has bound to its receptor, activation of one or several proteolytic enzymes may constitute an early step in stimulus-secretion coupling in mast cells. This theory was initiated by Austin and co-workers, who demonstrated that diisopropylfluorophosphate (DFP), an irreversible inhibitor of serine esterases, inhibited IgE-dependent histamine release from chopped guinea-pig lung [78], rat mast cells [79,80] and human lung fragments [81]. They also reported that histamine release was inhibited only when DFP was present at the time of challenge by the stimulus. It was later observed that the inhibitory effects of DFP were greatly diminished when the cells were pre-incubated with DFP and then washed to remove the inhibitor prior to challenge by the stimulus. This finding suggested that serine esterases in resting cells are present in the inactive form. They also found that DFP was ineffective when added after the stimulus. Thus, these findings suggest that IgE-dependent activation of mast cells leads to the priming of serine esterases necessary for histamine secretion, the pro-esterase being unaffected by DFP.

Further evidence for the involvement of serine esterases in initiating mediator release was provided when α -chymotrypsin [82,83] and rat mast cell chymase [84] were found to induce mediator secretion from rat mast cells. However, later work indicated that chymase and anti-IgE induced mediator release occurred via different mechanisms. Ishizaka and co-workers [85] demonstrated that chymotrypsin and trypsin inhibitors blocked IgE-mediated release of histamine and increased phospholipid methylation in rat mast cells. They also observed that chymotrypsin

and IgE-mediated release was inhibited by serine esterase inhibitors. Thus, the above findings suggest that one or more serine esterases is involved in IgE-dependent histamine release. However, the protease involved has not yet been identified, nor has the mode of its action.

1.7.2 THE ROLE OF CALCIUM IONS.

For some time it has been appreciated that calcium ions play an important role in secretion. Indirect evidence linking the presence of calcium ions and histamine secretion was initially reported by Mongar and Schild [86], who observed that maximal anaphylactic release of histamine from isolated guinea-pig lung tissue only occurred if calcium ions were present in the surrounding medium.

In the early 1970's, it was shown that histamine release could be induced by direct microinjection of calcium ions into rat mesenteric mast cells [87]. This observation indicated the necessity of calcium ions to induce, if not initiate, histamine release from mast cells. Thus calcium ions were thought of as being second messengers as they transduce the ligand-receptor signal to within the cell and, in doing so, triggered off processes which lead to mediator release.

Attempts have been made to measure intracellular calcium levels after IgE-dependent stimulation. This was achieved experimentally using radio-isotopic ^{45}Ca in the extracellular medium. Foreman and co-workers found that ^{45}Ca accumulated within the mast cell during IgE-dependent stimulation [88]. This method of monitoring intracellular levels of calcium has been extensively used by Ishizaka and co-workers. Their findings from human lung mast cells, basophils and mouse mast cells indicated that increased accumulation of ^{45}Ca preceded or was concurrent with histamine release [89-92].

Calcium ionophores such as A23187 and ionomycin have been used extensively to increase our understanding of the role played by calcium ions in mediator release. Ionophores are organic molecules, capable of binding to specific metal ions such as calcium. Once bound to calcium, these molecules are able to traverse the plasma

cell membrane and enter the cell, thus causing a rise in intracellular calcium levels. This method of causing an increase in cellular calcium levels can only occur if calcium ions are present in the extracellular medium [93].

The importance of calcium ions in mediator release from mast cells was demonstrated by Pearce and co-workers in the early 1980's. Their work involved substituting lanthanum ions for calcium ions in membrane binding sites. Lanthanum ions have a similar ionic radius to that of calcium and, owing to a higher valency, were able to compete with calcium ions in order to bind to the membrane sites initially occupied by calcium ions. Thus, lanthanum was found to inhibit IgE-dependent histamine release from rat peritoneal mast cells [94].

Further studies on the role played by calcium has been facilitated by the use of the fluorescent tetracarboxylate calcium chelator, quin-2. It was seen that after antigen challenge on rat mast cells, an increase in fluorescence indicated an increase in the concentration of cytosolic calcium ions [96].

1.7.3 CALCIUM POOLS INVOLVED IN EXOCYTOSIS.

Once the need for extracellular calcium ions in mediator release was identified, the role of intracellular calcium ions in the same process was investigated. There are two basic mechanisms by which mast cells can increase their cytosolic free calcium ion levels: via release of calcium ions from intracellular stores or by the entry of extracellular calcium ions through membrane calcium channels [97,98].

Antigen stimulation of the mast cell causes an increase in the calcium ion permeability of the mast cell membrane by allowing calcium membrane channels to open. An influx of calcium ions from superficial sites on the cell membrane results in exocytosis [95-99]. It is assumed that lanthanum ions bind to these sites, thus inhibiting exocytosis [95]. Evidence for this hypothesis has been derived experimentally, where it was observed that rat mast cells in the presence of a calcium-free medium, previously having been isolated in the presence of calcium ions, displayed a decrease in the level of histamine release in response to

stimulation [100].

Certain secretagogues have been found to induce substantial histamine release in the absence of extracellular calcium ions [101]. Such secretagogues include compound 48/80, peptide 401 and polylysine [94,95]. The release is attributed to the mobilization of intracellular pools of calcium ions or sequestered calcium ions attached to the inner surfaces of the plasma cell membrane or cellular organelles. It has also been experimentally shown that pre-treating mast cells in a calcium-free medium containing ethylenediaminetetraacetic acid (EDTA), a calcium chelating agent, reduces the responsiveness of the cells to subsequent stimulation by the above secretagogues [94,95].

1.7.4 THE ROLE OF CALMODULIN.

Once the intracellular levels of calcium have risen, how mediator release is initiated is still not fully understood. Previous studies on other cell systems have indicated that many of the effects of calcium are mediated through a calcium-binding protein, termed calmodulin [102,103].

Calmodulin is a 17,000 MW protein which binds reversibly to calcium with a high affinity. Each molecule possesses four calcium ion binding sites. Binding of calcium to all four sites causes a conformational change that activates the protein. Thus, calmodulin regulates the activities of a large number of key enzymes involved in secretion; adenylate cyclase, methyl transferase, phospholipase A₂ and cyclic nucleotide phosphodiesterases [95,102,103]. Calmodulin also regulates its own functioning, by controlling the intracellular levels of calcium [104-107].

The protein activates the calcium pumps of both the plasma membrane and the sarcoplasmic reticulum, thus reducing the cellular calcium concentration by promoting its extrusion into extracellular membrane-linked pools or by sequestering the ions into internal pools.

Upon mast cell activation, there is a rapid calcium-dependent phosphorylation of

proteins with molecular weights of 68,000, 59,000 and 42,000 [108]. This phosphorylation precedes or occurs simultaneously with histamine release from the mast cell. Further studies of protein phosphorylation have revealed that the termination of histamine release requires the phosphorylation of another protein of molecular weight 78,000. Thus, phosphorylation of specific proteins may control the initiation and termination of histamine release from mast cells. However, the specific involvement of calmodulin with these regulatory proteins has yet to be fully understood.

Previous studies using calmodulin inhibitors observed that histamine release was inhibited from mast cells and basophils [109-111], thus indicating that calmodulin may be significantly involved in the release process.

1.8 THE ROLE OF PHOSPHOLIPIDS.

1.8.1 METHYLATION OF PHOSPHOLIPIDS.

Stimulation of a variety of cells including mast cells may initially involve plasma membrane components. In this regard, a variety of stimuli which activate mast cells via IgE-receptors, such as antigen, anti-IgE and concanavalin A, have been reported to increase the methylation of the phospholipid, phosphatidyl- ethanolamine (PE), a major component of the plasma cell membrane. The concept of PE methylation was first described in the early 1960's using liver microsomes [112,113].

By the early 1980's, a sequential pathway of biochemical events leading to the methylation of endogenous phospholipids was put forward, (Fig.1.3). According to this pathway, two membrane-bound methyltransferases (I and II) sequentially convert PE to phosphatidylcholine (PC), via the formation of phosphatidyl-N-monomethyl-PE and phosphatidyl-N,N-dimethyl-PE. Methyltransferase I is situated on the cytoplasmic face and transfers a methyl group from the donor S-adenosylmethionine (AdoMet) to give rise to the first intermediate in the pathway, phosphatidyl-N-monomethyl-PE. Methyltransferase II, located in the plasma cell membrane facing outwards, catalyses two successive N-methylations of phosphatidyl-N-monomethyl -

PE to form PC. During this pathway the methylated phospholipids are translocated across the membrane and, in so doing increases the fluidity of the membrane [114,115].

Evidence for a central role of PE methylation in mast cells and basophil activation has been derived from two lines of study. Firstly, radiolabelled studies have shown increased transfer of [³H-methyl] groups from [³H-methyl] AdoMet into cellular membrane phospholipids to occur rapidly following IgE-receptor cross-linking. Secondly, pharmacological agents which inhibit AdoMet-dependent methylation also inhibit various biochemical changes associated with cell activation. Moreover, inhibitors of methyltransferases, such as 3-deazaadenosine (3-DZA) and homocysteinethiolactone (HC) were found to block phospholipid methylation, calcium influx and histamine release [116-119].

As stated previously, stimulus-dependent increases in phospholipid methylation have been shown to occur in rat peritoneal mast cells activated by concanavalin A, anti-IgE, C3a and C5a, [120-122]; in mouse mast cells activated by anti-IgE, antigen and bradykinin, [96,123,124]; in human mast stimulated with antigen and anti-IgE, [125] and in human cultured basophils activated with anti-IgE [126]. From such studies, it was seen that increases in phospholipid methylation were accompanied by stimulus-dependent increases in ⁴⁵Ca accumulation, cAMP synthesis, phosphoinositide metabolism and histamine release [85,126]. Thus, it was proposed that phospholipid methylation played a vital role in initiating secretion from mast cells [85,115]. However, it should be noted that phospholipid methylation was undetectable in rat mast cells stimulated with compound 48/80 and the calcium ionophore A23187 [121]. This observation indicates that phospholipid methylation is only associated with IgE-dependent histamine secretion.

The precise mechanism as to how phospholipid methylation affects adenylate cyclase, the regulation of calcium channels and the enzymes of phosphoinositide metabolism in stimulated mast cells has yet to be fully elucidated. Also, Ishizaka and co-workers suggested that phospholipid methylation played a key role in IgE-dependent histamine release, other independent research groups failed to obtain

reproducible results [127-129] regarding the importance of phospholipid methylation during mediator release from mast cells.

1.8.2 PHOSPHOINOSITIDE METABOLISM.

The resultant behaviour of a stimulated cell is dependent on signalling systems which transfer external information into internal signals. Such signals, for example a rise in intracellular calcium ions, are known as second messengers. Second messengers control nearly all cellular activities such as metabolism and secretion [130].

It has been shown that activation of cell-surface receptors, resulting in the mobilisation of calcium pools, initiates the metabolism of phosphoinositides [130-132]. Initially, phosphatidylinositol (PI) breakdown to diacylglycerol (DAG) was measured by analyzing the incorporation of radiolabelled [^{32}P] phosphate and [^3H] inositol into PI, phosphatidylcholine (PC) and phosphatidic acid (PA). This finding lead to many groups reporting that a variety of secretagogues induce accumulation of [^{32}P] into rat mast cell PI [133-135]. Later work by Ishizaka and co-workers confirmed that an increased uptake of [^{32}P] into membrane phospholipids occurs after IgE-dependent stimulation of human cultured basophils.

As stated before, stimulation of cell-surface receptors initiates the hydrolysis of the membrane-bound lipid PI, which results in the formation of two second messengers; DAG and inositol 1,4,5-trisphosphate (IP_3) [130,136]. Phosphorylation of PI by ATP-dependent specific kinases results in the sequential formation of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bis-phosphate (PIP_2) [137]. A membrane enzyme phospholipase C (PLC) is then activated and in turn causes PIP_2 breakdown to give DAG and IP_3 .

Recent studies by Gruchalla and co-workers have suggested an alternative route to DAG formation. Their results indicate that cleavage of the terminal phosphodiester bond on PC by the enzyme phospholipase D gives rise to PA. PA is then converted into DAG [138], during IgE-receptor mediated stimulation of rat peritoneal mast

cells. They also suggested that PA conversion to DAG from PC is the major pathway by which DAG is formed during mast cell activation. However, the role of phospholipase D in DAG formation has yet to be reported by other independent groups.

IP_3 has been shown to cause mobilisation of calcium from intracellular stores [130], such as the endoplasmic reticulum, via a specific receptor. Detailed studies using permeabilised cells have demonstrated that submicromolar concentrations of IP_3 are required for half-maximal release of calcium from its intracellular stores [139]. IP_3 is then converted into IP_4 , which also may participate in regulating the cytosolic calcium concentration.

DAG acts by stimulating protein kinase C (PKC) [140]. PKC is a phospholipid-dependent protein and its presence has been identified in many mammalian organs [141]. Several observations have been put forward, suggesting that PKC activation by DAG may play a vital role in stimulus-secretion coupling. Many groups have reported that IgE-dependent stimulation induces a rapid formation of DAG in a variety of mast cells [142] which precedes or occurs simultaneously with histamine release.

Experimentally the effects of DAG on PKC can be mimicked by using phorbol esters such as phorbol myristate acetate (PMA) [143]. In order to explore the possible involvement of PKC in protein phosphorylation, rat mast cells were treated with PMA, which directly activates PKC by substituting for DAG [144,145]. At concentrations less than 10 ng/ml, PMA was found to be a poor histamine releaser but potentiated histamine secretion stimulated by low concentrations of anti-IgE and calcium ionophores [146,147], thus suggesting a positive role in histamine release for PKC. Sagi-Eisenberg and co-workers have shown that the other phorbol ester, tetradecanoyl phorbol acetate (TPA) blocked intracellular increases in calcium but potentiated antigen-induced serotonin release [148]. Further studies, including work on mast cells, have shown that phorbol esters can inhibit PI breakdown and calcium mobilisation [149]. This phenomenon may be due to the direct action of phorbol esters, rather than PKC interfering with the calcium signal.

1.8.3 ARACHIDONIC ACID METABOLISM.

Further metabolism of membrane phospholipids by the enzyme phospholipase A₂ or by PLC together with diacylglycerol lipases, gives rise to arachidonic acid (AA). AA is further metabolised via the cyclooxygenase or lipoxygenase pathway, to produce physiologically relevant chemicals which are released after activation of the mast cell or basophil by a specific stimulus. Metabolites of AA will be discussed in section 1.9.7.

Exogenous PLA₂ was reported to induce histamine release from mast cells and basophils [150,151]. In addition, it has also been reported that PLA₂ inhibitors, such as mepacrine, potently inhibited IgE- and A23187- induced histamine release from rat peritoneal mast cells and basophils [152,153].

Recent studies into whether AA metabolism occurs concurrently with histamine release have indicated that, in many instances, the lipoxygenase pathway of AA metabolism fails to commence, although histamine release occurs readily [154,155]. Thus, the findings suggest that AA metabolism via both of the two enzymic pathways is not always initiated but depends on the type of stimulation received by the mast cell or basophil.

1.8.4 THE ROLE OF cAMP.

Adenosine 3'-5'-(cyclic) monophosphate (cAMP) is now thought of as a second messenger involved in signal transduction within the cell. It is now known that cyclic nucleotides are involved in coupling reactions within the mast cell during activation of the cell. Experimental evidence has indicated that IgE-mediated activation of rat mast cells [156-158], human basophils [159,160] and human lung mast cells [125] is associated with increases in cAMP concentrations.

Following stimulation of mast cells, a transient increase in intracellular cAMP levels occurs, reaching a maximum value between 15-45 seconds. It was observed that the

cAMP decrease to baseline levels occurs within 2-3 minutes following stimulation. It was also observed that peak levels of cAMP preceded or were concurrent with the onset of ^{45}Ca accumulation and histamine release. Thus, the changes in cellular cAMP levels may be involved in stimulus-secretion coupling [157].

The relationship between phospholipid methylation and cAMP levels was investigated using methyltransferase inhibitors, such as 3-deazaadenosine (DZA). It was observed that DZA did not diminish IgE-dependent increases in cAMP levels in rat mast cells [161], human lung mast cells [128] or in human basophils [162], at concentrations which inhibit histamine release by greater than 50%. Moreover, DZA was found to increase both baseline and stimulus-increased levels of cAMP, possibly by the inhibition of cAMP phosphodiesterase [163]. Thus, increased cAMP levels are generally inhibitory to mediator secretion from mast cells.

Berridge and co-workers put forward a theory involving cAMP and the regulation of intracellular calcium levels. This putative interaction was divided into two directions: monodirectional and bidirectional [164]. In the former system, the levels of cAMP are enhanced due to the direct effect of the stimulus on adenylate cyclase, thus increasing the uptake of calcium by the activated cell. This leads to an enhancement of mediator secretion from the activated cell. In bidirectional systems, cAMP is thought to prevent calcium-induced mediator release by activating calcium ATPase membrane pumps. These pumps selectively extrude calcium ions from within the cytosol into intracellular calcium pools or into the external medium.

The question of whether the transient rise in cellular levels of cAMP occurs to facilitate or inhibit mediator release has long been in doubt. Schild, in 1936, observed that high doses of adrenalin, which alter cAMP levels, inhibited antigen-induced histamine secretion from isolated guinea-pig lung tissue [165]. In the early 1970's, agents which either increased or decreased cellular cAMP levels were also found to inhibit histamine release from human lung tissue [166,167]. More recently similar results were obtained with human basophils [168,169] and dispersed human lung mast cells [170,171].

In the same context, similar studies were carried out on rat serosal mast cells. It was observed that no significant correlation existed between cellular cAMP levels and mediator release. β -agonists which increase cellular cAMP levels, were found not to inhibit IgE-dependent mediator secretion [172,173]. This lack of effect cannot be explained by an absence of β -adrenoceptors, since rat mast cells have around 40,000 binding sites/cell. Stimulation of these binding sites causes an increase in cellular cAMP levels [174]. Explanations of the above phenomenon still remain unclear. However, Ishizaka and co-workers have suggested that the ineffectiveness of β -adrenoceptors may occur due to a maintained rise in cAMP levels [85]. This has yet to be fully clarified. Moreover, it was observed that inhibitors of cAMP phosphodiesterase, such as theophylline and 3-isobutyl-1-methyl-xanthine (IBMX), effectively inhibited IgE-dependent mediator release by preventing the breakdown of endogenous cAMP [175]. Extensive studies by Holgate and co-workers [176] led to the suggestion that several stores of adenylate cyclase and hence cAMP exist in rat mast cells, which are differentially affected by certain stimuli and only some are involved in mediator release.

A few potential mechanisms by which cAMP may inhibit mediator release from mast cells have been postulated over the years. Firstly, increased levels of cellular cAMP inhibit IgE-dependent increases in phospholipid metabolism, thus lowering the availability of DAG, which in turn would have activated PKC [177]. Secondly, it has been observed that in the presence of isoprenaline and theophylline, depression of phospholipid methylation occurs [61].

The role of cAMP in mediator release is still not fully elucidated as several independent groups have been unable to reproduce the same results. Lichtenstein and co-workers [178] have been unable to demonstrate IgE-dependent accumulation of cellular cAMP, in contrast to the previous findings of Ishizaka and co-workers [125]. Also, it has been observed that non-immunological agents, which induce mediator release from rat mast cells, do not increase cellular levels of cAMP [179]. This observation suggests that if cellular cAMP has any role in IgE-dependent secretion, then this would occur in the early stages of stimulus-secretion coupling.

1.8.5 GUANOSINE TRIPHOSPHATE (GTP)-BINDING PROTEINS.

Ligand-receptor interactions at the cell surface result in the formation of intracellular signals. This phenomenon was found to be controlled by GTP-binding proteins (G-proteins) [180]. In the early 1980's, Gomperts and co-workers examined the role of G-proteins in inducing histamine release from rat peritoneal mast cells by injecting the non-hydrolysable GTP analogue, guanosine-3-thio-triphosphate (GTP- γ -S), into the cell [181]. This procedure resulted in the exocytotic release of histamine in the presence of additional calcium ions. Further studies indicated that a G-protein could act as the intermediate-linking factor between the membrane receptor and PLC. Thus, upon receptor activation, GTP- γ -S bound to the G-protein and in doing so activated PLC, resulting in the formation of IP₃ and DAG [182]. More recent studies have indicated the presence of two G-proteins that act in series to control stimulus-secretion coupling in mast cells [183]. This theory was put forward after it was experimentally observed that in the presence of calcium ions, GTP- γ -S induced histamine secretion and PI metabolism by binding to the G-protein and activating PLC in rat mast cells permeabilised with streptomycin-O. Moreover, it was also observed that GTP- γ -S still caused histamine release from rat mast cells in the presence of the aminoglycoside antibiotic neomycin, a PLC inhibitor. This observation suggested the putative involvement of a second G-protein, G_E, culminating in exocytosis. The exact role of the G_E-protein in exocytosis is awaited with anticipation.

1.8.6 CELLULAR PROTEIN PHOSPHORYLATION.

As mentioned before, stimulation of the mast cell activates several protein kinases, which in turn cause calcium-dependent phosphorylation of several proteins. Experimentally, it has been observed that up to four proteins of molecular weights 42,000, 59,000, 68,000 and 78,000 Dalton undergo phosphorylation after stimulation by a diverse range of stimuli, including anti-IgE, compound 48/80 and ionophore A23187 [184-186].

It was also observed that compound 48/80 induced rapid phosphorylation of the

42,000, 59,000 and 68,000 Dalton proteins. Phosphorylation of the 78,000 Dalton protein occurred more slowly [184] and pharmacologically-induced phosphorylation of that particular protein inhibited mediator release from mast cells [185]. Thus, these findings indicate that phosphorylation of 42,000, 59,000 and 68,000 Dalton proteins are somehow involved in "switching on" mediator release, whilst phosphorylation of the 78,000 Dalton protein occurs prior to the cessation of secretion [186].

1.9 MAST CELL MEDIATORS.

Upon immunological or non-immunological stimulation, mast cells secrete a diverse range of chemicals, some of which are preformed whilst others are newly generated. The majority of the secreted mediators, each within their own right, can induce immediate hypersensitivity reactions and initiate other pathological conditions.

1.9.1 PREFORMED MEDIATORS.

These mediators are preformed and stored within secretory granules. They are released upon mast cell activation. The following sections describes, in detail, the different types of preformed mediators found within mast cells.

1.9.2 HISTAMINE.

Histamine is widely distributed throughout the mammalian body. The majority of the amine is stored in mast cells within tissues while circulating histamine is contained in the basophil [40]. The granule-associated amine is formed by the action of the cytoplasmic enzyme histidine decarboxylase on the amino acid histidine (Fig.1.4). Histamine is then bound to the anionic side-chains of the proteoglycans that make up the granule matrix [39]. Human mast cells and basophils contain 1-3 pg of histamine per cell [187-189]. However, rat mucosal mast cells were found to contain 1-2 pg whereas rat serosal mast cells contain 10-30 pg of histamine per cell [190-192]. Once released from the mast cell, histamine is best suited to act locally near its site of release, as greater than 90% of the amine is

metabolised rapidly within 2-3 minutes by histamine N-methyl transferase and diamine oxidase. Further metabolism of methyl histamine to methyl imidazole acetic acid and ribosylation of imidazole acetic acid may occur. The catabolites of histamine are then excreted in the urine from the mammalian body [39], (see Fig.1.4). In some cases, small intact quantities of histamine have been detected in human urine samples [193].

As stated previously, histamine exerts its biological effects through three distinct histamine receptor subclasses, (H_1 , H_2 and H_3) [40]. The vasoactive properties of histamine were identified as early as 1910 by Dale and Laidlaw [4]. Contraction of smooth and bronchial muscle, increased vascular permeability and stimulation of nasal mucus production are all mediated by histamine via the H_1 receptor [194,195]. The effects mediated by histamine via the H_2 receptor include gastric acid secretion, airway mucus production, inhibition of skin and basophil histamine release and elevation of cellular cAMP levels [196]. Many biological effects, though mainly H_1 - or H_2 -receptor mediated, can result from the combined effects of the two receptors. Such effects include chemokinesis of eosinophils and neutrophils, complement $C3_b$ receptor expression on eosinophils, wheal and flare cutaneous responses and prostaglandin I_2 production by endothelial cells [197,198]. H_3 -receptor mediated effects are thought to regulate the synthesis and release of histamine in the brain [199].

1.9.3 SEROTONIN.

5-hydroxytryptamine (5-HT) or serotonin, is found only in rodent mast cells, together with histamine [200]. This amine was found to be absent in human mast cells. Once released in rodents, it acts as a potent bronchoconstrictor. Moreover, exogenously applied 5-HT was seen to act as a potent vasoconstrictor in human skin.

1.9.4 PROTEOGLYCANS.

It is the presence of highly sulphated proteoglycans found within the mast cell

which enables them to stain metachromatically with basic dyes. There are two major types of glycosaminoglycans found in mast cells: heparin and chondroitin sulphates. These glycosaminoglycans are attached within the mast cell to small peptide cores, hence the name proteoglycans [192]. The exact function of mast cell proteoglycans has yet to be fully established. It is known that proteoglycans bind to histamine within the secretory granules and in so doing may facilitate the uptake and packaging of histamine into the secretory granules. Mast cell proteoglycans also regulate the stability and activation of other enzymes present in the mast cell.

Heparin consists of a protein core made up of alternating serine and glycine residues. Glycosaminoglycans (GAGs) are attached to every second or third serine residues by a unique sequence of sugars, consisting of a glucuronic acid-galactose-galactose-xylose linkage which occurs predominantly in proteoglycans [201]. Heparin can be synthesised by human skin and lung mast cells [202,203].

Chondroitin sulphates are glycosaminoglycans with $\beta_{1,4}$ linked disaccharide units of glucuronic acid in $\beta_{1,3}$ linkage to galactosamine. Different classes of chondroitin sulphates have been identified in both human and rat mast cells, the key factor being the location of the mast cell within the body. This will be discussed later in detail.

1.9.5 NEUTRAL PROTEASES.

Neutral proteases (so called as they perform optimally at neutral pH) are enzymes which catalyse the cleavage of peptide bonds. Two types of proteases, chymase and tryptase have been identified in human mast cells [204,205]. They form the major protein constituents of the secretory granules, comprising 20-50% of the total cellular protein. Chymase and tryptase were found to be absent in basophils, thus forming a specific marker for human mast cells [204,206].

Tryptase is a tetrameric serine endoprotease of 134,000 Dalton, with subunits of 31,500 and 33,000 Dalton, each with an active enzymatic site [205,207]. Initially, Tanaka and co-workers suggested that tryptase obtained from skin mast cells was

different to that found in lung mast cells [208]. However, this is now known to be untrue. Lung mast cells contain up to 10 pg of tryptase per cell. The exact biological role of tryptase is still not fully elucidated, although potential roles of the protein have been investigated *in vitro*. Schwartz and co-workers [209] found that tryptase acts on the complement component C3 to form the anaphylatoxin C3a, but has no effect on C5. Anaphylatoxin C3a has the ability to induce smooth muscle contraction.

Chymase, unlike tryptase is not present in all mast cells, but preferentially found in skin and colonic submucosal mast cells [210]. Human chymase is a monomeric serine endoprotease of 30,000 Daltons. Like tryptase, human chymase is bound to the granular proteoglycan and stored in the mast cell secretory granules.

1.9.6 CHEMOTACTIC MEDIATORS.

Mast cells also release a variety of chemicals which are chemotactic for eosinophils and neutrophils, after activation by certain stimuli. The eosinophil chemotactic factor for anaphylaxis (ECF-A) and neutrophil chemotactic factor for anaphylaxis (NCF-A), once released from mast cells, will induce movement of eosinophils and neutrophils to the site of injury. Both chemotactic factors are involved in the pathogenesis of asthma [196].

1.9.7 NEWLY SYNTHESISED MEDIATORS.

Mast cells have the capacity to synthesise and release a range of lipid mediators following immunological or non-immunological stimulation, both *in vitro* and *in vivo*. All the newly formed lipid mediators collectively known as eicosanoids, possess inflammatory properties to varying degrees. Eicosanoids are derived from arachidonic acid (AA) which itself is generated by the action of PLA₂ on membrane phospholipids. Liberated AA is then rapidly metabolized via either of two pathways: via the enzyme cyclooxygenase to form a range of prostaglandins (PGs) and thromboxanes (TXs) or by a lipoxygenase enzyme to form a range of leukotrienes (LTs) (see Fig.1.5) [196]. Once released from the cell, the eicosanoids exert their

biological actions in the surrounding tissues.

Mast cells generate PGE_2 , $\text{PGF}_{2\alpha}$, PGI_2 , PGD_2 , TXA_2 and TXB_2 via the unstable intermediates PGG_2 and PGH_2 . Rat serosal and human lung mast cells were found to generate large quantities of PGD_2 (50-60 ng/cell), after stimulation by calcium ionophores, anti-IgE and antigen [217]. Once liberated, PGD_2 causes smooth muscle contraction and increased vascular permeability. Recent studies have also indicated that PGD_2 may cause systemic flushing and hypotension in humans with mastocytosis [213].

Mast cells also generate LTC_4 , LTD_4 and LTE_4 via 5-HPETE and LTA_4 . LTC_4 , LTD_4 and LTE_4 comprise what was formerly known as the slow reacting substance of anaphylaxis (SRS-A). LTs possess a variety of potent biological actions, including induction of bronchoconstriction, dose-dependent wheal and flare reaction in human skin and increased oedema in guinea-pigs [214].

1.10 MAST CELL HETEROGENEITY.

Over the years, the rat serosal mast cell has been extensively used as a tool in investigating many biological and pharmacological phenomena. However, recent improvements in enzymatic dispersion techniques have enabled scientists to isolate tissue mast cells of experimental animals and humans [215-221]. Thus, comparative studies were carried out in detail on the isolated mast cell and basophils.

It was observed that histaminocytes from different species, and even from different tissues within the same species, exhibited a marked heterogeneity in their biochemical, functional and histochemical properties.

The above phenomenon of mast cell heterogeneity was first reported in 1906 by Maximow [222], who observed that mast cells from different tissues within the rat displayed variations in their staining properties. A significant breakthrough on the theme of mast cell heterogeneity occurred during the mid 1960's, when Enerbäck and co-workers identified two distinct types of mast cells found in the

gastrointestinal tract of the rat [223,224]. His findings pointed out that mast cells situated in the lower layer of the intestinal tissue resemble those mast cells found in other connective tissues and the serosal cavity, and they were thus termed connective tissue mast cells (CTMC). However, those mast cells located in the mucosa of the intestinal wall, mucosal mast cells (MMC), displayed different properties, (Table 1.3). In brief, MMC were seen to be smaller in size compared to CTMC. They possess fewer granules, and thus have a lower histamine and 5-HT content than CTMC. MMC contain chondroitin sulphate, more recently identified as mainly being chondroitin sulphate di-B, with smaller amounts of chondroitin sulphate A and E but no heparin [225].

1.11 HISTOCHEMICAL DIFFERENCES BETWEEN MAST CELLS.

Mast cell heterogeneity in the rat has been further characterised largely based on histochemical properties. This technique has now been extended to human mast cells. The major histochemical property of the mast cell is its ability to exhibit metachromasia, when stained with basic dyes such as toluidine blue. The metachromatic staining is largely due to the interaction between the dye and anionic proteoglycans contained within the mast cell. It was also observed that only CTMC stained metachromatically with safranin and berberine dyes. Thus, histochemical techniques have verified that two distinct mast cell classes exist within the rat.

The subject of mast cell heterogeneity is not as clear cut in humans as it is in the rat. Many groups have reported that the conditions of staining the cells is imperative in order to get reproducible results. Strobel and co-workers reported that the number of mucosal type mast cells that could be identified in the human jejunum was dependent on the nature of the fixative used [227]. Another group reported that mast cells stained to indicate that they were of the "mucosal" type were in fact located in connective tissue. Thus, several problems exist in using the labels "connective tissue" and "mucosal" to describe mast cell subclasses in man defined solely on a histochemical basis.

1.12 DIFFERENCES IN PREFORMED AND NEWLY SYNTHESISED MAST

CELL MEDIATORS.

The mast cell is capable of secreting a variety of chemical compounds upon stimulation. Different mast cell populations vary in the types and amounts of mediators they contain. Thus, the existence of a biochemical diversity provides another marker of heterogeneity among this cell type [228].

The amount of histamine contained in mast cells varies considerably. Rat peritoneal mast cells contain up to 20 pg of histamine per cell, whereas skin and intestinal mast cells contain 4 and 1 pg per cell respectively [229]. 5-HT was only found in rodent but not human mast cells [230].

Human mast cell heterogeneity on the basis of their proteoglycan content is not as clear cut as it is in the rat. However, human lung mast cells were found to contain both heparin and chondroitin sulphates in the ratio of 2:1 [231,232]. Conversely, human mucosal mast cells from the stomach and colon were reported to contain chondroitin sulphates but no heparin [223]. Further studies on the proteoglycan content of human mast cells can be continued, once purified preparations have been isolated.

Two neutral proteineases (tryptase and chymase) have been identified in human mast cells by Schwartz and co-workers [234]. These enzymes are released following Ige-mediated activation of the mast cell. Further work by Schwartz and co-workers have defined two human mast cell subtypes based on their tryptase and chymase content. The TC mast cell contains both proteineases, and is predominantly found in the skin and intestinal submucosa. However, 10% of human lung mast cells were found to be of the TC subtype. T mast cells contain only tryptase and are found in the intestinal mucosa and the majority of lung mast cells are of this subtype [235].

Mast cells also produce a variety of eicosanoids, resulting from the oxidative metabolism of AA, which are synthesised de novo following stimulation. Mast cells from different species and tissues have been shown to be heterogeneous in their

ability to synthesise eicosanoids. Table 1.4 summarises the predominant eicosanoids produced by various mast cells and basophils following immunological activation.

It can be thus assumed that a biochemical heterogeneity does indeed exist between mast cell subtypes. Hence, this manifestation implies that activation of mast cells from different tissues and species should also be heterogeneous.

1.13 FUNCTIONAL DIFFERENCES BETWEEN MAST CELLS.

Mast cells can be activated by a variety of immunological and non-immunological stimuli. It has been well documented that mast cells from different species and tissues can vary widely in their responsiveness to both stimuli and inhibitors of degranulation [236,237]. Table 1.5 summarises the responses of human and rat mast cells to various secretagogues. From such studies, it is difficult to relate functional responsiveness of a given cell type to its biochemical make up.

Functional mast cell heterogeneity may also extend to inhibition of histamine release from a given mast cell type by anti-allergic drugs and other compounds. Table 1.6 summarises the responses of human and rat mast cells to various inhibitors. The varying ability of drugs to inhibit the release process has obvious clinical implications. Moreover, it may also indicate the different mechanism of the release process in various histaminocytes.

1.14 ORIGINS OF MAST CELL HETEROGENEITY.

Significant developments have been made of late in our understanding of mast cell ontogeny and differentiation. Mast cell heterogeneity is thought to arise due to distinct pathways of development, cellular differentiation and local environmental factors.

Experimental evidence has suggested that mast cells and basophils originate from bone marrow haemopoietic precursor cells [238]. Basophils mature within the bone marrow like the other circulating granulocytes [239], whereas immature mast cells

invade tissues via the blood system, where they proliferate into mast cells. It has been observed that in rodents, mucosal mast cell proliferation is T-lymphocyte dependent [240,241].

It is assumed that the anatomical microenvironment which a mast cell precursor (immature mast cell) finds itself in has a profound effect on the phenotype that cell will express [242]. Moreover, mast cells in the rat which become MMC or T mast cells in humans, may originate from other mast cell types. This phenomenon has yet to be fully clarified. An alternative theory suggests that mast cell precursors are influenced by the tissue which they invade, thus undergoing a tissue-dependent differentiation.

Since this project investigates the effects of platelet activating factor on different cell types, namely mast cells, basophils and neutrophils, the following sections describe in detail certain aspects and properties relating to this particular compound.

1.15 PLATELET ACTIVATING FACTOR.

1.15.1 HISTORICAL ASPECTS.

During the late 1960's, Barbaro and Zweifler demonstrated the release of histamine into rabbit plasma during an acute allergic reaction [243]. It had been previously demonstrated in the rabbit that platelets were the major source of histamine among the blood elements [244], but it was not known whether histamine release during antigen challenge represented a direct or indirect consequence of antigen-antibody recognition. It was subsequently demonstrated independently by two groups that histamine release in sensitised rabbits undergoing antigen challenge was a consequence of IgE-dependent activation of basophils that, in turn, released a soluble product capable of eliciting platelet activation [245,246]. Benveniste and co-workers coined the term "platelet activating factor" (PAF), and thus began a detailed study of PAF which is still commencing.

Benveniste and co-workers then went on to initiate a detailed study involving the full characterisation of the biologically active constituent responsible for platelet activation [247]. On the basis of its physicochemical properties, they suggested that the active compound was likely to be a phospholipid. The chemical structure was then identified in 1979 by three independent groups as 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine [248-250], (Fig.1.6). This phospholipid has also been referred to as PAF-acether [248], acetyl glyceryl ether phosphorylcholine [249] and antihypertensive polar renomedullary lipid [250]. However, the original nomenclature (PAF) [251] will be used throughout this thesis.

1.16 SYNTHESIS OF PAF.

Benveniste and co-workers reported the partial synthesis of PAF in 1979 [248] and more recently, the total synthesis of this molecule has been achieved [252]. Further work on various cell types established that neither damage nor physical disruption of the cell yields substantial amounts of PAF, thus suggesting that the phospholipid is neither preformed nor stored [253]. It was also later found that the release of PAF is sensitive to changes in calcium ion concentrations, thus indicating that an enzymatic process is involved in its synthesis [254].

Further elucidation of the synthetic pathway of the phospholipid led to the observation that coincident formation of PAF and a non-acetylated compound, lyso-PAF, occurred [255,256]. Lyso-PAF is produced by a variety of inflammatory cells including platelets [256], macrophages [257,258] and neutrophils [259,260]. It was also established that lyso-PAF formation could be abolished in the presence of phospholipase A₂ (PLA₂) inhibitors such as bromophenacyl bromide and lipocortin [261,262], thus showing that a calcium-dependent PLA₂ enzyme is involved in the cellular synthesis of PAF. Experimentally it was shown that the addition of synthetic lyso-PAF and acetyl-CoA to rat macrophages resulted in the formation of PAF [263], hence indicating that lyso-PAF is a cellular precursor of PAF. This finding has been further supported by the observation that thrombin-activated platelets incorporate radiolabelled acetate into PAF [264]. Lyso-PAF is then converted into PAF by an acetyltransferase enzyme [265]. This enzymatic formation of PAF from

lyso-PAF has been observed in numerous cell types [257,266,267], (Fig.1.7).

More recently a second pathway has been proposed for PAF formation involving the transfer of phosphorylcholine from ether-linked phospholipids (plasmogens) catalysed by the enzyme choline phosphotransferase [268,269].

1.17 METABOLISM OF PAF.

In the presence of plasma, PAF is metabolised both *in vivo* and *in vitro* due to the action of the enzyme acetyl hydrolase (phosphatidyl-2-acetyl-hydrolase). The enzyme cleaves the acetate moiety at the *sn*-2 position of the phospholipid resulting in the formation of lyso-PAF [270]. Thus, lyso-PAF is the primary metabolite and precursor of PAF. Moreover, experimental evidence suggests that there is a constant cycle of PAF production and metabolism (Fig.1.7). Lyso-PAF can be further metabolised enzymatically by the removal of the *O*-alkyl group, to give fatty aldehydes and glyceryl-3-phosphorylcholine [271].

1.18 CELLULAR ORIGINS OF PAF.

Since PAF release was first demonstrated *in vivo* [246], its secretion from a wide range of mainly inflammatory cells following suitable stimulation has been reported (Table 1.7).

It has been observed that if a cell synthesises appreciable quantities of PAF *de novo* following stimulation, it is not necessary for the cell to release PAF into the extracellular medium. Schleimer and co-workers [272], found that purified human lung mast cells generate PAF when stimulated with anti-IgE, but this is not released with other newly synthesised mediators such as PGD₂ and LTC₄. However, two independent groups reported conflicting experimental results regarding PAF release from human basophils following stimulation. Camussi and co-workers reported such [273], whereas Betz and co-workers found no effect [274]. However, stimulated bone marrow-derived mast cells from mouse mastocytoma cell lines have been shown to release PAF [275]. Thus a species variation in the ability of mast cells

to release PAF following activation exists.

Platelets, neutrophils, human macrophages and eosinophils are all rich sources of PAF, which is readily released following IgE-mediated stimulation [254,256,266].

1.19 PHARMACOLOGICAL EFFECTS OF PAF.

Ever since the positive identification of PAF, its effects in the mammalian system have been studied in detail. PAF causes severe bronchoconstriction in several species, including guinea-pigs [276], rabbits [277] and man [278-280]. PAF was identified by Patterson and co-workers [281] as being the most potent spasmogen in both experimental animals and man, considerably more so than histamine or LT's. PAF-induced bronchoconstriction may also involve the activation of neutrophils in humans, thus resulting in the release of LT's which in turn may potentiate the bronchoconstrictor effects of PAF [282]. Recent studies by Cuss and co-workers found that in humans after PAF administration by inhalation, potent bronchoconstriction is induced with recovery occurring after one hour. However, tachyphylaxis occurs rapidly, rendering the airways unresponsive to cumulative doses of PAF [278].

The mechanism of increased responsiveness of the respiratory airways (bronchial hyperresponsiveness) to different stimuli remains to be fully elucidated. PAF is able to induce a prolonged increase in bronchial responsiveness in man, guinea-pigs and monkeys. Inhaled PAF causes bronchial hyperresponsiveness in both normal and asthmatic human subjects [283]. The increase in responsiveness was found to occur three days after PAF inhalation, and persisted for up to four weeks. Owing to the fact that inhaled PAF is inactivated fairly quickly, this led to the theory that PAF exerts its effects on the airways through a series of secondary events, such as the activation of other inflammatory cells which in turn release other mediators thus prolonging the response. Another theory arose from such experiments, which suggested that PAF may be chemotactic for other cells such as eosinophils and neutrophils, which when primed prolong the inflammatory response by releasing other mediators. It has been experimentally shown that PAF is

selectively chemotactic for eosinophils in human skin and in primate lung tissue [284,285]. Since eosinophils are excellent sources of PAF, newly released PAF may in turn attract more eosinophils, thus completing a cycle and prolonging bronchial hyperresponsiveness [266].

In addition to releasing PAF when activated, eosinophils also produce and secrete major basic protein (MBP), eosinophil cationic protein and eosinophil peroxidase, all of which induce damage of the airway epithelium [286].

As mentioned previously, PAF has the ability to recruit and activate a range of inflammatory cells [287,288]. It is not known whether PAF is chemotactic for human basophils or whether its presence causes an increase in tissue mast cell numbers. PAF induces aggregation of these inflammatory cells and in so doing induces them to release their inflammatory mediators such as the lipoygenase and cyclooxygenase products of AA.

PAF-induced cellular activity is not restricted to the airways but can occur in other tissues such as the skin. Experimental results have shown that four hours after an intradermal injection of PAF in normal human subjects, neutrophil infiltration predominates, whereas after twenty-four hours the cellular infiltrate contains both neutrophils and mononuclear cells [289]. In contrast, selective eosinophil infiltration predominates in atopic human subjects undergoing the same experimental procedure [284]. Thus, atopic individuals respond differently to PAF in comparison with normal subjects. These observations suggest that PAF may play a central role in the induction and maintenance of eosinophil functions in allergic diseases [251]. PAF also increases vascular permeability in several tissues at concentrations 1000 times less than that of histamine [290-292]. It has also been experimentally determined that PAF-induced vascular permeability can be inhibited by PAF antagonists, thus indicating the presence of PAF receptors on the vascular endothelium [292]. PAF receptors will be discussed later in detail. Intradermal injection of PAF into the skin produces a wheal and flare reaction [289], thus indicating the vasoconstrictor properties of PAF in systemic tissues.

1.20 PAF RECEPTORS.

The mechanism by which PAF produces its various effects on target tissues is still debated. A number of groups have reported that high-affinity binding sites exist for PAF and such sites have been identified on platelets [293,294], neutrophils [294] and macrophages [295]. The binding of PAF to its receptor appears to be specific and is inhibited by PAF antagonists.

Following binding of PAF to its receptor, several biochemical reactions are initiated. Firstly, activation of PLC occurs, which results in the degradation of phosphoinositides to give IP_3 and DAG. It has been well documented that both these chemicals are able to act as second messengers in bringing about a variety of intracellular events [296,297]. Thus, DAG activates PKC leading to the phosphorylation of several intracellular proteins involved in physiological processes such as secretion or contraction [298] and IP_3 initiates the release of calcium ions, which in turn regulate other intracellular events [130].

The actual structure of the PAF receptor is still not fully elucidated, although Braquet and co-workers did suggest a putative structure in 1986 [299]. As yet, there have been no recent developments in this field.

1.21 PAF ANTAGONISTS.

Recently, a number of different classes of drugs have been identified as having antagonistic activity against PAF. These compounds are proving to be useful tools in elucidating the role of PAF in health and disease.

The antagonists of PAF include analogues of the phospholipid, natural products and synthetic structures. The first PAF antagonist developed was a synthetic analogue CV3988. This compound incorporates an octadecyl carbamate in position 1, a methylether in position 2 and a thiazolium ethyl phosphate group attached to the positively charged nitrogen atom in the PAF molecule. However, it proved to be clinically unsuitable owing to its poor stability and partial agonist activity [300].

The first natural product kadsurenone, described as having PAF antagonistic properties was isolated from the Chinese herb *Piper futokadsura* [301]. Extracts of this herb had been employed in China for use in anti-asthmatic and anti-rheumatic therapy. Kadsurenone (a benzofuranoid neolignan) is essentially formed by the oxidative dimerization of hydroxyalkoxyphenylpropane. Following the isolation of kadsurenone, another class of natural products, ginkgolides, were discovered to possess PAF antagonistic properties. The ginkgolides were isolated from the leaves of the Ginkgo biloba tree [302], and include compounds such as BN50730, BN52021 and BN52063. These compounds are structural isomers of terpenes. The ginkgolides are unique cage C₂₀ molecules, incorporating a tertiary butyl (*t*-Bu) group and six five-membered rings including a spiro nonane system, a tetrahydrofuran cycle and three lactonic groups.

Synthetic compounds have been developed which also exhibit marked PAF antagonistic activities. Such agents include the thienotriazolodiazepine WEB2086 [303].

All the PAF antagonists described are capable of inhibiting the wide array of PAF-induced pathological effects both in vivo and in vitro to varying degrees.

Deeming and co-workers reported the ability of PAF antagonists to inhibit PAF-induced bronchial hyperactivity and allergic bronchoconstriction in experimental animals [304]. Also, BN52021, kadsurenone and WEB2086 have all been reported to inhibit the bronchospasm accompanying anaphylactic shock in sensitised guinea-pigs [305-307]. The activity of PAF antagonists in inhibiting antigen-induced hyperactivity is yet to be determined, but will obviously be of interest in ascertaining the role of PAF in asthma.

1.22 THE ROLE OF PAF IN HEALTH AND DISEASE.

There is increasing evidence which suggests that PAF may contribute to the overall features observed in human anaphylaxis. However, experimental evidence using live

human subjects is impossible to obtain. Following an infusion of PAF into experimental animals such as dogs and guinea-pigs, it was observed that PAF mimicked many features of anaphylaxis, including a fall in circulating neutrophils and platelets [307].

Urticaria can be described as a localized cutaneous form of anaphylaxis. It can be induced by allergies or physical stimuli, such as extremes of cold. Eosinophil and neutrophil chemotactic factors have been identified in the exudate from the urticarial lesion [308]. In addition, a PAF-like phospholipid was also isolated from the exudate [309]. The presence of PAF may contribute to the hypotension observed in patients with cold urticaria.

Since PAF potently induces microvascular leakage and protein extrusion in the skin, it may be plausible to suggest a possible role for PAF in the pathogenesis of eczema [310]. This phenomenon has yet to be fully elucidated. PAF has also been implicated in psoriasis, in which increased levels of the phospholipid have been reported, although the exact role of PAF in this disease remains to be determined [311].

1.22.1 THE ROLE OF PAF IN ASTHMA.

Concepts regarding the pathogenesis of asthma have changed considerably during the past few years, but still remain unclear. Initial work by Sir Henry Dale and co-workers suggested an interrelationship between chemical mediators, anaphylaxis, allergy and smooth muscle contractions, all of which contributed collectively to the overall pathogenesis of asthma [312]. Since then, detailed studies undertaken by several groups have increased our awareness of asthma and many theories have been put forward regarding the aetiology of this disease.

Asthma may be simply defined as a reversible obstruction of the intrathoracic airways. Hallmarks of this disorder include smooth muscle contractions, a non-specific increase in bronchial responsiveness, increased vascular permeability and an increase in inflammatory response [313]. Several actions of PAF on the

respiratory airways support a possible role for this mediator in asthma [314]. Moreover, PAF mimics many of the pathological features of asthma. Table 1.8 summarises the pathological features of asthma.

1.22.2 EFFECTS OF PAF RELEVANT TO ASTHMA.

Table 1.9 summarises the effects of PAF relevant to the pathology of asthma. In brief, PAF is a potent inducer of airway microvascular leakage which may be an important feature of asthma. It is also a powerful chemotactic agent for eosinophils and neutrophils which, when stimulated, may release more PAF, thus giving rise to continued inflammation. In addition, the presence of PAF leads to impaired mucociliary clearance, which is also a feature of asthma [316].

It is obvious from the properties of PAF that it could be a major mediator in the pathogenesis of asthma. However, further evidence for the involvement of PAF in human asthma still remains to be obtained.

1.23 THE FUTURE OF PAF.

The exact role of PAF in asthma may yet be fully evaluated when clinical trials involving newly synthesised PAF antagonists for use in man start. With this in mind, results from such experiments may provide a novel approach to the treatment of bronchial asthma.

1.24 AIMS OF THIS STUDY.

It has been well documented that PAF is an extremely potent endogenously produced phospholipid that has been implicated in the pathogenesis of asthma and allergic diseases. PAF is produced by a variety of cells found abundantly in the mammalian body.

The aim of this study was to investigate and characterize the effects of exogenous PAF on isolated histaminocytes. Also, to a smaller extent, the effects of PAF on

isolated human neutrophils were investigated. Mast cells, as stated earlier, play a critical role in the aetiology of asthma and allergy. Therefore it struck us as being important to establish whether a relationship exists between these particular cells and PAF, a compound which closely mimics the pathogenesis of asthma and allergy.

Fig.1.1 Basic structure of immunoglobulin. Each molecule comprises two heavy (H) and two light (L) chains with constant (C) and variable (V) amino acid sequences. Adapted from ref. 54.

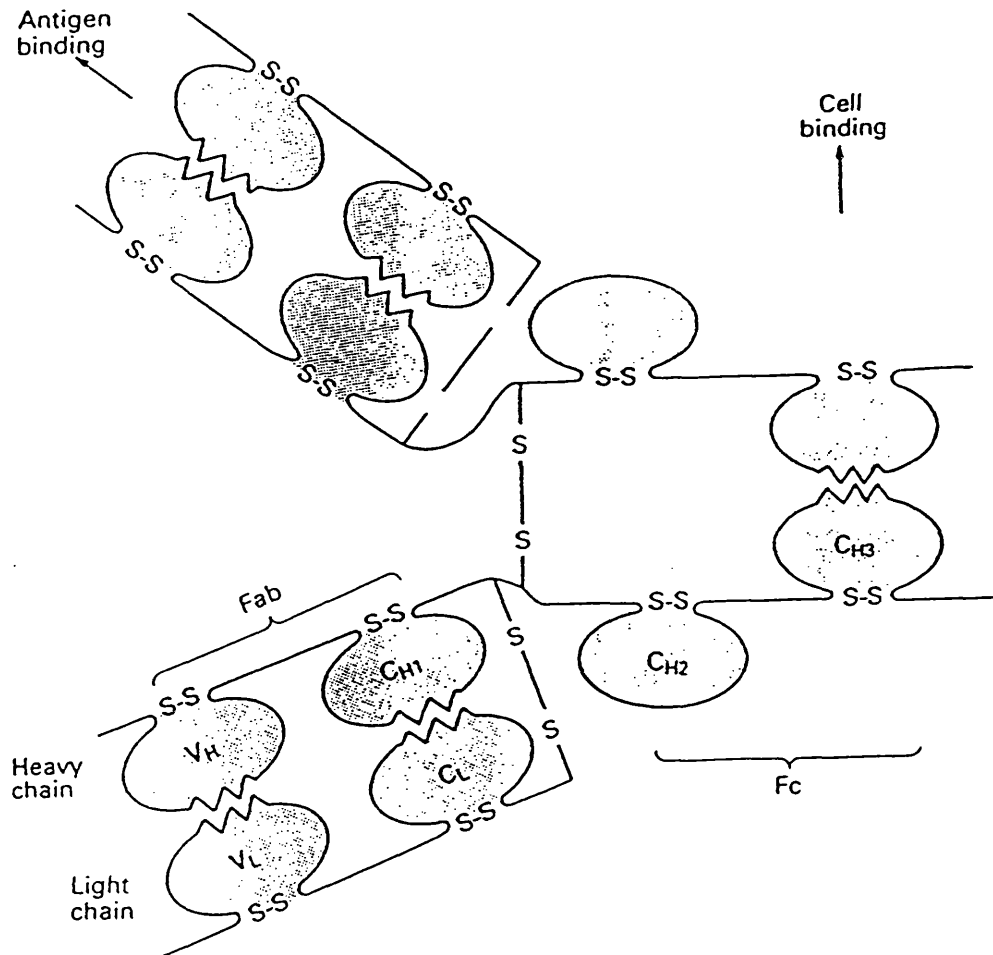


Fig.1.2 Diagram of the high affinity receptor for IgE, showing the relationship between the subunits in the membrane. Shaded areas represent carbohydrate. Source: H. Metzger, J-P. Kinet, R. Perez-Montfort, V. Rivnay and S.A. Wank: Progress in Immunology, P493, Academic Press, New York, (1983).

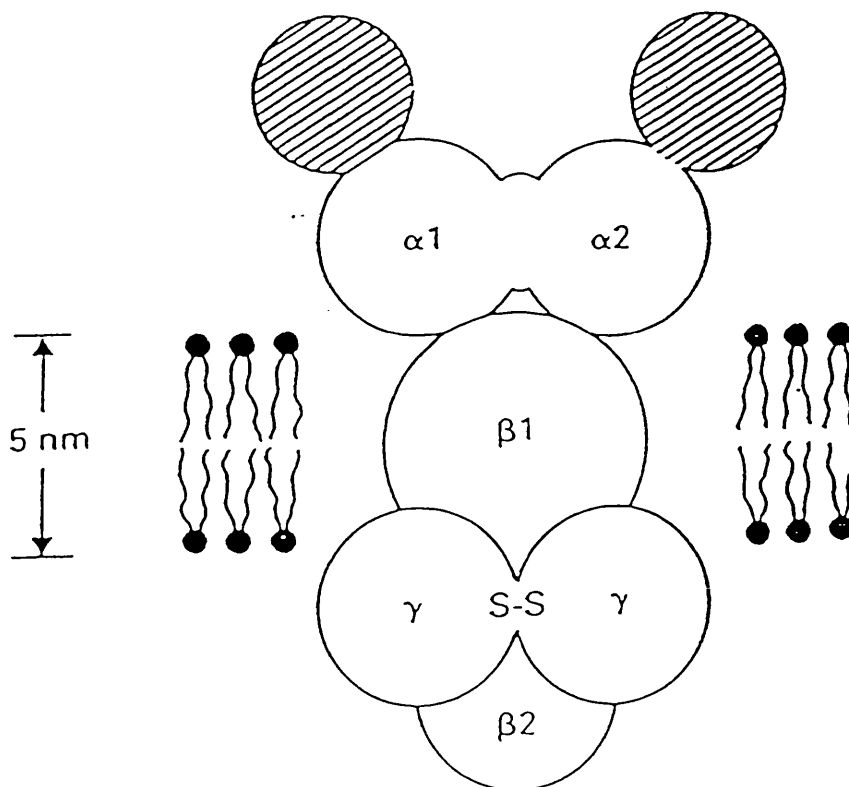


Fig.1.3 Sequence of reactions involved in phospholipid methylation in rat mast cells.

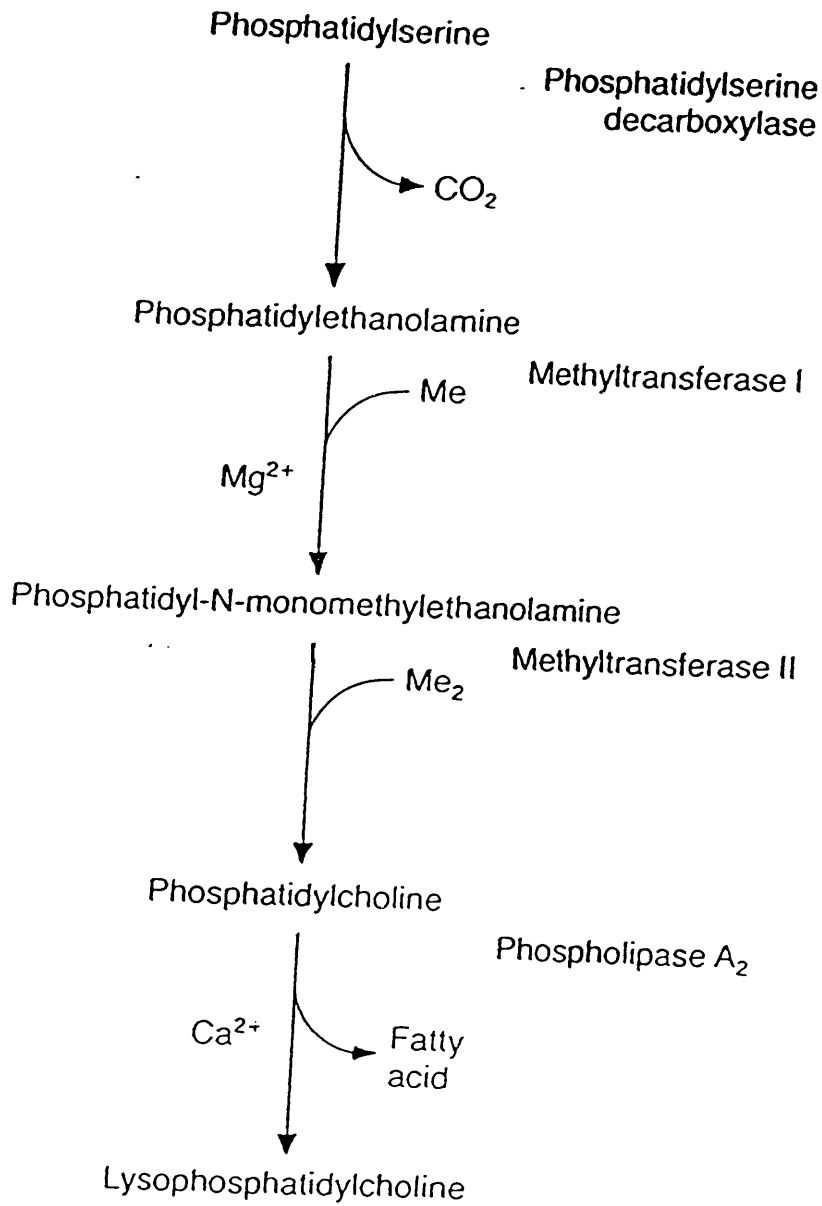


Fig.1.4 Synthesis and catabolism of histamine. Percentage recovery of histamine and its metabolites in the urine after 12 hours following intradermal [^{14}C] histamine in human males.

Compiled from ref. 39.

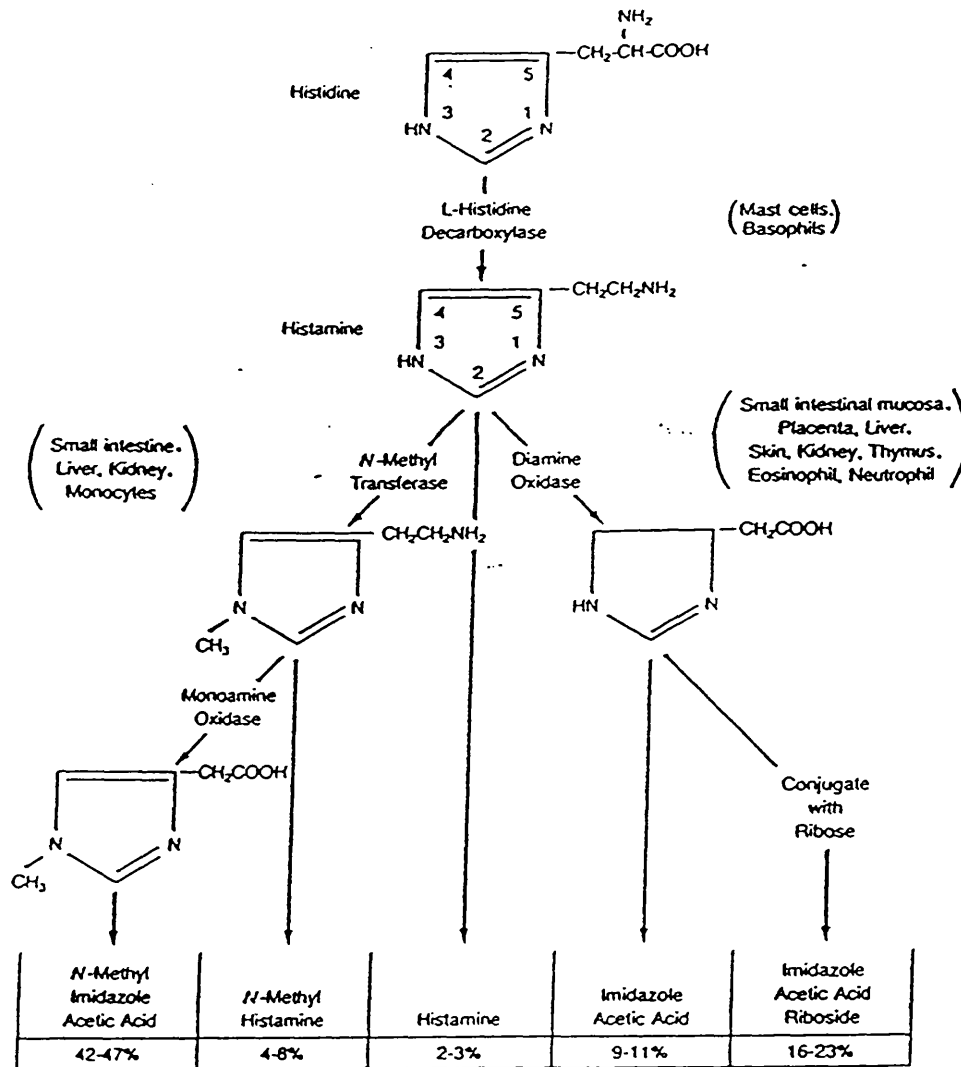
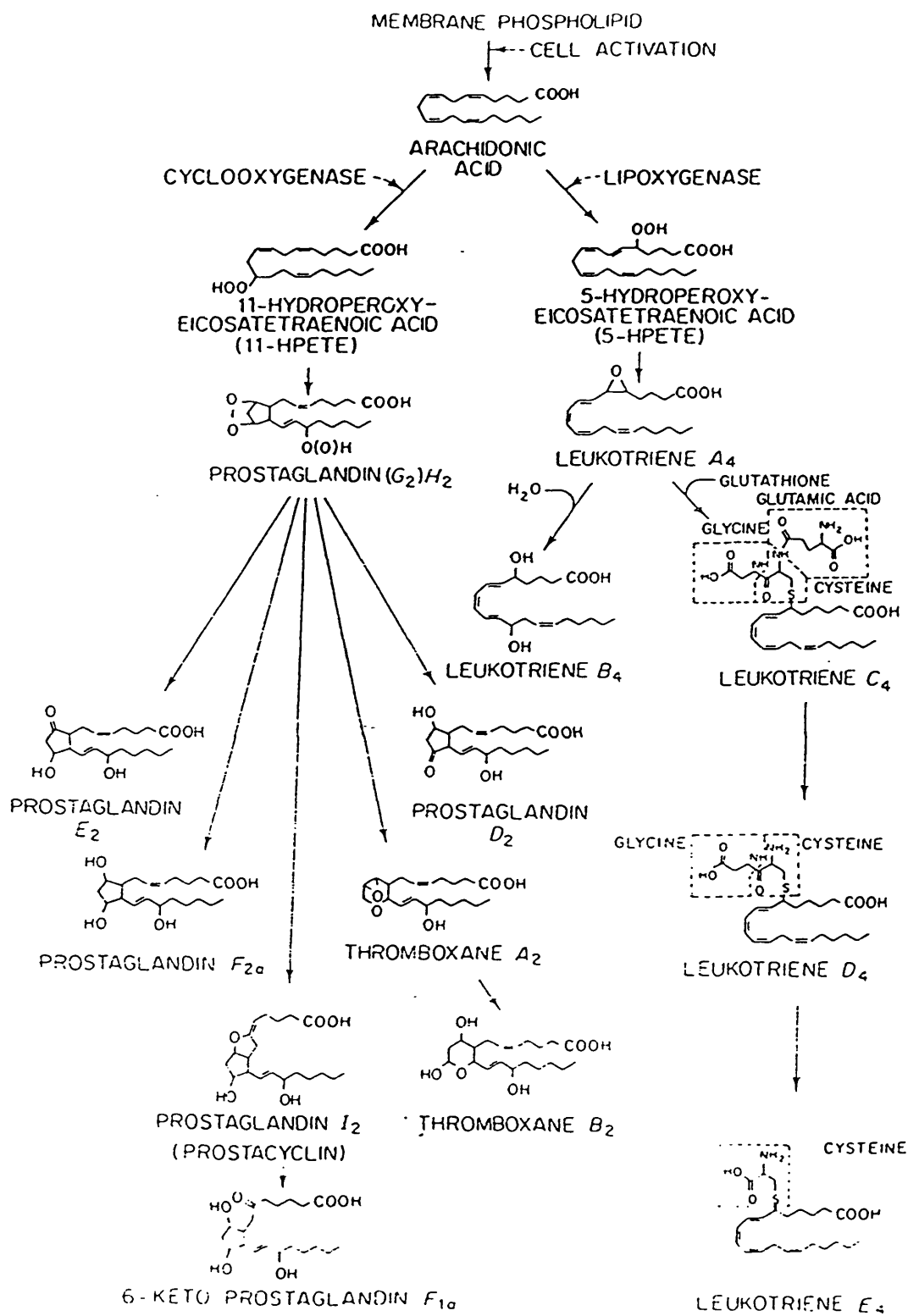


Fig.1.5 Arachidonic acid metabolism.



Source: F.L. Pearce: Asthma Reviews, 1, 95, (1987).

Fig.1.6 Diagrammatic representation of PAF, illustrating the location of the ether bond (position 1) and the acetyl side chain (position 2).
Adapted from ref. 376.

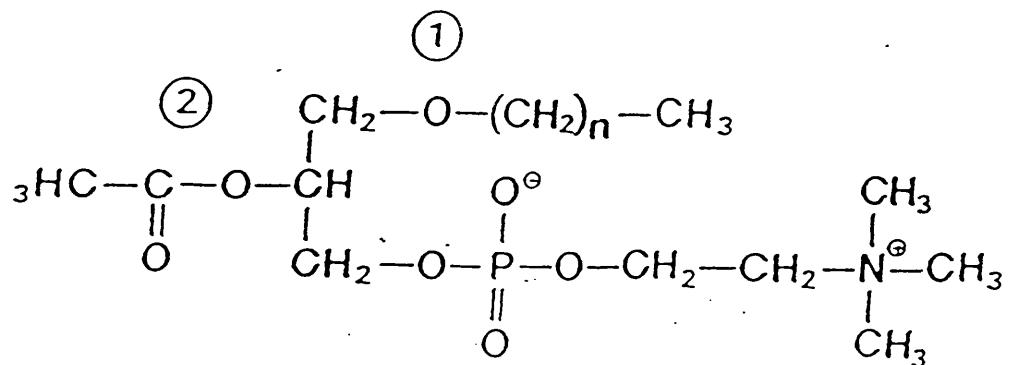


Fig.1.7 Sequence of reactions involved in the formation and breakdown of PAF.
Adapted from ref. 376.

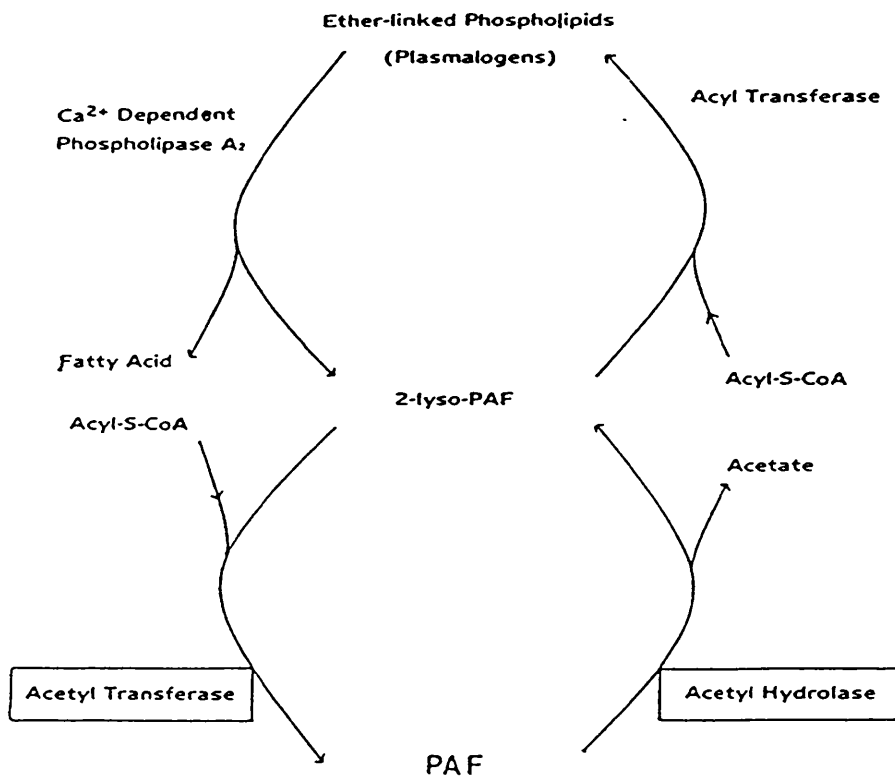


Table 1.1 Symptoms and pathological features of anaphylaxis and proposed mediators.

Symptoms	Pathological features	mediators
Urticaria, laryngeal and intestinal oedema	Vascular permeability	Histamine Eicosanoids
Flushing, headache and hypotension	Vasodilatation	Histamine Eicosanoids
Palpatations	Arrhythmias	Histamine
Rhinorrhea and bronchorrhea	Mucus secretion	Histamine Eicosanoids

Adapted from ref. 39.

Table 1.2 Agents which cause histamine release from mast cells.

Selective liberators	Examples
Polybasic cations	compound 48/80, peptide 401, polylysine, polymyxin.
Calcium ionophores	A23187, ionomycin
Anaphylatoxins	C3 _a , C4 _a , C5 _a

Adapted from ref. 63-70.

Table 1.3 Some properties of mucosal and connective tissue mast cells from the gastrointestinal tract of the rat.

Mucosal mast cells	Connective tissue mast cells
Small (9.7 μM), few granules	Large (19.6 μM), many granules
Chondroitin sulphate	Heparin
Low histamine (1.3 pg/cell) and low 5-HT content	High histamine (15 pg/cell) and high 5-HT content
Short lifespan (<40 days)	Long lifespan (>6 months)
Berberine negative	Berberine positive
Does not counterstain with safranin	Counterstains with safranin
Low density of surface IgE-receptors	High density of surface IgE-receptors

Compiled from ref. 224 and 226.

Table 1.4 Ability of human basophils and various mast cells (MC) to produce eicosanoids upon immunological stimulation.

Cell type	Eicosanoid (ng/10 ⁶ histaminocytes)	
	LTC ₄	PGD ₂
Human intestinal MC	31	21
Human lung MC	50	60
Human basophil	80	-
Murine bone marrow MC	90	6
Rat peritoneal MC	<1	50

Compiled from ref. 226.

Table 1.5 Responses of human and rat mast cells (MC) to various secretagogues.

Secretagogue	Rat (MC)		Human (MC)		
	serosal	mucosa	lung	intestine	skin
Antigen	+	+	+	+	+
Ionophore	+	+	+	+	+
Comp. 48/80	+	-	-	-	+
Substance P	+	+	-	-	+
Morphine	+	-	-	-	+

+, mediator released observed; -, no effect. Ref. K.E. Barret and F.L. Pearce: Histamine and Histamine Antagonists (Ed. B. Uvnäs), P104, Handbook of Experimental Pharmacology, 97, (1991).

Table 1.6 Inhibition of histamine release from human and rat mast cells (MC) by anti-allergic drugs.

Drug	Rat (MC)		Human (MC)	
	serosal	mucosal	lung	intestinal
DSCG	+	-	±	±
Theophylline	+	-	+	+
Isoprenaline	±	-	+	+
Quercetin	+	+	+	+

+, inhibition of histamine release observed; ±, variable inhibition of some magnitude; -, no inhibition observed;
DSCG, disodium cromoglycate.

Compiled from ref. 242.

Table 1.7 Cellular sources of PAF.

Cells	Species	Stimulus
Platelets	Rabbit	A23187
Neutrophils	Human	C5 _a , A23187, FMLP
Eosinophils	Human	C5 _a , A23187,
Monocytes	Rabbit	A23187
Macrophages	Rat Human	A23187 antigen
Basophils	Human	Anti-IgE, A23187, C5 _a
Mast cells	Human lung	Anti-IgE

Adapted from ref. 251.

Table 1.8 Pathological features of asthma and proposed mediators.

Pathological feature	Proposed mediator
Bronchospasm	Histamine, LTC ₄ , LTD ₄ , LTE ₄ , PG's, TXA ₂ and PAF.
Mucosal oedema	Histamine, LTC ₄ , LTD ₄ , LTE ₄ , PGE and PAF.
Airway inflammation	Eosinophil chemotactic factor, neutrophil chemotactic factor, LTB ₄ and PAF.
Mucus secretion	LTC ₄ , LTD ₄ , LTE ₄ , and prostaglandin generating factor.

Adapted from M. Kaliner: Chest 87, 25, (1985).

Table 1.9 Effects of PAF relevant to asthma.

-
1. Most potent inducer of microvascular leakage.
 2. Human eosinophil and neutrophil chemoattractant.
 3. Induces bronchoconstriction.
 4. Induces sustained bronchial hyperresponsiveness.
 5. Causes protein exudation into airway lumen.
 6. Reduces mucocilliary clearance.
-

Adapted from ref. 315.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS.

Throughout this study male Sprague Dawley rats (200-450 g), BKW mice of either sex (30-50 g) and Dunkin Hartley guinea-pigs (400-600 g) were obtained from closed, random-bred colonies kept at the Joint Animal House, University College London.

2.2 HUMAN TISSUE.

Macroscopically normal human lung tissue was supplied by the Middlesex Hospital at the time of resection generally for bronchogenic carcinoma. Human foreskin from circumcision was obtained from University College Hospital and the Middlesex Hospital. Macroscopically normal human colonic tissue was obtained following surgery for colonic carcinoma from University College Hospital. Peripheral blood was obtained by venipuncture from normal donors by a qualified medical practitioner.

2.3 CHEMICALS AND REAGENTS.

2.3.1 COMPOUNDS FOR BUFFERS.

Bovine Serum Albumin	Sigma, London
Calcium chloride	Hopkins & Williams
Ethylenediaminetetraacetic acid (EDTA)	BDH Chemicals

Glucose	BDH Chemicals
Heparin (5000 units/ml)	Weddel Pharmaceuticals
N-2-hydroxyethyl piperazine-N'- 2 ethane sulphonic acid	Sigma, London
Hydrochloric Acid	Fisons
Potassium Chloride	Fisons
Sodium Chloride	BDH Chemicals
Sodium Dihydrogen Phosphate	Sigma, London

2.3.2 HISTAMINE LIBERATORS.

Platelet Activating Factor	Novabiochem
Compound 48/80	Sigma, London
Concanavalin A	Sigma, London
Sheep Anti-Serum to Rat IgE	Miles
Rabbit Immunoglobulins to Human IgE	Dako-Immunoglobulins

2.3.3 β -GLUCURONIDASE LIBERATORS.

Platelet Activating Factor A23187	Novabiochem Calbiochem
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2.3.4 INHIBITORS OF HISTAMINE RELEASE.

Disodium Cromoglycate	Fisons
Nedocromil Sodium	Fisons
Isoprenaline	Sigma, London
Quercetin	Sigma, London
Theophylline	Sigma, London
Dibutyryl cAMP	Sigma, London
8-Bromo cAMP	Sigma, London
Isobutyl methyl xanthine	Sigma, London

2.3.5 METABOLIC INHIBITORS.

Antimycin A	Sigma, London
2-Deoxy-D-glucose	Sigma, London

2.3.6 MATERIALS FOR HISTAMINE ASSAY.

Butan-1-ol	Fisons
n-Heptane	Fisons
Methanol	May & Baker
<i>o</i> -Phthalialdehyde (OPT)	Sigma, London
Perchloric Acid (72%)	May & Baker
Triton X-405	Sigma, London
Sodium Hydroxide	BDH Chemicals
Hydrochloric Acid	BDH Chemicals

2.3.7 MATERIALS FOR β -GLUCURONIDASE ASSAY.

4-methylumbelliferyl- β - glucuronide	Sigma, London
Sodium Hydrogen Carbonate	BDH Chemicals
Sodium Carbonate	BDH Chemicals

2.3.8 PLATELET ACTIVATING FACTOR ANTAGONISTS.

BN50730	Gift from P. Braquet.
BN52021	Gift from P. Braquet.
WEB2086	Gift from Bohrenger Ingelheim, England

2.3.9 RADIOACTIVE MATERIALS.

Prostaglandin D ₂ [³ H] Radioimmunoassay (RIA) kit	Amersham, UK
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2.3.10 MISCELLANEOUS.

Collagenase, Type 1A	Sigma, London
Charcoal, Granular Activated	BDH Chemicals
Cytochalasin B	Sigma, London
Dextran 70 (MW 10,000)	Fisons
Dextran (MW 298,000)	Sigma, London
Dimethyl Sulphoxide	Hopkins & Williams
Hyaluronidase	Sigma, London
Sterile Syringes	Sabre
Percoll	Pharmacia Fine

Chemicals

Alcian Blue	BDH Chemicals
Trypan Blue	BDH Chemicals

2.4 BUFFERS.

2.4.1 Full Hepes Buffers (FHB).

In this study all experiments were carried out in Full Hepes Tyrode Buffer (FHB), or modifications of this buffer.

The constituents of FHB are listed below:

NaCl, 137 mM
HEPES, 10 mM
D-Glucose, 5.6 mM
KCl, 2.7 mM
NaH ₂ PO ₄ .H ₂ O, 0.4 mM
CaCl ₂ .2H ₂ O, 1.0 mM

2.4.2 Calcium and magnesium free (CMF) buffer:

FHB - 1.0 mM CaCl

2.4.3 2x Calcium buffer:

CMF + 2 mM CaCl

2.4.4 2x EDTA buffer:

CMF + 0.2 mM EDTA

2.4.5 Heparinized FHB:

FHB + 50 units/ml heparin

2.4.6 Isotonic Saline:

NaCl 153 mM (9 g/l)

The pH of the buffers was adjusted to 7.3-7.4 by the addition of 4 M sodium hydroxide using a pH meter.

2.5 ISOLATION OF MAST CELLS.

2.5.1 PERITONEAL AND PLEURAL MAST CELLS.

The rat was anaesthetized using 95% CO₂ and then sacrificed by applying pressure to the neck. Abdominal skin was cut away and 10 ml of FHB containing heparin (0.05 ml/50 ml FHB) was injected into the peritoneal cavity. The cavity was massaged gently for 2 min and then opened by a midline incision. The peritoneal fluid was slowly retrieved using a plastic pipette. The cell suspension was centrifuged at room temperature (RT) (MSE, minor "S", 1000 rpm, 150 g, 2 min). The supernatant was discarded and the cells washed with pre-warmed FHB and resuspended in the required volume of FHB.

In order to isolate mouse peritoneal mast cells, the same procedure as above was

followed except 5 ml of heparinized FHB was injected into the peritoneal cavity and massaged only for 1 min.

Pleural mast cells were obtained by injecting 5 ml of heparinized FHB into the chest cavity through the diaphragm. The diaphragm was opened and the cell suspension was gently retrieved.

2.5.2 RAT MESENTERIC AND LUNG MAST CELLS.

The mesentery was dissected from the small intestine. Any attached lymph nodes were removed. The lungs were removed from the chest cavity and dissected free of major airways and blood vessels. After washing both tissues in warmed FHB, both the lung and the mesentery were cut into small pieces with scissors and further cut using an automated McIlwain tissue chopper. The chopped tissue was further dispersed in prewarmed-BSA-FHB (37 °C) containing collagenase (120 units/ml) for 60 min in a shaking water bath.

At the end of the digestion period, the tissue fragments were further disrupted through a syringe (10 ml). The final suspension was then filtered through absorbent gauze to remove any undigested tissue fragments. The cells were isolated by centrifugation (MSE coolspin, 1000 rpm, 150 g, 3 min, 4 °C). After washing the cells twice with warmed FHB, the cells were resuspended in the required volume of FHB.

2.5.3 GUINEA-PIG MESENTERIC AND LUNG MAST CELLS.

A similar procedure to that described for the dissociation of the rat tissue was used apart from the following modifications. The prewarmed BSA-FHB (37 °C) contained a higher concentration of the enzyme collagenase (160 units/ml) and the digestion time was increased to 90 min.

2.5.4 RAT SKIN MAST CELLS.

Skin covering the abdomen (area 3 cm by 3 cm) was shaved in order to remove the fur. The skin was then cut away from the abdomen and the underlying fat was removed. The tissue was cut manually into small fragments with scissors (approx. 1 mm). The tissue was then digested in prewarmed BSA-FHB containing collagenase (160 units/ml) and hyaluronidase (500 units/ml) for 4x 60 min. in a shaking water bath at 37 °C. The cells were isolated as explained previously.

2.5.5 HUMAN LUNG MAST CELLS.

Macroscopically normal lung obtained from resection was dissected free of major airways and blood vessels. The tissue was cut into small fragments with scissors. The tissue fragments (approx. 1 mm) were washed in FHB for 2x 10 min in order to remove excess blood. The fragments were then digested in prewarmed BSA-FHB containing collagenase (120 units/ml) for 90 min at 37 °C with stirring using a magnetic stirrer. At the end of the digestion period the cells were isolated from the supernatant as explained previously.

2.5.6 HUMAN COLONIC MAST CELLS.

The mucosal tissue (normal) was recovered following surgery for colonic carcinoma. The mucosal layer was separated from the muscle layer using blunt dissection. Chopped tissue was washed for 2x 10 min in prewarmed FHB. The tissue was then digested in prewarmed BSA-FHB containing collagenase (120 units/ml) for 2x 60 min at 37 °C with constant stirring. The cells were isolated from the supernatant as explained previously.

2.5.7 HUMAN CUTANEOUS MAST CELLS.

Excess underlying fat was carefully removed from human foreskin tissue samples using blunt dissection. The tissue was cut into small fragments (1 mm²) manually using scissors. The chopped tissue was then dispersed in FHB containing collagenase (160 units/ml) and hyaluronidase (500 units/ml) at 37 °C for 3x 2 hours, with constant stirring. At the end of the first two hours, the tissue mixture

was filtered through gauze and the cells were pelleted by centrifugation (1000 rpm, 4 min, 4 °C) and retained in FHB at 37 °C. The filtrate was then dispersed for a further two hours, after which the above procedure was repeated. At the end of the third digestion period the tissue was further disrupted by expression through a 20 ml sterile syringe and the cells recovered as explained above. All the cell preparations were pooled together, washed once more and the required volume of FHB was added to the cell sample ready for experimental use.

2.5.8. HUMAN BASOPHILS.

Blood was collected from healthy human volunteers by a qualified medical practitioner. As soon as the required volume of blood (20 ml) had been extracted, heparin (50 units/ml) was added to each sample to prevent coagulation. One part (ie. 5 ml) of dextran (6%) containing glucose (30 mg/ml) was mixed with four parts of blood and the mixture was left to stand for 60-90 min at room temperature. The top layer which was rich in platelets and leukocytes was gently recovered and centrifuged (MSE minor "S", 1000 rpm, 150 g, 3 min, RT) to sediment the basophils leaving the platelets suspended in the supernatant. The supernatant was discarded and the cell pellet was washed twice with prewarmed FHB.

2.5.9 ISOLATION OF HUMAN NEUTROPHILS.

Peripheral blood (40 ml) was obtained by venipuncture from healthy human donors by a qualified medical practitioner. The sample blood was immediately mixed with 0.1 M EDTA and dextran (6% in isotonic saline) and left to stand at room temperature for 40 min. After this period of time had elapsed, the top layer (orange in colour) was gently aspirated and centrifuged (1500 rpm, 6 min, 4 °C) to sediment the neutrophils. After discarding the supernatant the pellet was washed again in FHB. Again the supernatant was discarded. At this stage the neutrophil rich pellet appeared pink in colour, due to the presence of erythrocytes. To eliminate the erythrocytes 5 ml of ice cold distilled water was added to the cell pellet and gently shaken for 1 min. An equal volume of 2x FHB was added to the mixture in order to regain the correct FHB concentrations. The suspension was centrifuged (1500

rpm, 6 min, 4 °C) to sediment the neutrophils, leaving the lysed erythrocytes in the supernatant. After discarding the supernatant, the white neutrophil pellet was washed twice in FHB. The cells were then counted and resuspended in the required volume of FHB ready for experimental use.

2.6 ACTIVE SENSITISATION OF RATS.

2.6.1 SENSITISATION OF RATS WITH *Nippostrongylus brasiliensis*.

Sprague Dawley rats (180-200 g) were given a subcutaneous injection in the hind leg with the third stage larvae (L3, 2,500) of *N. brasiliensis* (life cycle Fig. 2.1) in sterile physiological saline. The larvae (L3) were obtained from cultures as described in section 2.6.2. On several occasions they were provided by Mr. Pedley, Department of Agricultural Zoology, University of Leeds. The rats became sensitised three weeks after injection and remained so for a further three to four weeks.

2.6.2 PREPARATION OF THIRD STAGE LARVAE (L3).

On the seventh day after injecting the rats, the faeces of the treated rats were collected. The faeces (15 g) were moistened with tap water and gently ground with an equal weight (15 g) of activated charcoal. The mixture was transferred to petri dishes (Fig.2.2) and kept in an incubator (25 °C) for 14 days. The larvae were isolated by pouring the contents of the petri dishes over two layers of gauze lined with lens tissue. The larvae were allowed to sediment (approx. 60 min) through warm tap water into a graduated glass test tube (Fig.2.3). The larvae were washed twice with sterile physiological saline and resuspended in the same medium, ready for injection.

2.7 PURIFICATION OF PERITONEAL MAST CELLS.

In certain experimental procedures such as the measurement of prostaglandin D₂ levels highly purified preparations of mast cells are required. Rat peritoneal mast cells were purified by gradient centrifugation over Percoll. In brief, peritoneal cells

from 3-5 sensitised rats were collected as described in section 2.5.1 and mixed. The cells were washed in cold FHB and the pooled pellets were resuspended in heparinized BSA-FHB. Percoll (90%) was gently added to the cell concentrate and 1 ml of BSA-FHB was carefully layered over the mixture thus forming a percoll-FHB interface. The mixture was then centrifuged (MSE chilspin, 1000 rpm, 4 °C) for 25 min. At the end of the given period of time, it was observed that cell pellet formation had occurred at the bottom of the tube. The supernatant was gently removed by aspiration and the cell pellet resuspended in BSA-FHB. The cells were then washed twice with FHB and then resuspended in the correct volume of FHB required for the experiment.

The above purification procedure was found to yield mast cells of 90-99% purity when stained with Alcian blue and counted using an improved Neubauer haemocytometer.

2.8 METHODOLOGY FOR HISTAMINE RELEASE EXPERIMENTS.

2.8.1 EFFECT OF HISTAMINE LIBERATORS ON ISOLATED MAST CELLS AND BASOPHILS.

Cells (250 µl) were aliquoted into disposable Sarstedt tubes which already contained 200 µl of FHB. The cell suspension was left to equilibrate in a water bath (37 °C) for 5 min. 50 µl of the test secretagogue at ten times the required concentration was added to the cell samples. After 10 min (basophils 30 min), the reaction was quenched by the addition of 1.5 ml or 500 µl of ice-cold FHB. The samples were centrifuged (peritoneal: 100 g, 4 °C, 2min, others: 150 g, 4 °C, 4min) and the supernatant from each sample was decanted into correspondingly labelled Sarstedt tubes. Either 1 ml or 2 ml of FHB was added to the resultant cell pellet in the reaction Sarstedt tubes.

Histamine content was determined in both the cell pellet and the supernatant tubes. Histamine release was expressed as a percentage of the total amount present in the cell.

Hence:

$$\text{Histamine Release (HR) (\%)} = 100 \{(\text{HS}) / (\text{HS} + \text{HCP})\}$$

Where HS represents the amount of histamine in the supernatant and HCP the corresponding amount in the cell pellet.

The value of (HR) was corrected for the spontaneous release of histamine which occurred in the absence of any histamine liberator. Values are given as mean \pm SEM.

2.8.2 EFFECT OF METABOLIC BLOCKERS.

Cells were resuspended in a glucose-free buffer and allowed to equilibrate in a water bath at 37 °C for 5 min. 200 μ l of the cell suspension was then added to four different buffers (250 μ l, FHB, glucose-free, 2-deoxyglucose, 5 mM and antimycin-A, 1 μ M) and allowed to pre-incubate for 20 min. Cells were then challenged with one concentration of specific secretagogues for 10 min. The reaction was quenched by the addition of ice-cold FHB.

2.8.3 KINETICS OF HISTAMINE RELEASE.

Rat peritoneal mast cells (RPMC) were incubated with one concentration of a secretagogue for various periods of time (1 min - 60 min) and reactions were quenched with ice-cold FHB.

2.8.4 EFFECT OF TEMPERATURE ON HISTAMINE RELEASE.

RPMC were allowed to equilibrate at 0, 25, 37 and 45 °C for a period of 10 min. The cells were then challenged with one concentration of various secretagogues for a further 10 min, after which the reaction was quenched with ice-cold FHB.

2.8.5 EFFECT OF CALCIUM FREE AND EDTA BUFFERS.

The procedure explained in 2.8.1 was repeated on RPMC in calcium free and EDTA buffers.

2.8.6 EFFECT OF pH.

RPMC pellets were resuspended in prewarmed FHB at different pH values (6.0, 6.5, 7.0, 7.5, 8.0). The cells were allowed to equilibrate (3 min, 37 °C) before being challenged by one concentration of different secretagogues. The reactions were terminated by the addition of ice-cold FHB.

2.8.7 EFFECT OF INHIBITORS OF HISTAMINE RELEASE.

Cells (160 µl) were allowed to preincubate together with a specific inhibitor (240 µl) at 37 °C for a given period of time, depending on the type of inhibitor being studied, after which the secretory stimulus was added. The reaction was terminated after 10 min, as before. In some cases the cells and the secretory stimulus were added together and allowed to incubate in the presence of a given inhibitor.

The results were expressed in terms of the percentage inhibition of the control histamine release, ie. histamine release induced by a given concentration of the secretory stimulus in the absence of any inhibitor and thus given as:

$$100\{(Ra-Rp)/(Ra)\}$$

where Ra denotes histamine release in the absence of any inhibitor and Rp denotes the release of histamine in the presence of a particular inhibitor. Values are given as mean ± SEM.

2.8.8 SYNERGISTIC HISTAMINE RELEASE.

Cells (400 µl) were allowed to incubate in the presence of two secretory stimuli (50 µl) for 10 min after which the reaction was terminated by the addition of ice-cold FHB (500 µl). In these set of experiments, the concentration of PAF chosen was that which itself does not cause any histamine release. The other stimuli used

were anti-IgE, concanavlin A and compound 48/80, each of which induced histamine release in a dose-related manner.

In all of the above experimental procedures the percentage histamine release was calculated as explained in section 2.8.1.

2.9 HISTAMINE ASSAY.

The histamine assay procedures used throughout this study are modifications of the technique initially employed by Shore and co-workers [317].

2.9.1 AUTOMATED ASSAY.

This procedure basically involves the isolation of histamine from the sample and, in doing so, eliminates any substances which would otherwise interfere with the histamine assay. The samples firstly were acidified by the addition of perchloric acid (72%, 33 μ l/ml). The samples were vortexed and centrifuged (MSE coolspin, 2000 rpm, 4 °C, 20 min), in order to sediment any cellular debris which would otherwise impair the function of the commercial autoanalyzer (Techicon Autoanalyzer II). After centrifugation, the supernatants were poured into sample cups and assayed for their histamine content using the autoanalyzer.

Briefly, the samples were made alkaline and histamine was extracted into salt-saturated butan-1-ol, leaving the aqueous waste products which were removed. The organic phase was washed and the insoluble waste products removed. The organic phase was further separated by washing with a less alkaline solution. The medium was made less polar by the addition of n-heptane. This step enabled histamine to be extracted into dilute hydrochloric acid. The amine was then coupled with OPT (Fig.2.4) under alkaline conditions. The newly formed adduct produced a fluorescence which was detected by a fluorimeter connected to a chart recorder.

2.9.2 MANNUAL METHOD.

A manual method for assaying histamine was used only when the mast cells originated from the rat peritoneal cavity. Samples were made alkaline by the addition of sodium hydroxide (1 M) followed by OPT (1% in methanol), mixed vigorously by vortexing and left to react for 4 min. The reaction was quenched by the addition of hydrochloric acid (3 M). The samples were poured into plastic cuvettes and the fluorescence directly measured using a spectrofluorimeter (Perkin Elmer, LF5B). The wavelengths of excitation and emission were set at 360 nm and 440 nm respectively.

2.10 PROCEDURE FOR PROSTAGLANDIN D₂ ASSAY.

2.10.1 EXPERIMENTAL PROCEDURE FOR PGD₂ RELEASE.

These experiments were carried out on rat peritoneal mast cells of which purified preparations were required. Cells from 3-5 sensitised rats were pooled together and purified following the technique described in section 2.7. Once purified, the cells were counted and an appropriate amount of FHB added to the cells in order to ensure that after the cells were aliquoted into the reaction tubes, each sample contained approx. 1×10^5 cells. Samples were incubated at 37 °C and then challenged with certain secretagogues for the required period of time. After the reaction was terminated, samples were centrifuged (1000 rpm, 2 min, 4 °C) and aliquots of the supernatants (500 µl) were quickly pipetted into appropriately labelled Eppendorf tubes and snap-frozen in liquid nitrogen. The rapid snap-freezing process prevents degradation and further synthesis of PGD₂. The Eppendorf tubes were stored at -70 °C until required. The remaining supernatants were separated from the cell pellets and both fractions were assayed for histamine as described in section 2.9.

2.10.2 PROCEDURE FOR PGD₂ ASSAY.

PGD₂ release was determined using a commercially available radioimmunoassay (RIA) kit. The assay is based upon competition between unlabelled PGD₂ and a fixed amount of tritium labelled PGD₂ (³H]PGD₂), to bind to a limited quantity of a specific antibody which has a high affinity for the prostaglandin. The amount

of radioactive PGD₂ bound to the antibody will be inversely proportional to the amount of the non-radioactive prostaglandin added. By measuring the amount of antibody-bound radioactive PGD₂, one can determine the quantity of non-radioactive PGD₂ contained in the sample being tested.

Together with the experimental samples, a set of PGD₂ standards (100 µl of each) were pipetted into individually labelled polystyrene tubes (12x 75 mm). In addition to the above tubes, three other tubes were prepared and labelled: total count (TC), non-specific binding (NSB) and zero standard (B₀). 100 µl of the radioactive prostaglandin ([³H]PGD₂) was added to each tube, followed by 100 µl of the antibody except into the NSB tube. Finally diluted assay buffer was added to the sample and standard tubes (100 µl), 200 µl into both the TC and B₀ tubes and 300 µl into the NSB tube. All tubes were mixed thoroughly by vortexing and left overnight to incubate at 4 °C. The following day 500 µl of dextran-coated charcoal was added to each tube, except the TC tube to which 500 µl of assay buffer was added instead. The tubes were vortexed gently, left to stand in an ice-bath for 10 min, then centrifuged (2000 rpm, 10 min, 4 °C). This procedure enables the separation of antibody-bound PGD₂ from the unbound prostaglandin, by the adsorption of the free prostaglandin onto the dextran-coated charcoal. After centrifugation, the supernatants were gently decanted into labelled scintillation vials leaving the charcoal pellets intact. Finally, 5 ml of scintillant (Optiphase "Safe", LKB) was added to each vial and the contents were gently mixed. The radioactivity of each sample was measured using a β-scintillation counter (Packard Model 3255 Tris-carb Liquid Scintillation Spectrometer, 4 min). By measuring the radioactivity in each sample the amount of radioactive PGD₂ bound to the antibody was quantified.

Once the average counts per minute (cpm) for each sample were obtained, the percent of PGD₂ bound could then be calculated using the following relationship.

$$\%B/B_0 = \{(\text{sample cpm} - \text{NSB cpm}) / B_0 \text{ cpm} - \text{NSB cpm}\} \times 100$$

A standard curve was plotted, thus enabling the amount of unlabelled PGD₂

contained in the sample to be read off the curve. The results were then expressed as ng PGD₂/10⁶ mast cells.

2.11 METHODOLOGY FOR β -GLUCURONIDASE RELEASE EXPERIMENTS.

2.11.1 EFFECT OF β -GLUCURONIDASE LIBERATORS.

Having successfully isolated the human neutrophils to approx. $92 \pm 2\%$ purity, an appropriate volume of FHB was added to the cells to ensure that after the cells (800 μ l) were aliquoted into the reaction tubes, each tube contained approx. 5×10^6 cells. The cells were preincubated with 100 μ l of cytochalasin B for 10 min, after which the cells were challenged with 100 μ l of secretory stimuli for 20 min. The reactions were terminated by placing each tube in an ice-bath for 2 min and centrifugation (2000 rpm, 10 min, 4 °C). After centrifugation, 50 μ l of the supernatant from each sample was removed and placed in correspondingly labelled plastic tubes. The samples were then assayed for their β -glucuronidase content, which will be explained in section 2.12. The results were expressed in a similar fashion to histamine release.

2.11.2 EFFECT OF METABOLIC BLOCKERS.

The procedure described in section 2.8.2 for mast cells was followed, except the total cell volume was increased to 400 μ l. Each sample was then tested for its β -glucuronidase content.

2.11.3 EFFECT OF INHIBITORS ON β -GLUCURONIDASE RELEASE.

The procedure described in section 2.8.7 for mast cells was followed, except the cell and inhibitor volumes were increased to 450 μ l and 500 μ l respectively. Each sample was then assayed for its β -glucuronidase content.

2.12 β -GLUCURONIDASE ASSAY.

Having previously placed 50 μ l of supernatant into correspondingly labelled tubes, a further 50 μ l of 4-methylumbelliferyl- β -glucuronide (substrate) was added to each tube. The tubes were incubated at 37 °C for 20 min. In the presence of free β -glucuronidase, the substrate is cleaved thus liberating a fluorophore. The reaction was quenched by the addition (5 ml, 1:1 ratio) of sodium hydrogen carbonate (1 M) and sodium carbonate (1 M). The fluorescence was directly measured using a spectorfluorimeter (Perkin Elmer, LF5B). The excitation and emission wavelenghts were set at 356 nm and 500 nm respectively.

2.13 STATISTICAL ANALYSIS.

All values are given throughout this thesis as means \pm SEM for the number (n) of experiments noted, unless otherwise stated. The points on the graphs represent the mean value and the vertical bars denote SEM.

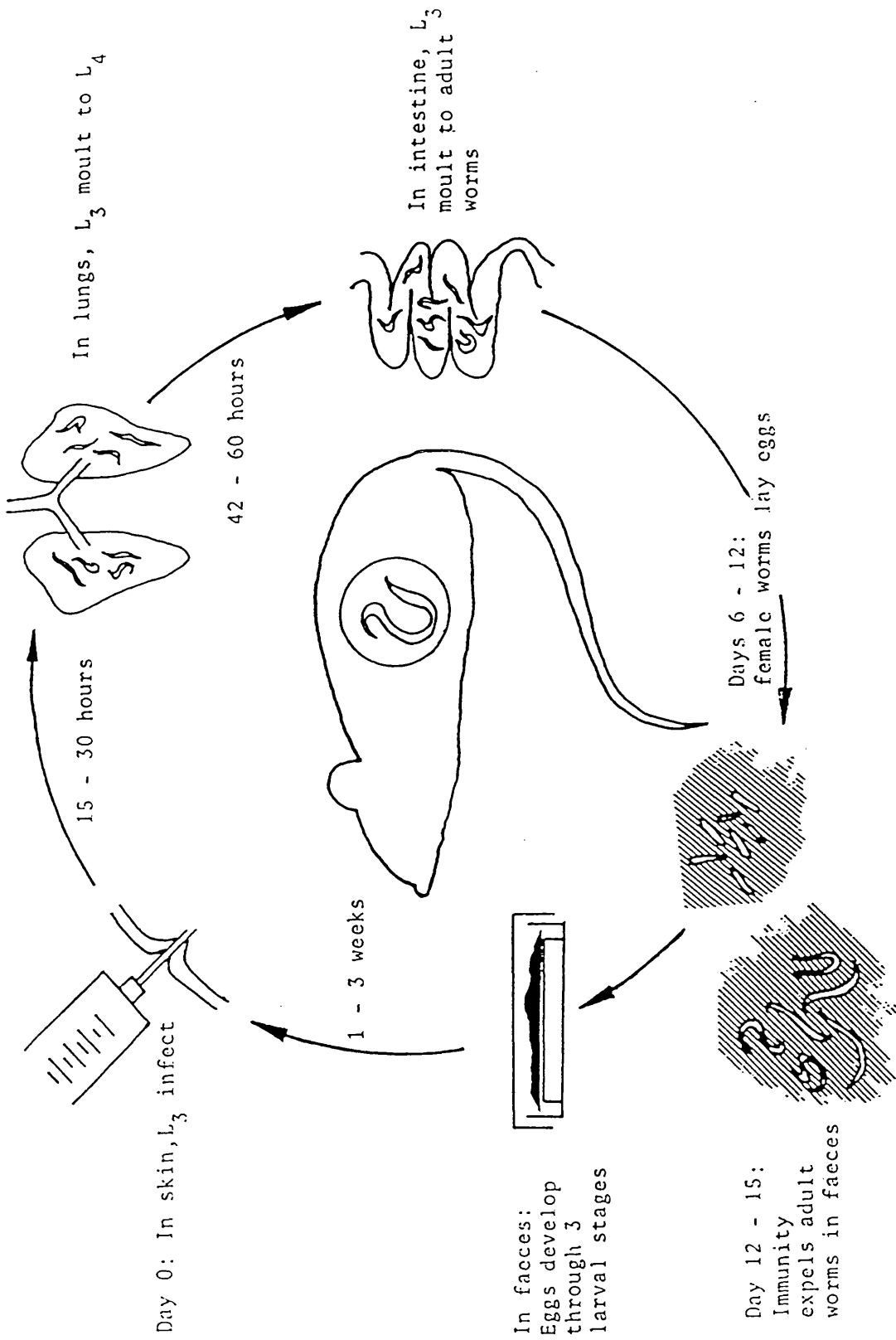


Fig.2.1 Life cycle of the nematode *Nippostrongylus brasiliensis* in the rat.

Preparation of third stage larvae

Mashed mixture of faeces containing eggs
and equal amount of charcoal

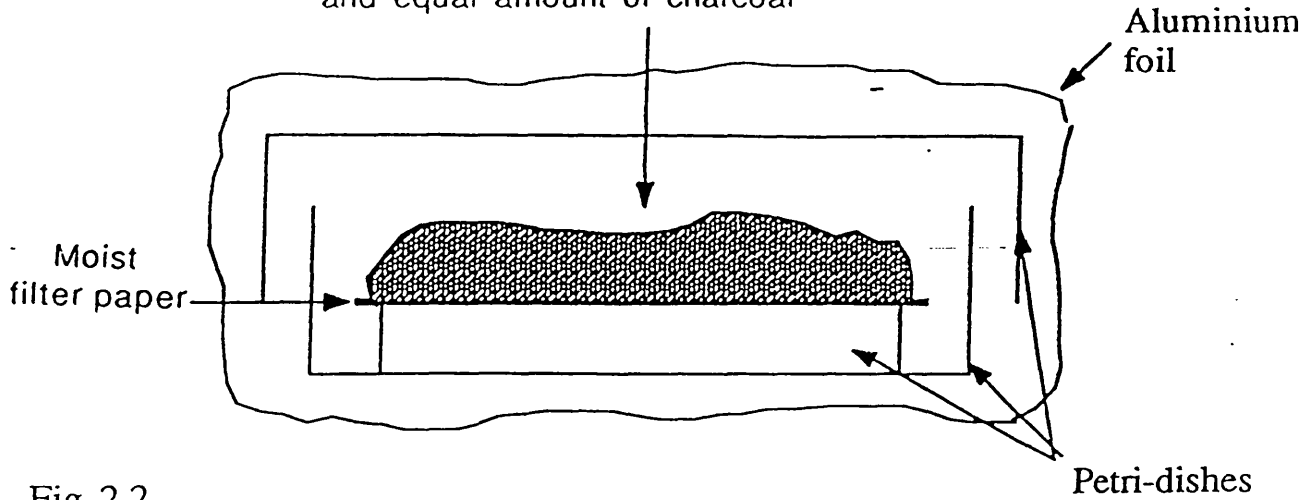


Fig 2.2

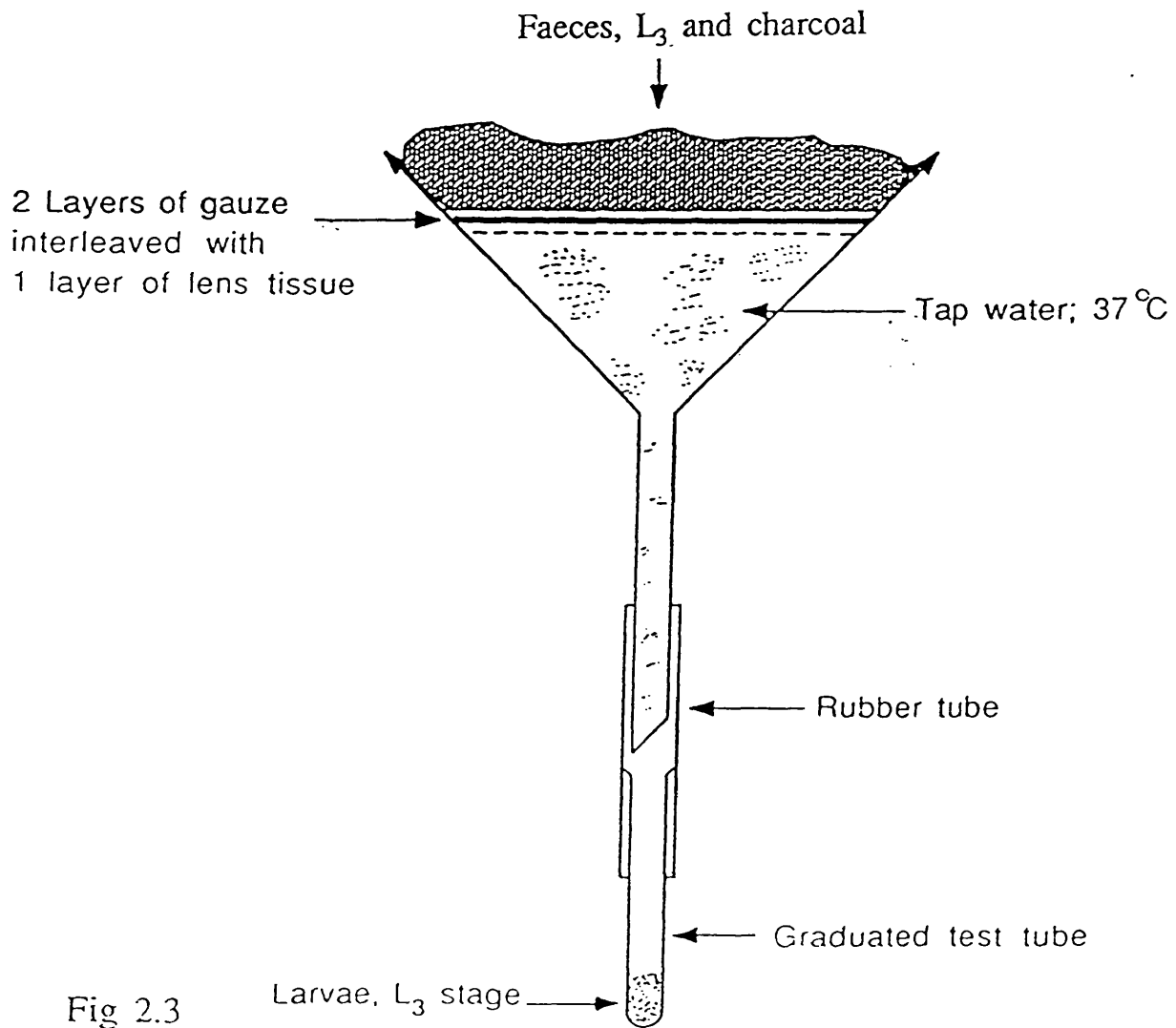


Fig 2.3

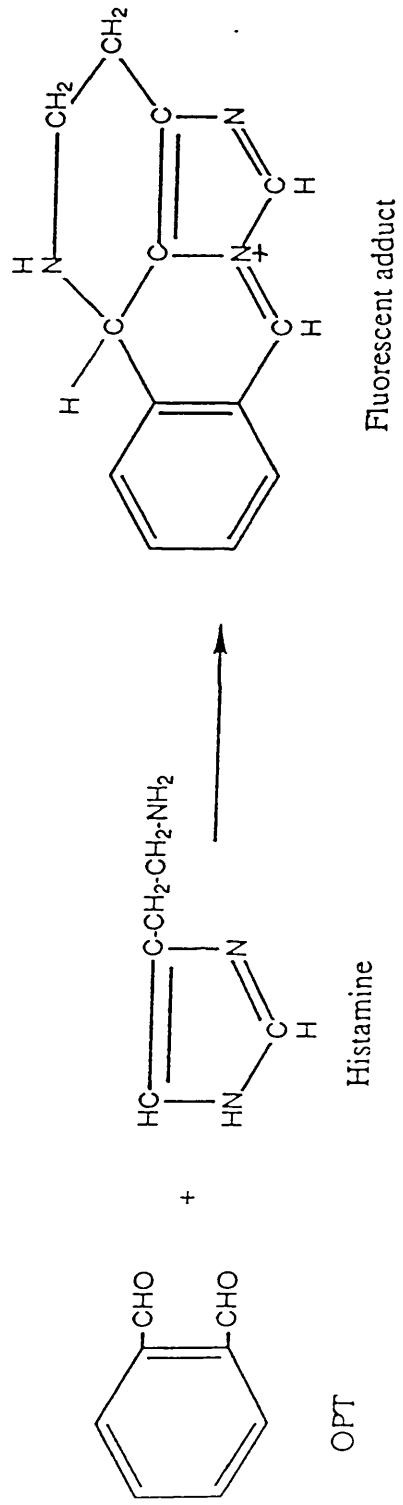


Fig.2.4 Reaction of histamine with OPT

CHAPTER THREE

THE EFFECT OF PAF ON RAT PERITONEAL MAST CELLS

3.1 INTRODUCTION.

The following chapter investigates the action of exogenous PAF on mast cells isolated from the peritoneal cavity of the rat. Any studies looking at the effects of a particular drug on mast cells are usually carried out firstly on rat peritoneal mast cells before moving on to tissue histaminocytes, as they are easily acquired and do not involve long dispersion procedures.

3.2 METHODS AND MATERIALS.

All methods described in this section are clearly outlined in chapter two.

3.3 RESULTS.

3.3.1 CHARACTERISTICS OF THE EFFECTS OF PAF ON HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS.

3.3.1.1 EFFECTS OF DIFFERENT MOLECULAR WEIGHTS OF PAF.

PAF (C16) produced a dose-dependent release of histamine from rat peritoneal mast cells (RPMC) within the concentration range of 1- 100 μM (Fig.3.1). PAF C17 and C18 (1- 100 μM) also produced similar dose-related releases of histamine from RPMC (Fig.3.2). Optimal release of histamine was seen to occur in both studies at PAF concentrations of 100 μM . Similarly, negligible release of histamine was observed at a concentration of 1 μM PAF in both studies. Throughout the

remainder of this study PAF (C16) was exclusively used, unless otherwise stated.

3.3.1.2 EFFECTS OF EDTA AND CMF BUFFERS.

Brief pretreatment of RPMC in EDTA and CMF buffers did not affect the overall release of histamine induced by PAF (C16, 1- 100 μM), as compared to the release observed in FHB (Fig.3.3). Contrary to Fig.3.3, histamine release induced by compound 48/80 (0.025- 1 $\mu\text{g/ml}$) from RPMC following the above procedure was significantly depressed in the absence of added calcium as compared to the release observed in FHB (Fig.3.4).

3.3.1.3 EFFECT OF METABOLIC INHIBITORS.

In the presence of the metabolic inhibitor antimycin A (AA) together with glucose (2x G) and 2-deoxyglucose (2x DG), histamine release evoked by 5 μM PAF was seen to be almost abolished. The same pattern of histamine release was observed with compound 48/80 (1 $\mu\text{g/ml}$). However, histamine release induced by 10 μM PAF was seen to be unaffected by the omission of glucose or addition of 2-deoxyglucose and antimycin A (Fig.3.5).

3.3.1.4 EFFECT OF TEMPERATURE ON THE RELEASE PROCESS.

Histamine release induced by 5 μM PAF from RPMC was seen to be optimal at 37 °C (physiological temperature) but depressed at 0, 20 and 45 °C. The same pattern of histamine release was also observed with various other secretagogues (Fig.3.6).

3.3.1.5 KINETICS OF THE RELEASE PROCESS.

Histamine release induced by 5 μM PAF from RPMC was seen to reach a maximum value within 10 min, after which no increase in histamine release was found to occur (Fig.3.7). In contrast, maximum histamine release mediated by 10 μM PAF was seen to be complete after 40 min.

3.3.1.6 VARIATION OF pH ON THE RELEASE PROCESS.

Histamine releases from RPMC stimulated by 5 and 10 μM PAF were found to be largely unaffected by changes in the pH ranging from 6.0 to 8.0 of the external media. In contrast, histamine release evoked by anti-IgE (dilution 1/300) and compound 48/80 (1 $\mu\text{g}/\text{ml}$) were seen to be optimal at the physiological pH 7.0 but depressed at the extremes of pH (Fig.3.8).

3.3.2 PROSTAGLANDIN D₂ (PGD₂) RELEASE.

A PGD₂ standard curve was initially constructed (Fig.3.9). This curve was used to determine the amount of PGD₂ produced by purified RPMC in each sample. The PGD₂ content of each sample was then converted into ng/10⁶ mast cells.

PAF (0.1-100 μM) generated a dose-dependent release of PGD₂ from purified RPMC (Fig.3.10). Similarly, compound 48/80 (0.05- 1 $\mu\text{g}/\text{ml}$) also induced a dose-related release of PGD₂ from the same cell type (Fig.3.11). Histamine releases induced by both secretagogues were also studied from the same cell populations (Table 3.1).

3.3.3 SYNERGISTIC EFFECTS OF PAF WITH OTHER SECRETAGOGUES.

PAF (1 μM) which causes negligible histamine release from RPMC was seen to potentiate anti-IgE and concanavalin A (Con A) mediated histamine release (Fig.3.12 and 3.13), but not that induced by compound 48/80 (Fig.3.14).

3.4 DISCUSSION.

As mentioned earlier, PAF is released upon stimulation of a variety of inflammatory cells including rabbit and cultured bone marrow-derived mouse mast cells [318,319], but not by human lung or rat mast cells, although they synthesise the molecule following stimulation. As the increasing importance of PAF in asthma and allergy

has become apparent, it is therefore vital to establish its effect on mast cells.

Initial studies on any new compound thought to play positive or preventative roles in the aetiology of a particular disease are usually carried out on animal models before moving onto human volunteers or isolated human tissues.

The present study indicates that PAF (C16) induces histamine release from RPMC in a dose-related manner, with a maximum release of approx. 90%. A comparative study involving PAF with an increasing number of carbon atoms attached to the alkyl chain, produced similar results. The number of carbon atoms on the alkyl chain of the native PAF molecule ranges between 16 and 18, but PAF (C16) predominates in humans. Thus, throughout this study PAF (C16) has been exclusively used, unless otherwise stated.

It has been firmly established that the presence of extracellular calcium ions are normally essential for maximum, non-cytotoxic histamine release following stimulation from RPMC [95]. Following such stimulation of RPMC by compound 48/80, it was observed that histamine release in the absence of extracellular calcium ions was substantially diminished compared to that which occurred in the presence of the ion. In contrast, the absence of extracellular calcium ions did not greatly affect histamine release evoked by increasing concentrations of PAF. Preincubation of RPMC in the presence of EDTA buffer (no external calcium ions) initiates the mobilization and release of internal stores of calcium ions, thus allowing some histamine release to occur. Regarding PAF-induced histamine release from RPMC, in the presence of EDTA, an enhanced histamine release of $18 \pm 4\%$ was seen to occur at a concentration of $1 \mu\text{M}$ PAF, whereas histamine release at this concentration in FHB did not exceed $3 \pm 1\%$. Moreover, the same pattern of an enhanced release of histamine from RPMC at low concentrations of compound 48/80 was also observed.

Antimycin A, a potent inhibitor of oxidative phosphorylation, may be employed as a tool to determine whether mediator release induced by a specific secretagogue occurs either by disrupting the entire cell membrane (cytotoxic mechanism) or by

a specific membrane or ligand-receptor interaction (non-cytotoxic mechanism). PAF (5 μ M) induced histamine release was seen to be almost abolished in the presence of 2-deoxyglucose and antimycin A. This outcome thus indicates a non-cytotoxic mechanism of histamine release. Similar results were obtained with compound 48/80 (1 μ g/ml). However, histamine secretion evoked by PAF (10 μ M) remained constant in the different buffers tested, oblivious to the presence of antimycin A. Thus, one can suggest that histamine release from RPMC at this particular concentration of PAF and above occurs via a cytotoxic mechanism.

PAF mediated histamine release from RPMC was found to be depressed at extremes of temperature. Similar results were observed for secretion evoked by anti-IgE and compound 48/80. These results suggest that histamine release evoked by both 5 and 10 μ M PAF may occur via an enzymatic pathway. Recent studies have shown that PAF in other systems can initiate the metabolism of polyphosphoinositides giving rise to DAG and IP₃, which in turn regulate cellular functions [320]. PI breakdown involves specific enzymes such as phospholipase C. As this mechanism is dependent upon enzymatic activity, extremes of temperature will impair their function, thus affecting the overall PI metabolism and cellular functions.

Histamine release induced by both 5 and 10 μ M PAF from RPMC was observed to occur in a manner not dependent on the pH of the external medium. In contrast, release evoked by anti-IgE and compound 48/80 was found to be strongly dependent on the pH of the incubation medium. In both of the latter cases the response was optimal at the physiological pH (7.0) but depressed at both extremes.

Further studies on the effect of PAF on RPMC involved investigating whether PAF stimulated other biochemical pathways, such as the metabolism of AA. Recent studies by Levine have indicated that PAF stimulates AA metabolism in rat liver cells [321], hence this study was extended to mast cells. PAF (0.1-100 μ M) produced dose-related releases of newly synthesised PGD₂ and histamine, with maximum values of 15.3 ± 5 ng/10⁶ cells and $87.1 \pm 3.6\%$ respectively from purified RPMC. Similar results were obtained with compound 48/80. PGD₂ is the major metabolite of AA metabolism via the cyclooxygenase pathway in RPMC as

LT's are generated in comparatively small amounts ($<1 \text{ ng}/10^6 \text{ cells}$).

The present study has already pointed out that histamine release from RPMC induced by PAF ($10 \text{ } \mu\text{M}$ and above) occurs in a cytotoxic manner. However, it is assumed that within this species the physiological levels of PAF do not exceed $1 \text{ } \mu\text{M}$ except possibly in pathological conditions. Thus, certain questions arose as to whether low concentrations of PAF, which themselves cause little or no histamine release, might interact with classical antigen-antibody interactions, leading to an increase in histamine release from RPMC. Taking the above into consideration, it was experimentally observed that PAF ($1 \text{ } \mu\text{M}$) significantly potentiated anti-IgE mediated histamine release from RPMC. Histamine release evoked by anti-IgE was unaffected by 0.01 and $0.1 \text{ } \mu\text{M}$ PAF. Similar results were obtained using the plant lectin Con A, which also causes histamine release from RPMC by binding to the carbohydrate moieties within the IgE antibody molecules [322,323]. Thus, the above findings suggest that low concentrations of PAF may interact with the IgE receptor system resulting in potentiation of histamine release induced by agents which cross-link adjacent IgE antibodies.

Histamine release induced by compound 48/80 from RPMC seemed to be unaffected by the presence of PAF. Compound 48/80 is a non-cytotoxic liberator of certain mediators including histamine from mast cells. It is thought to act via ill-defined receptors on the mast cell membrane.

The above results may indicate a possible mode of action of PAF on RPMC. One can suggest that low concentrations of PAF, ie. those which cause little or non-cytotoxic histamine release ($< 5 \text{ } \mu\text{M}$), may do so by somehow interacting with the IgE receptor or the IgE antibody molecules already bound to its receptor or by interaction with the cell membrane. Moreover, at higher concentrations of PAF ($> 10 \text{ } \mu\text{M}$) cytotoxic release of histamine occurs, probably due to disruption of the mast cell membrane. These studies have yet to be fully expanded.

In summary, exogenous PAF was found to be a potent inducer of histamine release from RPMC. The release of histamine from RPMC was found to be calcium and

pH independent, cytotoxic at concentrations above 10 μM , and maximal at the physiological temperature. The non-cytotoxic release process evoked by 5 μM PAF was seen to be essentially complete within 10 min. PAF induced a dose-related release of PGD_2 from purified RPMC. Finally, PAF (1 μM) potentiated both anti-IgE and Con A induced histamine release but proved ineffective towards compound 48/80 induced histamine release from RPMC.

Following the initial work on RPMC, the project was extended to investigate the effect of PAF on isolated tissue mast cells from different locations within a variety of species.

Fig.3.1 The effect of PAF (C16) on histamine release from rat peritoneal mast cells. n=4

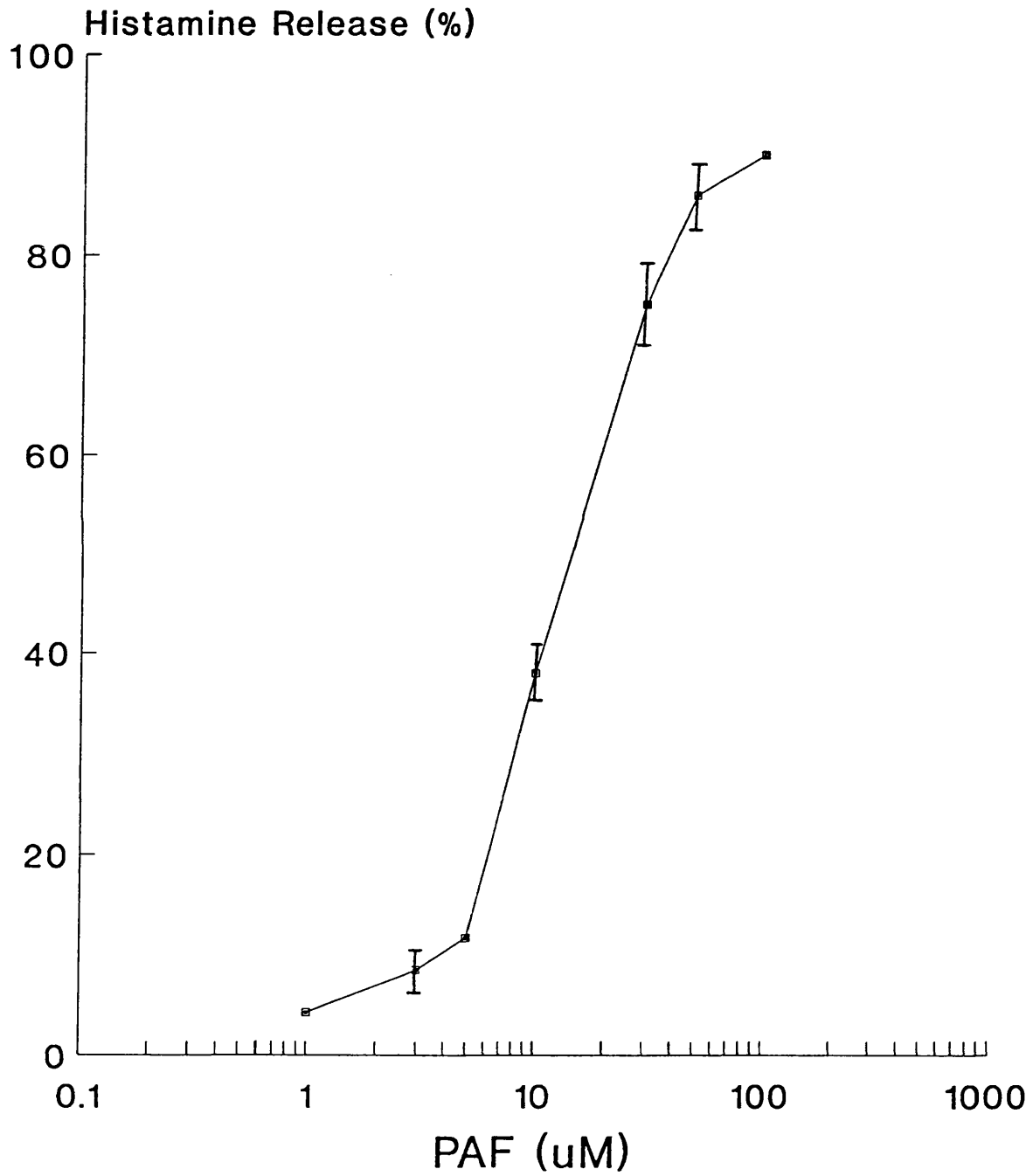


Fig.3.2 The effect of PAF C16, C17 and C18 on histamine release from rat peritoneal mast cells. n=4

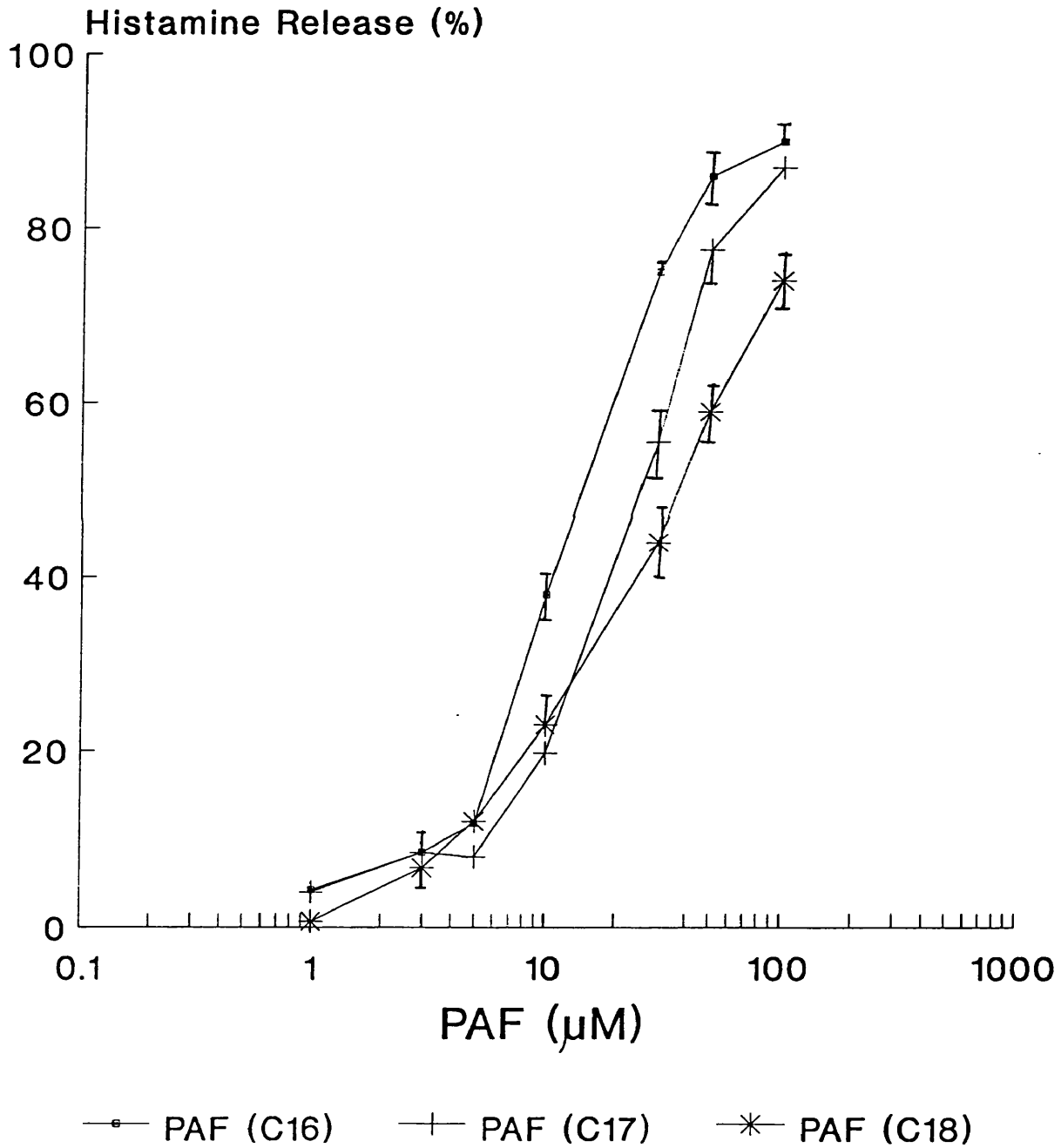


Fig.3.3 The effect of different media on histamine release from rat peritoneal mast cells induced by PAF. n=4

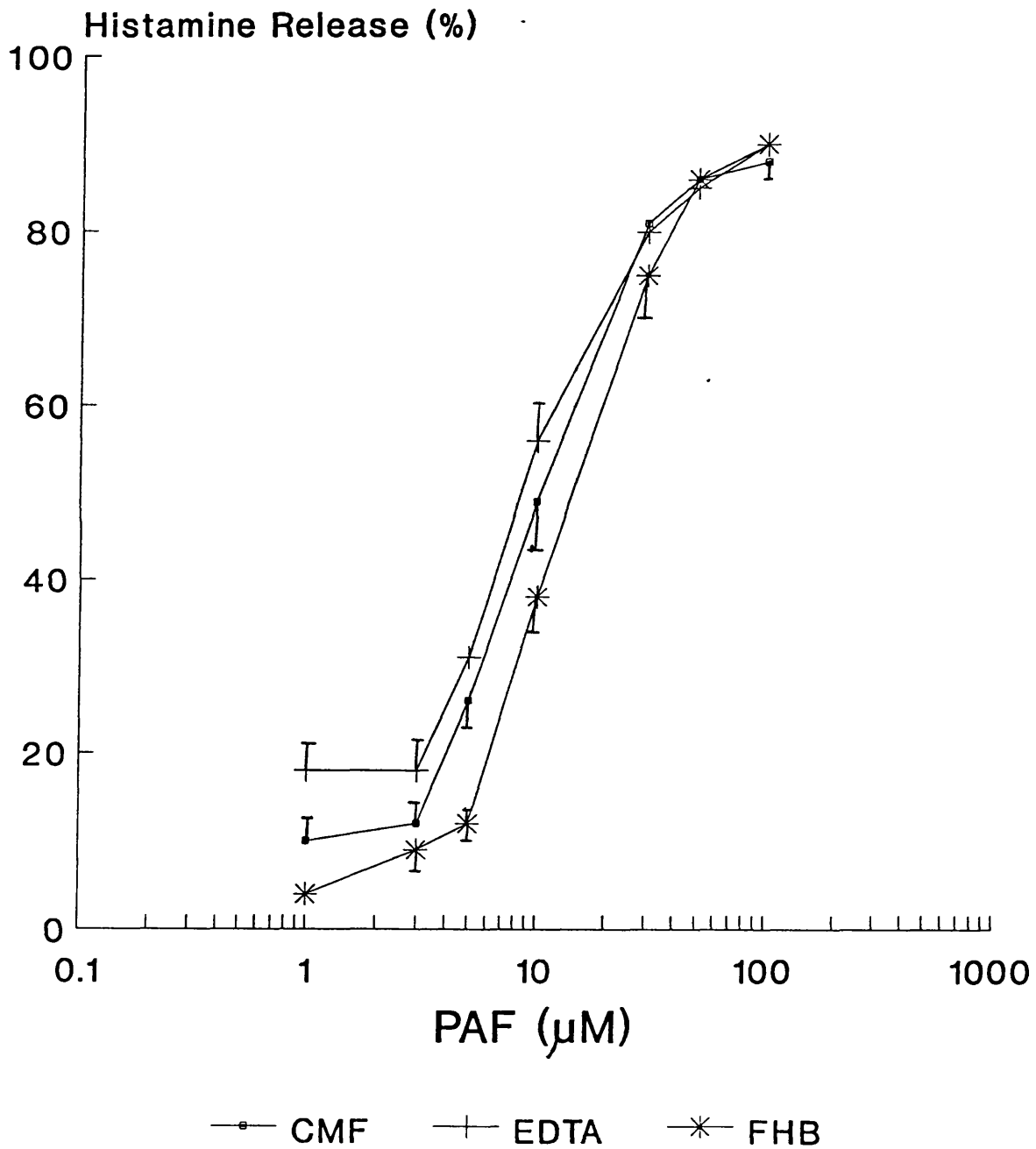


Fig.3.4 The effect of different media on histamine release from rat peritoneal mast cells stimulated with compound 48/80. n=4

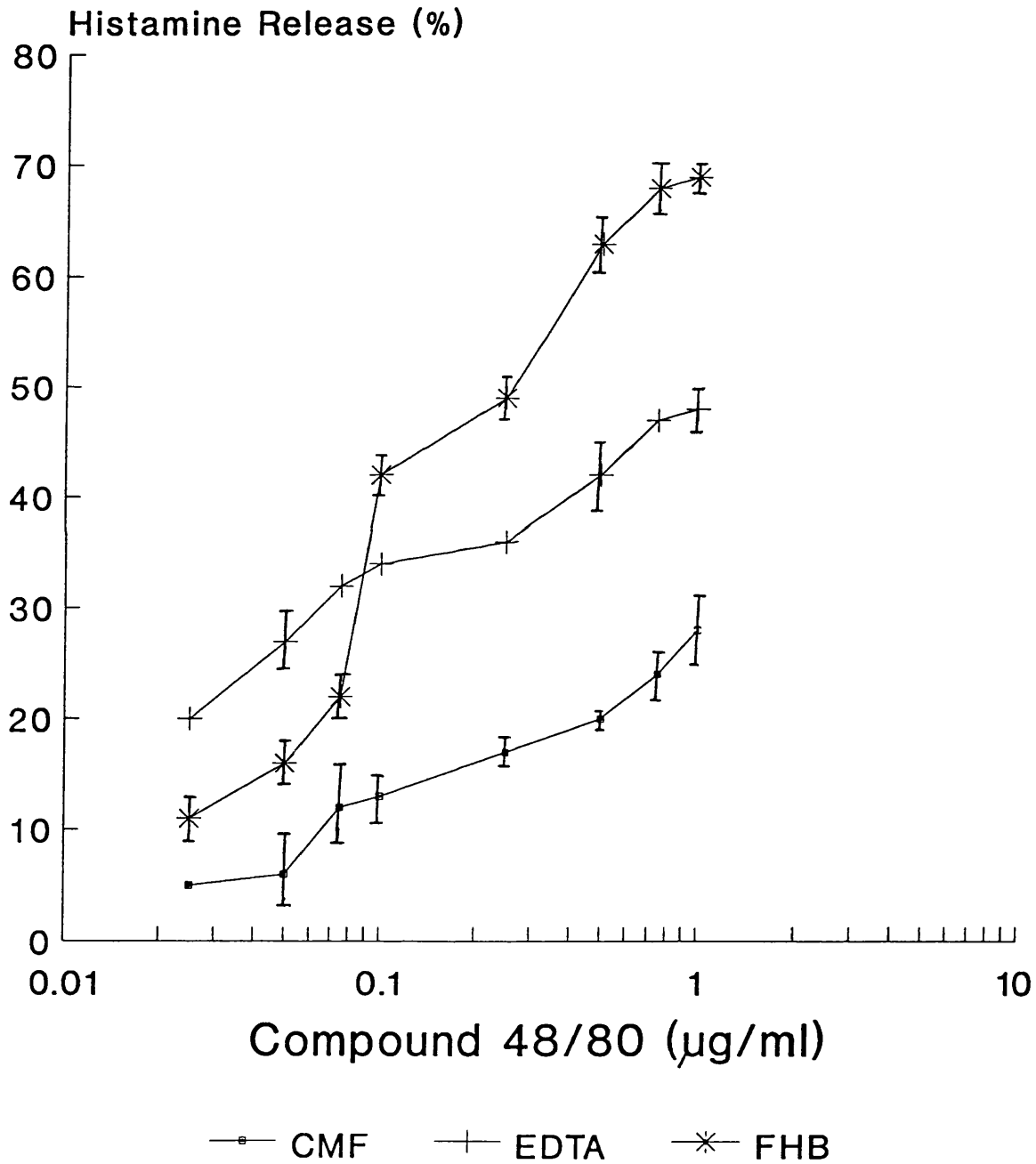
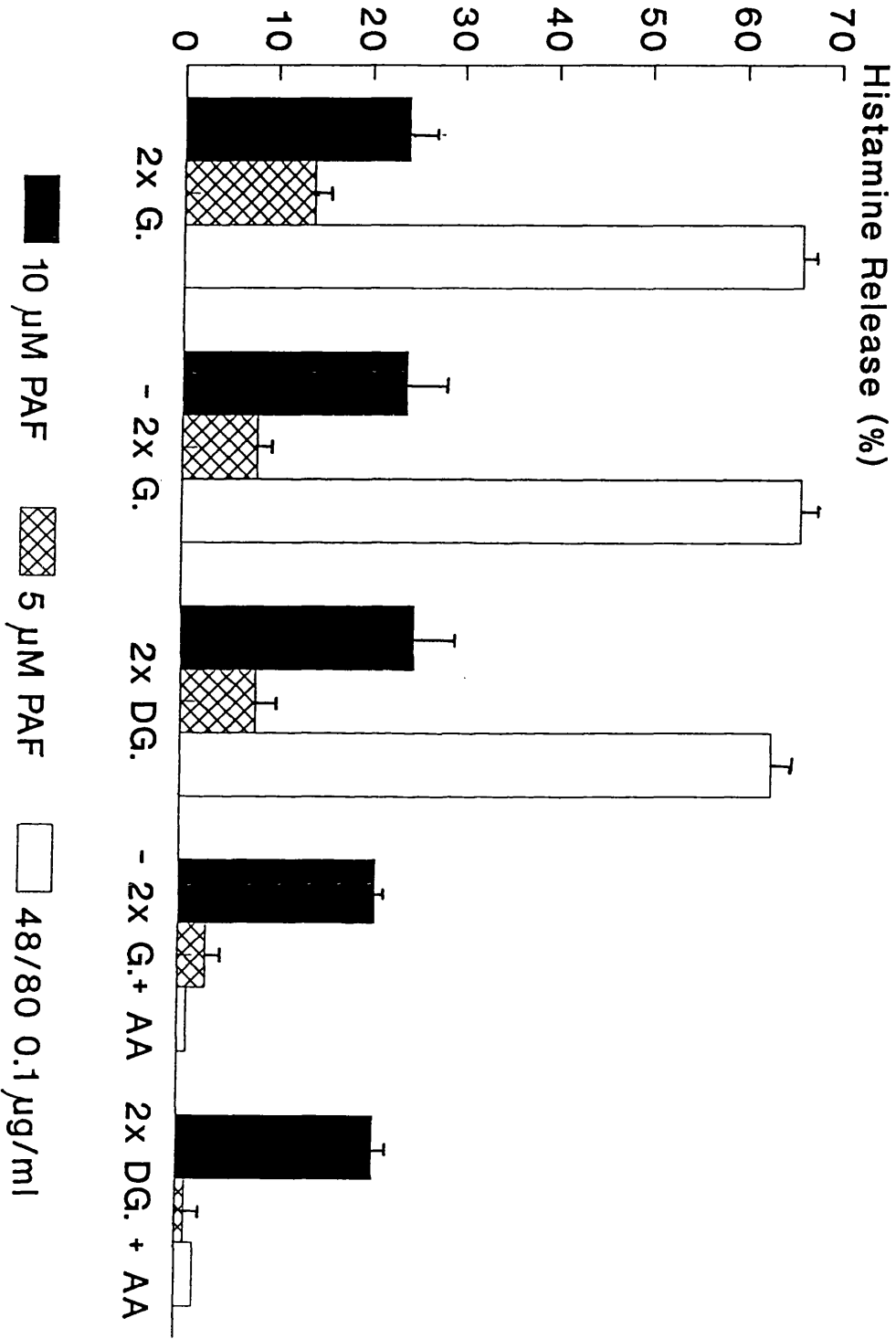


Fig. 3.5 The effect of metabolic inhibitors on histamine release from rat peritoneal mast cells induced by PAF (5 and 10 μ M) and compound 48/80 (1 μ g/ml). n=4



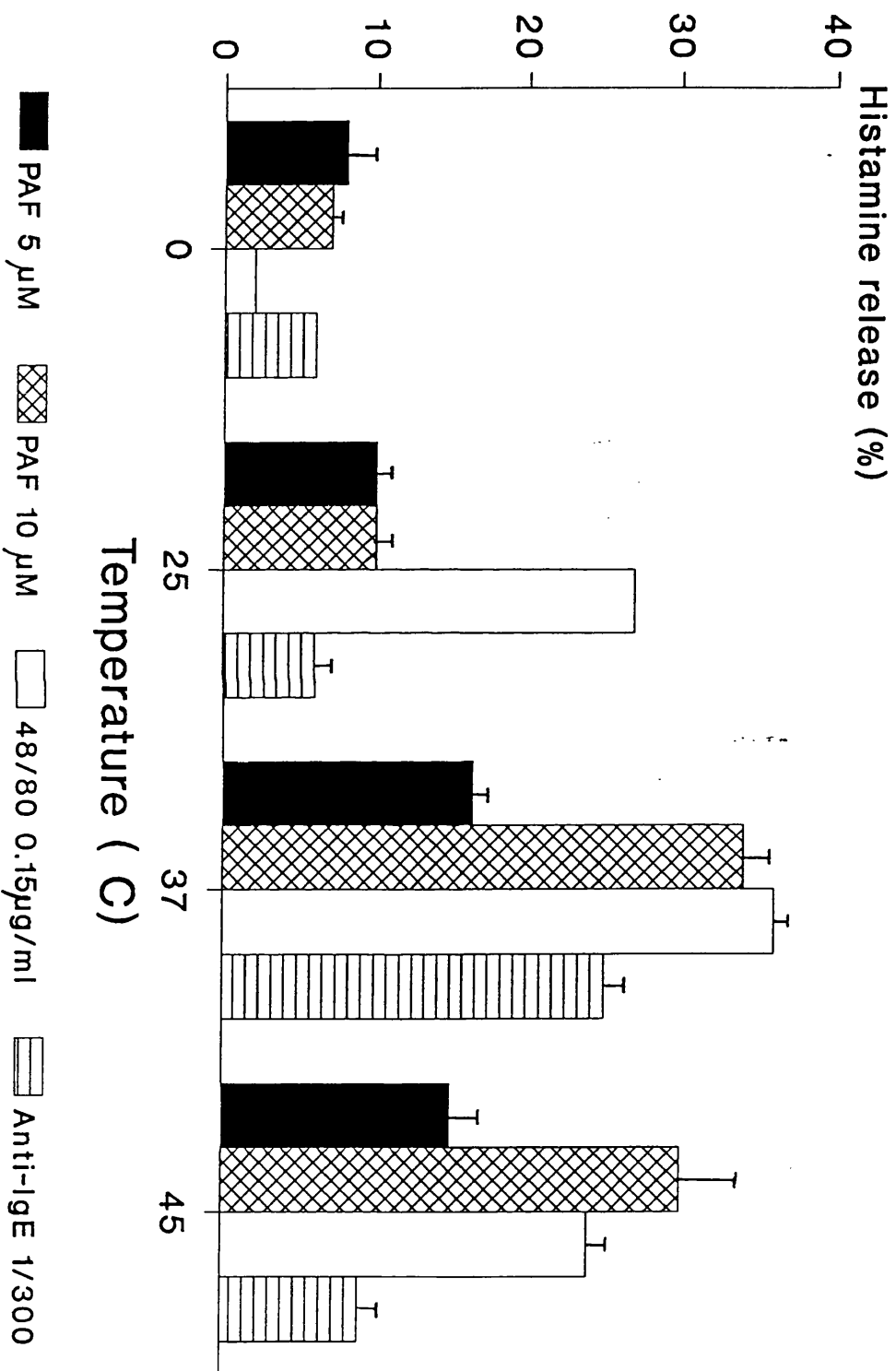


Fig.3.6 The effect of temperature on histamine release from rat peritoneal mast cells induced by PAF (5 and 10 μ M), compound 48/80 (0.15 μ g/ml) and anti-IgE (dilution 1/300). n=4

Fig.3.7 Kinetics of histamine release induced by PAF (5 and 10 μ M) from rat peritoneal mast cells. n=4

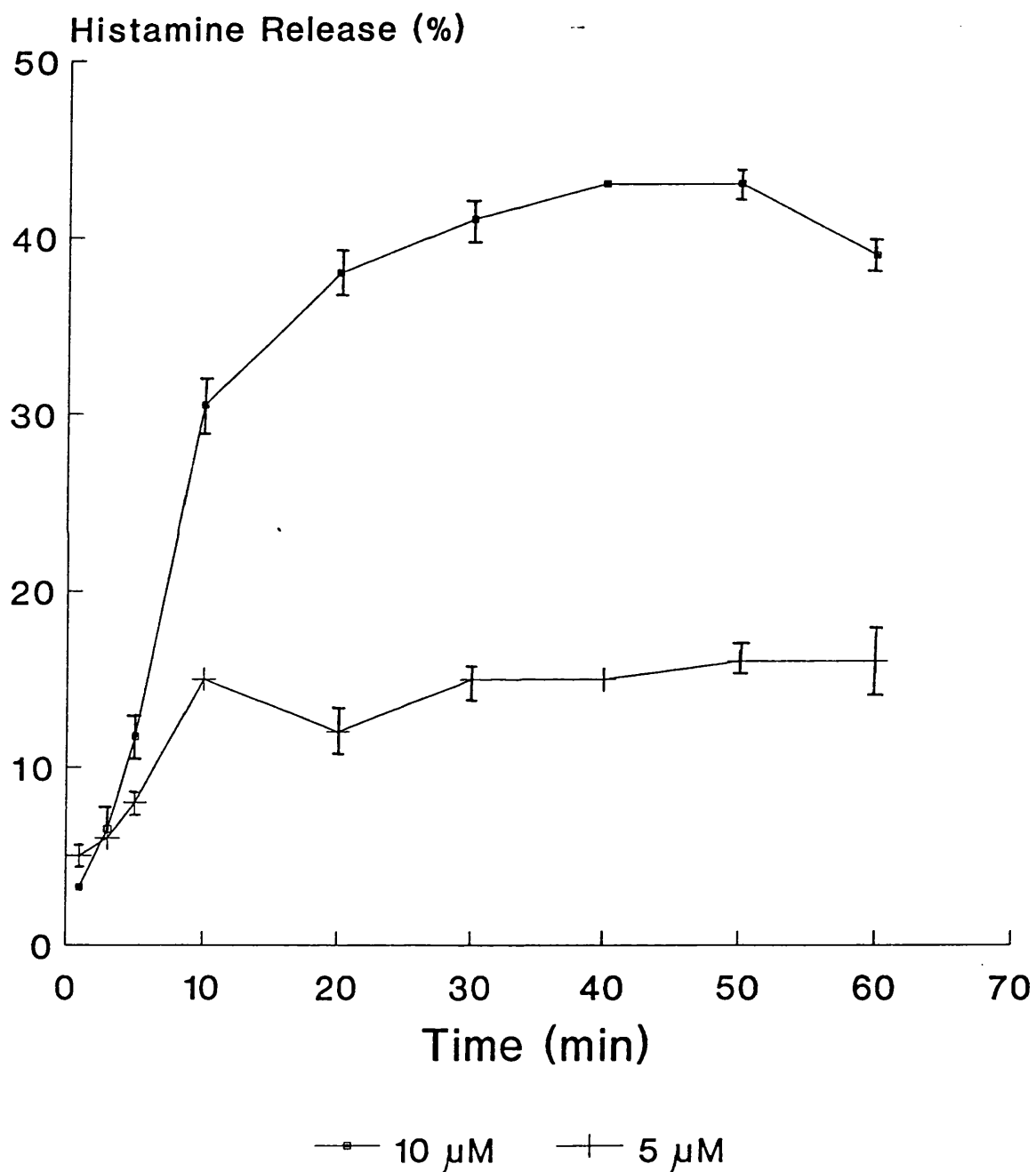


Fig.3.8 Effect of pH on histamine release from rat peritoneal mast cells induced by PAF (5 and 10 μ M), anti-IgE (dilution 1/300) and compound 48/80 (1 μ g/ml). n=4

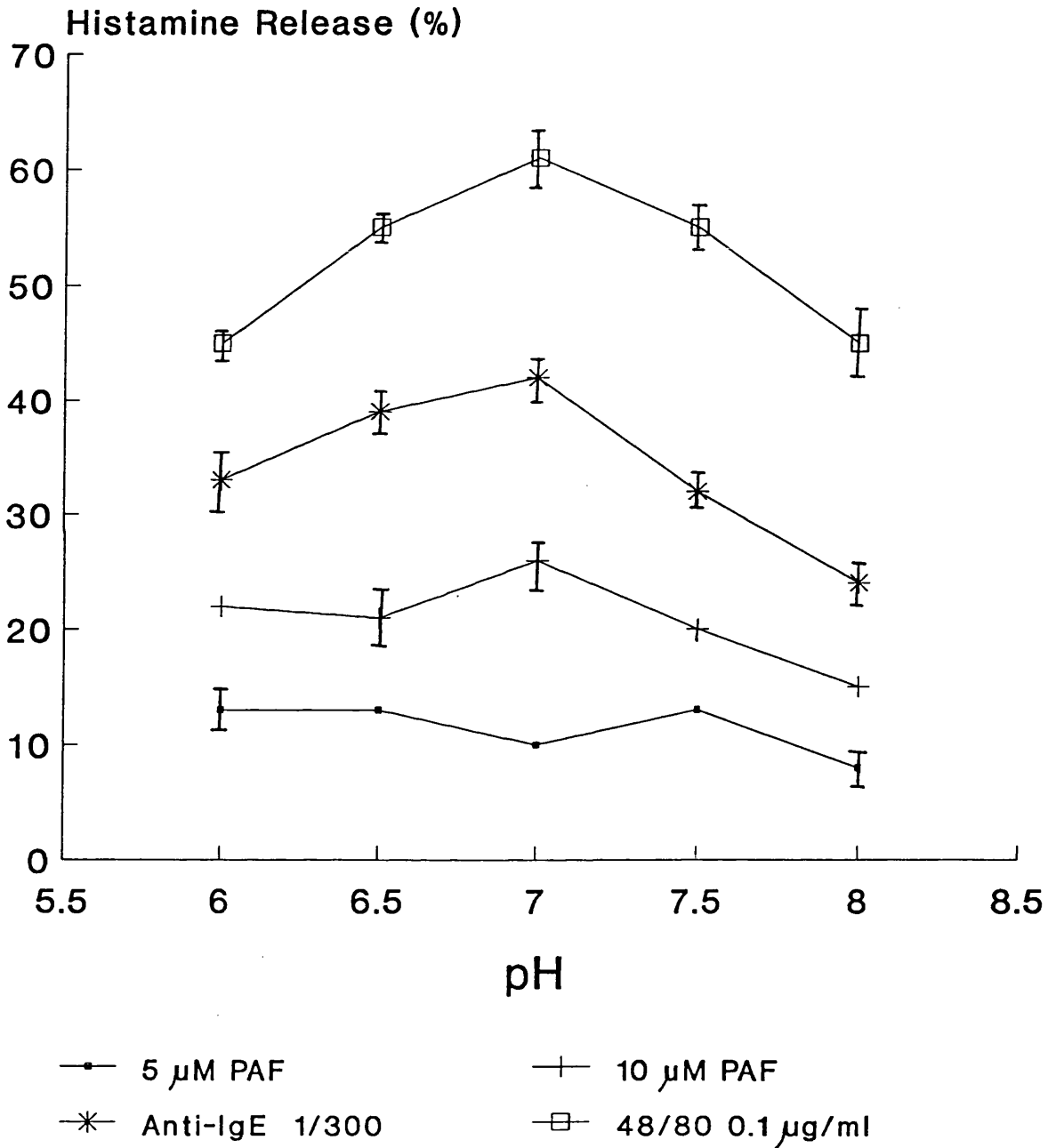


Fig.3.9

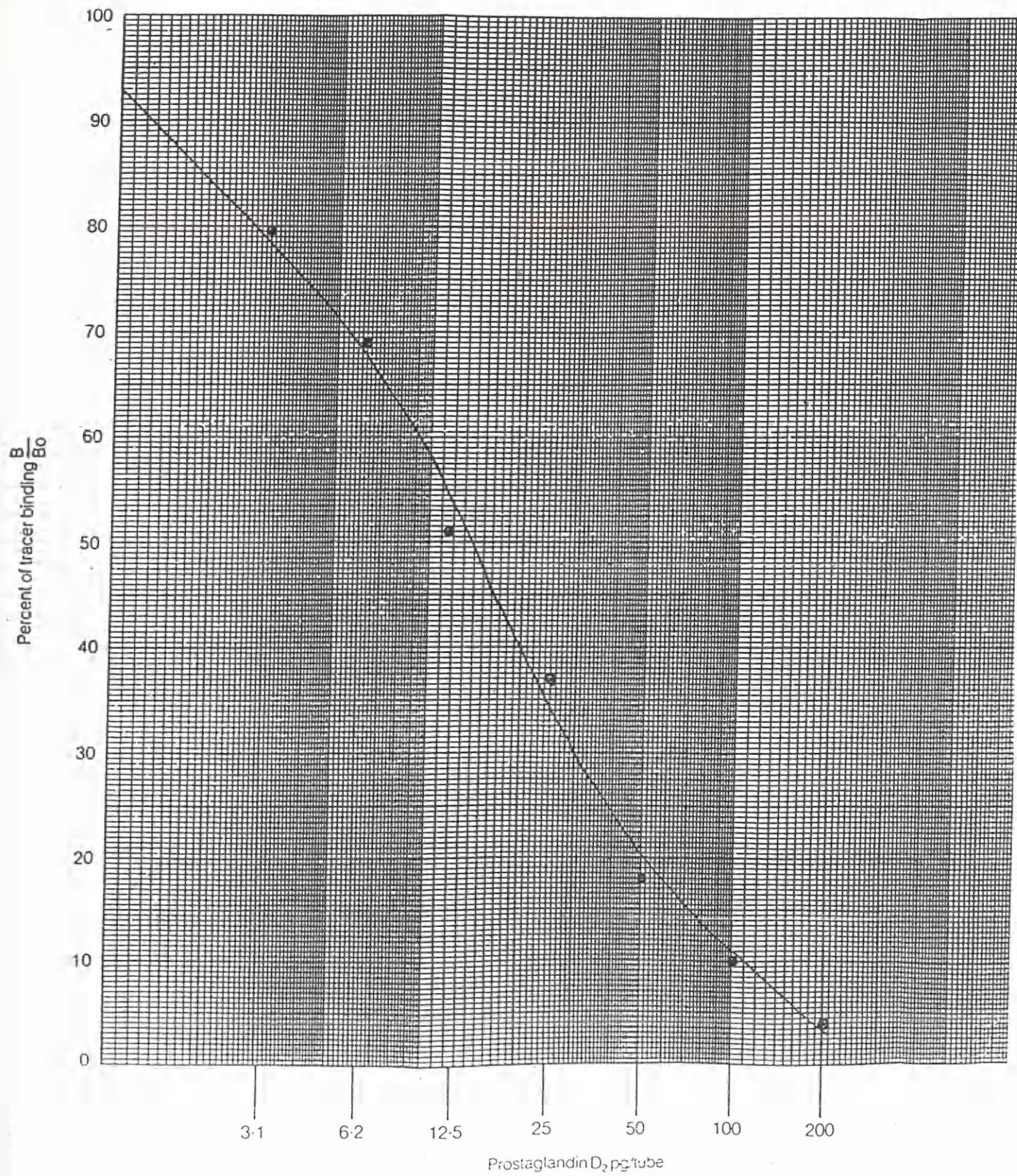
Prostaglandin D₂ standard curve

Fig.3.10 Effect of PAF on PGD₂ release from purified rat peritoneal mast cells. n=3

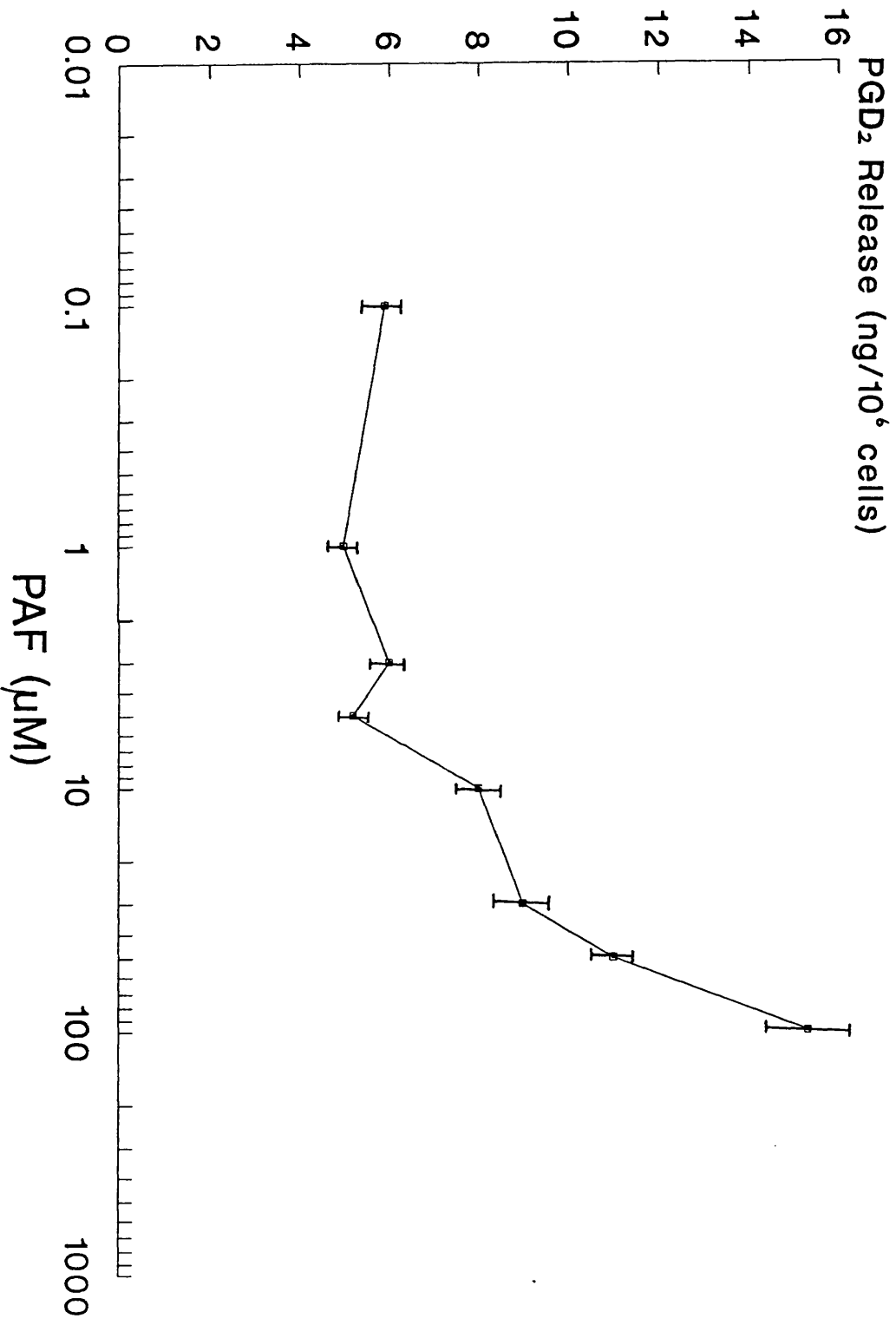


Fig.3.11 Effect of compound 48/80 on PGD₂ release from purified rat peritoneal mast cells. n=3

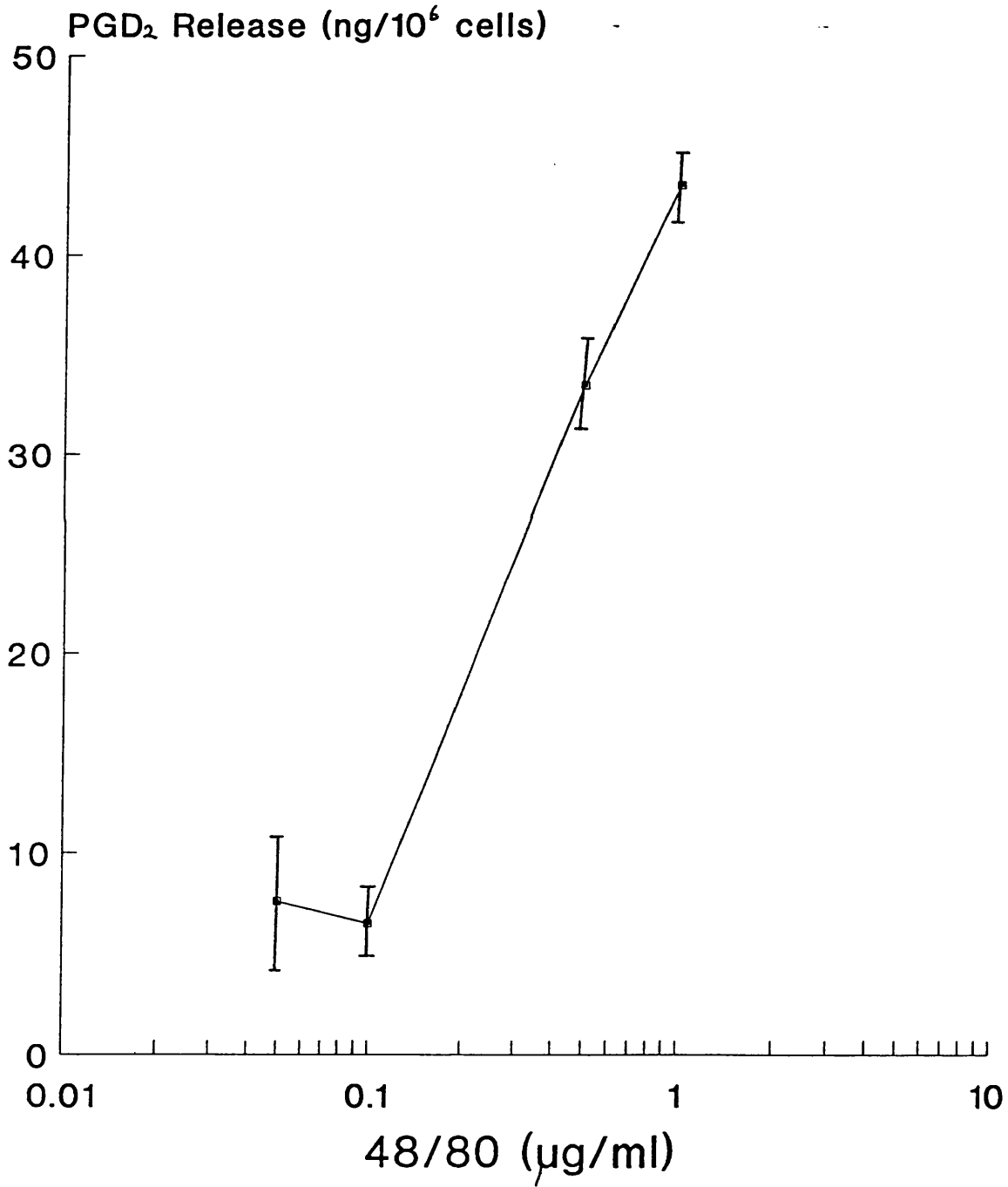
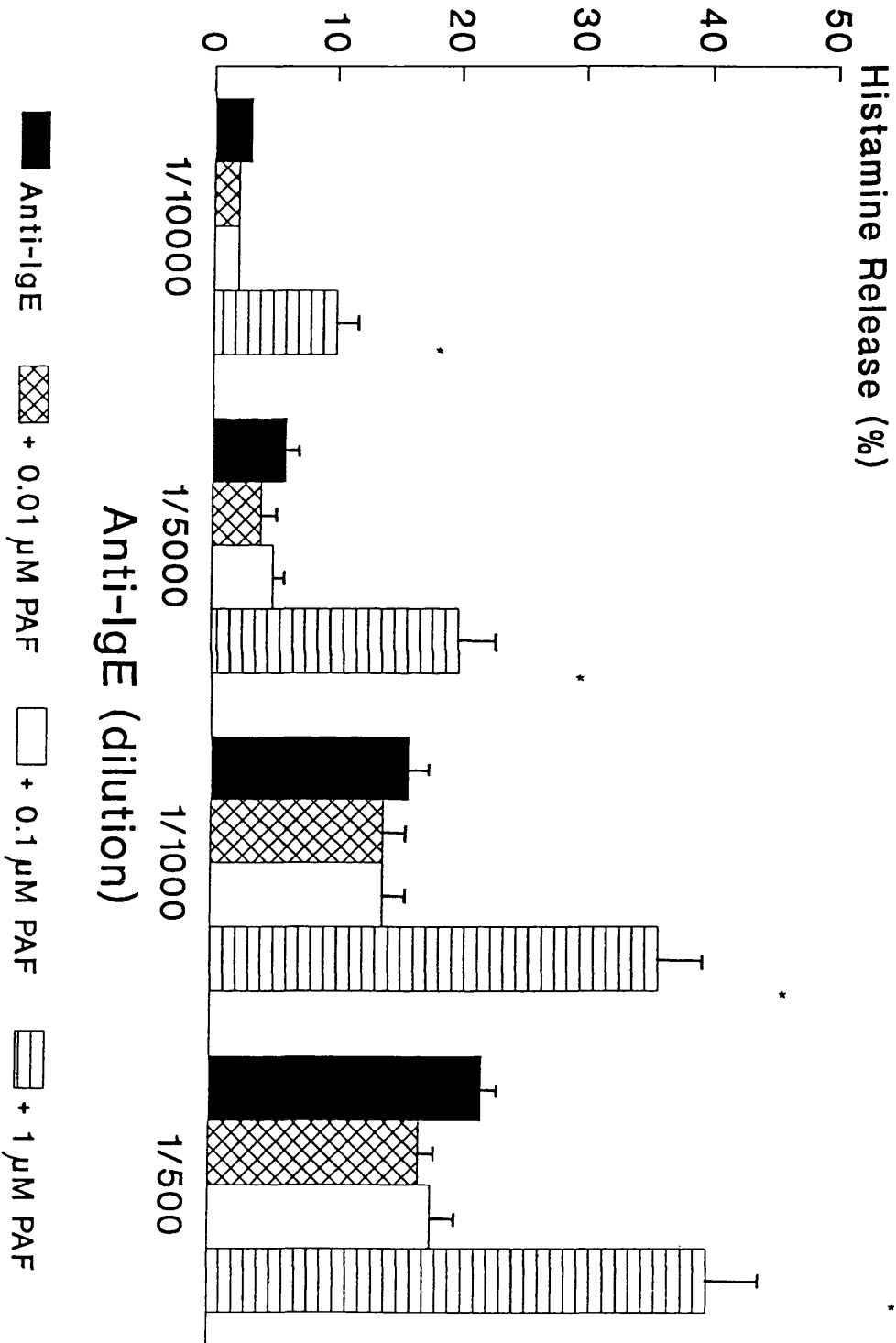
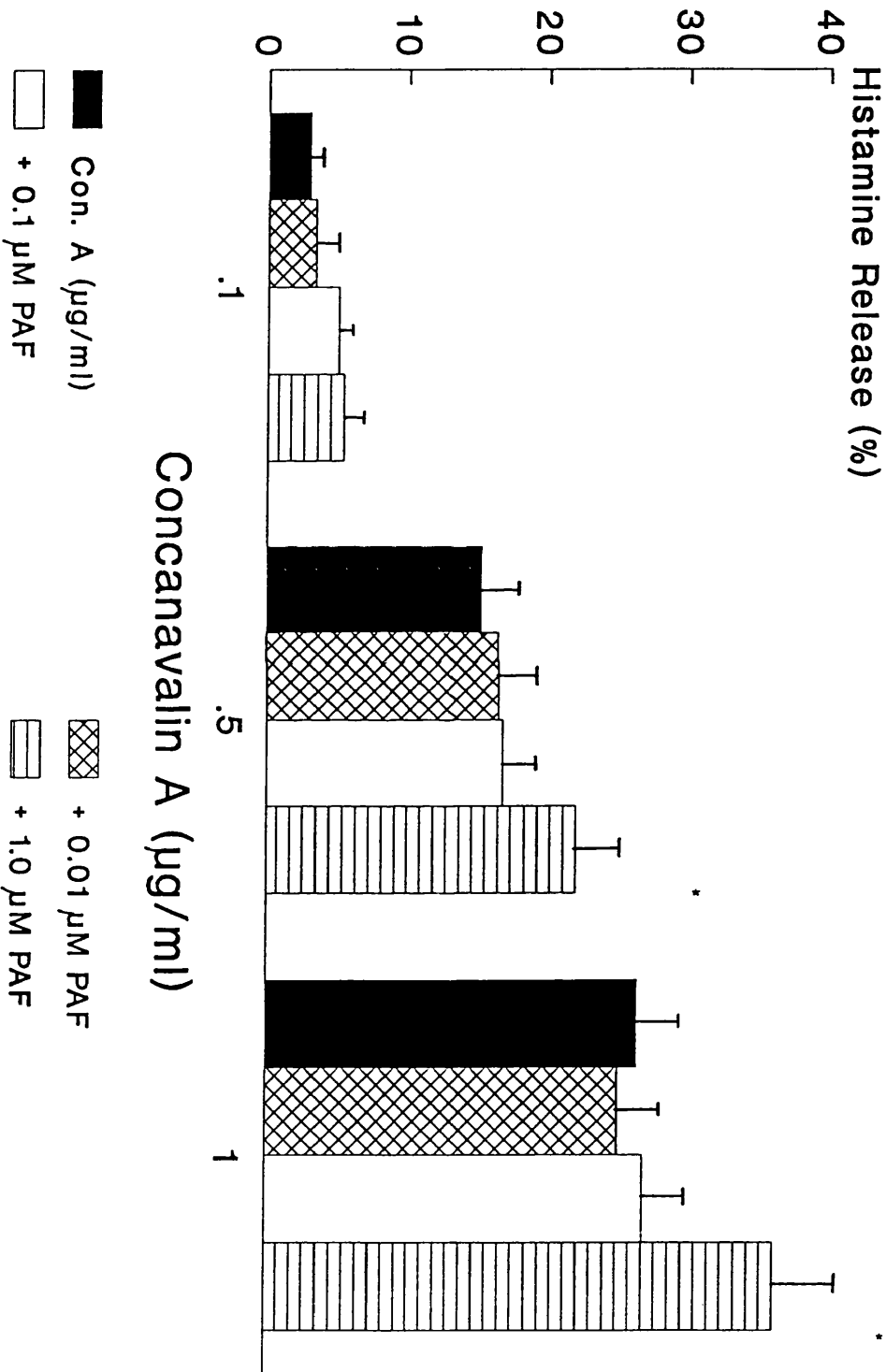


Fig.3.12 Effect of PAF on anti-IgE mediated histamine release from rat peritoneal mast cells. n=4



*P<0.05 as compared to control release.

Fig.3.13 Effect of PAF on concanavalin A mediated histamine release from rat peritoneal mast cells. n=4



* P<0.05 as compared to control release.

Fig.3.14 Effect of PAF on compound 48/80 mediated histamine release from rat peritoneal mast cells. n=4

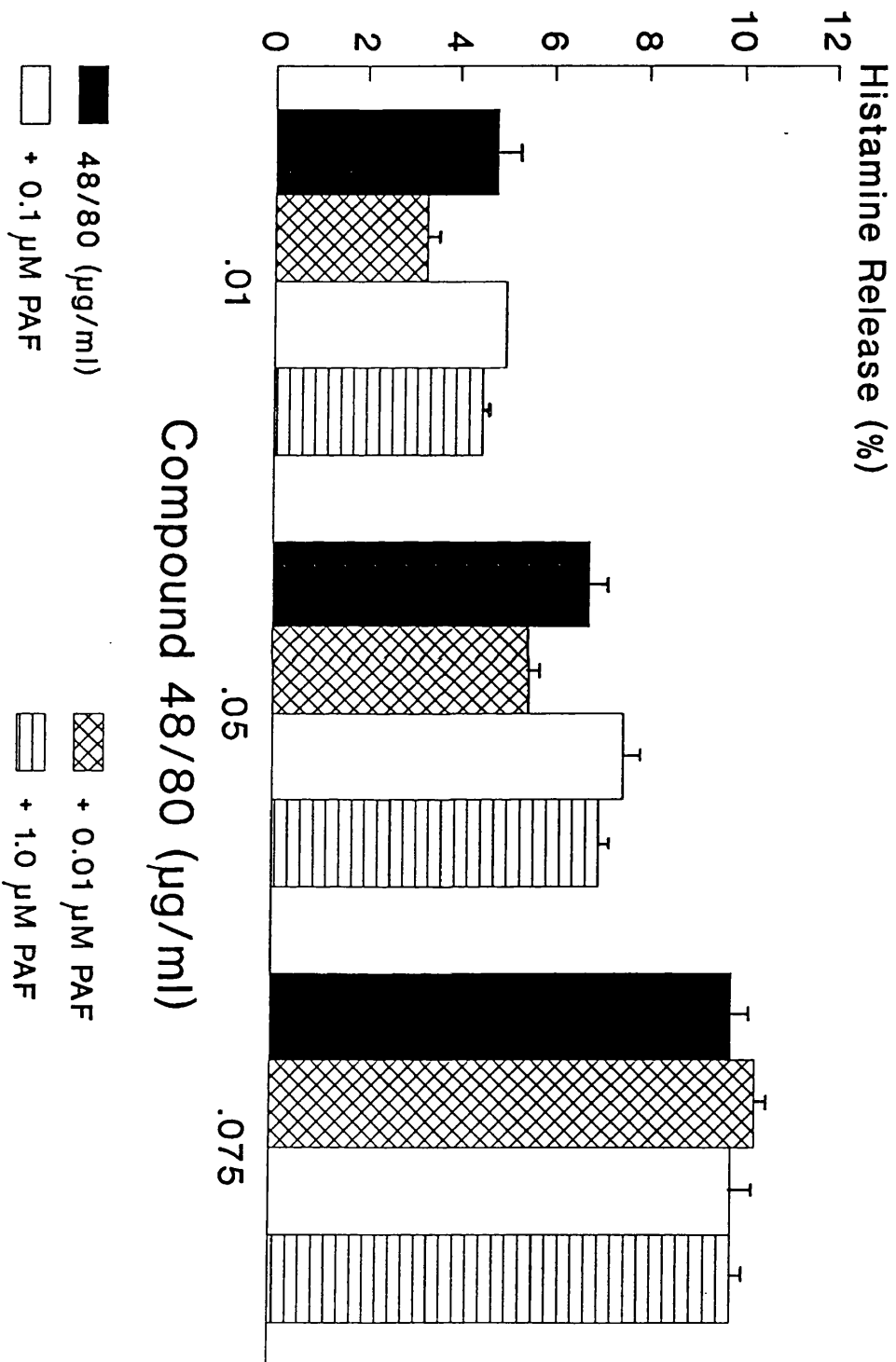


Table 3.1 Effect of PAF (C16) and compound 48/80 on histamine release from purified rat peritoneal mast cells. n=3

PAF (C16, μ M)	Histamine Release (%)
0.1	3.0 \pm 1.7
1.0	9.3 \pm 3.6
3.0	35.4 \pm 3.2
5.0	55.1 \pm 3.5
10	67.4 \pm 3.3
30	83.3 \pm 5.3
50	86.2 \pm 3.1
100	87.1 \pm 3.6

Compound 48/80 (μ g/ml)	Histamine Release (%)
0.05	12.3 \pm 1.7
0.1	42.1 \pm 3.5
0.5	61.0 \pm 1.7
1.0	71.1 \pm 2.3

Values are means \pm SEM for 3 experiments.

CHAPTER FOUR

THE EFFECT OF PAF ON A VARIETY OF HISTAMINOCYTES ISOLATED FROM DIFFERENT SPECIES.

4.1 INTRODUCTION.

A great deal of research spanning over fifty years has increased our knowledge and understanding of the manifestations involved in allergy and inflammation. Allergy, implies an autotoxic, frequently spreading reaction to a normally benign substance (antigen). In order that the acute allergic response occurs, two antigenic exposures are required, the first of which confers susceptibility rather than protection and the second of which brings about the allergic response. In contrast, an inflammatory response tends to consist of firstly localizing and then neutralizing the noxious antigen [324]. However, what is of interest to scientists is the fact that both reactions involve many of the same cell types, mediators and mechanisms.

The causes of inflammation are diverse and include, in addition to immunological reactions such as allergy, infectious, physical and chemical responses. Inflammation is manifested by the release of chemicals from cells within tissues eg. mast cells and migratory cells eg. eosinophils. Inflammatory mediators maybe distinguished from hormones and neurotransmitters by the term 'autacoids' introduced in 1980 by Douglas [325]. They include histamine, 5-HT, PGE₂, PGD₂, LTB₄, LTD₄, PAF and interleukin-1 (IL-1). All the above mediators originate from cells which include mast cells, basophils, neutrophils, eosinophils, macrophages and endothelial cells. Some of the afore mentioned mediators are preformed while others are synthesised *de novo* within the cells, following stimulation [326].

The outcome of allergic inflammation in a particular tissue depends upon both the

primary (resident) and secondary (migratory) cells involved in the reaction. Hence, the cellular structure of the tissue and the mediators released determines the overall response.

As mentioned previously in chapter one, PAF is released from proinflammatory cells and induces inflammatory responses in the tissues of a variety of animal species and humans, besides being also able to mimic the main clinical features of asthma [327].

Intradermal injections of PAF into guinea-pig skin induce a rapid increase in plasma protein extravasation, platelet accumulation and an increase in the number of inflammatory cells such as neutrophils. In the guinea-pig, PAF is exceedingly more potent than histamine in inducing plasma protein extravasation [328]. Recent studies by Calignano and co-workers have demonstrated increased levels of PAF in those areas of guinea-pig skin exposed (3 min) to ultra violet (UV) light [329]. Thus, one can propose that UV irradiation stimulates the *de novo* generation of PAF. Moreover, these results may be extended to include human subjects, as the dangers of over exposure to the UV rays emitted by the sun are becoming evermore apparent. It may be suggested that excessive sunbathing may induce PAF generation, thus possibly leading to the development of psoriasis and other skin disorders later on in life. However, this aspect of study has yet to be fully investigated.

In man, an intradermal injection of PAF elicits an inflammatory response reminiscent of that induced by antigen in a suitably sensitised individual. The response consists of acute oedema formation visible by the presence of a wheal and flare on the skin surface near the point of injection. A question arises as to whether exogenous PAF stimulates cutaneous mast cells to produce the inflammatory response or whether it acts directly on the vascular endothelium. Recent studies have shown that in the presence of the H₁-receptor antagonist chlorpheniramine, the acute flare response induced by PAF is reduced, thus indicating the involvement of histamine [310]. More recent studies have show that in the presence of another H₁-receptor antagonist cetirizine, PAF levels found in the wheal and flare response in human skin induced by antigen challenge are lower than

the levels of the phospholipid detected in the absence of cetirizine [330]. It has yet to be established whether stimulated human cutaneous mast cells *in vivo* release PAF along with other well known mediators. Thus, it can be suggested that cetirizine inhibits the formation or release of PAF from either cutaneous mast cells or other inflammatory cells such as eosinophils. Moreover, it can also be proposed that cetirizine displays PAF antagonistic properties, which have yet to be experimentally established. Conversely, it is possible that PAF antagonists may possess H₁-receptor antagonistic properties. This has also yet to be experimentally established. These results point towards a possible interaction between cutaneous mast cells and PAF in eliciting an inflammatory response.

As mentioned previously, PAF is an important mediator in the aetiology of bronchial asthma. However, the exact role of PAF in eliciting the symptoms of asthma remain unclear. PAF has already been identified as a potent bronchoconstrictor and causes contraction of smooth muscle in the parenchyma of guinea-pig lung. The effect of PAF on human lung *in vivo* has yet to be fully investigated. However, *in vitro* experiments on human lung airways have shown that PAF stimulates mucus secretion, which is associated with bronchial asthma [331]. It has also been experimentally proven that bronchoalveolar lavage (BAL) fluid from asthmatic patients contains larger amounts of PAF and its precursor lyso-PAF, compared to BAL from normal subjects. Another finding which indicates the importance of PAF in asthma was reported by Chan-Yeung and co-workers who found that plasma PAF levels increased in asthmatic patients following antigen-induced bronchoconstriction [332]. One of the problems facing scientists involved in studying the role of PAF in bronchial asthma is the lack of a specific or sensitive assay for this particular phospholipid. This is due to the similarity between PAF and lyso-PAF and the fact that PAF is a compound with a variable number of carbon atoms in it, hence it has proved difficult to develop radio-immunoassays to PAF.

Mast cells are present in several tissues and secrete inflammatory mediators following stimulation. This chapter examines the relationship between exogenous PAF and isolated tissue mast cells, as both PAF and mast cell mediators have the ability to play key roles in the pathogenesis of inflammation and asthma.

As mentioned previously in chapter one (section 1.10), mast cells are widely distributed throughout the mammalian body. They exhibit a marked heterogeneity, the causes of which are still not fully understood. During the last twenty years, many research groups have studied and subsequently improved enzymatic dispersion procedures for isolating tissue mast cells from both human and animal specimens.

Chapter four examines the response of different isolated tissue mast cells from a variety of species and human basophils in the presence of exogenously applied PAF.

4.2 METHODS AND MATERIALS.

All experimental procedures employed in the following section are thoroughly described in chapter two.

4.3 RESULTS.

4.3.1 THE ACTION OF PAF ON VARIOUS HISTAMINOCYTES ISOLATED FROM THE RAT AND MOUSE.

Fig.4.1 graphically demonstrates that PAF induced a dose-dependent release of histamine from rat pleural, mesenteric, lung and skin and mouse peritoneal mast cells. It was observed that rat pleural mast cells were the most responsive, followed by mesenteric, lung and finally cutaneous mast cells.

PAF induced a dose-related release of histamine from mouse peritoneal mast cells, comparable to that from rat peritoneal mast cells (Fig.4.1).

4.3.2 EFFECT OF METABOLIC INHIBITORS ON TISSUE MAST CELLS OF THE RAT.

In the presence of the metabolic inhibitors antimycin A and 2-deoxyglucose,

histamine release induced by 5 μ M PAF from rat mesenteric mast cells was reduced when compared to the release of histamine in the absence of the above inhibitors (Fig.4.2). Histamine release induced by compound 48/80 (1 μ g/ml) from the same cell type was seen to be totally abolished in the presence of the same inhibitors (Fig.4.2).

In contrast, PAF (10 μ M)-induced histamine release from rat lung mast cells was not at all affected by the presence of antimycin A and 2-deoxyglucose. However, histamine release induced by compound 48/80 (10 μ g/ml) from the same cell population was greatly reduced in the presence of the inhibitors (Fig.4.3).

Similar results were obtained when rat cutaneous mast cells were challenged by 50 μ M PAF and 10 μ g/ml compound 48/80 (Fig.4.4).

4.3.3 EFFECT OF PAF ON TISSUE MAST CELLS OF THE GUINEA-PIG.

Fig.4.5 clearly indicates that both guinea-pig mesenteric and lung mast cells actively released histamine in a dose-related manner in the presence of exogenous PAF.

4.3.4 EFFECT OF METABOLIC INHIBITORS ON GUINEA-PIG TISSUE MAST CELLS.

Histamine secretion evoked by PAF (10 μ M) from mesenteric mast cells was seen to be totally unaffected by the presence of both antimycin A and 2-deoxyglucose, whereas histamine release induced by anti-IgG (1/300 dilution) from the same cell type was greatly reduced in the presence of the inhibitors (Fig.4.6).

Fig.4.7 represents similar results obtained when guinea-pig lung mast cells were used.

4.3.5 EFFECT OF PAF ON HUMAN HISTAMINOCYTES.

PAF (1-100 μ M) induced dose-related releases of histamine from mast cells isolated

from human skin, lung and colonic mucosal tissues (Fig.4.8). Similarly, PAF (1-100 μM) induced a dose-dependent release of histamine from isolated human basophils (Fig.4.9).

4.3.6 EFFECT OF METABOLIC INHIBITORS ON HISTAMINE RELEASE INDUCED BY VARIOUS SECRETAGOGUES FROM HUMAN HISTAMINOCYTES.

PAF (10 μM) induced histamine release from isolated human cutaneous mast cells regardless of the presence of antimycin A and 2-deoxyglucose (Fig.4.10).

Histamine release from human basophils stimulated with PAF (30 μM) also occurred in the presence of the same metabolic inhibitors. In contrast, histamine release from the same cell type induced by human anti-IgE (1/10000 dilution) was almost completely abolished under these conditions (Fig.4.11).

4.3.7 SYNERGISTIC EFFECTS OF PAF AND ANTI-IgE ON HISTAMINE RELEASE FROM HUMAN LUNG MAST CELLS AND BASOPHILS.

In the presence of both anti-IgE and PAF (at concentrations of the latter which themselves do not induce histamine release), histamine release from human lung mast cells was found to be similar to the release induced by anti-IgE alone (Fig.4.12). Similar results were obtained with human basophils (Fig.4.13).

4.4 DISCUSSION.

This study clearly indicates that PAF (1-100 μM) elicits histamine release from isolated histaminocytes of the rat, guinea-pig, mouse and human. Thus, the action of exogenous PAF is neither tissue nor species selective.

The effect of PAF on rat mesenteric, lung and cutaneous mast cells appeared largely to involve a cytotoxic mechanism, as determined by the persistence of much of the histamine release in the presence of antimycin A and 2-deoxyglucose. Moreover,

this cytotoxic histamine release was only induced by concentrations of PAF which are physiologically impossible to achieve, except possibly in extreme pathological conditions. However, one must not ignore the fact that non-cytotoxic histamine release (approx. 4-12%) induced by low concentrations of PAF (1-3 μ M) does occur especially from rat peritoneal, pleural and mesenteric mast cells. Thus, the small amounts of histamine released could contribute towards certain pathological conditions, together with other inflammatory mediators.

Similar results were obtained regarding the action of PAF on tissue mast cells of the guinea-pig.

PAF (1-100 μ M) produced dose-dependent releases of histamine from human lung, skin and colonic mucosal mast cells and isolated basophils. However, histamine release (approx. >18-20%) was again essentially via a cytotoxic mechanism.

The reported levels of PAF detected in the fluid from human skin after repeated antigen challenge vary between different research groups. Michel and co-workers detected levels of up to 100 nM in the fluid from skin chambers placed over denuded skin blister sites following repeated antigen challenge [330]. In contrast, similar work carried out by Shalit and co-workers [333] reported the presence of a higher concentration of PAF (approx. 90 μ M). Both groups quantified the levels of PAF by a rabbit-platelet aggregation assay [334]. Hence, it is possible for levels of PAF found in the skin of certain individuals to reach high concentrations. If this is the case, then such levels of PAF would indeed cause histamine release from skin mast cells. However, follow-up work on this particular aspect has yet to be carried out.

It is possible that exogenous PAF acting on isolated human lung mast cells may, in fact, cause these cells to release cell-associated PAF synthesised *de novo* following stimulation. Whether PAF possesses this positive feedback mechanism in mast cells has yet to be established. It is well known that PAF can activate other inflammatory cells such as neutrophils, eosinophils, monocytes and macrophages which in turn generate and release more PAF. Thus, if the levels of PAF within the

tissue are high enough it would be able to stimulate nearby mast cells, resulting in mediator release. The above postulation applies mainly to *in vivo* situations and *in vitro* studies of this nature have yet to be carried out. Moreover, it is becoming blatantly apparent that PAF alone is not responsible for the overall inflammatory response, but works together with other allergic mediators released from a variety of activated inflammatory cells.

It was experimentally observed that low concentrations of PAF do not potentiate anti-IgE mediated histamine release from human lung mast cells or basophils. These findings are in contrast to those obtained from rat peritoneal mast cells. Thus, PAF in the human system appears not to act on or with receptor-bound IgE molecules on the surface of the mast cell membrane. One can assume from these results that PAF is more likely to be interacting with the cell membrane.

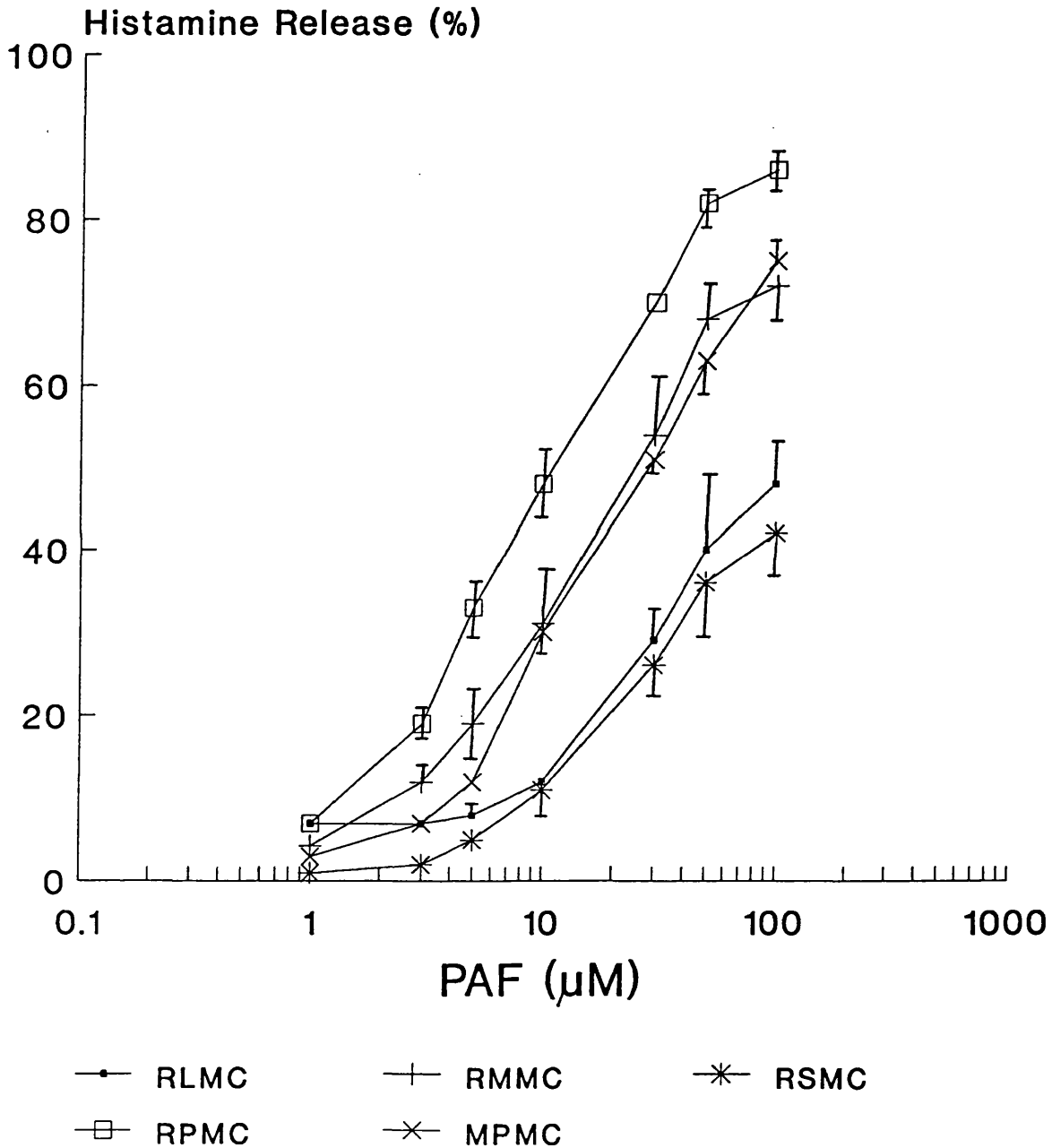
In conclusion, PAF induces histamine release in a dose-related manner from isolated histaminocytes of different species. At higher concentrations of PAF the release was essentially cytotoxic. Anti-IgE mediated histamine release from human lung mast cells and basophils was not potentiated by a low concentration of PAF (1 μ M).

4.5 ABBREVIATIONS FOR FIG.4.1-4.13

RMMC	Rat mesenteric mast cells.
RLMC	Rat lung mast cells.
RSMC	Rat skin mast cells.
MPMC	Mouse peritoneal mast cells.
GPMMC	Guinea-pig mesenteric mast cells.
GPLMC	Guinea-pig lung mast cells.
HSMC	Human skin mast cells.
HCMC	Human colonic mucosal mast cells.
HLMC	Human lung mast cells.

Fig.4.1 Histamine release induced by PAF from isolated mast cells from the rat pleural cavity (RPMC), mesentery (RMMC), lung (RLMC), skin (RSMC) and mouse peritoneum (MPMC).

n=4-5



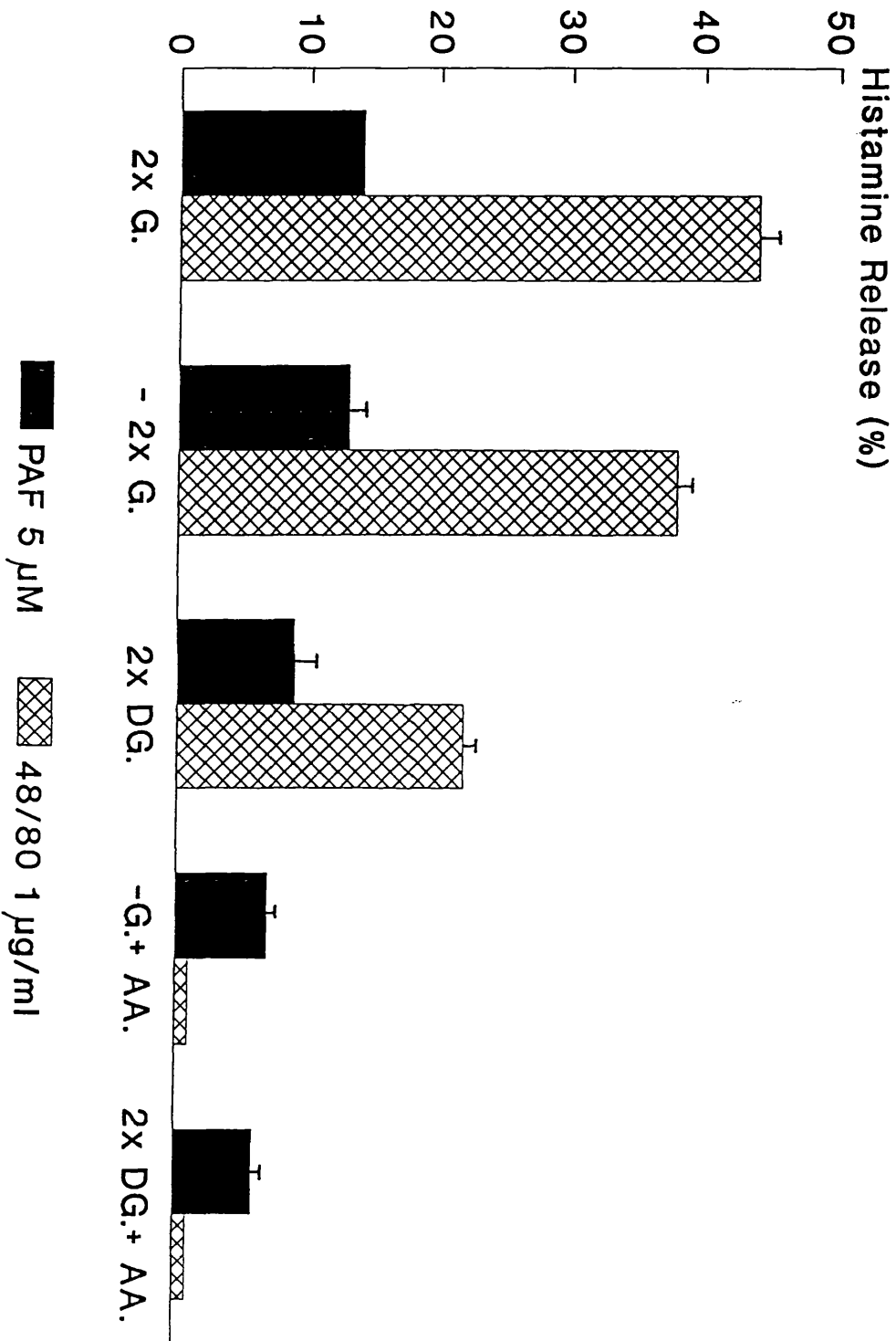
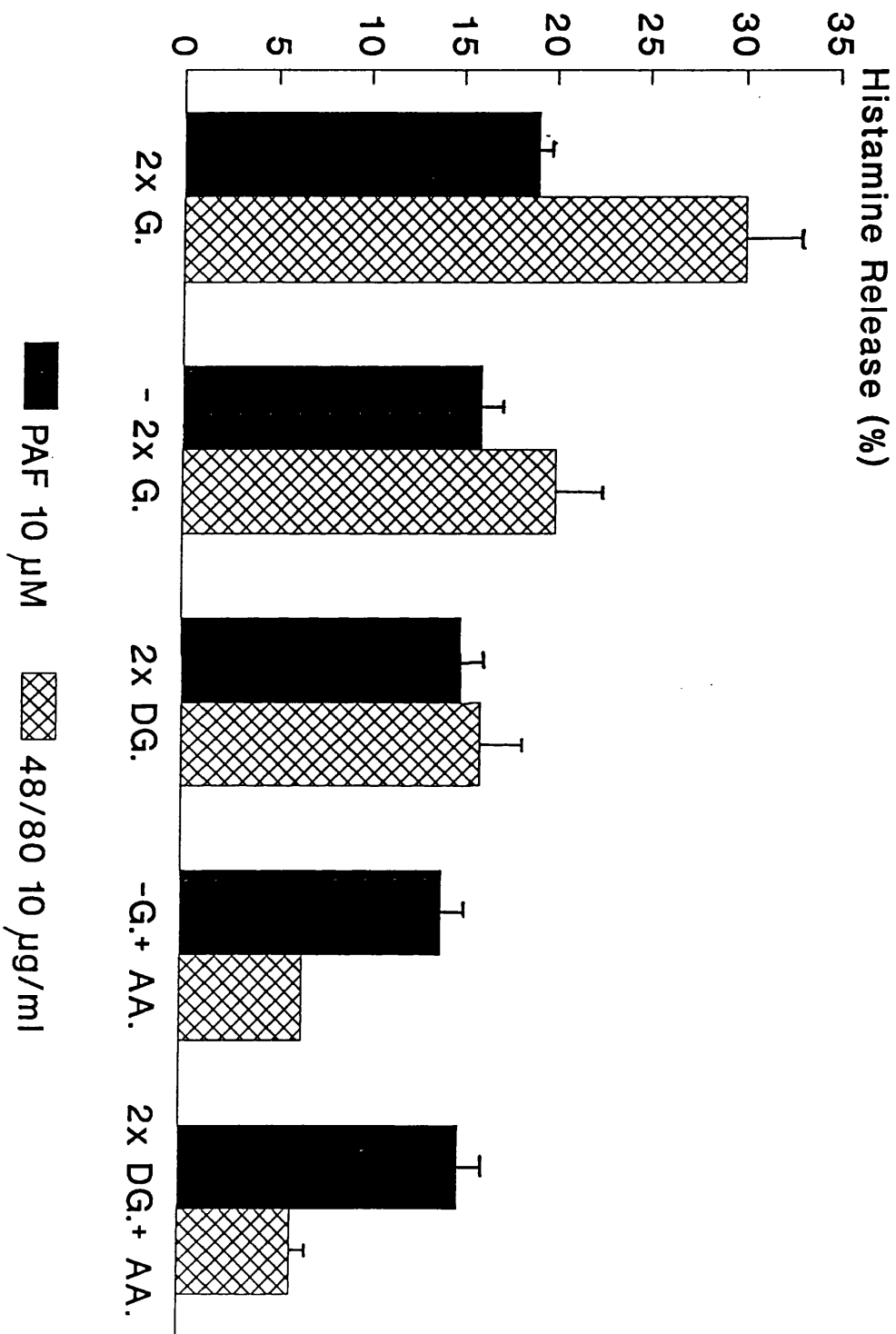


Fig.4.2 The effect of metabolic inhibitors on histamine release from rat mesenteric mast cells (RMMC) induced by PAF (5 μ M) and compound 48/80 (1 μ g/ml). n=3

Fig.4.3 The effect of metabolic inhibitors on histamine release from rat lung mast cells (RLMC) induced by PAF (10 μ M) and compound 48/80 (10 μ g/ml). n=4



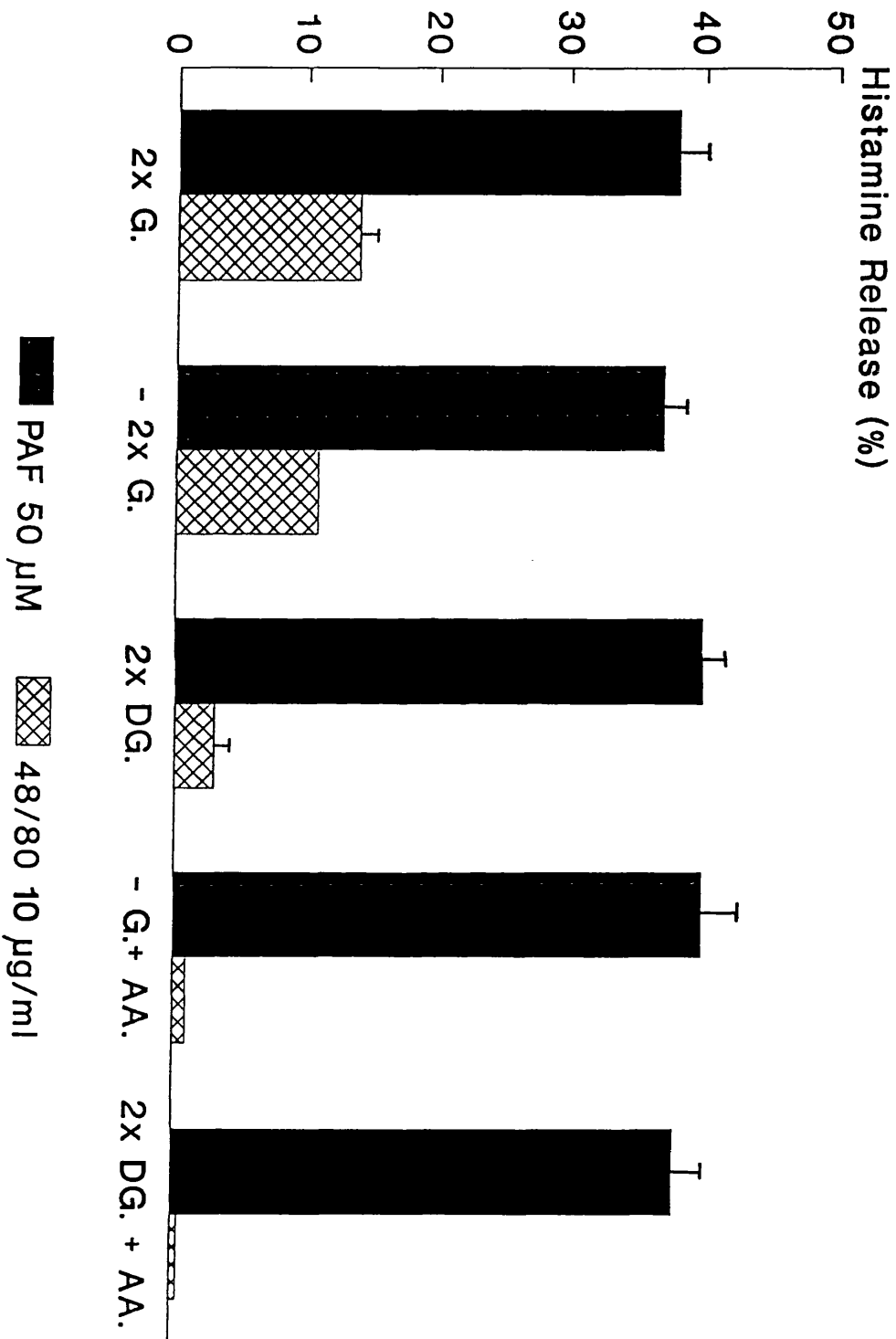
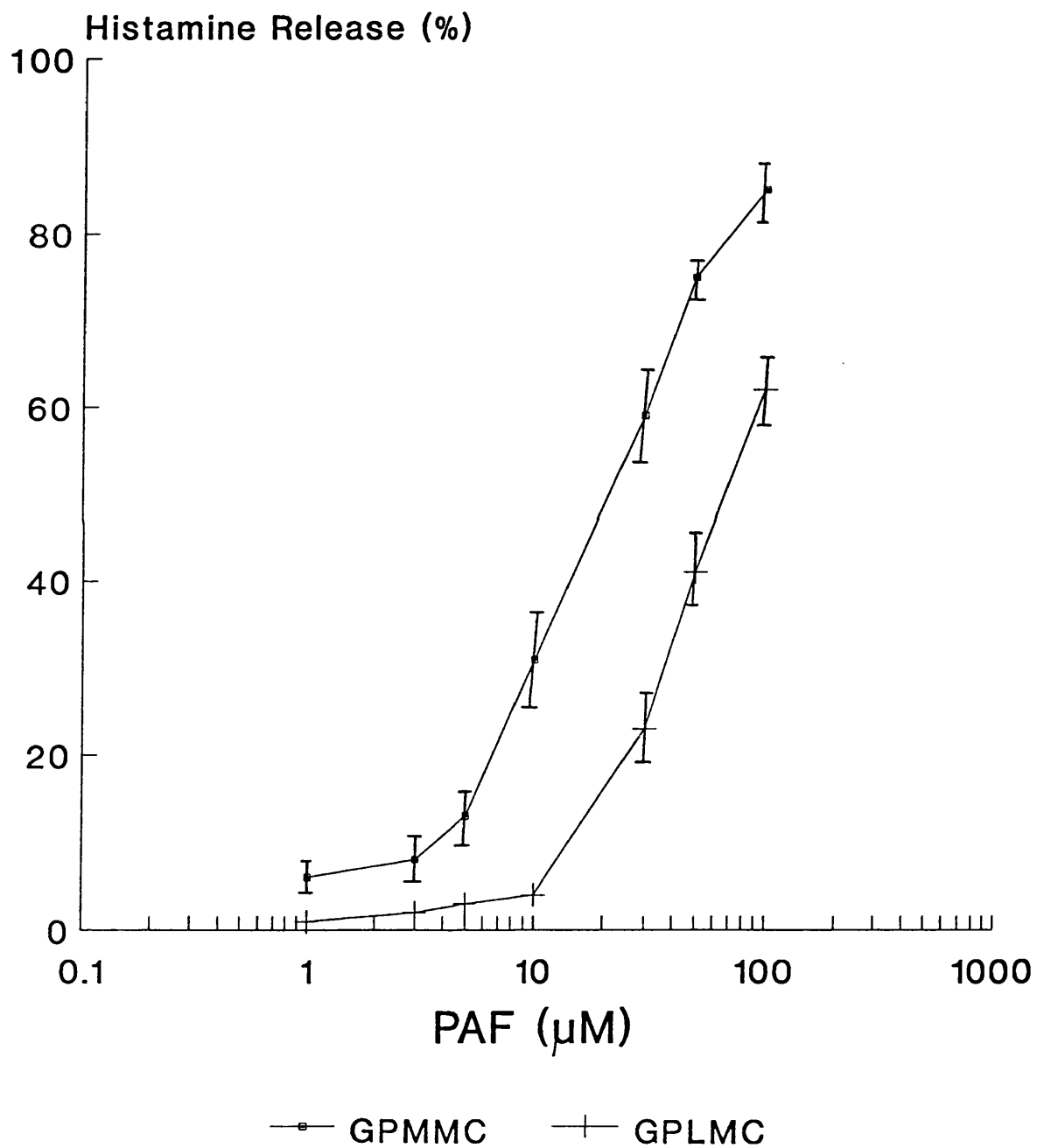


Fig.4.5 The action of PAF on isolated mast cells from the guinea-pig mesentery (GPMMC) and lung (GPLMC). n=4



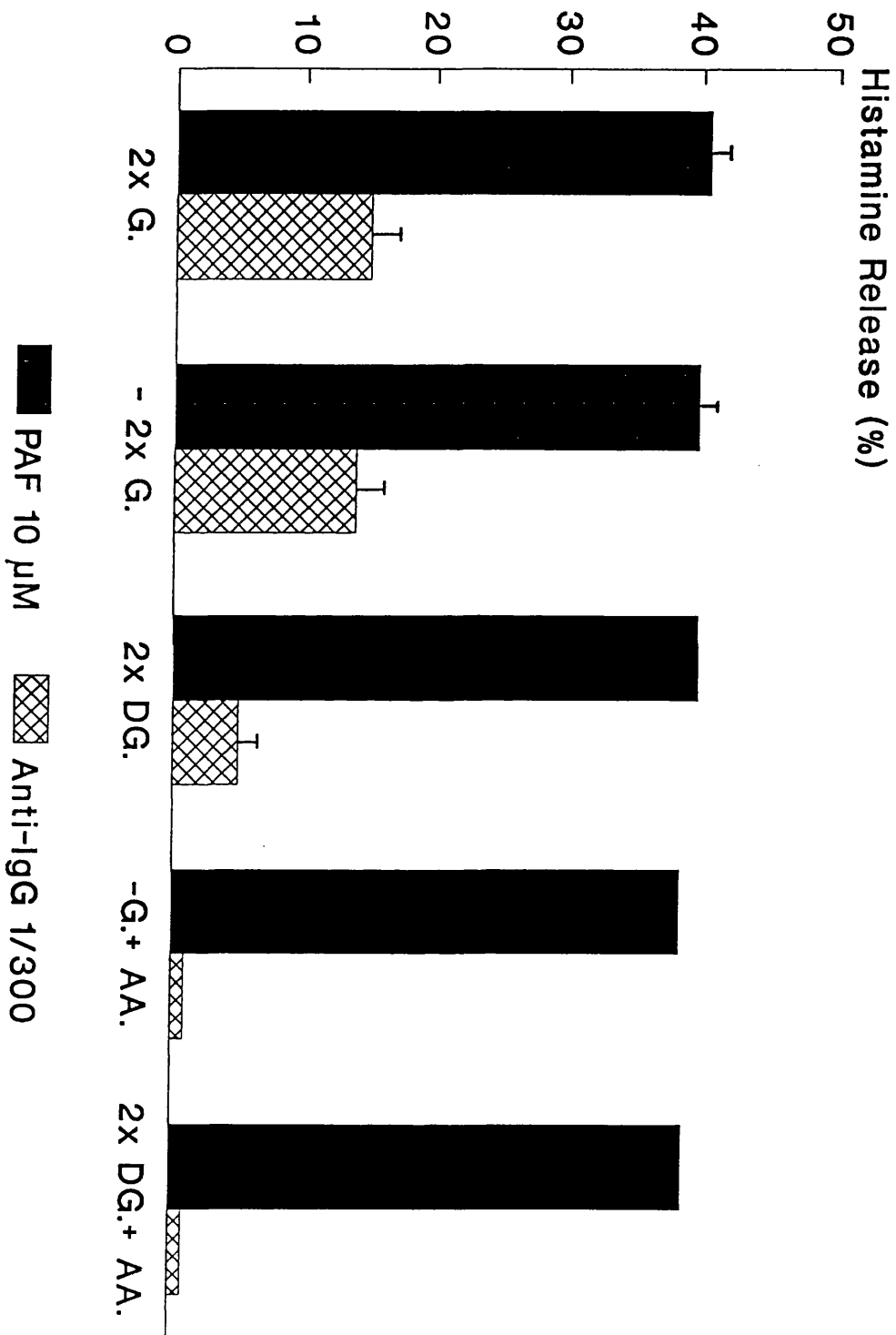


Fig.4.6 The effect of metabolic inhibitors on histamine release from guinea-pig mesenteric mast cells (GPMMC) induced by PAF (10 μ M) and anti-IgG (1/300 dilution). n=4

Fig.4.7 The effect of metabolic inhibitors on histamine release from guinea-pig lung mast cells (GPLMC) induced by PAF (50 μ M) and anti-IgG (1/300 dilution). n=4

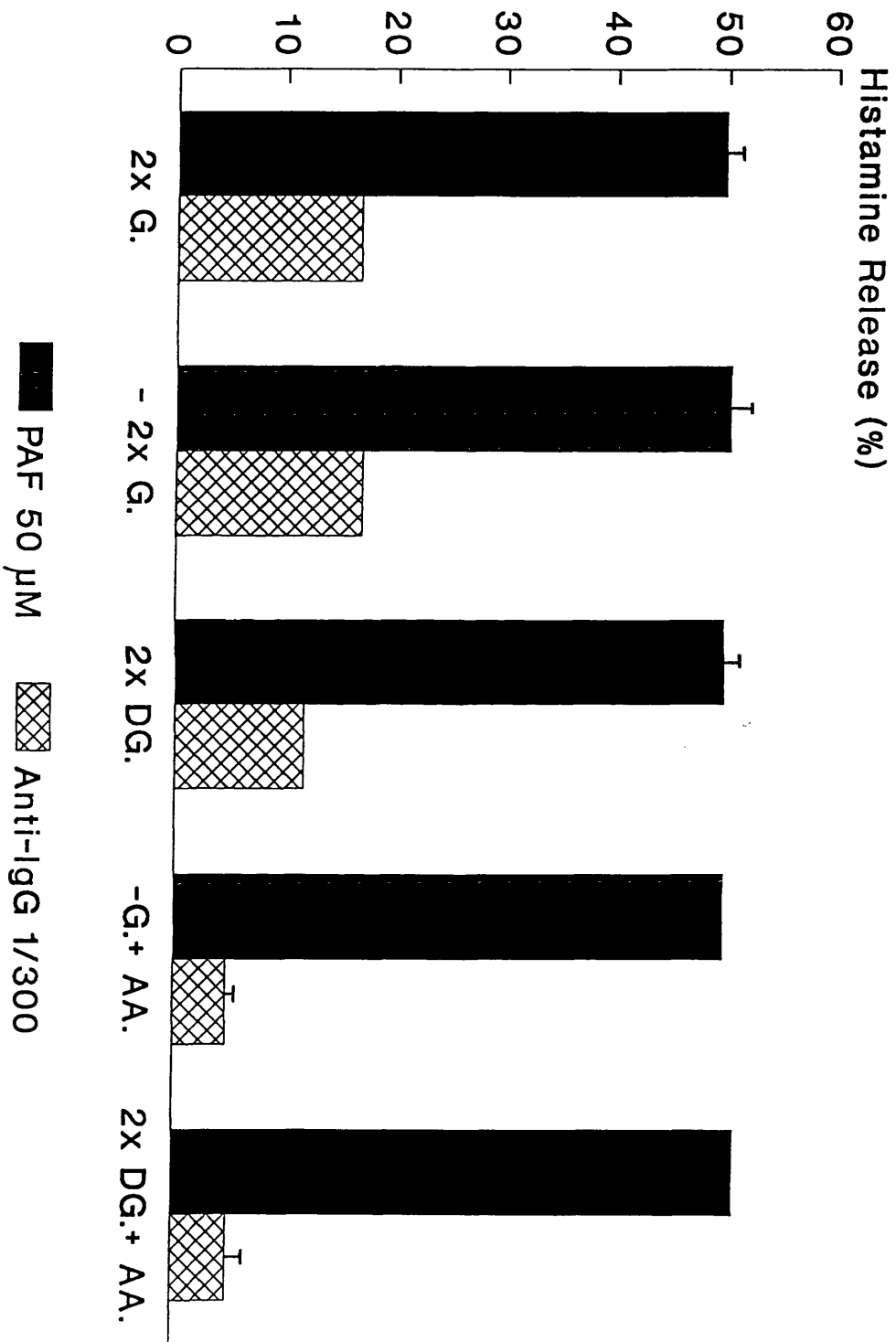


Fig.4.8 The effect of PAF on mast cells isolated from human skin (HSMC), colonic mucosa (HCMMC) and lung (HLMC).
n=3-4

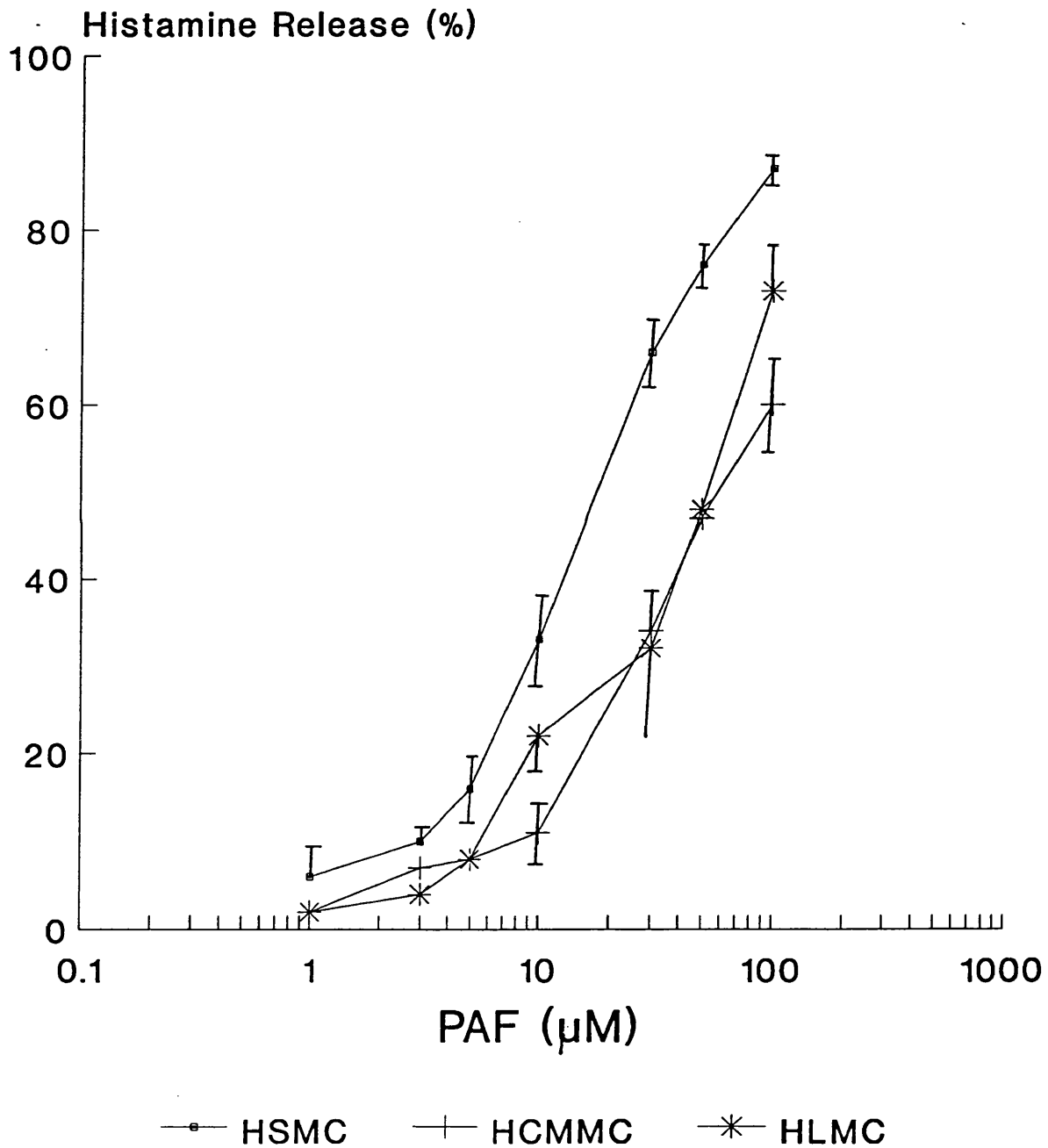
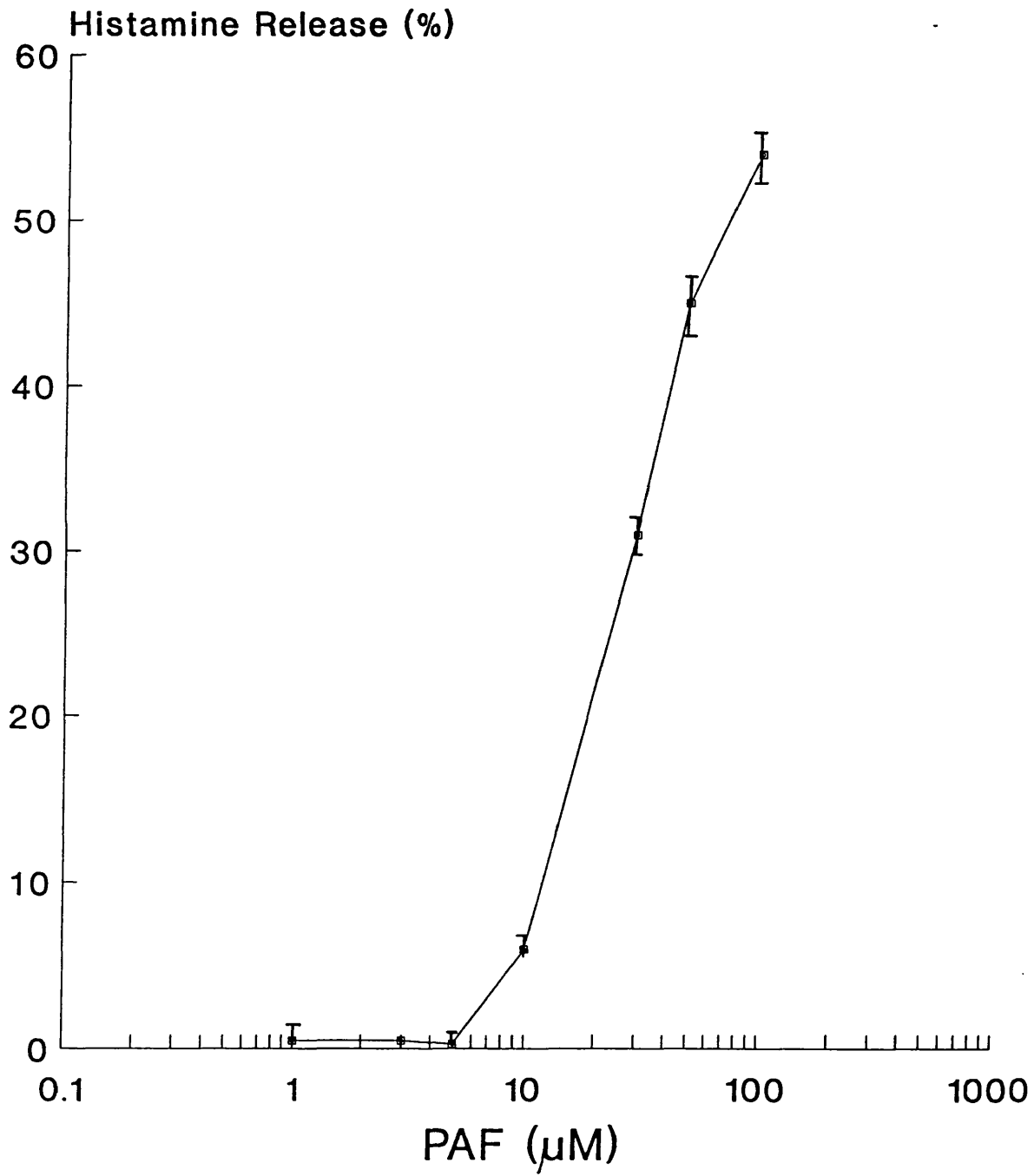


Fig.4.9 The effect of PAF on human basophils. n=4



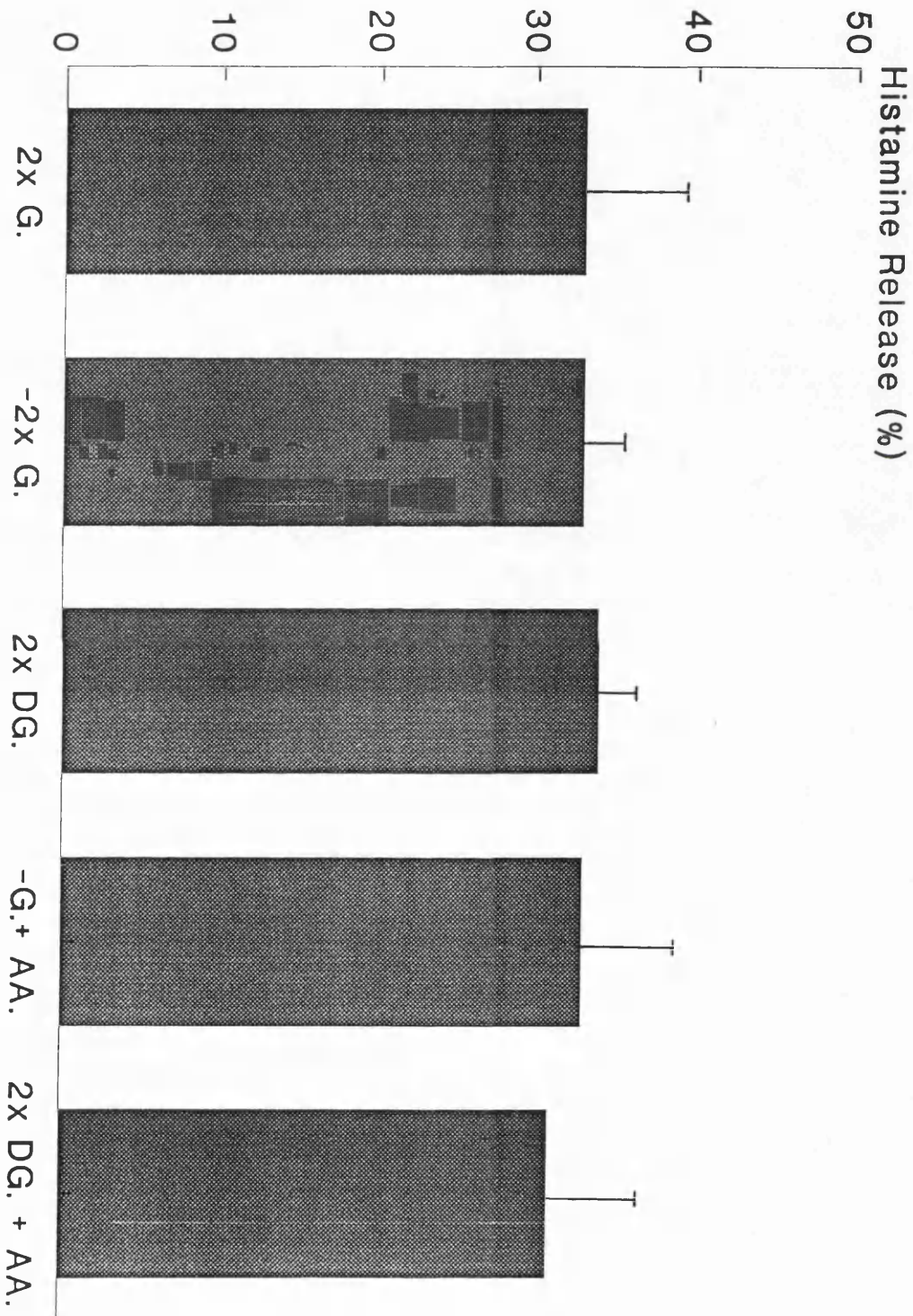


Fig.4.11 The effect of metabolic inhibitors on histamine release from human basophils induced by PAF (30 μ M) and anti-IgE (1/10000 dilution). n=4

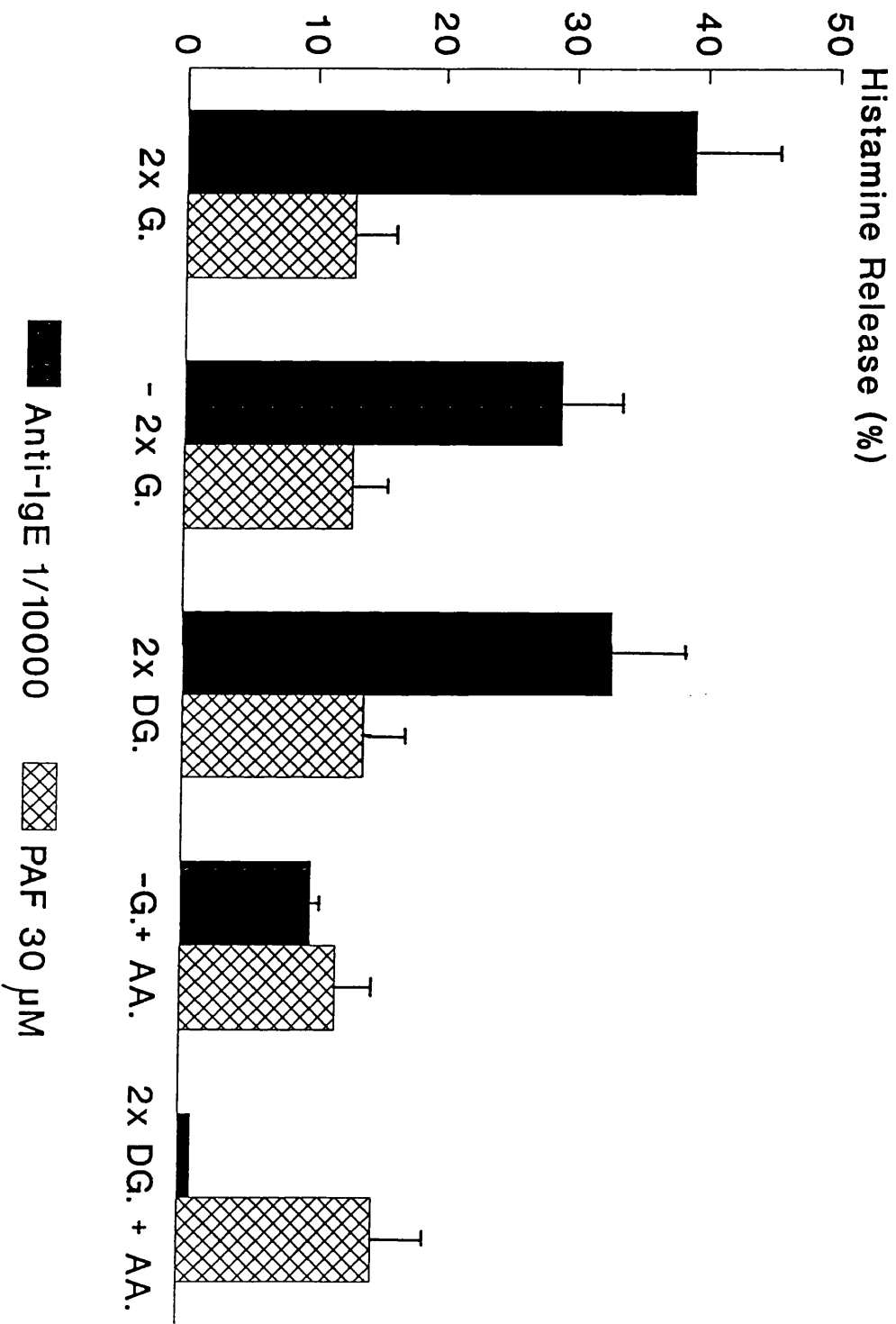


Fig.4.12 The effect of PAF on anti-IgE mediated histamine release from human lung mast cells (HLMC). n=6

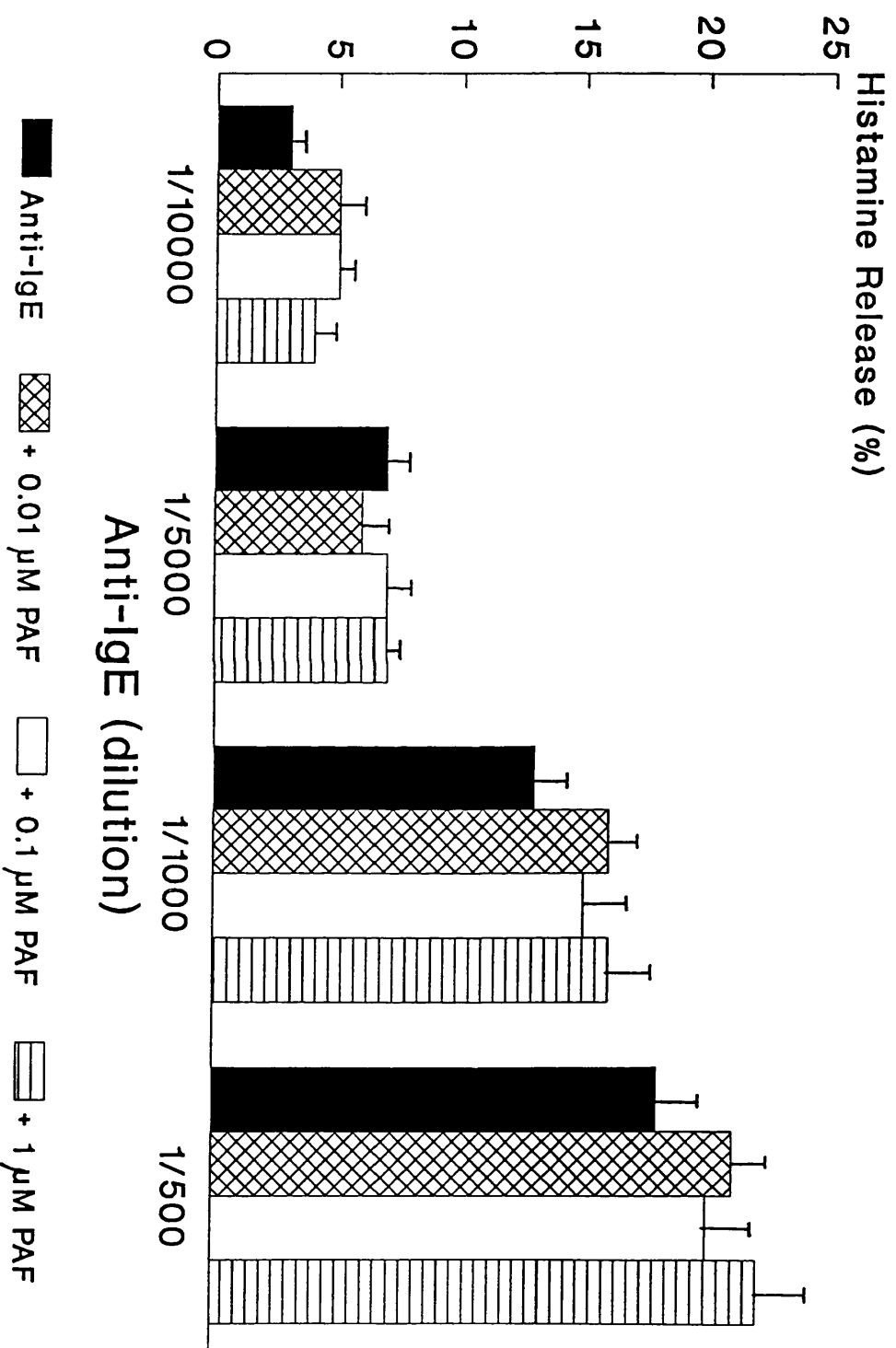
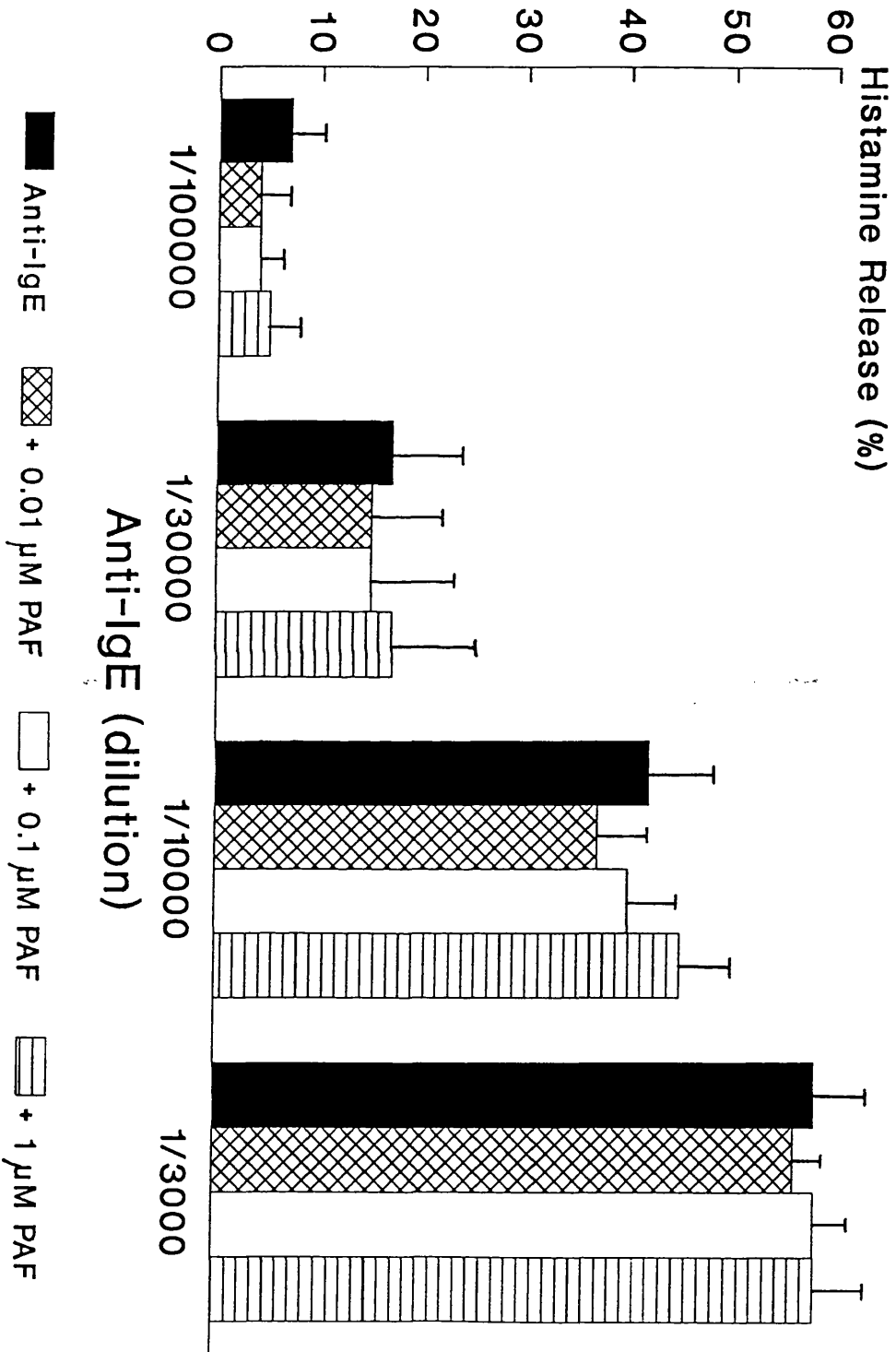


Fig.4.13 The effect of PAF on anti-IgE mediated histamine release from human basophils, n=4



CHAPTER FIVE

INHIBITION OF PAF AND ANTI-IgE INDUCED HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS BY A VARIETY OF ANTI-ALLERGIC DRUGS AND SELECTIVE PAF ANTAGONISTS.

5.1 INTRODUCTION.

This chapter is divided into two parts. Part one looks at the inhibitory effects of a variety of anti-allergic compounds in preventing histamine release from rat peritoneal mast cells stimulated by anti-IgE and PAF. Part two primarily studies the effects of three PAF antagonists BN52021, BN50730 and WEB2086 in this system.

5.2 ANTI-ALLERGIC COMPOUNDS.

As mentioned previously in chapter one, mast cells play a key role in many allergic conditions. Owing to their ideal situation within mammalian bodies, they are able to interact to the highest possible degree with invading antigens. A vast amount of evidence exists implicating the activation of mast cells prior to the development of many diseases with an allergic basis [335]. The possible use of drugs which protect mast cells and prevent mediator release would, therefore, appear to be of great therapeutic benefit. A diverse range of drugs exist which, during the last seventy years or so, have been prescribed to patients with allergic disorders. Many of these drugs are structurally unrelated and have different modes of action.

The following sections look briefly at a range of antiallergic compounds and their ability to inhibit histamine release from rat peritoneal mast cells stimulated by anti-IgE and PAF.

Disodium cromoglycate (DSCG) is an anti-allergic drug used prophylactically in the treatment of allergic diseases such as asthma, rhinitis, conjunctivitis and allergic reactions to food [336-341]. DSCG is a derivative of the naturally occurring anti-spasmodic compound, khellin (a furanochromone), and was successfully synthesised during the mid-1960's [342]. Following clinical trials, it was first marketed in the U.K. in 1968 as an anti-asthmatic drug.

In the early 1970's, Garland reported that DCSG was an effective inhibitor of IgE-mediated histamine release from rat peritoneal mast cells [343]. Further studies later indicated that DSCG also selectively inhibited IgE-mediated histamine release from mast cells isolated from the lung but not those from the colonic mucosa of the rat [344,345].

The exact mode of action of DSCG has yet to be fully elucidated and accepted by all, as over the last twenty years or so several research groups have put forward possible mechanisms of action based upon their own experimental results. Proposed mechanisms include the possibility of DSCG exerting its effect on mast cells by stabilizing the cell membrane [62], or by sequestering extracellular calcium ions or finally by inhibiting cAMP phosphodiesterase [346,347]. However, a more specific mode of action of DSCG was put forward by Theoharides and co-workers [185], who suggested that the effect of DSCG on rat mast cells was associated with the phosphorylation of the 78,000 Dalton protein, which is thought to regulate the termination of the secretory response.

Drugs which elevate the intracellular cyclic AMP (cAMP) levels also bring about the cessation of secretion. β_2 -agonists such as isoprenaline interact with cell membrane associated β -adrenoceptors, triggering off a series of biochemical reactions culminating in an increase in intracellular cAMP levels. β -agonists are potent inhibitors of mediator release from human lung mast cells [348]. In contrast, β_2 -agonists such as isoprenaline do not produce a marked inhibition of IgE-mediated histamine release from rat mast cells.

Another group of drugs which prevent mediator release from stimulated mast cells by inducing an increase in cAMP levels are the methyl xanthines such as theophylline and 3-isobutyl-1-methyl xanthine (IBMX).

The flavonoids are a group of naturally occurring compounds, whose structure is based on 2-phenyl-4-chromone, otherwise known as flavone [349]. Fewtrell and Gomperts first investigated the action of quercetin, a widely used flavonoid, on histamine release from rat mast cells [350]. They concluded that quercetin regulates histamine release from rat mast cells by blocking Na^+/K^+ -ATPases, ie. by increasing the efficiency of the ATP-dependent ion pumps. It was also proposed that quercetin increases the efficiency of the Ca^{2+} -ATPases, responsible for maintaining low intracellular levels of calcium, thus preventing histamine release.

The above are just a few examples of drugs which prevent mediator release from stimulated mast cells. Most of the mentioned drugs are, or used to be, in therapeutic use. As insights into mast cell biochemistry and its role in allergy are increasing, it is hoped that more effective and specific drugs may be developed which could prove to be therapeutically useful.

5.3 PAF ANTAGONISTS.

As mentioned previously in chapter one, PAF possesses a wide spectrum of inflammatory properties. PAF, injected intravenously, elicits a variety of effects including bronchoconstriction and increased vascular permeability. Direct administration of PAF into the lung triggers off an inflammatory response which comprises mucus secretion, broncho-constriction and cellular accumulation. PAF exerts its effects by interacting with specific PAF receptors. PAF antagonists have been shown to block PAF-induced pathologies in both laboratory animal models and human volunteers.

The aim of this part of chapter five was to investigate the role of selective PAF antagonists in modulating histamine release from rat peritoneal mast cells stimulated by PAF and anti-IgE.

5.4 METHODS AND MATERIALS.

Experimental details relating to this section of study are contained in chapter two.

5.5 RESULTS.

5.5.1 RESULTS PERTAINING TO PART ONE.

5.5.1.1 THE EFFECT OF ANTI-ALLERGIC COMPOUNDS ON HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS STIMULATED BY PAF AND ANTI- IgE.

The anti-allergic chromone DSCG and its more potent congener nedocromil sodium both potently inhibited histamine release from rat peritoneal mast cells stimulated with anti-IgE (Fig.5.1 and 5.4). However, preincubation of the cells with DSCG and nedocromil sodium (10 and 30 min) before challenge with anti-IgE decreased the inhibitory actions of both drugs (Fig.5.2, 5.3, 5.5 and 5.6).

DSCG and nedocromil sodium produced dose-related inhibition of histamine release from rat peritoneal mast cells stimulated by PAF (Fig.5.1 and 5.4). After preincubation (10 min) of the cells with DSCG, the chromone produced a similar inhibitory effect as observed in Fig.5.1, following stimulation by PAF (Fig.5.2). In contrast, nedocromil sodium ceased to exhibit any inhibitory action under those conditions (Fig.5.5). After preincubation (30 min) of the cells with DSCG, the chromone failed to inhibit histamine release following stimulation with PAF (Fig.5.3). In contrast, the inhibitory effects of nedocromil sodium were re-established to a similar degree as observed in Fig.5.4 (Fig.5.6).

The effects of DSCG and nedocromil sodium in inhibiting histamine release from rat peritoneal mast cells following stimulation were extended, in this case lyso-PAF being the stimulus. Lyso-PAF, the metabolic precursor of PAF and found to be biologically inactive in certain systems, was seen to induce histamine release

from rat peritoneal mast cells in a dose-related manner (Table 5.1).

Simultaneous addition of DSCG produced weak inhibition of histamine release from rat peritoneal mast cells challenged by lyso-PAF (Fig.5.1), and nedocromil sodium was observed to have no effect (Fig.5.4). Preincubation (10 min) of the cells with DSCG and nedocromil sodium prior to stimulation by lyso-PAF produced dose-related inhibition of histamine release (Fig.5.2 and 5.5). Preincubation (30 min) of the cells with DSCG prior to challenge by lyso-PAF, produced a dose-related inhibition of histamine release (Fig.5.3). Similar results were obtained with nedocromil sodium (Fig.5.6). It is apparent from the above results that the inhibitory action of both anti-allergic drugs increases with preincubation time.

Fig.5.7 indicates that the β_2 -agonist isoprenaline was ineffective in inhibiting histamine release from rat peritoneal mast cells stimulated by anti-IgE. In contrast, isoprenaline produced a dose-related inhibition of histamine release from the same cell type following stimulation by PAF.

8-bromo cAMP and dibutyryl cAMP, which are cell permeable analogues of cAMP, potently inhibited histamine release from rat peritoneal mast cells following stimulation by anti-IgE. 8-bromo cAMP similarly inhibited histamine release due to PAF while dibutyryl cAMP was rather less active against this stimulus (Fig.5.8 and 5.9).

The phosphodiesterase inhibitors IBMX and theophylline both potently inhibited anti-IgE and PAF mediated histamine release from rat peritoneal mast cells (Fig.5.10 and 5.11).

Quercetin produced a marked dose-dependent inhibition of histamine release from rat peritoneal mast cells following stimulation by anti-IgE but was much less active against PAF (Fig.5.12).

5.5.2 RESULTS PERTAINING TO PART TWO.

5.5.2.1 THE EFFECT OF SELECTIVE PAF ANTAGONISTS ON HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS STIMULATED BY ANTI-IgE AND PAF.

Simultaneous addition of the PAF antagonist WEB2086 failed to inhibit histamine release from rat peritoneal mast cells stimulated by anti-IgE. However, a very weak inhibitory effect was observed when the cells were stimulated by PAF (Fig.5.13). Preincubation (10 min) of the cells with the antagonist produced a weak inhibition of histamine release from cells stimulated by PAF, but was totally ineffective against cells stimulated by anti-IgE (Fig.5.14). Preincubation (30 min) of the cells with the antagonist produced a very slight inhibition of histamine release from cells stimulated by anti-IgE but had only a weak and variable effect on PAF (Fig.5.15).

Simultaneous addition of the PAF antagonist BN50201 produced a weak inhibitory effect against anti-IgE mediated histamine release from rat peritoneal mast cells, but was totally inactive against PAF (Fig.5.16). Preincubation (10 min) of the cells with the drug produced a similar (see Fig.5.16) inhibition of histamine release from rat peritoneal mast cells stimulated by anti-IgE and caused an inconsistent pattern of inhibition from cells stimulated by PAF (Fig.5.17). Preincubation (30 min) of the cells with the antagonist produced a dose-related inhibition of histamine release from peritoneal cells stimulated by anti-IgE but had a negligible effect on cells stimulated by PAF (Fig.5.18).

Simultaneous addition of the PAF antagonist BN50730 produced a dose-dependent inhibition of histamine release from rat peritoneal mast cells stimulated by anti-IgE (Fig.5.19). However, this inhibition decreased with increased preincubation time of the cells with the antagonist (Fig.5.20 and 5.21).

The afore mentioned antagonist produced a pronounced and dose-related inhibition of histamine release from peritoneal cells stimulated by PAF only after preincubation (10 min) of the cells with the antagonist (Fig.5.20). In contrast, the same antagonist seemed relatively inactive in inhibiting PAF-induced histamine release at other times (Fig.5.19 and 5.21).

Significant inhibition (>10%) of histamine release from rat peritoneal mast cells stimulated by lyso-PAF only occurred at the highest concentration of BN50730 used following preincubation (30 min) of the cells (Fig.5.21). At other times, minimum inhibitory effects were observed (Fig.5.19 and 5.20).

5.6 DISCUSSION.

DSCG was found to be a potent inhibitor of IgE-mediated histamine release from rat peritoneal mast cells. However, the phenomenon of tachyphylaxis or self-inhibition was observed as the preincubation time of the cells with DSCG was increased. The exact mode of action of DSCG remains unclear. Extensive studies by Pecht and co-workers reported the presence of a specific glycoprotein found in rat basophilic leukaemia cells (RBL). The glycoprotein was referred to as the cromolyn binding protein (CBP). This protein was thought to lie in the mast cell membrane and control the influx of calcium ions into the cell by regulating the calcium gating mechanism [351-354]. DSCG was proposed to bind to the CBP, thus preventing the influx of calcium ions and hence degranulation. However, the RBL cell type are the only cells in which a CBP has been identified. Moreover, RBL cells are totally unresponsive towards DSCG [355].

Chapter one explains in detail the concept of how protein phosphorylation is thought to regulate exocytosis from mast cells. It has been proposed that DSCG induces phosphorylation of the 78,000 Dalton protein thought to be responsible for the natural termination of secretion, this event occurring 30-60 seconds after stimulation [184,186]. The emergence of tachyphylaxis is thought to be due to dephosphorylation of the 78,000 Dalton protein. Hence, the interaction between DSCG and the 78,000 Dalton protein seems to be the most widely accepted mode of action of the drug.

DSCG also inhibited PAF-induced histamine release from rat peritoneal mast cells and tachyphylaxis was again observed after prolonged preincubation (30 min). In contrast, inhibition of histamine release by DSCG from cells stimulated with lyso-

PAF increased as the preincubation time of the cells with the drug was increased.

Basran and co-workers have demonstrated that DSCG inhibited PAF-induced wheal and flare reaction in human skin. Other than its well documented effects in inhibiting histamine release from mast cells, few pharmacological properties of DSCG have been reported. Thus, DSCG not only inhibits an inflammatory response by protecting mast cells, but can also block PAF-induced reactions via an alternative mechanism which has yet to be fully studied [359].

Similar results were observed when nedocromil sodium was used as an inhibitor against IgE-mediated histamine release from rat peritoneal mast cells. Inhibition of histamine release by nedocromil sodium from mast cells is thought to involve a similar mode of action to DSCG. However, in this case tachyphylaxis was more pronounced. In the case of PAF-induced histamine release from rat peritoneal mast cells, tachyphylaxis was complete after preincubation (30 min) of the cells with nedocromil sodium. In contrast, the inhibitory effects of nedocromil sodium again increased with preincubation time following stimulation by lyso-PAF.

The β_2 -agonist isoprenaline failed to elicit any appreciable inhibition of histamine release induced by anti-IgE from rat peritoneal mast cells, despite the existence of a large number of high affinity β_2 -receptor sites present on the mast cell membrane [174]. In contrast, isoprenaline modestly inhibited PAF-induced histamine release from rat peritoneal mast cells. The reason for this may be due to an interaction between PAF and isoprenaline at the cell plasma membrane.

The present study has shown that compounds which raise intracellular levels of cAMP such as 8-bromo cAMP, dibutyryl cAMP, IBMX and theophylline inhibited both anti-IgE and PAF-induced histamine release from rat peritoneal mast cells. The results observed when anti-IgE was used as the stimulus were consistent with previous findings [175,344].

The flavonoid quercetin potently inhibited IgE-mediated histamine release and to a much lesser extent PAF-induced histamine release from rat peritoneal mast cells.

Some time ago, it was suggested that quercetin exerted its inhibitory effects by interacting with the 78,000 Dalton protein [185]. However, this postulate regarding the mode of action of quercetin has yet to be fully studied.

In conclusion to the results pertaining to part one of this study, histamine release induced by IgE from rat peritoneal mast cells was effectively inhibited by a diverse range of anti-allergic compounds. With regard to histamine release induced by PAF from rat peritoneal mast cells, all the above compounds studied inhibited histamine release but to varying degrees.

The PAF antagonist WEB2086 actively inhibits a diverse range of PAF- induced pharmacological actions both *in vivo* and *in vitro* [356] (see Table 5.2 for IC_{50} values). However, this study has shown that the antagonist was incapable of producing consistent inhibition of histamine release from rat peritoneal mast cells stimulated with PAF. It has been well documented that PAF exerts its effects via distinct PAF receptors that have been identified on several tissues. The existence of such receptors on mast cells has yet to be studied. In contrast, the antagonist very weakly inhibited anti-IgE mediated histamine from rat peritoneal mast cells but only after perincubation (30 min) of the cells with the drug.

BN52021, another PAF antagonist studied (see Table 5.3 for IC_{50} values) seemed effective in inhibiting PAF-induced histamine release from rat peritoneal mast cells only after preincubation (10 min) of the cells with the drug. In contrast, inhibition of anti-IgE mediated histamine release was seen to increase as the preincubation time of the cells with the drug was increased.

In terms of *in vivo* activity, the PAF antagonist BN50730 is the most potent antagonist to be synthesised yet [358] (see Table 5.4 for IC_{50} values). In the present system the antagonist inhibited anti-IgE mediated histamine release from rat peritoneal mast cells, an effect which decreased as the preincubation time of the cells with the drug was increased. However, PAF-induced histamine release was potently inhibited only after preincubation (10 min) of the cells with the antagonist.

The present results indicate that PAF-induced release of histamine (non-cytotoxic) from rat peritoneal mast cells can be inhibited by a range of anti-allergic compounds. Inhibition of release by selective PAF antagonists at times is both weak and variable. This indicates that PAF is not stimulating mast cells via specific receptors but is more likely to be interacting with the cell membrane. The proposed mode of action of PAF on mast cells is further supported by the observation that lyso-PAF, which is inactive in many other biological systems, induces histamine release from rat peritoneal mast cells in a similar fashion to PAF. However, the precise mechanism by which PAF-induced histamine release is inhibited from mast cells has yet to be fully elucidated. This may serve as a useful base to study further the role of PAF and its supposed interaction with mast cells in allergic disorders and eventually may lead to the development of novel anti-allergic drugs.

5.6 ABBREVIATIONS FOR FIG. 5.1-5.21

DSCG	Disodium cromoglycate.
RPMC	Rat peritoneal mast cells.

Fig.5.1 The effect of DSCG on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The drug was added to the cells with the stimulus. Control releases were 15.0 \pm 2.5% (PAF), 14.0 \pm 2.0% (lyso-PAF) and 28.0 \pm 7.2% (anti-IgE). n=4

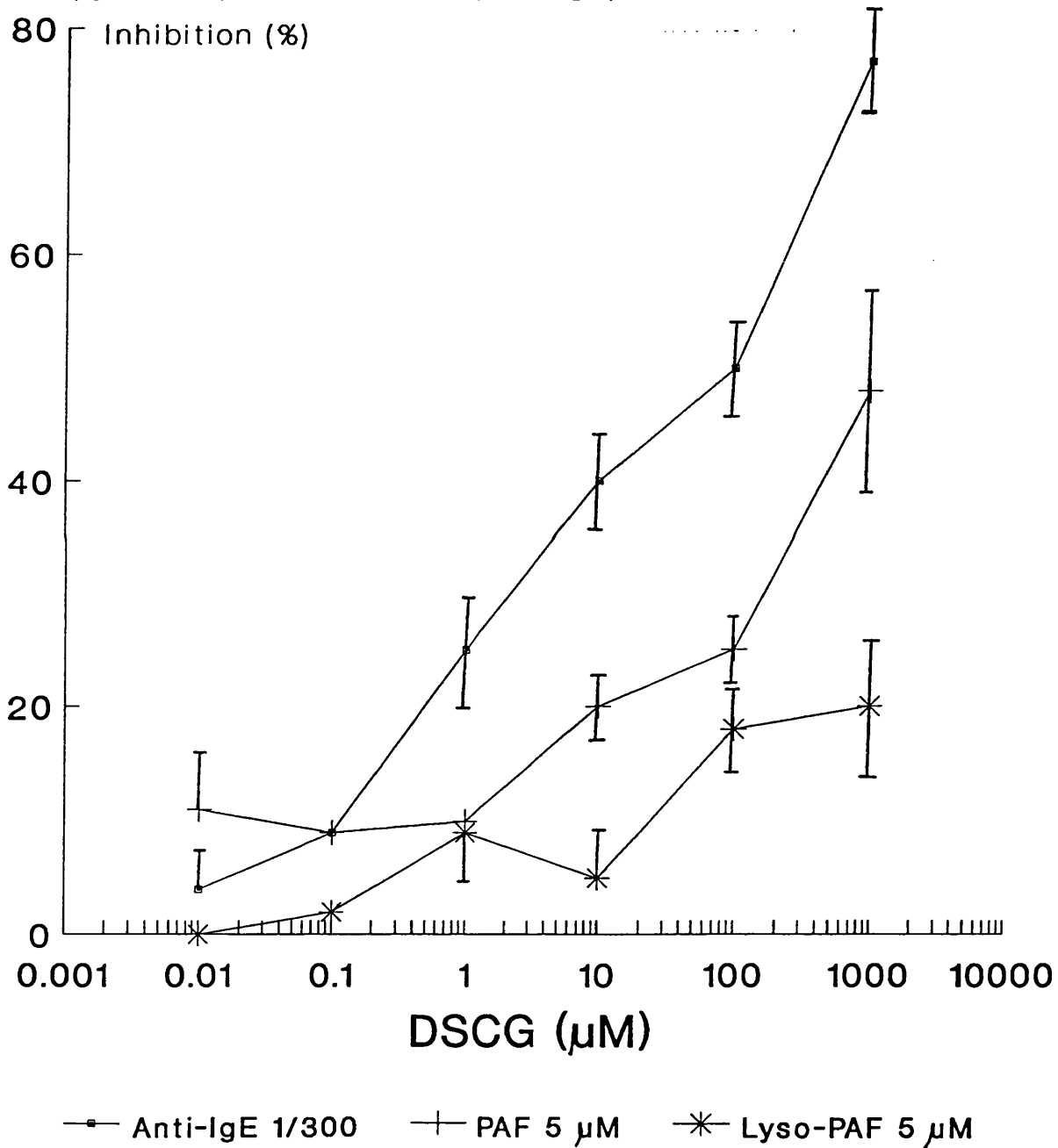


Fig.5.2 The effect of DSCG on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated for 10 min with the drug before the stimulus was added. Control release were $16.0 \pm 2.0\%$ (PAF), $14.0 \pm 1.0\%$ (lyso-PAF) and $30.0 \pm 3.0\%$ (anti-IgE). n=4

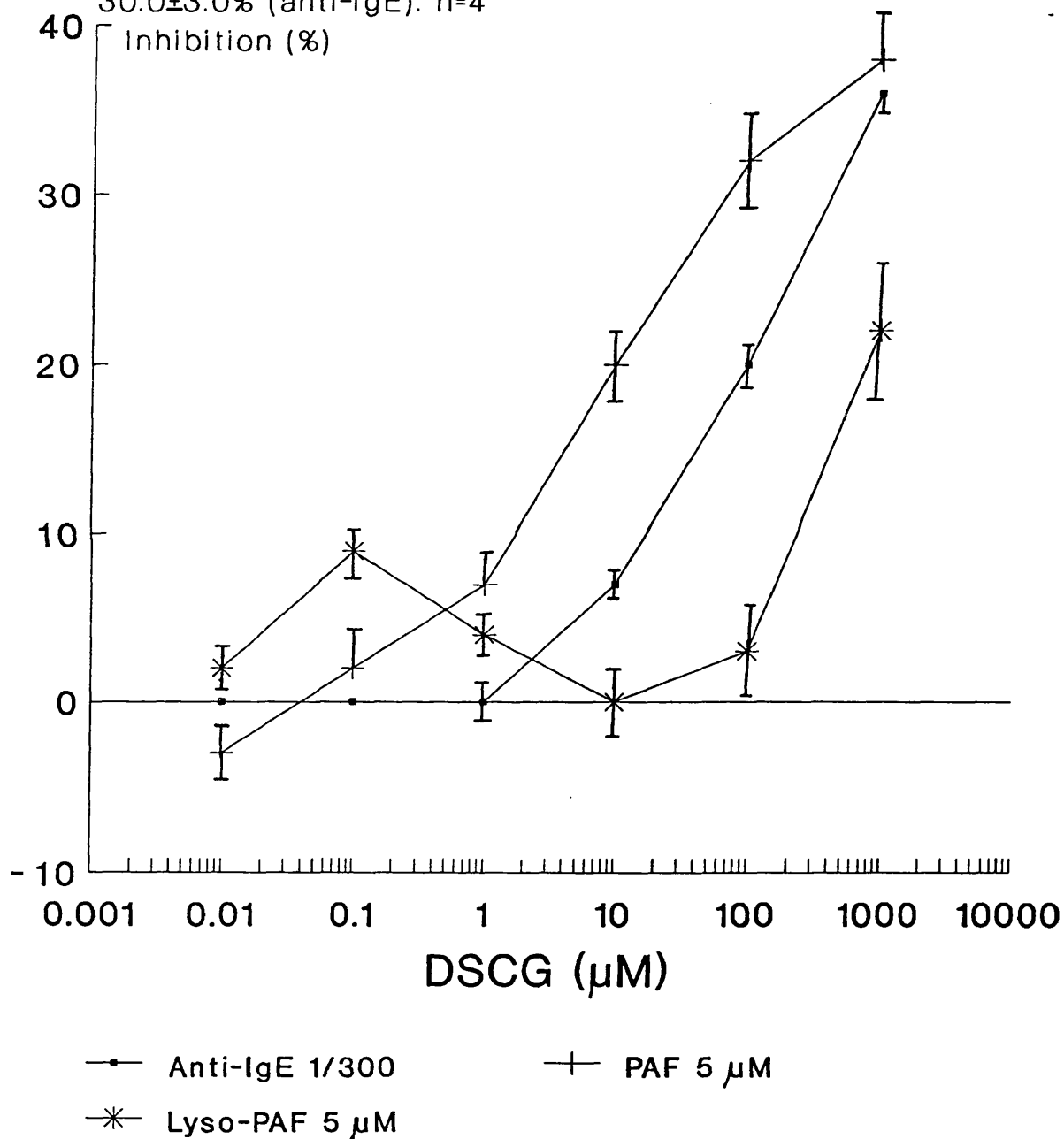


Fig.5.3 The effect of DSCG on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated for 30 min with the drug before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF), $13.0 \pm 2.0\%$ (lyso-PAF) and $20.0 \pm 3.0\%$ (anti-IgE). n=4

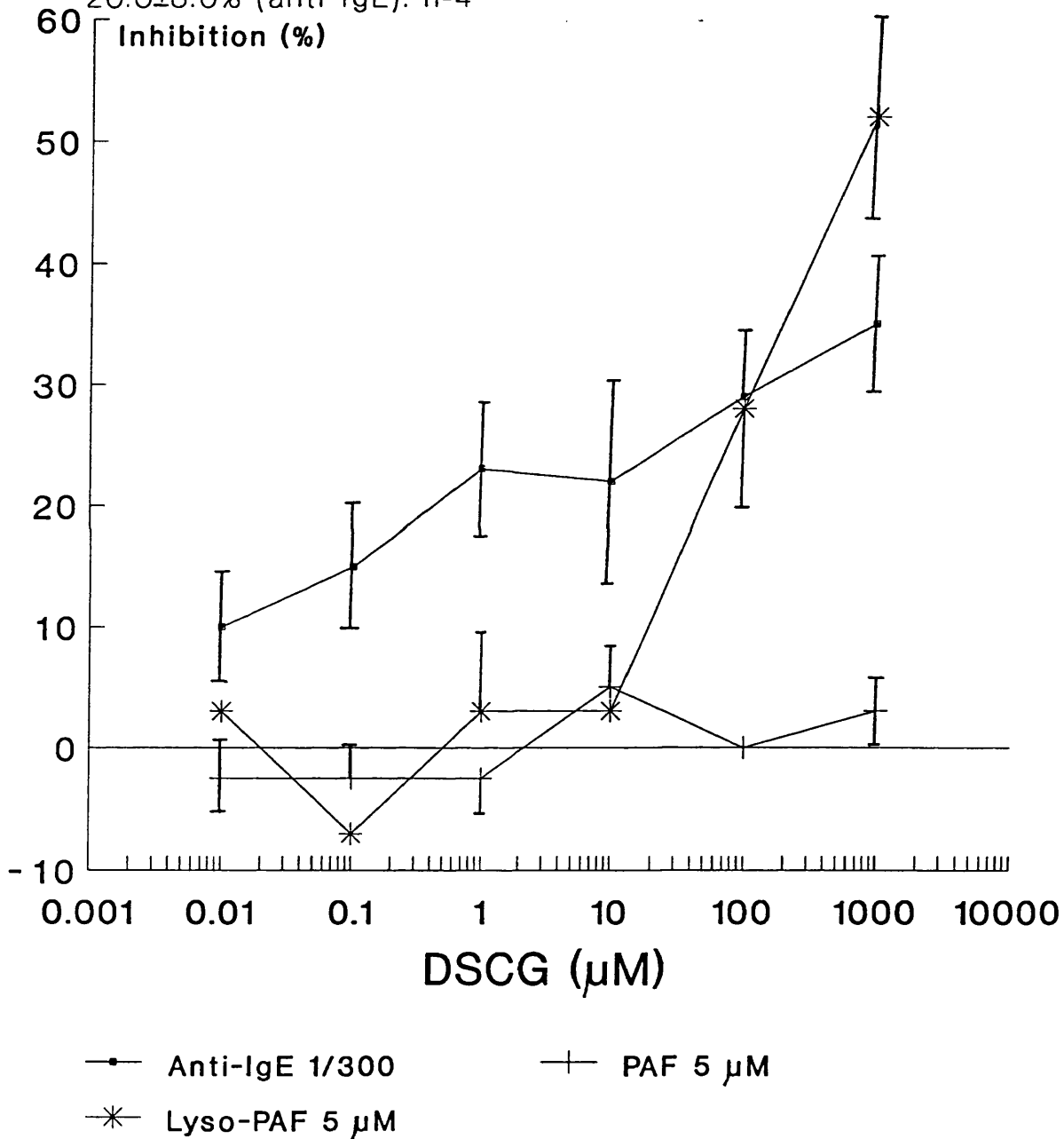


Fig.5.4 The effect of nedocromil sodium on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The drug was added to the cells simultaneously with the stimulus. Control releases were $15.0 \pm 2.5\%$ (PAF), $14.0 \pm 2.0\%$ (lyso-PAF) and $28.0 \pm 7.2\%$ (anti-IgE). n=4

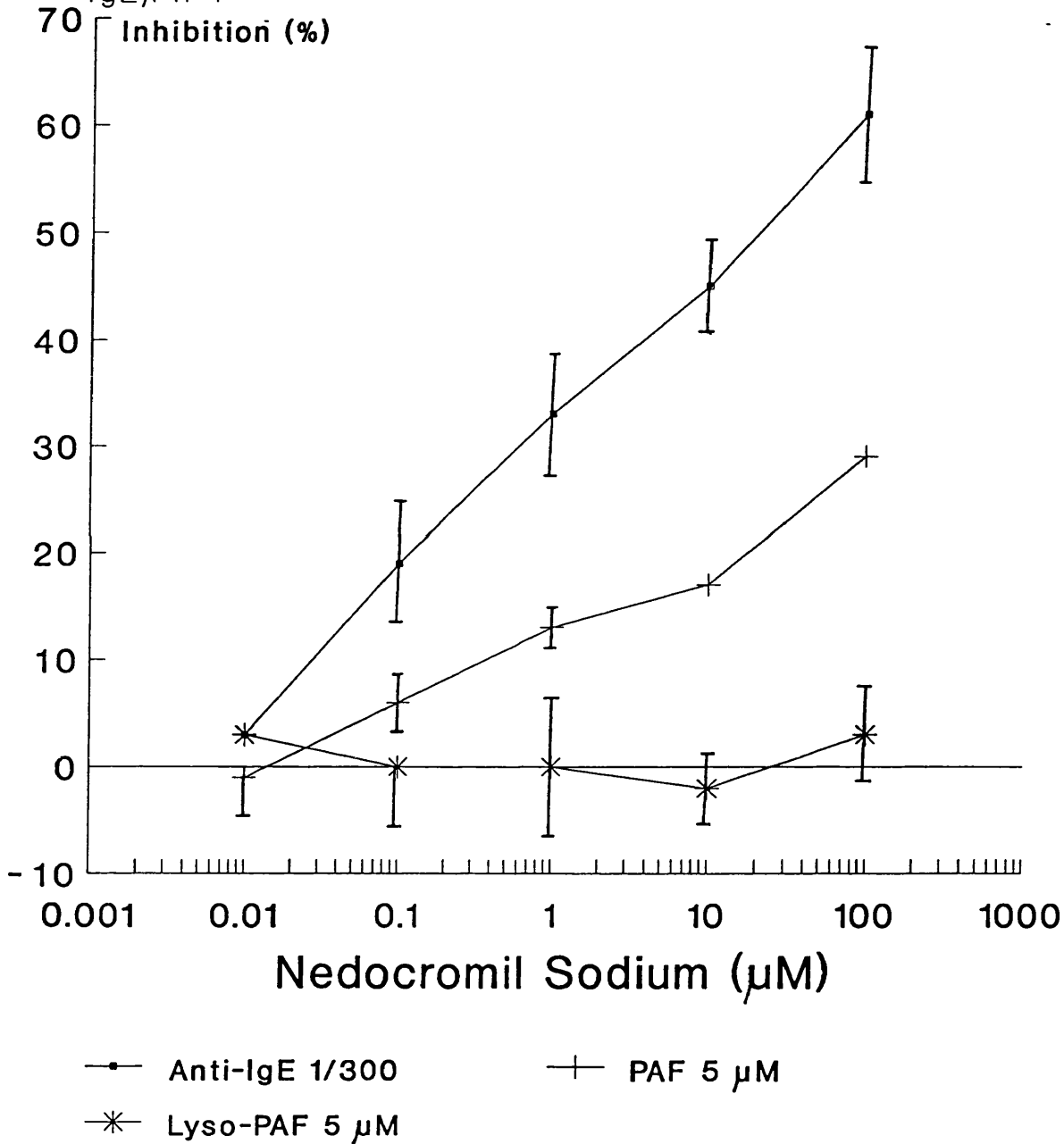


Fig5.5 The effect of nedocromil sodium on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated for 10 min with the drug before the stimulus was added. Control releases were $16.0 \pm 2.0\%$ (PAF), $14.0 \pm 1.0\%$ (lyso-PAF) and $30.0 \pm 3.0\%$ (anti-IgE). n=4

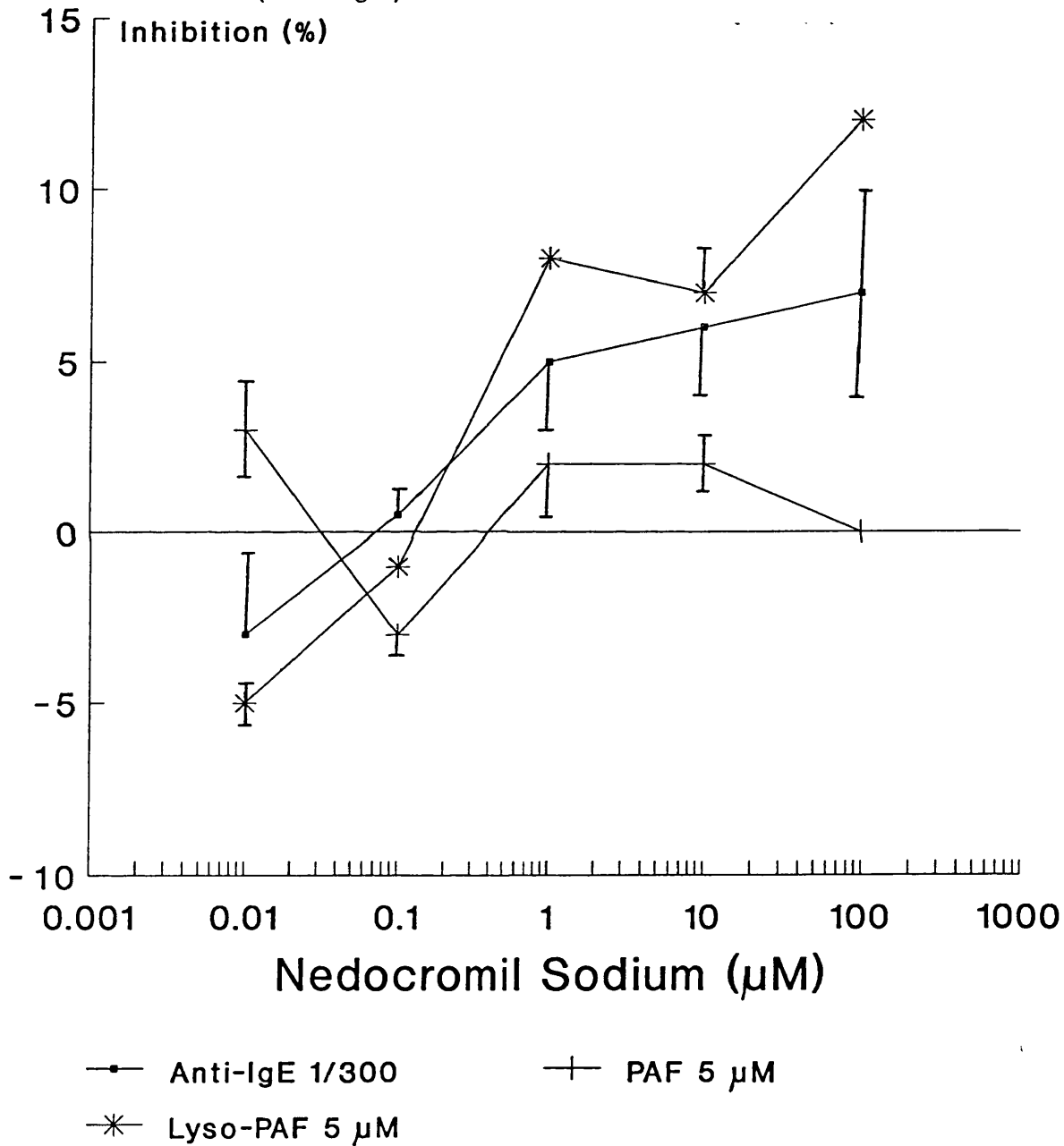


Fig.5.6 The effect of nedocromil sodium on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF), $13.0 \pm 2.0\%$ (lyso-PAF) and $20.0 \pm 3.0\%$ (anti-IgE). n=4

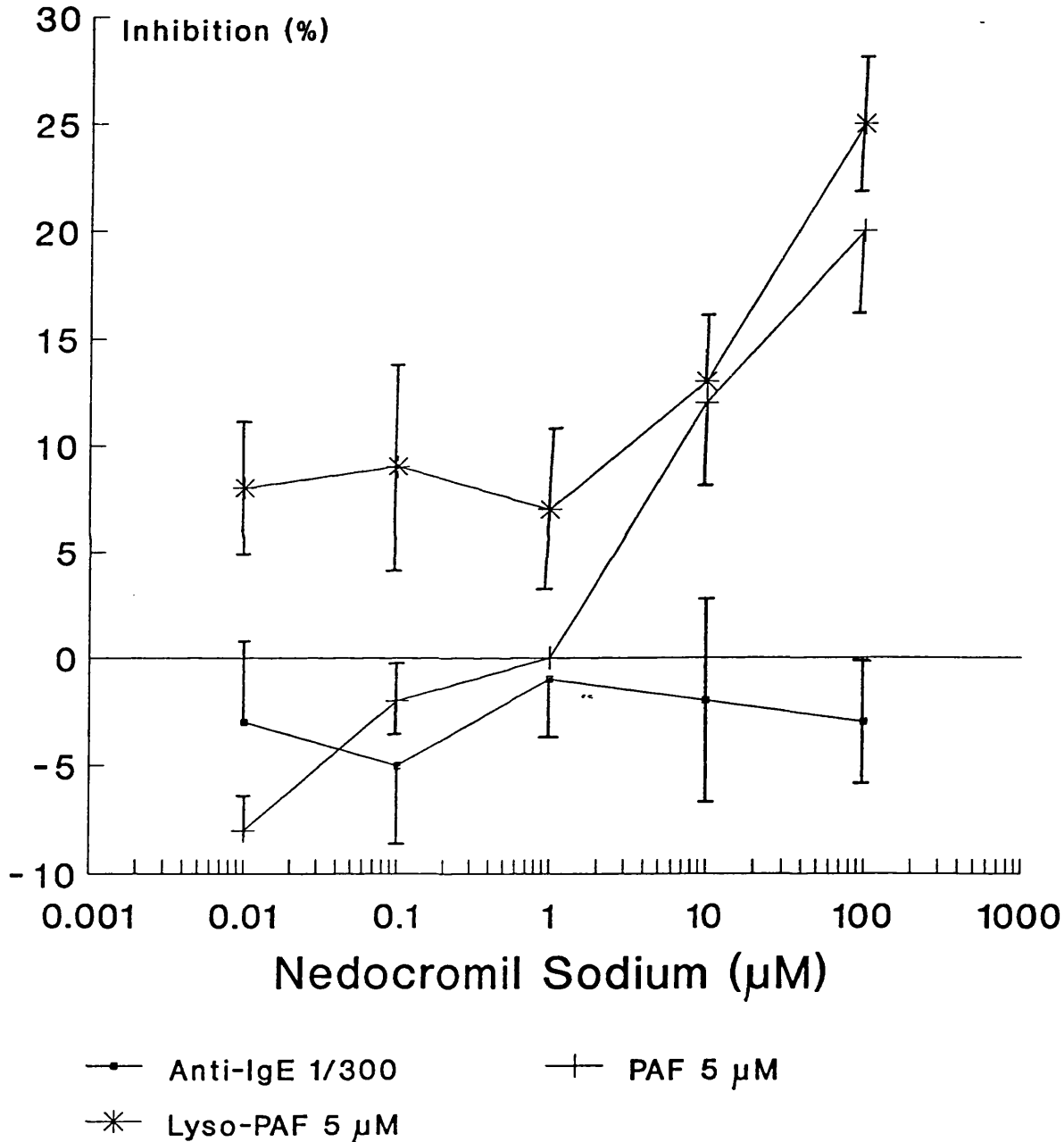


Fig.5.7 The effect of isoprenaline on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF) and $29.0 \pm 4.5\%$ (anti-IgE). n=4

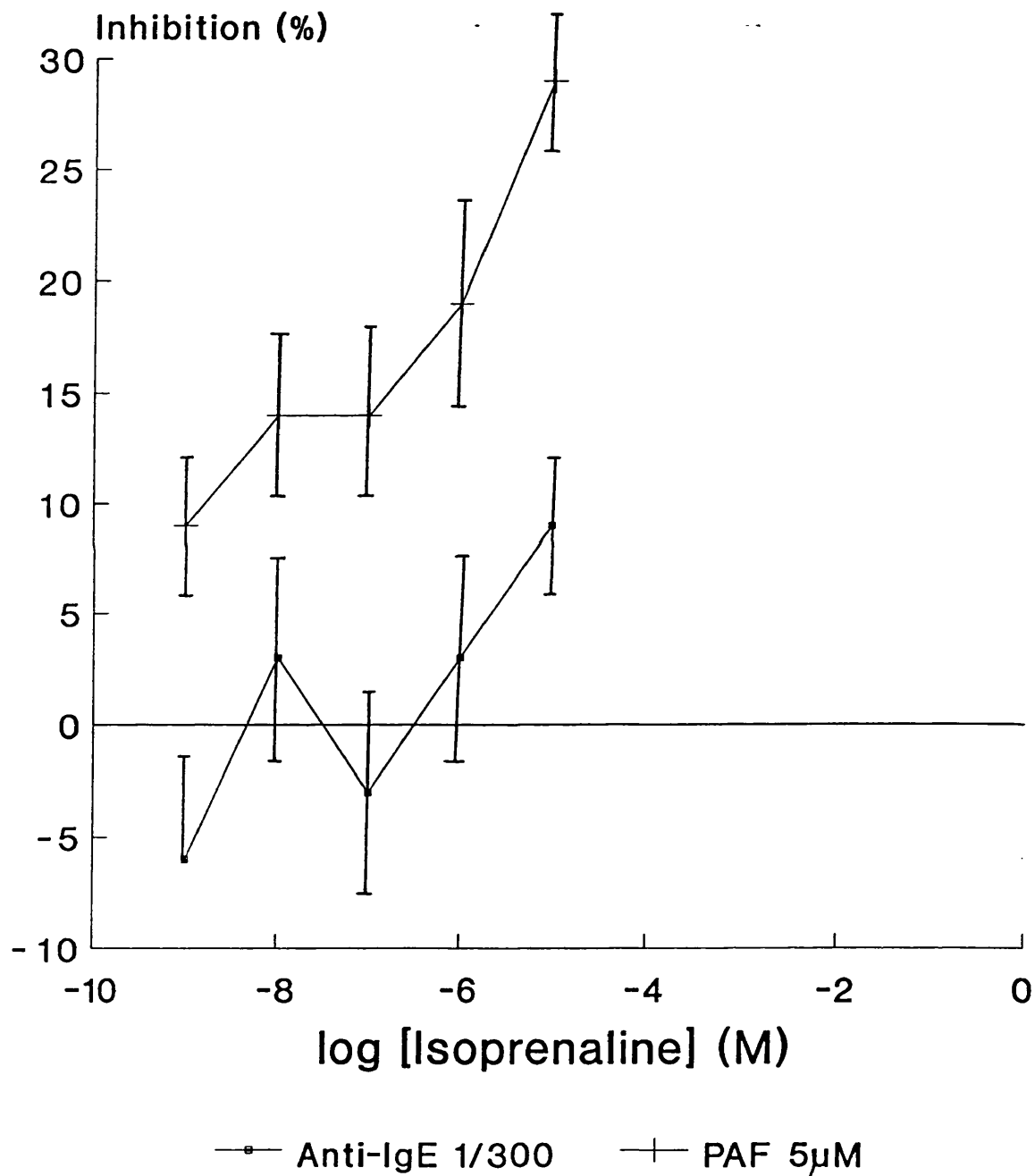


Fig.5.8 The effect of 8-bromo cAMP on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were $14.0 \pm 1.7\%$ (PAF) and $26.0 \pm 2.9\%$ (anti-IgE). $n=3-4$

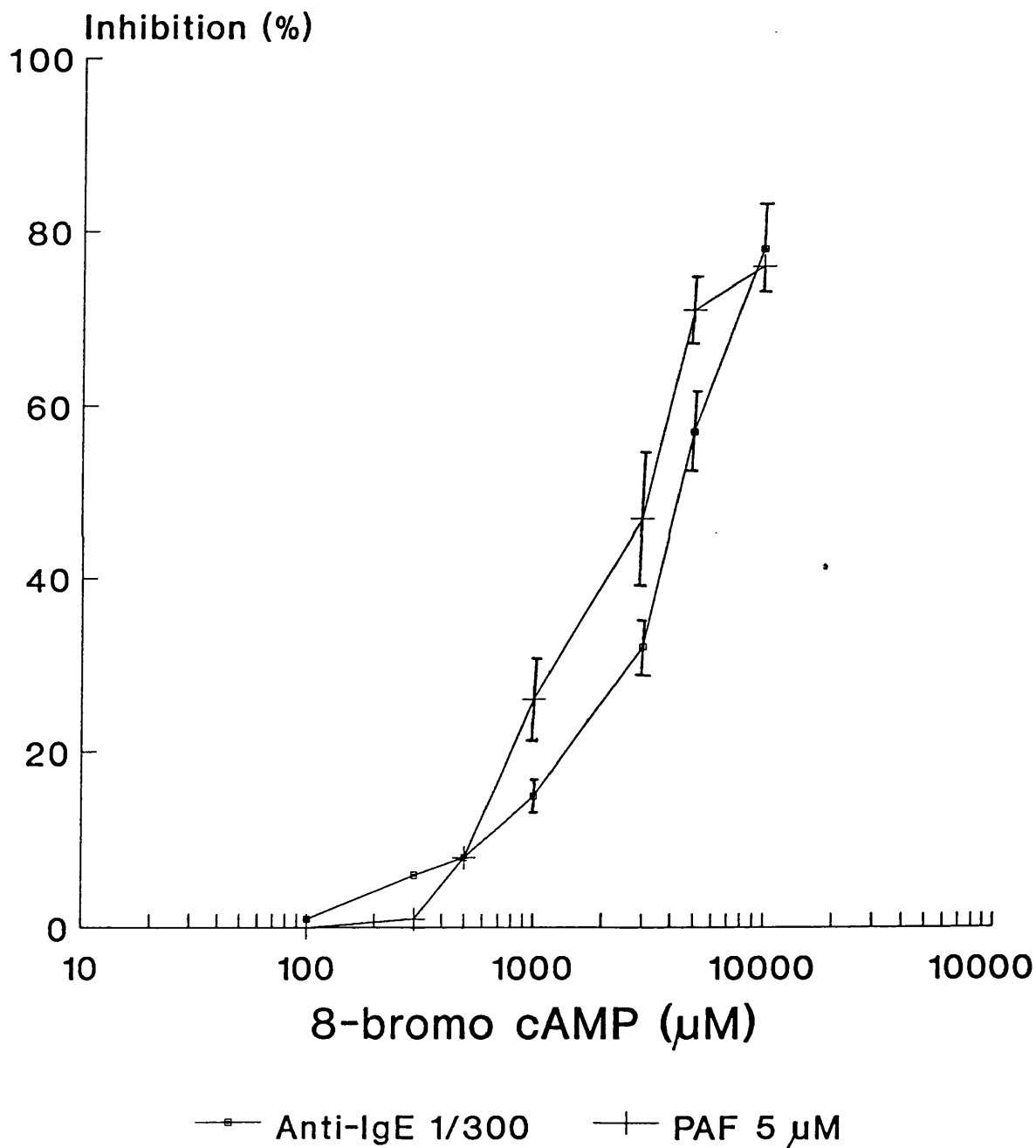


Fig.5.9 The effect of dibutyryl cAMP on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were $14.0 \pm 1.7\%$ (PAF) and $26.0 \pm 2.9\%$ (anti-IgE). n=4-6

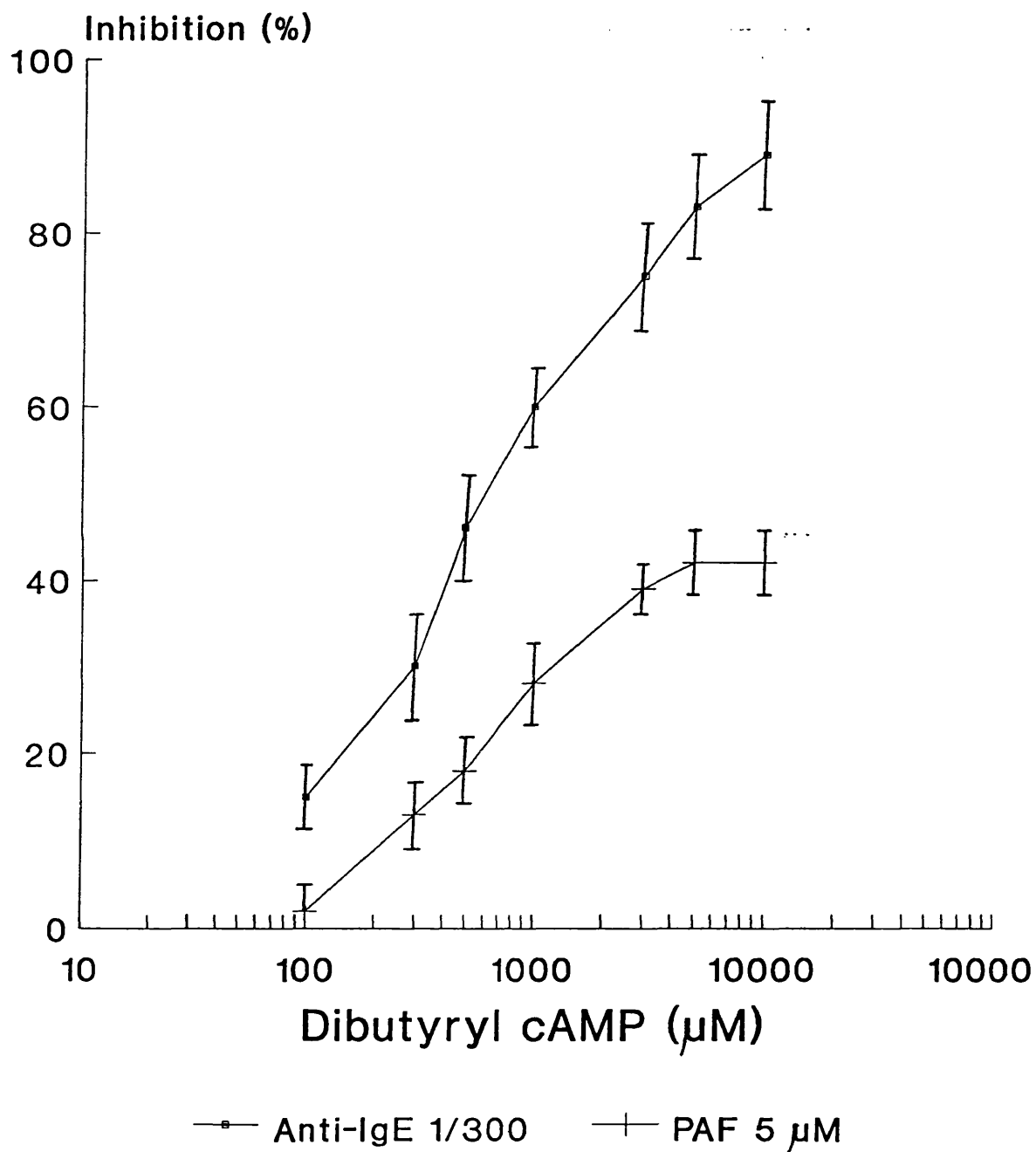


Fig.5.10 The effect of IBMX on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF) and $29.0 \pm 4.5\%$ (anti-IgE). $n=3$

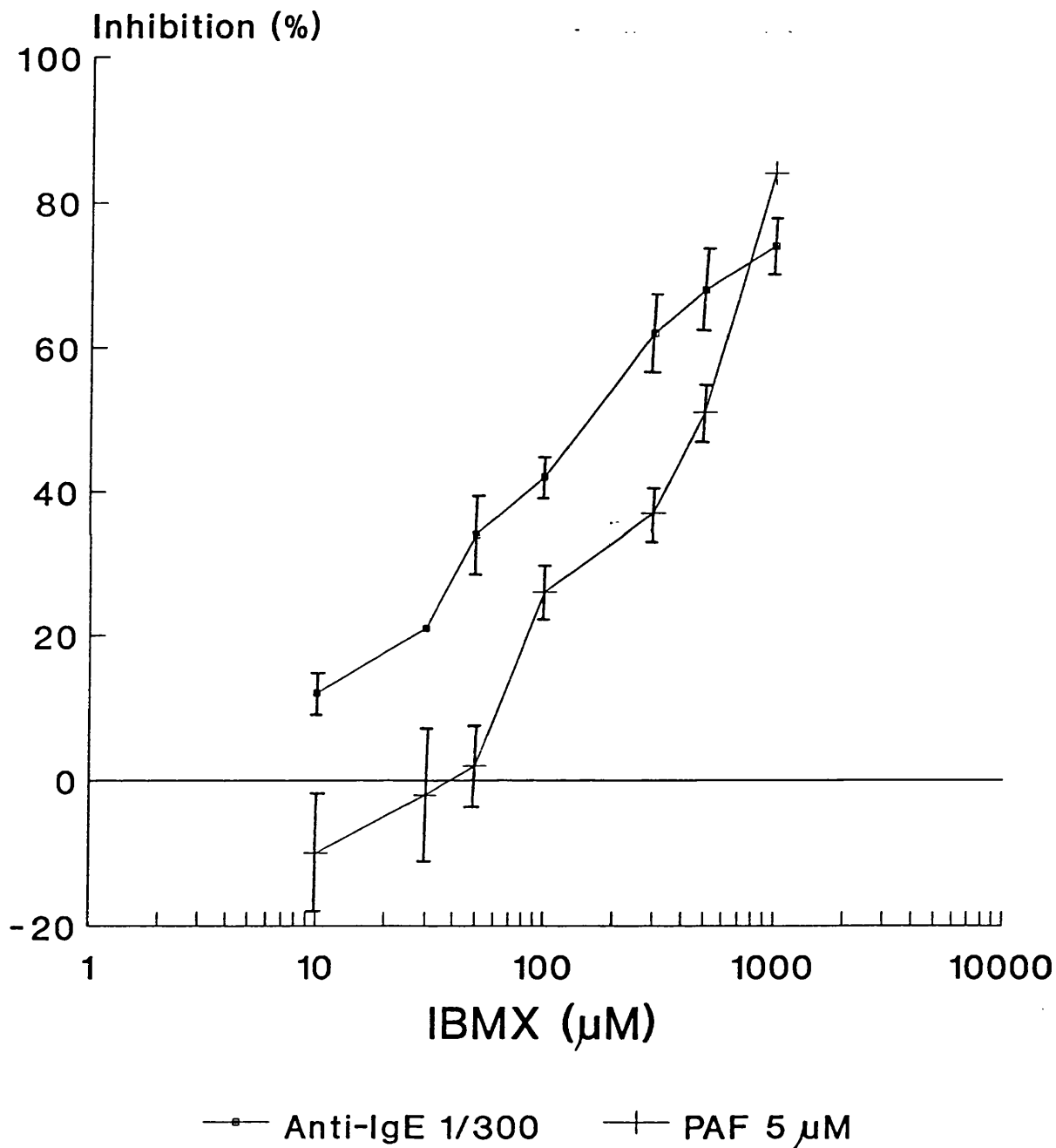


Fig.5.11 The effect of theophylline on histamine release from RPMC stimulated with PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF) and $29.0 \pm 4.5\%$ (anti-IgE). n=4

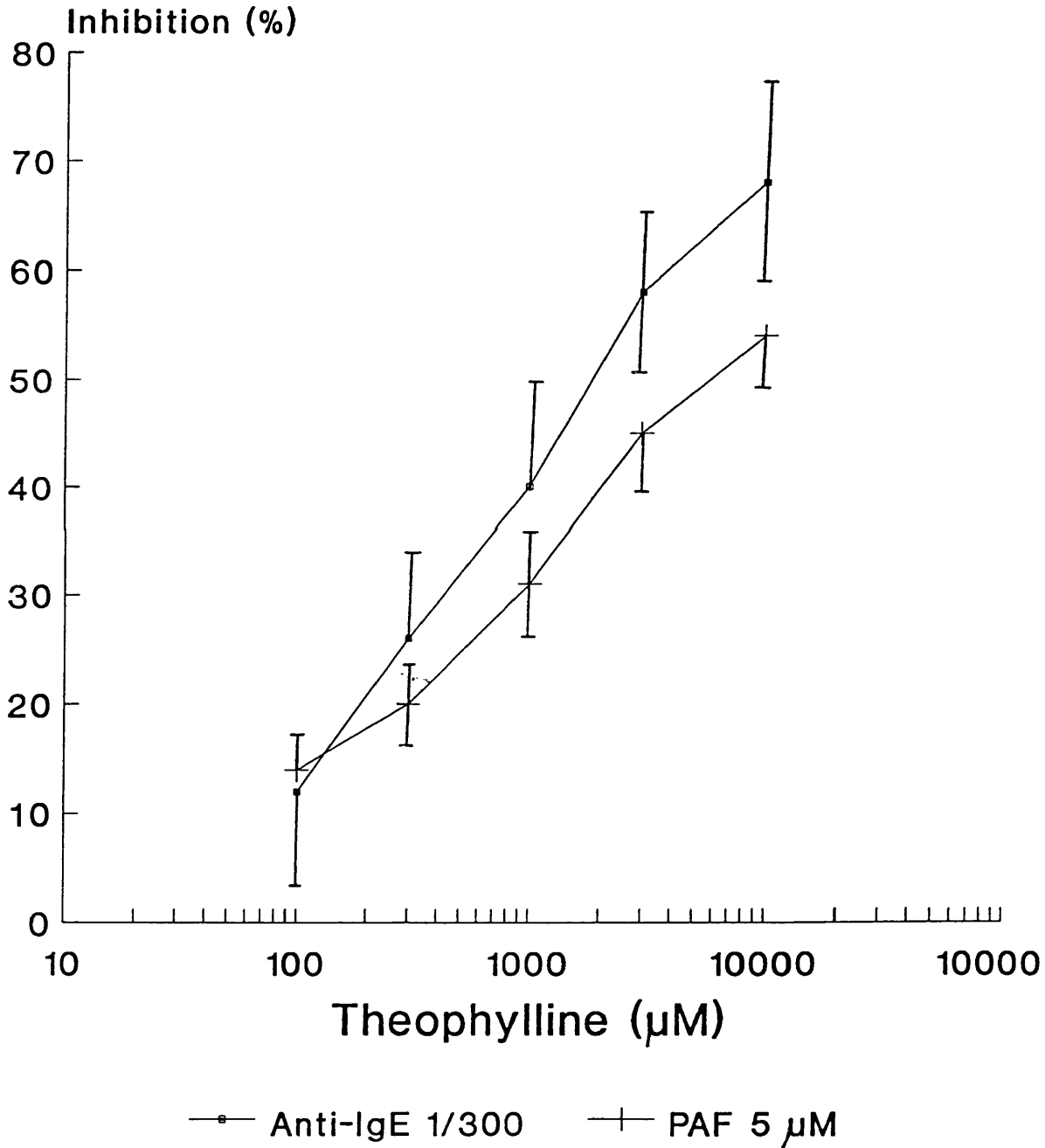


Fig.5.12 The effect of quercetin on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/300 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $15.0 \pm 3.0\%$ (PAF) and $29.0 \pm 4.5\%$ (anti-IgE), $n=4$

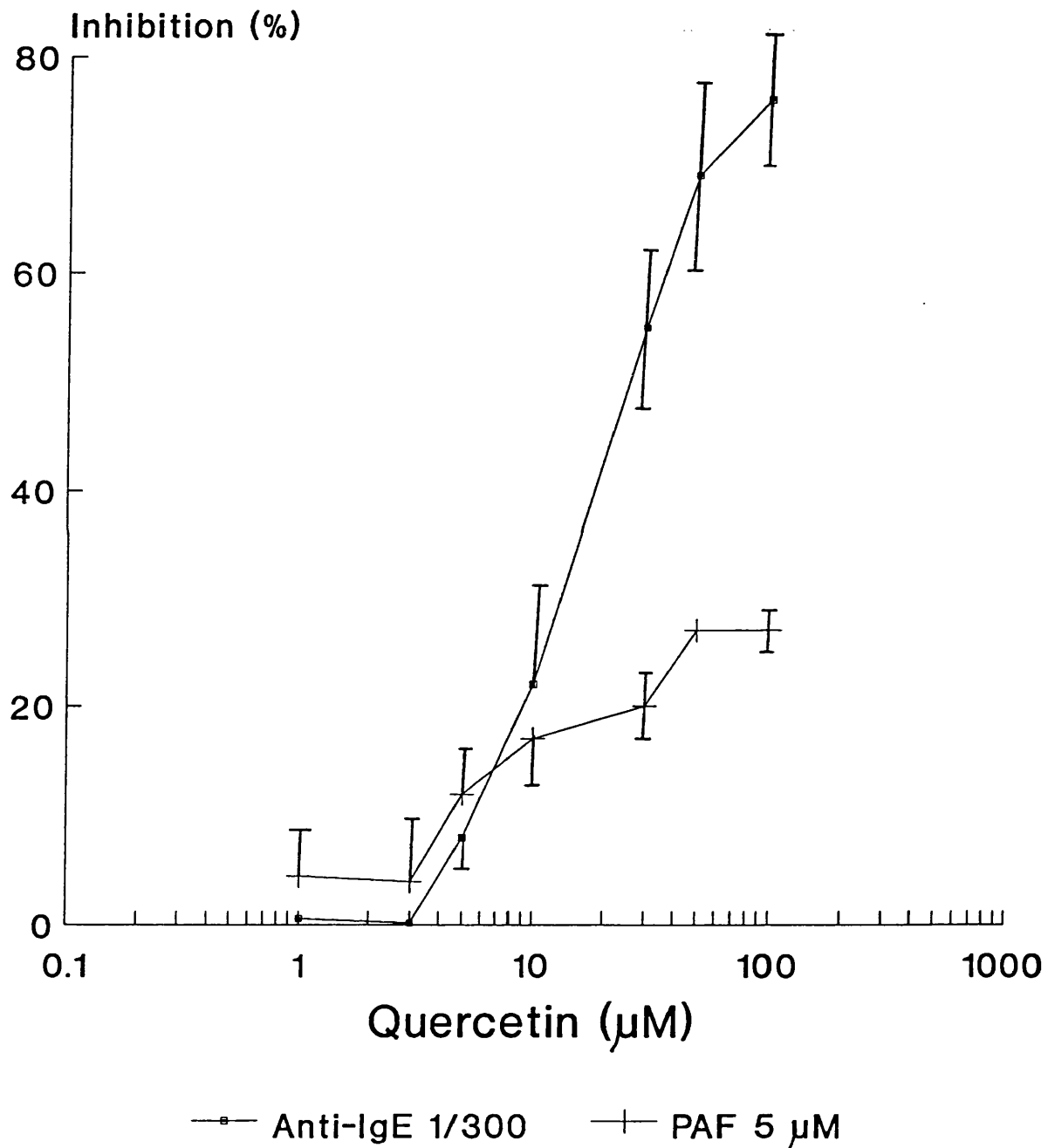


Fig.5.13 The effect of WEB2086 on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/250 dilution). The drug was added to the cells simultaneously with the stimulus. Control releases were 15.0 \pm 3.3% (PAF) and 36.0 \pm 4.2% (anti-IgE). n=4

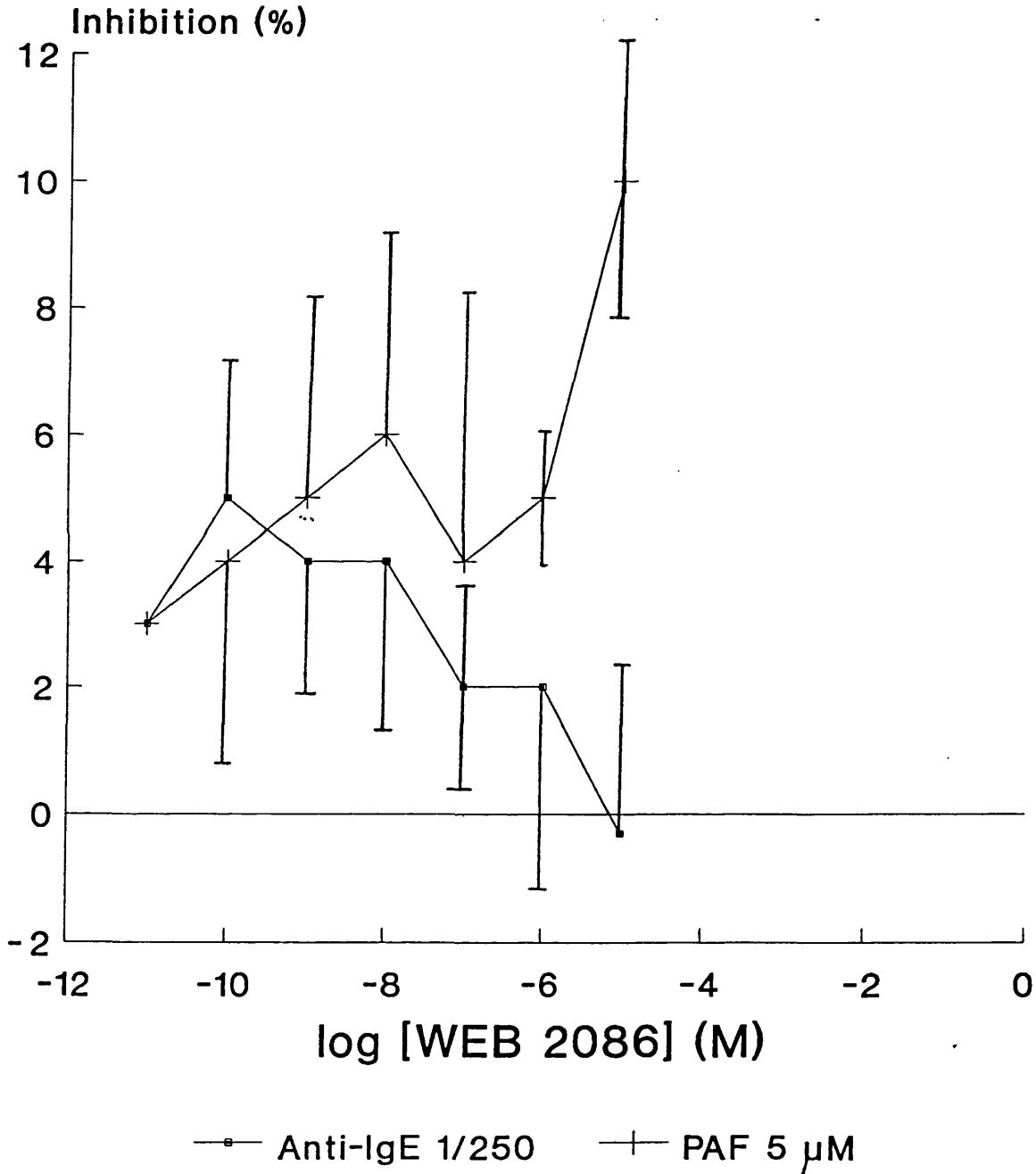


Fig.5.14 The effect of WEB2086 on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $14.7 \pm 2.5\%$ (PAF) and $30.4 \pm 3.9\%$ (anti-IgE). n=4

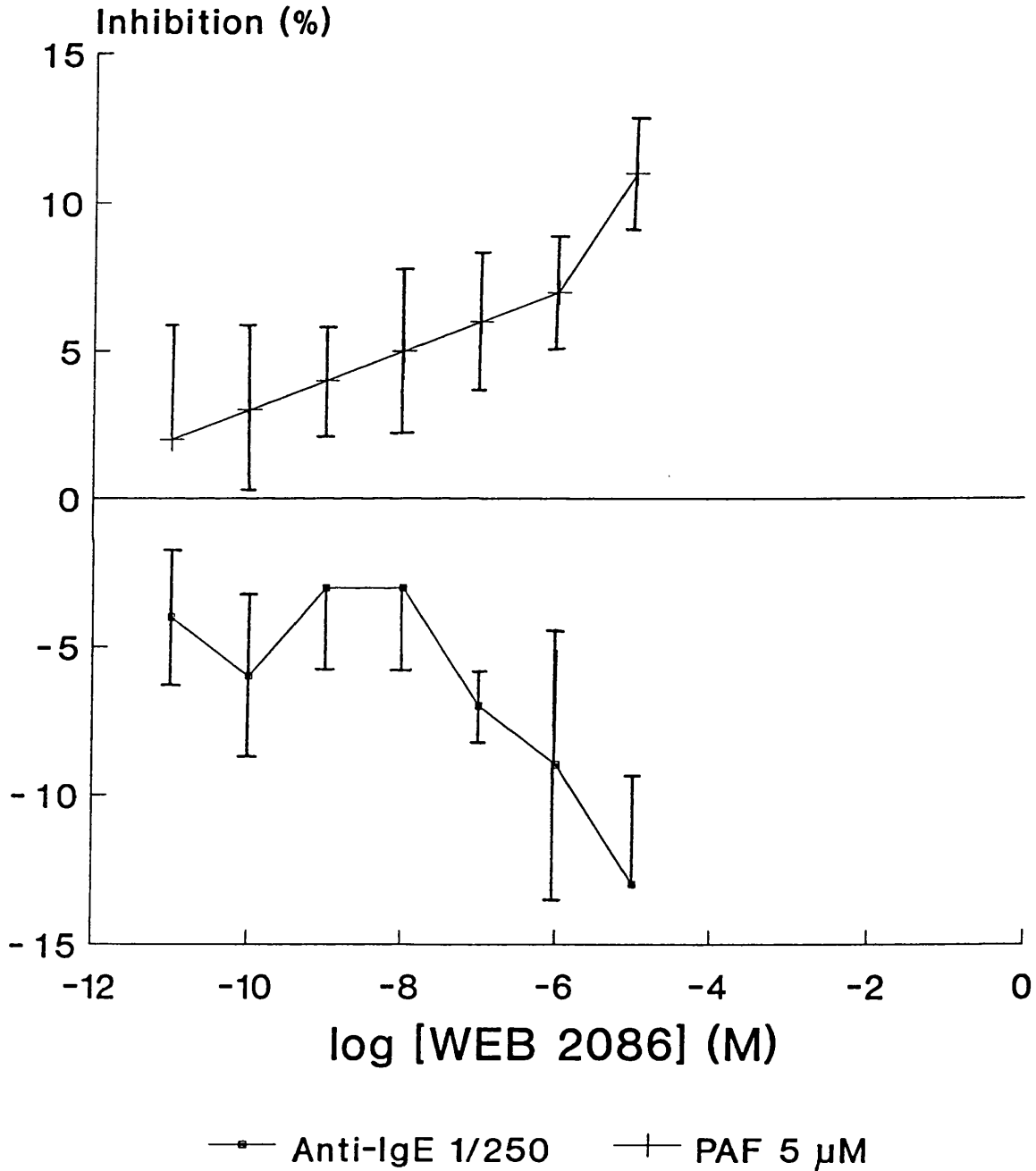


Fig.5.15 The effect of WEB2086 on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were $13.0 \pm 1.6\%$ (PAF) and $26.2 \pm 2.0\%$ (anti-IgE). n=4

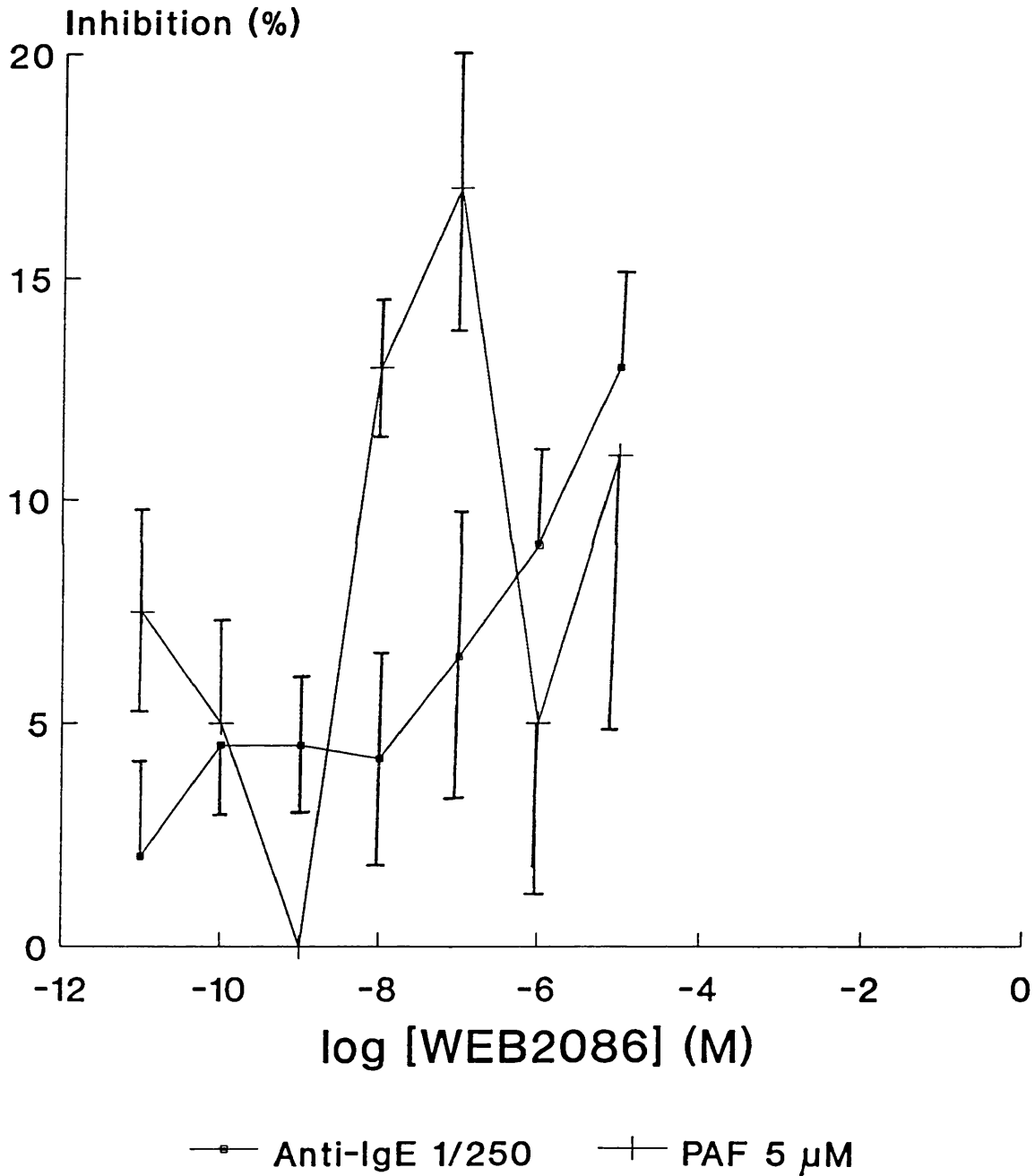


Fig.5.16 The effect of BN52021 on histamine release from RPMC stimulated by PAF (5 μ M) and anti-IgE (1/250 dilution). The drug was added to the cells simultaneously with the stimulus. Control releases were 15.0 \pm 3.3% (PAF) and 36.0 \pm 4.2% (anti-IgE). n=4

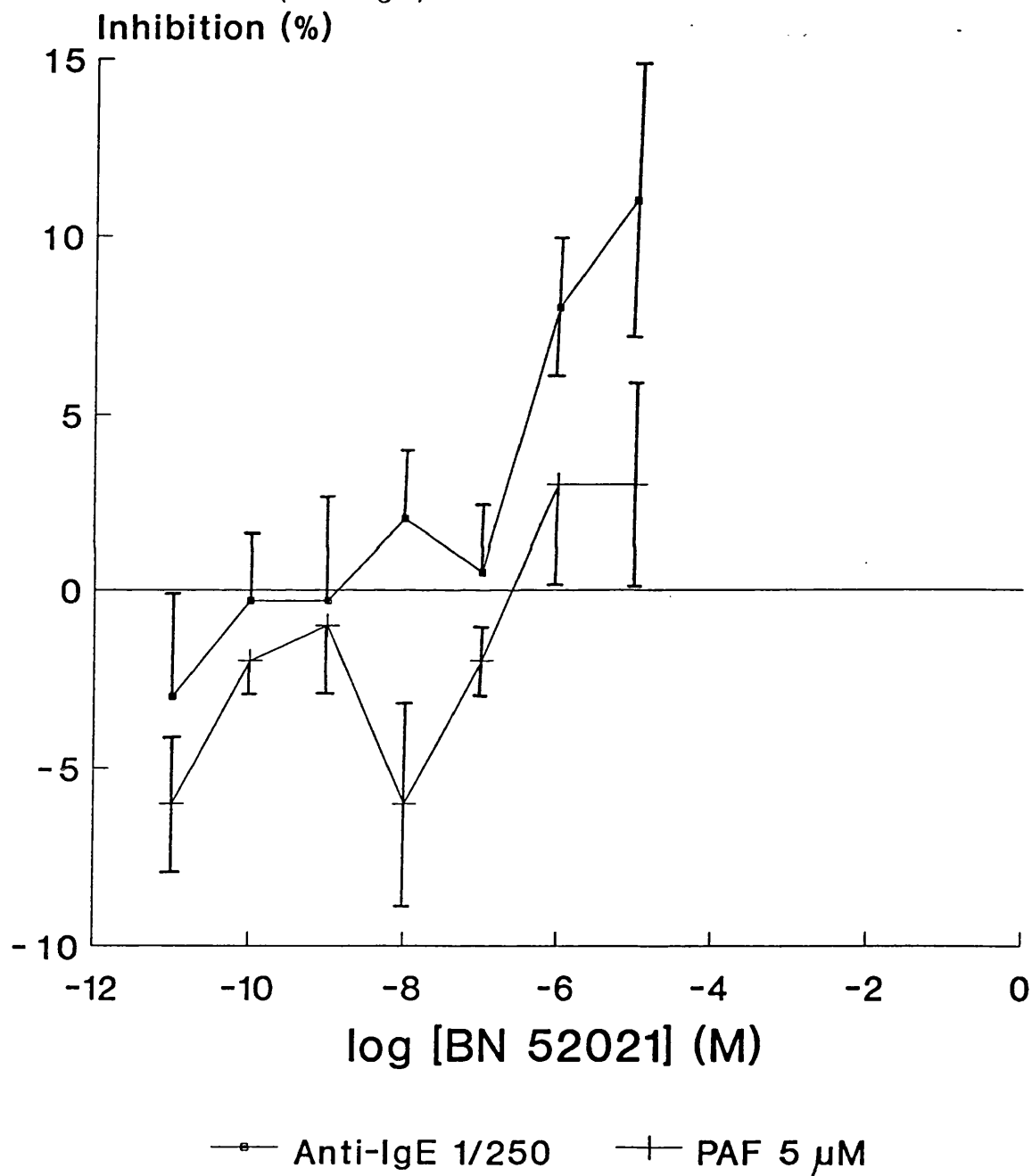


Fig.5.17 The effect of BN52021 on histamine release from RPMC stimulated with PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated for 10 min with the drug before the stimulus was added. Control releases were $14.7 \pm 2.5\%$ (PAF) and $30.4 \pm 3.9\%$ (anti-IgE). n=4

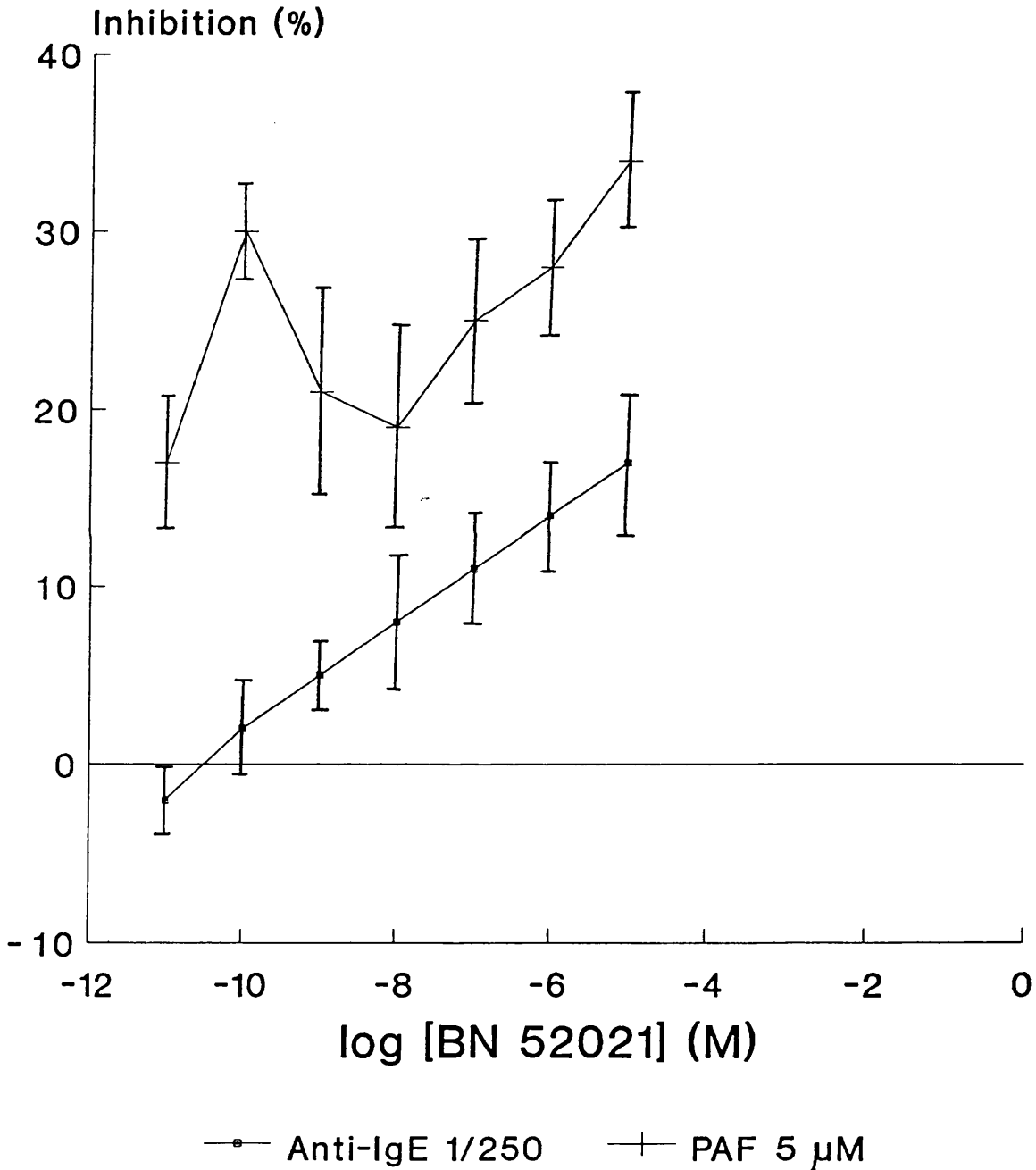


Fig.5.18 The effect of BN52021 on histamine release from RPMC stimulated with PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were $13.4 \pm 1.6\%$ (PAF) and $26.2 \pm 2.0\%$ (anti-IgE). n=4

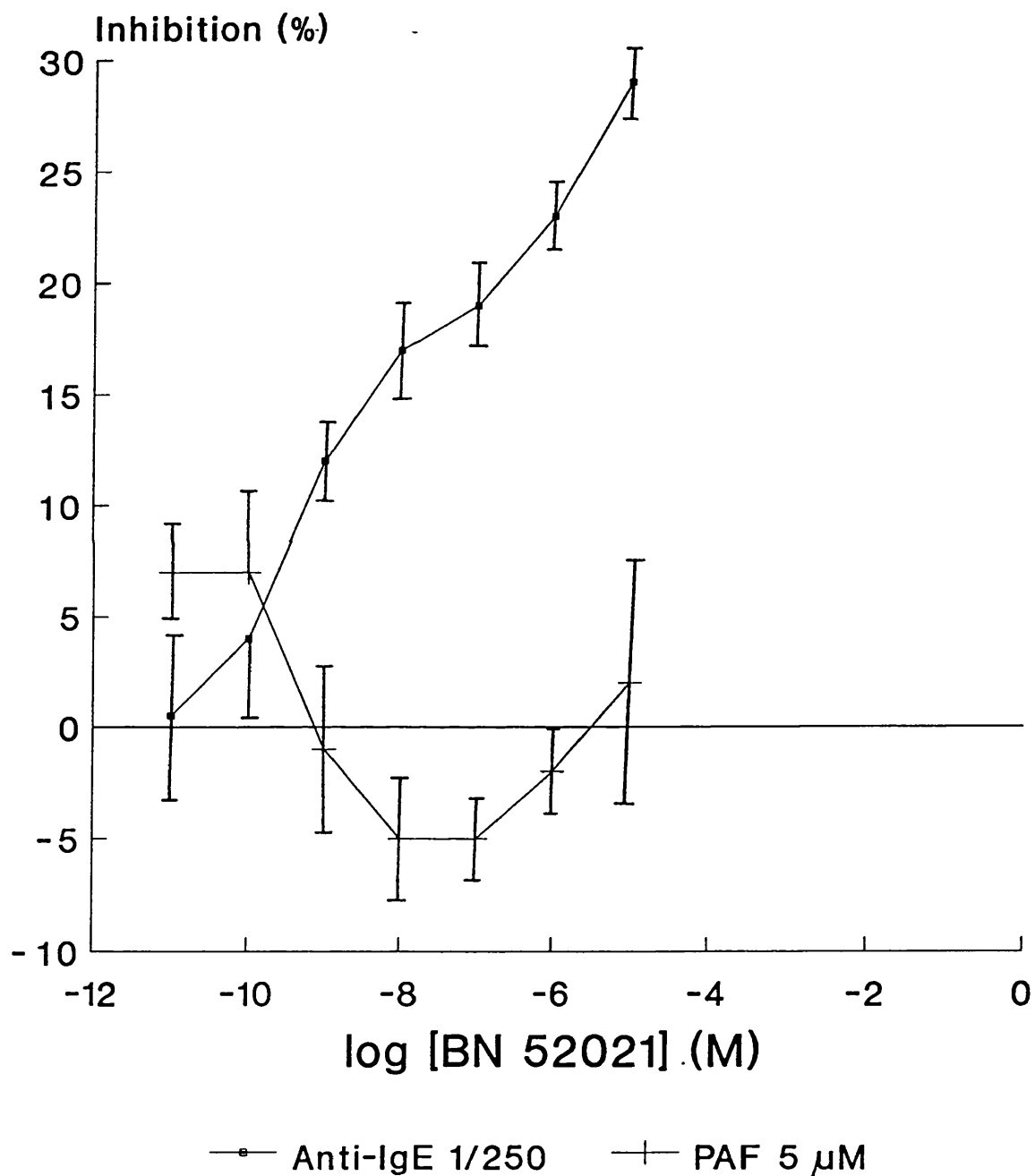


Fig.5.19 The effect of BN50730 on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/250 dilution). The drug was added to the cells simultaneously with the stimulus. Control releases were $15.0 \pm 3.3\%$ (PAF), $14.0 \pm 2.0\%$ (lyso-PAF) and $36.3 \pm 4.2\%$ (anti-IgE). n=4

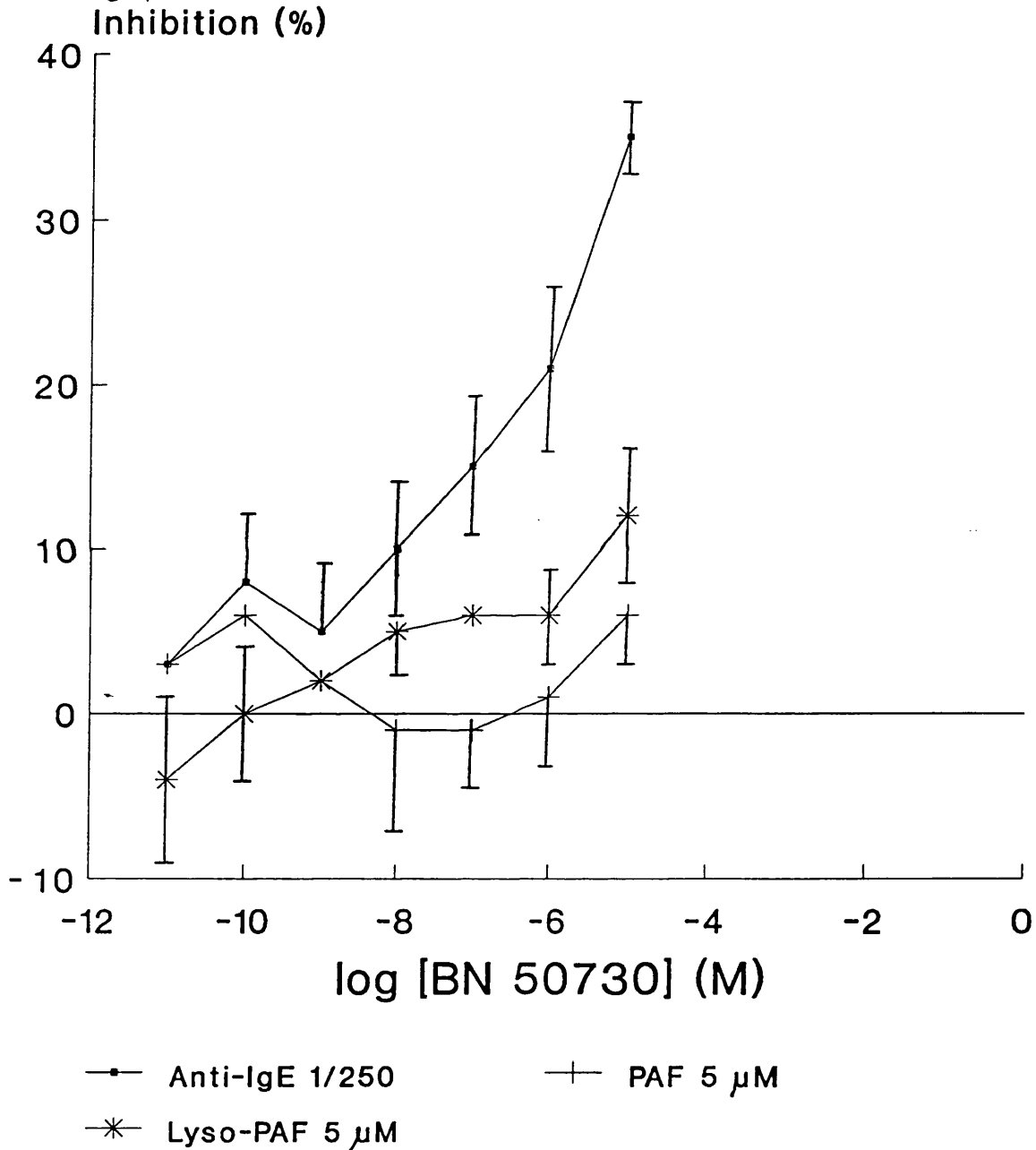


Fig.5.20 The effect of BN50730 on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated with the drug for 10 min before the stimulus was added. Control releases were $14.7 \pm 2.5\%$ (PAF), $13.3 \pm 1.0\%$ (lyso-PAF) and $30.0 \pm 4.0\%$ (anti-IgE). n=4

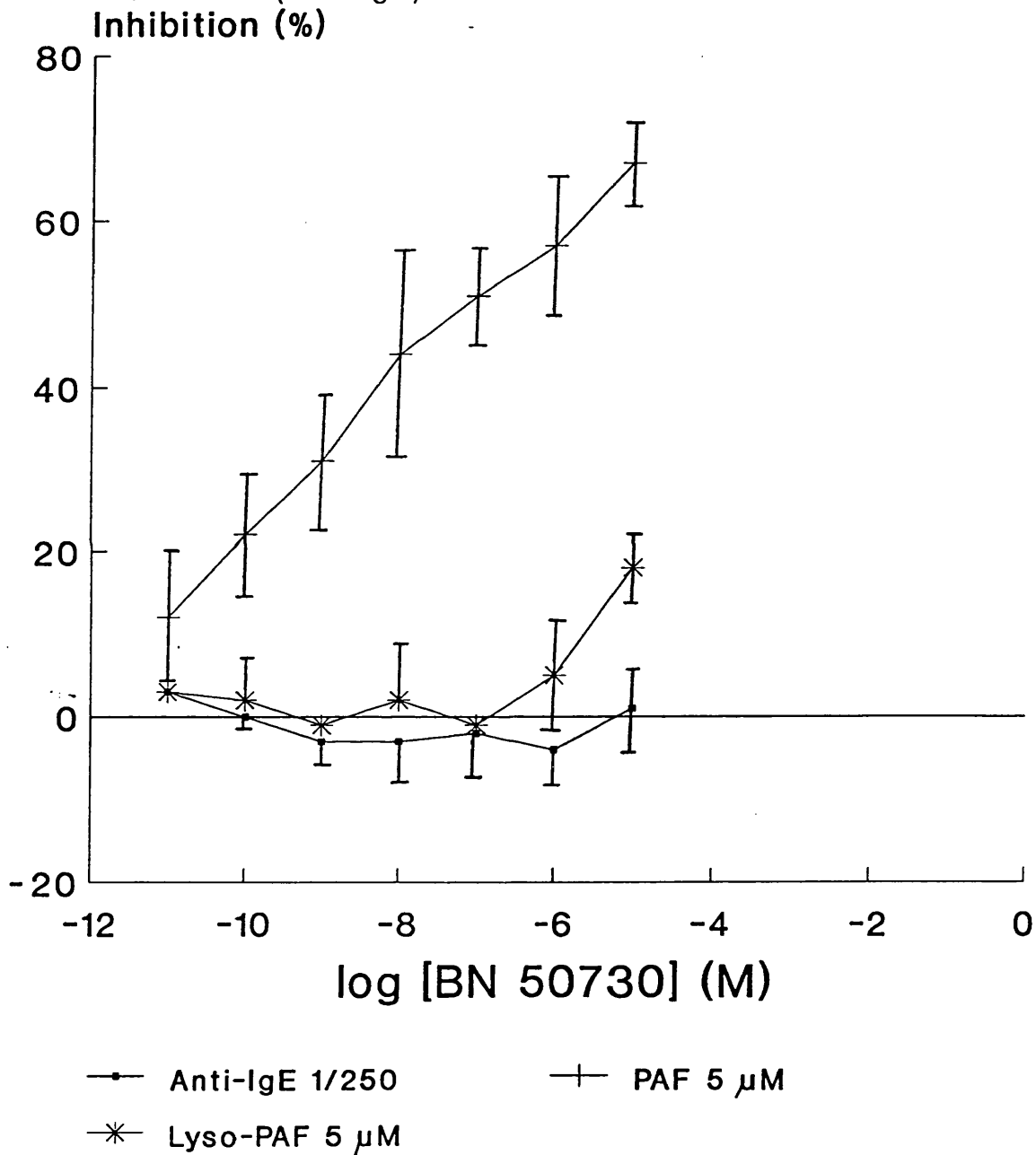


Fig.5.21 The effect of BN50730 on histamine release from RPMC stimulated with PAF (5 μ M), lyso-PAF (5 μ M) and anti-IgE (1/250 dilution). The cells were preincubated with the drug for 30 min before the stimulus was added. Control releases were 13.4 \pm 1.6% (PAF), 15.3 \pm 1.0% (lyso-PAF) and 26.0 \pm 2.0% (anti-IgE). n=4

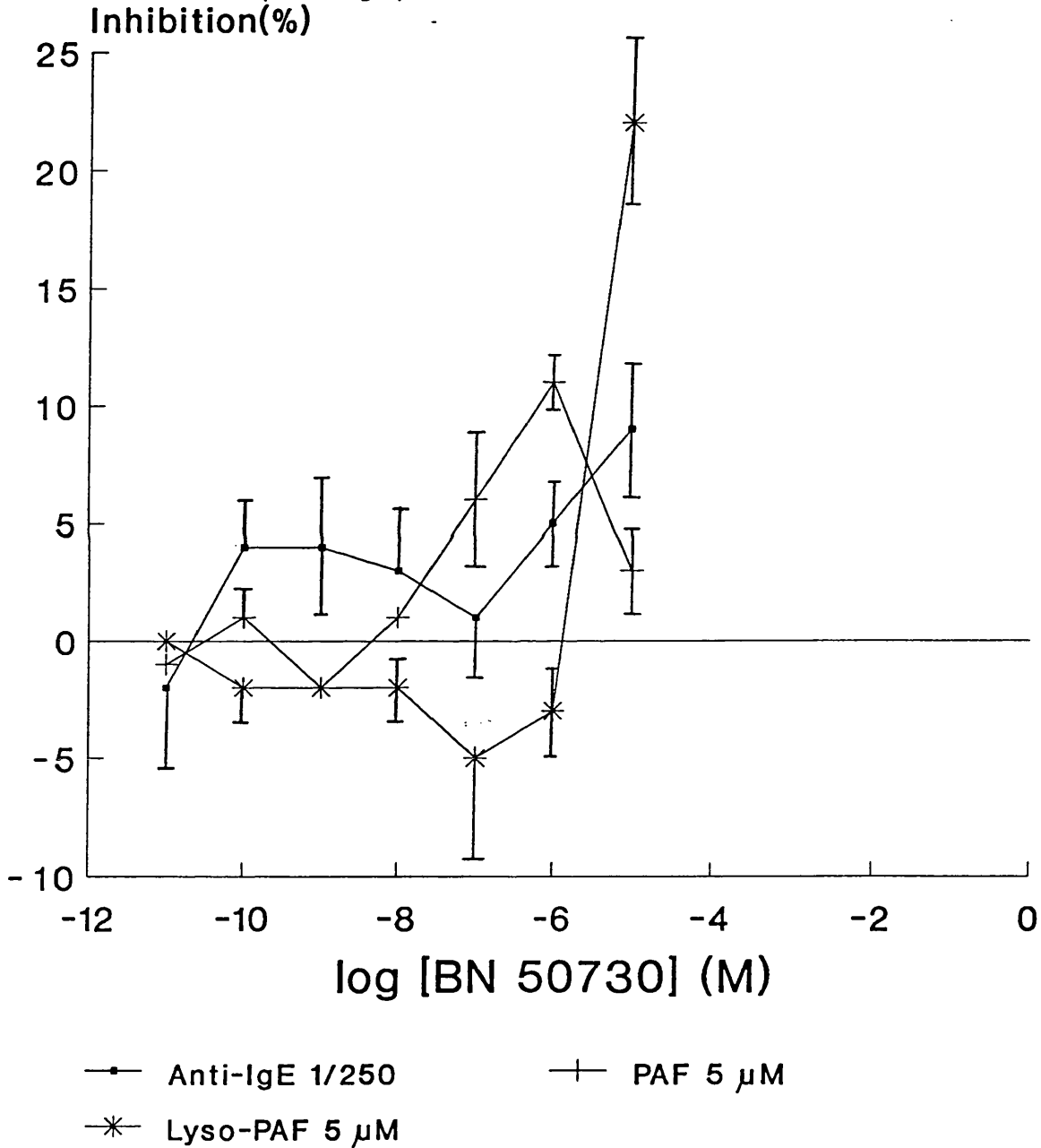


Table 5.1 The effect of Lyso-PAF on histamine release from rat peritoneal mast cells. n=4

Lyso-PAF (μM)	Histamine Release (%)
1	5.3 ± 1.1
10	28.5 ± 2.3
100	73.0 ± 5.0

Values are means \pm SEM for 4 experiments.

Table 5.2 Some IC_{50} values of the PAF antagonist WEB2086 in various PAF mediated effects.

Effect	IC_{50} value
1. PAF-induced platelet aggregation in vitro.	0.17 μ M
2. PAF-induced human neutrophil aggregation in vitro.	0.36 μ M

Compiled from ref. 356.

Table 5.3 Some IC_{50} values of the PAF antagonist BN50201 in various PAF mediated effects.

Effect	IC_{50} value
1. PAF-induced neutrophil chemotaxis in vitro.	1 nM
2. PAF-induced platelet aggregation in vitro.	0.3 μ M
3. PAF-induced contractions of guinea-pig lung parenchymal tissue in vitro.	1 nM

Compiled from ref. 375 and 376.

Table 5.4 Some IC_{50} values of the PAF antagonist BN50730 in various PAF mediated effects.

Effect	IC_{50} value
1. PAF-induced platelet aggregation in vitro.	0.2 μ M
2. PAF-induced bronchospasm in the guinea-pig.	2.7 nM

Compiled from ref. 358.

CHAPTER SIX

THE EFFECT OF PAF ON ISOLATED HUMAN NEUTROPHILS.

6.1 INTRODUCTION.

The human neutrophil is a polymorphonuclear leukocyte derived from pluripotential stem cells within the bone-marrow. Neutrophils are characterized by possessing a nucleus which consists of several lobes joined together by thin strands of nuclear material. The cell itself is highly motile, and is usually the first cell to appear at the site of injury mainly in response to the presence of various chemotactic factors.

Neutrophils, besides ingesting and digesting the injurious stimuli, are also capable of releasing a number of chemicals which may have important roles in the pathogenesis of several allergic disorders. These include tissue-damaging O_2 metabolites, acid hydrolases, serine proteases, anti-bacterial enzymes and cationic proteins. In addition to secreting a variety of chemicals, *de novo* synthesis of phospholipid metabolites such as LTB_4 and PAF are initiated.

6.2 BIOCHEMICAL EVENTS FOLLOWING NEUTROPHIL STIMULATION.

6.2.1 BREAKDOWN OF PHOSPHOINOSITIDES.

As stated previously in chapter one, a breakdown of polyphospho- inositides (PPI) occurs following cellular activation. Dougherty and co-workers reported the formation of phosphatidylinositol-4,5-bisphosphate (PIP_2) from PPI following stimulation of the neutrophil. Further breakdown of PIP_2 mediated by the enzyme phospholipase C (PLC) results in the formation of IP_3 and DAG [360]. IP_3 then goes on to mobilize internal calcium stores, while DAG activates the enzyme PKC.

The above pathways contribute towards the overall response of the activated neutrophil.

6.2.2 THE ROLE OF CALCIUM IONS.

Experimental studies have identified calcium as an important transducer in certain neutrophil-mediated responses such as chemotaxis and degranulation. Increased intracellular calcium ion levels also activate the enzyme phospholipase A₂ (PLA₂) which is involved in the metabolism of membrane-bound phospholipids.

6.23. METABOLISM OF MEMBRANE-BOUND PHOSPHOLIPIDS.

Following stimulation of the human neutrophil, methylation of membrane-bound phospholipids is initiated by the presence of PLA₂. Formation of arachidonic acid (AA) occurs, which is further metabolised via the lipoxygenase pathway to form LTB₄. PLA₂ activity also gives rise to the formation of PAF from other membrane-derived phospholipids [254].

6.3 RESPONSES OF THE NEUTROPHIL.

The earliest response of the neutrophil, once it has reached the site of inflammation, is to adhere to the endothelium of small venules. Once activated, the neutrophil undergoes exocytosis and releases its granular contents into the surrounding tissue. The neutrophil is also capable of engulfing certain micro-organisms by a process known as phagocytosis. Thus, neutrophils are capable of a wide variety of actions all of which contribute to the overall pathogenesis of disease as well as host defence.

6.4 THE ROLE OF NEUTROPHILS IN ALLERGY AND INFLAMMATION.

It has been well documented that neutrophils are the first cells to reach the site of inflammation. The emigration of neutrophils from blood to tissues involves the chemotactically mediated transmigration of these cells across endothelial and

epithelial barriers [361]. Once the cells have reached their destination, they accumulate rapidly, undergo stimulation and thus prolong the physiological disorder. During IgE-mediated reactions especially in the skin, neutrophil infiltration occurs rapidly at the site of injury. A similar situation was found to exist in the lungs of asthmatic experimental animals and in humans following allergen or exercise induced asthma [362-364].

Thus, the neutrophil together with other inflammatory cells may be involved to a greater or lesser extent in the pathogenesis of allergic disorders such as asthma. However, no current evidence exists for believing that this cell plays a unique role.

6.5 THE EFFECT OF PAF ON NEUTROPHILS.

PAF is able both chemically to attract and stimulate human neutrophils, thus indicating a possible involvement of the two in the pathogenesis of allergic disorders [365]. Casale and co-workers demonstrated the ability of PAF to induce neutrophil migration across the epithelial barriers of cultured cells [361]. Recent studies have shown that PAF also induces neutrophil aggregation, adherence, degranulation, LT synthesis and superoxide anion production [366,367].

Conflicting evidence exists regarding the ability of PAF to stimulate its own synthesis from neutrophils via specific PAF membrane receptors [368]. Sissons and co-workers reported that exogenous PAF does not elicit its own synthesis from human neutrophils, whereas Doebber and co-workers reported otherwise [369,370]. However, more recent studies have confirmed that PAF does indeed further its own formation via a receptor-mediated mechanism from human neutrophils [368].

The capacity of PAF to stimulate its own synthesis from neutrophils would result in a prolonged half-life *in vivo*. This prolonged half-life could contribute to the capacity of PAF to induce prolonged inflammatory reactions *in vivo*.

6.6 AIMS OF THIS STUDY.

It is becoming evermore apparent that inflammatory reactions do not involve just one cell type, but consist of an intricate network linking different cells and chemical mediators. Such a complex network is now thought to be critical for the orchestration of in vivo inflammatory reactions.

The aim of this study was to investigate the ability of PAF to induce degranulation of isolated human neutrophils (measured as β -glucuronidase release) as both have been strongly implicated in allergic disorders within their own rights.

6.7 METHODS AND MATERIALS.

Chapter 2 contains the experimental techniques relevant to this particular study.

6.8 RESULTS.

6.8.1 THE EFFECT OF PAF ON HUMAN NEUTROPHILS.

Exogenous PAF (0.001-10 μ M) induced β -glucuronidase release in a dose-related manner from isolated human neutrophils (Fig.6.1). Maximum release of the enzyme ($13.2 \pm 1.5\%$) occurred at the highest concentration of PAF (10 μ M).

6.8.2 THE EFFECT OF IONOPHORE A23187 ON HUMAN NEUTROPHILS.

The ionophore A23187 induced a dose-related release of β -glucuronidase from human neutrophils (Fig.6.2). The effect of A23187 on the above cell type was included in the present study as means of a control.

6.8.3 THE EFFECT OF METABOLIC BLOCKERS ON β -GLUCURONIDASE INDUCED BY PAF.

In the presence of the metabolic blockers antimycin A and 2-deoxyglucose, β -glucuronidase release induced by 1 and 10 μ M PAF was reduced when compared to the release which occurred in the absence of the two metabolic inhibitors

(Fig.6.3).

6.8.4 THE EFFECT OF METABOLIC BLOCKERS ON β -GLUCURONIDASE RELEASE INDUCED BY A23187.

A similar pattern of release was observed as in section 6.8.3 (Fig.6.4).

6.8.5 THE EFFECT OF BN50730 ON β -GLUCURONIDASE RELEASE FROM HUMAN NEUTROPHILS.

The PAF antagonist BN50730 was observed to prevent β -glucuronidase release induced by PAF (5 μ M) from human neutrophils, but potent inhibition was observed only at the highest concentration of the drug tested (10 μ M) (Fig.6.5).

6.8.6 EFFECT OF DISODIUM CROMOGLYCATO ON β -GLUCURONIDASE RELEASE FROM STIMULATED HUMAN NEUTROPHILS.

Fig.6.6 indicates that disodium cromoglycate (10-1000 μ M) did not prevent β -glucuronidase release from human neutrophils stimulated by PAF (5 μ M).

6.9 DISCUSSION.

The present study indicates that PAF stimulates isolated human neutrophils resulting in degranulation, as measured by β -glucuronidase release. The release induced by 10 μ M PAF was found not to occur via a cytotoxic mechanism as it was significantly reduced in the presence of the metabolic inhibitors antimycin A and 2-deoxyglucose. 2-deoxyglucose blocks glycolysis, whereas antimycin A inhibits oxidative phosphorylation. The two inhibitors together block overall ATP production from occurring within the cell, thus preventing the normal cellular events involved in degranulation. Hence, PAF-induced β -glucuronidase release occurs via the normal degranulation pathways involved in mediator release from this cell type.

The present study has indicated that relatively high concentrations (1 μ M and

above) of PAF were required to induce β -glucuronidase release (approx. >10% of the total enzyme content) from neutrophils. Similarly, Bruijnzeel and co-workers reported the optimal chemotactic concentration of PAF for human neutrophils was 1 μ M [375]. They also reported that the chemotactic potency of the drug decreased at a concentration of 10 μ M. Although the effects of PAF described by the present study and other independent research groups were obtained in vitro, they might be relevant to the in vivo situation. However, it is not possible to confirm the exact role of PAF in inflammatory conditions until the levels of the phospholipid in vivo have been detected by reproducible methods.

Many independent research groups have experimentally identified the presence of specific PAF receptors on the plasma membranes of target cells involved in allergy and inflammation, which include human eosinophils, neutrophils, macrophages and monocytes [371-373].

The PAF antagonist BN50730, in terms of in vivo activity was found to be the most potent and broadly effective triazolothienodiazepine-based antagonist synthesised [358]. The present findings show that the antagonist (10 μ M) actively prevents degranulation of human neutrophils stimulated by PAF.

Braquet and co-workers have reported that BN50730 is an effective PAF antagonist against a range of PAF-induced conditions. The antagonist optimally inhibited PAF-induced platelet aggregation in vitro at a concentration of 0.4 μ M. Compared to the present findings, ie. BN50730 potently prevents degranulation of human neutrophils at a concentration of 10 μ M, it is apparent that the antagonist is highly effective at lower concentrations in preventing platelet aggregation. Interestingly, the present study also observed that optimal inhibition of histamine release from rat peritoneal mast cells by the same antagonist also occurred at a concentration of 10 μ M. However, one must note that a species variation exists within the present study and further work regarding the effect of BN50730 on human mast cells is clearly indicated.

The mode of action of this antagonist probably involves interaction with the PAF

receptor situated on the outer surface of the cell membrane, thereby preventing the binding of PAF itself. Thus, this and previously mentioned properties (see chapter one) of the antagonist indicates the potential therapeutic values of this drug in a diverse range of pathologies, particularly those concerning the broncho-pulmonary system and allergic disorders. However, the results of proposed clinical trials involving a range of PAF antagonists are eagerly awaited.

The ability of PAF to modulate inflammatory processes is illustrated by its effects on human neutrophils. Besides inducing degranulation as mentioned above, it also causes neutrophil chemotaxis, aggregation, adherence, LT and its own synthesis and superoxide anion production [366,374]. All these effects initiate, contribute towards and may prolong the inflammatory response. Bruijnzeel and co-workers reported that disodium cromoglycate (DSCG) inhibited the chemotactic response of human neutrophils in vitro, induced by PAF [375]. The present study has indicated that DSCG does not prevent β -glucuronidase release from human neutrophils stimulated by PAF. However, the potential therapeutic use of DSCG in pathologies such as asthma must not be totally dismissed as it does prevent the mobilization of human neutrophils by inflammatory mediators like PAF. However, until the above system has been successfully modified to fit into the in vivo situation, the therapeutic value of DSCG in preventing the mobilization of human neutrophils into different tissues remains pure speculation.

In conclusion, the present findings have shown that PAF induces a dose-related, non-cytotoxic release of β -glucuronidase from isolated human neutrophils. Similar results were observed when ionophore A23187 was used as the control stimulus. Mediator release was inhibited by the PAF antagonist BN50730. The anti-allergic chromone DSCG was ineffective in preventing degranulation. The results from further in vivo diagnostic studies involving PAF antagonists and their putative role in modulating neutrophil behaviour are anticipated with great interest, as they could lead to possible therapeutic uses of PAF antagonists in allergic disorders.

Fig.6.1 The effect of PAF on human neutrophils. n=5

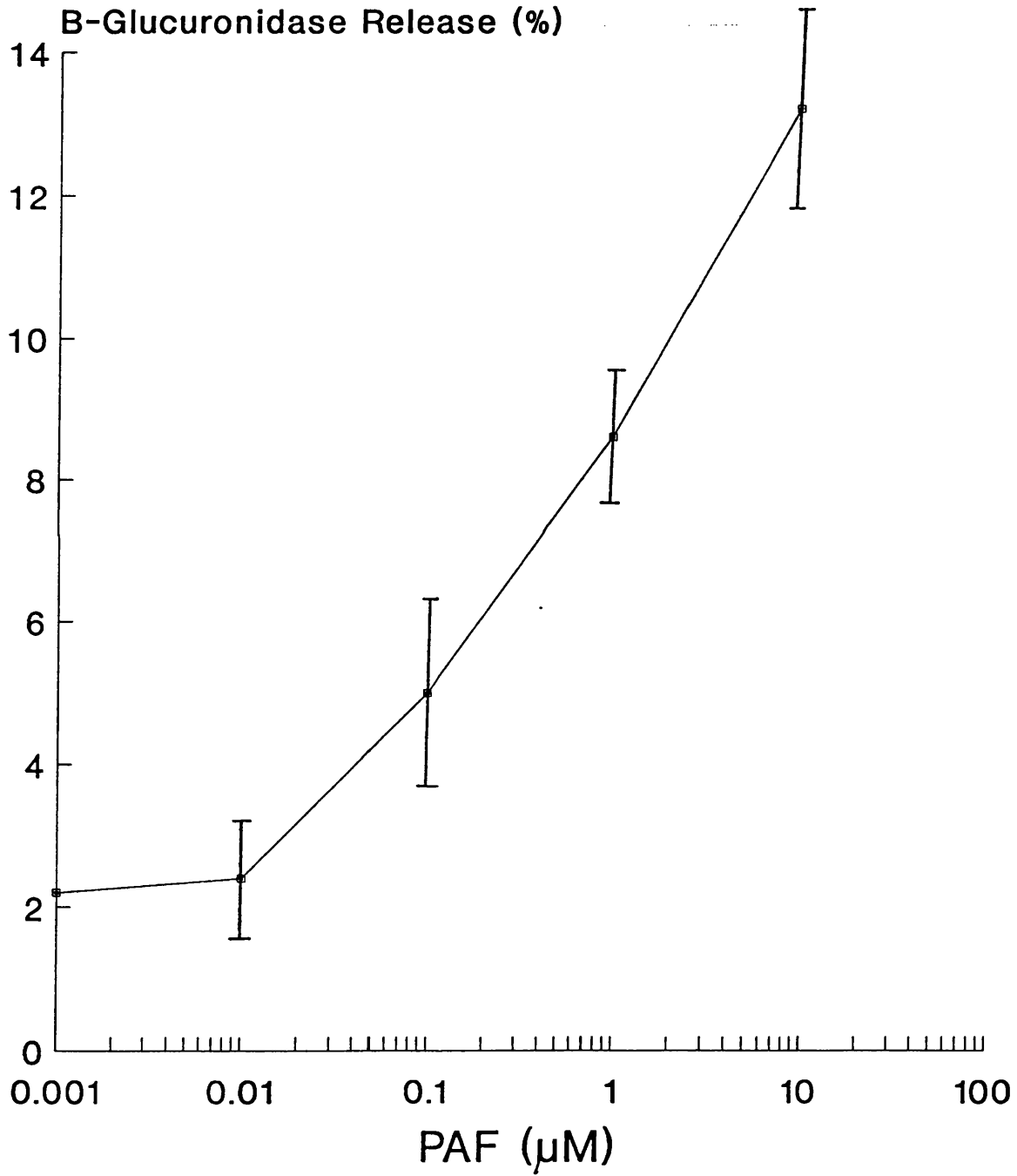


Fig.6.2 The effect of A23187 on human neutrophils. n=5

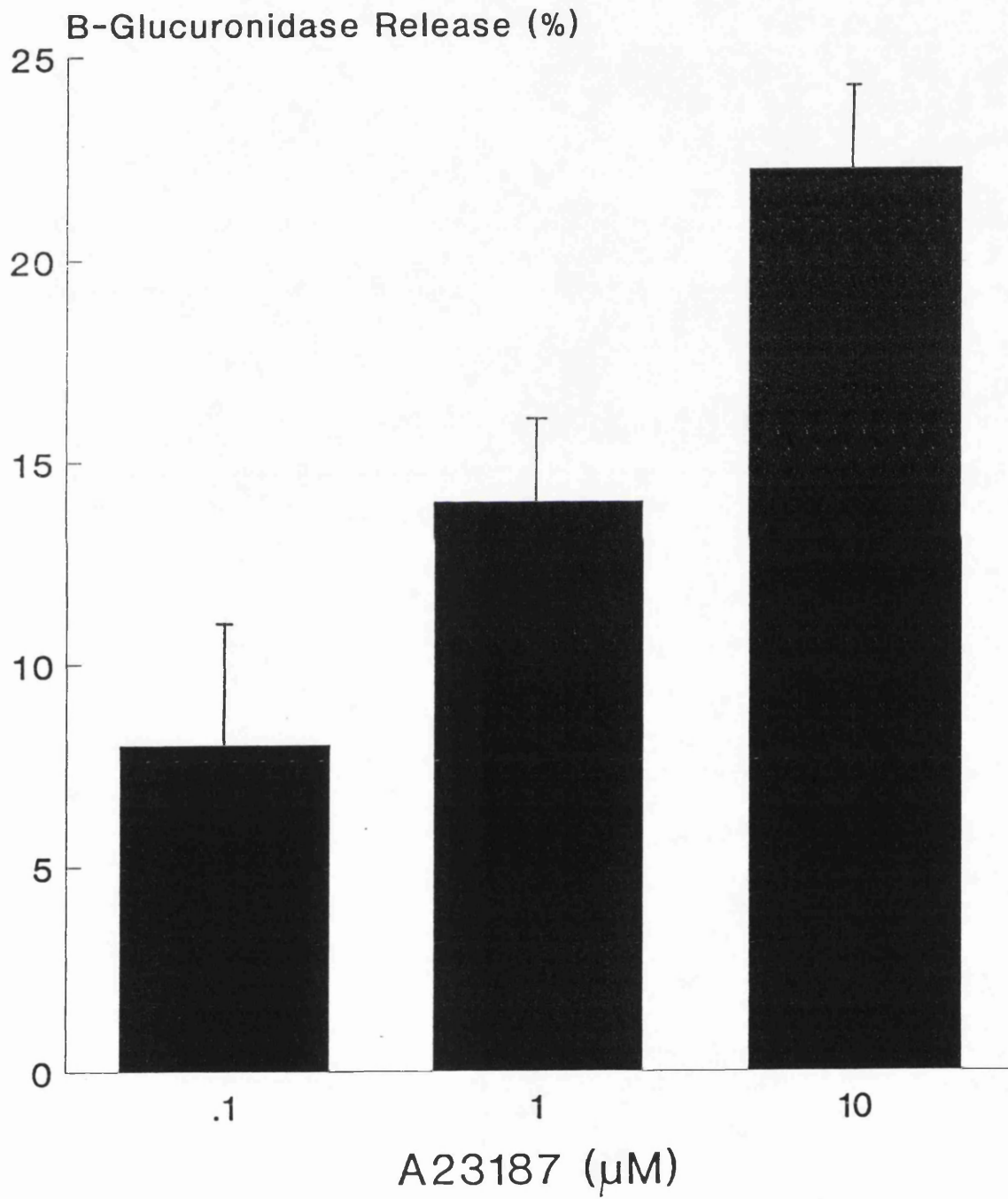


Fig.6.3 The effect of metabolic blockers on B-glucuronidase release from human neutrophils stimulated with PAF. n=3

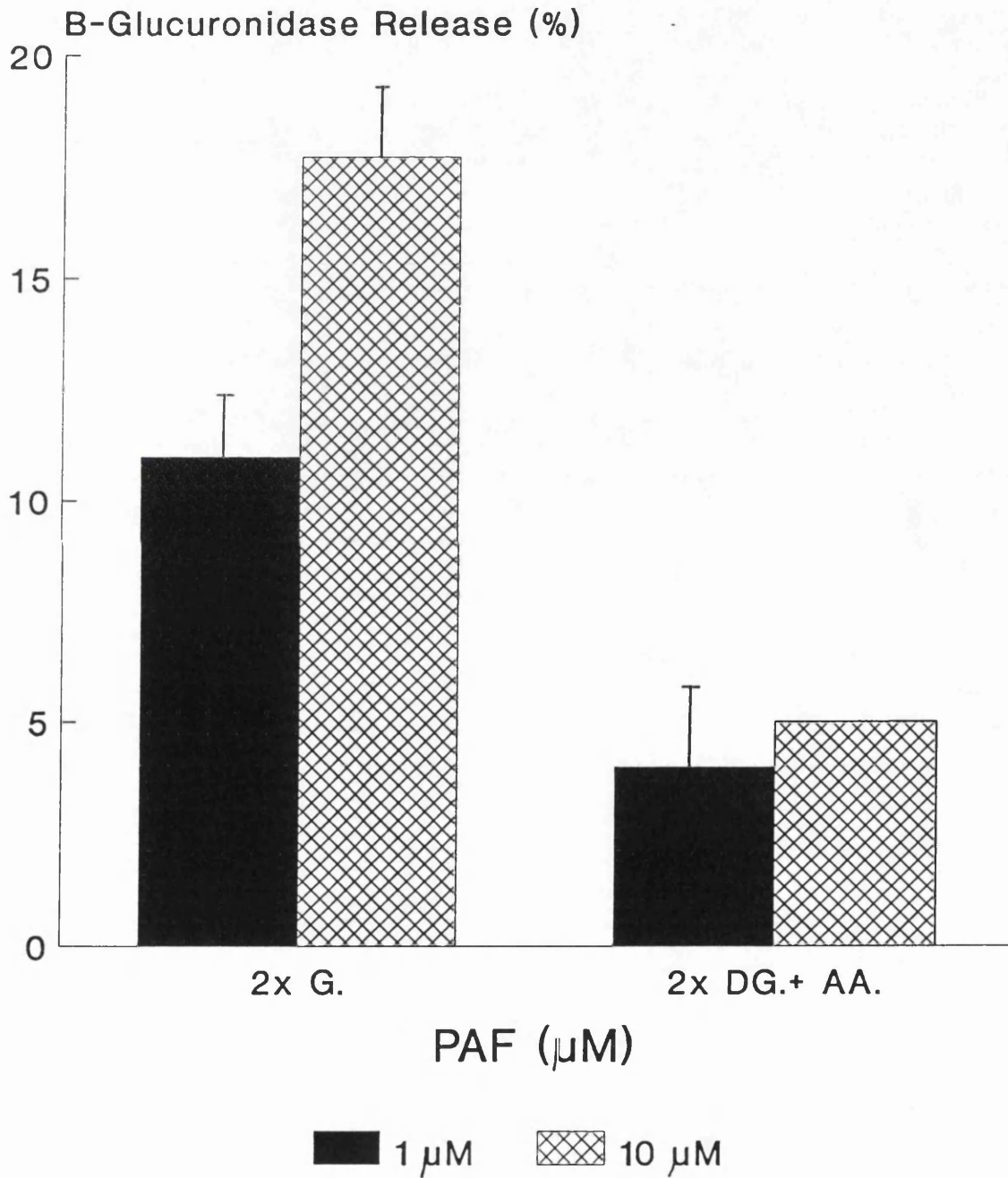


Fig.6.4 The effect of metabolic blockers on B-glucuronidase release from human neutrophils stimulated with A23187. n=3

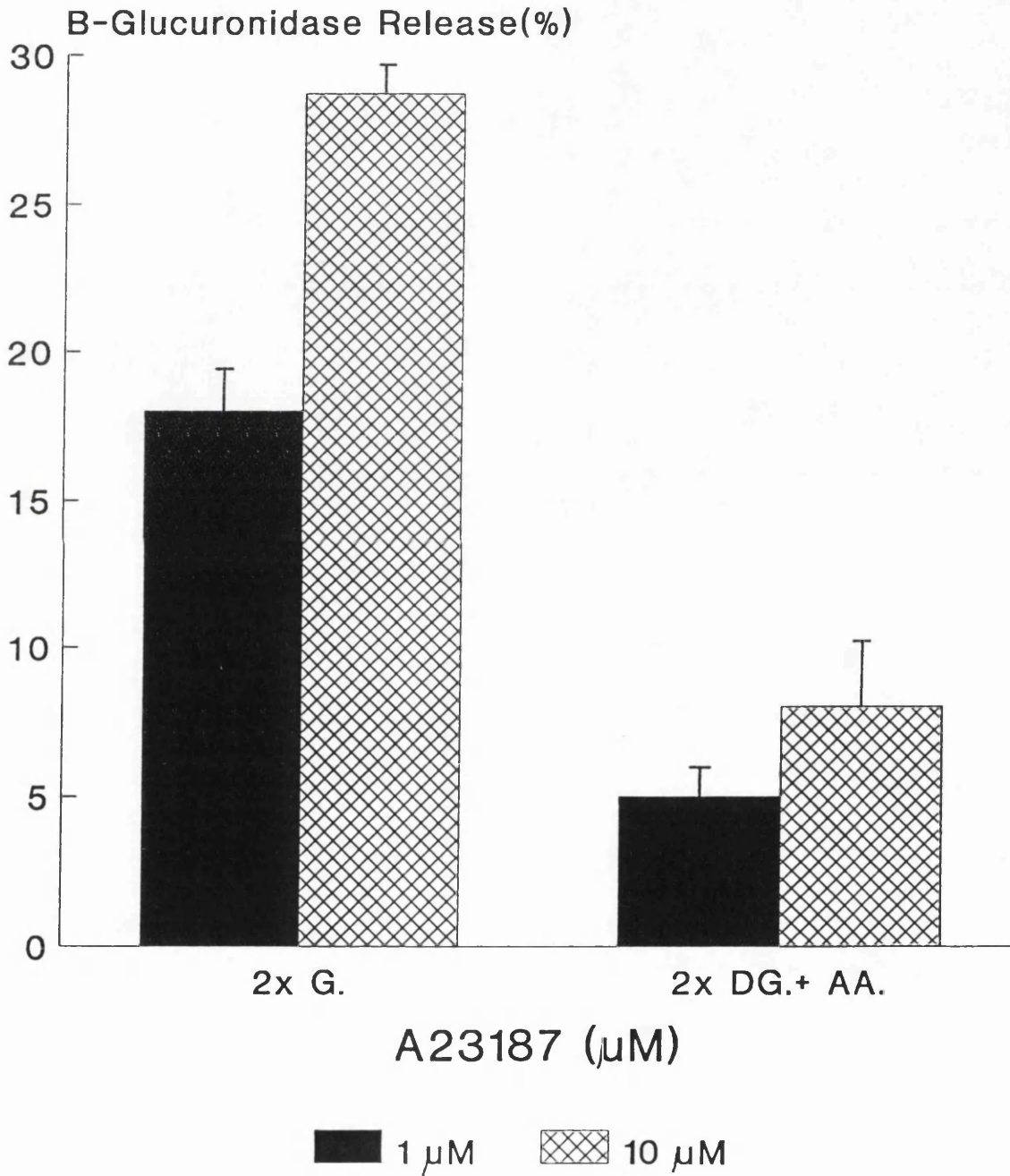


Fig.6.5 The effect of BN50730 on B-glucuronidase release from human neutrophils stimulated with PAF (5 μ M). n=3

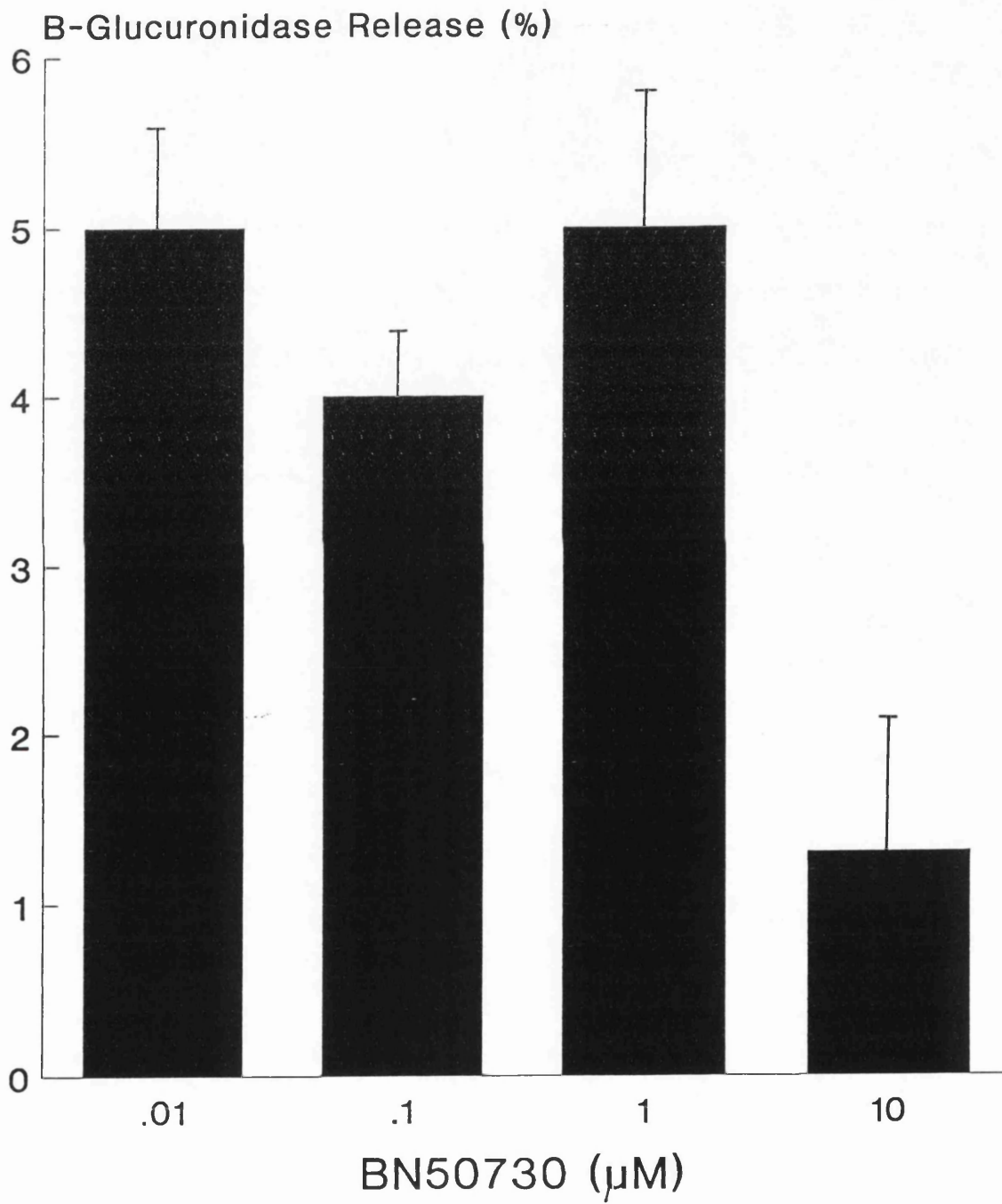
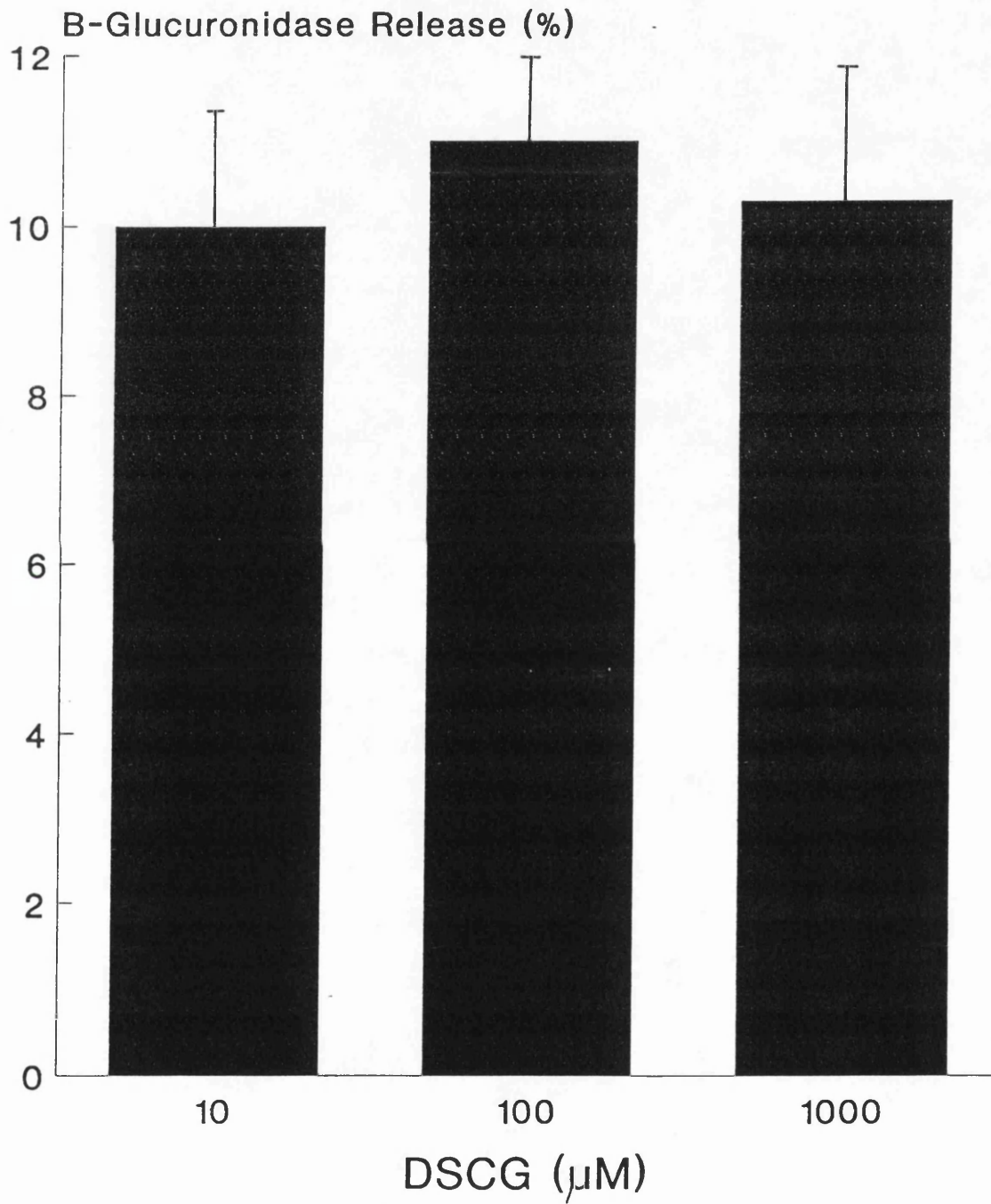


Fig.6.6 The effect of disodium cromoglycate (DSCG) on B-glucuronidase release from human neutrophils stimulated with PAF (5 μ M). n=3



CHAPTER SEVEN

GENERAL CONCLUSIONS

PAF is a potent biologically active phospholipid with a wide spectrum of proinflammatory properties. It is synthesised from membrane phospholipids by the sequential action of phospholipase A₂ and acetyl transferase. The phospholipid is not preformed but synthesised *de novo* following cellular stimulation. PAF is generated by a variety of cells, including eosinophils, macrophages, mast cells, endothelial cells, platelets and neutrophils. Although human mast cells can synthesise PAF, it is not released but appears to be stored intracellularly [374].

As well as synthesising PAF, the above cells are also targets for the action of PAF. *In vitro*, PAF is a potent chemoattractant for granulocytes. Exposure of granulocytes to PAF results in degranulation and the release of lysosomal enzymes and cationic proteins [376]. *In vivo* PAF elicits vasoconstriction, systemic hypotension, thrombocytopenia and neutropenia. Direct administration into the lung triggers an inflammatory response involving oedema formation, cell accumulation and mucus secretion. Similarly, injection into the skin elicits plasma leakage and cell recruitment. Of interest to the pathogenesis of asthma, PAF can induce bronchoconstriction, increased vascular permeability and an increase in bronchial hyperresponsiveness lasting for several weeks.

PAF exerts its effects through interaction with specific receptors found in several cells. A diverse range of PAF antagonists have been synthesised during the last ten years or so. Recently, many research groups have demonstrated the ability of particular antagonists to inhibit PAF-induced actions relating mainly to asthma in several laboratory animals. However, the therapeutic use of these particular drugs in asthma will not be fully appreciated until clinical trials have taken place.

In the present study, exogenous PAF (1-100 μM) evoked a characteristic, dose-dependent release of histamine from rat peritoneal mast cells. The release process was cytotoxic at high concentrations (10 μM) but not at lower concentrations (5 μM). Both cytotoxic and non-cytotoxic histamine releases were found to be calcium independent, depressed at extremes of temperature and refractory to changes in the pH of the external medium. Non-cytotoxic histamine release induced by PAF (5 μM) was essentially complete within 10 min, but cytotoxic release (induced by 10 μM PAF) was much slower.

Analogues of PAF (C17, C18 and lyso-PAF) all induced dose-related releases of histamine from rat peritoneal mast cells.

PAF (1 μM) significantly potentiated both anti-IgE and Con.A induced histamine release from rat peritoneal mast cells but had no effect on the release mediated by compound 48/80.

Exogenous PAF (0.1-100 μM), in addition to secretion of preformed mediators, also induced the metabolism of AA, resulting in the release of PGD_2 in a dose-related manner from purified rat peritoneal mast cells.

PAF (1-100 μM) evoked characteristic dose-related releases of histamine from rat pleural, mesenteric, pulmonary and cutaneous mast cells, guinea-pig mesenteric and pulmonary mast cells and human pulmonary, cutaneous and colonic mucosal mast cells. Isolated human basophils responded in a similar fashion.

Histamine release induced by PAF (5 μM) from rat mesenteric mast cells was found not to occur cytotoxically. In contrast, histamine release evoked by PAF (10 and 50 μM) from rat pulmonary and cutaneous mast occurred via a cytotoxic mechanism respectively.

Both guinea-pig mesenteric and pulmonary mast cells released histamine in the presence of PAF (10 and 50 μM) respectively, via a cytotoxic mechanism.

Histamine release from human basophils and cutaneous mast cells occurred cytotoxically at concentrations of PAF above 30 and 50 μM respectively.

Thus, the effect of PAF on tissue histaminocytes is not species selective. The above findings suggest that PAF interacts with the mast cell membrane resulting in mediator release, rather than stimulating the cell through a specific receptor. In support of this, lyso-PAF, which is inactive in many receptor mediated PAF actions, also induces histamine release from mast cells, thus indicating a non-specific mode of action of the phospholipid.

PAF (1 μM) was found not to potentiate anti-IgE induced histamine release from human lung mast cells or basophils.

Non-cytotoxic histamine release induced by PAF and lyso-PAF (5 μM) from rat serosal mast cells was inhibited by the anti-allergic chromone DSCG and its more potent congener nedocromil sodium. Given the putative mode of action of PAF and lyso-PAF, it is possible that DSCG and nedocromil sodium exert their inhibitory effects by a general stabilizing action on the mast cell membrane.

Other anti-allergic compounds including phosphodiesterase inhibitors, cAMP analogues, a naturally occurring flavonoid and a β_2 -agonist all inhibited PAF-induced histamine release to varying degrees.

Studies on the role of PAF in allergy and inflammation have been greatly facilitated by the use of specific PAF antagonists. These compounds can inhibit the binding of PAF to its receptor in various tissues and cells [376].

The present study has shown that the PAF antagonist WEB2086 exerts a very weak inhibitory action towards PAF-induced histamine release from rat peritoneal mast cells. A variable pattern of inhibition of histamine release was observed by the PAF antagonist BN52021. Similar inhibition by the PAF antagonist BN50730 was also observed. BN50730 also inhibited lyso-PAF induced histamine release but only after

preincubation of the cells with the drug.

It has been well documented that PAF antagonists exert their inhibitory effects by interacting with PAF receptors. Thus, the failure of the above drugs to inhibit potently histamine release from mast cells following stimulation by PAF in a consistent pattern, indicates the absence of specific PAF receptors on the mast cell membrane. Hence, it can be concluded that PAF interacts with the mast cell membrane resulting in mediator release.

PAF (0.001-10 μ M) stimulates human neutrophils to undergo degranulation resulting in β -glucuronidase release. The PAF receptor antagonist BN50730 prevents degranulation of the cells. The present findings have confirmed previous reports that PAF stimulates human neutrophils via specific receptors as mediator release is blocked by the antagonist.

Currently, newly synthesised PAF antagonists are undergoing clinical trials for their potential use as anti-asthmatic drugs. In addition, drugs which inhibit intracellular PAF synthesis may prove to be therapeutically useful in reducing the levels of PAF generated during allergic reactions. This approach has yet to be fully studied.

It has been estimated *in vivo* that the amount of histamine released during the most severe asthma attack is likely to be less than 1% of the total lung content of the amine. The levels of PAF are increased during inflammatory reactions owing to the infiltration of cells such as eosinophils and neutrophils. These cells when stimulated by PAF, synthesise and release newly formed PAF thus potentiating the original levels of the phospholipid in the surrounding tissue. Hence, increased levels of PAF may then stimulate nearby mast cells, resulting in mediator release. PAF (at concentrations which cause <5% release of the total amine content *in vitro*) may in fact contribute to the overall inflammatory response *in vivo*.

Therefore, further studies regarding the role of PAF, PAF antagonists and PAF synthesis inhibitors in chronic asthma and allergic conditions are clearly indicated.

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