SOME STUDIES ON HISTAMINE RELEASE FROM MAST CELLS STIMULATED WITH SOMATOSTATIN AND OTHER SECRETAGOGUES

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ABSTRACT

A variety of polybasic compounds and neuropeptides release histamine from rat serosal mast cells. In the present study, the effect of somatostatin on different mast cells was examined in detail. In this way, it was hoped to elucidate further the mechanisms involved in the activation of mast cells by polyamines and neuropeptides and the possible importance of the latter effect in allergy and inflammation.

Somatostatin was shown to be an effective histamine liberator from rat peritoneal mast cells. The process was non-cytotoxic and resembled that evoked by other polybasic agents such as compound 48/80 and substance P in being extremely rapid, independent of extracellular calcium ions and unaffected by added phospholipids. The release was not inhibited by antagonists of the dextran receptor and was independent of cell-fixed antibody. However, secretion was selectively blocked by antagonists of the purported polyamine receptor, benzylammonium chloride (BAC) and benzyl dimethyltetradecyl ammonium chloride (BDTA), and by the C-terminal octapeptide fragment of substance P, SP_{4-11}. Cross-desensitisation was observed between somatostatin and other polyamines but not between the neuropeptide and immunologic stimuli.

The effect of somatostatin was species and tissue specific. Rat pleural mast cells responded in similar fashion to those from the peritoneum while the latter cells from the hamster and mouse were considerably less reactive. Tissue mast cells of the rat showed graded responsivity while such cells from the guinea pig, the pig and the human were essentially unreactive. Where studied, the relative responsiveness of different mast cells was mirrored by fluorescence binding studies.

Varying concentrations of somatostatin did not strikingly modulate immunologic or pharmacologic histamine release from human basophils or murine mast cells.

The precise conditions required for BDTA to act as a selective antagonist of polyamine-induced release were established and detailed studies showed the antagonism to be surmountable.

The release induced by somatostatin and other polyamines was inhibited by treatment of the cells with neuraminidase and by pertussis toxin, suggesting that sialic acid may be involved in the binding of such agonists to the cell membrane and that a G-protein(s) may be involved in the transduction mechanism.

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LIST OF ABBREVIATIONS

AA	-	arachidonic acid
ADP	-	adenosine diphosphate
ATEE	-	N-acetyl-tyrosine ester
ATP	-	adenosine triphosphate
BAA	-	N-benzoyl-arginine amide
BAC	-	benzalkonium chloride
BAEE	-	N-benzoyl-arginine-ethyl ester
BDTA	-	benzyldimethyltetradecylammonium chloride
BMMC	-	bone marrow-derived mast cell
BPB	-	p-bromophenylacyl bromide
BSA	-	bovine serum albumin
BTEE	-	N-benzoyl-tyrosine-ethyl ester
CBP	-	cromolyn binding protein
CGRP	-	calcitonin gene-related peptide
CMF	-	calcium and magnesium free
CTC	-	chlortetracycline
CTMC	-	connective tissue mast cell
cyclic AMP	-	adenosine 3',5'-cyclic monophosphate
cyclic GMP	-	guanosine 3',5'-cyclic monophosphate
DAG	-	1,2,-diacylglycerol
DDA	-	2',5'-dideoxyadenosine
DFP	-	diisopropylfluorophosphate
DMSO	-	dimethyl sulphoxide
DNP	-	dinitrophenol
DSCG	-	disodium cromoglycate
ECF-A	-	eosinophil chemotactic factor of anaphylaxis
EDTA	-	ethylenediaminetetracetic acid
EFS	-	electrical field stimulation
ETI	-	5,8,11-eicosatriynoic acid
ETYA	-	5,8,11,14-eicosatetraynoic acid
FMLP	-	F-met-leu-phe
GDP	-	guanosine diphosphate
GI	-	gastrointestinal
GIP	-	gastric inhibitory peptide
GSL	-	glycosphingolipid
GTP	-	guanosine triphosphate
GTP-γ-S	-	guanosine 5'-O-(3-thiophosphate)
HEPES	-	N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid

HETE	-	hydroeicosatetraenoic acid
HPETE	-	hydroperoxyeicosatetraenoic acid
HTYA	-	5,8,11,14-henicosatetraynoic acid
IAP	-	islet activating protein
IgA	-	immunoglobulin A
IgE	-	immunoglobulin E
IgG	-	immunoglobulin G
IP	-	inositol monophosphate
IP_2	-	inositol 1,4-bisphosphate
IP_3	-	inositol 1,4,5-trisphosphate
ITYA	-	4,7,10,13-icosatetraynoic acid
LT	-	leukotriene
lyso-PS	-	lyso-phosphatidyl serine
MMC	-	mucosal mast cell
MT	-	methyltransferase
NANA	-	N-acetyl neuraminic acid
NANC	-	non-adrenergic non-cholinergic
NCF	-	neutrophil chemotactic factor
NDGA	-	nordihydroguairetic acid
NK	-	neurokinin
OAG	-	1-oleoyl-2-acetyl-glycerol
OPT	-	o-phthaldialdehyde
PAF	-	platelet activating factor
PC	-	phosphatidyl choline
PE	-	phosphatidyl ethanolamine
PF4	-	platelet factor 4
PG	-	prostaglandin
PHI	-	peptide histidine isoleucine
PHM	-	peptide histidine methionine
PI	-	phosphatidyl inositol
PIA	-	N^6-(L-2-phenyl-isopropyl)-adenosine
PIP	-	phosphatidyl inositol monophosphate
PIP_2	-	phosphatidyl inositol 4,5-bisphosphate
PMA	-	phorbol-12-myristate-13-acetate
PMN	-	polymorphonuclear
PMSF	-	phenylmethylsulphonyl chloride
PS	-	phosphatidyl serine
RBL	-	rat basophilic leukaemia cell
RPMC	-	rat peritoneal mast cell
SCG	-	sodium cromoglycate

SEM	-	standard error of mean
SL-	-	spin labelled-
SP	-	substance P
SPA	-	[D-Pro^{4},D-Trp^{7,9,10}]-SP$_{4-11}$
SPF	-	specific pathogen free
SRS-A	-	slow reacting substance of anaphylaxis
TAME	-	N-p-toluenesulphonyl-arginine methyl ester
THIQ	-	tetrahydroisoquinoline
TLCK	-	N-tosyl L-lysine chloromethyl ketone
TPA	-	12-O-tetradecanoylphorbol-13-acetate
TPC	-	tetradecylpyridinium chloride
TPCK	-	N-tosyl L-phenylalanine chloromethyl ketone
VIP	-	vasoactive intestinal peptide
WE	-	worm equivalents

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 *THE MAST CELL*

The mast cell has been studied extensively yet, its precise function and physiological role remain obscure. Certainly, its location, particularly at sites of possible invasion by foreign organisms (connective tissue of the skin, gastrointestinal, respiratory and genito-urinary tracts, sclera of the eye etc.), would point to a possible role in defense mechanisms [1-4]. Upon appropriate activation the mast cell may liberate any of an impressive array of pre-formed mediators (e.g. histamine) and newly synthesised compounds (e.g. leukotrienes). These factors are potent constituents of the inflammatory response [4-7].

Lymphocytes are integral components of the immune system. Upon contact with antigenic material, B lymphocytes differentiate into a subclass of antibody-secreting plasma cells and another population of B lymphocyte memory cells. T lymphocytes of both 'helper' and 'suppressor' subclasses regulate the production of immunoglobulin E (IgE) [8,9].

The mast cell is capable of high affinity binding to IgE, through specific receptors for the immunoglobulin on the membrane [10]. Adjacent antibody molecules can be cross-linked by the same antigen that provoked their production. This cross-linking will lead eventually to exocytosis. Exocytosis encompasses mast cell degranulation, whereby granule associated mediators are discharged into the extracellular environment [11-15].

There is strong evidence for the involvement of mast cells in the elimination of helminthic parasites [16,17]. An important consequence of this purported physiological role for the mast cell, is the apparent dysfunction in responses to normally innocuous substances (e.g. pollen) in selected individuals. In these cases, IgE is produced in response to certain agents and the subject is said to be sensitised to that agent. On subsequent exposure to the allergen, mast cell activation occurs resulting in the release of mediators. These responses are superfluous and potentially harmful. The rationale proposed for this dysfunction is a breakdown in the recognition system, where T cells may discriminate incorrectly between harmful or harmless agents [9].

1.1.1 Mast cell derived mediators

Mast cell activation culminates in exocytosis and the release of preformed and/or newly generated mediators. The granular constituents may differ according to the species and tissue of origin [5-7,18].

1.1.1.1 **Preformed mediators**

In most instances, the highly sulphated proteoglycan heparin, is present in the granules. However, less sulphated proteoglycans such as chondroitin sulphates have also been identified [19,20]. A proteoglycan comprises a peptide core substituted with covalently-linked, glycosaminoglycan side chains. The side chains are unbranched, highly

acidic carbohydrate polymers, composed of repeating, sulphated-disaccharide monomeric units [21].

The biogenic amine histamine or ß-imidazolyethylamine is most often chosen to demonstrate the process of degranulation, although serotonin is also present in rodent mast cells [5,6]. Histamine is synthesised in mast cells from histidine by the action of histidine decarboxylase and stored in the granules until required [22]. The amine possesses both pro- and anti-inflammatory activity in addition to several chemotactic properties. Pro-inflammatory effects are mediated via H_1-receptors and include smooth muscle contraction, increased vascular permeability, bronchospasm, increased mucus production and enhanced neutrophil and eosinophil chemotaxis [5,6].

Other granule-associated mediators include eosinophil, neutrophil and lymphocyte chemotactic factors, of which eosinophil chemotactic factor of anaphylaxis (ECF-A) and neutrophil chemotactic factor (NCF) are the most prominent [5-7].

A number of enzymes are also present within mast cell granules. Some are secreted in parallel with histamine, others remain complexed with the granule matrix and dissociate more slowly [6,23]. Most significant is a neutral serine protease with chymotrypsin-like specificity. This has been designated Protease I, or chymase, for the connective tissues of the rat. A similar, but immunologically distinct enzyme, termed Protease II, is present in the mast cells of the mucosa [23-27]. In human lung mast cells, the major neutral protease possesses trypsin-like activity [28]. This protease has also been detected in some rat connective tissues [29].

Other granular enzymes include ß-hexosaminidase, ß-glucuronidase, carboxypeptidase A and aryl sulphatase [5,30]. A prekallikrein-like activator and a Hageman factor activator have also been identified [31,32].

1.1.1.2 **Newly generated mediators**

Mast cells are able to generate a variety of metabolites upon suitable activation. Of these, the products of arachidonic acid (AA) metabolism, namely prostaglandins (PG's) and leukotrienes (LT's), are the most important [4,33,34]. The generation of the platelet activating factor, PAF acether, is probably of similar importance [18].

Mast cell activation produces a slow-acting, spasmogenic agent traditionally termed the slow reacting substance of anaphylaxis (SRS-A). This is now recognised to be a mixture of LTC_4, LTD_4 and LTE_4. LT's such as these can induce spectacular smooth muscle contraction and increased vascular permeability. In human bronchi, the effects are some 1000-fold more potent that the action of histamine [35,36]. The PG' are also potent spasmogens, in addition to increasing vascular permeability [35,37].

PG's and LT's can produce synergistic pro-inflammatory effects illustrating an interaction distinct from that with other mediators. This may serve to intensify the overall development of inflammatory events [4].

1.1.2 Mast cell heterogeneity

Mast cells derived from different species and even from different tissues within the same species, may vary substantially in their responses to histamine liberators and release antagonists [3,19,38]. Furthermore, this heterogeneity may extend to histochemical, ultra-structural and cytochemical properties [39-42].

Three distinct subclasses of cells have been identified in the rat which contain histamine, exhibit IgE-receptors and stain metachromatically. The serosal cavities and connective tissues produce heparin-containing mast cells, which are non-replicating. Mucosal tissue supports cells containing over-sulphated proteoglycans, which can proliferate upon appropriate stimulation [43]. Mucosal mast cells (MMC) increase in number following, for example helminth infection, but this probably does not involve the division of end cells, rather the multiplication and/or the recruitment of undifferentiated precursors.

Connective tissue mast cells (CTMC) are located in the lower layers of the gastrointestinal wall and mucosal mast cells are found in the lamina propria [41]. Morphologically, MMC are smaller than CTMC, have fewer granules of variable size and contain less histamine [19,41]. MMC contain a chymotrypsin-like, neutral serine protease, designated rat mast cell protease II, where as CTMC contain protease I [27,44]. CTMC are susceptible to the inhibitory effects of anti-allergic compounds such as disodium cromoglycate (DSCG), nedocromil sodium and theophylline, to which MMC remain resistant [45]. A distinctive feature of MMC is their characteristic granule metachromasia. They exhibit typical absorbance spectra after staining with toluidine blue, but lack appreciable affinity for the dyes berberine and safranine [46,47]. Studies have shown that the activation of these distinct subtypes may tend to produce different products of AA metabolism. The MMC generate mainly LT's and the CTMC predominantly PG's [48-50].

Within the lung and intestine of man, at least two types of mast cell have been described which bear some histochemical similarities to the CTMC and MMC of the rat. However, the distinction between the two human cell types is less clearly defined [51]. Moreover, cells resembling MMC are found in the submucosa and muscle and therefore, not confined merely to the mucosa of human intestine [52]. Additionally, this cell type is widely distributed throughout the parenchyma of human lung outnumbering the CTMC populations [51,53]. It is clear, that the histochemical criteria for distinguishing between subpopulations of rat mast cells are probably restricted to this species. Therefore, the terms *mucosal* and *connective tissue* mast cells should not be extrapolated to other species without caution.

The functional heterogeneity of mast cells takes on far greater meaning when it is appreciated that mast cells from different tissues and species respond differently to various agents [3,19,38,54]. Of paramount importance to this study , is the heterogeneity of mast cells in their responses to compound 48/80. In the rat, although all mastocytes respond to anaphylactic stimulation, only CTMC respond to compound 48/80 agonism [55,56]. Recent studies have shown that even in adult rats, the peritoneal mast cells are heterogeneous and

can be distinguished by a number of functional properties, including their responsiveness to compound 48/80 [57]. This topic is discussed in greater detail in the following sections. In summary, it would seem that the ability of a agent to elicit or inhibit mediator secretion only bears real significance if the studies are conducted on the specific mast cell system with which the agent is thought, or intended, to interact.

1.1.3 The IgE receptor

Basophils and mast cells exhibit high affinity binding to immunoglobulin E (IgE) [10,58-60], but much weaker associations with immunoglobulin G (IgG) and immunoglobulin A (IgA) [61]. The reaction of cell bound IgE and antigen induces the release of chemical mediators from mast cells [21,58-60,62]. IgE-receptors on the surface of mast cells and basophils belong to a family of molecules named Fc_ϵ-receptors, which are found on mast cells involved in immune responses. IgE-receptors are not confined to the mast cell [63,64] or basophil [65,66], but have been characterised on lymphocytes [67], macrophages [68] and eosinophils [69], although the nature and full significance of such binding is unknown.

Fc_ϵ-receptor aggregation on mast cell and basophil membranes is thought to initiate a biochemical chain of events culminating in degranulation [70,71]. IgE binds to the Fc_ϵ-receptor at its Fc portion [72]. However, the precise location within this fragment remains unclear. Suggestions have been made that sites within the C_3 and the C_5 domains are involved [73]. More recently, a single interaction with the C_3 domain is favoured [71,74]. An indirect mechanism for Fc_ϵ-receptor aggregation is effected by the subsequent cross-linking of Fab regions of adjacent, cell-bound IgE with multivalent antigen [75,76].

Experiments with fluorescently labelled, monomeric IgE have demonstrated that the Fc_ϵ-receptor is univalent, laterally mobile and diffusely distributed on the mast cell membrane [77,78]. On the assumption that the maximum number of bound IgE molecules is equivalent to the number of Fc_ϵ-receptors, studies have shown that each rat mast cell carries $2\text{-}5 \times 10^5$ molecules of receptor, while the range for rat basophilic leukaemia (RBL) cells and human basophils was found to be $0.3\text{-}1.0 \times 10^6$ molecules per cell [79].

The IgE receptor is a glycoprotein with a content of about 13% carbohydrate. The overall molecular weight of the receptor is about 87kDa, divided amongst subunits (α 45kDa; β 33kDa; γ 9kDa) [10,80,81]. The α, β and γ subunits are all further subdivided into α_1, α_2, β_1, β_2; the two γ chains being identical [83,84]. The α subunit is directed to the surface of the cell and binds IgE [10]. The β and γ subunits cannot be labelled from the cell surface and are presumed to be buried in the cell membrane [82,84]. The functions of these subunits are, at present, unknown [85]. Little data of equal depth is available on IgE receptors in other types of mast cell, but similarities between the rat and other species is likely since human and rodent IgE may interact with the same receptor [86,87].

1.1.4 Membrane receptor activation

Simple binding of the IgE molecule to its receptor on a mast cell or basophil leucocyte does not activate the cell to secrete histamine. It is the binding of specific antigen to the cell-fixed IgE which initiates activation.

Monovalent antigens do not cause mast cell activation, only bi- and polyvalent antigens induce degranulation. The cross-linking of adjacent receptors seems to be the key to effective cell stimulation. Bi- or polyvalent antigens cross-link adjacent IgE molecules by combining with the Fab regions, indirectly causing Fc_ϵ-receptor aggregation. Anti-IgE, which is IgG antibody directed against the Fc_ϵ heavy chains of IgE, can also cross-link receptors although at a site different to that of antigen. The lectin concanavalin A activates mast cells and basophils by binding to IgE-associated carbohydrate and cross-linking adjacent molecules of IgE.

The concept that histamine release is proportional to the dimeric cross-linking of receptors is not wholly consistent with observed data. Basophils from certain subjects fail to release histamine, despite the certainty that cross-links are formed. This may represent the uncoupling of Fc_ϵ-receptors from the induction mechanisms needed to transfer the information from cross-linking to mediator secretion processes. Secondly, both qualitative and quantitative characteristics of histamine release vary, depending on the type of cross-linking ligand. It is uncertain how the size of the receptor aggregate, produced by cross-linking, influences the response. However, data indicates that aggregate size does have some sort of influence on release levels [88].

1.2 *HISTAMINE SECRETION*

1.2.1 Calcium

Mongar and Schild [89] were the first to demonstrate a requirement for extracellular calcium in the anaphylactic release of histamine from mast cells in guinea pig lung fragments. Secretion was found to be calcium dependent in the human basophil [90], rabbit leucocytes [91], human cutaneous mast cells [92] and cells from the guinea pig mesentery [93]. In most cases, the reduction of extracellular calcium was sufficient to reduce the extent of release by significant amounts.

Studies on rat peritoneal mast cells have shown that histamine secretion is severely impaired after immunologic challenge in the absence of calcium. However, alkaline earth cations such as strontium (10mM) and barium (16mM) could substitute for calcium (1mM), but only at higher concentrations [94].

In view of the apparent association between calcium ions and mediator release from mast cells, Foreman *et al.* [95] investigated cation fluxes following cellular activation. Studies on ^{45}Ca fluxes indicated that the time course, magnitude and pH effects on calcium influx correlated to the degree of histamine liberation. Interestingly, the addition of the phospholipid phosphatidyl serine (PS) to the incubation medium potentiated ^{45}Ca uptake

and histamine release. The increase in calcium uptake under the effects of PS was proportional to the increased level of secretion.

Related work by Ishisaka *et al.* [96] illustrated an increase in membrane permeability on activation, as a consequence of Fc_ϵ-receptor cross-linking and that calcium entry from the extracellular milieu induced exocytosis. The increased permeability of the membrane to calcium declines quite rapidly after initial challenge [95]. If mast cells are presented with antigen in the absence of extracellular calcium and ^{45}Ca reintroduced at discrete intervals after challenge, both ^{45}Ca uptake and histamine secretion will decrease as the time interval between calcium reintroduction and challenge is increased.

Despite this, a number of workers have expressed reservations on the validity of relating ^{45}Ca uptake with a discrete functional role for the cation in the release process [2,97-99]. The bases of argument centre on the possibility that increased ^{45}Ca uptake may reflect non-specific binding to sites that are exposed as a consequence of exocytosis. More importantly, it does not distinguish between an increased influx and a decreased efflux of the ion, nor between a net increase in intracellular calcium and an exchange of the labelled for unlabelled cation across the cell membrane [2,100].

Furthermore, the ^{45}Ca uptake studies indicate an increase in cytosolic calcium concentration from a resting level of approximately $10^{-8}M$ into the millimolar range after activation. Fluxes of this magnitude are incompatible with a messenger or signalling function of calcium since the cells possess a greater sensitivity to calcium elevations. Also, increases into the micromolar range are thought to be operative in stimulus-secretion coupling [99]. Such pyrrhonistic arguments cannot detract from the fact that optimal anaphylactic release is promoted in the presence of extracellular calcium. Rather, they question the validity of ^{45}Ca uptake studies, *per se*, as a means of confuting the functional significance of calcium in the release process [2,99].

Ionophores are compounds capable of elevating cytosolic calcium concentrations by carrier-assisted, passive diffusion, thereby circumventing any biochemical events at the cell membrane level. The ionophores A23187 and ionomycin possess some specificity for calcium and can produce a non-cytotoxic, dose-dependent release of histamine from mast cells [101-104].

Mast cells can be permeabilized by ATP [105], Sendai virus [106] or liposomes loaded with calcium [107], which result in degranulation. Visual monitoring of exocytosis by microscopy revealed that the degranulation was localized to discrete areas of the mast cell, probably corresponding to points of fusional contact thus puncturing the membrane with concomitant cation entry.

Mast cells were found to undergo exocytosis when exposed to high concentrations of calcium (16-110mM), after prolonged incubation in calcium-free media. Calcium deprivation was considered to render the plasma membrane more permeable to the cation, such that upon its reintroduction in high concentration, it provoked a secretory response. Magnesium, however, was unable to mimic the effect [108].

The lanthanum ion La^{3+}, has an ionic radius similar to that of calcium and strontium. Its trivalent state should allow binding to any calcium sites on mast cell membranes with greater affinity. La^{3+} has been shown competitively to antagonise the adherence of calcium to a superficial site [109,110].

La^{3+} and other lanthanide ions are capable of inhibiting release stimulated by basic inducers in the presence of calcium, but not in the absence of the cation [109]. Basic compounds are thought to mobilize internal calcium stores in circumstances of calcium deprivation. Another study confirmed these data, but also proved La^{3+} to be the least effective of the lanthanide ions in abrogating mediator release induced by these agents [110]. In addition, it was found that the inhibition obtained in the presence of calcium was immediate and did not require preincubation of the cells. The inhibitory effects observed in the absence of the cation increased steadily, on extended preincubation with lanthanum. This suggests that penetration into less accessible sites in the membrane by lanthanum could lead to a displacement of bound calcium ordinarily at the disposal of the secretagogue. Alternatively, the lanthanide ions may exert a general stabilizing effect on the cell membrane, so preventing the mobilization of sequestered stores [2,110].

1.2.1.1 **Extracellular calcium**

The extent to which calcium levels are raised upon stimulation have been quantitatively assessed, using fluorescent the Ca^{2+}-indicator quin-2 [111]. These studies have established unequivocally a critical role for a rise in cytosolic calcium as a precedent for exocytosis [112]. Quin-2 is introduced into the cells as the acetoxymethyl ester derivative, which is nonpolar and traverses the cell membrane. Once in the cytosol, it is hydrolysed to the parent tetracarboxylate anion, which is membrane impermeable [113]. It is then possible to monitor discrete fluctuations in intracellular calcium, an increase in fluorescence corresponding to a rise in calcium levels.

The resting intracellular calcium level in RBL 2H3 cells was found to be of the order of 10^{-7}M, rising into the micromolar range upon stimulation with antigen [112]. La^{3+} blocked both the calcium signal and histamine release associated with cellular activation by antigen. This effect is consistent with a mechanism of action for La^{3+} as an antagonist of calcium entry into the cell. Moreover, cells challenged with antigen in the absence of extracellular calcium failed to release histamine and did not generate a calcium signal. Essentially identical results have been obtained in studies conducted on mast cells [114].

The physiological concentration of calcium in the extracellular space is 10^{-3}M, at least four orders of magnitude greater than the intracellular concentration. This differential creates a large concentration gradient, augmented by an electrochemical gradient, which promotes an inward flow of calcium into the cell. Under resting conditions, cellular homeostatic control mechanisms arrest calcium entry via favourable gradients. Besides promoting the sequestration of calcium into internal stores, recent experiments have shown that extrusion of calcium ions is facilitated through the operation of a sodium-calcium antiporter [115]. The basal efflux of radiolabelled calcium from the cells preloaded with the isotope was

found to be essentially unaffected by metabolic inhibitors, thereby excluding the immediate involvement of an ATP-dependent calcium pump. However, the process was entirely dependent on extracellular sodium ions. In order to maintain its biological integrity, the relative impermeability of the mast cell membrane is paramount [2,77].

Anaphylactic stimulation of the mast cell produces a transient rise in calcium permeability, facilitating cation entry [2,116]. This effect is coupled with a temporary cessation of calcium efflux, enhancing the rise in cytosolic calcium and hence providing a cooperative mechanism for augmenting the secretory response [115].

Studies involving a variety of secretagogues have demonstrated that calcium is in rapid equilibrium with the cell membrane [2]. It has been proposed that calcium binding to superficial sites receptive to the cation could be an initial step prior to its internalization and the consequent series of events leading to exocytosis [117,118].

1.2.1.2 **Intracellular calcium**

Basic inducers such as compound 48/80 can still elicit a potent response from mast cells in the absence of extracellular calcium. Immunologic stimuli do provide a token response in calcium-free media [2,119]. Under these circumstances, the elevation of cytosolic calcium levels is usually attributed to the utilization of intracellularly bound stores of the cation. Basic inducers appear to be more adept at mobilizing these stores than the immunologic ligands [120].

The depletion of intracellular calcium can be brought about, by prolonged incubation (1-3 hours) with a calcium chelating agent such as EDTA in a calcium-free medium, or by suboptimal concentrations of calcium ionophores such as A23187. Following this treatment, the mast cell becomes refractory to stimulation [104,121,122]. The combination of ionophore and calcium chelator appears to be the most effective, in terms of depleting the cells of their more firmly bound stores [123]. Restoration of the cells' responsivity to an applied stimulus is possible, by reconstitution in a calcium-containing medium. Restored secretion is comparable to that obtained with control cells [121-124].

1.2.1.3 **Membrane bound calcium**

In many cell types, the two most common sites associated with calcium sequestration are the mitochondrial apparatus and the cell membrane. However, lanthanum ions are progressively inhibitory and are thought not to traverse the membrane into the cytosol. Therefore, the calcium stores are most likely to be deeply buried within, or on, the membrane's cytosolic surface. Brief preincubation with EDTA (5 minutes) enhances the secretion produced by most inducers in the absence of extracellular calcium.

In contrast, supraoptimal concentrations of calcium inhibit histamine release by apparent membrane stabilization [2]. By analogy with smooth muscle, regulatory sites may then exist on the cell membrane which, when saturated, would reduce cellular responsivity to ligand challenge. Hence, removal of calcium from these regulatory sites, by brief exposure to a calcium chelator, may destabilize the membrane and provide an explanation for the enhanced secretion observed.

Therefore, three major calcium pools have been identified that are associated with mast cells. A superficial membrane site on the cell surface, an intracellular store of the cation and a regulatory site on the membrane, which on occupation tends to stabilize but on vacation renders the cell more responsive.

1.2.1.4 **Calcium channels**

Studies with calcium antagonists would not support the existence of discrete, voltage-dependent calcium channels in mast cells [124]. However, even in the absence of calcium ions, IgE-receptor aggregation causes depolarization of the plasma membrane. This suggests that other ions can traverse a putative channel. The supposition has been extended to the idea of a channel, which in the absence of calcium is rather unspecific but the presence of calcium ions can modify it to become selective for calcium [125].

Mazurek *et al.* [126] attempted to localise the site of action for the anti-allergic drug, SCG, on the basis that cromolyn exerts its inhibitory effects by blocking calcium uptake. A specific calcium-dependent binding site was demonstrated on the surface of mast cells and RBL 2H3 cells, through which the pharmacological activity of cromolyn was expressed [126].

Although the controversies surrounding these studies are manifest [127], a cromolyn binding protein (CBP) was isolated [128] and subsequently incorporated into the membrane of RBL 2H3 cell variants, which are naturally defective in binding cromolyn and unable to elicit normal calcium influx or degranulation, in response to IgE stimulation [129]. The fusion completely restored the capacity for calcium uptake and degranulation after immunologic stimulation. Thus, substantial evidence has been provided for the existence of a specific membrane calcium channel, in some way related to the CBP, permitting calcium influx during the secretory response.

1.2.1.5 **Calmodulin**

In non contractile cells, calmodulin is the intracellular receptor for calcium which modulates calcium-regulated processes [130-133]. Calmodulin has been detected in mast cells [134] and basophils [135]. In fact, the widespread occurrence of calmodulin, together with a primary structure highly conserved throughout nature, suggests that it is a protein of significant functional importance [130-133]. A scheme for the mode of action of calmodulin involves the binding of calcium to the protein, to induce a conformational change. This structural modification constitutes a rise in the helical content by approximately 10%. Calmodulin interaction with a given enzyme also brings about these events, as follows:

$$CaM + nCa^{2+} \rightleftharpoons CaM.Ca^{2+}n \rightleftharpoons CaM^{*}.Ca^{2+}n$$

$$CaM^{*}.Ca^{2+}n + ENZYME \rightleftharpoons (CaM^{*}.Ca^{2+}n).ENZYME \rightleftharpoons (CaM^{*}.Ca^{2+}n).ENZYME^{*}$$

where * indicates structural modification.

Calmodulin possesses four binding sites for calcium but the full significance and nature of these sites remains obscure. If the sites exhibit different binding affinities, the protein could be predisposed to binding calcium, at specific sites, dependent on the availability of the cation. Several enzymes may well be under calmodulin control, so it is necessary to ensure the correct enzyme is activated in a given situation.

It is possible that differing levels of calmodulin occupancy (n=1-4), determined by calcium availability, could determine specific enzyme activation. Therefore, elevations of intracellular calcium would tend to activate the calmodulin-regulated enzyme(s), whereas a reduction in cytosolic calcium would lead to a dissociation of the active calcium-calmodulin complex. Several, ill-characterized proteins may bind to calmodulin and thereby regulate its function. Of these, calcineurin is the most widely studied. This protein inactivates calmodulin by combining with it to form an inactive conjugate [136].

A number of neuroleptic drugs, principally those of the phenothiazine family, are able to combine with the calcium-calmodulin complex. These compounds prevent the interaction of the complex with its target proteins and so inhibit calcium-dependent reactions. Most significantly, these agents prevent histamine release from mast cells and basophils [135,137,138].

These results provide preliminary evidence for the involvement of calmodulin in histamine release. However, several enzymes implicated in mast cell function are regulated by calmodulin, including cyclic nucleotide phosphodiesterase, adenylate/guanylate cyclase, various ATPases, phospholipase A_2, myosin kinase and several kinases involved in specific protein phosphorylations [139,140]. Activation of adenylate cyclase leads to the generation of cyclic AMP, a process reversed by phosphodiesterase. Calmodulin may be involved in the intracellular regulation of the nucleotide, due to its ability to regulate these two enzymes. In the mast cell, immunologic activation instigates a transient rise in cyclic AMP, which rapidly declines. This occurs prior to secretion. As calcium enters the cell, it passes through the membrane and in doing so, may activate membrane bound adenylate cyclase, with an associated elevation of cyclic AMP. Excess calcium may activate phosphodiesterase and the degradation of the newly formed cyclic nucleotide [130,132].

Intracellular calcium levels may be regulated by a calmodulin-dependent protein kinase, responsible for promoting calcium sequestration into internal stores. The activity of calmodulin found in the cytosol can be influenced thereby [141]. Hence, calmodulin may be considered self-regulatory. Microtubules may be important in the translocation of the granule to the periphery during exocytosis [142]. Myosin kinase is associated with microtubular function and thought to initiate contraction by phosphorylating a major protein on the light chain of myosin [143,144].

In many different cell types, the phosphorylation of particular proteins may well be the critical event in stimulus-secretion coupling. Mast cell activation initiates phosphorylation of three proteins of molecular weight 48, 55 and 59kDa [145]. In addition, the phosphorylation of a 78kDa protein is coincident with its involvement in the termination of

secretion. Protein kinase C may be responsible for the phosphorylation of the 48kDa protein [146]. Specific, calmodulin-dependent cytosolic and membrane protein kinases may be involved in the phosphorylation of other key mast cell proteins. Thus, a synergistic interaction between the diacylglycerol (DAG) and the calcium-signal pathway can be proposed.

Berridge suggested that the hydrolysis of phosphatidyl inositol 4,5 diphosphate may represent a 'bifurcation' in the signal pathway [147], resulting in the formation of two separate second messengers. One limb of the pathway depends on DAG, which activates protein kinase C to phosphorylate specific cellular proteins. The other pathway depends on calcium, which acts through calmodulin to phosphorylate a separate group of proteins. If this is so, an element of versatility is conferred, necessary to introduce subtle variations in control mechanisms. The two pathways may contribute to the final response by acting co-operatively or synergistically.

In summary, tentative evidence exists for calmodulin involvement in mast cell secretion and the possible inclusion of a synergistic pathway in the mechanism of histamine release remains an attractive possibility.

1.2.2 Phospholipids

1.2.2.1 **Phosphatidyl serine**

Goth and coworkers [148,149] first reported that phosphatidyl serine (PS) enhanced anaphylactic histamine release from rat peritoneal mast cells. The release evoked by the basic inducer, compound 48/80, was unaffected by the addition of PS. The observed potentiations were quite specific to PS and not produced by ethanolamine, choline or inositol phospholipids. Various mastocytes were heterogeneous in their responses to PS; enhancement of the anaphylactic reaction was greater in rat peritoneal mast cells than in the rat mesentery and only minimal in rat or guinea pig lung [150]. PS was totally ineffective in human basophil leucocytes [151].

Mixed peritoneal mast cells will release a small amount of histamine in response to dextran stimulation [152] and the reaction is considerably potentiated by the addition of exogenous PS [119,149,153]. However, PS is not required for dextran-induced histamine release from peritoneal cells of Wistar rats [94] or mast cells from rat tissues such as skin [154] or mesentery [155].

In rat peritoneal cells, PS acts almost immediately to increase the magnitude of histamine release. Calcium is necessary for the enhanced release induced by dextran [94] or by the antibody-antigen reaction. As such, PS is presumed to potentiate secretion by facilitating the uptake of calcium, during anaphylactic stimulation [116]. Acidic phospholipids, such as PS and phosphatidyl inositol (PI), are essential components of cell membranes and have been shown to interact with calcium [156]. While PS and PI bind calcium and possibly other divalent ions, the neutral phospholipids do not [157]. Studies have shown that the carboxyl group is important in the biological activity of PS [158] and calcium binding is a

function of the degree of ionization of phosphate and carboxyl groups [159]. This may account for the inactivity of phosphatidyl ethanolamine (PE), the decarboxylated derivative of PS.

1.2.2.2 **Phospholipid methylation**

Fc_ϵ-receptor cross-linking appears to initiate the methylation of some membrane phospholipids. Evidence indicates that following receptor cross-linking, membrane PS is decarboxylated to yield PE [160-162]. Membranes appear to contain two methyl transferases (MT's) requiring S-adenosyl-L-methionine as a receptor, the first located in the inner section of the membrane bilayer and the second in the outer section [162]. The first enzyme (MT I) methylates PE to yield phosphatidyl-N-monomethylethanolamine. This is then the substrate for the second enzyme (MT II), which converts it to phosphatidyl choline (PC) [162].

The net result appears to be the conversion of PS from the inner layer of the membrane to PC in the outer layer [160]. PC may be a substrate for phospholipase A_2, which converts it to lyso-PC and AA [163].

The methylation reactions have been shown to precede histamine secretion [164-168] and are temporally related to calcium influx into the mast cell [76,116]. Also, inhibitors of MT's prevent histamine secretion and calcium influx [169,170]. However, the dose-response curves for these inhibitors do not exclude the possibility that inhibition of MT activity is not related to the inhibition of calcium movement and histamine secretion [171,172].

The major difficulty is to ascertain how this system generates a signal to the cell, since all it appears to do is produce PC in the outer membrane leaflet and this phospholipid already forms the bulk of the membrane. It is said that the process can increase membrane fluidity, but this does not resolve the question of membrane signal transduction.

1.2.2.3 **Phosphatidyl inositol turnover**

Increasing evidence suggests that hydrolysis of membrane phosphoinositides is a critical event in stimulus-secretion coupling in mast cells [173-175]. The initial view was that the primary event, following interaction of a calcium dependent agonist with its receptor, was the breakdown and resynthesis of PI. Original studies were performed by Hokin and Hokin [176] and extended by Michell, who proposed that breakdown of membrane PI is an essential step in the formation and control of calcium channels [177]. Early investigations in mast cells were made by measuring the incorporation of [^{32}P] or [^{3}H] inositol into PI, PC and phosphatidic acid [174,175]. More recent studies assayed PI metabolism directly, by measuring the degradation of the pre-labelled lipid, principally because the activation of any of several pathways of lipid metabolism can lead to an increase in PI labelling [178] without an increase in PI breakdown.

Kinetic studies have shown large increases in the levels of inositol 1,4,5-trisphosphate (IP_3) and inositol 1,4-bisphosphate (IP_2) upon stimulation [179]. The very rapid formation of inositol phosphates suggested that IP_3's may function as the second messengers of calcium mobilizing receptors, to release internal calcium [147]. IP_3 induced calcium liberation from

the endoplasmic reticulum of permeabilized pancreatic acinar cells. The release mechanism was specific for IP_3, since the effect was not observed with IP_2 or inositol monophosphate (IP) or inositol 1,2, cyclic phosphate [180-181].

Various proposals have been presented to explain the mechanism by which these lipids might operate to regulate the permeability of the plasma membrane, including the opening of a channel [182,183] or the formation of phosphatidic acid that may function as a calcium ionophore [182]. Alternatively, the agonist may modulate calcium permeability indirectly through an intracellular messenger, that can be either calcium [184] or IP_3 [180,185].

Nakamura and Ui have demonstrated that the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) is the primary event in phosphoinositide metabolism in mast cells [186]. The transient breakdown of PIP_2 on mast cell stimulation with compound 48/80 was coincident with the rapid generation of the inositol phosphates and DAG. ^3H-labelled IP_3 was formed as a direct product of the metabolism of PIP_2, reaching maximum levels within 5s and then being hydrolysed to IP_2 and IP in succession. The IP is recycled back through free inositol for re-synthesis to PI. The substrate supply of PIP_2 is limited by the activity of the inositol lipid kinases, which sequentially phosphorylate PI to the monophosphate (PIP) and then to PIP_2.

In spite of these results, studies by Majerus *et al.* [187] have demonstrated that in platelets, all three phosphoinositides, PI, PIP, PIP_2 are substrates for two, distinct, isolated phospholipase C enzymes. However, only PI metabolism is absolutely calcium-dependent. Thus, on cellular activation, phospholipase C may catalyse PIP_2 hydrolysis and the subsequent generation of IP_3, which triggers an increase in intracellular calcium. Calcium is itself a prerequisite for phospholipase C hydrolysis of PI.

These data infer that the production of IP_3 and DAG are separate events, since the majority of DAG is derived from PI. Furthermore, greater quantities of DAG are produced than IP_3, whereas the original scheme predicts equimolar amounts of each metabolite. It would seem that the early but small calcium-independent hydrolysis of PIP_2 may sometimes be masked by a massive increase in calcium-dependent lipid metabolism as part of an amplification loop responsible for the formation of large quantities of AA metabolites. Thus, calcium-independent and calcium-dependent changes in phosphoinositide metabolism may coexist within the same cell [147].

Michells' hypothesis suggests that PIP_2 breakdown should occur at intracellular calcium concentrations present in the resting cell. Receptor-mediated breakdown of inositol lipids must precede (and thus be independent of) any increase in the intracellular level of calcium.

Experiments in rat brain [188], RBL 2H3 cells [189] and intact mast cells [174] have established that PIP_2 breakdown is calcium-independent. IP_3 formation and the generation of an intracellular calcium signal were found kinetically coincident upon antigen stimulation of RBL cells. The antigen-induced calcium signal could be selectively blocked by La^{3+},

without affecting stimulated phosphoinositide breakdown. A23187 activation caused only a small increase in phosphoinositide metabolism.

Neomycin is a phospholipase C antagonist [190] and has been applied to the mast cell [191]. The investigators suggested the participation of a guanosine triphosphate (GTP)-binding protein in calcium-mediated release. Mast cells, transiently permeabilized in the presence of the GTP analogue, guanosine 5'-O-(3-thiophosphate) (GTP-γ-S), will secrete histamine on subsequent calcium addition [192].

The islet activating protein (IAP) from pertussis toxin selectively interacts with N_i, the guanine regulatory protein responsible for the inhibition of adenylate cyclase in mast cells [193-195]. N_i communication between membrane receptors and the catalytic unit of adenylate cyclase is lost, as a result of IAP catalysed, ADP-ribosylation of a 41kDa protein. This protein is a GTP-binding subunit of N_i [193-198]. Histamine release induced by a variety of agents was also regulated by pretreatment of the cells with pertussis toxin [199]. In addition to histamine secretion, pertussis toxin abolished compound 48/80-induced transient breakdown of PIP_2, rapid generation of inositol phosphates, ^{45}Ca inflow and AA liberation from PC. No such effects were observed on stimulation with A23187. Inhibition was calcium independent and thus receptor-mediated. IAP-susceptible breakdown of PIP_2 is then unlikely to result from, or depend on, increases in intracellular calcium.

The phosphoinositide phosphodiesterase of human neutrophil plasma membranes can be activated simply by adding GTP analogues in the presence of physiological resting calcium concentrations [191]. Phosphodiesterase may be subject to control by the binding of GTP in exchange for guanosine diphosphate (GDP). This occurs at a receptor-controlled guanine nucleotide binding protein (N-protein) in it's cellular environment. Consequently, activation of this putative N-protein motivates phosphodiesterase to hydrolyse PIP_2.

DAG is transiently produced in cell membranes as a result of PIP_2 hydrolysis. It serves as a second messenger to activate protein kinase C, which phosphorylates certain proteins [146,200,201]. Whilst IP_3 is released into the cytosol, DAG is retained in the organic phase of the membrane [146,147].

Protein kinase C is a calcium and phospholipid-dependent enzyme, detected in a variety of tissues [202]. In mast cells, it is located in the cytosol, with phosphate acceptor proteins of the enzyme [203], where it is presumably inactive. This enzyme has a hydrophilic cata-lytic domain and a hydrophobic membrane-binding domain [146]. The production of DAG results in the binding of protein kinase C to the cell membrane, presumably to PS [187]. A small amount of DAG then dramatically increases protein kinase C calcium-affinity, fully activating the enzyme without any change in calcium levels [200,201, 204,206].

Protein kinase C is also a target for phorbol esters, such as 1-oleoyl-2-acetyl-glycerol (OAG) and 12-O-tetradecanoylphorbol-13-acetate (TPA). These compounds are structural analogues of DAG, which can be intercalated into the membrane and substitute for DAG by directly activating the enzyme [146,207,208]. The phosphorylation of four cytosolic

proteins (48, 55, 59 and 78kDa), concurrent with PI hydrolysis and histamine release, suggested a role for protein kinase C in stimulus-secretion coupling in mast cells [145]. TPA-induced phosphorylation of the same spectrum of proteins but without an associated rise in intracellular calcium. TPA has also been shown to promote histamine release independently, in the absence of extracellular calcium [209]. Synergy is observed between TPA and histamine secretagogues that utilize extracellular calcium [145]. The phorbol ester may then also enhance the cellular calcium sensitivity. Accordingly, low concentrations of TPA, which synergize with antigen induced release in RBL 2H3 cells, also blocked calcium influx but not the influx elicited by A23187 [210].

Exogenous PS [145] or lyso-PS [209], in conjunction with TPA, enhanced histamine release induced by calcium dependent agents. Thus, the calcium dependent pathway, promoted by the lipids or protein kinase C activation by TPA alone, can induce release from mast cells. Together, these agents act synergistically.

Finally, it has been reported that one minute after mast cell stimulation with compound 48/80, a sharp increase in the phosphorylation of a 78kDa protein occurs [145]. Other secretagogues have a much slower time course of histamine release and did not show significant phosphorylation of the 78kDa protein within one minute. Studies on the time course of compound 48/80-induced protein phosphorylation [211] and SCG [212] have suggested that the phosphorylation of this protein is related to the mechanism by which histamine secretion is terminated. Therefore, the phosphorylation of three of these proteins may be involved in the induction of exocytosis, whilst the fourth is associated with the cellular mechanism for physiological termination and pharmacologic inhibition of the secretory response.

To summarize, mast cell stimulation leads to the hydrolysis of PIP_2 to DAG and IP_3. These two metabolites then function as second messengers to activate two, independent but parallel pathways, that may also be responsible for AA release and guanyl cyclase activation [177,213,214]. DAG functions within the plane of the membrane to increase protein phosphorylation by activating protein kinase C. IP_3 is released into the cytosol to mobilize calcium from intracellular stores and possibly to increase the permeability of the plasma membrane to calcium. These two signal pathways seem to operate synergistically to stimulate the activation of the cellular response.

1.2.3 Arachidonic acid generation and metabolism

Mast cell activation causes the secretion of preformed, granule-associated mediators and initiates the *de novo* synthesis of certain lipid-derived substances [2,4,5,215]. Of particular importance are the cyclooxygenase and lipoxygenase metabolites of (AA) [4,216]. These products posses potent inflammatory activity and may also play a role in modulating the release process itself [4,5,217,218].

A major source of AA is derived from membrane phospholipids, where activation of specific phospholipases can liberate the free fatty acid. It has been proposed that the early

biochemical events in stimulus-secretion coupling involve the sequential methylation of phospholipids to PC, facilitating an influx of calcium necessary for the activation of phospholipases [116]. PC is then metabolized to lyso-PC and AA by the activation of phospholipase A_2 [163].

Inhibition of histamine release from activated mast cells is possible on the application of purported phospholipase A_2 inhibitors such as p- bromophenacyl bromide (BPB) [186,219-221], mepacrine [186,221,222] and tetracaine [223]. Histamine release induced by the ionophore chlortetracycline (CTC) was unaffected [222] by mepacrine and exogenously applied phospholipase A_2 produces exocytosis which can be inhibited by BPB [221].

Cyclooxygenase products include PG's and thromboxanes, whereas lipoxygenase pathways generate the LT's, hydroperoxyeicosatetraenoic acids (HPETE's) and the related products, hydroeicosatetraenoic acids (HETE's). In the rat peritoneal mast cell, the majority of liberated AA is apparently metabolized via the cyclooxygenase pathway to yield significant quantities of PGD_2 by either immunologic or non-IgE directed ligands [220,221] Treatment with indomethacin and other non-steroidal anti-inflammatory drugs (NSAID) prevented this generation of PGD_2, presumably as a consequence of their known inhibition of cyclo-oxygenase, but left histamine release unaffected [219,221,223].

The AA analogue 5,8,11,14-eicosatetraynoic acid (ETYA), which inhibits both cyclo-oxygenase and lipoxygenase, prevented both PG generation and histamine secretion [223,219,224]. Moreover, the addition of HETE's was found to augment secretion provoked by IgE-mediated secretagogues [225]. Collectively, these results pointed to one or more lipoxygenase products being important in mediating histamine secretion.

Nemeth and Douglas however, have suggested that ETYA and other inhibitors such as 5,8,11,14-henicosatetraynoic acid (HTYA) and 4,7,10,13-icosatetraynoic acid (ITYA), which are thought to be more specific inhibitors of lipoxygenase, are not, in fact, acting as antagonists of either pathway. These compounds block immunologically induced histamine secretion but not that induced by pharmacologic agents such as compound 48/80, unless ultra-high concentrations of the antagonists are employed [226,227]. This would indicate a point of action for these drugs, early on in the sequence of biochemical events and one peculiar to IgE-directed ligands.

Basophils produce minor quantities of AA metabolites in comparison to the mast cell [18]. Furthermore, human basophils grown *in vitro* failed to generate any AA metabolites [228], suggesting that this is not a component of the release process. However, inhibition of immunologically and non-immunologically induced secretion from isolated basophils was obtained with BPB [217,218,223], mepacrine [217,218], ETYA [217,218,223,229] and the lipoxygenase inhibitor 5,8,11-eicosatriynoic acid (ETI). Indomethacin either had no effect or potentiated release [217,218, 229]. An extensive study, utilizing eighteen inhibitors of cyclooxygenase, lipoxygenase, PG isomerases and thromboxane synthetases, has shown that only lipoxygenase inhibitors, such as nordihydroguairetic acid (NDGA), prevented both mediator secretion from basophils and mast cells [223].

The major arachidonate metabolite of RBL cells was found to be PGD_2 [167,230], although the generation of LT's and HETE's have been reported [229,231] and a LTC_4 synthetase has been detected [232]. Purified human lung mast cells generate equimolar quantities of PGD_2 and LT's, predominantly LTC_4, but significant amounts of LTB_4 are also produced [232,233].

Rat and mouse peritoneal mast cells generate PGD_2 and contain heparin. The mouse mast cell, derived from bone marrow, contains chondroitin sulphate and generates LTC_4 predominantly [48], some LTB_4 [49] and little or no PGD_2. A possible correlation may exist between AA metabolite production and proteoglycan content [234].

Another class of lipid-derived mediators of which platelet activating factor, PAF-acether, is the prototype, is generated from lung mast cells, mouse bone marrow derived mast cells and rabbit basophils [18,235,236]. PAF-acether has an inflammatory potential equal to that of the LT's and it is possible that these two mediators are metabolically linked [237]. PAF is not found in unstimulated mast cells but significant quantities can be recovered after IgE cross-linking and in association with the cells rather than in the supernatant fluid [238].

Finally, in some cells, the agonist dependent hydrolysis of PIP_2 may play an important role in controlling the release of AA in a separate pathway [214,239-241]. The fatty acid on the 2-position of the phosphoinositides is usually AA, thus DAG lipase may be responsible for releasing AA from DAG [241]. Alternatively, other workers have proposed that phosphatidic acid is degraded to AA by an acid-specific phospholipase A_2 [242]. It may be possible that the same situation exists in the mast cell where AA and its metabolites are derived from phosphoinositide hydrolysis, in addition to AA release from PC and other phospholipids.

1.2.4 Protease activation

Esterases containing serine residues at the active site can be irreversibly inhibited by diisopropylfluorophosphate (DFP). DFP inhibits antigen-induced secretion from chopped guinea pig lung, suggesting that a putative proteolytic enzyme, with serine esterase activity, is involved in exocytosis [243]. These studies were extended and DFP was found to inhibit mediator release from rat mast cells [244], chopped human lung [245] and basophil leucocytes [246]. DFP must be present at the time of challenge in order to suppress secretion, implicating the existence of a DFP-insensitive proenzyme which is activated on challenge.

Studies on purified rat mast cells demonstrated that DFP inhibited both phospholipid methylation and the transient rise in cyclic AMP, in addition to mediator release [76,116]. DFP also inhibits glucose utilization by mast cells and so it has been proposed that DFP may act by inhibiting metabolic energy production [247]. Therefore, DFP inhibition of mediator release may not be solely due to serine esterase antagonism. However, later studies with other serine esterase inhibitors and substrates of proteolytic enzymes, such as trypsin

and chymotrypsin, were performed. Inhibition of phospholipid methylation and cyclic AMP synthesis was observed by agents with anti-serine esterase activity [76,116].
Chymase [248] and tryptase [29] have been isolated from rat peritoneal mast cells. Chymase was found to elicit mediator release, elevate cyclic AMP levels and PGD_2 production [248].
It has been reported that chymase, heparin and some undefined protein in rat mast cell secretory granules remain associated with the mast cell surface after immunologic or A23187 activation [249]. Tryptase from secretory granules is released into the extracellular space, in a fixed ratio to histamine, after IgE-dependent activation of human pulmonary cells [28] and rat peritoneal mast cells [29]. These data suggest that tryptase acts in the extracellular environment and chymase on the cell surface of mast cells after stimulation. Chymase and tryptase are released as complexes with associated inhibitors; heparin and trypstatin. Therefore, a modulatory role for these inhibitors may exist [248].
It would seem that a membrane associated proteolytic enzyme is activated by Fcϵ-receptor bridging. The protease is involved in the activation of adenylate cyclase and MT's. However, phospholipid methylation is a phenomenon not yet affiliated with secretion in a causative capacity [250-253].

1.2.5 Cyclic nucleotides

The cyclic nucleotides, adenosine 3',5'-cyclic monophosphate (cyclic AMP) and guanosine 3',5'-cyclic monophosphate (cyclic GMP), play an integral role in the modulation of biological function in a variety of cell types [254-256]. Berridge classified cells into two systems and proposed that cyclic AMP enhanced the calcium signal in 'monodirectional systems' whereas in 'bidirectional systems' it opposes the calcium signal [254].
Early studies showed that adrenaline could inhibit antigen induced histamine release from sensitised guinea pig lung slices [257]. Pharmacologic agents that elevate cyclic AMP levels, such as β-adrenergic agonists, PGE_1 and the methylxanthine phosphodiesterase inhibitors, inhibit histamine release as well as the generation and release of SRS-A from sensitised primate lung fragments [258-261]. Conversely, α-adrenergic agonists and muscarinic cholinergic agonists, which elevate cyclic GMP levels, enhanced mediator release [262-264]. In mixed human leucocytes, agents which elevate cyclic AMP inhibit the immunologic release of histamine [265-267]. Thus, the mast cell appears to conform to a bidirectional system.
Later studies were performed on purified mast cells stimulated with a wide range of immunologic, pharmacologic and ionophoretic stimuli [76,268-276]. However, no consistent correlation between cyclic AMP alterations and histamine release emerged. Mast cell release induced by A23187, concanavalin A, antigen and anti-IgE was either suppressed [270,273-275], unaffected [275,276] or enhanced [273,274] by drugs that elevate cyclic AMP [273,274,277].

Immunologic stimulation of purified rat serosal mast cells produced a transient, monophasic rise in cyclic AMP levels within 15 seconds of activation [76,269,270]. A second peak was observed at 2-5 minutes. Increments of cyclic GMP followed cyclic AMP; a monophasic increase at 30 seconds and a second, smaller monophasic rise at 3-5 minutes [76,261].
Preincubation with indomethacin suppressed the conversion of AA into PGD_2 and PGF_2, ablated the second peak of cyclic AMP and both rises of cyclic GMP [243], but had no effect on histamine secretion [270]. This suggests that the initial rise in cyclic AMP is a primary event in coupled activation and secretion, whereas the other cyclic nucleotide changes are related to the generation of oxidative products of AA [261,269,278].
Theophylline produces an inhibition of mediator release that is directly correlated to mast cell cyclic AMP levels. PGD_2 elevates cyclic AMP levels without affecting mediator release [268,271,279]. The proposed rationale for these observations suggests that there are separate pools of cyclic AMP within mastocytes and that only certain pools are linked to the inhibition of histamine secretion [275,279]. Therefore, PGD_2 produces a non-inhibitory pool of the nucleotide.
Receptor linked adenylate cyclase is thought to be a ternary complex of receptor, G/F protein (R site) and a catalytic subunit (P site). Cell surface purinoceptors are subdivided into A_1-receptors which inhibit adenylate cyclase and A_2-receptors, which activate the enzyme [280,281]. Adenosine interacts with the R site, increasing adenylate cyclase activity. Methylxanthines, at low concentrations are competitive antagonists of adenosine. At higher concentrations (micromolar), adenosine interacts at the P site and inhibits adenylate cyclase activity [280,282].
Adenosine potentiates histamine release from rat mast cells [272,283-285], which is proportional to the increase in intracellular cyclic AMP. Adenosine does not induce histamine release alone, implying the necessity of coupling of the Fcϵ-receptor with enzyme. The P site active adenosine analogues, 2',5'-dideoxyadenosine (DDA) and 9-(tetra-hydro-2-furyl)-adenosine, attenuate the initial rise in cyclic AMP following stimulation, with parallel suppression of mediator release [284]. These findings indicate that Fcϵ-receptor activated adenylate cyclase, with the consequent early rise in cyclic AMP, is an essential event in the secretory process.
Cyclic AMP can mediate its effects through specific cyclic AMP-dependent protein kinases that are predominantly found in the cytosol of eukaryotic cells [278,286,287]. The enzyme is tetrameric, comprising an inhibitory, regulatory dimer (R) and two catalytic subunits (C). The type I and II regulatory dimers are immunologically distinct entities. The stoichiometry of activation is as follows:-

$$R_2C_2 + 4 \text{ cyclic AMP} \rightleftharpoons R_2(4 \text{ cyclic AMP}) + 2C$$

The catalytic subunit evolved participates in protein phosphorylation in the receptor polypeptide [286,287]. In the mast cell, 94% of protein kinase activity is cyclic AMP dependent and the ratio of type I to type II isoenzymes is 1:5 [288].

Theophylline causes a rise in cyclic AMP and inhibition of mediator release. Additionally, it antagonises protein kinase activity. PGD_2 did not affect either mediator secretion or protein kinase activity [261]. The P site active DDA was found to attenuate cyclic AMP production and protein kinase activation, with an associated reduction in mediator release [278,289].

There would seem to be a direct connection between Fc_ϵ-receptor aggregation, activation of adenylate cyclase, generation of cyclic AMP with subsequent kinase activation and mediator release [233].

Polybasic compounds and ionophores, compounds which are not involved in or bypass any membrane related adenylate cyclase activation, do not produce an increase in cyclic AMP levels after presentation to the cells. Rather, there is some evidence that a fall in nucleotide levels occurs soon after challenge [268,272]. Moreover, immunologic activation of RBL cells is not accompanied by elevated levels of cyclic AMP prior to secretion [166]. These findings imply that cyclic AMP elevation is not a prerequisite for mediator release from mast cells but may be important for some types of secretion.

Incongruously, agents which do not activate adenylate cyclase, such as compound 48/80, dextran and A23187, were potentiated by adenosine and suppressed by DDA without significant changes in the intracellular cyclic AMP levels [272,283,290,291]. Secretagogues which induce release without a preceding transient rise in cyclic AMP should not be susceptible to the modulatory effects of adenosine and its analogues. This is because R site agonists will only potentiate secretion by increasing an existing cyclic AMP signal and P site agonists inhibit secretion by attenuating the early rise in cyclic AMP [284].These studies cast doubt on the criteria used to implicate a role for adenylate cyclase and a transient rise in cyclic AMP as an essential event in stimulus-secretion coupling [261,278].

Vardey and Skidmore have reported that the enhancement of histamine secretion by PIA or adenosine is unaffected by theophylline [292]. Further examination revealed that, in the presence of theophylline, PIA was able to enhance IgE-mediated histamine release without increasing cyclic AMP levels [290]. Similar studies have demonstrated that the relative potencies of adenosine analogues and nucleotides, taken with the fact that the effect was antagonised by 8-phenyltheophylline (a potent purinoceptor antagonist) or theophylline, did not uphold purinoceptor mediation to rationalise adenosine enhancement [293]. Therefore, two functional receptors for adenosine may exist on the mast cell membrane, an A_2 purinoceptor linked to adenylate cyclase and an uncharacterised receptor, coupled independently to the enhancement of mediator release [290,293].

To conclude, it would seem certain that a transient increase in cyclic AMP does occur in response to immunologic stimulation but the bearing of this on the secretory process remains unclear. Studies on the time course of changes in cellular levels of cyclic AMP with respect to the kinetic profiles of basic peptides, antigen, anti-IgE and A23187, suggested that the two events may not be causally related [294]. It is possible that increases in cyclic

AMP may be involved in other cellular processes related to the termination of the immunologic secretory mechanism.

1.3 *COMPOUND 48/80*

1.3.1 History

In 1937, Ide and Buck [295] described the synthesis of a number of tetrahydroisoquinolines (THIQ's) which were prepared by cyclization of the appropriate phenethylamine (Fig. 1.31). Closure of the heterocyclic ring was achieved by using formaldehyde in acid as a source of methylene groups, to link the amine of the side chain to the aromatic ring. Later, other investigators described the hypotensive effects of some of the THIQ's [296]. One of them, 6-ethoxy-N-methyl THIQ, was especially potent. In a study of the purity of this product, it was found that in addition to the THIQ, it contained an unidentified byproduct which was responsible for the strong hypotensive effect [297].
Baltzy *et al.* [298] described their studies of the byproduct and reported that the yield of hypotensive activity could be greatly increased if the structure of the phenethylamine starting material was altered slightly. They found the optimum activity was obtained by reacting equimolar concentrations of formaldehyde and p-methoxy-N-methylphenethylamine. By countercurrent distribution, they separated fractions which appeared to contain the dimer, trimer, tetramer and higher oligomers. The hypotensive activity was associated with the trimeric and tetrameric members of the family and p-methoxy-N,N-dimethylphenethylamine was also quite active.
In 1966, DeGraw *et al.* [299] synthesised the dimer and trimer by an alternative route and reported them to be inactive. They concluded that the active members of the family must be higher molecular weight oligomers. Read and Lenney [300] later reported that the active constituents range , in free base molecular weight, from approximately 700 to 1400. This suggested that the degree of polymerization of the active constituents ranges from the tetramer to the octamer, with the average being the hexamer.
Since its discovery, a precipitate from this reaction mixture, known as compound 48/80, has been shown to be a histamine liberator [301] and is extensively used in the study of this and related phenomena because of its high potency in most systems. The physiologic stimulus for the release of histamine is provided by the combination of antigen with specific antibody fixed to the cell membrane. In contrast, secretion stimulated by compound 48/80 seems to operate via a non-IgE-directed pathway.

1.3.2 Calcium and compound 48/80

The most significant difference in these two, apparently separate induction mechanisms, is in their calcium requirements for activation. Compound 48/80 was reported to elicit histamine secretion in the absence of extracellular calcium [302]. The suggestion was made that the polyamine could mobilize intracellular calcium stores and cells deprived

of this reserve, by treatment with calcium chelators or A23187, became unresponsive [104,303,304].

Later studies on rat peritoneal mast cells reported that supramaximal concentrations of calcium (>1mM) were in fact inhibitory to compound 48/80 induced stimulation [305]. Similar results were obtained with peptide 401, the mast cell degranulating (MCD) peptide from bee venom [305].

Strontium was able to substitute for calcium but at higher concentrations and without inhibitory effects at supramaximal levels. Mast cells pretreated with EDTA still released histamine in response to the polyamines, provided that calcium, strontium or barium, but not magnesium, were added with the inducer. It then follows that all three cations are individually able to promote histamine release or that these ions displace bound calcium which is inaccessible to the action of both EDTA and the basic releasers.

Taken together, these results suggest a dual mechanism of action for compound 48/80 and peptide 401. These agents can clearly utilize intracellular calcium to initiate the release process but can also stimulate cells depleted of this store provided that calcium is present in the extracellular medium. Under the latter conditions, the inducers may evoke exocytosis by promoting calcium uptake, presumably by activating specific calcium gates [307] in the membrane in a way similar to antigen and other agents. The mechanism by which the basic releasers mobilize intracellular calcium was not clear. Cochrane and Douglas [307] suggested that calcium may be bound to the inner plasma membrane, in a manner which requires or is facilitated by the binding of sodium to the outer surface of the cell. Cationic agents like compound 48/80 and peptide 401 might then displace sodium from these sites, with an accompanying liberation of intracellular calcium and release of histamine.

Dainaka *et al.* [308] demonstrated a spike increase of intracellular $^{45}Ca^{2+}$ and a decrease of total $^{45}Ca^{2+}$ within a few seconds after exposure of $^{45}Ca^{2+}$-equilibrated mast cells to compound 48/80, without any significant granular extrusion. It is possible that the polycation is able to utilize membrane-associated Ca^{2+} effectively for mediator release by acutely mobilizing it for transference into the cells. This causes a small increment in the size of the exchangeable intracellular Ca^{2+} compartment. Some cation is partially discharged from the cells. The resulting transient increase in intracellular Ca^{2+} would seem to cause the release of histamine.

1.3.3 Arachidonic acid

AA inhibited compound 48/80 induced calcium mobilization in $^{45}Ca^{2+}$ -preloaded mast cells [308]. This observation led to the proposal that the formation of an electrostatically stable complex could take place between membrane associated $^{45}Ca^{2+}$ and AA or its metabolites. This proposal was supported by several other observations. Firstly, AA increased the total $^{45}Ca^{2+}$ uptake in stimulated and unstimulated cells at 0°C but not the intracellular $^{45}Ca^{2+}$ uptake. Secondly, AA inhibition was Ca^{2+}-dependent but AA itself caused detectable histamine release in Ca^{2+}-depleted media. Lastly, lidocaine, a suppressor

of cellular Ca^{2+} binding and NGDA, an inhibitor of calcium flux, stimulated AA-induced histamine release. However, these agents inhibited compound 48/80- induced histamine secretion.

Goth and Knoohuizen [309] reported that, like IgE-mediated secretion [224,229], compound 48/80-induced release was also inhibited by ETYA, NGDA is also a lipoxygenase inhibitor [310] and was able to inhibit histamine release induced by melittin, a phospholipase A_2 activator [311]. The phospholipase A_2 inhibitor, mepacrine could inhibit compound 48/80- and AA-induced secretion [222,308]. These results suggested a lipoxygenase-dependent mechanism is possibly of importance in compound 48/80-induced release but cyclooxygenase metabolism is unnecessary, as indomethacin and aspirin are without effect.

1.3.4 Heterogeneity

Several other deviations from IgE-mediated secretion have been observed for compound 48/80. Perhaps of equal importance to the calcium requirements, is the marked tissue and species specificity of compound 48/80 action. The agent produced a severe anaphylactoid reaction in the rat, cat and dog [312-314] but has a limited or negligible effect in the rabbit, mouse, guinea pig, hamster, man and the monkey [312,315-319]. The systemic responses in the former species are accompanied by a marked degranulation of mast cells and a depletion of histamine in affected tissues, including the skin, mesentery, omentum, skeletal muscle, heart, lung, diaphragm and tongue [313,314,320]. However, mast cells in the mucosa of the gastrointestinal tract are completely unresponsive to compound 48/80 [55].

The species and tissue specificity shown by compound 48/80 *in vivo* is paralleled by studies on isolated cells *in vitro* [38]. Rat peritoneal and mesenteric mast cells respond strongly to the secretagogue, whereas mucosal mast cells from the rat intestine, guinea pig mesentery and human lung are almost totally unreactive [38]. Later studies have shown that mouse peritoneal mast cells are markedly less responsive than those of the rat, to the basic liberators compound 48/80 and peptide 401 [321]. Previous workers [322] found mouse peritoneal mast cells to be refractory to compound 48/80. Curiously, another polyamine, polylysine, shares many features in common with compound 48/80 but shows a broader spectrum of activity than compound 48/80 or peptide 401. For example, polylysine induces histamine release from human basophils which are refractory to the other basic stimulators [3,323].

The response of the mouse mast cell to compound 48/80 and peptide 401 was of further interest, in that it was enhanced when calcium ions were removed from the external environment. Brief preincubation of rat mast cells with a chelating agent may remove calcium ions from regulatory binding sites in the cell membrane, thus facilitating release into the cytosol of more firmly sequestered stores of the cation [2]. This may then initiate exocytosis. The regulatory pool of calcium may be less firmly bound in the mouse cell and

subject to removal in simple, calcium-free media. Calcium in the extracellular medium (>1mM) can induce secretion in its own right from mouse peritoneal mast cells [324]. Therefore, the alternative possibility is that calcium ions may directly and more readily compete for the presumably anionic receptors for polycations on the membrane.

1.3.5 Phospholipids

Goth and coworkers [148,149] reported that anaphylactic histamine release from rat peritoneal mast cells was enhanced by exogenous phospholipids. The release by dextran and ovomucoid was also enhanced but not that of compound 48/80. This is presumably due to the difference in calcium requirements for these agents.

Stimulation of mast cells with either A23187 or compound 48/80 leads to the *de novo* synthesis but not the breakdown of PI [325]. Later studies showed that compound 48/80 stimulation led to the transient breakdown of PIP_2, which was coincident with the rapid generation of inositol phosphates [186]. The degree of compound 48/80 breakdown of polyphosphoinositide was not affected by calcium depletion. Exogenous pertussis toxin was inhibitory to the breakdown, in either the presence or absence of extracellular calcium [199].

A marked increase in the phosphorylation of a 78kDa protein occurs, one minute after stimulation with compound 48/80 [145]. Studies on the time course of this phosphorylation [211] suggested that this is a mechanism by which histamine release is switched off, as it is also activated by cromolyn. A more recent communication reports that on stimulation with compound 48/80, rat mast cells show elevated phosphorylation of a 35kDa protein and dephosphorylation of a 15kDa protein [326].

The same profile was seen when the cells were stimulated with the phorbol ester, phorbol-12-myristate-13-acetate (PMA). PMA directly activates protein kinase C by substituting for DAG [327,328]. There is some evidence that polyamines can interact with the catalytic domain of protein kinase C directly [329]. Polyamines, particularly spermine, could inhibit the phosphorylation activity of protein kinase C, by binding to its catalytic domain. It may then be, that polyamines such as spermine could function as multipotential regulators of protein kinase C, operating *in vivo*. The same workers [330] also demonstrated that micromolar concentrations of polyamines could prevent the association of protein kinase C on the cell membrane, thus supporting the argument. Collectively, these results suggested the involvement of protein kinase C, which was substantiated by the release of phosphatidic acid [326].

In contrast, anti-IgE induced secretion involved two additional protein phosphorylations (68kDa and 56kDa). It appears, therefore, that compound 48/80 induces secretion by a different mechanism to that of IgE-mediated stimuli. The hydrolysis of inositol phospholipids and the concomitant IP_3 formation seems to be an integral and sufficient signal to induce secretion. These conditions do not suffice for IgE-mediated stimulation.

1.3.6 Binding studies

Studies using both unlabelled and spin labelled compound 48/80 have shown that although compound 48/80 does not cause a permanent change in the fluidity of membrane lipids [331], it does bind to both mast cells and mastocytoma cells with high affinity [332]. Further investigations [333], using electron spin resonance (esr) microscopy showed that unlike spin-labelled stearic acid, spin-labelled compound 48/80 (SL-48/80) could bind to mastocytoma cells in a manner that was unaffected by the non-ionic detergent, Triton X-100. SL-fatty acids such as stearic acid are thought to intercalate among membrane lipids. In contrast, sodium dodecylsulphate (SDS), a protein denaturant, altered the SL-48/80 binding site, causing an apparent increase in the motion of the spin label.

The investigators also studied the light scattering properties of intact cells, isolated membranes and liposomes in the presence and absence of compound 48/80. The amine was able to bind to a variety of biological preparations ranging from intact cells to membrane lipid-derived liposomes. There was no evidence to suggest anything unique about its binding to mast cells. The data also indicated that compound 48/80 interacts in some way with the membrane proteins and that even at low concentrations, this interaction resembles thermal denaturation and detergent treatment. Although compound 48/80 can probably bind to a variety of protein sites, it is possible that it may modify the properties of specific molecules in a way that precipitates membrane fusion.

Barratt and Parsons [334] studied a different form of SL-48/80 from that previously examined. Additionally, they confined their investigations to purified rat mast cells. The authors reported $1x10^{10}$ SL-48/80 molecules were bound to the denatured mast cell membrane. This huge figure is in excess of the available surface area, assuming the cell surface to be a smooth sphere. Therefore, the apparently smooth cell surface (as seen with a scanning electron microscope) must contain very many ridges and pockets to accommodate the number of binding sites.

Another possibility exists; that some of the SL-48/80 molecules may penetrate to binding sites located within the mast cell membrane. Evidence from esr experiments on mastocytoma cells [332] showed that SL-48/80 molecules can bind at sites away from contact with ferricyanide ions in the aqueous medium. This infers that some of the binding sites are located deep within the membrane. Many of the sites are surface situated since NOR-48/80, attached to Sepharose beads, has been shown to bind to the exterior of the cells.

Granular extrusion was found to contribute extensively to the binding of SL-48/80. In addition, granule exit from the mast cell makes available the perigranular membrane, which also has the potential to bind a large number of compound 48/80 molecules [335].

Binding sites seemed significantly less plentiful on cells previously desensitised to ovalbumin. One explanation, is that some of the sites for the polyamine on the mast cell membrane could be sterically hindered due to the binding of specific IgE to the cell surface IgE-receptors [336]. Hertel *et al.* [337] offered a more likely rationale; that a new population of mast cells arises after sensitization. Although larger than normal mast cells, the new cells

contain a considerably lower number of granules, which might account for the larger part of the difference in SL-48/80 binding to normal and sensitised cells. This is, of course, presuming granule extrusion acts to assist polyamine binding.

The spin label studies had concluded the absence of a fluidity effect in the plasma membrane on stimulation by compound 48/80. Another report [338] investigated fluorescence polarization as a method to observe the interaction of compound 48/80 with phospholipidic liposomes [339], giving rise to an increase in membrane fluidity. However, large doses (5μg/ml) of compound 48/80 were required and the investigative system was far from comparable to a physiological counterpart.

In actuality, variations in membrane fluidity should be expected to accompany histamine release induced by the polyamine, since the inducer has been reported to enhance PI turnover [340]. This implies a change in the lipidic order of the plasma membrane. Results indicated a decrease in membrane fluidity upon stimulation.

The role of calcium ions was also examined. The anisotropic increase was antagonised by excess calcium, in a fashion similar to histamine release. The anisotropy effect was already maximal in the absence of extracellular calcium. These results might be consistent with two assumptions: firstly, histamine release may be a biphasic process, the initial step does not require calcium internalization, is monitored by the anisotropy effect and may correspond to the early interaction of compound 48/80 with the plasma membrane. Histamine release itself, would occur only thereafter. The second assumption is based on the observation that the calcium antagonist FR 7534 inhibited the anisotropy effect, which was calcium independent. Therefore, it is more likely that the mechanisms of the inhibition with these antagonists, involve interactions at the level of membrane phospholipids, as already suggested [341].

Within recent years, the available literature on compound 48/80 has become quite overwhelming and profuse. Therefore, the more recent observations, relevant to this thesis, will be discussed in detail, where appropriate.

1.4 *NEUROPEPTIDES AND THE IMMUNE RESPONSE*

1.4.1 Modulation of immunity and hypersensitivity by sensory neuropeptides

The control of immunologic responses has been viewed traditionally in terms of the mutual interactions among immunocompetent cells. These express surface determinants, secrete diverse regulatory principles and recognize comparable signals from other cells in the immune system. However, it has been observed that the nervous system, which was considered previously to lack immunocompetence, may alter immunologic responses specifically.

The possibility that peripheral nerves might serve to modulate local immunity was initially suggested by the discovery that neuropeptides in unmyelinated dorsal root C-fibres are

transported to distal nerve endings. From there, the peptides are released when axon reflexes are evoked by nervous stimuli and local tissue inflammation.

The sensory neuropeptides somatostatin and substance P can enhance or suppress humoral components of inflammation. The capacity for physiologically meaningful concentrations of neuropeptides to elicit such responses supported the possibility of their involvement in the modulation local immune functions. The anatomical distribution and immunologic effects of somatostatin and substance P provide the foundation for a model of peripheral sensory neurones capable of sensing and responding to complex local challenges.

1.4.1.1 **Distribution of neuropeptides**

The undecapeptide, substance P, was discovered incidentally by Gaddum and von Euler [342] while they were working on the distribution of acetylcholine in the gut. Substance P is widely distributed in the nervous system but is one of the few neuropeptides restricted to cells of neural crest origin [343]. In the central nervous system, substance P is found in several foci and appears to be involved in neural circuits that serve different physiological functions. The peptide also occurs in a subpopulation of primary afferent neurones in the peripheral nervous system [345] and in nerve plexi of the gastrointestinal tract [346,347].

In the peripheral nervous system, substance P exists in both central and peripheral processes of small diameter, unmyelinated, sensory neurones, termed C-fibres, which terminate in the superficial layers of the dorsal horn of the spinal cord [343]. Although substance P is considered to be a neurotransmitter at the central terminals of the C-fibres [348], up to 90% of the peptide synthesized in the cell bodies of these neurones is transported to the peripheral terminals by physiologic or electric stimulation of the neurones [350].

Somatostatin, a tetradecapeptide, was initially described to be a hypothalamic peptide, capable of blocking the release of growth hormone as well as other peptide hormones and mediators [351]. Somatostatin is also widely distributed in the central and peripheral nervous system but unlike substance P it occurs in nonneural tissues such as pancreatic islets [351].

In peripheral nerves, both neuropeptides are found exclusively in the C-fibres and small, unmyelinated A-δ-fibres and are first detectable at the same times during embryogenesis [352]. Somatostatin and substance P are found in different cell bodies in the dorsal root ganglia [343]. As with substance P, somatostatin is produced in the neuronal cell bodies in dorsal root ganglia, from which it is transported to peripheral terminals. A neurotransmitter role for somatostatin is not as well established as that for substance P. Nonetheless, somatostatin occurs in the synaptosomal fraction of axon terminals and is released *in vitro* upon electrical depolarization in the presence of calcium [353]. The ionic dependency of somatostatin release has been extensively studied in the hypothalamus [354]. Although marked tachyphylaxis characteristic of the effects of somatostatin have complicated neurophysiologic studies, it has been shown that the peptide suppresses impulse

transmission in the spinal cord in neurones that are excited by noxious inputs or local iontophoresis of substance P [355]. A functional interaction between substance P and somatostatin is also suggested by the ability of somatostatin to inhibit the release of substance P from peripheral terminals of primary afferent neurones [356].

Besides somatostatin and substance P, other neuropeptides also appear to be present in the spinal cord and periphery. Among these are bombesin, cholecystokinin and vasoactive intestinal peptide (VIP) [358,359]. More recently, the novel calcitonin gene-related peptide (CGRP) has been added to this list [360].

It would seem that many neuropeptides coexist. Substance P has been found to coexist with neurotensin-like peptides in single neurones of the rat hypothalamus [361]. VIP has been shown to coexist with peptide histidine methionine (PHM) in autonomic neurones in the human pancreas [362].

Both the slowly conducting C-fibres and the rapidly conducting A-δ-delta fibres contain neuropeptides and include a high percentage of neurones that carry nociceptive signals [343,357]. There is no apparent relationship between the neuropeptide content of such primary afferent fibres to sensory function. This had led to the hypothesis that the peripheral stores of neuropeptides in the fibres may serve predominantly to modulate activities of nonneural systems.

Physiologically meaningful concentrations of substance P and somatostatin have the capacity to modify lymphocyte function, mast cells and other leucocytes. This suggests that the transduction of afferent neurologic impulses into signals that are detectable by immuno-competant cells may be mediated by the neuropeptides.

1.4.1.2 Regulation of leucocyte functions by somatostatin and substance P

The diverse activities of substance P and somatostatin on lymphocytes, polymorphonuclear (PMN) leucocytes and monocytes may result in the local modulation by these and other neuropeptides of the immune response (Fig. 1.42). Local vasodilatation and increases in vascular permeability evoked by substance P, released from sensory C-fibres in specific tissues [365-367], may enhance the delivery and accumulation of a range of mononuclear leucocytes critical for the expression of immune responses.

The effects of substance P on human T lymphocytes is manifested by stimulation of both DNA and protein synthesis, as measured by the uptake of [^{3}H]-thymidine and [^{3}H]-leucine, respectively [368]. Such T lymphocyte effects are blocked by the substance P antagonist D-Pro^2,D-Phe^7,D-Trp^9-SP [369]. By using both radiolabelled substance P and a fluorescent conjugate of the peptide, specific receptors have been identified on a subset of blood T lymphocytes [370].The results of similar *in vitro* experiments have demonstrated that somatostatin specifically inhibits immunologically important activities of human T lymphocytes [371] and that the T lymphocytes have stereospecific receptors for somatostatin [372].

It is of interest to note that the opposing immunologic effects of somatostatin and substance P are analogous to the bidirectional regulatory activities of the same neuropeptides in the nervous system [355].

The phagocytic activity of both macrophages and PMN leucocytes is similarly augmented by substance P, due largely to the stimulatory effect of the amino-terminal substituent tetrapeptide [372,373]. Contrariwize, the carboxy-terminal peptide of substance P appears to be the principal determinant for the stimulation of PMN leucocyte chemotaxis, *in vitro* [374].

The primary, direct effects of somatostatin released from the peripheral terminals of C-fibres would be to prevent the recruitment of basophils, enhance monocyte/macrophage function and subdue T lymphocyte activation. Although somatostatin generally tends to oppose the effects of substance P, the rapid tachyphylaxis exhibited for responses to somatostatin would limit the duration of the biological antagonism to that of non-sustained responses unless the effective concentration of somatostatin increased with time.

1.4.1.3 A model for neurogenic inflammation

The role of somatostatin and other neuropeptides in the immune response is still largely unexplored. At first glance, the neuropeptides divide into two groups. Substance P, neurotensin, ß-endorphins and met- enkephalin appear to stimulate the immune response and mediate inflammatory reactions, while somatostatin and VIP attenuate the immune response. Substance P is able to stimulate phagocytosis by neutrophils, enhance lymphocyte proliferation and may induce an inflammatory response [373,375]. Neurotensin stimulates neutrophil chemotaxis and phagocytosis [367-378]. Thus, neurotensin and substance P, acting in concert, may help to initiate the inflammatory response.

A recent report correlated substance P innervation of joints with the severity of experimental arthritis; arthritic symptoms were induced by injecting substance P directly into the joint [375]. Other neuropeptides may be responsible for turning off the inflammatory response. Somatostatin could be envisioned to act centrally, in blocking substance P or neurotensin release from primary sensory neurones. Somatostatin and VIP might also act locally, binding to receptors on lymphocytes and thereby inhibiting lymphocyte proliferation [371,379,380]. VIP may then activate suppressor cell functions, ultimately turning off the inflammatory response.

Bipolar sensory neurones, having their cell bodies in dorsal root ganglia, seem to serve efferent as well as afferent functions. A century ago, it was appreciated that stimulation of the ends of a divided posterior root produced vasodilation in the limb which it served. Bayliss [381] showed that mechanical, thermal and chemical stimuli produced a vasodilator response mainly in skin blood vessels and that the mechanism involved sensory neurones having cell bodies in the dorsal root ganglia. Only sensory neurones appeared to be involved, there being no evidence to implicate the sympathetic nervous system. These observations suggested that antidromic impulses in sensory neurones may be involved in vascular dilation.

Developments of Bayliss' original, physiological observations have come from direct recording of C-fibre activity in man using tungsten microelectrodes [382]. Attention has focused on polymodal nociceptor units which, in some species, represent as much as 80% of all C-fibre inputs. These units are responsive to a range of stimuli. They are sensitive to a variety of chemicals, including several endogenous substances, responding with short-latency increases in firing when heated and exhibit slowly adapting responses to firm pressure.

In non primates, the unit receptive field occurs as small spots ($2mm^2$) but in primates, they may extend over an area up to 17mm in diameter ($>200mm^2$). These units become sensitised following antidromic stimulation in the same field or during local injury, intimating the release of one or more chemical mediators [383].

The first to suggest that neurones are involved in 'antidromic vasodilatation' was Bruce [384], who found that vascular dilation failed to occur after chronic denervation of the skin. The observations of Bayliss and Langley [385] proposed the concept of an axon-reflex and this led to the development of the hypothesis that sensory nerves could have a peripheral effector function by means of an impulse spread through antidromic transmission in an axon-reflex arrangement.

Lewis [386] developed this hypothesis in the skin and showed that the flare or vaso-dilatation is mediated by a neurogenic mechanism, probably involving axon-reflexes in sensory nerves. The flare spreads several centimetres from the point of injury in the skin and is part of the 'triple response' to injury. Lewis demonstrated that the triple response in the skin (initial reddening, development of flare, development of wheal) could be mimicked by the intradermal injection of histamine. Histamine will cause increased blood flow to the dermic arterioles and increase vascular permeability.

Further work demonstrated that the fibres carrying impulses which mediate peripheral vasodilation are unmyelinated C-fibres originating from the dorsal root ganglion [387]. There was some evidence to show that chemically induced inflammation depended on an intact, sensory nerve supply. Antidromic stimulation of peripheral nerves results not only in vasodilatation but also in increased vascular permeability and plasma extravasation [388]. However, there is also evidence that not all inflammatory stimuli invoke a neurogenic response [388,389].

In the periphery, C-fibres may be associated with a polymodal nociceptor and it has been proposed [390] that an arrangement exists in which C-fibres branch. One arborization is associated with the polymodal nociceptor, the input and the other arborization forms a neuroeffector junction with some target cell.

Overall, the data implies that sensory nerves may be activated in the periphery through polymodal nociceptors to produce axon-reflexes which result in inflammatory responses. The role for neuropeptides as the transmitters for neurogenic inflammation was proposed, initially for substance P [391], because of the likelihood that the C-fibres involved were

peptidergic. Additionally, Kiernan [392] demonstrated that nerve stimulation, causing vasodilation, results in the degranulation of mast cells in the skin.

1.4.1.4 Capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-noneamide) is the hot extract from red peppers and has a mechanism of action which is not completely understood. It does, however, cause the initial release and depletion of the neuropeptide content from some primary afferent neurones [393,394]. Capsaicin also inhibits the axoplasmic transport of neuropeptides in these nerves [395].

Application of capsaicin to the skin produces an acute inflammatory response, ostensibly by neuropeptide release, since prior denervation abolishes the response [388]. Repeated application will result in complete desensitization to the inflammatory effects of capsaicin and other inflammatory agents. Furthermore, this treatment renders the tissue unresponsive to additional thermal injury or to antidromic nerve stimulation [388,396]. It is pertinent to note that capsaicin exhibits selective action on neuropeptide-containing C-fibres linked to polymodal nociceptors since, after application, anaesthesia is incomplete and a continued response to pressure and temperature changes occurs [397,398].

Injection of histamine into human skin desensitized to capsaicin produces the wheal but not the flare, further indicating the requirement of intact, neuropeptidergic nerves in the generation of the flare [394,399]. In rat

skin, capsaicin treatment has been shown to prevent heat-induced oedema [400].

In the lung, capsaicin increases the vascular permeability of airway mucosa and this response is susceptible to desensitization on repeated capsaicin application. Capsaicin-treatment of vagus nerves culminates in the partial prevention of anaphylactic broncho-constriction and increases in vascular permeability caused by cigarette smoke [401]. Capsaicin itself induces an atropine-resistant bronchoconstriction which exhibits tachyphylaxis [402].

Thus, in both lung and skin, experiments with capsaicin indicate that neuropeptide release causes inflammation and bronchoconstriction, while depletion of neuropeptides from these tissues attenuates inflammatory changes and bronchoconstriction induced by nerve stimulation and by chemical or thermal stimuli.

1.4.1.5 Substance P

Intradermal injection of substance P into human skin produces a flare and wheal reaction, comparable to that seen following a similar injection of histamine [403]. However, substance P is about 100-fold more potent , on a molar basis, than histamine in this system [404]. Comparison of other tachykinins with substance P, with respect to their ability to produce a wheal and flare, indicates that the mechanism of flare generation is different to that for wheal production [404]. Physalaemin, for example, is about twice as potent as substance P in producing a wheal in human skin but failed to produce a flare. Eledoisin-related peptide produces both flare and wheal but only one tenth as severe as that for substance P.

Structure activity studies of a number of related peptides have shown that the ability to produce vasodilatation depends upon the presence of N-terminal basic amino acids. In contrast, the wheal-producing action of the peptides is a property of the C-terminal sequence. Peptides like physalaemin or the octapeptide fragment of substance P (SP_{4-11}) have C-terminal homology with substance P but lack N-terminal basic amino acid residues and can elicit a wheal but no flare [404].

Pretreatment with an H_1-receptor antagonist will prevent a substance P induced flare, on intradermal injection into human skin [403-405]. The formation of the wheal is, however, only partially inhibited by antihistamine pretreatment. A combination of H_1 and H_2 antagonists also fails to inhibit the wheal response entirely. There are two possible explanations for these observations. Firstly, on intradermal injection, substance P may release histamine from mast cells local to the site of injection. The histamine so released may then stimulate polymodal nociceptors to activate an axon-reflex, producing a flare distant to the site of injection. In this instance, the flare would actually be produced by neuropeptides released from the sensory nerves through the axon-reflex.

A second possibility which may, in fact, operate with that just outlined, is one where the neuropeptide released from the sensory nerve by the axon-reflex does not itself act directly to produce the vasodilatation of a flare but acts indirectly, by the release of histamine from mast cells. In this case, it is histamine which is the actual mediator of the flare and there are several pieces of evidence to support this latter proposal.

1.5 *THE ROLE OF MAST CELLS IN NEUROGENIC INFLAMMATION*

Mast cells are predominantly located around the blood vessels in human skin and several studies have revealed that neuroeffector junctions exist between these mast cells and nerves in the skin and elsewhere [406-410]. It has been shown that fibres containing substance P form neuroeffector junctions with mast cells around blood vessels but rarely form such relationships with mast cells elsewhere in the tissues [408].

Recently, Stead *et al.* [411] have reported that 67% of rat intestinal, mucosal mast cells were touching subepithelial nerves and an additional 20% were within 2μm of nerves. The association was declared to be 5 times greater than that by chance alone. Moreover, the nerves in contact with mast cells were shown to contain substance P and/or CGRP. Electron microscopy illustrated that membrane-membrane contact occurred with 8% of the mast cells and unmyelinated axons. However, of several neuropeptides tested, only substance P was found to be active in inducing histamine release from rat intestinal mast cells [412].

Further implications for an involvement of mast cells include evidence that the antidromic stimulation of sensory nerves induces mast cell degranulation [392,413]. Nerve stimulation has also been shown to induce histamine release in the lung and gut, although there is no proof for the existence of histaminergic neurones in these tissues [414,415].

Depletion of mast cell histamine by pretreatment with compound 48/80 reduces the inflammatory response normally seen on antidromic stimulation of nerves [416], In addition, H_1 antagonists of histamine also prevent the vasodilatation and plasma extravasation which occurs on antidromic nerve stimulation [416,417].

These and supplementary data suggest that substance P and other neuropeptides such as somatostatin, can release histamine from tissue mast cells and indeed, injection of one of several neuropeptides into human or rat skin or into the perfused hind limb has been shown to release histamine into the circulation [418-420].

1.5.1 Histamine release from mast cells

As already mentioned, several neuropeptides release histamine from mast cells, with varying potencies. However, not all histamine-releasing neuropeptides produce a wheal and flare and *vice versa.* Apart from substance P [421,422], other mast cell active peptides include somatostatin [420,423-425], CGP [420], neurotensin [426-428], VIP [425,429], bradykinin [94] and several of the endorphins [412,430].

Stanworth *et al.* [431] had previously noted homology between some basic peptides causing histamine release and part of the amino acid sequence of IgE. They demonstrated that a synthetic decapeptide, representing the sequence 497 to 506 of the C4 domain of IgE, was a potent histamine releasing agent, as were several peptides of related structure but derived from sequences within the adrenocorticotropic hormone molecule.

All these peptides induced a noncytotoxic release of histamine from rat peritoneal mast cells. Stanworth postulated that a conformational change within the antibody molecule, on antigen binding, could expose a basic peptide sequence, that might participate in activating the mast cell to secrete histamine. Although such a mechanism appears unlikely, in view of the fact that mast cells can be activated in the absence of IgE, by cross-linking IgE receptors with an antibody [70], the participation of such a mechanism in IgE-mediated secretion cannot wholly be excluded. The biochemical differences between peptide-induced and IgE-mediated secretion are relatively subtle, making it difficult to reject the postulate with confidence.

SOMATOSTATIN Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-NH_2

S S

NEUROTENSIN Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-Tyr-Ileu-Leu-OH

SUBSTANCE P Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH_2

PHYSALAEMIN pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

NEUROKININ A His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2

NEUROKININ B Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

ELEDOISIN Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2

Fig. 1.51 Structure of somatostatin and other neuropeptides

One related and potentially very interesting observation was made by Hamberger [432], who reported that a small peptide (Asp-Ser-Asp-Pro-Arg) from the human epsilon chain-sequence could inhibit human Prausnitz-Küstner reactions. Subsequent studies, however, have failed consistently to confirm this and no testimony of the displacement of cell-bound IgE by this peptide has been obtained [433,434].

The extensive structure-activity studies conducted by Stanworth *et al.* [431] and Jasani *et al.* [435] have revealed several features of peptide molecules, that determine their effectiveness in releasing histamine from mast cells. The presence of a cluster of basic amino acids, usually lysine and arginine residues, at the N-terminal end is essential for activity, together with a blocked carboxyl group at the C-terminal end. Also, many of the active peptides have hydrophobic residues, including phenylalanine and tryptophan, in the C-terminal portion of the molecule. These are features, of course, assigned to substance P, somatostatin and other peptides. Structure-activity studies have been performed, confirming the necessity of N-terminal amino acids for substance P-induced histamine liberation [421]. The octapeptide SP_{4-11} is devoid of histamine-releasing activity, as is physalaemin, which is similar to substance P in the C-terminal region but contains no basic amino acid residues. The peptide comprising the first four amino acid residues of substance P, SP_{4-11} (Arg-Pro-Lys-Pro) releases histamine from mast cells but is only about 0.06 times as active as substance P itself [421]. Thus, the C-terminal sequence of substance P is also important for the maximum expression of histamine releasing activity. Progressive shortening of substance P in the series of peptides SP_{1-9}, SP_{1-8}, SP_{1-7} and SP_{1-6}, leads to a corresponding reduction in the potency of the molecules for the release of histamine from rat peritoneal mast cells. Moreover, certain amino acid substituents in the C-terminal sequence have been shown to increase the activity of the peptide on rat mast cells [421,436]. For example, [D-$Trp^{7,9}$]-SP_{1-11} is about 10 times more active than substance P.

Substance P receptors are known to exist in a variety of smooth muscle preparations and in salivary glands. Receptors mediating substance P effects have been classified into P and E types, on the basis of the relative activities of agonist peptides in these tissues [437]. The rat mast cell receptor, however, appears to belong to neither of these types. Studies with a hybrid molecule, formed from the N-terminal sequence of substance P and dodecylamine, indicate that the properties of substance P which relate to histamine release are the specific structure of the N-terminal region, in which basic amino acids are essential and a non-specific hydrophobic property of the C-terminal sequence.

Apart from an understanding of the substance P interaction with the mast cell membrane, these structure-activity studies revealed an interesting parallel between the ability of peptides to release histamine from rat mast cells and their ability to produce a flare in human skin. The histamine release data is, therefore, compatible with the other evidence cited above which has been used to argue that substance P released from sensory nerves on activation by an axon-reflex will itself release histamine from mast cells. The liberated amine produces vasodilatation, which in the skin is observed as a flare. No parallel exists

between the ability of peptides to induce wheal or plasma extravasation and their ability to liberate histamine [421,404]. This, too, is consistent with the observation that local anaesthetic prevents flare but not wheal responses induced by peptides [404].
It must be emphasised that this model is not universally true and in lung tissue, for example, the bronchoconstrictor and increased vascular permeability effects of substance P both appear to be mediated directly by the peptide, with little or no contribution from histamine released by mast cells [402,438].

1.5.2 Peptide antagonists

Considerable efforts have been exerted in an attempt to find peptide antagonists, with relatively little success. The main emphasis has been laid, once more, on antagonists for substance P. Antagonists in general have been confined to substituted, substance P-like peptides, which have low affinity and poor selectivity [439,440]. The P and E type classification [437] of substance P receptors has not been supported by the antagonist studies. Occasionally, the affinity of the antagonist for the substance P 'receptors' varies, depending on which particular antagonist has been used [440]. Many of the substance P antagonists developed in smooth muscle were found subsequently to be agonists at the substance P 'receptor' in human skin and the rat mast cell [421,436]. Nevertheless, some antagonists have provided useful information. [D-Pro^4,D-Trp^{7,9,10}]-SP$_{4-11}$ (SPA) competitively antagonises histamine release induced by substance P from rat mast cells [441]. SPA will also inhibit the flare but not the wheal induced by the intradermal injection of substance P into human skin [425].
The substance P antagonist [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{1-11}$ has been shown to prevent heat-induced oedema in rat skin [442] and also the increased vascular permeability that occurs in airways stimulated with cigarette smoke [443]. Furthermore, this substance P antagonist inhibits the non-cholinergic component of bronchial smooth muscle contraction induced by stimulation of the vagus nerve and the increased vascular permeability of the airways that follows vagal stimulation [366]. Trigeminal stimulation produces inflammatory changes in the eye and the above antagonist also blocks these responses [444].
A different antagonist, [D-Pro^2,D-Phe^7,D-Trp^9]-SP$_{1-11}$ has been shown to antagonise the vasodilatation of tooth pulp which occurs on antidromic stimulation of the sensory nerve to the tooth [445].
The substance P receptors designated P and E type give a rank order of potency: physalaemin > substance P > eledoisin > kassinin, for P type and the E type receptors exhibit the order eledoisin > kassinin > substance P > physalaemin. There are several notable exceptions to these classifications, of which the mast cell is just one [421,446-448].

1.6 *NEUROPEPTIDES AND AIRWAY DISEASE*

The lung is embryologically derived from the foregut. Therefore, it is not surprising that many peptides originally described in the gut have been found in the autonomic innervation of the lung [449]. It is now recognised that in addition to classic adrenergic and cholinergic neural mechanisms in airways, there is a third nervous system that is neither adrenergic nor cholinergic (NANC) [450,451].

In vitro studies of airways of several species, including man, have shown that electrical field stimulation (EFS), which excites intrinsic nerves, in the presence of adrenergic and cholinergic blockade, produces relaxation of airway smooth muscle [452-454]. The neural nature of this response is confirmed on its inhibition by tetrodoxin. In man, there is no evidence of direct sympathetic innervation [455-457] and there are almost no adrenergic nerve terminals in airway smooth muscle [454,458]. NANC bronchodilator responses have also been shown *in vivo* in cats after vagal nerve stimulation [459] and in humans after laryngeal stimulation [460]. There is also some evidence in guinea pig and occasionally human airways that EFS produces atropine-resistant contractions of airway smooth muscle [461].

The neurotransmitter in NANC nerves is as yet undefined. In airway smooth muscle, unlike the gut, neither adenosine nor ATP fulfil the criteria for a neurotransmitter and purinergic blockers have no effect on NANC responses [462,463].

It is thought that VIP and PHM are the transmitters in NANC inhibitory nerves and that substance P and other tachykinins may be involved in NANC excitatory nerves. VIP receptors are found in the smooth muscle of proximal airways but there are no receptors in bronchiolar smooth muscle. VIP receptors are also found in the airway epithelium and submucosal glands [464]. *In vitro*, VIP is a potent relaxant of airway smooth muscle of several species [452,465,466]. For example, VIP is a potent bronchodilator of cat airways [459]. In humans, inhaled VIP had no effect on resting airway tone. However, it does have a small protective effect against histamine-induced bronchoconstriction [467].

Peptide histidine isoleucine (PHI) and PHM are two peptides structurally related to VIP. PHI was originally isolated from porcine tissue and is termed so because the C-terminal amino acid is isoleucine and the N-terminal residue is histidine [468]. In humans, the C-terminal acid is methionine (PHM). PHI is encoded by the same gene as VIP and both are synthesized from the same prohormone [469]. VIP and PHI coexist in airway nerves of several species [470] and therefore PHI has a similar distribution [471]. *In vitro*, PHM is a relaxant of human bronchi, is of similar potency to VIP and like VIP, has no effect on bronchioles [457]. *In vitro*, PHM is a less potent vasodilatator than VIP but equipotent to VIP as a relaxant of airway smooth muscle [472]. *In vivo*, PHM has no effect on airway calibre.

Substance P contracts airway smooth muscle of several species, including man, *in vitro* [402,473,474]. Capsaicin also has a contractile effect on airway smooth muscle, suggesting the release of substance P. Substance P antagonists can inhibit the contractile response to

the peptide, although the specificity of these agents has been questioned [473]. *In vivo*, substance P infusion causes bronchoconstriction in animals [475]. In man, infused substance P produces transient bronchoconstriction followed by bronchodilatation accompanied by marked flushing, hypotension and tachycardia. It is likely that the cardiovascular effects, with subsequent reduction in vagal tone, lead to the bronchodilation seen at higher doses [476]. Inhaled substance P has no significant effect on airway calibre [476]. The gene that codes for substance P also codes for the related peptide neurokinin A and a similar peptide, neuropeptide B has also been isolated. These peptides have been localized to afferent nerves of the airways [477]. In human airway smooth muscle, neurokinin A and eledoisin are more potent than substance P, suggesting that the SP-E receptor is dominant [474]. Neurokinin A also causes bronchoconstriction when infused in normal subjects [478].

CGRP is localized to airway nerves in animals, where it may be co-localized with substance P [479]. It has also been described in human airways [480]. *In vitro*, CGRP is a potent constrictor of human airway smooth muscle, being significantly more potent than substance P and produces equivalent contraction to carbachol [480].

Somatostatin and cholecystokinin have been identified in the airways. However, they are present in concentrations too small to localize [449]. Galanin, a 29 amino acid peptide, has been localized to motor nerves of several animal species [481] but it has no functional effect on guinea pig
trachea [482] and its role in airway function is not clear.

Gastrin-releasing peptide is thought to be the mammalian form of bombesin and has been localized in the respiratory tract of several species [483]. Neuropeptide Y (substance K) is a 36 amino acid peptide localized primarily to blood vessels, with very little immunoreactivity seen in association with airway smooth muscle [484]. Neuropeptide Y is a potent constrictor of vascular smooth muscle and may be a co-transmitter of noradrenaline [485]. Its principal role seems to be in regulating vascular rather than airway tone. It has no effect on human airway smooth muscle but is a potent constrictor of the human pulmonary artery.

Substance P, neurokinins and CGRP are capable of producing many of the pathophysiological features of asthma; the contraction of airway smooth muscle, oedema, plasma extravasation and mucus hypersecretion. Damage to airway epithelia, which may occur even in patients with relatively mild asthma [486], may expose afferent nerve endings which could be stimulated by various inflammatory mediators. Attention has focused on myelinated 'irritant' receptors in airway epithelium as the executors of a mechanism for asthma via reflex, cholinergic pathways. More recently, non-myelinated C-fibre afferents have been described in bronchi [487]. In animals, these afferents can be stimulated with capsaicin, which on inhalation by healthy, human subjects causes cough and transient bronchoconstriction [488].

Bradykinin is produced in inflammatory reactions and human lung mast cells have been shown to release a kininogenase which could generate bradykinin locally [489]. Bradykinin has a potent bronchoconstrictor effect when inhaled by asthmatic subjects but has little effect in normal subjects [490,491] or on isolated bronchi. In the dog, bradykinin is a potent and selective stimulant of bronchial C-fibres [492]. The observation that this peptide may be inhibited by substance P antagonists suggests that it may release sensory neuropeptides from C-fibre endings exposed by epithelial damage. This may activate an axon-reflex with the release of sensory neuropeptides which then produce, or at least contribute to, the pathology of asthma [494]. Local reflexes might then spread the response to other airways and act as amplifying mechanisms.

Although the link between epithelial damage and the pathology of asthma might be explained by local reflex mechanisms, it is difficult to understand why asthmatic patients should differ from normal subjects and why bronchial hyperresponsivity persists.

From the data presented, it would appear that sensory neuropeptides may contribute to airway disease by direct effects on bronchial smooth muscle and vasculature and indirectly, via postganglionic vagal endings and mast cell activation.

1.7 *TISSUE AND SPECIES SELECTIVITY OF NEUROPEPTIDE-MAST CELL INTERACTIONS*

Most of the studies with neuropeptides have been performed *in vivo* or on isolated rat peritoneal mast cells. The functional heterogeneity of mast cells has already been discussed in detail (1.1.2) but not with respect to neuropeptide activity.

Evidence that the neuropeptides may be involved in inflammatory reactions in the skin derives from their effects when injected intradermally and from the use of capsaicin to deplete the neuropeptides from primary sensory afferents [394]. Substance P, somatostatin and VIP all induce a wheal and flare reaction when injected into human skin [404,497]. In contrast, CGRP causes a local, persistent erythema and neurokinin A, a local vasodilatation, neither of which are accompanied by flare reactions [496,497]. Incrimination of a neuropeptide as the transmitter of the flare derives from observations that pretreatment of the skin with capsaicin reduced this response, but not the wheal, on subsequent injection of histamine [404,495].

Recently, several studies on isolated human skin mast cells have been performed. Mast cells of human skin are closely associated with both dermal nerve endings and blood vessels and the evidence suggests that their secretory functions are regulated by the nervous system. Somatostatin, substance P and VIP all release histamine from human, dispersed skin mast cells [498], with characteristics similar to those described for rat mast cells [412].

Histamine secretion from human skin mast cells, induced by these peptides, is concentration-related over the range 1μM to 30μM. The release process is dependent on intact mechanisms for glycolysis and oxidative phosphorylation, indicating a non-cytotoxic, active process. This conclusion is further supported by the observation that histamine secretion

is inhibited by SPA [498]. The time course of release is rapid, being complete within 30 seconds. This is much faster than substance P-induced histamine release demonstrated *in vitro* from human skin slices. However, physical barriers to peptide and histamine diffusion may complicate the observations [499].

Removal of extracellular calcium reduces substance P-induced secretion by less than half and has little effect on secretion induced by somatostatin and VIP. This indicates that peptides may activate secretion by either mobilization of intracellular calcium stores or by a calcium-independent mechanism. These findings are similar to those in rat peritoneal mast cells, where the absence of extracellular calcium does not affect the release process induced by somatostatin, substance P or VIP. Depletion of intracellular calcium by prolonged incubation of rat peritoneal mast cells with EDTA does, however, lead to a 70-80% reduction in histamine release [412].

The low histamine releasing activity of physalaemin, eledoisin and neurokinins A and B, relative to substance P, suggests that the human skin, mast cell activation site is a receptor distinct from the tachykinin NK-1 (SP-P), NK-2 (SP-E) or NK-3 (SP-N) types, previously described by Lee *et al.* [500]. Additionally, the stepwise removal of N-terminal amino acids of substance P progressively reduces histamine-releasing activity, supporting the hypothesis that the integrity of this portion of the molecule is essential for secretion. Nonetheless, the C-terminal portion also has a role in the full expression of activity, possibly increasing the lipophilicity of the peptides [421].

In terms of time-course and calcium-dependency, histamine release from human skin mast cells induced by somatostatin, substance P and VIP is similar to that previously described for compound 48/80, poly-L-lysine and morphine [502]. Furthermore, relatively high concentrations of each secretagogue are required to induce 15-20% histamine release. In contrast, CGRP, neurotensin, bradykinin and lysylbradykinin, none of which readily induce wheal and flare reactions, caused little or no histamine release from human skin mast cells but are active on the rat peritoneal mast cell.

Further information on the nature of the mast cell receptor for basic stimuli has been gained by the use of SPA [441]. In addition to inhibiting substance P-induced release, SPA also inhibited histamine release from human skin mast cells induced by somatostatin, VIP, compound 48/80, morphine and poly-L-lysine but not that induced by anti-IgE or the calcium ionophore A23187 [498]. These results suggest that human skin mast cells possess binding sites of low affinity and low specificity, for neuropeptides and compounds with similar physicochemical properties. These sites are distinct from those involved in the initiation of IgE-dependent mast cell activation.

Knowledge of the mechanisms of neuropeptide-induced mediator secretion should prove important in understanding the role of skin mast cells. The ability of skin mast cells to release mediators in response to neuropeptide stimulation, is evidence in favour of neuro-immune interactions within human skin. The close association between dermal mast cells, nerves and blood vessels [410,502] suggests that the development of skin mast cells may

have been influenced by their local environment to produce a cell capable of fulfilling a homeostatic rather than a defensive role.

It would appear that the model of neurogenic inflammation mediated via an axon-reflex may occur in the skin, as mast cells isolated from this tissue release histamine in response to neuropeptide stimulation. However, this model has been extended to other localities such as the lung, gut and other mast cell-containing tissues. Studies on isolated mast cell systems other than the skin have yielded somewhat contradictory results.

Human basophil leucocytes and isolated human lung mast cells are resistant to the activity of substance P, bombesin, beta lipotropin, alpha and gamma endorphins, leucin enkephalins, peptide 401, compound 48/80 and other basic stimuli [412,430,503,504]. Moreover, these agents do not affect significantly the anti-IgE-induced histamine release from these particular mast cells [505]. Later studies have also shown that substance P is without effect on mast cells isolated from human tissue, such as tonsils, adenoids, intestinal muscle and intestinal mucosa/submucosa [506].

Similar results were obtained using morphine, compound 48/80 and poly-L-lysine [506], suggesting that the peptide sensitivity extends to other basic lipophilic substances. Thus, in a range of human mast cells, sensitive to immunologic stimuli, the neuropeptides and polyamine stimuli are incapable of eliciting a response. The notable exception to this trend are mast cells from the skin. The reasons for this discrepancy are unclear but it may reflect a specific action of these agents or, more simply, further evidence for the heterogeneity of mast cells. However, under these circumstances, it would appear that the model for neurogenic inflammation begins to breakdown when applied to systems other than the skin.

The apparent tissue selectivity of neuropeptide action is also species specific. Studies on guinea pig lung and mesentery show that isolated mast cells from these tissues are completely refractory to substance P [504], compound 48/80 and peptide 401 [507]. Additionally, whilst rat peritoneal mast cells are the most responsive to peptide agonism, mast cells from tissues other than the peritoneum are less responsive or totally insensitive. Aspects of this topic are covered extensively in chapter 5 and data presented in this thesis aspires to confirm and extend much of the existing literature.

In summary, sensory neuropeptides share mast cell activity with compound 48/80 and other basic stimuli in terms of their species and tissue specificity. The implications of this, for the general application of the axon-reflex to systems other than the skin and murine mast cells, are potentially far-reaching.

1.8 *AIMS OF THE PRESENT WORK*

The work presented here employed somatostatin as an example of a neuropeptide also capable of eliciting histamine release from rat peritoneal mast cells. The aim of the project was to fully investigate the histamine releasing properties of this peptide by monitoring the release of the amine from rat mast cells. The knowledge gained from the reaction profile of somatostatin was used to compare its activity to other secretagogues such

as compound 48/80 and substance P and a selection of immunologic stimuli so that somatostatin could be safely assigned to the polyamine group of ligands. Somatostatin was also used to stimulate a variety of histamine-containing cells from various tissues and species. Data from these experiments was hoped to provide further evidence for the heterogeneity of mast cells and also to examine further the possible role of neuropeptides in neurogenic inflammation.

Investigations into a possible modulatory role for somatostatin were conducted with the aim of understanding its role in the allergic response with respect to its already defined role as a biogenic peptide.

Lastly, experiments with various inhibitors and other pharmacologic agents were performed in order to attempt to shed more light on the precise method of membrane activation of somatostatin and other polyamines and to clarify some of the speculation surrounding the exact mechanism of membrane interaction and subsequent cell activation brought about by these agents.

CHAPTER 2

MATERIALS AND METHODS

2.1 *MATERIALS*

2.1.1 Tissue and cell sources

2.1.1.1 **Animals**

Closed, random-bred colonies of male Sprague-Dawley rats (200-400g), Dunkin-Hartley guinea pigs (400g) of either sex, male Porton mice (60-100g) and male Syrian hamsters (130-180g) were obtained from the Joint Animal House, UCL, whose invaluable assistance is also gratefully acknowledged. Extra animals and specific pathogen free (SPF) rats were acquired from Charles River, Kent.

2.1.1.2 **Human Tissue**

Whole venous blood (20ml) was obtained from healthy volunteers. Human lung and other tissues were supplied by coworkers from the Middlesex hospital.

2.1.2 Buffers

All buffers utilized in this study were modifications of full Tyrode's-based solution, buffered with N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES). The pH of the buffers was adjusted by the addition of sodium hydroxide (4M) or hydrochloric acid (3M). The range of buffers and their specific constituents are listed in Fig. 2.12.

2.1.3 Secretagogues

2.1.3.1 **Immunologic stimuli**

anti-guinea pig IgG	-	Miles Laboratories
anti-human IgE	-	Generous donation from the
	-	Middlesex hospital
anti-rat IgE	-	Miles Laboratories
Concanavalin A	-	Sigma London Chemical Co. Ltd.

2.1.3.2 **Polyamine and peptide stimuli**

Somatostatin	-	Sigma London Chemical Co. Ltd.
Substance P	-	Peninsula Laboratories
		Europe Ltd.
CGRP	-	Bachem
Eledoisin	-	"
Neurotensin	-	Sigma London Chemical Co. Ltd.
NKA	-	Bachem
NKB	-	"
PHM	-	"

BUFFER	NaCl mM	Glucose mM	HEPES mM	KCl mM	$NaHPO_4$ mM	$CaCl_2$ mM	OTHER mM	pH
Full Tyrode's	137	5.6	10	2.7	0.4	1.0		7.4
CMF Tyrode's	137	5.6	10	2.7	0.4			7.4
2 x Ca Tyrode's	137	5.6	10	2.7	0.4	2.0		7.4
2 x EDTA Tyrode's	137	5.6	10	2.7	0.4		0.2mM EDTA	7.4
BSA Tyrode's	137	5.6	10	2.7	0.4	1.0	0.1% w/v BSA	7.4
Heparin Tyrode's	137	5.6	10	2.7	0.4	1.0	50 IU/ml	7.4
Glucose-free Tyrode's	137		10	2.7	0.4	1.0		7.4

Fig. 2.12 Constituents of Experimental Buffers

VIP	-	Sigma London Chemical Co. Ltd.
Compound 48/80	-	" " " " "
Poly-L-lysine	-	" " " " "
Polymyxin B	-	" " " " "

2.1.3.3 **Other secretagogues**

Dextran	-	Fisons plc
F-Met-Leu-Phe	-	Sigma London Chemical Co. Ltd.
Ovalbumin	-	" " " " "

2.1.4 Inhibitors

Antimycin A	-	Sigma London Chemical Co. Ltd.
BAC	-	" " " " "
BDTA	-	Fluka
Choline	-	Sigma London Chemical Co. Ltd.
Decamethonium	-	Sigma London Chemical Co. Ltd.
2-deoxyglucose	-	" " " " "
DSCG	-	Fisons plc
ETYA	-	Generous donation from Roche
Gallamine	-	Sigma London Chemical Co. Ltd.
Hexamethonium	-	" " " " "
Isoprenaline	-	" " " " "
Nedocromil sodium	-	Fisons plc
Pertussis toxin	-	Generous donation from M. M. Dale.
PMSF	-	Sigma London Chemical Co. Ltd.
Polyglucose (MW 2600)	-	Aldrich Chemical Co.
Salbutamol	-	Sigma London Chemical Co. Ltd.
SP_{4-11}	-	Generous donation from J. C. Foreman
Theophylline	-	Sigma London Chemical Co. Ltd.
TLCK	-	" " " " "
TPCK	-	" " " " "

2.1.5 Enzymes

Collagenase (TYPE IV)	-	Sigma London Chemical Co. Ltd.
Neuraminidase (from Cl. Perfringens)	-	" " " " "
Trypsin	-	" " " " "

2.1.6 Phospholipids

PS	-	Lipid Products.
Lyso-PS	-	Sigma London Chemical Co. Ltd.

2.1.7 Immunofluorescence materials

FITC-conjugated goat anti-rabbit IgG	-	ICN Biochemicals Ltd.
Rabbit anti-somatostatin	-	" " "
Acetate contact adhesive	-	Generous donation from C. King.
Glycerol/PBS	-	" " " "
Glass coverslips (32mm x 32mm)	-	Chance Propper Ltd., Fisons
Ground glass slides	-	Scientific Supplies

2.1.8 Other materials

BSA	-	Sigma London Chemical Co. Ltd.
Centrifuge tubes (10ml, polystyrene)	-	Sarstedt
Freund's incomplete adjuvant	-	ICN Biochemicals Ltd.
Heparin	-	CP Pharmaceuticals.
OPT	-	Sigma London Chemical Co. Ltd.
Percoll	-	Pharmacia Ltd.
Pipettes (disposable)	-	Sterilin
Toluidine Blue	-	Raymond A. Lamb
Universals (polystyrene)	-	Sterelin

2.2 *METHODS*

2.2.1 Isolation of Histaminocytes

2.2.1.1 **Murine peritoneal mast cells**

Animals were anaesthetized with diethyl ether and killed by decapitation and exsanguination under running water. Subsequently, this procedure was revised and animals were killed by an excess of nitrous oxide. Skin from the midline was removed and heparin Tyrode's buffer (10ml, 37 °C) was injected into the peritoneal cavity. The abdomen was gently massaged for two minutes, after which the cavity was exposed by a midline incision. The exudate was removed into polystyrene centrifuge tubes with polythene disposable pipettes. In experiments on purified cells, greater volumes of heparin buffer were used and the cavity was washed several times with additional buffer.

The crude cell suspensions were then centrifuged (MSE Chilspin 1000rpm, 150g, 3min, RT) after which the supernatants were discarded and the cell pellets washed by resuspending in experimental buffer (3ml) prewarmed to physiological temperature. The

cells were centrifuged once more and the supernatants again discarded. The final step was the resuspension of the cells in the volume and buffer required for the experiment.
Identical steps were taken in isolating mouse and hamster cells, with modifications to injection volumes (3-5ml). One rat supplied sufficient cells for an average of 30 samples, one mouse 4-6 samples, one hamster 15-20 samples. Cells obtained from SPF animals were isolated as soon as possible after the arrival of the animals, in order to reduce antibody formation.

2.2.1.2 **Rat pleural mast cells**

Pleural cells were recovered after isolation of peritoneal cells from the same animal. Heparin Tyrode's (6ml, 37 °C) was injected through the diaphragm (3ml each side) and the thorax massaged for 2min. The rib cage was then cut away carefully and the cells recovered by disposable pipette. The procedure was then as for peritoneal cells.

2.2.1.3 **Tissue mast cells**

Mast cells from the lung and mesentery were obtained by enzymic dispersion of the tissue. Samples of lung were dissected free of pleura and major airways and mesentery samples were separated from lymph nodes. The remaining tissues were washed with full Tyrode's buffer and chopped into 1mm^2 sections, using a McIlwain tissue chopper. The chopped tissue was then subjected to a 60-90 minute incubation , with magnetic stirring, in a suitable volume (100ml/2g tissue) of full Tyrode's buffer containing BSA (1mg/ml) and collagenase (type IV, 80 units/ml for rat tissues, 160 units/ml for guinea pig tissues, human lung and rat heart). The incubation medium was maintained at physiological temperature. Plastic and siliconized glassware was used throughout.
After incubation, the samples were passed through a syringe (10 ml) several times to ensure disruption of mucoid clumps. The incubate was then filtered through a double layer of gauze moistened with BSA-Tyrode's. Cells were recovered from the filtrate by centrifugation (MSE Chilspin, 1000rpm, 150g, 3min, 4 °C). The cell pellet was washed once more in BSA-Tyrode's and resuspended in the final volume and buffer befitting the experiment.

2.2.1.4 **Human basophil leucocytes**

Human basophils were isolated by dextran sedimentation. Whole venous blood (20ml) was collected from healthy donors into a plastic syringe and dispensed into a universal (20ml) containing heparin (3-4 drops), dextran (1% w/v, 5ml) and glucose (150mg). The contents were mixed gently and the sample left to stand uncapped for 30-40 minutes. When all erythrocytes had sedimented out, the top plasma layer was removed by disposable pipette into reaction tubes. The sample was washed after centrifugation (MSE Chilspin 1000rpm, 150g, 3min, 37 °C) in full Tyrode's buffer and finally resuspended in the volume and buffer required for the investigation.

2.2.2 Purification of mast cells

Cells obtained by peritoneal lavage contain 2-5% mast cells. The remainder comprises macrophages, neutrophils, eosinophils and erythrocytes. Contamination by

lysosomal enzymes from macrophages may alter the results of certain experiments by digesting surface components of perigranular and plasma membranes or the granule matrix itself.

Percoll (polyvinyl pyrrolidone (PVP)-coated silica particles) is of low osmolarity and viscosity and has been found to produce mast cell suspensions of high purity and yield. Morphological and biological activity is preserved after Percoll purification.

Percoll solutions (90%) were prepared to a composition of 9 parts Percoll to 1 part Tyrode's (x10 concentration) buffer. The solution was mixed thoroughly and aliquots (4ml) were frozen (-20 °C) in reaction tubes until required. Storage periods did not exceed 3 months. One aliquot was sufficient for the purification of cells from two large rats.

Cells from crude extracts were centrifuged and resuspended in volumes commensurate with 1ml per 4ml aliquot of Percoll. The cells were distributed throughout the Percoll by pipette and covered with 1ml of buffer applied gently enough to form a surface layer. The tubes were centrifuged (MSE Chilspin 1200rpm, 200g, 25min, 4 °C), causing mast cells to migrate to the bottom of the tube through the density gradient. The solution lying above the purified cells was removed by vacuum and the cells were resuspended in full Tyrode's (3ml), centrifuged (MSE Chilspin 100rpm, 150g, 2min, RT), pelleted and resuspended finally in the appropriate buffer.

2.2.3 Metachromatic staining with toluidine blue

Mast cells were counted by mixing a cell suspension (100μl) with a stock solution of toluidine blue (0.1% in 0.9% saline, 10μl). Following incubation in a water bath (37 °C, 5min), the stained preparation was examined microscopically using a haemocytometer (Neubauer improved) and cell counts were made as follows:

$$\text{No. of cells/ml} = \text{Total counts in 9 squares} \times \text{dilution factor} \times 1/\text{Total volume} \times 1000$$

where:

Depth of Haemocytometer	=	0.2mm
Total area .	=	9.0mm^2
Hence, total volume	=	1.8mm^3
Dilution factor	=	110/100

Mast cells were readily identified by their metachromatic (violet) staining, in contrast to the other cells which stain blue. This technique also facilitates purity determination.

2.2.4 Sensitization of animals

2.2.4.1 Sensitization of rats

SD rats were sensitized to the nematode *Nippostrongylus brasiliensis* by subcutaneous injection of 2500 larvae (L_3 stage) in 0.9% sterile saline into the thigh. Maximum sensitivity

was obtained after 21 days and levels of responsivity to secretory allergen were sustained for up to 2 weeks.

A culture cycle was maintained by collecting faeces on the eighth day after injection. A slurry of faeces (14g) were maintained in a petri dish for ten days (29°C), with regular hydration using an atomizer. The larvae were recovered by filtration through gauze and washed several times. The larvae were counted microscopically and adjusted to a final density of 6250 worms/ml. Rats were injected with 0.2ml worm suspension immediately.

2.2.4.2 **Sensitization of guinea pigs**

Guinea pigs were sensitized on reaching maturity by intra-peritoneal injection of ovalbumin (20μg/ml) in Freund's incomplete adjuvant. The injection solution was constituted as follows. Ovalbumin (400mg) was dissolved in sterile saline (10ml). To this, Freund's incomplete adjuvant (10ml) was added and the mixture was agitated to ensure uniformity. The preparation was used immediately (1ml) for injection. Sensitization was achieved 10 days after injection and lasted for a maximum of one month.

2.2.5 Preparation of reaction agents

2.2.5.1 **Water-soluble agents**

Most compounds used in the study were water soluble and thus dissolved in experimental buffer or glass distilled water. Agents were freshly prepared before each experiment, except for compound 48/80 and somatostatin, which could be stored temporarily (4 °C, 1 week). Swift use of peptide preparations was essential to ensure maximum activity. This was especially critical with the peptides substance P and VIP, which could hydrolyse within 30 minutes of preparation.

Final solutions were either x10 or x2 experimental concentrations, depending on solubility. Most peptide concentrations are quoted as micrograms per millilitre. When the need to quote concentrations in molar terms arose, the molecular weights and peptide purity were used to safeguard accuracy. The molecular weights of the polymers are rather ill-defined but representitive values may be chosen. For compound 48/80, the molecular weight of the trimer is cited (520), because it is the predominant polymeric unit. Poly-L-lysine was quoted as MW 52,000, at the supplier's recommendation.

2.2.5.2 **Non water-soluble agents**

i. Solubility in DMSO

Most non water-soluble agents were dissolved in a minimal volume of dimethyl sulphoxide (DMSO) and sequentially diluted with buffer. DMSO was used as a solvent for Antimycin A, ETYA, PMSF and TPCK. ETYA was stored under nitrogen in solid and solvated forms at all times.

ii. BDTA and BAC

30mg BDTA or BAC were added to 1666μl of ethanol. 20μl of this solution were made up to 12ml with experimental buffer to produce a 30mg/ml stock. Subsequent dilutions

were made in buffer. The final concentration of ethanol must not exceed 0.5μg/3ml, otherwise toxic effects may become apparent.

iii. Phospholipids

PS was obtained commercially as a solution (20mg/ml) in chloroform/ methanol (3:1 ; v/v). Lyso-PS was obtained in solid form and dissolved in a mixture of chloroform/methanol (3:1; v/v, 20μg/ml). In both cases, 50μl of the organic solution was pipetted into a glass test tube and evaporated to dryness in a stream of nitrogen (O_2-free, 37 °C, dry). On gentle rotation of the tube, a thin, even film was deposited on the sides of the vessel. CMF-Tyrode's buffer (1ml) was added and a suspension formed by vortexing. 15μl and 100μl of this solution in a final volume of 1ml yielded concentrations of 15μg/ml for PS and 0.1μg/ml for lyso-PS, respectively.

iv. Preparation of secretory allergen

Seven days after the injection of 5000 *N. brasiliensis* larvae, the host rats were killed and the abdominal cavity exposed. The small intestine was removed by dissection and the adult worms were easily identified by their red colour. The gut was cut along its length using vein scissors and laid upon layers of gauze. The worms were recovered by migration through the gauze, on elution with sterile saline (0.9%, 25 °C). The worms were washed five times by settling under gravity and resuspending in sterile saline. After the final wash, the worms were transferred to a plastic beaker and the worm-sterile saline mixture allowed to incubate in a shaking water bath (5-6h, 37 °C). The supernatant of the incubate contained the allergen and was expressed as worm equivalents (WE), as defined by Ogilvie [508]. Stock solutions were prepared at a concentration of 1WE/ml and stored at -20 °C until required.

2.2.6 Histamine release experiments

2.2.6.1 General procedure

For most experiments, the reaction volume was confined to 200μl. The majority of this volume was the cell suspension (100-180μl), as the drugs were applied in minimal volume (20μl). Tubes were customarily prepared containing the active agents and the cell suspension added subsequently to each sample at timed intervals. Agents were added after the cells only when preincubation with another compound was necessary.

In experiments where differential effects of various media were being investigated, mast cells were resuspended in CMF-Tyrode's buffer, before addition to the sample tubes containing modified media of equal volume and twice the concentration of the test components. The cells and new media were allowed to equilibrate for five minutes before the addition of the secretagogue.

From the instant of contact between the cells and agonist, the reaction was allowed to proceed for 10 minutes at physiological temperature. All experiments included a sample to determine spontaneous histamine release. This was achieved by exposing the cells to identical handling as that of the test cells, in the absence of any reagents. Typically,

spontaneous release values did not exceed 5%, however a limit of 10% total histamine content was determined to be acceptable.
Secretion was terminated by the addition of ice cold buffer (1.8ml) to each tube. This process arrested release by cooling the cells and diluting the agonists to ineffective concentrations. The cells were then sedimented (MSE Chilspin 1000rpm, 150g, 3min, 4 °C) and the supernatants transferred to appropriately labelled tubes and retained. The cell pellets were resuspended in buffer (2ml) and heated in a water bath (100 °C, 10min), to release residual cellular histamine by membrane disruption. The supernatants and cell pellets were then ready for assay of the histamine content.
Preincubation of the cells with inhibitors prior to the addition of the agonist was altered to suit the particular antagonist. The usual time period for preincubation was 10 minutes.
Experiments on human basophil leucocytes accommodated the slow response of this cell type by extending the incubation period (30min).
Variations in general protocol are quoted where appropriate.

2.2.6.2 **Addition of phospholipids**

PS and its lyso derivative potentiate release induced by immunologic stimuli. To allow the incorporation of the lipids into the cell membrane, a minimal volume of the respective compound was added to the equilibration medium 2 minutes prior to challenge by the secretagogues.

2.2.6.3 **Somatostatin as an inhibitor**

Experiments described in chapter 4 focus on using somatostatin as an inhibitor. The concentrations of the peptide were very low (10^{-9}-10^{-15}M). To ensure that the cited concentration remained true, all serial dilutions were made with new pipette tips. This procedure should curb major errors by limiting the adhesion of peptide to the walls of a single pipette. Serial dilutions performed without this precaution cannot guarantee peptide concentrations.

2.2.7 Histamine assay

The assessment of histamine levels in both the supernatant and cell pellet fractions of each sample utilizes a method developed and described by Shore *et al.* [508]. The technique depends upon the generation of a highly fluorescent adduct by the condensation of histamine with *o*-phthaldialdehyde (OPT), under basic conditions. The adduct is stabilized by acidification and the fluorescence relative to a histamine-free sample was assessed spectrofluorometrically.

2.2.7.1 **Manual spectrofluorometric assay**

Each sample (approx. 2ml) of cell pellet or supernatant was made strongly alkaline by the addition of NaOH (200μl, 1M). OPT (100μl, 0.1 w/v in methanol) was then administered with thorough vortexing and the condensation reaction allowed to proceed for 4 minutes at room temperature, to provide maximum fluorescence. The samples were then acidified to pH 2.0 by the addition of HCl (100μl, 3M), followed by thorough mixing.

Acidification terminates the reaction and stabilizes the fluorescence product. The stabilization is not absolute and samples were measured as quickly as possible after condensation as fluorescence starts to decay within 10 minutes, particularly in the light. Fluorescence could be maintained for slightly longer periods if necessary, by storage of the samples in the dark.

Histamine levels were then measured on a Perkin Elmer spectrofluorometer. A sample was transferred to a clean plastic cuvette and the fluorescence at 440nm was measured following excitation at 360nm. For each assay, a blank, comprising buffer alone, was processed as described above and all sample readings were subsequently corrected for this value.

Histamine release was expressed as a percentage of the amine present in the supernatant, with respect to the total histamine content of the supernatant plus that of the cell pellet. This can be expressed mathematically:

$$\% \text{ Histamine release} = \frac{\text{Histamine in supernatant}}{\text{Histamine in supernatant plus Histamine in cell pellet}} \times 100$$

The spontaneous histamine release was calculated in the same fashion and subtracted from release values obtained with experimental samples.

Where an antagonist of histamine liberation was under investigation, results were expressed as the percentage inhibition obtained, with respect to the release observed in the absence of the inhibitor. This control release was calculated as above and usually the mean of two release values for each study was used.

Once more, this can appear in mathematical terms:

$$\%\text{Inhibition of release} = \frac{\text{Histamine release in absence of inhibitor} - \text{Histamine release in presence of inhibitor}}{\text{Histamine release in absence of inhibitor}} \times 100$$

2.2.7.2 Automated assay of histamine

In certain instances the manual assay is not possible, for example when the levels of histamine are low or there is an excess of protein and debris following enzymic dispersion. There may also be problems of an experimental reagent interfering with fluorescence (coloured compounds, etc.). Under these circumstances, an automated, commercially available apparatus (Technicon Autoanylyser II) was employed. This technique, through a series of practical steps, relies on the principles of preferential solubility in organic and aqueous solvents.

Experimental samples (approx. 2ml) were acidified with perchloric acid (30μl, 72% w/v) and thoroughly mixed. This treatment serves to precipitate any protein, which could interfere with efficacy of extraction by causing blockages in the fine bore, sample-tubing. The samples were then centrifuged (MSE Chilspin 2000rpm, 300g, 10min, RT) to sediment the precipitates and the supernatants transferred to sample cups (2ml) and placed in sequence for automated assay.

Data was obtained in graphical form as a series of peaks rising from a base line. The height of each peak was proportional to histamine content and so these values were put into the equations above and a percentage release or inhibition value was obtained.

2.2.8 Statistical analysis of results

All values in this thesis are given as the means ± the standard error of the mean (SEM) for the number (n) of experiments performed. The points on the graphs are the means from the number (n) of experiments noted and the vertical bars represent SEM. Statistical analysis of results was generally (Chapter 4) carried out using a paired t-test for related measures. In addition, in appropriate cases (Chapter 7), a Student's t-test for the analysis of two independent means was performed. Values of $p < 0.05$ were considered to be statistically significant.

2.2.9 Schild Plots

Read and coworkers [509] illustrated a competitive interaction between compound 48/80 and BDTA for binding sites on the mast cell membrane. The essential feature of competitive antagonism is that the effect of the antagonist can be overcome by raising the agonist concentration. The degree of the shift to the right of the agonist log-concentration-response curve is proportional to the antagonist concentration. The affinity of the antagonist for the receptors is inversely proportional to the antagonist-receptor dissociation constant, K_A.

The negative logarithm of the molar concentration of the agonist, for which the ratio of equi-effective concentrations of agonist in the presence and absence of the antagonist is two, has been designated by Schild [605] as the pA_2 value. Mathematically, it is expressed thus:

$$pA_2 = -\log[A]_2 = \log (1/[A]_2)$$

The plot of $\log(x-1)$ against $\log[A]_x$ is a straight line, where x is the ratio of equiactive concentrations of the agonist in the presence and absence of the antagonist and $[A]_x$ represents the concentration of the antagonist. Therefore, when $x = 2$, the intercept on the log[A]-axis gives the value of $\log K_A$ or $-pA_2$.

When pA_2 values for an antagonist are determined using a series of agonists acting on the same receptor but having different potencies, the values are virtually identical. A high degree of specificity of antagonism is indicated by a high pA_2 value.

2.2.10 Immunofluorescence of mast cells

Certain dyes will absorb radiation such as ultraviolet, become excited and thereupon are capable of emitting radiation which ceases almost immediately after the withdrawal of the exciting radiation. Such dyes are known as fluorochromes. The fluorochrome, Fluoroscein, is excited by ultraviolet/blue light to emit visible, yellow-green light.
Fluorochromes can be converted to derivatives containing chemically active groups, such as isothiocyanate. In these forms, they can be readily coupled or conjugated with proteins such as antibodies, without destroying their specificity [510]. A. H. Coons showed that such conjugates combine with antigen present in a tissue section and that the bound antibody could be visualized in the ultraviolet microscope, through emission of fluorescence. In this way, the distribution of antigen throughout a tissue or within cells can be demonstrated. The method is also applicable to the detection of antibodies directed against antigens already known to be present in a given tissue or cell preparation [511].
Three procedures are open to the investigator.

1. The antibody directed against the tissue substrate is itself conjugated with the fluorochrome and applied directly.
2. Unlabelled antibody is applied directly to the tissue substrate and visualization brought about by treatment with fluorochrome-conjugated, anti-Ig serum.
3. The 'sandwich' test, a double layer procedure designed to visualize specific antibody. Receptor sites are stimulated with antigen and specifically bound antigen is then located by the addition of a fluoroscein-labelled antibody to the antigen.

A combination of the latter two tests were used to investigate specific binding sites for somatostatin on the mast cell membrane.
Purified cells from the rat, hamster and mouse peritoneum and those obtained by enzymic dispersion of guinea pig lung and mesentery were condensed to a volume of 1ml per sample. The samples were transferred to an ice bath and exposed to the metabolic inhibitor antimycin A (10μM) and somatostatin (100μg/ml) in a minimal volume (20μl each). This treatment should allow somatostatin adherence to its binding site to proceed without impediment, while histamine liberation should be prohibited by the cold and the metabolic antagonist.
The incubation medium was full Tyrode's buffer. Cell samples were allowed to incubate for 20 minutes and then centrifuged (MSE Chilspin 1000rpm, 100g, 3min, 4 °C), resuspended

in 1ml and the washing procedure repeated twice more. The cell samples were then 'blocked' by incubation in BSA-Tyrode's for 30 minutes. Such treatment assists the prevention of non-specific fluorescence by allowing BSA to occupy any available protein binding sites exposed on the cell surface. Cells were pelleted by centrifugation (MSE Chilspin 1000rpm, 100g, 3min, 4 °C) and the supernatants discarded.

The cells were then prepared for exposure to the primary antibody. The primary antibody in these experiments was a polyclonal, rabbit antiserum to somatostatin (rabbit anti-somatostatin IgG). The antiserum had been preadsorbed before addition to the sample. Preadsorbtion is critical in preventing non-specific binding of anti-somatostatin to other mast cell membrane constituents.

A supply of purified mast cells is excluded from challenge to somatostatin and instead, incubated with the primary antibody only (20-fold dilution, 50μl, 10^{-6} cells/ml). The cells were allowed to incubate for 10 minutes and then sedimented by centrifugation (as above). The cells were discarded and the supernatant (approx. 1ml), containing the primary antibody, was used to resuspend the cells after blocking with BSA. The reaction was allowed to proceed (10min, 4 °C) and terminated by pelleting and repeated washing (x3). The cells were pelleted once more after the last wash, in readiness for the secondary antibody.

The secondary antibody, FITC-conjugated anti-rabbit IgG, had also been preadsorbed with purified mast cells in the same way as for the primary antibody. The supernatant (approx. 1ml) containing secondary antibody (50-fold dilution, 20μl) was used to resuspend the cell pellet. All stages of the experiment employing the secondary antibody were conducted in the dark, to prevent fading of the fluorochrome. The cells were allowed to incubate with the secondary antibody (5min, 4 °C), before pelleting and washing. After the final wash, cells were resuspended in a final volume of 1ml and left in the dark and cold until visualization. Visualization took place within 15 minutes of application of secondary antibody, to prolong the intensity of observed fluorescence.

In addition to preadsorbtion of the primary and secondary antibodies, further controls were included in the study. These included samples treated as above but omitting somatostatin and others in which the primary antibody was excluded. On visualization, these controls should not fluoresce and any staining is a reflection of non-specific antibody binding. Several pilot experiments had also been performed to establish the optimum quantities of antibodies required to obtain maximum fluorescence.

Before viewing was possible, the cells had to be transferred to microscope slides. Viewing living cells presents several problems, not least of which is the migration of cells across the field of view, resulting in blurred photomicrographs of long exposure. To overcome these problems, cells were administered in a small drop of buffer to the centre of the slide and allowed to evaporate partially for a few minutes. The slides had been previously treated with micro-thin layer of a non-aldehydic, acetate, contact-adhesive (kindly donated by Conrad King, Zoology, UCL), around the boundaries of the cover slip.

The semi-dried cells were then mixed gently with a drop of mounting medium and the coverslip applied. Gentle pressure ensured adhesion of the slip to the slide at the edges and distributed the cell/mounting medium mixture in a thin film within that area. The mounting medium is known as glycerol/PBS (kindly donated by C. King, originally produced and patented at City University, London). The density of the medium impedes cellular drifting but ensures continued hydration. An additive, PBS, acts as an anti-fade agent to prolong fluorescence. After mounting, the slides were stored on ice in the dark in readiness for viewing.

Viewing was performed with a Zeiss (West Germany) universal fluorescence microscope, mounted on a Kohler illumination base. The microscope was fitted with a IV Fi epifluorescence condenser and an HB 50W super-pressure mercury lamp, 2-Planapo 63/1.4 Ph_3 oel and 1-Planapo 40/1.0 Ph_3 oel mI objectives and a x10 eyepiece. 63 corresponds to the initial magnification, 1.4 to numerical aperture, Ph to the presence of a phase ring and oel to the requirement of oil on the coverslip. Both oil-immersion objectives were used with Zeiss oil (refractive index 1.515).

Photomicrographs were taken with an MC63 photomicrograph counter, fitted to the fluorescence microscope, on Kodak Tri-X pan film (monochrome, 400 ASA). Cells were photographed in light field on automatic exposure and in dark field (to detect fluorescence) on manual exposure. Manual exposure times were typically 40 seconds, with brackets of 0.75s, to assure clear results. The negative film was processed by the Central Photographic Unit UCL, and the photographic plates produced by V. E. Kassessinoff on Ilford multigrade III variable contrast paper.

CHAPTER 3

SOME CHARACTERISTICS OF HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS STIMULATED WITH SOMATOSTATIN.

3.1. *INTRODUCTION*

3.1.1 Somatostatin and the mast cell

The first studies with somatostatin and mast cells were performed to examine any inhibitory effect of the peptide on histamine release induced by a variety of agents [512]. It had been proposed that somatostatin acts on secretory cells by altering a late stage of the granule secretory process at a step which is calcium dependent [513]. This prompted studies into the possible inhibitory role for somatostatin in mast cell degranulation. The results were, however, disappointing. The peptide was inactive as an agonist or antagonist of mast cell histamine liberation at the concentration investigated (10^{-6}M).

Theoharides and Douglas [514] were the first to report the histamine releasing properties of somatostatin. Mediator release from rat peritoneal mast cells was dependent on intact mechanisms for glycolysis and oxidative phosphorylation, temperature and independent of exogenous calcium, but dependent on cell-associated calcium depletable by long exposure to a chelating agent. The authors promptly noted the similarity in release characteristics between the peptide and the classic mast cell secretagogue, compound 48/80.

In addition to the natural, cyclized form of the molecule [515] which is a Q-shaped molecule of 15 amino acids, there is a linear form, dihydrosomatostatin [512] which also liberates histamine. Both forms of the peptide are active at concentrations just above those investigated during the inhibition studies [512]. The stimulant effect of somatostatin was comparable in potency to that of polymyxin B [514]. Somatostatin activity was therefore attributed to an appropriate orientation of its positively charged moieties at the amino terminus and at lysine residues in positions 4 and 9.

Theoharides *et al.* extended their initial studies on the reaction characteristics of somatostatin-induced histamine secretion [424]. The inhibitory effects of dibutyryl cyclic AMP and 8-bromo cyclic AMP on secretion were reported and cyclic AMP levels were seen to drop in the course of the secretory response. Somatostatin bound to Sepharose beads retained its stimulatory ability, inferring action at the mast cell surface.

Later studies [423] reported that the structural considerations of somatostatin that are seemingly important for stimulation of mast cells are to be distinguished from those that define inhibitory activity in other systems. Substitution of Lys^9 by its D-analogue profoundly reduced inhibitory activity [516,517], whereas it increased mast cell activity four fold [423]. Moreover, substitution of Lys^4 by the D-analogue had little effect on inhibitory activity but reduces mast cell stimulation by 80%. Construction of 3D models of somatostatin and compound 48/80 indicated that the potency of somatostatin as a histamine releaser may be related to a spatial orientation of its cationic amino acids. These amino acids approximated

to the possible orientation of the first, third and sixth cationic ethylamine groups of the compound 48/80 hexamer.

Compound 48/80 and A23187 cause a prompt increase in the incorporation of radioactive phosphate into three specific mast cell proteins of molecular weights 42, 59 and 68kDa [211]. Compound 48/80 induces an additional protein band at 78kDa, showing slow kinetics. Cromolyn initiates a selective incorporation of radioactive phosphate into this band suggesting a termination step [518]. Somatostatin has been shown to produce the same but weaker pattern of phosphorylation noted with compound 48/80 [519].

Somatostatin has also been reported to induce secretion from human leucocytes *in vitro* [520]. This was not observed with substance P or compound 48/80. The larger form of somatostatin, somatostatin-28, showed a lower potency attributable to the lower isoelectric point. The release depended on the cationic moieties of the peptides and was unaffected by the addition of exogenous phosphatidyl serine.

There is some evidence that somatostatin does inhibit histamine release from gastric carcinoid tumours stimulated with pentagastrin [521]. The mechanism of action remains unclear. Goetzl and Payan [522] reported that sub-releasing concentrations of somatostatin could inhibit mediator release from human basophils and rat leukaemic basophils (RBL). Goetzl and coworkers also reported that RBL cells appeared to contain somatostatin-like factors and substance P-like factors are found in mucosal type mast cells of rodents, mononuclear phagocytes and some malignant lines of lymphocytes. No signals were seen in human B or T lymphocytes, erythrocytes, PMN leucocytes or platelets [523]. Somatostatin-14-like immunoreactivity was also found in porcine PMN leucocytes and porcine and human blood mononuclear leucocytes [524]. The import of these observations is totally unknown.

The aim of the present chapter was to examine in more detail the histamine secretion from rat peritoneal mast cells stimulated with somatostatin. The basic characteristics of the release process, the nature of the peptide binding site and the effect of various inhibitors were examined. These results were compared with those obtained in parallel studies with immunologic ligands and other polycationic stimuli. In this way it was hoped to categorise the release induced by somatostatin and to identify similarities with and differences between the agonists. In particular, we sought to determine the extent to which the response induced by somatostatin resembled that evoked by other polyamines.

3.2 *RESULTS*

3.2.1 Basic characteristics of the release process

3.2.1.1 **Histamine release induced by somatostatin**

Somatostatin (1 - 100μg/ml) produced a dose-dependent release of histamine from isolated rat peritoneal mast cells (Fig.3.211). The maximal release was in excess of 60% at optimal concentrations of somatostatin. The release from a purified population of RPMC showed a similar profile, with only a slight displacement to the right (data not shown).

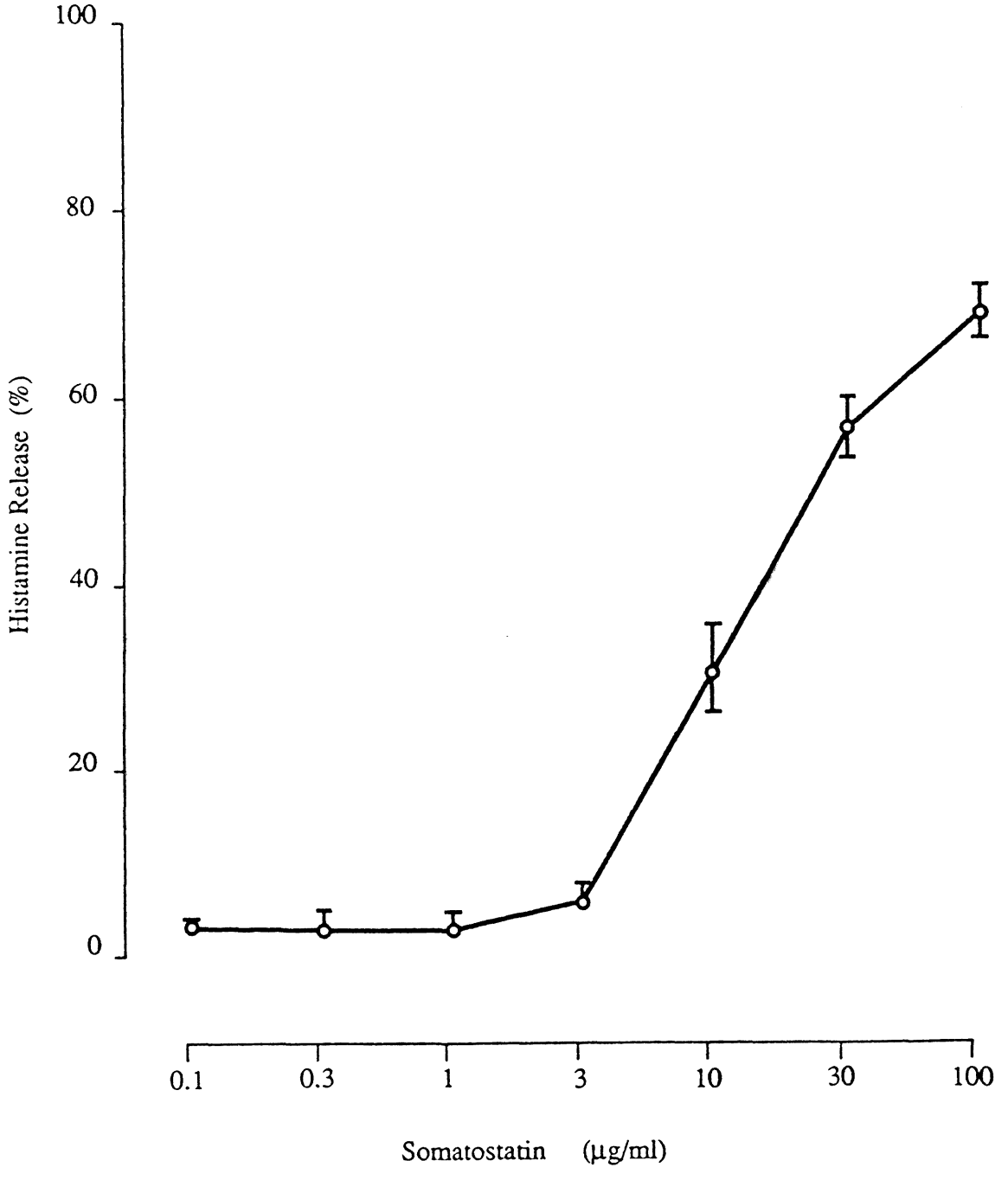


Fig. 3.211 Histamine release from rat peritoneal mast cells induced by somatostatin

All values ± SEM (n=5)

3.2.1.2 Effect of pH on histamine release

Cells were incubated within a physiologically acceptable range of pH (6.0 - 8.0, at 0.5 intervals). Somatostatin (100μg/ml) appeared generally unimpaired by changes in extracellular pH (Fig.3.212a). At more acidic pH, a slight depression in response was noted, but not deemed significant. For comparison, similar studies were performed, in parallel, with antigen (20WE/ml,± PS 15μg/ml) (Fig.3.212b). Optimal release was observed at pH 7.0, falling off at either extreme of the range used. The addition of PS had little effect in the response to pH, although the overall release was naturally increased and the maximum shifted to pH 6.5.

3.2.1.3 Kinetics of the release process

To determine the kinetics of the release process, aliquots of cells were incubated in full Tyrode buffer. The cells were then stimulated with the secretagogue and the reaction terminated at fixed time intervals with ice-cold buffer (2ml). This procedure served to rapidly cool the sample and dilute the agonist to essentially ineffective concentrations.
The kinetic profile for somatostatin (100μg/ml) was very rapid, virtually all release occurred within 30s and the process was 90% complete within 10s (Fig.3.213).

3.2.1.4 Temperature dependence of the release process

Somatostatin (100μg/ml) showed maximal responses at room temperature (22 °C), and at physiological temperature (37 °C) (Fig.3.214). Release at 0 °C was negligible. At 45 °C, release in the absence of external calcium and on brief pretreatment with EDTA was abolished. However, exocytosis in the presence of extracellular calcium was significantly inhibited, but by no means negated. Identical experiments were conducted on compound 48/80 (1μg/ml) and polylysine (10μg/ml). These compounds showed similar profiles to that of somatostatin, (data not shown) but with less evidence of significant release at the highest temperature in the presence of calcium.

3.2.1.5 Effects of metabolic inhibitors

Parallel experiments were conducted in the presence and absence of extracellular calcium, to investigate the action of metabolic inhibitors. Release induced by somatostatin (100μg/ml) was slightly greater in the absence of glucose, particularly in the presence or absence of external calcium (Fig.3.215).
Antimycin A (1μM) abolished all response to somatostatin. The effect of 2-deoxyglucose (5mM) was negligible when singly employed, but in conjunction with antimycin A, total inhibition was observed.
The action of metabolic inhibitors was not affected by extracellular calcium.

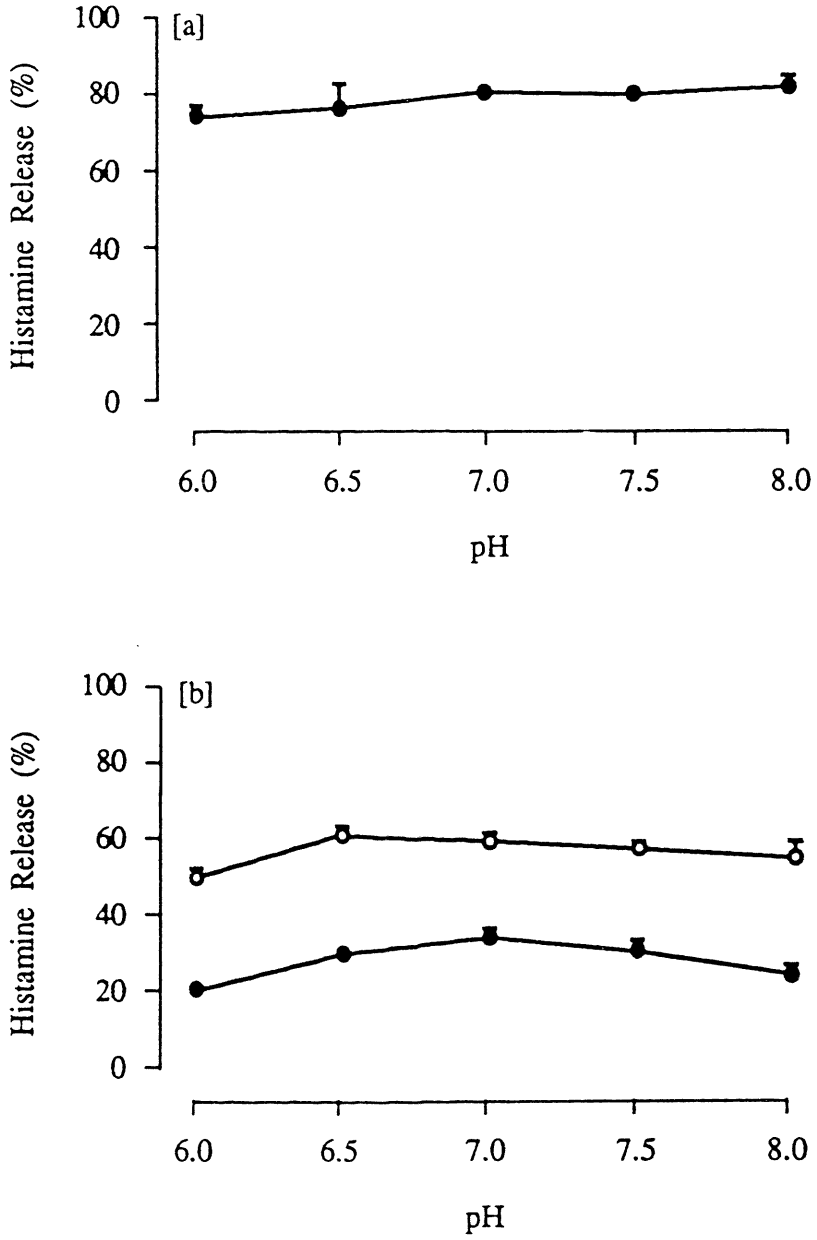


Fig. 3.212 Effect of pH on histamine release from rat peritoneal mast cells induced by
[a] Somatostatin (100μg/ml)
[b] Antigen (20WE/ml) either plus PS (15μg/ml, open symbols) or minus PS (closed symbols)

All values ± SEM (n=4)

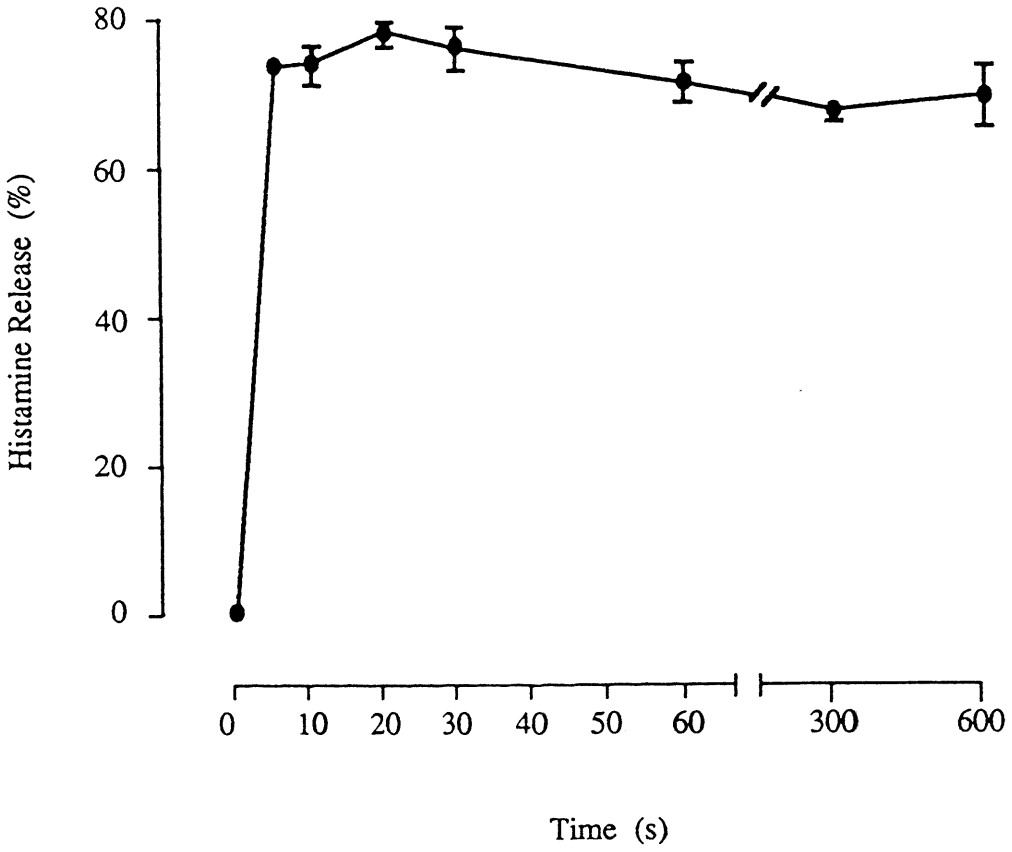


Fig. 3.213 Kinetics of histamine release from rat peritoneal mast cells induced by somatostatin (100μg/ml)

All values ± SEM (n=3)

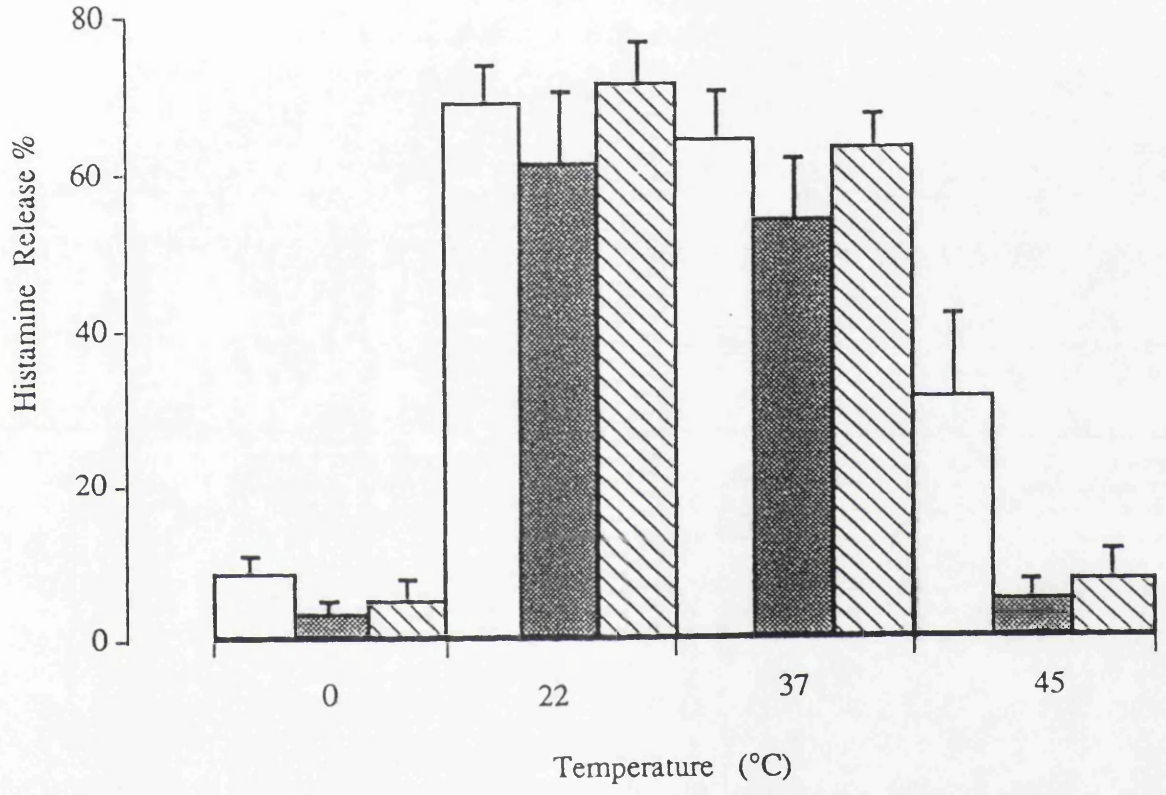


Fig. 2.214 Temperature dependence of histamine release from rat peritoneal mast cells induced by somatostatin (100μg/ml) in the absence (dotted columns) and presence of calcium (1mM, open columns) or after brief pretreatment with EDTA (10^{-4}M, hatched columns)

All values ± SEM (n=6)

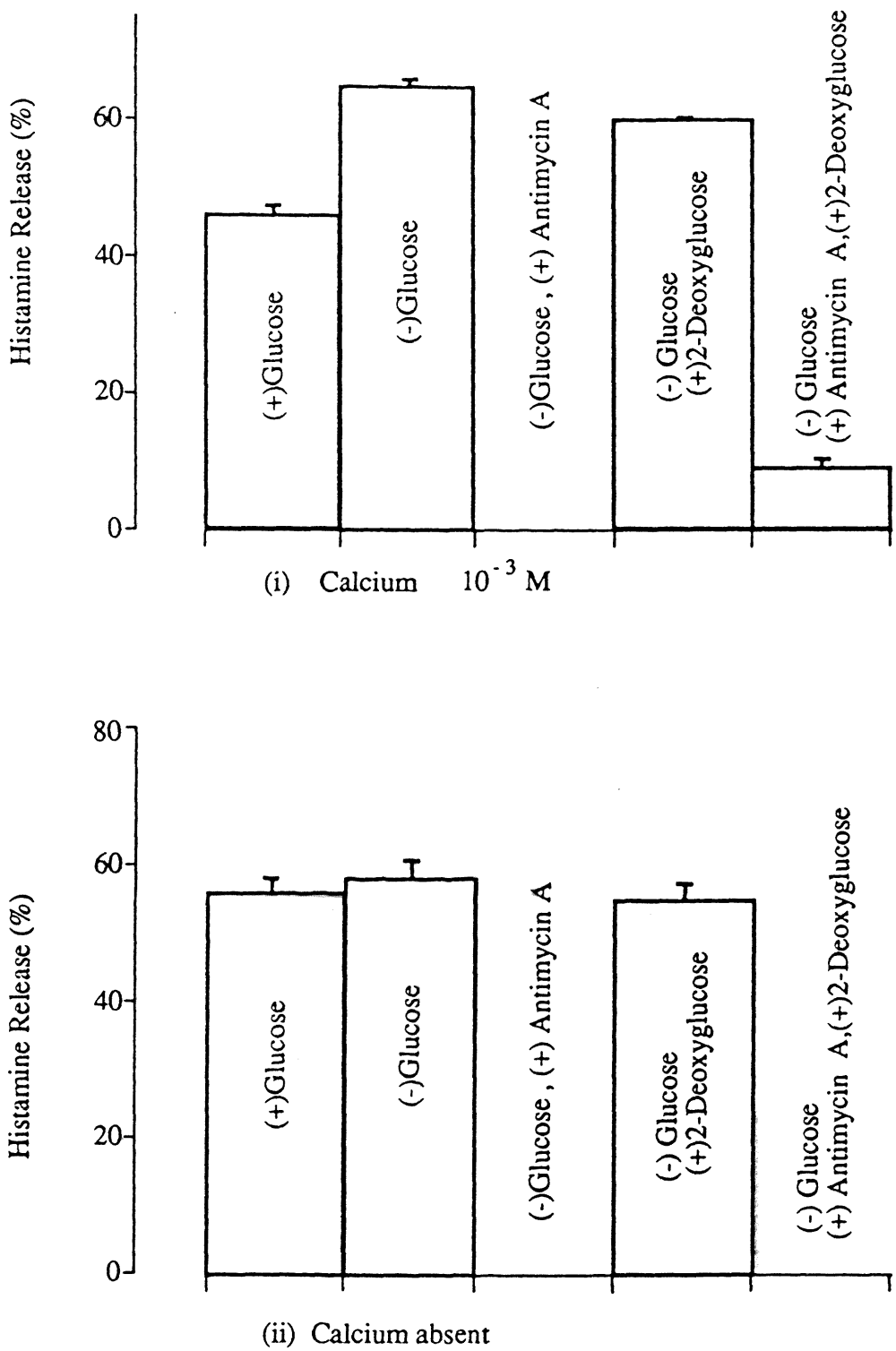


Fig.3.215 Effects of the metabolic inhibitors Antimycin A (1μM) and/or 2-Deoxyglucose (5mM) on histamine release from rat peritoneal mast cells induced by somatostatin (100μg/ml), either in the presence (i) or in the absence (ii) of calcium (1mM)

All values ± SEM (n=7)

3.2.1.6 **The significance of extracellular calcium**

Studies were conducted on somatostatin (0.1-100μg/ml), compound 48/80 (0.025-1μg/ml) and polylysine (0.1-10μg/ml). Cells were challenged in the presence and absence of extracellular calcium (1mM), or after brief pretreatment with the calcium chelator EDTA (5min, 10^{-4}M).

Somatostatin responded readily in the absence of external calcium, although maximal release appeared depressed (Fig.3.216.a). Maximal release on incubation with EDTA also seemed less than that in calcium. However, a greater response was observed at low and mid-range concentrations of the stimulus.

Similar results were obtained with compound 48/80 (Fig.3.216.b) and polylysine (Fig.3.216.c), in accordance with the literature.

3.2.1.7 **Effects of extracellular cations**

The effects of supramaximal concentrations of calcium and strontium ions were investigated. Cells obtained from the peritoneal washings of rats were preincubated in various concentrations of calcium and strontium (0-10mM) for twenty minutes before challenge. Experiments were conducted with somatostatin (100μg/ml) and included compound 48/80 (1μg/ml) for purposes of comparison.

Results show that histamine secretion induced by both agonists was evident with elevating cation concentration up to 1mM (Figs.3.217a,b). In excess of this concentration, calcium appears inhibitory. Strontium ions substituted well for calcium, but were non-inhibitory at supramaximal concentrations.

There was no appreciable difference between the responses of either stimulus under these experimental conditions.

3.2.1.8 **Effects of phospholipids**

The effects of PS, (15μg/ml) and lyso-PS, (1μg/ml) were examined on release induced by somatostatin (10μg/ml) and anti-IgE (1000-fold dilution). Histamine liberation induced by somatostatin was unaffected by the addition of phospholipids (Fig.3.218). In contrast, secretion brought about by anti-IgE was greatly enhanced by PS and also by lyso-PS as expected.

3.2.2 Investigations on the nature of the binding site

3.2.2.1 **Comparison with dextran**

RPMC were preincubated with glucose (0-15mM), or low molecular weight polyglucose (MW2600, 0-10mM). Cells were then challenged with either dextran (10μg/ml), or somatostatin (30μg/ml). Wherever dextran acted as the stimulus, lyso-PS (1μg/ml) was included.

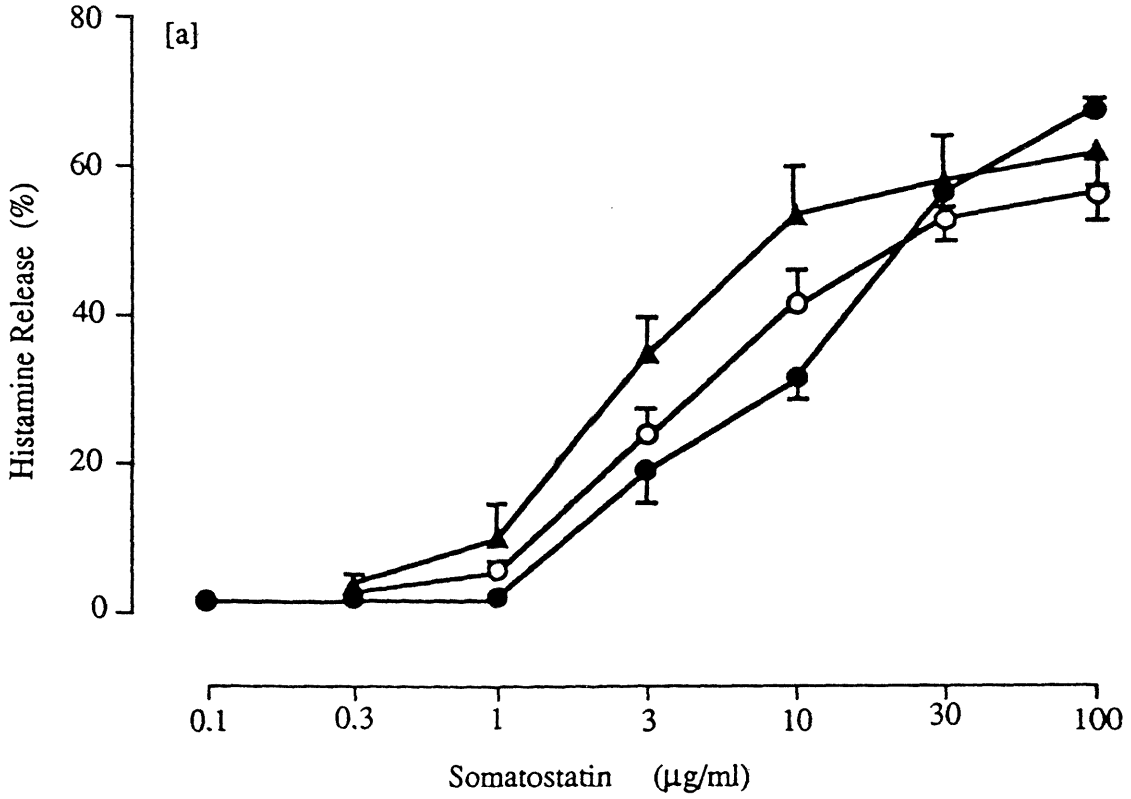
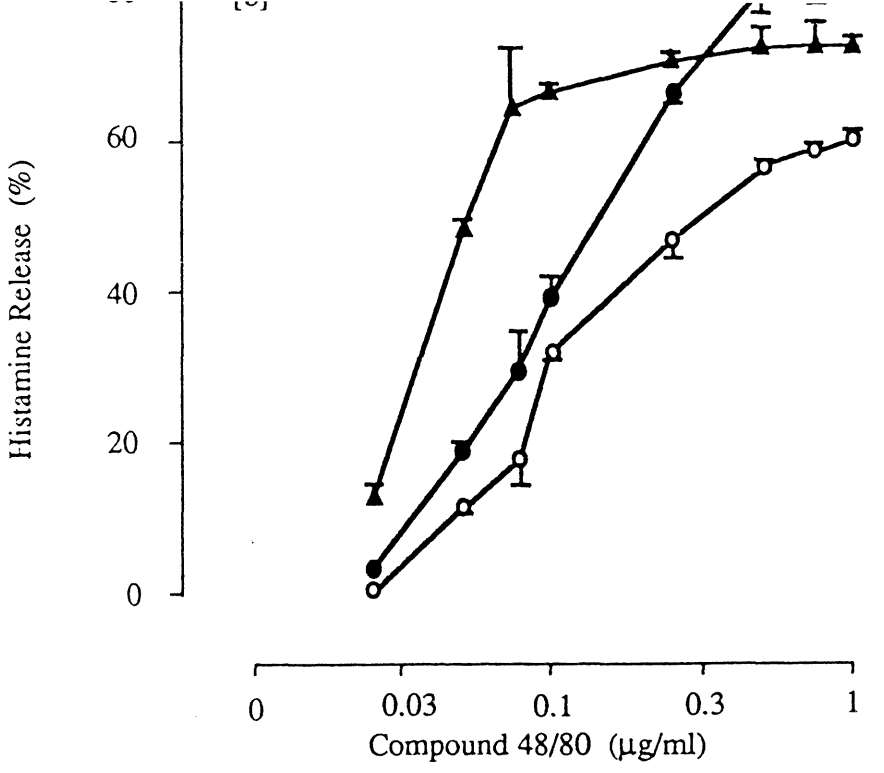


Fig. 3.216a The significance of extracellular calcium on histamine release from rat peritoneal mast cel ls induced by somatostatin. Experiments were conducted in the absence (open circles) or presence of calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles)

All values ± SEM (n=3)



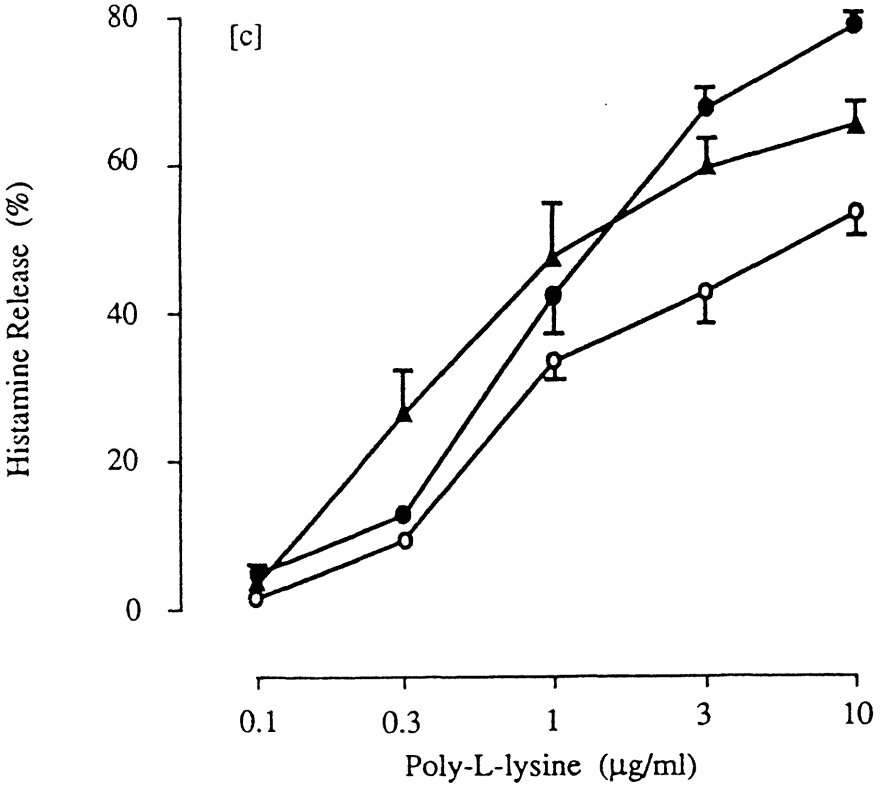


Fig. 3.216b,c

The significance of extracellular calcium on histamine release from rat peritoneal mast cells induced by [b] compound 48/80 and [c] polylysine. Experiments were conducted in the absence (open circles) or presence of calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles)

All values ± SEM (n=3)

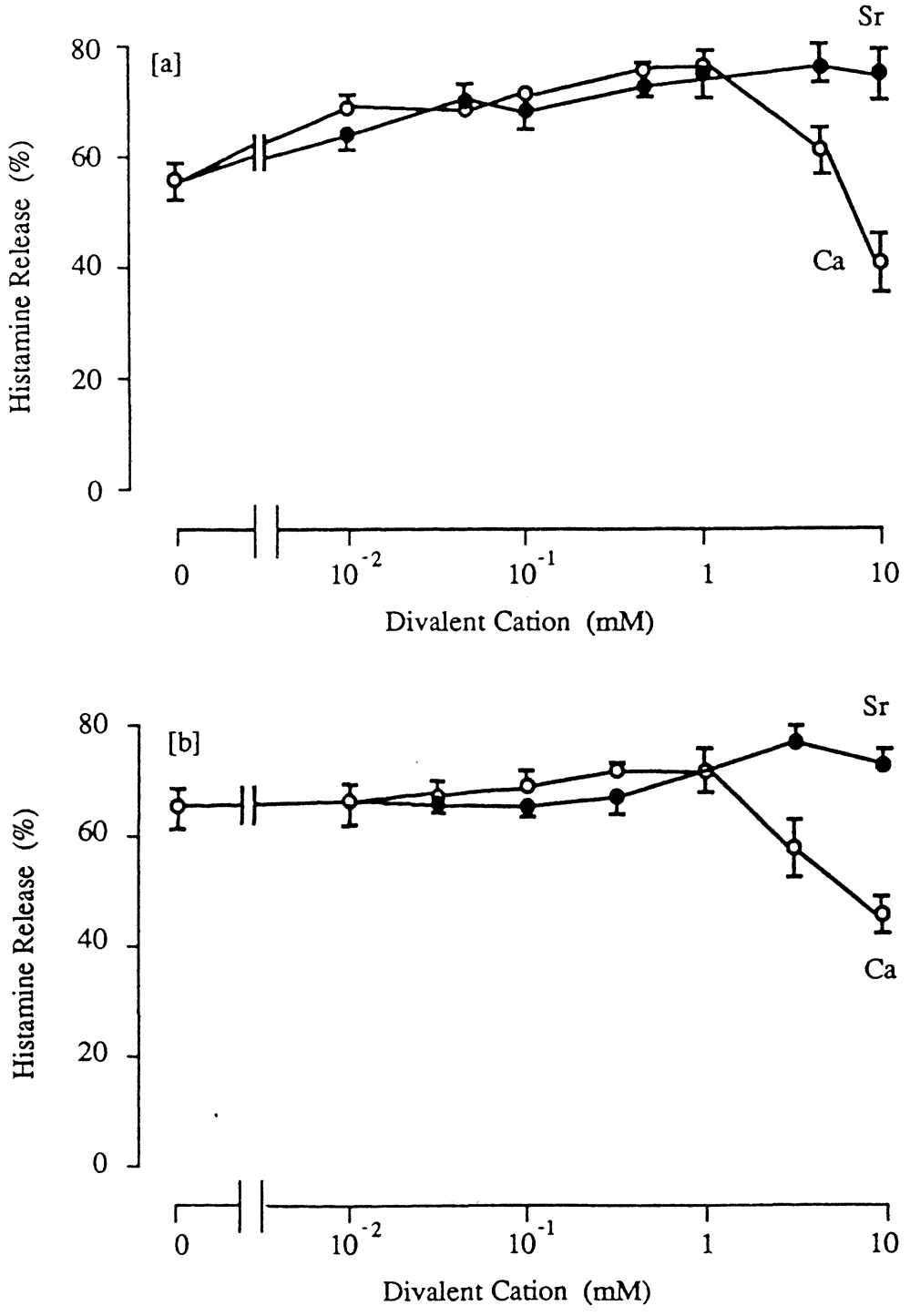


Fig. 3.217a,b Effects of the extracellular cation calcium (open circles) and strontium (closed circles) on histamine release from rat peritoneal mast cells induced by
[a] somatostatin (100μg/ml)
[b] compound 48/80 (1μg/ml)

All values ± SEM (n=4-6)

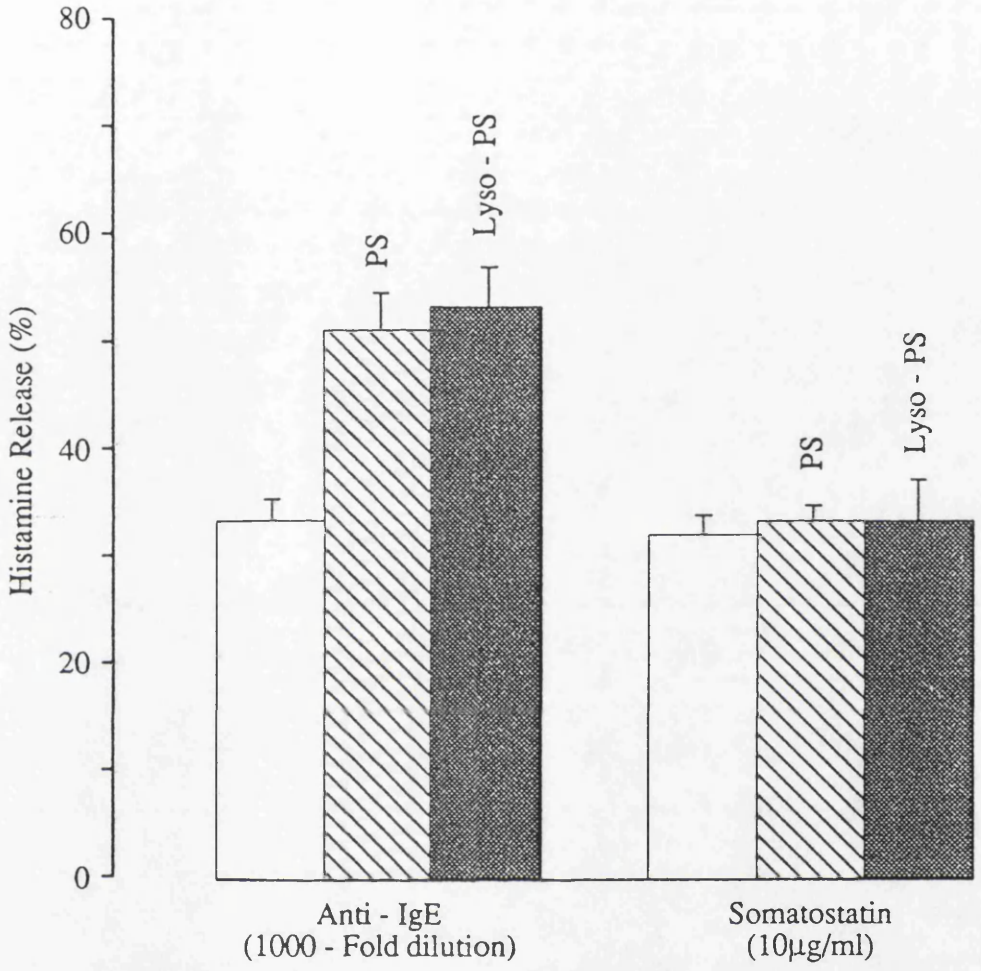


Fig. 3.218 Effects of the phospholipids PS (15μg/ml) and lyso PS (1μg/ml) on histamine release from rat peritoneal mast cells induced by somatostatin (10μg/ml) and anti-IgE (1000-fold dilution)

All values ± SEM (n=4)

Dextran-induced release was effectively inhibited by increasing concentrations of both glucose and polyglucose (Fig.3.221). This was not the case with somatostatin, whose activity was unimpaired by these agents.

3.2.2.2 **The significance of cell-fixed antibody**

Peritoneal mast cells were obtained from two separate rat populations. Firstly, from normally housed rats, and secondly from specific pathogen free (SPF) rats. The latter are bred and maintained in sterile environments to preclude the possibility of antibody formation. Experiments were conducted simultaneously utilizing somatostatin (1-100μg/ml) and anti-IgE (3×10^{-2}-3×10^{-4} dilutions), either in the presence or absence of PS (15μg/ml). Cells isolated from the SPF animals showed a marked decrease in response to anti-IgE, but not to somatostatin (Fig.3.222). Indeed, somatostatin exhibited a slight potentiation of release in this cell population. Typical release profiles were obtained in cells from normal animals. The addition of PS elevated the response in both cell types when stimulated with anti-IgE, but had no effect on somatostatin- induced secretion.

3.2.2.3 **Effects of polyamine inhibitors**

Experiments were performed using benzylammonium chloride (BAC), and one of its more potent analogues, benzyldimethyltetradecyl ammonium chloride (BDTA). Cells were added to buffers containing varying concentrations of either BAC or BDTA (0.1-3μg/ml) *and* the secretagogue (somatostatin 30μg/ml, compound 48/80 0.3μg/ml, substance P 30μg/ml, concanavalin A 10μg/ml, anti-IgE 100-fold dilution). This procedure facilitates simultaneous contact of the cells with the agonist and antagonist.
Both of the antagonists selectively inhibited the release induced by somatostatin, compound 48/80 and substance P (Fig.3.223a,b). The liberation of histamine stimulated by the immunologic stimuli was unaffected by low concentrations of the inhibitors but was actually potentiated at higher concentrations of the antagonists.

3.2.3 Action of various inhibitors

3.2.3.1 **Effects of anti-allergic drugs**

The action of DSCG, nedocromil and theophylline was investigated on a variety of polybasic and immunologic stimuli.
Agonist concentrations were chosen to provide closely correlated unblocked release values (antigen 10WE/ml, anti-IgE 1000-fold dilution, concanavalin A 10μg/ml, somatostatin 7.5μg/ml, substance P 15μg/ml, compound 48/80 0.3μg/ml).

i: DSCG
DSCG (0.1-1000μM) produced a dose-related inhibition of histamine release induced by all the agonists investigated (Fig.3.231i). The lone factor that distinguishes the

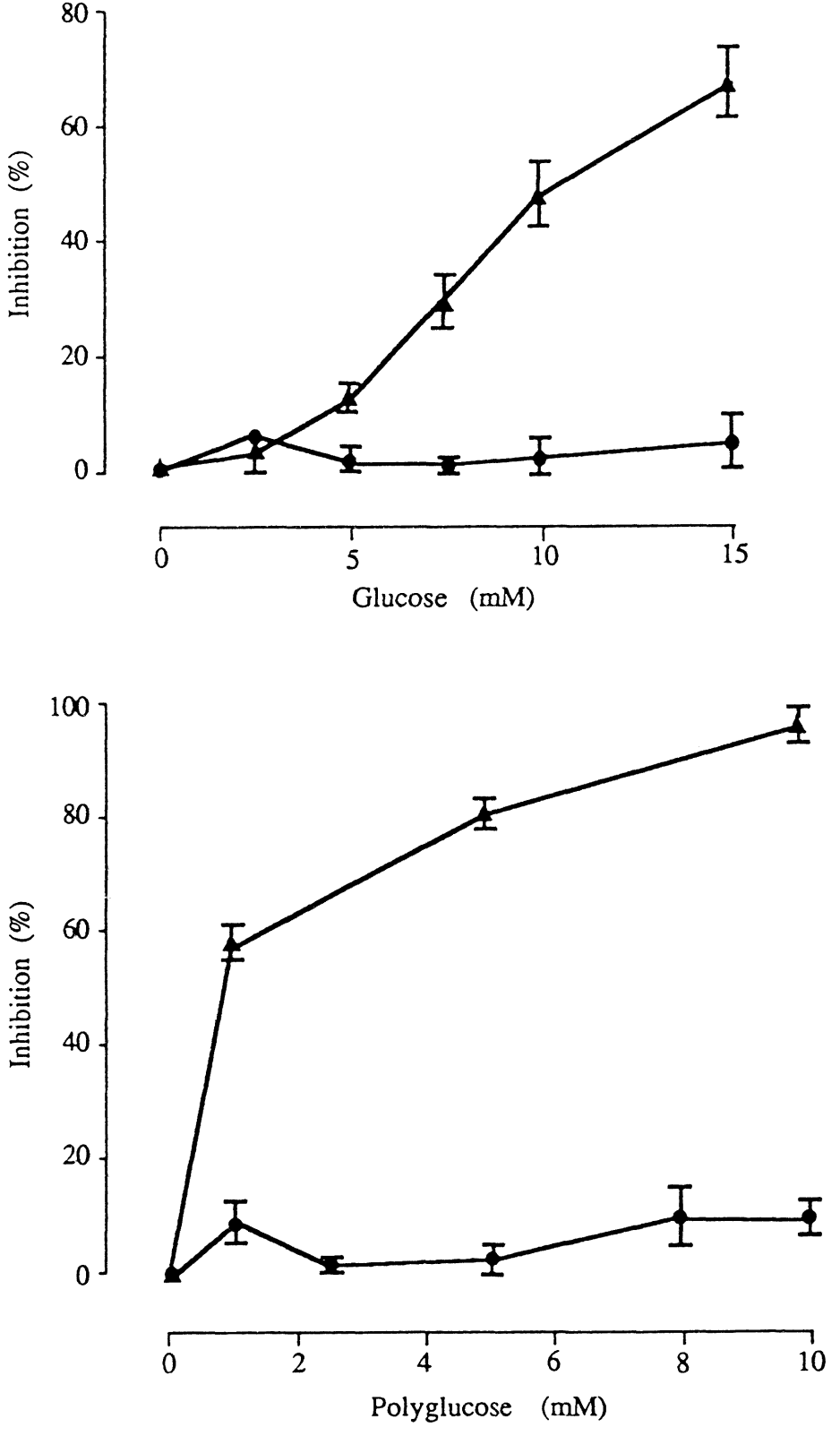


Fig. 3.221 A comparison between the effects of glucose and polyglucose (MW2600) on the inhibition of histamine release from rat peritoneal mast cells induced by somatostatin (30μg/ml, control release 67.5 ± 3.2, circles) and dextran (10μg/ml, lyso PS 1μg/ml, control release 69.7±3.7, triangles)

All values ± SEM (n=4)

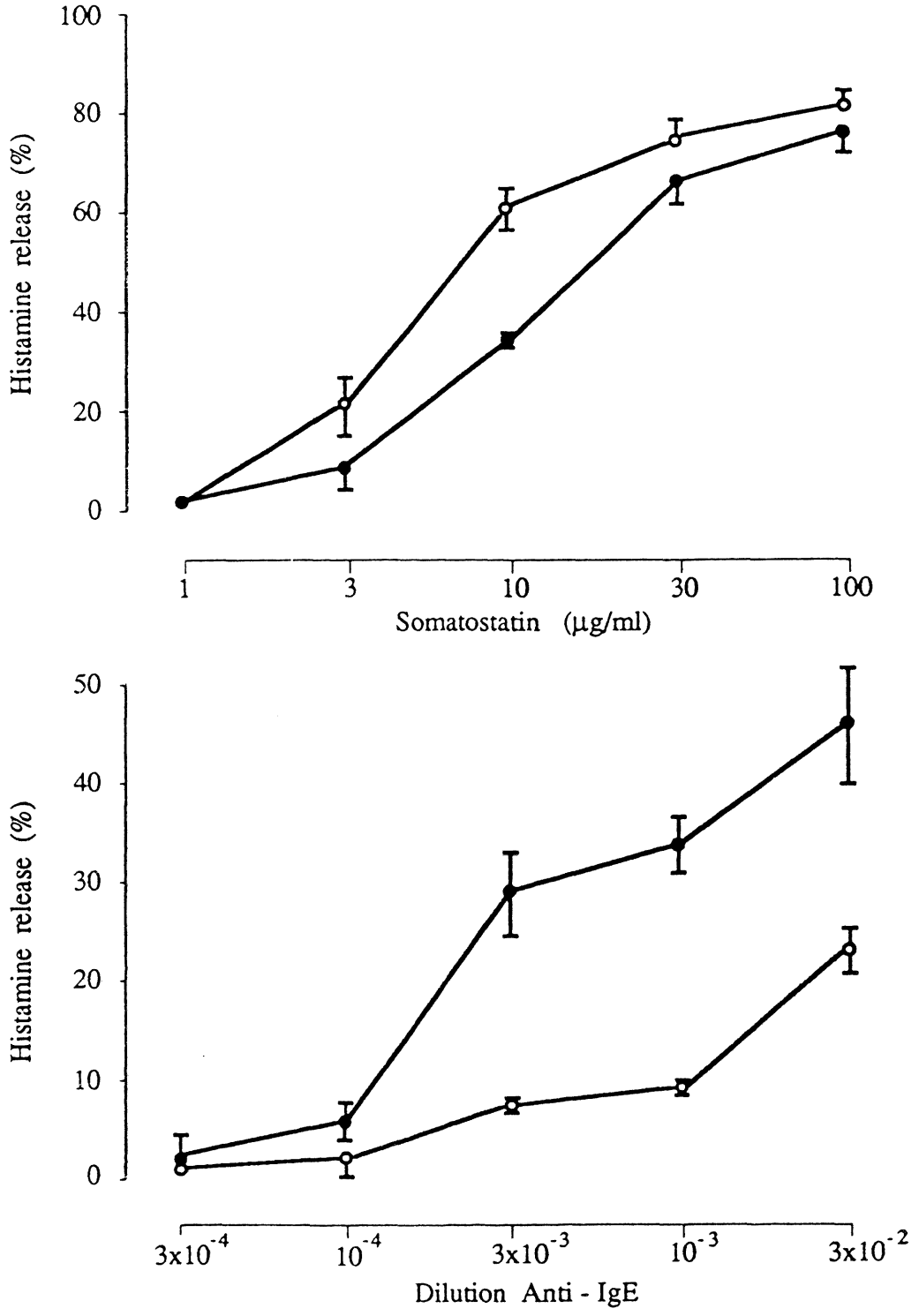


Fig. 3.222 Relevance of cell-fixed antibody on histamine release induced by somatostatin and anti-IgE from peritoneal mast cells of normally housed (closed symbols) and SPF rats (open symbols)

All values ± SEM (n=4-5)

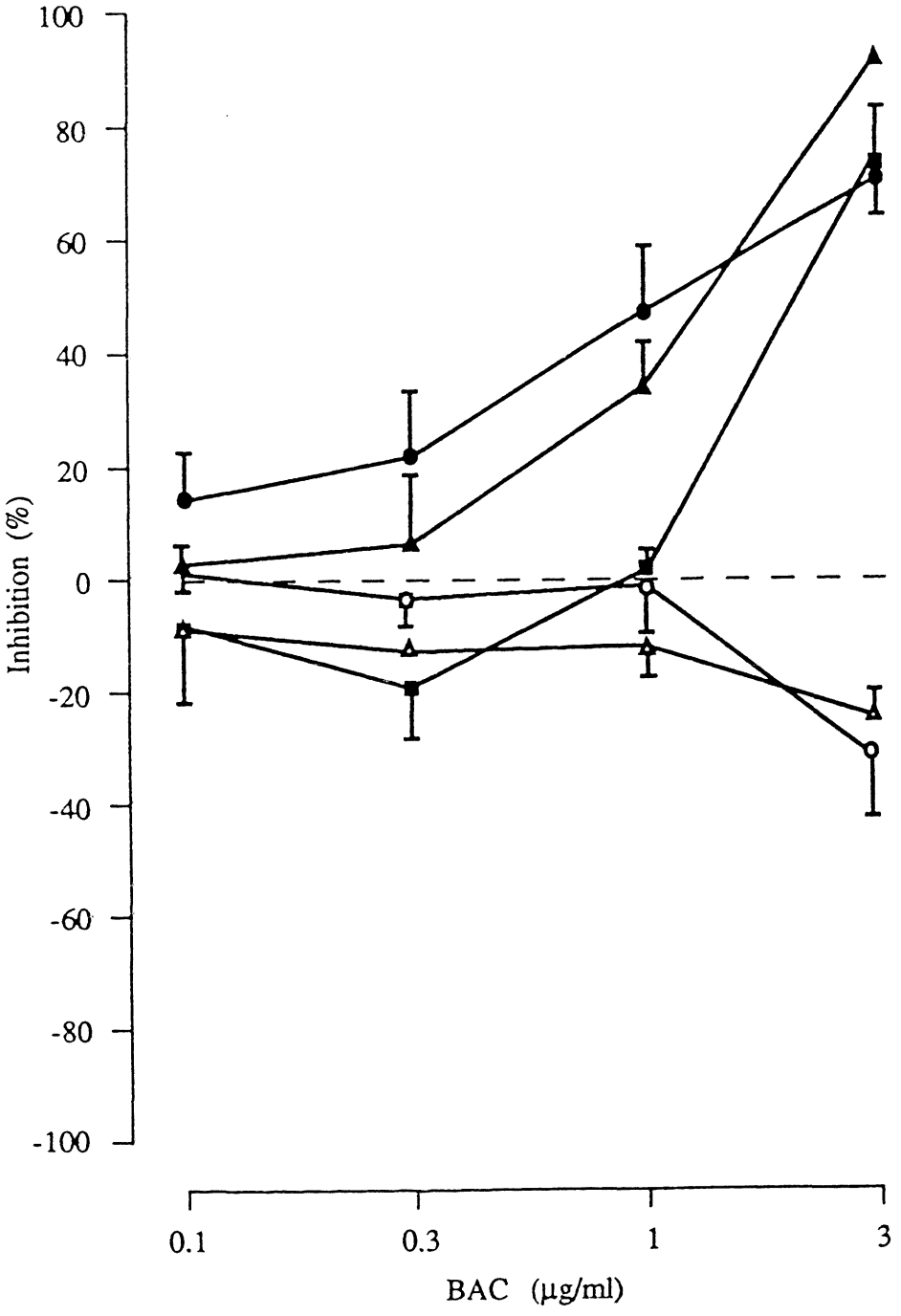


Fig. 3.223a Action of BAC on the inhibition of histamine release from rat peritoneal mast cells induced by compound 48/80 (0.3µg/ml, control release 56.3±2.3, closed squares) somatostatin (30µg/ml, control release 63.0±3.9, closed circles) substance P (30µg/ml, control release 51.3±4.1, closed triangles) concanavalin A (10µg/ml, control release 37.1±2.2, open triangles) anti-IgE (100-fold dilution, control release 31.7±1.1, open circles)

All values ± SEM (n=3-5)

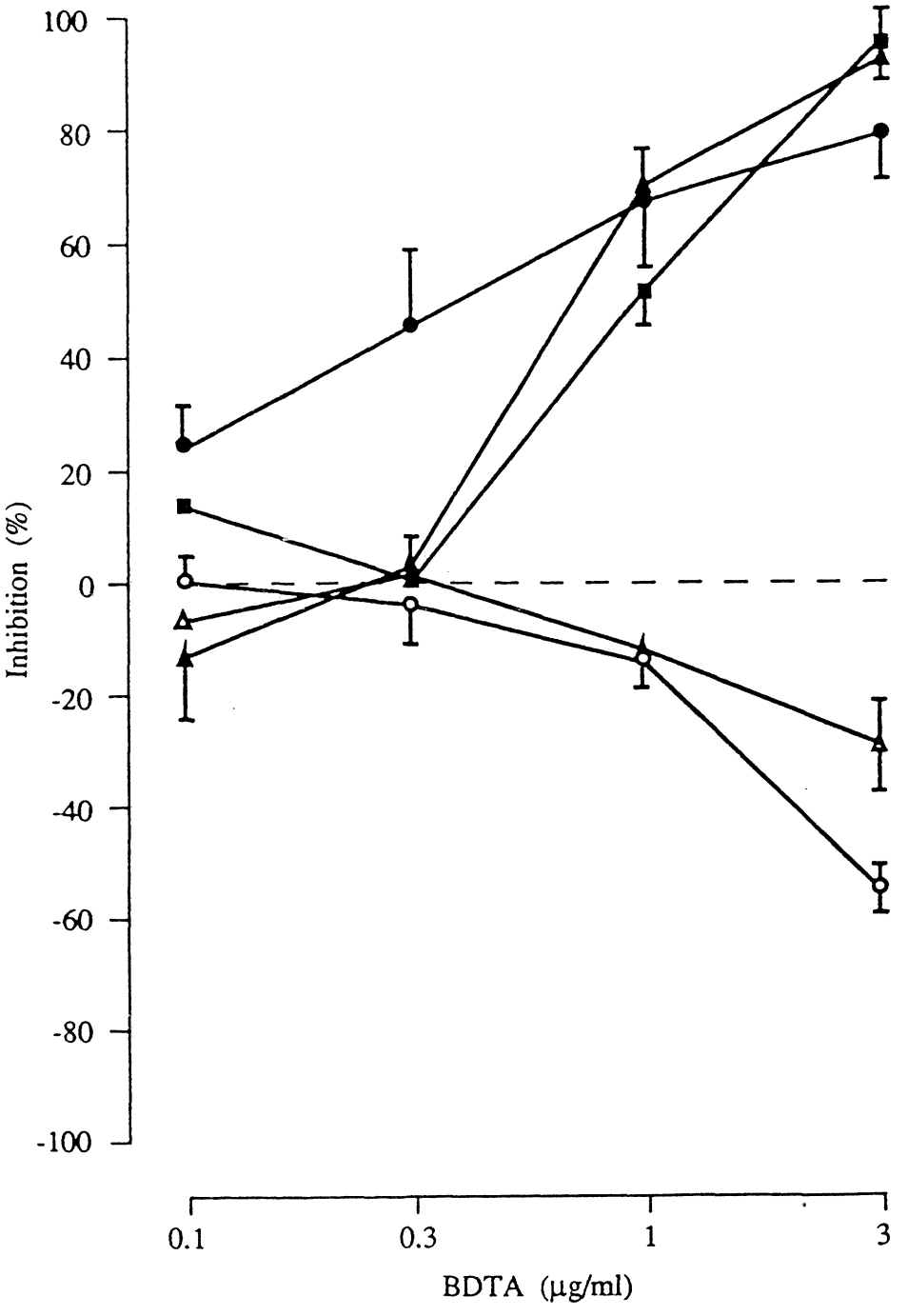


Fig. 3.223b Action of BDTA on the inhibition of histamine release from rat peritoneal mast cells induced by compound 48/80 (0.3µg/ml, control release 54.7±3.1, closed squares) somatostatin (30µg/ml, control release 65.3±3.7, closed circles) substance P (30µg/ml, control release 48.8±4.4, closed triangles) concanavalin A (10µg/ml, control release 37.7±1.8, open triangles) anti-IgE (100-fold dilution, control release 34.2±2.0, open circles)

All values ± SEM (n=4-6)

immunologics from the polybasics is the extent of inhibition, both in terms of the maximum response and the concentration of antagonist required to exhibit inhibition.

ii: Nedocromil

Nedocromil (0.1-100μM), like DSCG, demonstrated dose-dependent inhibition of all the agonists tested (Fig.3.231ii). Nedocromil seems to be a more potent antagonist of histamine release than DSCG. Far greater inhibition is observed at lower concentrations. The polybasic compounds exhibit classical sigmoid curves, with peak inhibition ranging from 40-60%. In contrast, the immunologic stimuli demonstrated prominent inhibition at lesser concentrations of antagonist, and almost complete inhibition of anti-IgE and concanavalin A induced release.

iii: Theophylline

Experiments were conducted on release induced by somatostatin and concanavalin A. Once more, agonist concentrations were adjusted for comparable responses. Both agonists were inhibited by theophylline (Fig.3.231iii). The action of this inhibitor resembled that of the other antagonists, insofar as somatostatin was less affected than concanavalin A. Again, concanavalin A-induced release is susceptible to inhibition at lower antagonist concentration and shows a greater overall inhibition.

3.2.3.2 Effects of ß-agonists

The actions of two clinically used ß-agonists, isoprenaline and salbutamol, were studied on several secretagogues. To extend the inquiry, experiments with salbutamol were designed to elucidate whether preincubation was of pertinence.

i: Isoprenaline

Isoprenaline ((−)isoproterenol (+) bitartrate salt, 10^{-9}-10^{-5}M) was preincubated for fifteen minutes before challenge. The results show that this compound is almost ineffective in this system (Fig.3.232i). In fact, the treated cells were sometimes slightly more responsive than the untreated control. However, this effect can be regarded as insignificant.

ii: Salbutamol

Salbutamol (10^{-9}-10^{-5}M) had little or no effect in this system. Both immunologic and polyaminic stimuli were equally unaffected, (Fig.3.232ii) irrespective of preincubation time (0,15min.). As with isoprenaline, treated cells were often more responsive to the secretagogues than the controls. This feature was neither consistent, profound nor significant.

3.2.3.3 Effects of 5,8,11,14-eicosatetraynoic acid (ETYA)

The action of ETYA upon histamine release induced by somatostatin (7.5μg/ml, 100μg/ml) was researched. Compound 48/80 (0.05μg/ml) and substance P (15μg/ml) were included in the study as examples of other polyamines. Histamine liberation stimulated by

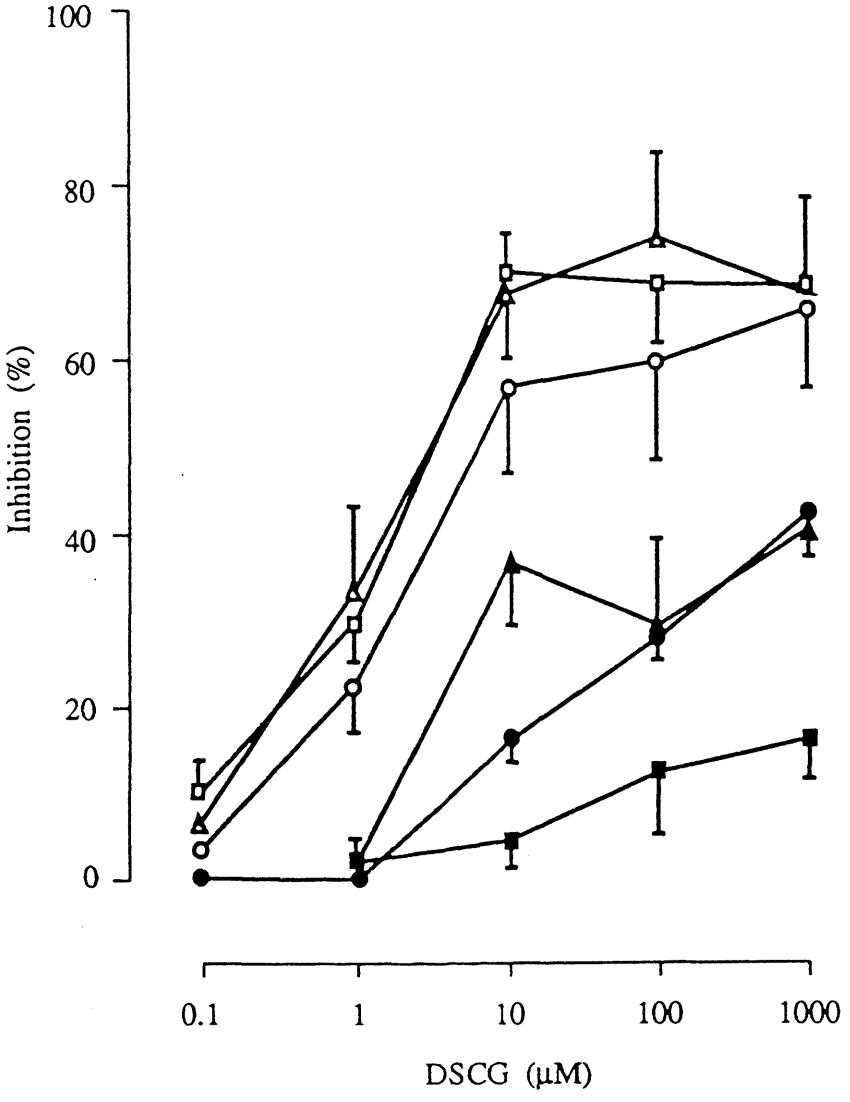


Fig. 3.231i DSCG inhibition of histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 56.8±4.1, closed circles) compound 48/80 (0.3μg/ml, control release 58.0±1.4, closed squares) substance P (15μg/ml, control release 53.3±3.7, closed triangles) antigen (10WE/ml, control release 55.0±6.9, open circles) anti-IgE (1000-fold dilution, control release 48.6±3.5, open squares) concanavalin A (10μg/ml, control release 54.2±6.7, open triangles)

All values ± SEM (n=4)

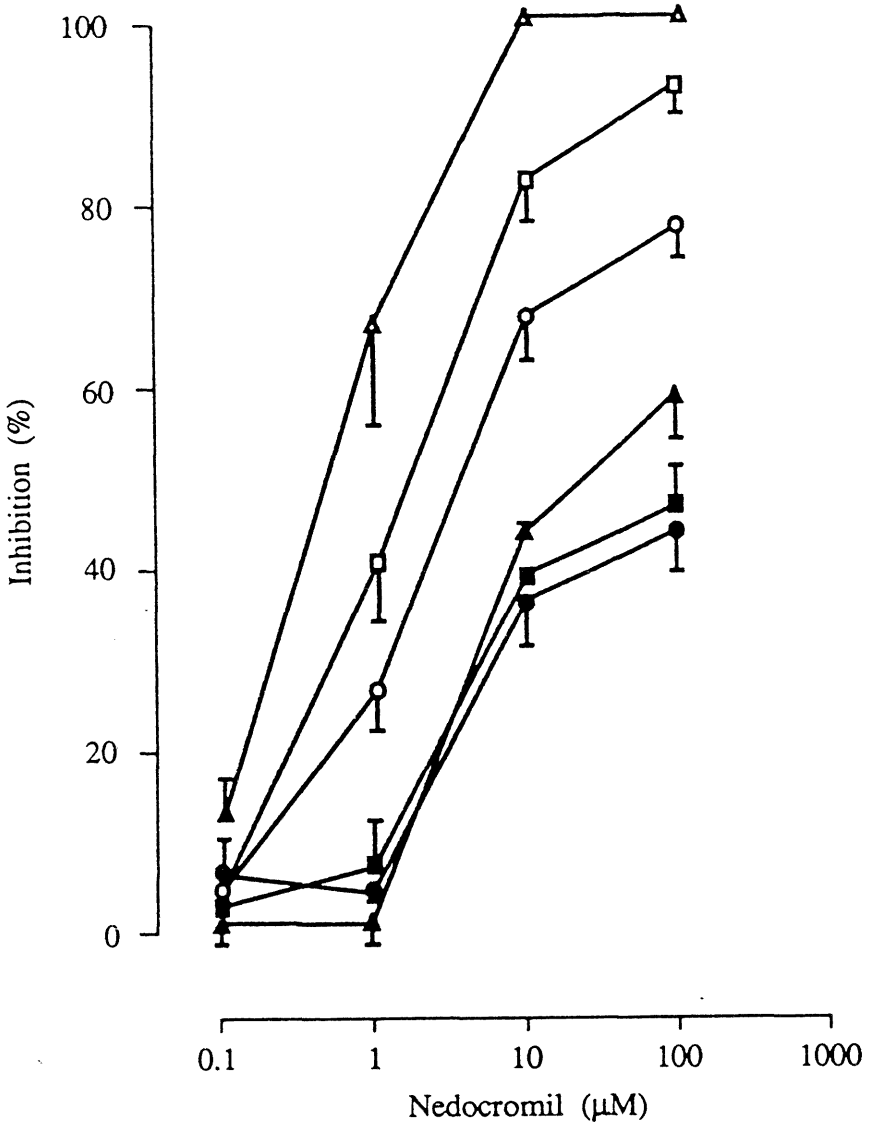


Fig. 3.231ii Nedocromil inhibition of histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 42.4±3.6, closed circles) compound 48/80 (0.3μg/ml, control release 46.2±2.3, closed squares) substance P (15μg/ml, control release 41.5±2.5, closed triangles) antigen (10WE/ml, control release 51.0±4.2, open circles) anti-IgE (1000-fold dilution, control release 39.8±3.0, open squares) concanavalin A (10μg/ml, control release 41.8±2.6, open triangles)

All values ± SEM (n=4)

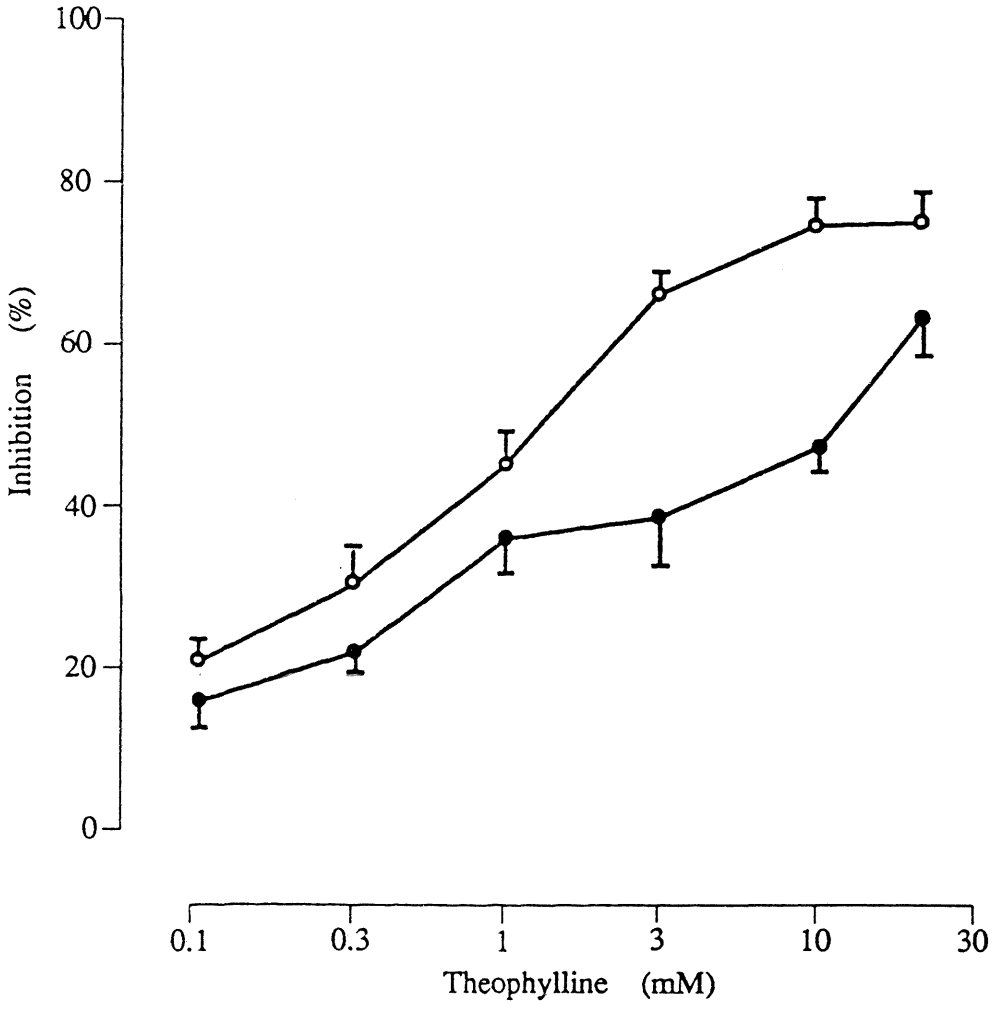


Fig. 3.231iii Theophylline inhibition of histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 42.1±3.3, closed circles) concanavalin A (20μg/ml, control release 39.7±3.1, open circles)

All values ± SEM (n=4)

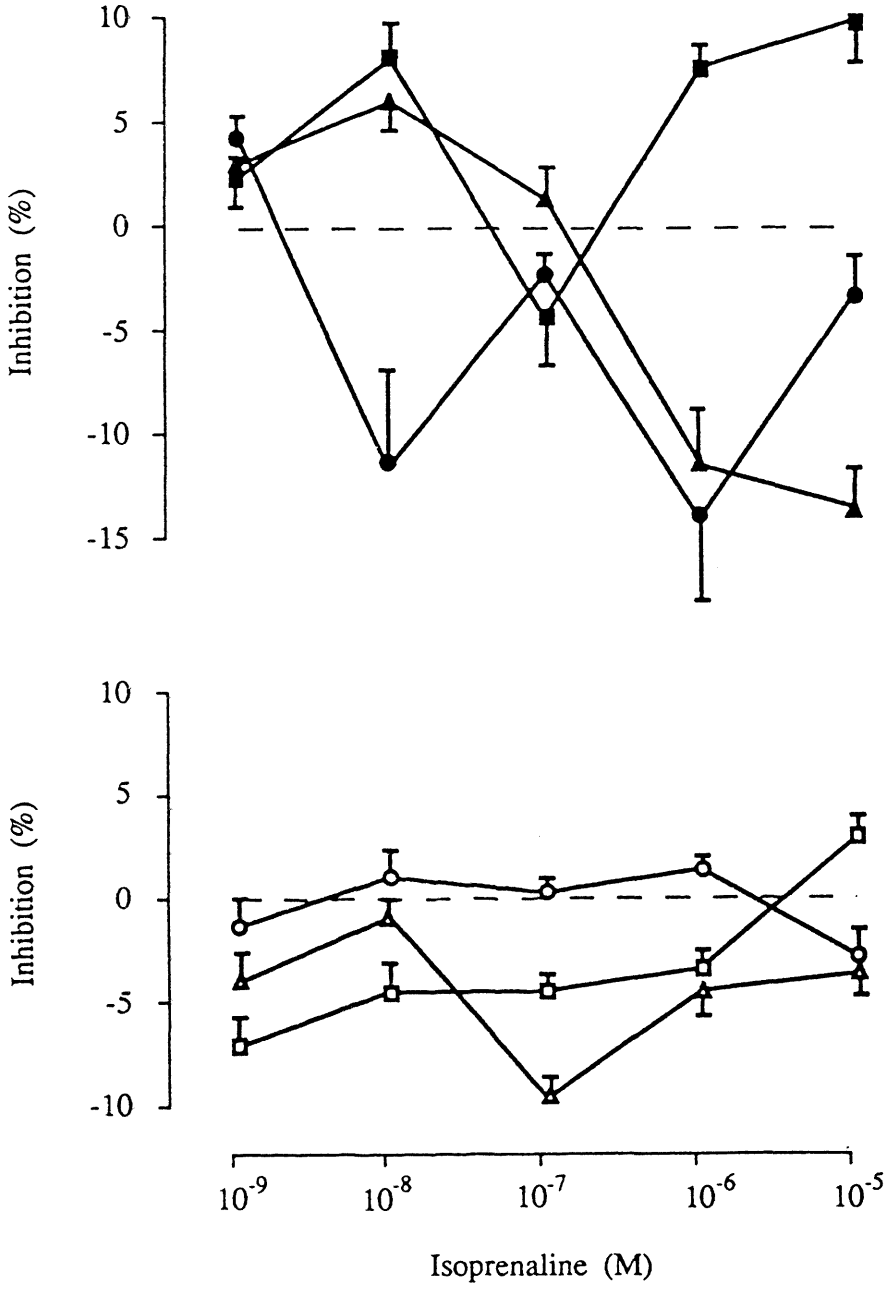


Fig. 3.232i Effect of isoprenaline on histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 37.9±4.1, closed circles) compound 48/80 (0.1μg/ml, control release 45.4±3.3, closed squares) substance P (15μg/ml, control release 34.6±4.2, closed triangles) antigen (15WE/ml, control release 48.4±3.7, open circles) anti-IgE (300-fold dilution, control release 34.1±5.4, open squares) concanavalin A (20μg/ml, control release 38.8±2.8, open triangles)

All values ± SEM (n=4)

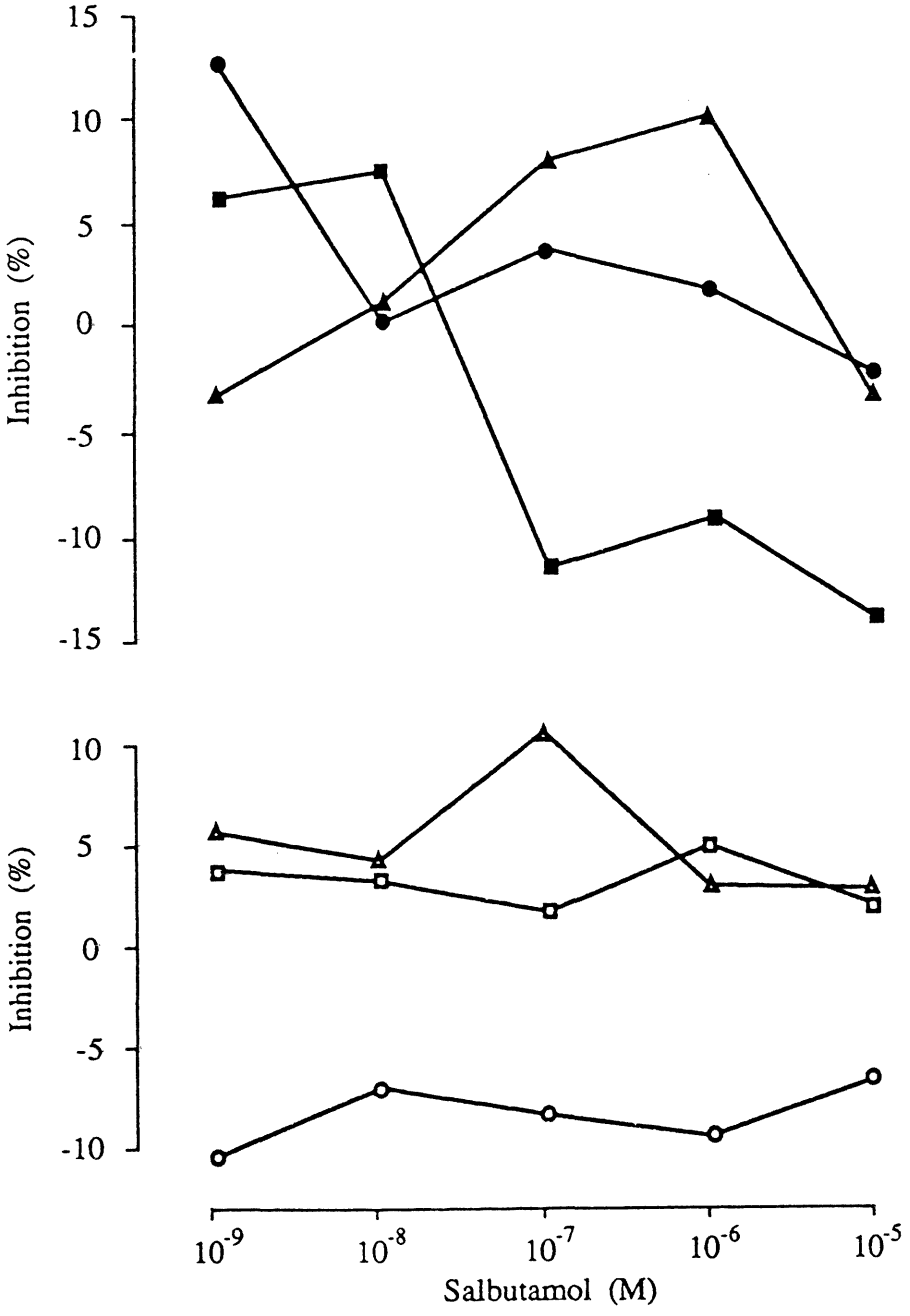


Fig. 3.232iia Effect of preincubation (15min) with salbutamol on histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 47.2±2.4, closed circles) compound 48/80 (0.1μg/ml, control release 39.5±1.8, closed squares) substance P (15μg/ml, control release 42.5±2.6, closed triangles) antigen (15WE/ml, control release 47.9±3.4, open circles) anti-IgE (300-fold dilution, control release 48.9±2.3, open squares) concanavalin A (20μg/ml, control release 47.3±3.1, open triangles)
Error bars have been omitted for clarity but SEM did not exceed ± 10.0

All values ± SEM (n=4)

anti-IgE (300-fold dilution), antigen (10 WE/ml) and concanavalin A (20μg/ml) was also examined.

ETYA (5-100μM) proved an effective inhibitor of immunologically induced secretion (Fig.3.233a). Inhibition of polyamine induced secretion was notable at low concentrations of agonist but substantial secretion induced by somatostatin (ca. 70%) was considerably less antagonised. ETYA would then appear dependent on the strength of the stimulus (Fig.3.233b).

3.2.3.4 Action of substance P fragment, substance P_{4-11}

Experiments were conducted using a fragment of substance P, lacking the N-terminal sequence found in the native peptide. Cells were preincubated for ten minutes with substance P_{4-11} (15μg/ml), and then challenged with somatostatin (7.5μg/ml), substance P (15μg/ml) and compound 48/80 (0.3μg/ml). For the purposes of comparison and control, anti-IgE (300-fold dilution) and substance P_{4-11} (15μg/ml) respectively, were also included in the study.

Histamine release induced by somatostatin and the other polybasic secretagogues was significantly inhibited by substance P_{4-11} (Fig.3.234). The activity of all three agonists was arrested, substance P_{4-11} showing no obvious selectivity in antagonising particular polycation-induced releases. Interestingly, the response evoked by anti-IgE was unabated, and even showed a slight, but statistically insignificant potentiation. The histamine releasing activity of substance P_{4-11} itself was deemed to be negligible at the concentration employed.

3.2.4 Desensitisation - Further studies on the polyamine binding site(s)

3.2.4.1 The effects of cold on the activated state induced by somatostatin and antigen

Histamine liberation induced by somatostatin and antigen was completely inhibited at 4 °C. Mast cells previously sensitised to *N.brasiliensis*, and following stimulation with antigen (20WE/ml) at 4 °C, produced a marked reduction in the response to this agonist upon warming to 37 °C (Fig.3.241). In contrast, the response of cells stimulated with somatostatin (10μg/ml) at 4 °C was much more persistent following the transfer of these cells to physiological temperature. Normal levels of stimulation were observed in cells treated identically but stimulated at 37 °C throughout, and were used to establish control release data.

3.2.4.2 Cross-desensitisation to antigen

Maximum desensitisation of cells to a second stimulus of antigen was attained by incubation of the cells with the allergen in a calcium-free medium for sixty minutes. Calcium ions were then introduced into the system in minimal volume to a final concentration of 1mM and the cells re-stimulated with antigen (20WE/ml), or with one of several other agonists (somatostatin, 10μg/ml; substance P, 30μg/ml; compound 48/80, 0.1μg/ml; anti-IgE, 1000-fold dilution; concanavalin A, 10μg/ml).

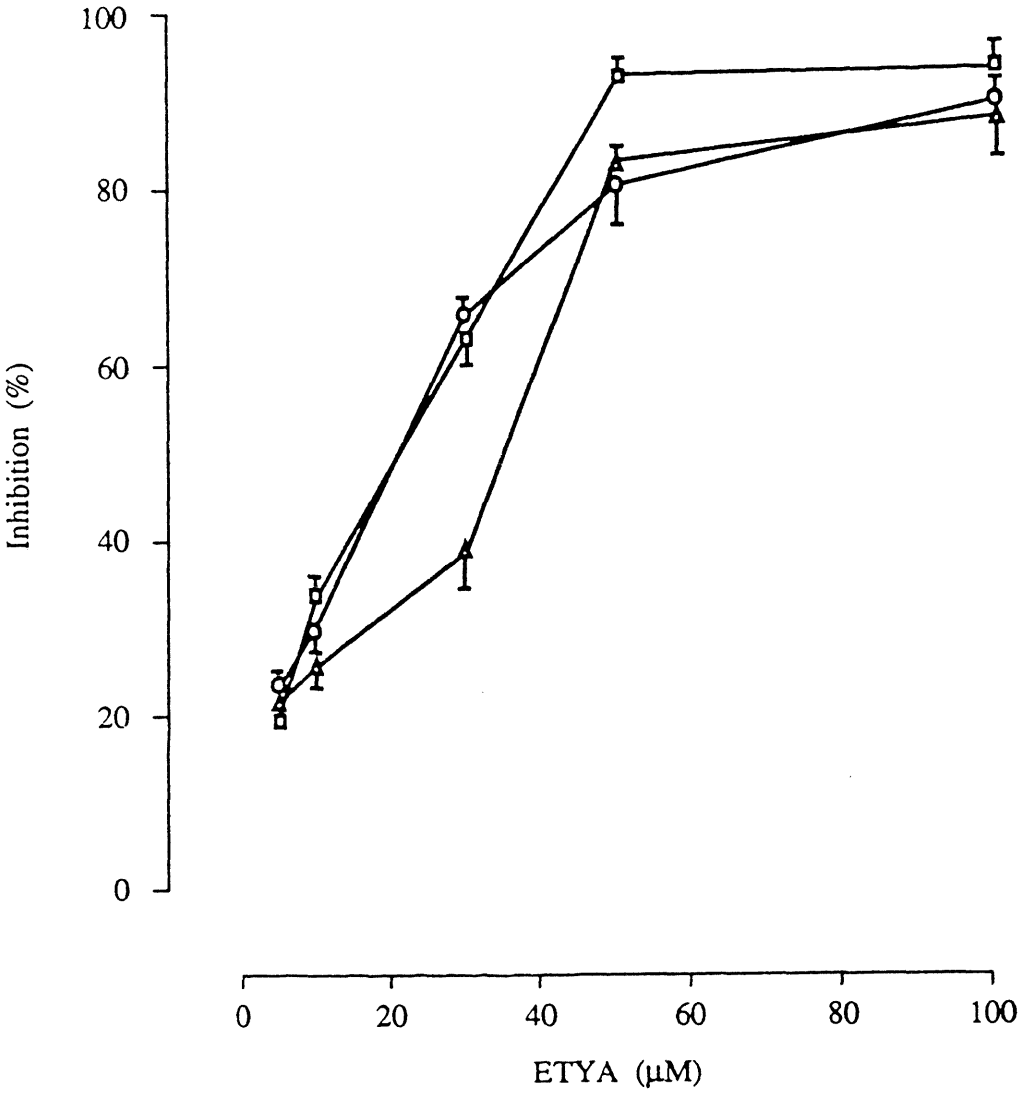


Fig. 3.233a Effect of ETYA on immunologically-induced histamine release from rat peritoneal mast cells.
antigen (10WE/ml, control release 37.3±3.7, open circles)
anti-IgE (300-fold dilution, control release 39.6±4.1, open squares) concanavalin A (20μg/ml, control release 41.3±3.3, open triangles)

All values ± SEM (n=4)

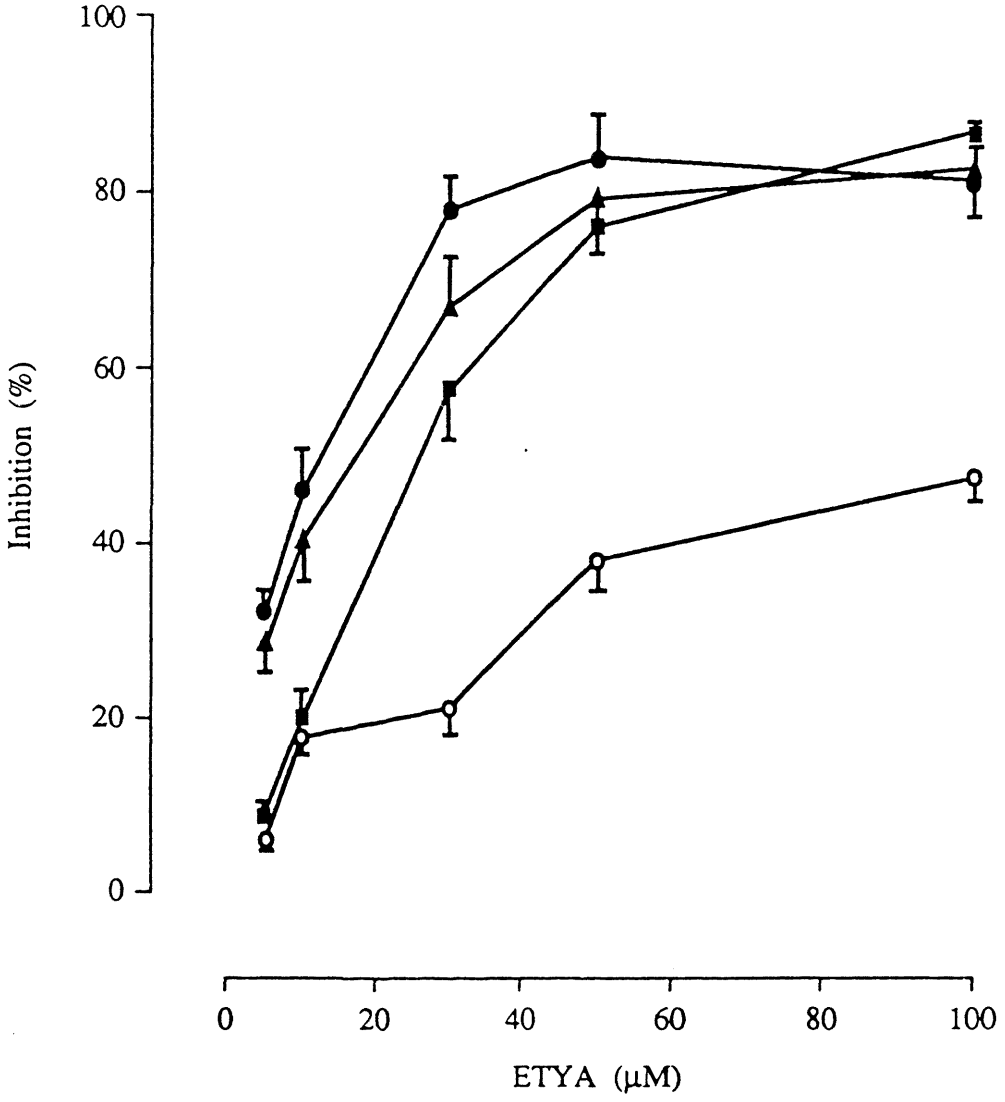


Fig. 3.223b Effect of ETYA on polyamine-induced histamine from rat peritoneal mast cells.
somatostatin (7.5μg/ml, control release 39.3±2.6, closed circles) somatostatin (100μg/ml, control release 76.2±3.6, open circles) substance P (15μg/ml, control release 41.3±2.9, closed triangles) compound 48/80 (0.05μg/ml, control release 36.7±2.4, closed squares)

All values ± SEM (n=4)

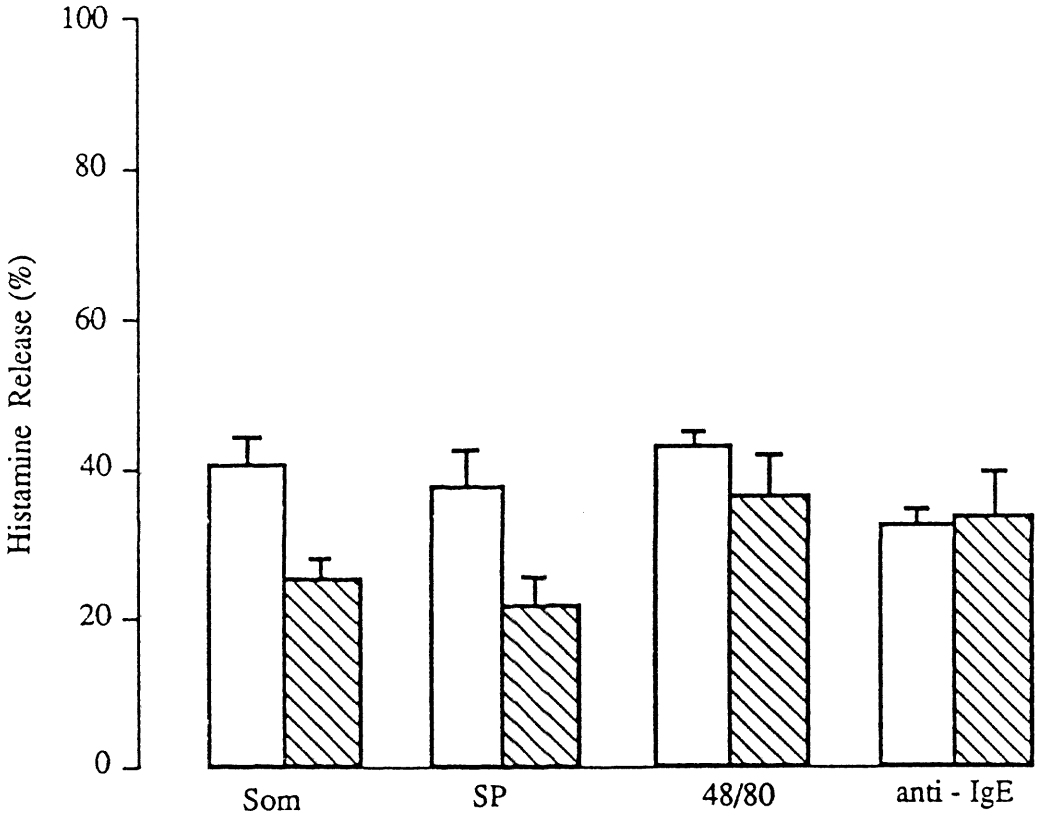


Fig. 3.234 Action of SP_{4-11} on histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml), substance P (15μg/ml), compound 48/80 (0.3μg/ml) and anti-IgE (300-fold dilution). Control releases are represented by open columns, agonists plus SP_{4-11} (15μg/ml) are represented by hatched columns.

All values ± SEM (n=4)

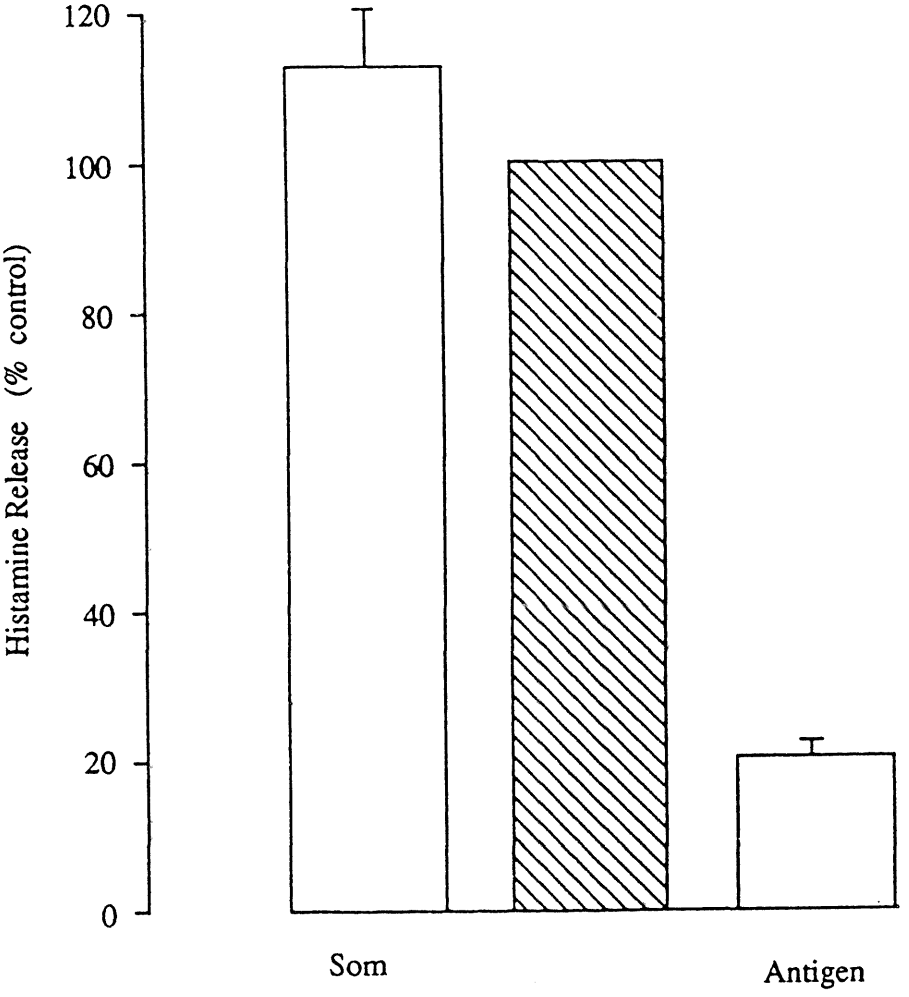


Fig. 3.241 Effects of cold on the activated state measured by histamine release from rat peritoneal mast cells induced by Somatostatin (SOM, 10μg/ml, control release 29.9±3.4) and antigen (10WE/ml, control release 27.2±2.1). Values (open columns) are presented as % of the control release (hatched columns, ± SEM)

(n=4)

Such treatment rendered the cells almost totally desensitised to antigen (Fig.3.242). This desensitisation was extended to the other immunologic stimuli, concanavalin A and anti-IgE. Cross-desensitisation of this type was not apparent with somatostatin, substance P or compound 48/80, their reactivity remaining virtually unimpaired.

3.2.4.3 **Cross-desensitisation to somatostatin**

Primary desensitisation to somatostatin involved overcoming two main hinderences. Firstly, the activated state induced by somatostatin was shown to be long-lived and secondly, the histamine liberation process is independent of external calcium. Previous studies have shown that somatostatin and similar agonists, can be completely and reversibly inhibited by theophylline (10mM). Consequently, cells were incubated with this antagonist and somatostatin (10μg/ml), washed free of the inhibitor and then re-challenged with somatostatin or with another ligand (see 3.2.4.2). Experimental provisions were made to establish that cells treated with theophylline in the absence of the initial stimulus were still fully responsive after washing. Initial treatment with somatostatin rendered the cells partially desensitised to somatostatin, comparably cross-desensitised to substance P and compound 48/80, but fully responsive to antigen, anti-IgE and concanavalin A (Fig.3.243).
Cumulatively, these two experiments show that there is no cross-desensitisation, in either direction, between somatostatin and any of the immunologic stimuli tested. Cross-desensitisation is observed, however, between somatostatin and other polybasic ligands.

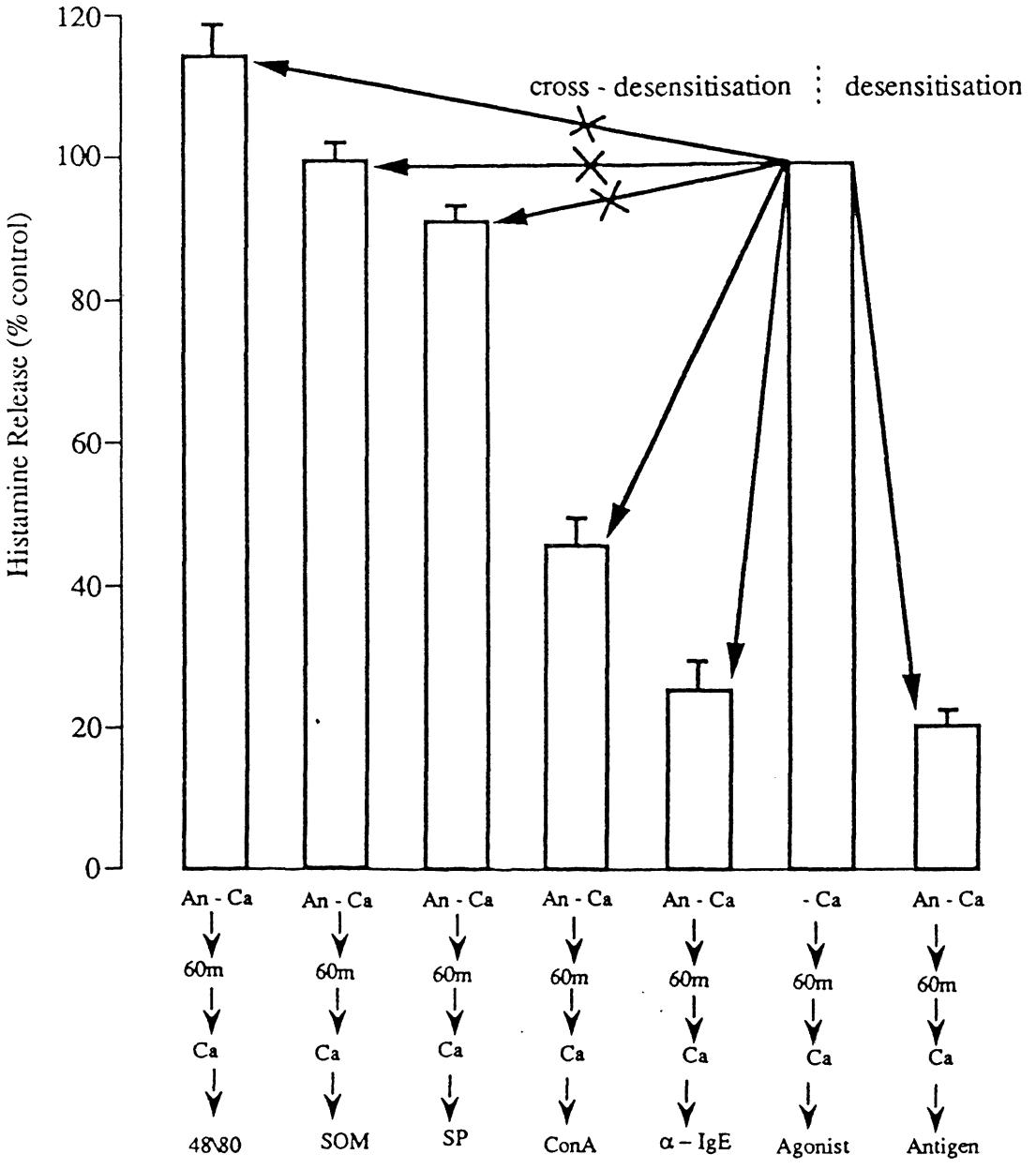


Fig. 3.242 Cross-desensitisation to antigen.
antigen (An, 20WE/ml, control release 41.2±3.4)
anti-IgE (α-IgE, 1000-fold dilution, control release 42.2±4.1) concanavalin A (Con A, 10μg/ml, control release 39.7±4.1) compound 48/80 (0.1μg/ml, control release 32.8±2.4) somatostatin (SOM, 10μg/ml, control release 32.2±1.7) substance P (SP, 30μg/ml, control release 37.3±2.2)
Experiments were performed on rat peritoneal mast cells and values are expressed as % of control release ± SEM

(n=4)

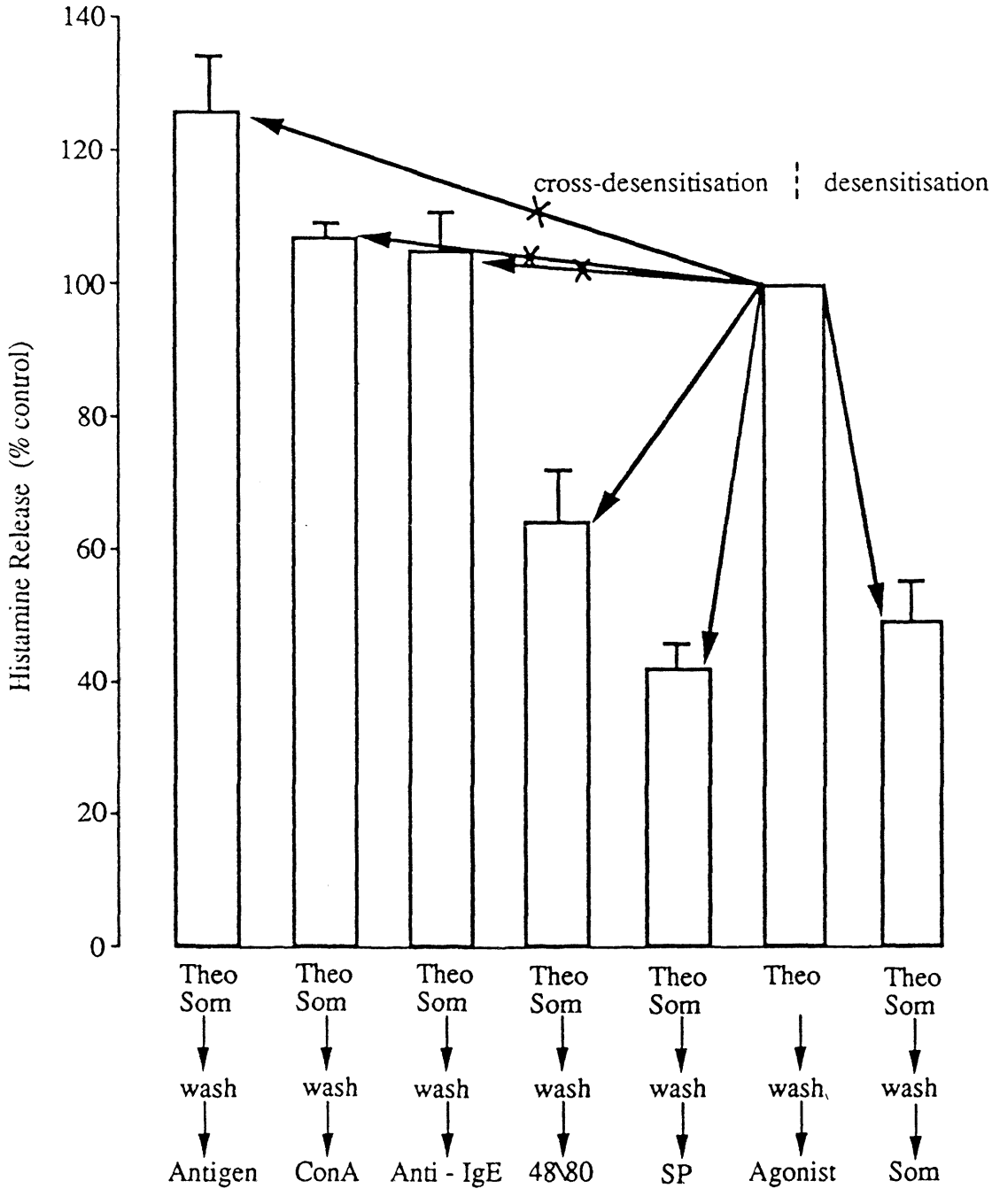


Fig. 3.243 Cross-desensitisation to somatostatin.
Theophylline (Theo, 10mM) compound 48/80 (0.1μg/ml, control release 38.3±5.1) somatostatin (SOM, 10μg/ml, control release 41.4±6.1) substance P (SP, 30μg/ml, control release 40.2±5.7) Experiments were performed on rat peritoneal mast cells and values are expressed as % of control release ± SEM

(n=4)

3.3 *DISCUSSION*

The data presented confirm and extend the findings of Theoharides and Douglas [514] that somatostatin is an effective histamine liberator from rat peritoneal mast cells. Results strongly indicate that somatostatin behaves in a similar fashion to the classic mast cell degranulator, compound 48/80. The other biologically active peptide included in the study, substance P, also exhibits a comparable response pattern. The apparent similarity of somatostatin to other polybasic ligands was made manifest at the earliest stages of the investigation. By virtue of this, the dissimilarity to the immunologically directed ligands was equally evident.

The secretion of histamine from rat peritoneal mast cells was non-cytotoxic, as it was arrested by extremes of temperature and the action of metabolic inhibitors. The reactivity of somatostatin (and other basic secreagogues) was only slightly impaired by physiologically acceptable changes in pH. A characteristic of basic inducers such as compound 48/80 [121] and polylysine [121] is an extremely rapid rate of histamine release, which is essentially complete within 10 seconds. Immunological stimuli tend to initiate comparatively slower rates of secretion [121]. Thus, the rapid rate of somatostatin-induced secretion is reminiscent of compound 48/80-induced histamine release.

Studies conducted in the presence and absence of external calcium also yielded interesting results. Most agonists, with the exception of dextran, are capable of inducing a residual, sub-maximal release of histamine in the absence of added calcium, a phenomenon usually attributed to the mobilization of intracellularly sequestered stores of the cation [2,122]. The magnitude of this effect varies from secretagogue to secretagogue, and polybasic ligands such as compound 48/80 [305], peptide 401 [305] and polylysine [121] exhibit near maximal release under these conditions. Somatostatin demonstrates close parallels to these agents in response to external calcium availability. Presumably, somatostatin also has the ability to effect easy displacement of calcium from the stores accessible to these agents.

Brief pretreatment with EDTA is thought to enhance mobilization of intracellular calcium stores, by chelation of surface-bound calcium, and generally to increase histamine secretion under conditions of calcium deprivation. This action enhanced the release induced by somatostatin, compound 48/80 and polylysine above the value obtained with these ligands in a calcium-free medium. Maximal release, after pretreatment with EDTA, is less than that obtained in the presence of calcium. It is then possible to suppose that some influx of external calcium into the cytosol is essential for the maximum release of histamine.

Supramaximal concentrations of extracellular calcium proved inhibitory to histamine secretion induced by somatostatin and compound 48/80. Variation in the concentration of extracellular calcium is thought to invoke the existence of a calcium regulatory binding site on the mast cell membrane (see 1.2.1). Occupancy of this site is considered to stabilize the cell membrane. This stability is lost, and the cell responsivity increases, when the site remains unoccupied. On this assumption, depressed release levels would be expected at high cation concentrations. However, in the case of polyamine stimuli, the inhibitory nature

of supraoptimal concentrations of calcium could arise from direct competition between the two positively charged species for binding sites on the mast cell membrane. This poses the question of whether the polyamines utilise the same, or similar binding sites as calcium. The added component of competitive inhibition, therefore, would go far to explaining the very definite inhibitory effect of excess calcium observed with this type of agonist.

Strontium ions were able to substitute for calcium in the histamine release process induced by somatostatin and compound 48/80. Unlike calcium, however, strontium is not inhibitory at high concentrations. On the premise that calcium binds to a site on the mast cell membrane, then strontium should bind at the same site. Results reported by Foreman and Mongar [525] on antigen-induced histamine release, indicate that the affinities and efficacies of these ions for ion binding sites are not the same. Thus calcium, whilst having a greater affinity for the receptor than strontium, possesses a smaller efficacy. So, at optimum calcium concentrations for histamine release (that is, sufficient receptor occupancy for membrane stability, but maintaining ample cellular responsivity), the response should be less than that with equivalent strontium concentrations. This effect is not clearly observed, but a lower affinity for the receptor would reconcile the lack of inhibition noted at higher concentrations of strontium.

PS and its lyso-derivative selectively potentiate histamine release induced by IgE-directed ligands and related ligands such as dextran. Postulates advanced to rationalize this effect exploit the ability of the lipids to reduce membrane viscosity. Increased membrane fluidity may enhance the formation of calcium "channels", or else maintain the "channel" operative for prolonged periods of time. In view of the lengthy response invoked by immunologic stimuli, sustained calcium influx should elevate release. This type of response is observed with IgE-directed ligands, but not with somatostatin and other polycations. Recalling the assertion that polycation-induced secretion is independent of external calcium, taken with a rapid release process, the actual benefits of sustained calcium influx and (or) enhanced "channel" formation are uncertain. Thus, the insensitivity of polyamine-induced release to phospholipids may reflect the difference in calcium dependency, compared to immunologically induced secretion.

Throughout the investigation into the basic characteristics of exocytosis, somatostatin and substance P consistently behaved like compound 48/80 and the other polybasic agents. The mechanistic similarities were confirmed by direct comparison with dextran and anti-IgE. Dextran is assumed to cross-link specific glucoreceptors on the mast cell membrane. As such, its action is reportedly inhibited by high concentrations of glucose and low molecular weight glucose polymers [526]. These findings were upheld in the present study, but neither agent had any effect on the liberation of histamine stimulated by somatostatin. Moreover, release evoked by somatostatin was faster than that of dextran [118], and strontium was able to substitute for calcium, a feature not noted with dextran [526].

Any possibility that somatostatin may act via cell-fixed antibodies, in a manner analogous to the anaphylactic reaction, was then investigated. Normal rat peritoneal mast cells possess significant quantities of surface bound IgE and IgG immunoglobulins. When these cells are challenged with antibodies directed against these proteins, exocytosis occurs. In contrast, cells obtained from SPF animals are relatively free of cell bound antibody. Cells obtained from normally housed animals responded well to both somatostatin and anti-IgE. However, cells obtained from SPF animals were considerably less responsive to anti-IgE and slightly hyperresponsive to somatostatin.

A token IgE presence may reside on the mast cell membrane. This would account for the nominal response observed with apparently antibody-free cells on stimulation with anti-IgE. The full response on stimulation with somatostatin is indicative of a release mechanism which is IgE-independent. The apparent hyperresponsivity of cells from SPF animals to challenge with somatostatin is in all probability a steric effect. The absence of large, bulky immunoglobulin molecules may facilitate greater binding of somatostatin to the mast cell membrane.

To conclude this area of investigation, experiments were conducted with a supposedly polyamine-specific antagonist. Read and coworkers [509,527] have described a family of positively charged quaternary ammonium compounds, named benzalkonium chlorides (BAC). These compounds exhibit selective, inhibitory activity against polyamine-induced histamine secretion. The antagonism was not extended to other types of stimuli, typically immunologic, ionophores and cytotoxic releasers.

The tridecyl derivative, BDTA, was shown to be the most potent of the BAC series [509]. Its interaction with compound 48/80 and other polybasic secretagogues was determined to be competitive and surmountable. On this evidence, BAC (particularly BDTA), was claimed specifically to inhibit polybasic compounds and it was suggested that their use could provide evidence for the existence of a polyamine receptor site.

Data presented in this thesis confirm these findings and antagonism of somatostatin activity was noted. One important experimental finding proved to be of interest. In order for BDTA (BAC) to act selectively, the antagonist and agonist must be added simultaneously (data cited in chapter 6). Only under these conditions, could BDTA be said to act as a selective, competitive inhibitor of polyamine induced histamine release. If the agonist was added several minutes after preincubation with the antagonist, the inhibition of release was found to be non-specific and surmountable. In such circumstances, BDTA probably acts as a membrane surfactant, affecting membrane fluidity and stability by virtue of its detergent properties. Many authors do not adhere to this protocol as laid down by Read and consequently fail to observe selectivity.

Such conflicts have led to much argument about the use of BDTA. Sub-sequently, conclusions drawn from the original experiments have fallen into disrepute and are under serious speculation. Data presented in this thesis would seem to favour the initial reports implicating BDTA as a reliable probe, but only under strict experimental conditions.

In direct contrast to the polyaminic stimuli, immunologically directed ligands such as concanavalin A, anti-IgE and antigen are far from antagonised by BDTA. A distinct, dose-dependent augmentation of the response is observed with all three stimuli. It would appear that, at concentrations above 0.3μg/ml, BDTA begins to exhibit its toxic effects by affecting membrane stability. On the assumption that polyamines and BDTA compete for the same binding sites, BDTA alone would have unrestricted access to those sites. Thus, the absence of inhibition (and concomitant potentiation) on stimulation with immunologic agents could be ascribed to the lack of interaction between BDTA and the IgE receptor with accompanying membrane destabilization. If this assumption is correct, it would infer two, distinct and discrete mechanisms of release for polycations and immunologic stimuli.
To summarize, the above series of experiments have clearly defined a mechanistic pathway for polyamine induced histamine release which is distinct from that of dextran and IgE-directed ligands. However, the actual mechanism itself remains obscure.
The mode of action of antiallergic agents such as sodium cromoglycate, nedocromil sodium and theophylline in the therapeutic management of conditions such as human bronchial asthma is undoubtedly complex but may be attributed, at least in part, to the inhibition of the release of chemical mediators from mast cells and other inflammatory cells.
In the present study, DSCG, nedocromil and theophylline inhibited release induced by polybasic and immunologic stimuli. Although actual histamine release levels were comparable, polybasic agents were less sensitive to the antagonists than the IgE-directed ligands. Greater concentrations of anti-allergic drugs were required to elicit significant inhibition of polyamine induced secretion. Also, the maximum effect is less than that with the immunologic agents. These observations seem to point to a difference in the mechanisms of polyamine and immunologic histamine liberation. However, a further interpretation exists; the rapidity and potency of the polycation stimulus may affect the ability of anti-allergics to operate as effectively as observed with IgE-directed ligands.

In contrast to the above data, the ß-agonists salbutamol and isoprenaline were ineffective in preventing histamine release induced by both immunologic and polycationic ligands. This may also indicate, as has been previously suggested [529], that the rat peritoneal mast cell is devoid of functional ß-adrenoceptors.
In the rat peritoneal mast cell, the majority of liberated arachidonic acid would appear to be metabolized via the cyclo-oxygenase pathway. Such metabolism yields significant quantities of PGD_2 on stimulation by either immunologic or polyamine agonists [220,221]. Only inhibitors of the lipoxygenase pathway have been found to inhibit histamine secretion [219-221,223,530]. ETYA was previously believed to antagonise both cyclo-oxygenase and lipoxygenase pathways. More recently, Nemeth and Douglas have suggested that ETYA and other inhibitors such as HTYA and ITYA, inhibit at a point early on in the sequence of biochemical events and one peculiar to IgE-directed secretagogues [226,227]. The authors

noted that abnormally high concentrations of the drug were required to inhibit histamine release induced by pharmacologic agents.
In agreement with this report, results from the present study show ETYA to be considerably less active in suppressing release induced by high concentrations of somatostatin. On reduction of the unblocked release levels to proportions seen with immunologic stimuli (ca. 40%), the inhibitory action of ETYA was greatly enhanced. Similarly, ETYA effectively abolished secretion evoked by suboptimal concentrations of somatostatin. Therefore, the validity of the claim that ETYA specifically inhibits IgE-directed ligands is called into question. Data obtained with ETYA, for use as evidence for the existence of an IgE-directed ligand, should be refuted accordingly. Results presented in this study are in agreement with co-workers (H.L. Thompson PhD. thesis 1986 University of London, K.B.P. Leung PhD. thesis 1986 University of London).

The C-terminal, octapeptide fragment of substance P, SP_{4-11}, does not induce histamine release from rat peritoneal mast cells but rather acts as an antagonist of secretion induced by the peptide and other polycations [436]. In keeping with this observation, the fragment was found to block the release of histamine induced by compound 48/80 and somatostatin but not that produced by anti-IgE. These data further illustrate the differences between the two types of stimuli.
The final section of this chapter is centred on the outcome of various desensitisation techniques. Several different mechanisms of desensitisation have been reported to occur in Fc_{ϵ}-receptor, stimulus-secretion coupling. When mast cells are confronted with an agonist that cross-links IgE, but under conditions prohibitive to secretion (extracellular calcium deprivation or low temperature), the cells become refractory. Additionally, the cells do not respond to a second application of the cross-linking stimulus under conditions where normal responses are observed in untreated cells [151,89,531]. In contrast, cold desensitisation was not observed with somatostatin. Data already presented in this study have indicated that the stimulus associated with somatostatin and other polyamines is very persistent. This means that cells treated with these agents at 4 °C are able to elicit a full response upon warming to 37 °C. Hence, the apparent lack of decay in response to somatostatin stimulation infers that the agonist activates the mast cell by an alternative mechanism to antigen, one which is not subject to the limiting effects shown by the IgE receptor.
When mast cells are stimulated with an antigen in calcium-free media, the subsequent addition of calcium at various time intervals leads to a time-dependent decrease in the amount of histamine released [531,532]. The decay of the response to calcium can be permanent. A second antigen challenge, after the addition of calcium, may not restore the reactivity of the cells or result in histamine release. The cells are then said to be desensitised to the primary stimulus. It is of obvious interest to determine whether cells which have been desensitised to a given agent are subsequently capable of responding to

the action of a second agonist. The observation of cross-desensitisation under these conditions may then be taken to indicate that the two stimuli activate common pathways [306].

Cells desensitised to antigen were cross-desensitised to both anti-IgE and concanavalin A, as anticipated. However, there was no cross-desensitisation, in either direction, between antigen and somatostatin. Furthermore, there was no cross-desensitisation between antigen and compound 48/80 or substance P. These data provide a convincing argument for the mechanistic differences between polyamine- and IgE-mediated release. Perhaps of equal importance, is the verification of a single mechanism for the biogenic peptides and compound 48/80. The results discussed above were substantiated further by data from experiments where mast cells were desensitised to somatostatin.

The calcium-independency of polycation-induced degranulation precludes the use of calcium-free media to expedite desensitisation. To overcome this problem, polycation-induced secretion was antagonised with theophylline. Control experiments showed the cells to be appreciably desensitised to somatostatin and fully responsive after theophylline treatment alone. Cross-desensitisation between somatostatin and the other polybasic ligands was established but no desensitisation occurred between somatostatin and the immunologic stimuli.

Analysis of the latter two desensitisation experiments provides overwhelming evidence for the existence of two, distinct mechanisms of stimulation. Moreover, polyamine agonists collectively act at a receptor or binding site classically described as promiscuous. The structural requirements for activation of this binding site are extremely liberal, in biological terms [421,436,533].

To conclude, this chapter has focused on the investigation into the basic characteristics of histamine release induced by somatostatin. Throughout the study, comparisons to the other polycations were established. Concurrently, the disparity to the immunologic ligands was interpellated. It is now abundantly clear that the basic agonists utilise a binding site and mechanism quite distinct from that of the immunologic agonists.

CHAPTER 4

INVESTIGATIONS INTO THE ACTION OF SOMATOSTATIN AS A MODULATOR OF HISTAMINE RELEASE.

4.1 *INTRODUCTION*

The effect of sensory neuropeptides on different histaminocytes appears to depend on the cell type and the nature of the peptide. Goetzl and Payan [523] observed that mast cells derived from bone marrow progenitors of mice will release histamine and LTC_4 on stimulation with nM concentrations of substance P and other peptides. In contrast, the authors reported that 1-10nM somatostatin substantially inhibits the release of mediators from such mouse mast cells. Interestingly, the inhibition was only observed in cells that had been sensitised with mouse monoclonal IgE antibody to dinitrophenyl (DNP) compounds and challenged with DNP-bovine gamma-globulin. Somatostatin had no effect on sensitised cells that were stimulated with C5a or the ionophore A23187 [525]. In contrast,the same concentrations of somatostatin failed to inhibit the release of mediators from rat peritoneal mast cells challenged by IgE-directed ligands.

Only very high concentrations of somatostatin have been recorded to elicit mediator release from basophil leucocytes [521]. In general, these cells are totally unresponsive to neuropeptide challenge [523,this thesis]. Once more, Goetzl and Payan performed experiments to examine somatostatin antagonism in this system. They reported that physiologically meaningful concentrations of somatostatin did, in fact, inhibit immunologically induced secretion in human and rat-leukaemic basophils. This inhibition appeared to be selective, as release stimulated by C5a, platelet factor 4 (PF4) and ionophore A23187 was not disrupted [523].

Mediator release from unpurified human basophils challenged with anti-IgE, was significantly suppressed by somatostatin. Concentrations of $3x10^{-13}M$ to $10^{-9}M$ somatostatin were found to inhibit histamine release and $10^{-13}M$ to $10^{-11}M$ for the inhibition of LTD_4 release. These concentrations had no effect on the release evoked by PF4 or A23187 [523].

Therefore, somatostatin could be acting as a modulator of mediator release, being inhibitory at low concentrations and stimulatory at high concentrations. The authors extended their investigation to somatostatin analogues. Dihydrosomatostatin was 100-fold less potent than the native peptide, while D-Trp^8-somatostatin failed to inhibit the release of mediators from basophils, irrespective of the initial stimulus [523]. The inhibitory nature of somatostatin has not been assessed *in vivo*.

Data presented in this chapter are the results of attempts to repeat the above observations. The possible modulatory role for somatostatin was investigated further. The possibility of inhibition by somatostatin of histamine release induced by sister, polyaminic stimuli was examined. In conjunction, immunologically induced secretion was also researched. Both high and low concentrations of somatostatin were applied in experiments conducted on

human basophil leucocytes and murine peritoneal mast cells. Finally, the inquiry was extended to the possible synergistic effects of somatostatin in relation to other stimuli. The latter studies were performed in systems where somatostatin is relatively inactive as a secretagogue. This allowed high, non-releasing concentrations of somatostatin to be investigated.

4.2 *RESULTS*

4.2.1 The action of somatostatin on histamine release from the human basophil stimulated by various agonists.

4.2.1.1 **Histamine release induced by immunologic activation**

In comparing the effects of different agents and modulators on histaminocytes, it is essential that comparable levels of histamine secretion (ideally 25-35%) are used. Pilot experiments confirmed previous reports [534] that the dose response curve of human basophils to anti-IgE was bell-shaped. Such ideal responses may thus be produced by both high and low concentrations of anti-IgE. Initially, the effect of somatostatin was examined under both conditions.

Studies were performed on unpurified human basophil leucocytes. The cells were preincubated with varying concentrations of somatostatin (10^{-15}M-10^{-8}M) for five minutes and then challenged with anti-IgE for twenty minutes. Preliminary experiments showed that somatostatin alone did not induce histamine release over this concentration range. The extended incubation time is a reflection of the differing response of basophils compared to that of murine peritoneal mast cells.

Somatostatin (10^{-15}-10^{-8}M) was found to have no consistent effect on histamine release induced by either concentration of anti-IgE (Fig. 4.211i,ii). There was perhaps some tendency towards potentiation of the release at high dilutions (3000-fold) of the antiserum (Fig. 4.211i) and towards inhibition at low dilutions (30-fold) but the results were extremely variable and erratic and reached statistical significance in only one case.

4.2.1.2 **Pertinence of preincubation upon inhibition**

To investigate the effects of preincubation on inhibition, the lower of the anti-IgE concentrations was used. Cells were either challenged simultaneously with somatostatin (10^{15}-10^{-8}M) and anti-IgE (3000-fold dilution), or preincubated with the agonist for five minutes before the addition of somatostatin.

In each case (Fig. 4.212i,ii) there was some tendency towards potentiation of histamine release, particularly when the cells were preincubated with the peptide (Fig.4.212ii). However, the responses were again extremely variable and in no case reached statistical significance.

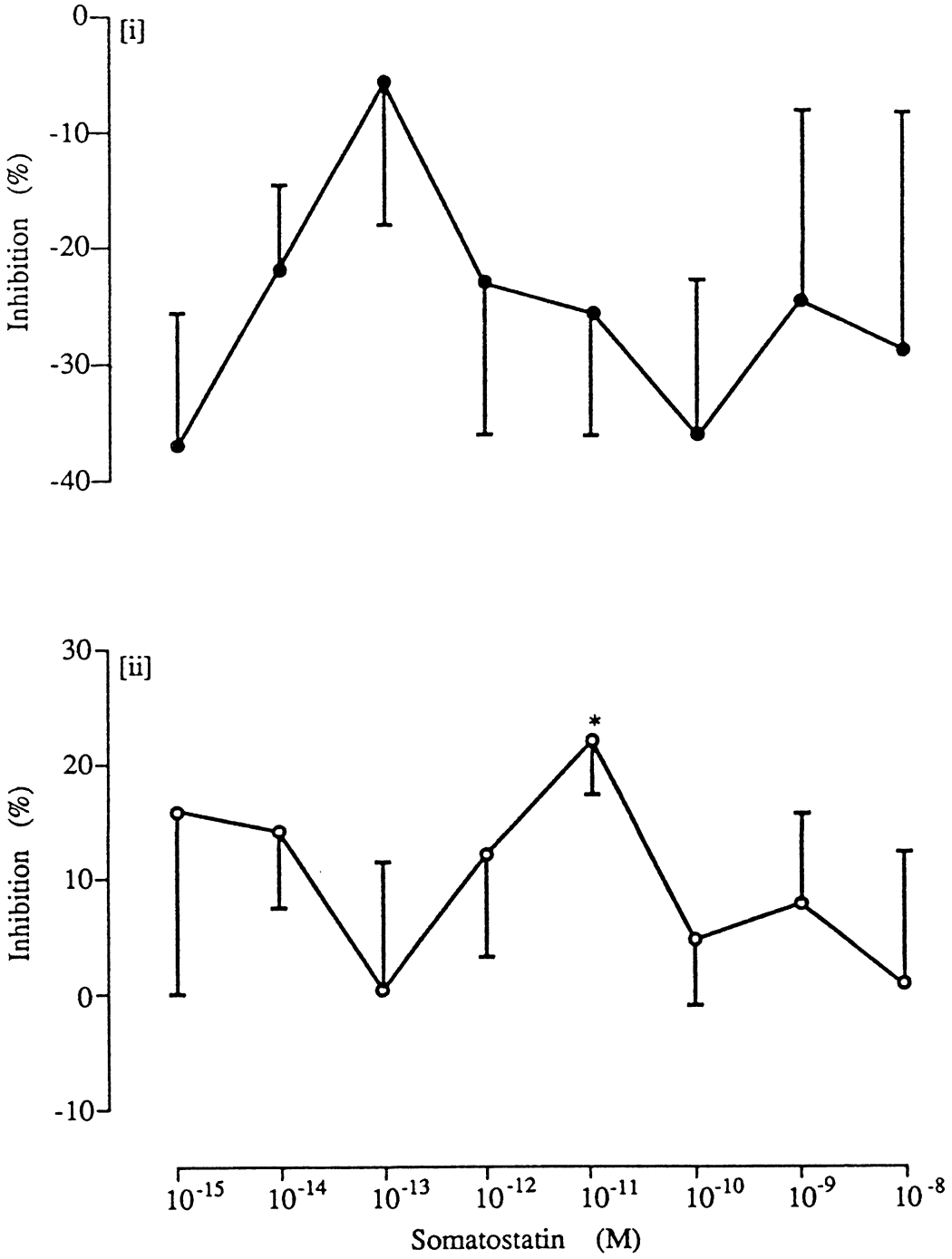


Fig. 4.211 Action of somatostatin on histamine release from human basophil leukocytes induced by
[a]anti-IgE (3000-fold dilution, control release 24.6±4.4, closed circles)
[b]anti-IgE (30-fold dilution, control release 23.9±4.2, open circles)

All values ± SEM *=$p<0.05$ (n=5)

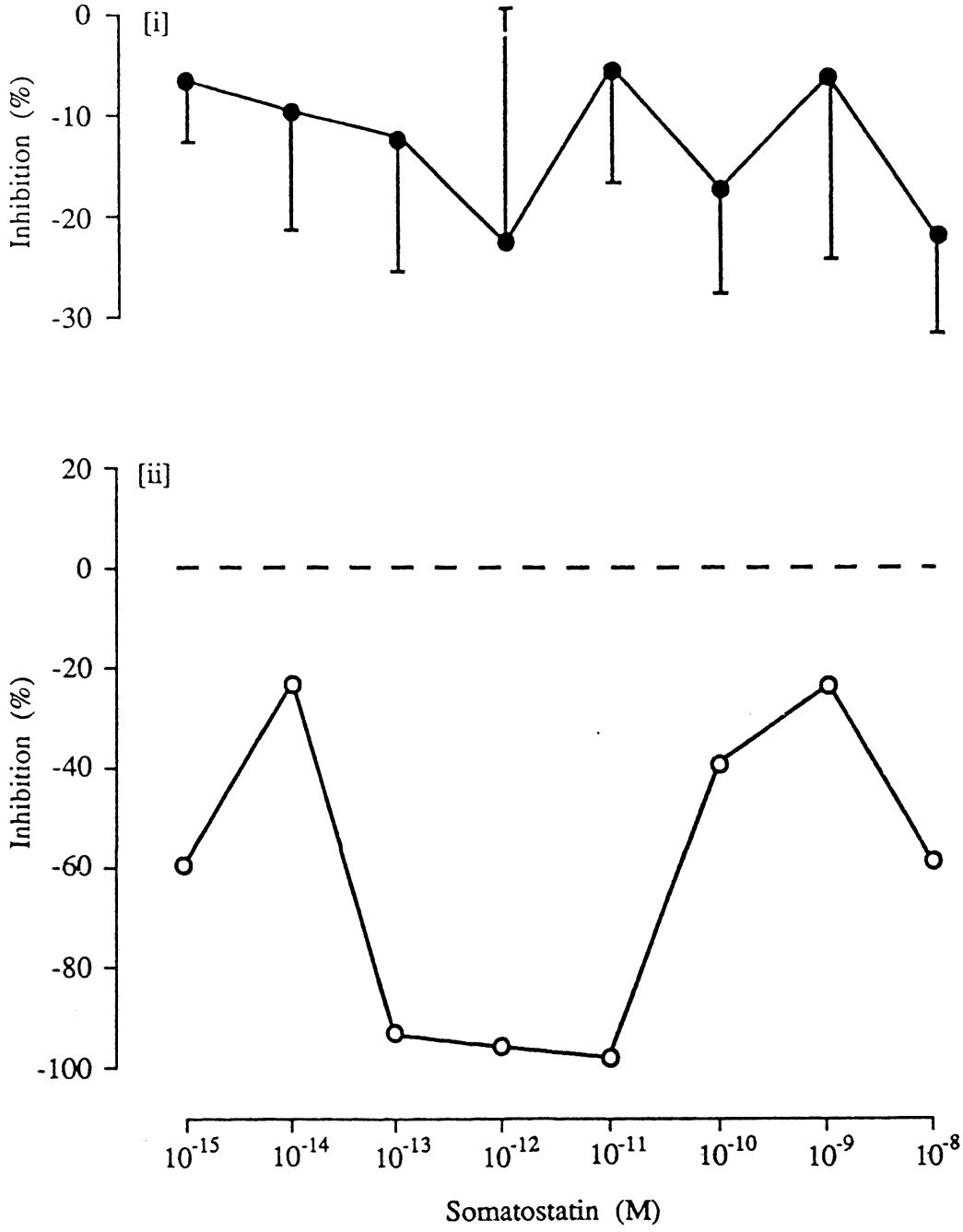


Fig. 4.212 Pertinence of preincubation (5min) of stimulus (anti-IgE, 3000-fold dilution, control release 25.3±4.7) on somatostatin inhibition of histamine release from human basophil leukocytes. All values ± SEM, $p>0.05$
[i] Simultaneous administration (closed circles).
[ii] Addition of somatostatin 5 minutes after addition of stimulus. Error bars omitted for clarity because often >100 for several points, $p>0.05$

(n=3)

4.2.1.3 **Release induced by poly-L-lysine**

Polylysine is known to elicit histamine release from human basophils. For this reason, as one of the only non-immunologic stimuli operative in this system (excepting ionophores), somatostatin (10^{-15}-10^{-8}M) was used to try and modulate polylysine-induced release. Cells were preincubated with somatostatin for five minutes before stimulation with polylysine (100μg/ml).

Somatostatin (10^{-15}-10^{-8}M) appeared to produce a very slight but significant inhibition of histamine release induced by polylysine (Fig.4.213i). Higher, sub-releasing concentrations of somatostatin (0.1-10μg/ml;6.1×10^{-7}M-6.1×10^{-6}M) failed to produce any further inhibition or significantly to affect the release induced by anti-IgE (Fig.4.213ii).

4.2.2 Effects of somatostatin on histamine release from rat peritoneal mast cells

4.2.2.1 **Anti-IgE and antigen-induced secretion**

The effects of somatostatin on histamine release from rat peritoneal mast cells stimulated with both anti-IgE and antigen were examined. Experiments were also conducted to investigate any possible effects of preincubation with the antagonist. Thus, some cells were preincubated with somatostatin (10^{-15}-10^{-8}M) for fifteen minutes, before stimulation with either anti-IgE (300-fold dilution) or antigen (3WE/ml).

There was some tendency for somatostatin to inhibit immunologic activation of rat peritoneal mast cells, particularly in the case of anti-IgE without preincubation (Table 4.221i) and of antigen with preincubation (Table 4.221ii). However, the majority of the responses were again highly variable and in no case reached statistical significance.

4.2.2.2 **Substance P-induced secretion**

For the purposes of comparison, any inhibitory activity of somatostatin on substance P-induced secretion was investigated. In keeping with the previous experiments, this activity was studied upon the preincubation and simultaneous challenge of reagents and cells.

As before, the effects were highly variable and no significant effect was observed in any case (Fig.4.222).

4.2.2.3 **Compound 48/80-, concanavalin A- and anti-IgE-induced release**

One final set of experiments were performed on peritoneal mast cells of the rat. These experiments were performed on cells from unsensitized animals, to determine the pertinence of available, cell-bound IgE. For comparative purposes, experiments on compound 48/80- and concanavalin A-induced secretion were included in the study. Extremely variable results were again observed, with intermittent, slight potentiation of release induced by compound 48/80 and some minor, erratic potentiation of that evoked by the IgE-directed ligands. However, all of the effects were small and not dose-related (Fig.4.223).

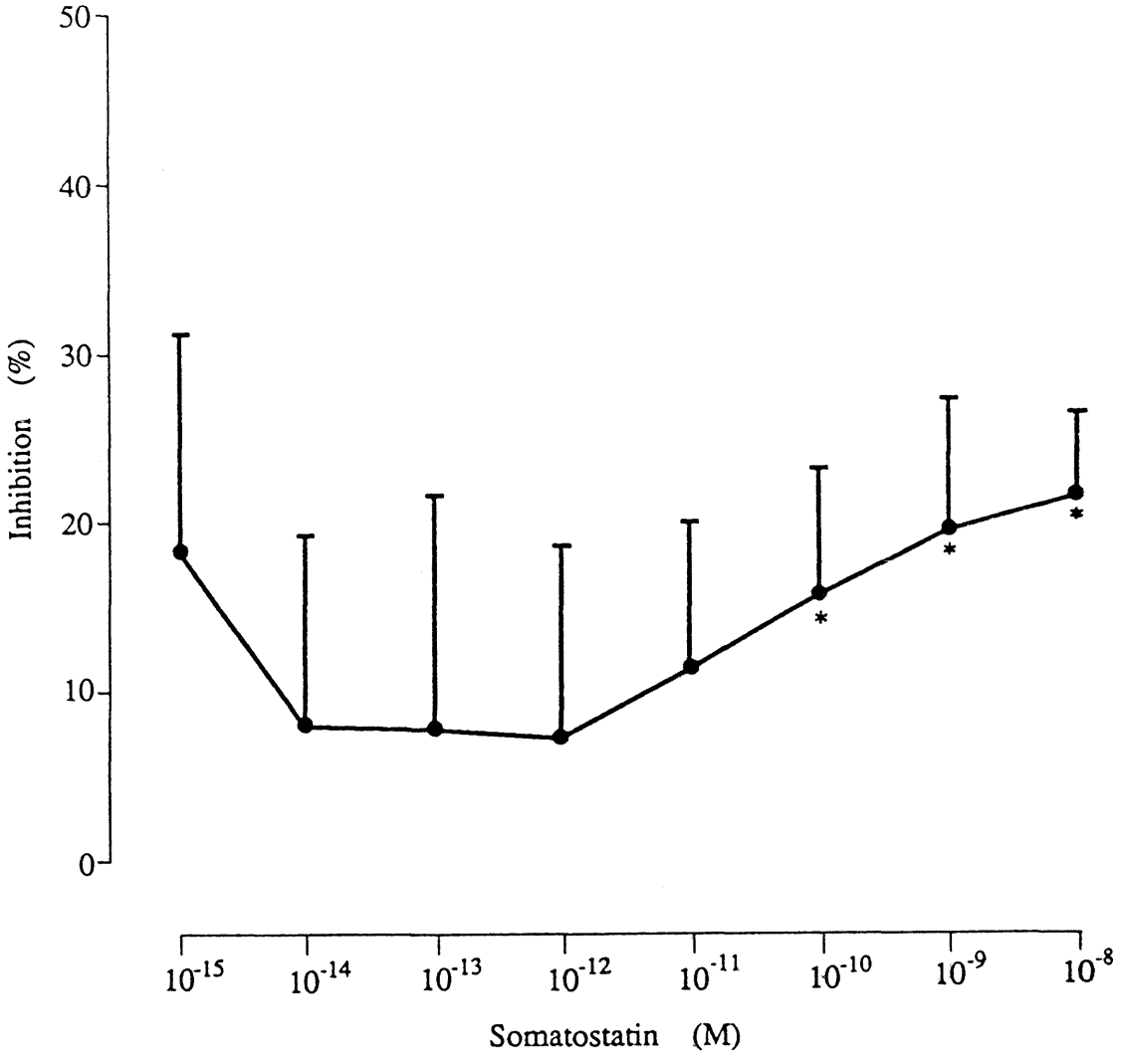


Fig. 4.213i Action of low concentrations of somatostatin on histamine release from human basophils stimulated with polylysine (100μg/ml, control release 28.2±6.4)

All values ± SEM *=$p<0.05$ (n=6)

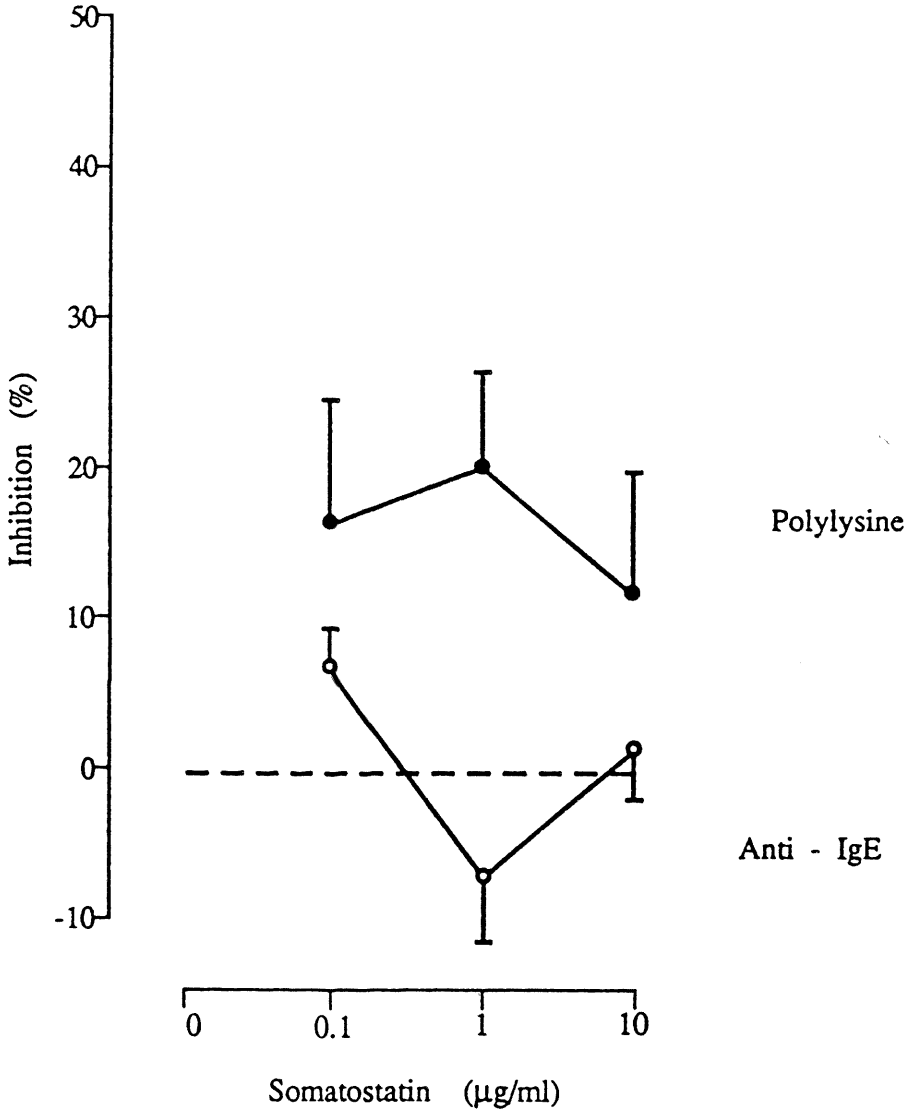


Fig. 4.213ii Action of higher concentrations of somatostatin on histamine release from basophils stimulated with polylysine (100μg/ml, control release 29.7±5.3, closed symbols) and anti-IgE (30-fold dilution, control release 31.1±3.7, open symbols)

All values ± SEM $p>0.05$ $(n=4)$

Somatostatin (M)	INHIBITION (%)	
	No Preincubation	Preincubation
0	30.1 ± 5.4	36.7 ± 7.0
10^{-15}	20.5 ± 12.7	−7.5 ± 17.4
10^{-14}	23.5 ± 12.7	−9.9 ± 14.9
10^{-13}	27.6 ± 15.1	7.7 ± 14.5
10^{-12}	23.6 ± 14.6	2.5 ± 13.9
10^{-11}	20.3 ± 11.2	4.3 ± 14.1
10^{-10}	18.0 ± 10.1	7.3 ± 14.8
10^{-9}	16.6 ± 9.9	3.8 ± 14.8
10^{-8}	−7.1 ± 20.4	1.0 ± 12.6

Fig. 4.221i Action of somatostatin on histamine release from rat peritoneal mast cells stimulated with anti-IgE (300-fold dilution)

All values ± SEM $p>0.05$ (n=8)

Somatostatin (M)	INHIBITION (%)	
	No Preincubation	Preincubation
0	40.2 ± 7.5	44.8 ± 6.2
10^{-15}	−1.3 ± 3.5	21.1 ± 12.3
10^{-14}	6.7 ± 4.2	11.0 ± 12.3
10^{-13}	−0.4 ± 6.8	1.7 ± 8.4
10^{-12}	−7.9 ± 5.8	1.0 ± 14.4
10^{-11}	1.2 ± 9.2	14.5 ± 14.5
10^{-10}	−3.7 ± 6.2	5.9 ± 11.7
10^{-9}	−0.5 ± 3.7	7.3 ± 13.0
10^{-8}	−19.9 ± 7.7	−2.2 ± 13.5

Fig. 4.221ii Action of somatostatin on histamine release from rat peritoneal mast cells stimulated with antigen (3WE/ml)

All values ± SEM $p>0.05$ (n=5)

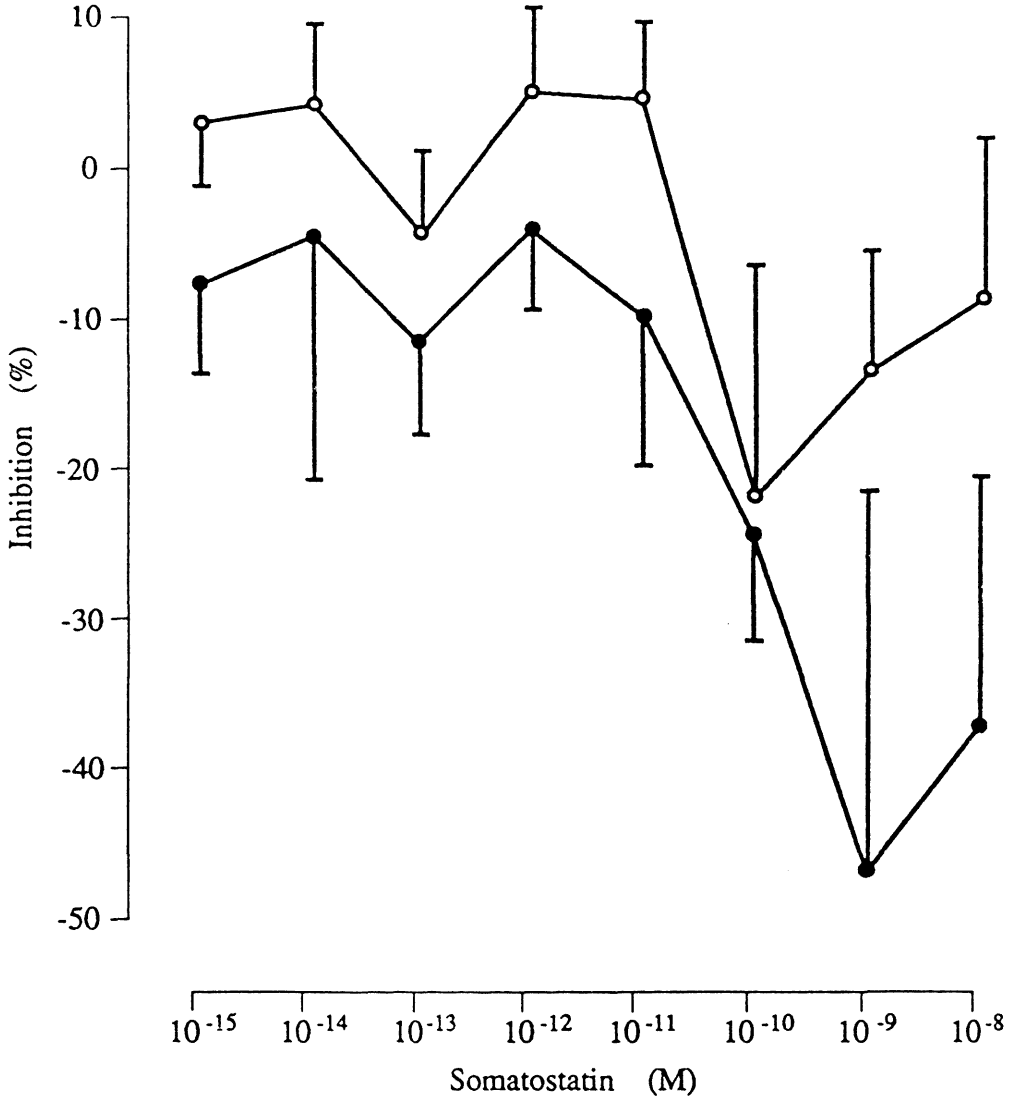


Fig. 4.222 Effects of somatostatin on histamine release from rat peritoneal mast cells stimulated with substance P (10μg/ml, control release 28.7±1.4) on simultaneous addition (closed symbols) and preincubation (15 min, open symbols)

All values ± SEM $p>0.05$ (n=4)

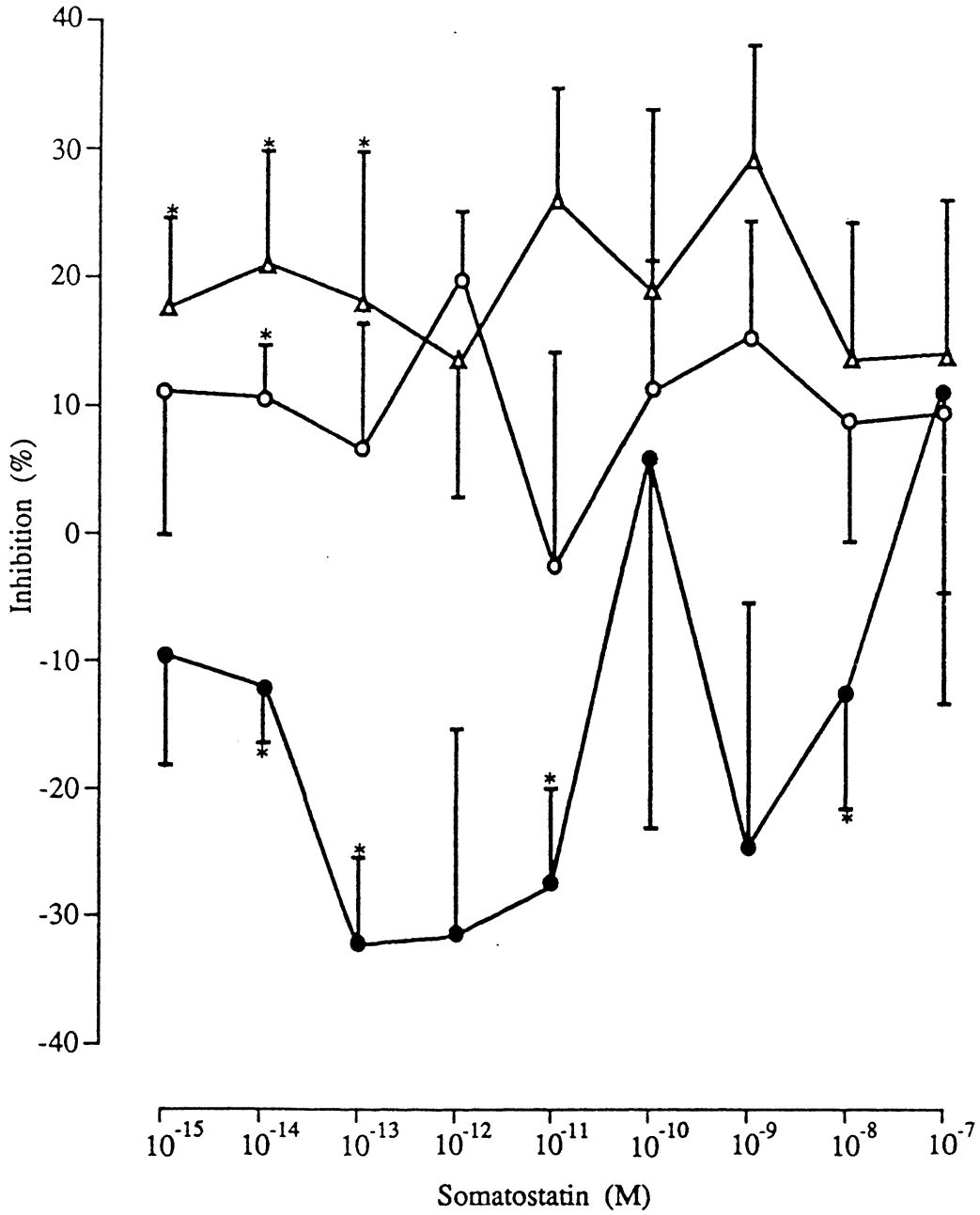


Fig. 4.223 Effects of somatostatin on histamine release from rat peritoneal mast cells stimulated by compound 48/80 (0.1μg/ml, control release 31.3±2.2, closed circles) anti-IgE (100-fold dilution, control release 35.6±3.4, open circles) concanavalin A (20μg/ml, control release 37.2±3.1, open triangles)

All values ± SEM *=$p<0.05$ (n=3)

4.2.3 Effects of somatostatin on histamine release from other murine peritoneal mast cells

The results presented above prompted the investigation of any effects in other murine peritoneal mast cells. Mouse and hamster cells are less responsive to the action of polycations, particularly in the presence of extracellular calcium [3].

4.2.3.1 **Mouse and hamster peritoneal mast cells**

Under conditions of simultaneous administration of reagents, somatostatin (10^{-15}-10^{-8}M) failed significantly to modulate secretion induced by anti-IgE (3000-fold dilution) from mouse peritoneal mast cells (Fig.4.231). Essentially similar results were observed on preincubation with the peptide (data not shown).

A single experiment (data not shown) was performed on hamster peritoneal mast cells. Extensive studies were not considered worthy, on the basis of previous data. Hamster peritoneal mast cells showed almost identical responses to those of mouse cells.

4.2.4 The investigation of any possible synergy between somatostatin and other secretagogues

4.2.4.1 **Synergy investigations in the human basophil**

As already mentioned, somatostatin is essentially inactive in the human basophil (also, chapter 5). This allows the use of relatively high concentrations of the peptide to be examined.

i: Anti-IgE-induced secretion

In this study, any possible synergy between somatostatin (1-100μg/ml) and anti-IgE (2000-fold dilution) was researched. Results show that somatostatin was itself only active at the top concentration used, eliciting a response of 3.8±2.8%. This value, on addition to the equivalent value also containing anti-IgE, show that there is no appreciable synergy between somatostatin and anti-IgE, in the human basophil (Fig.4.241i)

ii: F-Met-Leu-Phe-induced secretion

Certain formyl tripeptides, containing methionine, release histamine from human basophil leucocytes [535]. The mechanism of release induced by these agents is thought to be unrelated to the IgE-receptor activation of basophils. For this reason, any possible synergy between somatostatin (10^{-7}-10^{-4}M) and F-met-leu-phe (FMLP, 10^{-4}M) was studied. The data presented here suggest that there is no synergy between these two agents (Fig.4.241ii). Release values in excess of that for FMLP alone, correspond to the additive component of somatostatin- induced release, assessed separately.

Hence, somatostatin does not synergize with either anti-IgE or FMLP when these agents are used to stimulate human basophils.

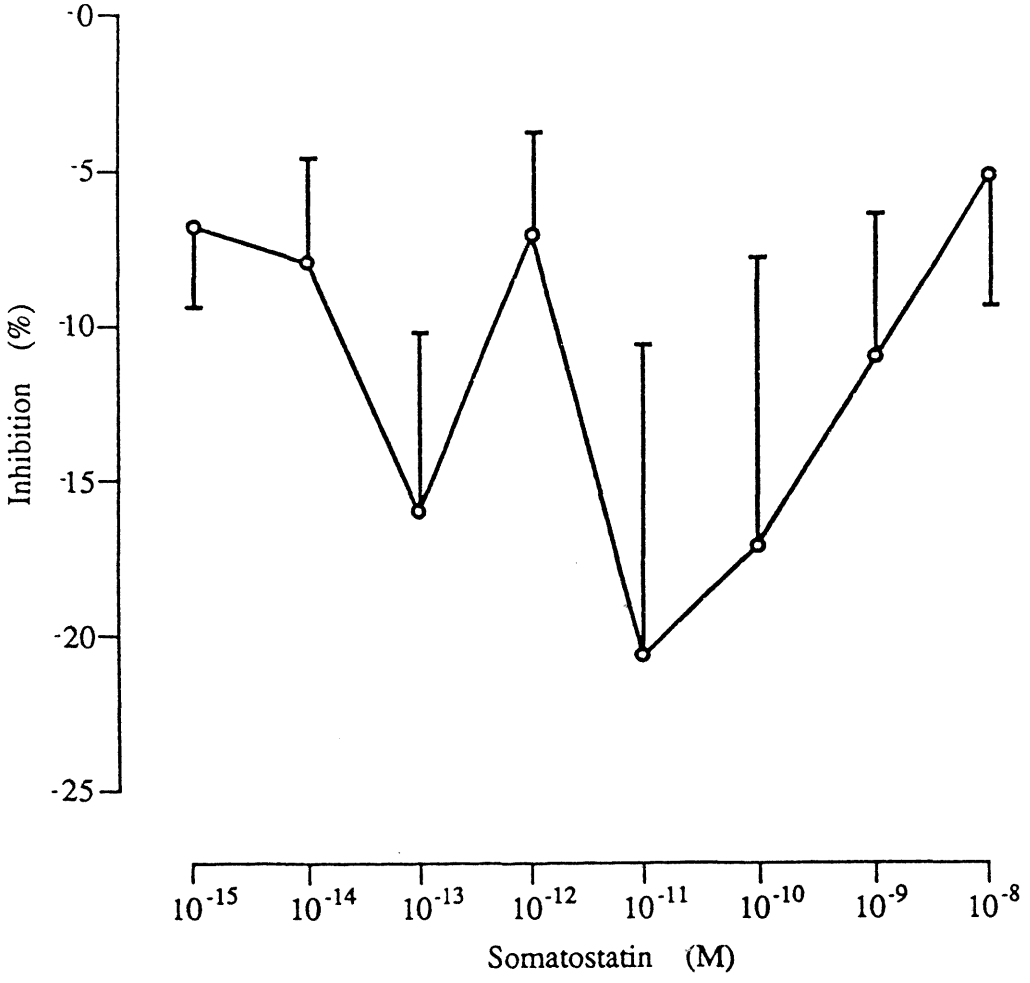


Fig. 4.231 Action of somatostatin on histamine release from mouse peritoneal mast cells stimulated with anti-IgE (3000-fold dilution, control release 37.7 ± 10.6)

All values ± SEM $p>0.05$ (n=3)

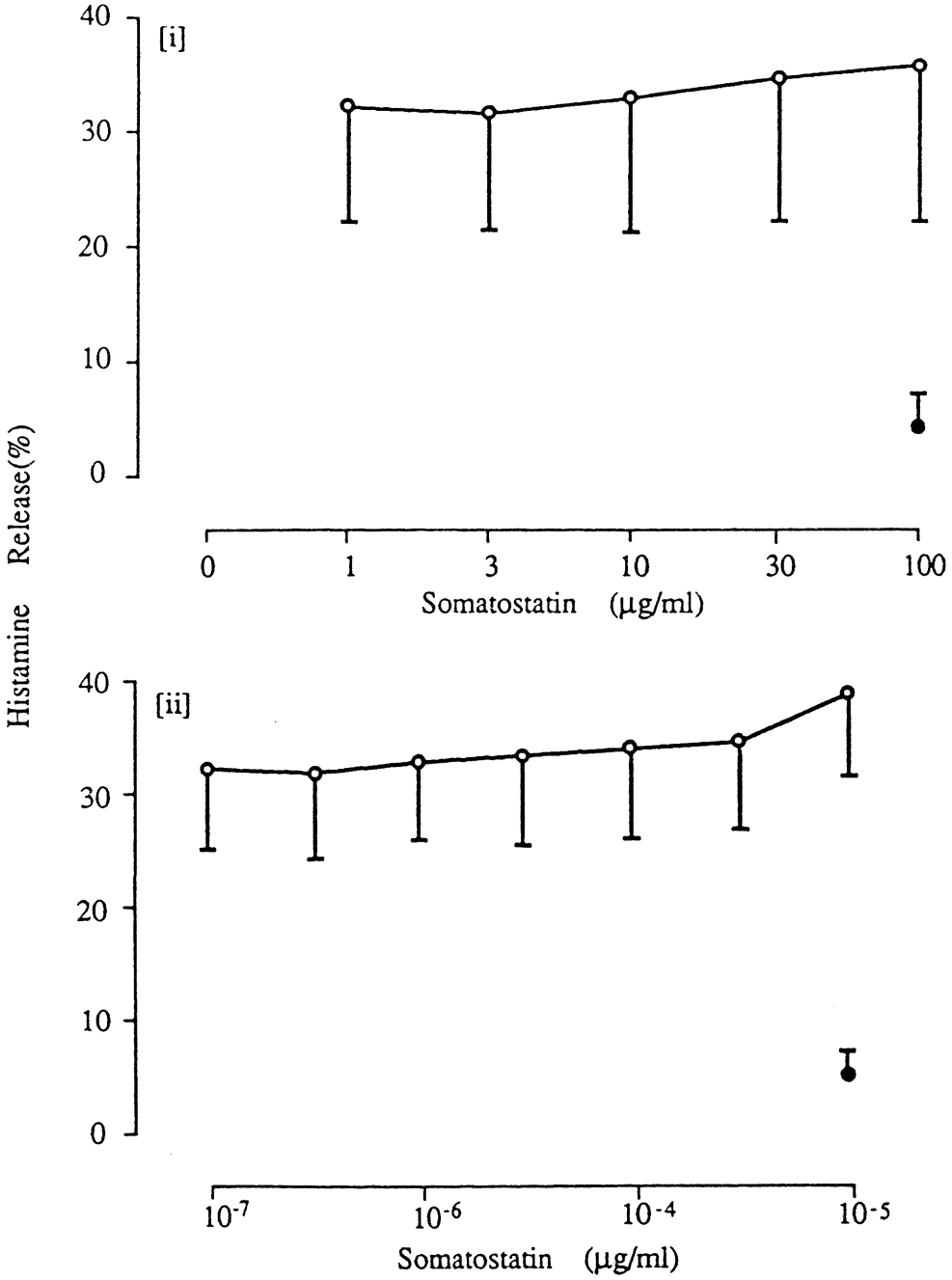


Fig. 4.241 Investigations into any possible synergistic effects between somatostatin and
[i] anti-IgE (2000-fold dilution)
[ii] FMLP (10^{-4}M)
Control releases are represented by closed symbols for comparison and all experiments were conducted on human basophil leukocytes.

All values ± SEM (n=4)

4.2.4.2 Synergy investigations in human lung mast cells

A study was performed to examine the possible synergy between somatostatin (10^{-6}-10^{-4}M, 1.6-164μg/ml) and anti-IgE (1000-fold dilution) on enzymically dispersed human lung mast cells. There is no expression of synergy between these agents in lung mast cell activation (Fig.4.242). In fact, the results seem to show an inhibition of release; combined stimulation yielding release values less than the additive response. This effect is not particularly profound and appraised as negligible.

4.2.4.3 Synergy investigations in the rat peritoneal mast cell

Studies with sub-releasing concentrations have already been performed in the rat mast cell. It was therefore interesting to investigate the action of higher concentrations of somatostatin (0.01-3μg/ml) on various concentrations of substance P-induced secretion (1-100μg/ml). Results show that there is some degree of antagonism between the peptides at higher release values (Fig.4.243). However, the predominant feature is a lack of synergistic activity in this system.

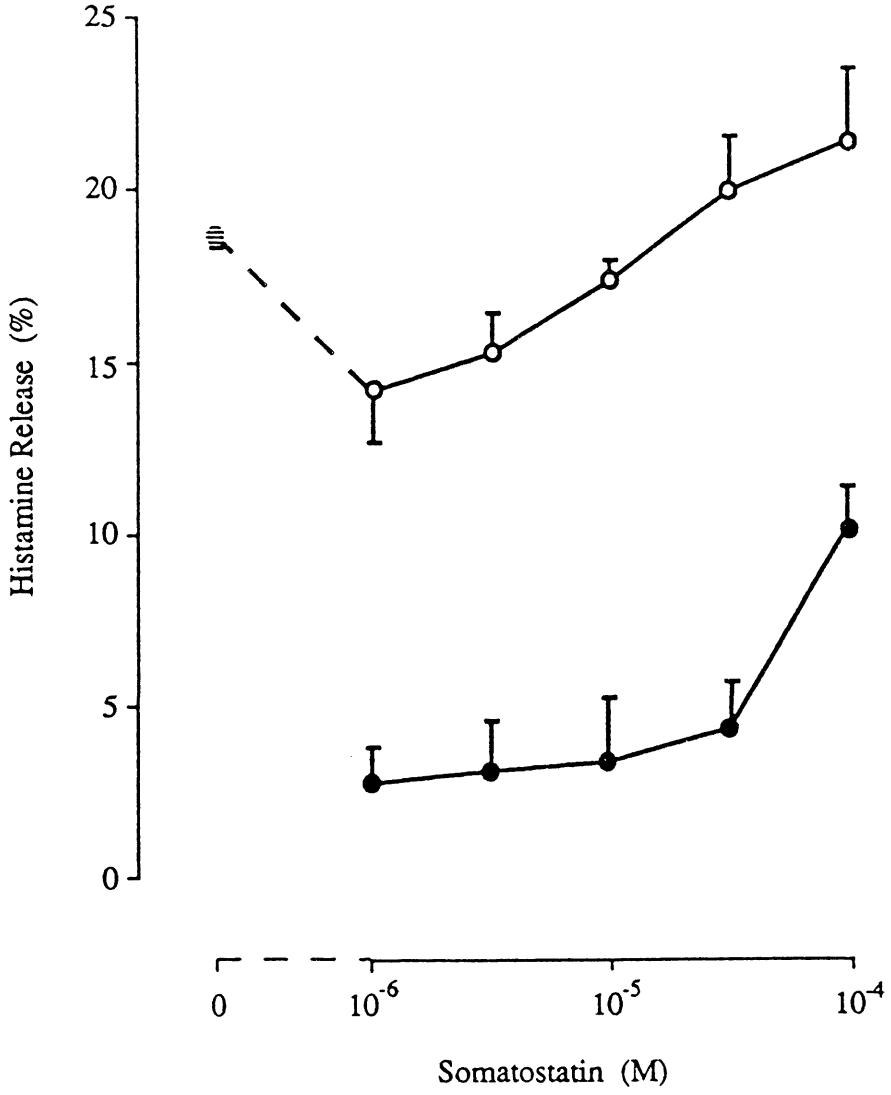


Fig. 4.242 Synergy investigations in the human lung mast cell between somatostatin and anti-IgE (1000-fold dilution, open symbols). Somatostatin induced secretion (closed symbols) is included for comparison.

All values ± SEM (n=3)

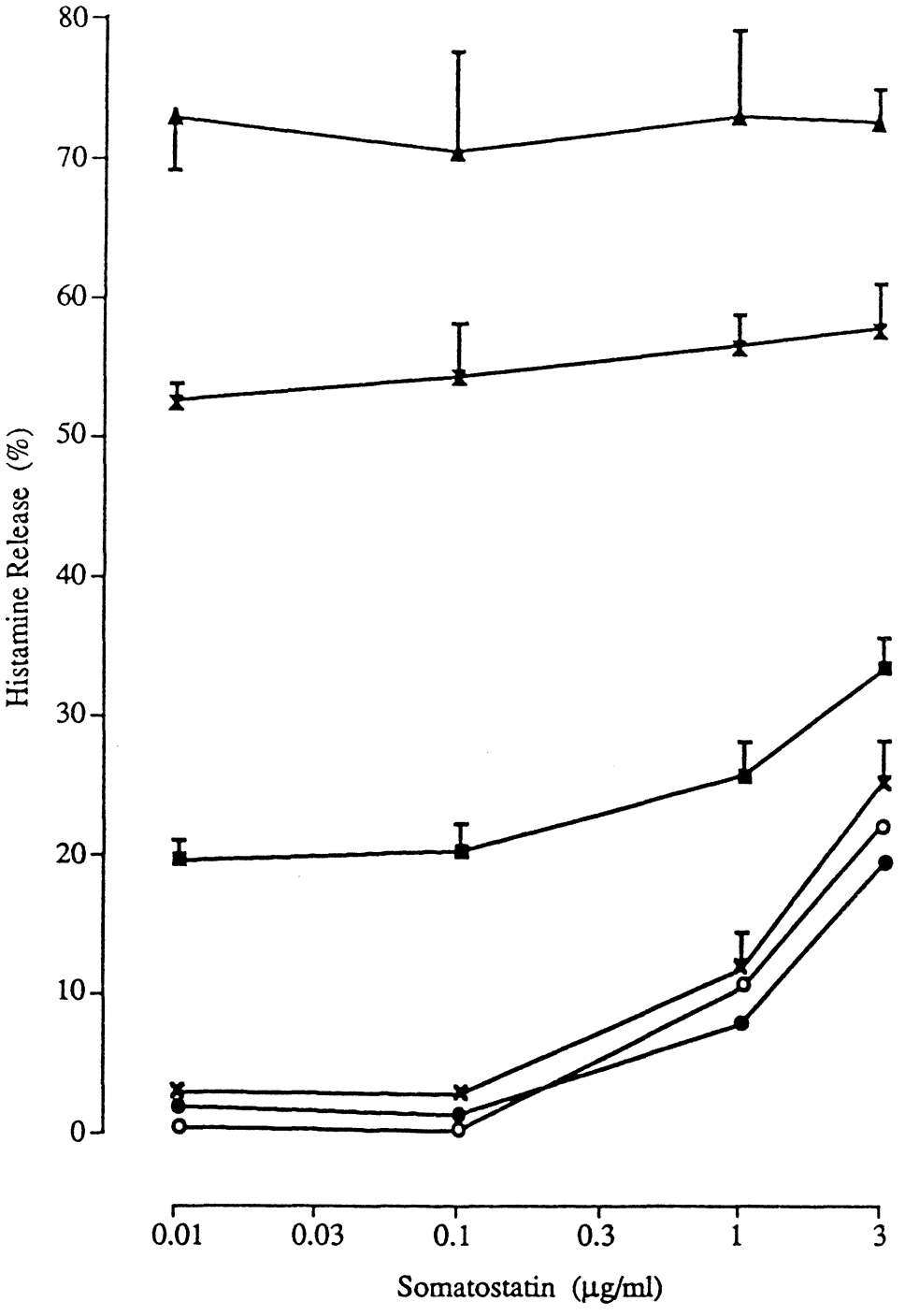


Fig. 4.243 Synergy investigations in the rat peritoneal mast cell between somatostatin and substance P (1μg/ml closed circles, 3μg/ml open crosses, 10μg/ml closed squares, 30μg/ml closed crosses and 100μg/ml closed triangles). Somatostatin induced release (open circles) is included for comparison.

Where error bars are omitted for clarity, SEM did not exceed ± 5.0 (n=4)

Somatostatin was first characterised as an inhibitor of the release of growth hormone [536]. Since then, the peptide has been shown to have an inhibitory effect in a variety of other systems. In particular, it may prevent the release of thyrotrophin [515,537], prolactin [515,537], insulin and glucagon [538,539], secretin [542], pepsin [543], gastric inhibitory peptide [544], human pancreatic polypeptide [545] and substance P from peripheral terminals of afferent neurones [356]. The peptide also specifically inhibits important activities of human T-lymphocytes [546]. It would then seem that, with the exception of specific mast cell activation, somatostatin is a predominantly inhibitory or modulatory peptide in most biological systems.

In keeping with this model, it has been reported that low concentrations of somatostatin inhibit the anti-IgE-induced release of histamine and LTD_4 from human basophil leucocytes [523]. However, this effect was confined to the immunologic stimulus and was not observed when the cells were stimulated with PF4 or the ionophore A23187 [523]. Moreover, the former findings could not be confirmed in the present study and somatostatin (10^{-15}-10^{-8}M) was found to have no striking effect on histamine release from human basophils stimulated with anti-IgE or polylysine. In addition, the peptide did not inhibit secretion from peritoneal cells of the rat, mouse or hamster following stimulation with anti-IgE and from the former cells upon activation with substance P, compound 48/80 or concanavalin A. Also, higher concentrations (10^{-7}-10^{-4}M) of somatostatin failed to potentiate the action of, or to exhibit any synergistic effect with, anti-IgE on human basophils and lung mast cells, FMLP on basophils, and substance P on rat mast cells.

In total, the above data show that somatostatin does not modulate, in either a positive or negative fashion, the responses of a range of histaminocytes to a diversity of stimuli. However, a characteristic feature of these experiments was that the observed responses were extremely variable and erratic. Such variability could mask small effects and may account for the differences between the present results and published data [523,524]. The reasons for the present erratic effects are by no means obvious and such responses seemed to be confined to this particular set of experiments. The variability might possibly be reduced, and statistically significant data obtained, by carrying out large numbers of additional experiments. However, such a programme did not seem to be justified in view of the negative results obtained.

CHAPTER 5

FURTHER INVESTIGATIONS INTO THE ACTION OF SOMATOSTATIN ON HISTAMINE-CONTAINING CELLS FROM VARIOUS TISSUES AND SPECIES

5.1 *INTRODUCTION*

A direct innervation of mast cells has been reported in various tissues, including the gut, mesentery and diaphragm [407,408,547]. The physiological proximity of mast cells and peptidergic, afferent nerves suggests a direct communication presumably through soluble mediators. Kiernan [413] and Lembeck and Holzer [416] proposed the involvement of mast cells in an axon reflex in which the flare reaction to noxious stimuli was mediated in part by substance P released from peripheral nerves.

Intradermal injection of substance P into the rat mimics the response of local injection of histamine or compound 48/80 [403]. This effect can be mostly inhibited by pretreatment with anti-histamines or capsaicin, both of which prevent the flare reaction in the skin [416]. Furthermore, antidromic nerve stimulation causes neurogenic oedema [548], mast cell degranulation, augmentation of histamine release and histamine-mediated modulation of neurotransmitter release [392,413].

Afferent nerve endings have been found in the airway mucosa of several species, including man [549]. A number of sensory neuropeptides have been localised to the lung and thus, the axon reflex of the skin was considered likely to occur in the lung [494]. Reports have also implicated an axon reflex mechanism in the gastrointestinal tract [550,551].

In the present study, an attempt was made directly to assess the possible role of somatostatin in neurogenic inflammation by examining its effect on mast cells from different sources. This investigation was considered essential in view of the known tissue and species specificity of other neuropeptides and polycations [3,38]. The study was also considered to be of interest in its own right as it would provide further information on the extent of mast cell heterogeneity. In addition, the relative binding of somatostatin to mast cell membranes was examined by fluorescence microscopy.

5.2 *RESULTS*

5.2.1 The effects of somatostatin on various histaminocytes

5.2.1.1 **Investigations in the presence and absence of calcium**

Experiments on various histaminocytes were conducted in the presence and absence of extracellular calcium. Additionally, a proportion of each cell population was briefly pretreated with EDTA (10^{-4}M, 5min), prior to challenge with somatostatin (0.1-100μg/ml).

i: Rat peritoneal and pleural mast cells

Somatostatin elicited a dose-dependent release of histamine from mast cells isolated from both peritoneal and pleural cavities (Fig.5.211ia,b). For both cell populations, the release was not dependent on extracellular calcium. Brief pretreatment with EDTA elevated release levels at lower peptide concentrations but maximum release was less than that observed in the presence of calcium.

No appreciable differences were observed between the two cell types, although the pleural cells were marginally more responsive than those of the peritoneum.

ii: Peritoneal mast cells of the mouse and hamster

Peritoneal mast cells from the mouse produced a dose-dependent release of histamine when stimulated with somatostatin. Curiously, mouse mast cells are not strongly responsive to the peptide in the presence of extra-cellular calcium. This is also the case with hamster peritoneal mast cells (Fig.5.211iia,b). Stimulation induced in conditions of calcium deprivation produced a significant response from both species, the release being greater than that in the presence of the cation. Brief pretreatment with EDTA produced release values in excess of those in calcium or calcium-free media.

iii: Mast cells isolated from rat lung and mesentery

Isolated mast cells from the lung and mesentery were less responsive than those of the pleural and peritoneal cavities of the rat. In this study, cells from the mesentery were more responsive than those of the lung (Fig.5.211iiia,b). Interestingly, these two cell populations show the greatest response to somatostatin on brief pretreatment with EDTA. Release observed in the absence of extracellular calcium was also greater than that observed in the presence of the cation.

iv: Human lung mast cells and basophil leucocytes

Somatostatin elicited a limited response from both human lung mast cells and basophils (Fig.5.211iva,b). Histamine liberation is observed only at maximum concentrations of the peptide and did not exceed 15% in either system. Once again, the absence of extracellular calcium produced a stronger response than in the presence of the cation. Responses were potentiated further still by brief pretreatment with EDTA.

v: Porcine lung mast cells

Porcine lung mast cells were very weakly reactive to challenge by somatostatin (Fig.5.211v) and maximal responses were of the order seen with human lung mast cells (≈10%).

5.2.1.2 Investigations in the presence of extracellular calcium

The following studies were performed in the presence of calcium only. This restriction was imposed mainly by limited cell numbers and the inclusion of control experiments.

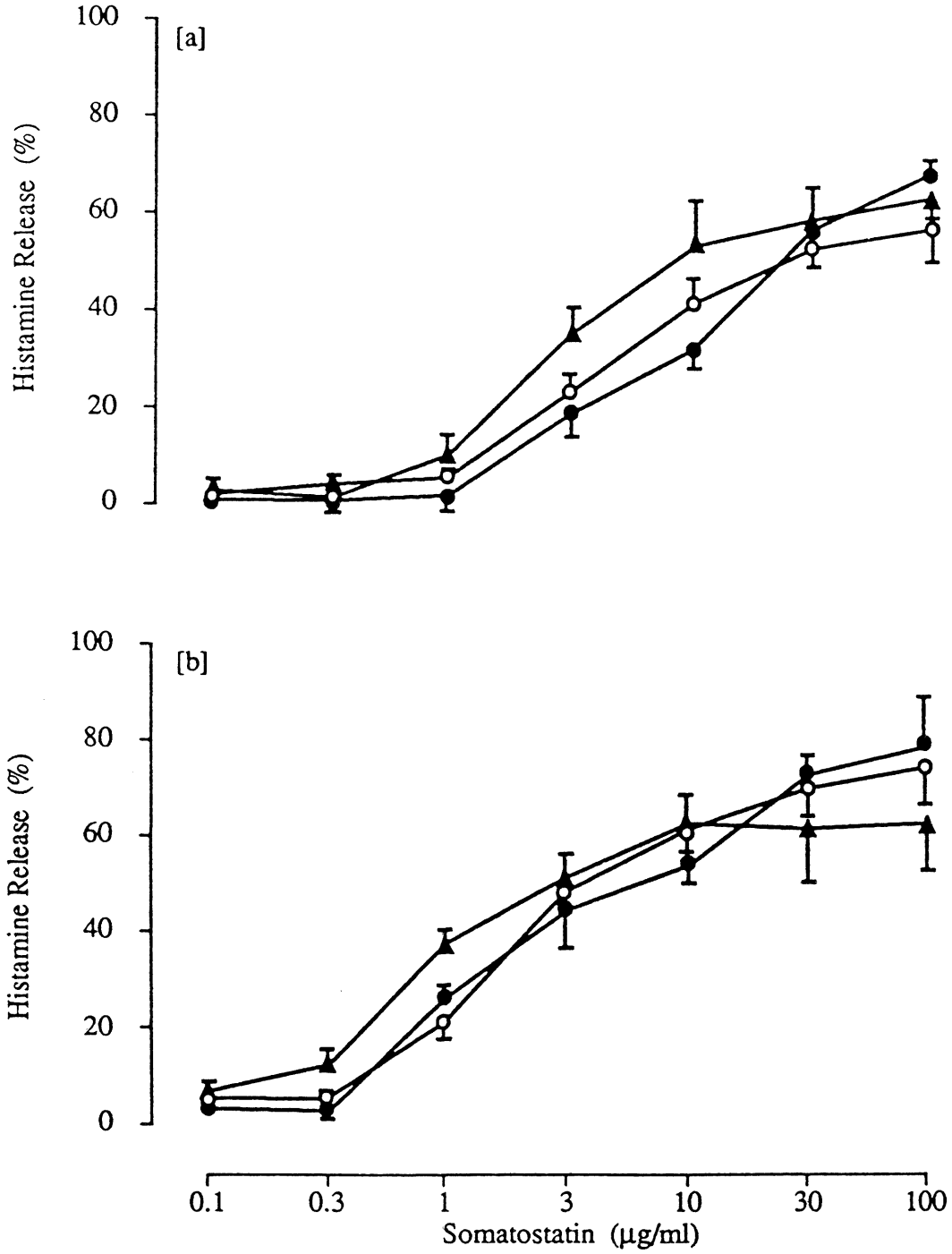


Fig. 5.211i Action of somatostatin on histamine release from rat [a] peritoneal mast cells [b] pleural mast cells. Experiments were conducted either in the absence (open circles) or presence of extracellular calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles).

All values ± SEM (n=4-5)

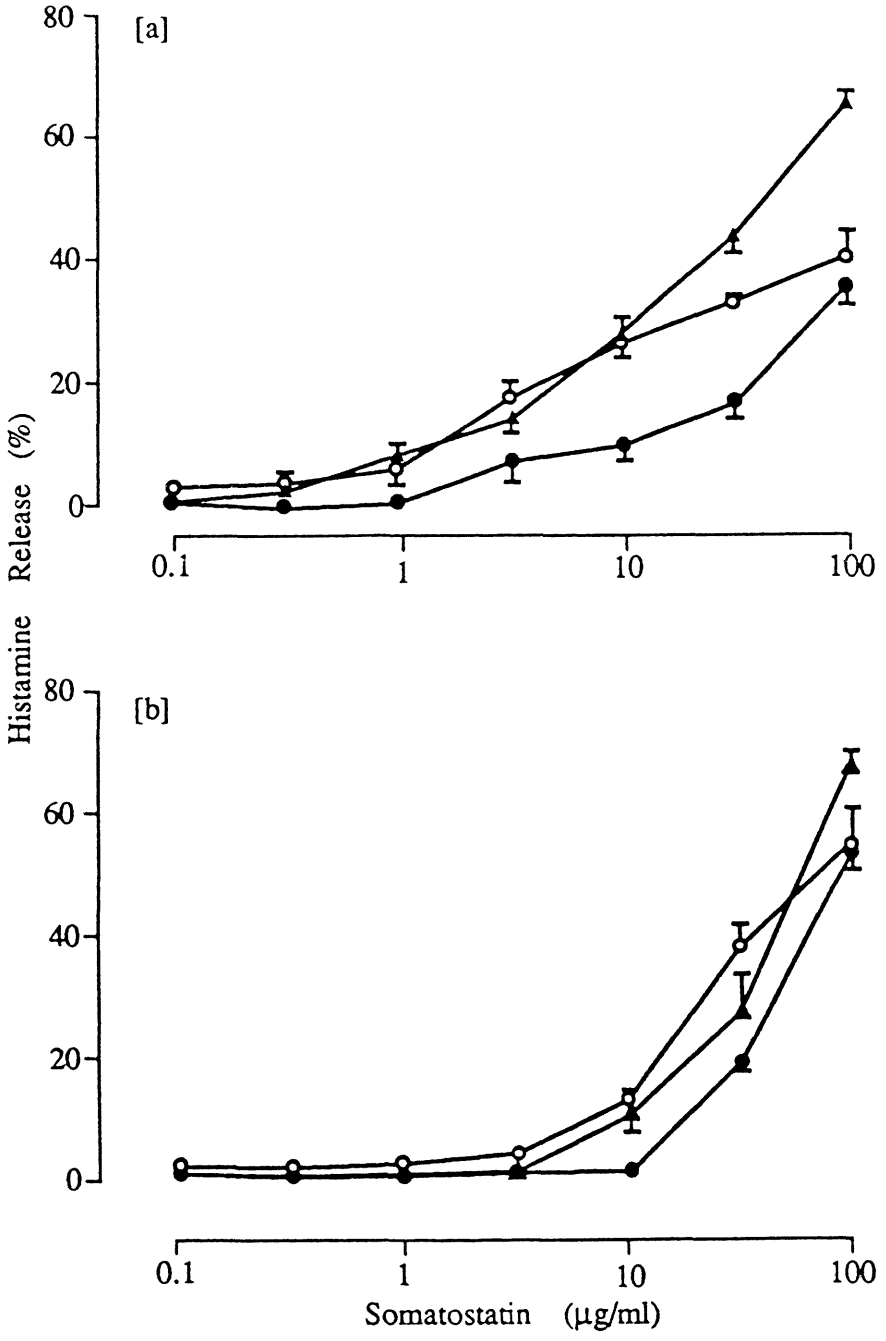


Fig. 5.211ii Action of somatostatin on histamine release from [a] mouse peritoneal mast cells [b] hamster peritoneal mast cells. Experiments were conducted either in the absence (open circles) or presence of extracellular calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles).

All values ± SEM (n=3-4)

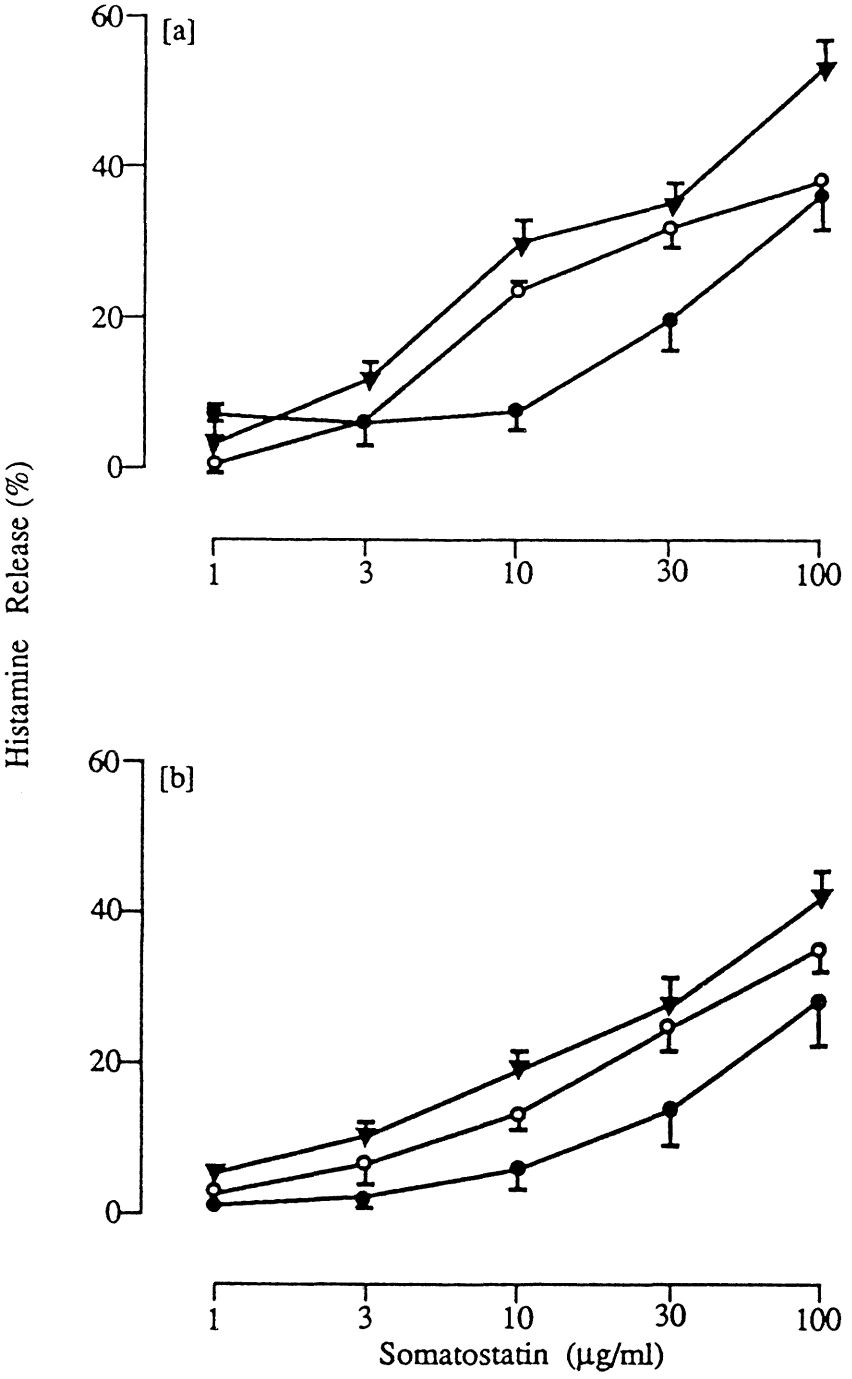


Fig. 5.211iii Action of somatostatin on histamine release from enzymically dispersed mast cells from [a] rat mesentery [b] rat lung. Experiments were conducted either in the absence (open circles) or presence of extracellular calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles).

All values ± SEM (n=5)

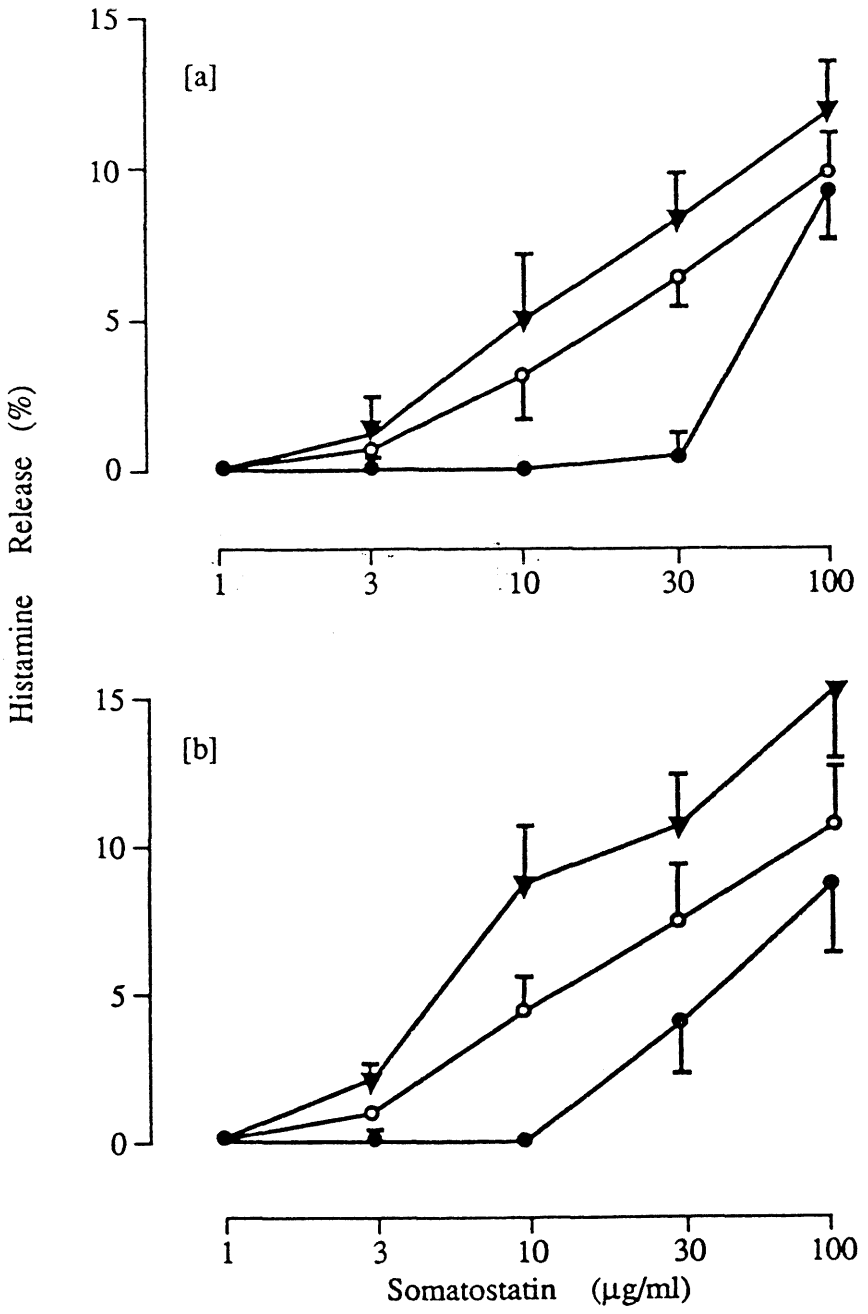


Fig. 5.211iv Action of somatostatin on histamine release from [a] enzymically dispersed human lung mast cells [b] human basophil leukocytes. Experiments were conducted either in the absence (open circles) or presence of extracellular calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles).

All values ± SEM (n=3-4)

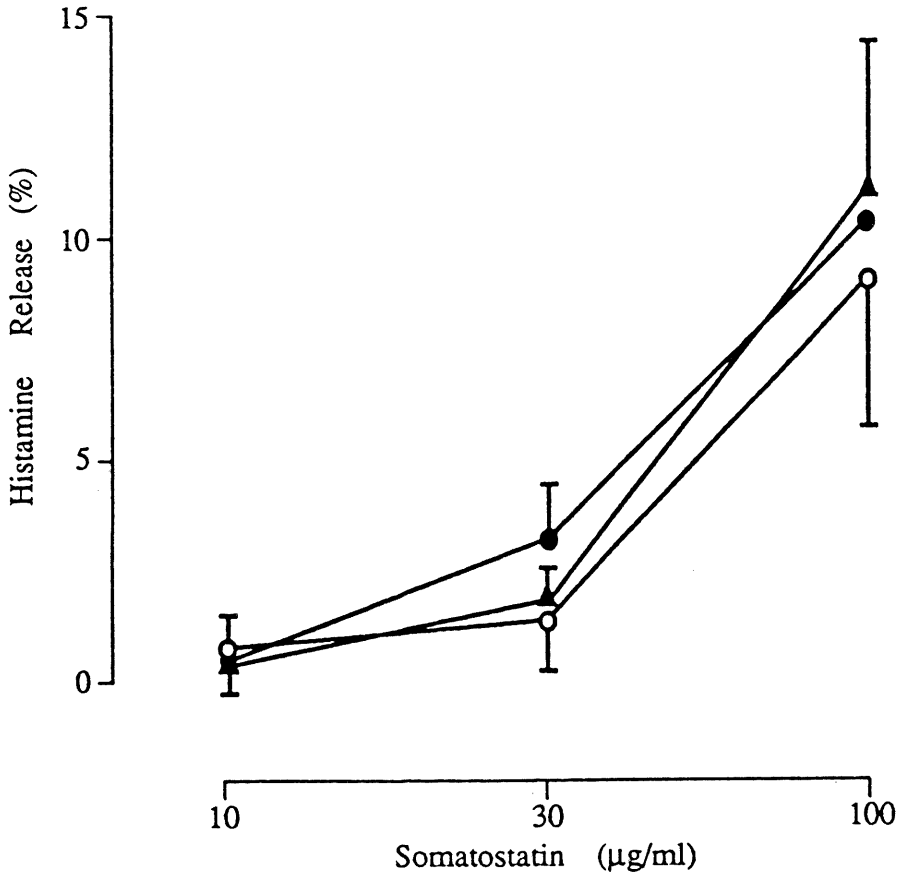


Fig. 5.211v Action of somatostatin on enxymically dispersed pig lung mast cells. Experiments were conducted either in the absence (open circles) or presence of extracellular calcium (1mM, closed circles) or after brief pretreatment with EDTA (10^{-4}M, closed triangles).

All values ± SEM (n=3)

i: Mast cells from rat heart and guinea pig lung, mesentery and heart.

Cardiac mast cells from the rat and all of the tissue mast cells from the guinea pig were unresponsive to somatostatin (Fig 5.212). The guinea pig cells responded normally to anti-IgG (10-1000 fold dilution) and to antigen ovalbumin (0.1-100μg/ml), releasing up to 40% of their histamine content, indicating that they were functionally intact (data not shown).

iii: Other histaminocytes

Extensive studies were not performed but single experiments on intestinal mast cells from man and the rat were executed. Results indicate that somatostatin is not active against rat intestinal mast cells but nominal responses (7.3±2.2%) from human gut cells were noted.

5.2.2 Comparison of various polyamines on rat peritoneal mast cells

Somatostatin (10^{-6}-10^{-4}M), substance P (10^{-6}-10^{-4}M), VIP (10^{-6}-10^{-4}M), CGRP (10^{-6}-10^{-4}M), PHM (10^{-6}-10^{-4}M), neurotensin (10^{-6}-10^{-4}M), compound 48/80 (10^{-7}-10^{-5}M) and poly-L-lysine (10^{-9}-10^{-7}M) produced dose-dependent histamine release from the rat peritoneal mast cell (Fig.5.22). Of these agents, polylysine was the most potent, in molar terms, followed by compound 48/80. The neuropeptides were less potent than the polyamines. Somatostatin and VIP were equipotent and roughly twice as active as substance P and PHM. CGRP and neurotensin were the least potent of the active agents tested. Neurokinins A and B (10^{-6}-10^{-4}M) and eledoisin (10^{-6}-10^{-4}M) were found to be without effect on the rat peritoneal mast cell.

5.2.3 Immunofluorescence of mast cells

A series of preliminary studies, confirmed microscopically, illustrated a 50-fold dilution of goat, FITC-conjugated, anti-rabbit IgG to yield the best fluorescence. In all experiments, non-fluorescent controls were conducted to preclude non-specific binding of either the primary or secondary antibody. Cells were initially challenged with somatostatin in the presence of a metabolic inhibitor (antimycin A, 1μM), before the subsequent addition of primary and secondary antibody.

The majority of somatostatin-treated rat peritoneal mast cells displayed strong fluorescent staining. However, a few cells failed to exhibit staining although they could be found adjacent to fluorescent counterparts. This effect was not monitored quantitatively (Fig.5.23i).

Purified hamster cells also displayed significant staining after treatment (Fig.5.23ii). There seemed to be a low density, universal staining of the entire membrane surface. However, distinct dense accumulations of fluorescence are evident in some surface aggregates. As seen with cells from the rat, there was also a nominal presence of non-fluorescent cells.

Mouse cells showed a lesser degree of staining than those of the rat or hamster (qualitative assessment). There was little evidence of overall staining, but localised aggregates were

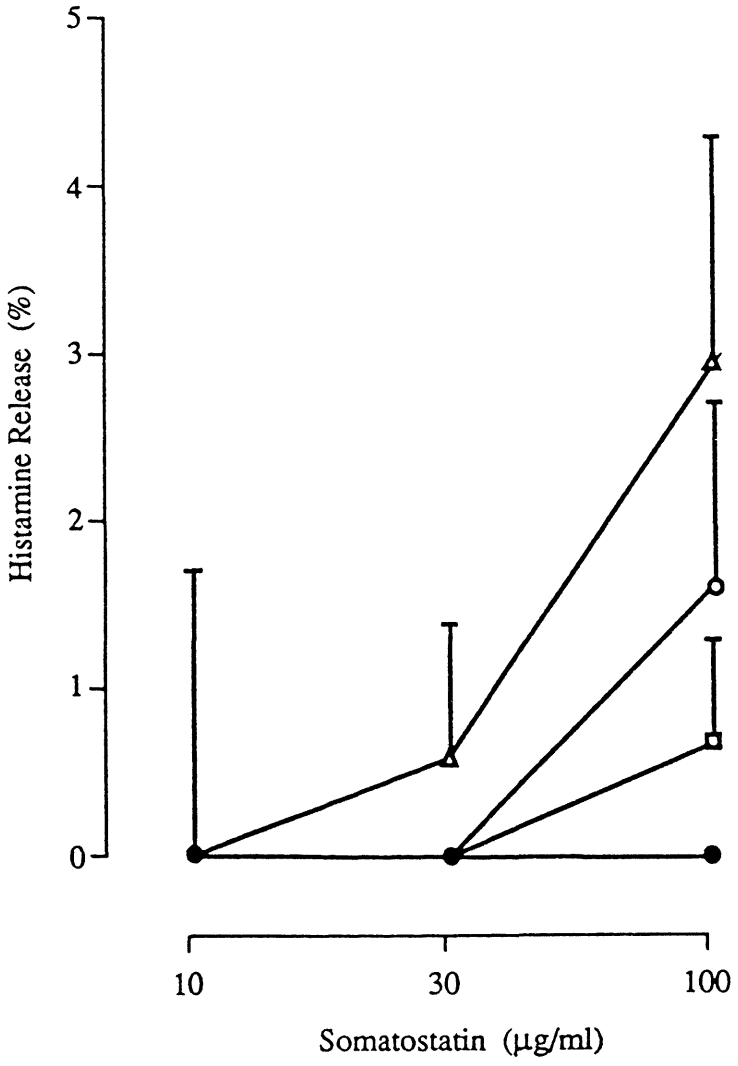


Fig. 5.212 Action of somatostatin on histamine release from mast cells of enzymically dispersed rat heart (open squares) and guinea pig lung (open circles), mesentery (open triangles) and heart (closed circles).

All values ± SEM (n=4-6)

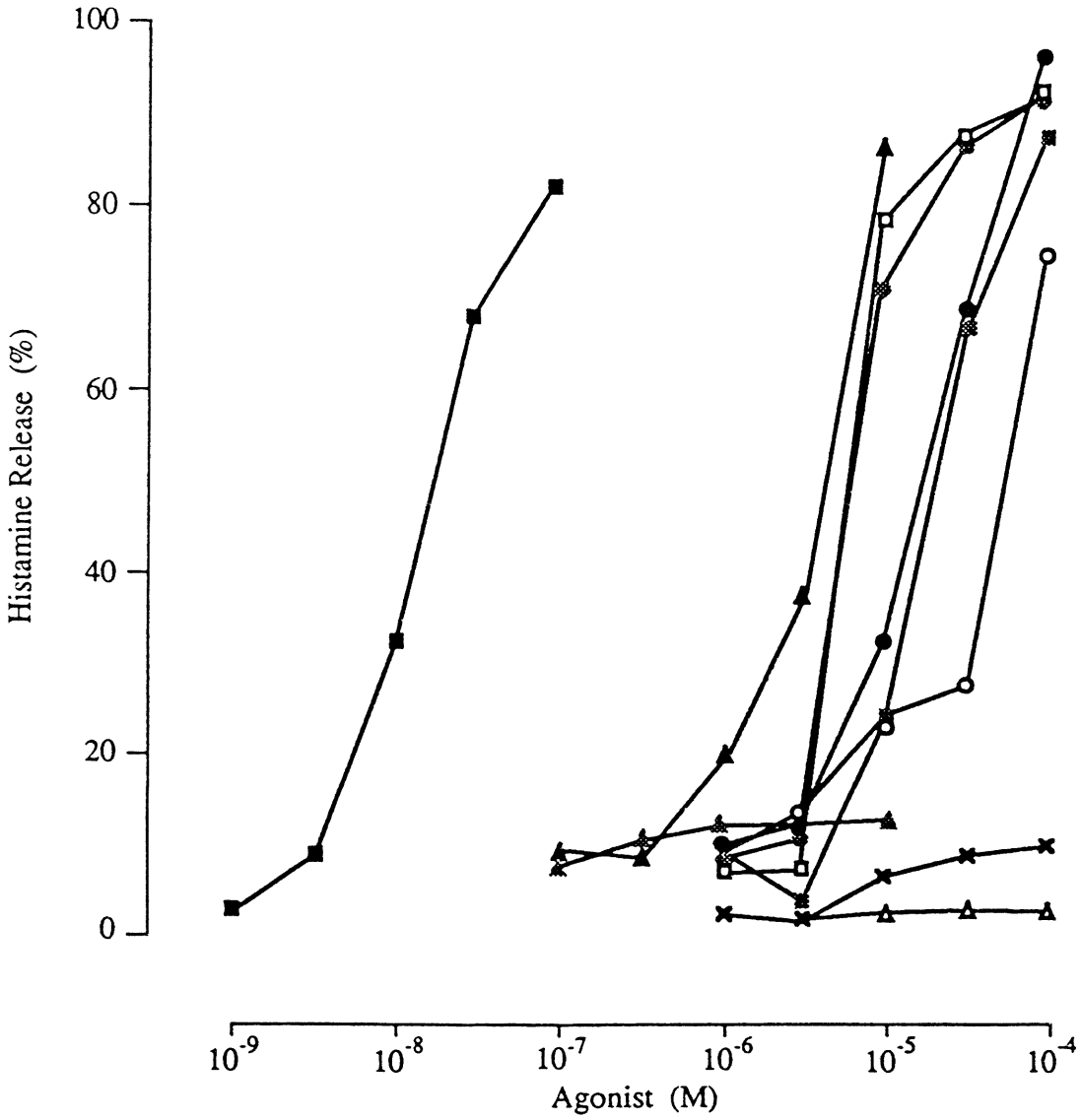


Fig. 5.22 Action of various polyaminic agonists on histamine release from rat peritoneal mast cells.
Somatostatin (hatched circles), substance P (closed circles), CGRP (open circles), PHM (hatched squares), polylysine (closed squares), VIP (open squares), neurotensin (hatched triangles), compound 48/80 (closed triangles), eledoisin (open triangles) and neurokinins A and B (open crosses). Error bars have been omitted for clarity but the SEM did not exceed ± 4.5. (n=4)

visible (Fig.5.23iii). Most surprisingly, several cells were apparently surrounded by a "cloud" of low intensity fluorescence. This effect seemed specific to cells from the mouse.
Mast cells from either the lung or the mesentery of the guinea pig were resistant to immunofluorescent staining after challenge with somatostatin. Due to the absence of fluorescence, photomicrographs of this system were not taken.

Fig. 5.22i Immunofluorescence of rat peritoneal mast cells treated with somatostatin, polyclonal rabbit anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[a] phase contrast, mangnification x63
[b] dark field, magnification x63

Fig. 5.23ii Immunofluorescence of hamster peritoneal mast cells treated with somatostatin, polyclonal rabbit anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[a] phase contrast, mangnification x63
[b] dark field, magnification x63

Fig. 5.23iii Immunofluorescence of mouse peritoneal mast cells treated with somatostatin, polyclonal rabbit anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[a] phase contrast, mangnification x63
[b] dark field, magnification x63

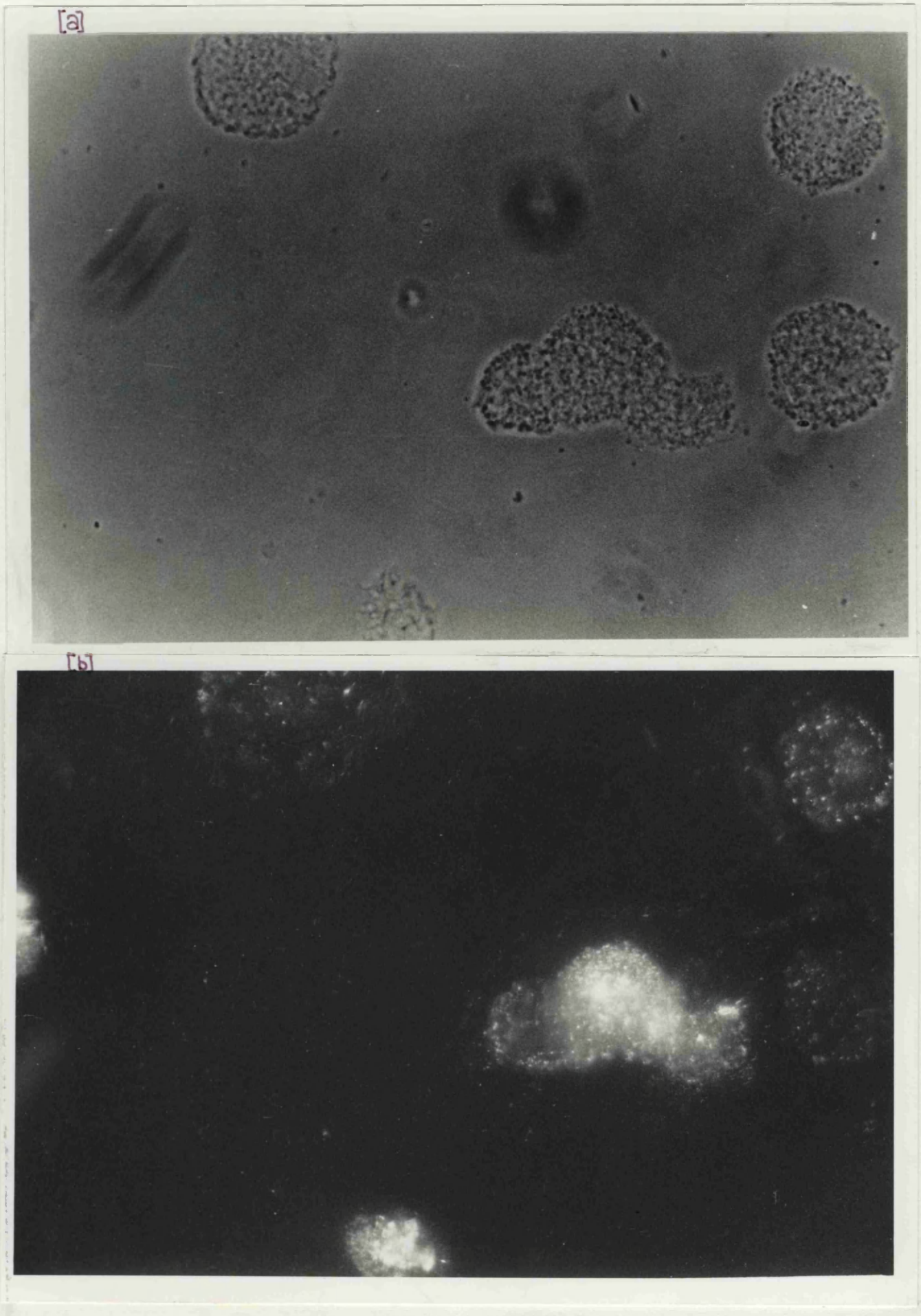


Fig. 5.23i

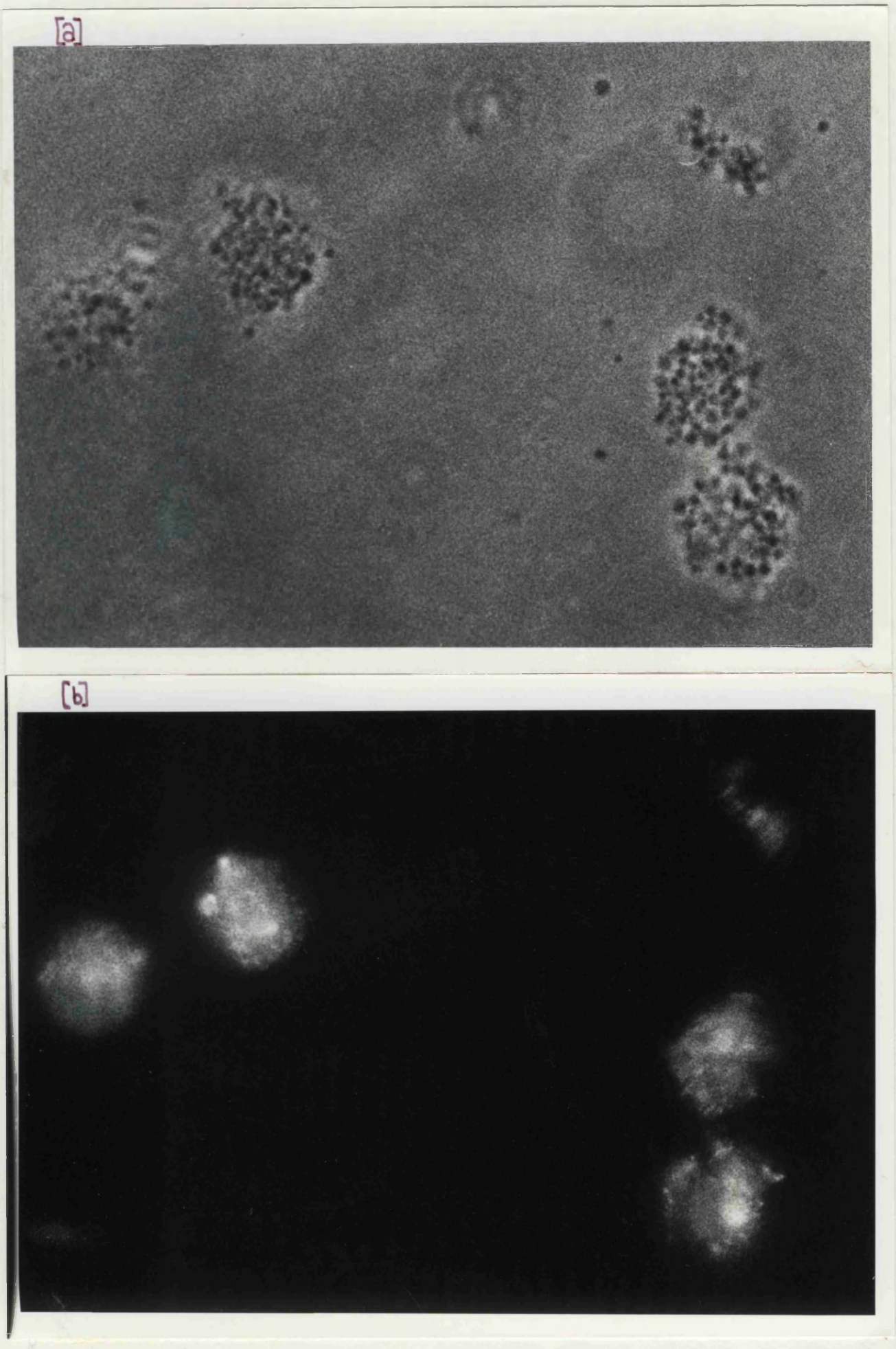


Fig. 5.23ii

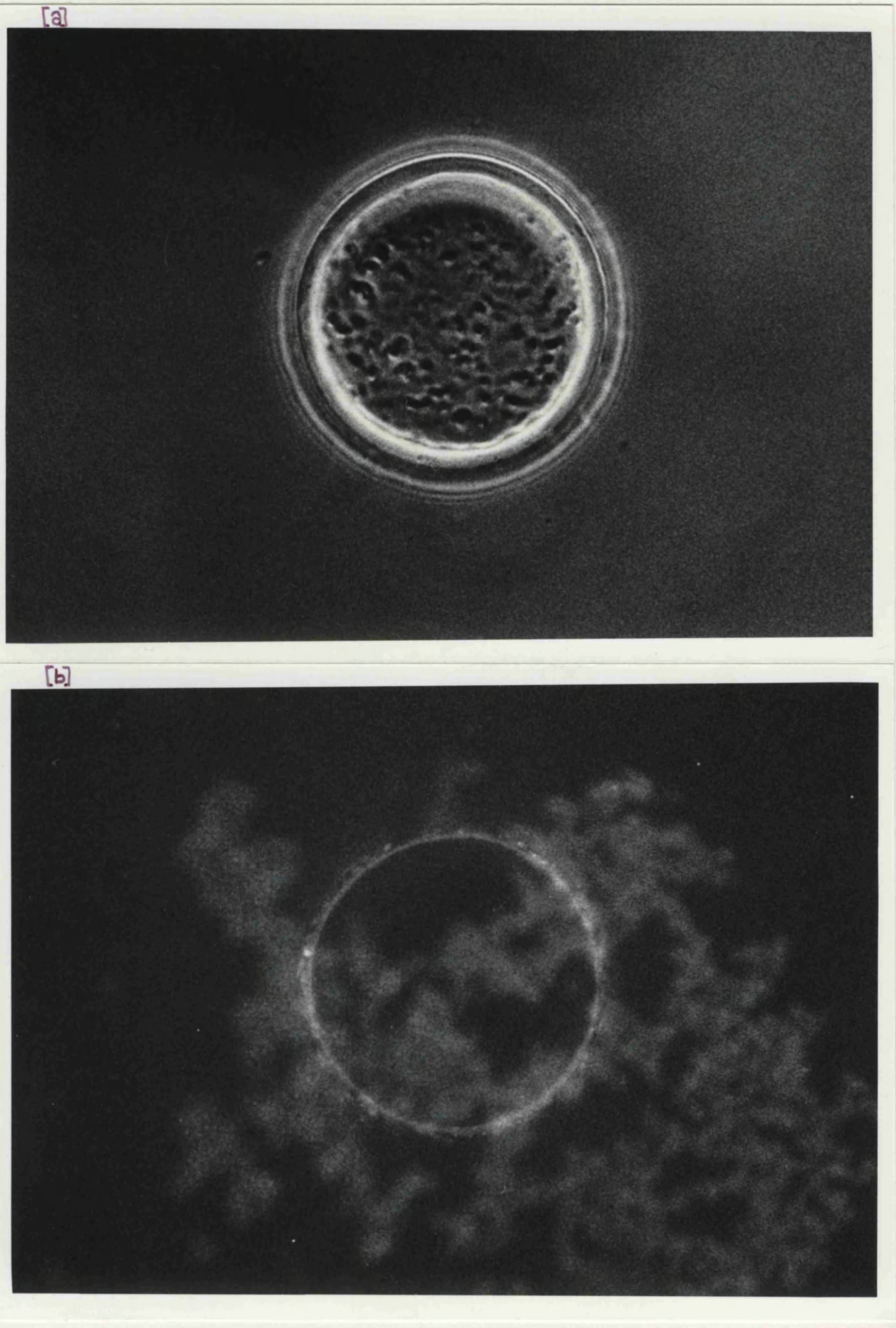


Fig. 5.23iii

5.3 *DISCUSSION*

It is now accepted that mast cells from different locations are functionally heterogeneous [3,38]. Studies performed with compound 48/80 have shown it to be a potent releaser of histamine from rat peritoneal mast cells and those of the pleural cavity [552]. It also produces significant release from rat mesenteric mast cells and, to a lesser extent, from pulmonary and cutaneous mast cells. However, cells from cardiac and intestinal sources are totally unresponsive. Tissue mast cells of the guinea pig are also completely unreactive, as are basophils and pulmonary mast cells of man, including mastocytes obtained by human bronchoalveolar lavage. Some response is, however, seen with human skin mast cells [553].

Somatostatin appears closely to resemble compound 48/80 in the selectivity of its action. Similar differential effects have also recently been observed with other polycations such as polylysine and substance P [554]. Under these circumstances, it is difficult to sustain a generalised model for neurogenic inflammation involving peptide-mast cell interactions in man, except possibly in the skin. Certainly, the lack of response from human lung and human bronchoalveolar lavage cells [552,this thesis] dismisses the possibility that the axon reflex occurs in the lung and is therefore not likely to participate in asthma or other aspects of airways disease. The so-called 'polyamine receptor' would then appear to exist largely on mast cells from murine tissues and human skin. Furthermore, within this limited selection of responsive cell types, a wide range of activities is manifest. Mast cells from the rat peritoneal and pleural cavities are the most responsive whereas those of the hamster and mouse are considerably less so. Mast cells from other tissues respond to polycation challenge even less readily.

Of the mastocytes tested, only rat peritoneal and pleural mast cells elicited optimal secretion on stimulation by somatostatin in the presence of extracellular calcium. This effect has been reported on compound 48/80- and peptide 401-induced histamine liberation from rat and mouse peritoneal mast cells [321]. Brief pretreatment with EDTA is considered to remove calcium ions from regulatory sites on the membrane, thus facilitating the release of more firmly sequestered stores of the cation into the cell cytosol [305]. This increase in intracellular calcium concentration may then initiate exocytosis. The regulatory pool of calcium may be less firmly bound in the mouse cell and subject to removal in simple calcium-free media. Alternatively, calcium ions could directly and more readily compete for the presumably anionic receptor for polycations on the mast cell membrane.

The relative responsiveness of the different mast cells to somatostatin was mirrored by fluorescence binding studies. Peritoneal mast cells of the mouse were not as strongly stained as those of the rat. Hamster peritoneal mast cells exhibited a low density, universal staining. However, discrete areas of intense fluorescence were evident. Universal staining is often the result of endocytosis of the FITC-complex. It is possible that the observed fluorescence may well be surface bound and not simply internal. A low density of binding sites may not initiate activation, even under conditions of full receptor occupancy and it may be that

some mechanism of 'receptor-aggregation' is also operative in polyamine-induced exocytosis. Localised regions of intense fluorescence would perhaps represent areas where receptor aggregation initiates secretion. Isolated guinea pig lung and mesenteric mast cells, which do not respond to somatostatin, did not stain with immunofluorescent antibodies to the peptide indicating that they probably do not bind the polyamine.

The immunofluorescent staining of mouse peritoneal mast cells produced a 'fall-out' of low intensity fluorescence around several of the cells. Control experiments precluded the possibility of non-specific staining and disrupted cells showed some evidence of granular staining. However, the antibodies were not blocked with disrupted mast cell incubates and so an element of non-specific binding to intracellular moieties cannot be excluded. It is perhaps of interest to note that somatostatin-like immunoreactivity has been reported in the human basophil [523] and that spin labelled compound 48/80 was shown to bind to the perigranular membrane of rat peritoneal mast cells. Therefore, this effect may be attributed to specific binding to polyamine receptors on the perigranular membrane or to the fact that the cells contain somatostatin which, on cellular disruption or degranulation, is expelled in some way into the extracellular space.

The ambient fluorescence noted in mouse mast cells may comprise dissociated somatostatin-FITC complexes. Somatostatin may be loosely bound to the membrane and easily dislodged by trauma or administered antibody. On the assumption that calcium and the polyamines compete for the same anionic binding sites, calcium may have a greater affinity for those sites than somatostatin in the mouse. Persistent extracellular calcium may competitively displace somatostatin which is already bound.

Experiments performed to explore the relative strength of the stimulus evoked by polyamines generated arresting results. The non-peptides, polylysine and compound 48/80 were more potent mast cell agonists than the neuropeptides. Of the series of agents tested, only neurokinin A and B and eledoisin failed to stimulate rat peritoneal mast cells. The rank order of potency for the agents was established to be polylysine > compound 48/80 > somatostatin = VIP > substance P = PHM > CGRP > neurotensin.

The ability of such a variety of agents to evoke histamine release from the rat peritoneal mast cell illustrates the non-specific nature of the putative polyamine receptor. Moreover, the relatively weak activity of the neuropeptides even against this receptive mast cell phenotype may mitigate further against the relevance of such interactions *in vivo*.

CHAPTER 6

INVESTIGATIONS INTO THE MECHANISM OF BENZYLDIMETHYLTETRADECYL AMMONIUM CHLORIDE-INHIBITION OF POLYAMINE-INDUCED HISTAMINE SECRETION

6.1 *INTRODUCTION*

To understand better the compound 48/80 induction system and to develop antagonists of polyamine-induced histamine release, Read and Kiefer [528] screened various chemical structures for inhibitory activity against compound 48/80. Benzalkonium chloride (BAC) is a family of quaternary ammonium compounds widely used as an antiseptic and disinfectant. The presence of both hydrophobic and cationic groups in the structure gives it surface active properties, which are thought to be responsible for its germicidal activity [555].

Read and Kiefer showed that BAC selectively inhibited histamine release induced by compound 48/80 and other polyamine secretagogues (bradykinin, curare, guanethidine, polylysine, polymyxin B, poly-tetrahydroisoquinoline, protamine, stilbamidine and substance P). BAC was inactive against secretion induced by immunologic agents (antigen, concanavalin A, dextran), ionophores (A23187, X-537A), enzymes (chymotrypsin, phospholipase C), lytic agents (decylamine, Triton X-100, fire ant venom) or monoamines (chlorpromazine, dextromethorphan, meperidine).

BAC induced histamine liberation itself at concentrations greater than 3μg/ml (7.5μM). BAC will liberate histamine from heat inactivated mast cells and, therefore, it appears that the effect is brought about by a nonspecific lytic action. The effect of inhibitory concentrations of BAC on different concentrations of compound 48/80 caused a parallel shift to the right of the compound 48/80 dose-response curves, with no loss of efficacy. This indicated an inhibition that was surmountable, which is suggestive of a competitive interaction at the polyamine receptor. The most potent of the BAC family is obtained when the alkyl side chain contained 14 to 16 carbon atoms. These data would support the suggestion that the inhibitory action is dependent on specific structural considerations and the lytic effect is related to non-specific surface activity.

In another communication [509], Read, Hong and Kiefer reported studies with BAC and several of its analogues. Although the most potent analogue was benzyldimethyltridecyl-ammonium chloride, the tetradecylammonium chloride (BDTA) is almost as potent and commercially available. The authors performed *in vitro* experiments on the inhibitory action of BDTA on histamine release induced by compound 48/80. On presentation of this data as a double reciprocal plot, the intersection of dose-response lines occurs at the ordinate. This is generally accepted as *prima facie* evidence of a competitive interaction (Lineweaver and Burk [556]).

The putative compound 48/80 receptor is located on the outer surface of the cell membrane [334] and it is assumed that the cell membrane is the site of this competitive interaction. Structure-activity studies proved that the benzyl group of BAC was not essential for inhibition to occur. However, an increase in the polarity of the benzyl group reduced inhibition. Removal of the positive charge on the quaternary ammonium and redistribution of hydrophobicity in the alkyl chain lowered the inhibition profoundly. Replacement of the nitrogen atom with either phosphorus or sulphur had no effect on the inhibitory potency but raised the toxicity of the molecule.

It would then appear that the polyamine receptor is susceptible to a wide range of molecular structures, provided there is an alkyl chain of sufficient length to allow for surface activity, facilitating anchorage in a hydrophobic environment. The binding of BAC to the membrane of mast cells seems to preclude the binding of compound 48/80. As such, the polyamine receptor appears more sensitive to the number of positive charges and their distribution, than to the shape of the carrier molecule.

The present study was designed to investigate further the nature of the inhibition produced by BDTA. In particular, the kinetics, the possible contribution of cytotoxicity, the persistence and surmountable nature of the antagonism was examined. The possible inhibition produced by other quaternary ammonium compounds was also examined and the displacement of somatostatin from the mast cell membrane by BDTA was tested by fluorescence microscopy.

6.2 *RESULTS*

6.2.1 Investigations into the kinetics of selective inhibition

The time course of BDTA (3μg/ml) inhibition on histamine release induced by various agents was examined. The agonists were added at time intervals after the introduction of BDTA to the cells and the resultant inhibition calculated. Histamine release induced by compound 48/80 (0.3μg/ml), substance P (15μg/ml) and somatostatin (7.5μg/ml) was almost completely inhibited by BDTA, independent of the time of addition of the agonist (Fig.6.21). In contrast, the release stimulated by the immunologic ligands such as anti-IgE (100-fold dilution) and concanavalin A (10μg/ml) was potentiated on simultaneous administration of the agonist and antagonist. However, this potentiation was progressively diminished on extended preincubation with the antagonist before challenge, culminating in potent inhibition on preincubation times of 30 seconds.

6.2.2 Investigation into BDTA inhibition as an effect of toxicity

Experiments were conducted to establish whether the inhibition produced by BDTA was a result of metabolic poisoning. Under these conditions, the effect might be expected to be reversed by increasing concentrations of extracellular glucose. Inhibition was produced by preincubating (5 min) the cells with BDTA before immunologic challenge, and by adding the compound simultaneously with polyamines. Under these conditions,

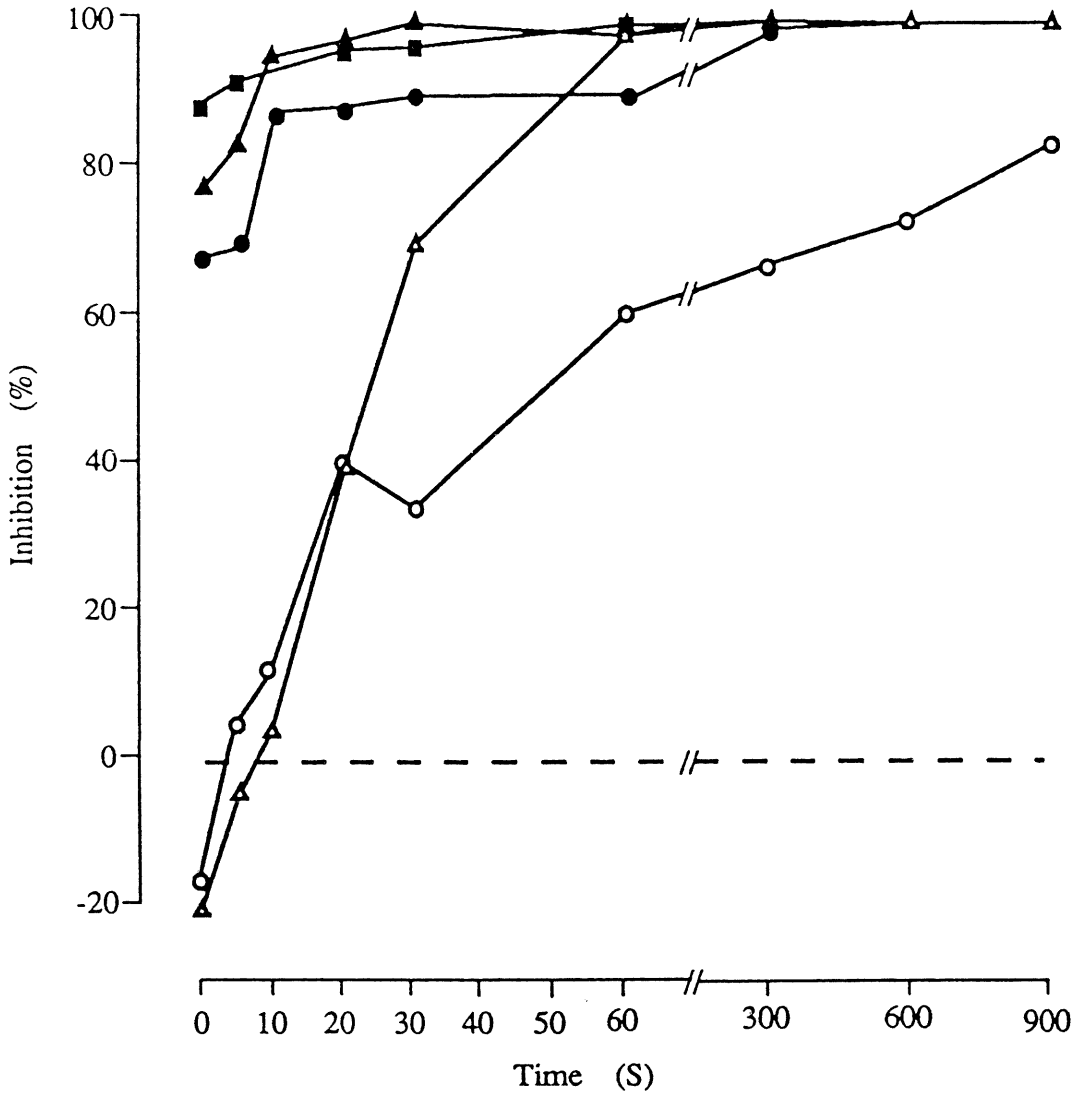


Fig. 6.21 Kinetics of BDTA (3μg/ml) inhibition of histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 41.1±1.9, closed circles) substance P (15μg.ml, control release 38.7±2.2, closed triangles) compund 48/80 (0.3μg/ml, control release 44.4±2.7, closed squares) anti-IgE (100-fold dilution, control release 36.3±3.1, open circles) concanavalin A (10μg/ml, control release 32.5±3.0, open triangles).
Error bars have been omitted for clarity but SEM did not exceed ± 6.2 (n=4)

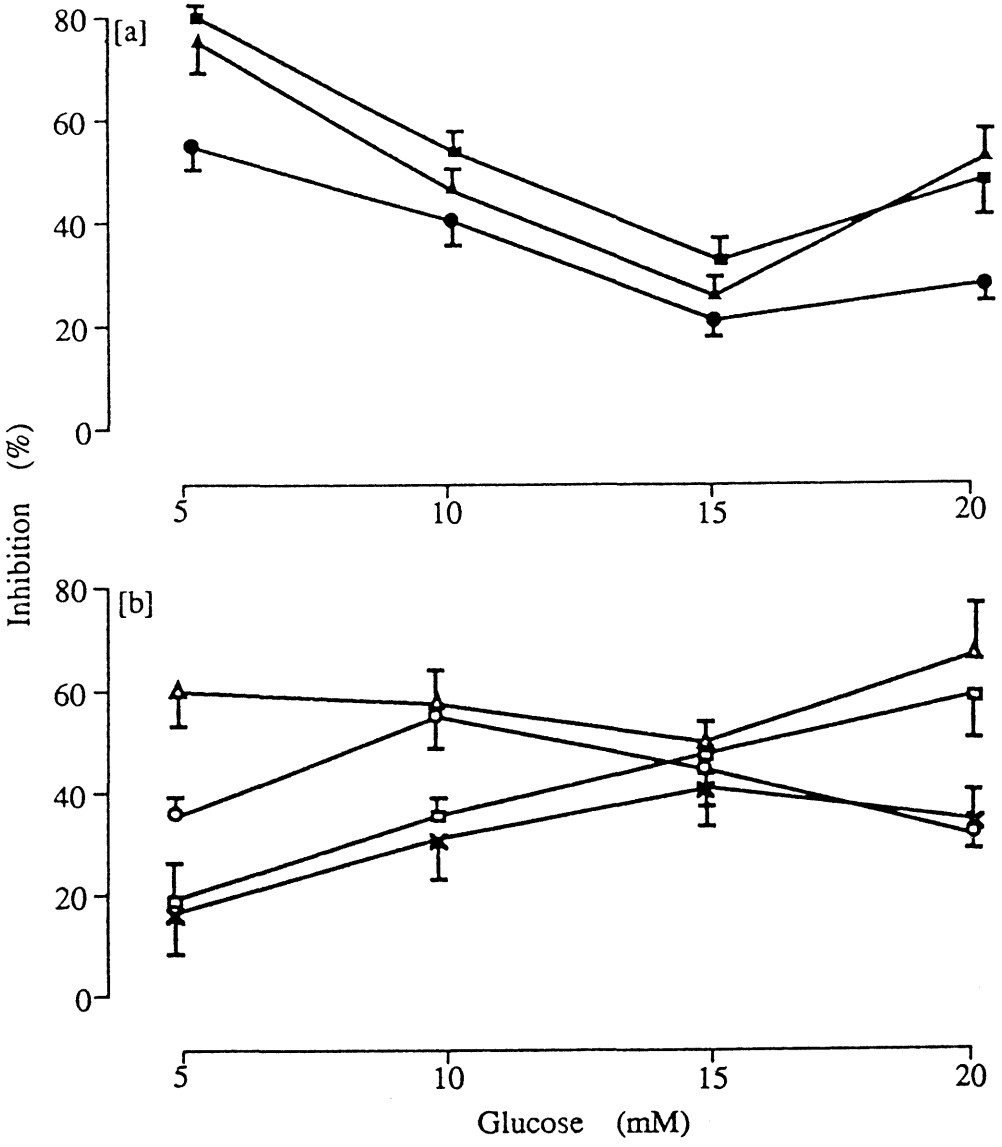


Fig 6.22 Investigation into BDTA (1μg/ml) inhibition of [a] immunologic and [b] polyamine-induced histamine release from rat peritoneal mast cells as an affect of toxicity.
Antigen (10WE/ml, control release 39.2±3.1, closed circles) Concanavalin A (20μg/ml, control release 40.3±2.7, closed triangles) Anti-IgE (300-fold dilution, control release 36.6±3.3, closed squares) Somatostatin (7.5μg/ml, control release 44.6±2.0, open circles) Substance P (15μg/ml, control release 48.1±2.7, open triangles) Polylysine (1μg/ml, control release 49.2±2.9, open squares) and compound 48/80 (0.1μg/ml, control release 41.1±3.2, open crosses)

All values ± SEM (n=4)

increasing amounts of glucose (5-15 mM) reduced the inhibition produced by BDTA in the cases of antigen, concanavalin A and anti-IgE, although this trend was reversed at higher concentrations of the monosaccharide (20 mM) (Fig.6.22). However, glucose had no consistent effect on polyamine induced secretion.

6.2.3 Investigations into the persistence of BDTA surface activity

Experiments were conducted to determine the effects of washing BDTA-treated mast cells, on histamine release induced by a variety of secretagogues. Cells were incubated with BDTA (5min), washed free of the inhibitor with buffer (2ml, x5) and then challenged with the secretagogue.

The inhibition of histamine liberation induced by compound 48/80 (0.2μg/ml), somatostatin (7.5μg/ml) and substance P (15μg/ml) was found to persist, even after repeated washing of the cells, prior to challenge. However, there was a some reduction in the inhibition produced by the top concentration of BDTA tested (Fig.6.23a). Similar results were obtained on stimulation of the cells with anti-IgE (300-fold dilution) and concanavalin A (20μg/ml) (Fig.6.23b).

6.2.4 Investigations into the competitive nature of BDTA antagonism

The dose-response relationships in the presence and absence of BDTA were determined for compound 48/80, polylysine, substance P and somatostatin. The action of BDTA on polymyxin B induced release was also examined. The concentrations of polymyxin B are quoted in micrograms per millilitre, due to an undefined molecular weight for the polymer. Figs.6.24a to 6.24e illustrate that all the polyamine agonists tested exhibit a shift to the right in their dose-response curves in the presence of varying concentrations of BDTA. This defines the inhibition as surmountable.

The Schild plots for the agonists investigated are shown in Figs.6.24f to 6.24j. Fig.6.24k lists the pA_2 values in tabular form, calculated for each agonist from the Schild plots.

Further experiments were performed on histamine release induced by compound 48/80 and polylysine in the presence and absence of varying concentrations of BDTA. These experiments were identical to those performed earlier, except in far greater detail with respect to the number of agonist concentrations investigated. The parallel-shift curves obtained showed the same, surmountable inhibition previously recorded. From these data (Figs.6.24l,m) Lineweaver-Burk plots were drawn for both agonists (Figs.6.24n,o). Data lines appear to intercept the ordinate at 0.01, the reciprocal maximal release. The slopes of the lines are seen to increase with the increase in BDTA concentration.

6.2.5 Effects of other quaternary ammonium compounds

To investigate further the structural requirements for inhibition of histamine release induced by polyamines and other stimuli, a range of additional quaternary ammonium compounds were tested. Choline (100,1000μM) had no effect on the histamine release

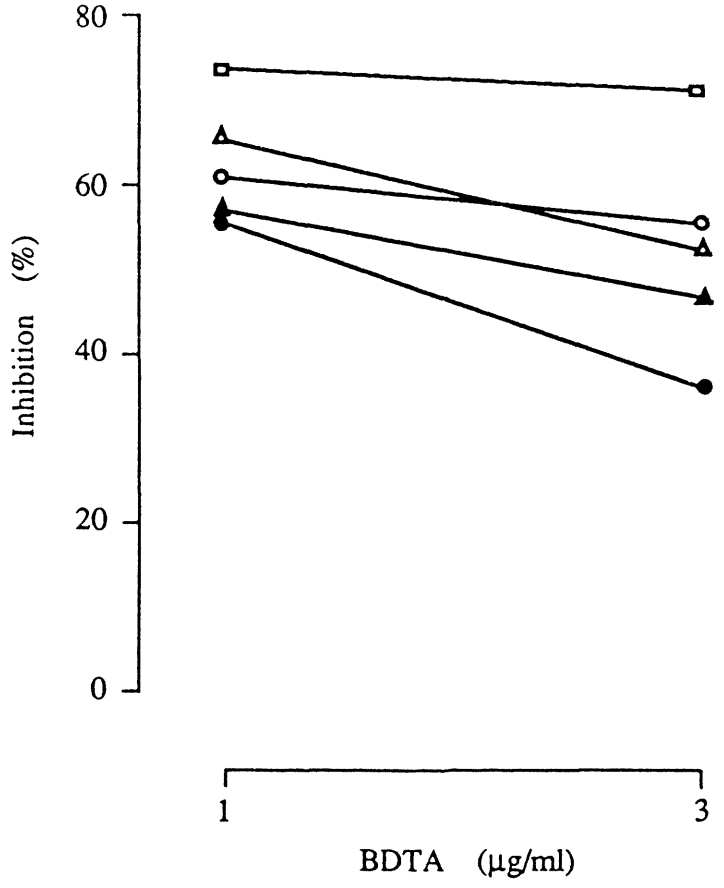


Fig. 6.23 BDTA inhibition of histamine release from rat peritoneal mast cells washed before challenge with [a] polyaminic ligands - somatostatin (7μg/ml, control release 46.7±2.7, open circles) substance P (15μg/ml, control release 48.3±3.1, open triangles) compound 48/80 (0.1μg/ml, control release 44.2±1.9, open squares)
[b] immunologic ligands - concanavalin A (20μg/ml, control release 39.8±2.6, closed triangles) and anti-IgE (300-fold dilution, control release 38.4±3.6, closed circles)
Error bars have been omitted for clarity but SEM did not exceed ± 7.3 (n=4)

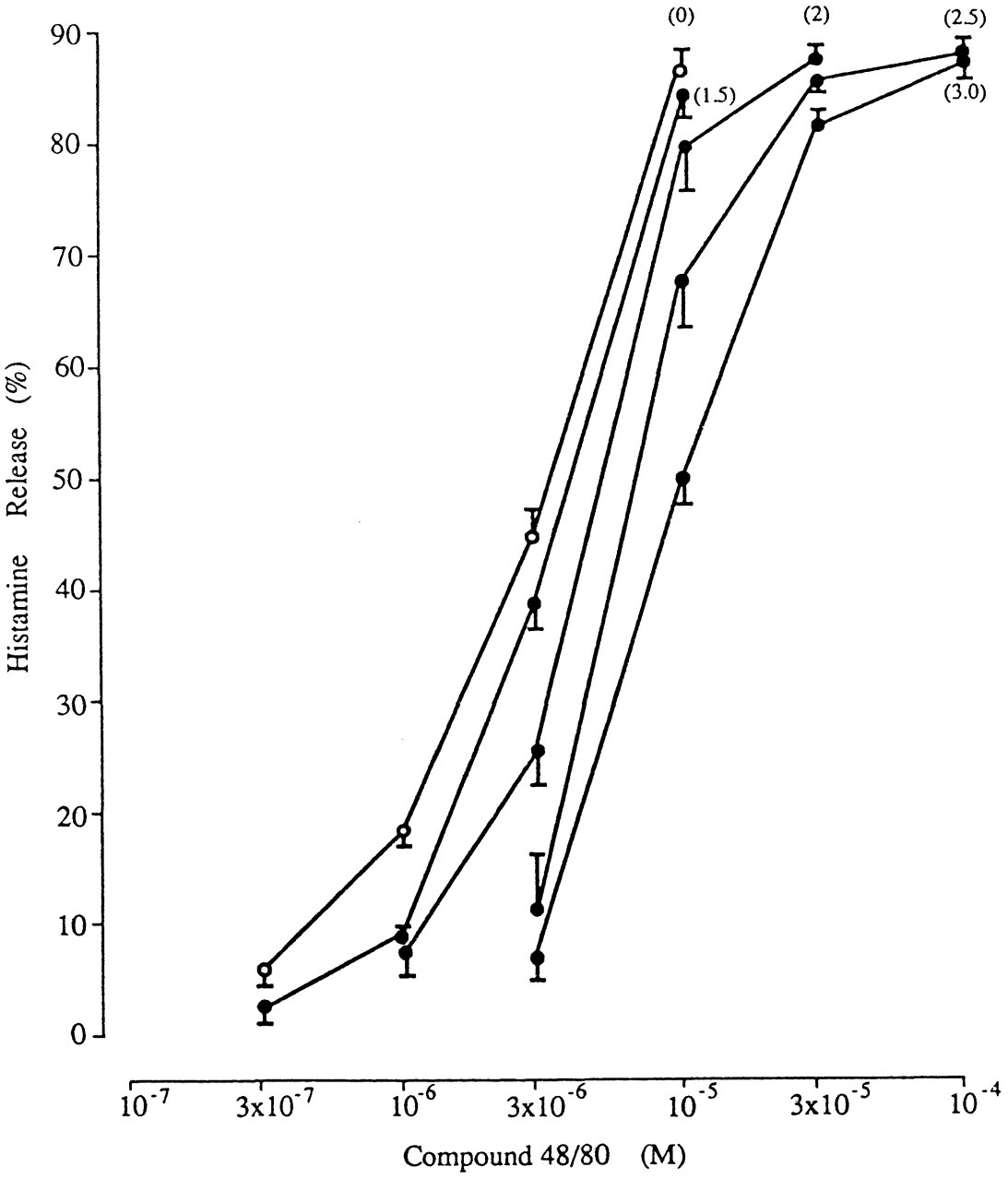


Fig. 6.24a Effects of varying concentrations of BDTA (μg/ml) on the dose response curve of histamine release from rat peritoneal mast cells induced by compound 48/80. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist.

All values ± SEM (n=9)

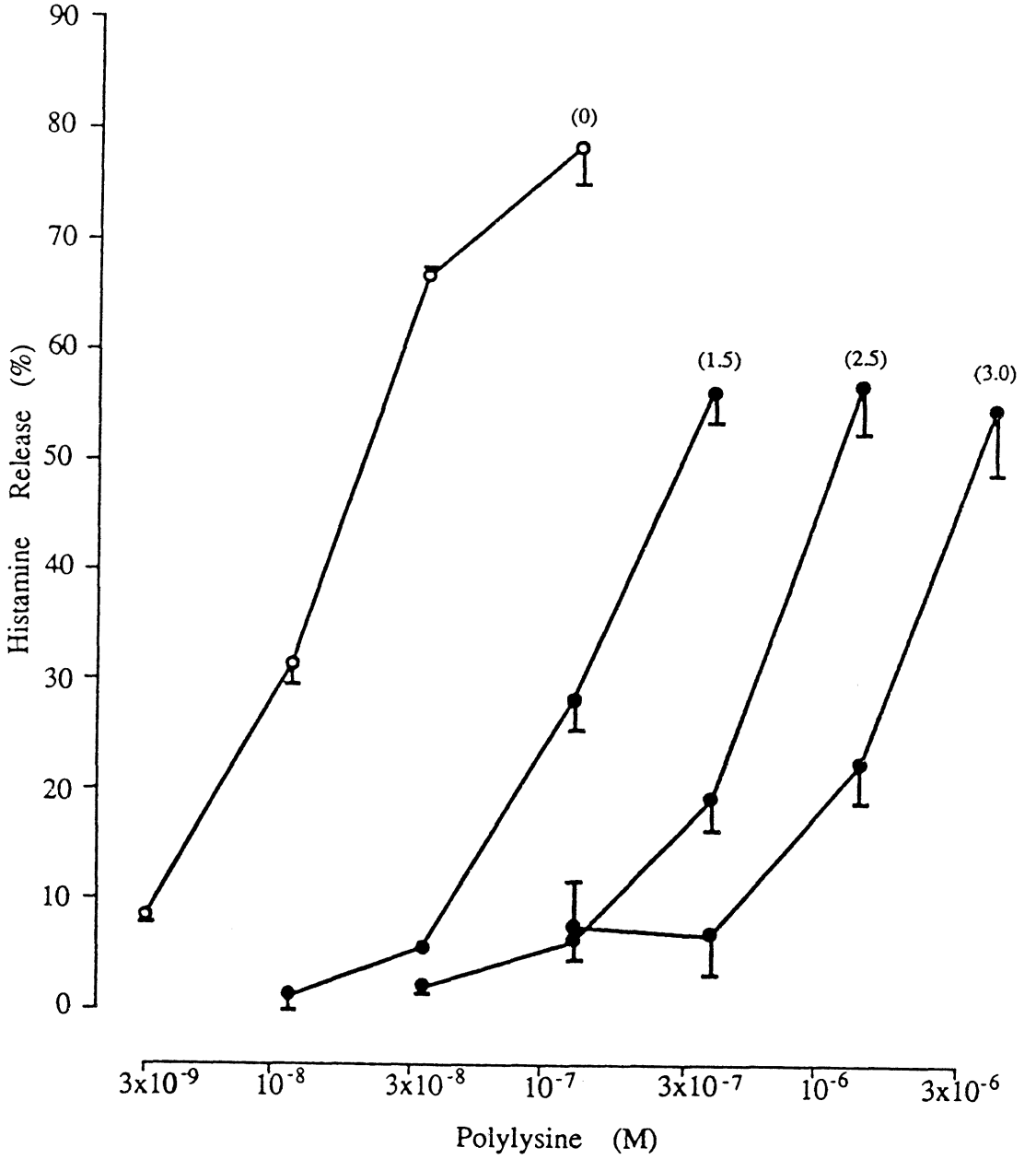


Fig. 6.24b Effects of varying concentrations of BDTA (μg/ml) on the dose response curve of histamine release from rat peritoneal mast cells induced by polylysine. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist.

All values ± SEM (n=11)

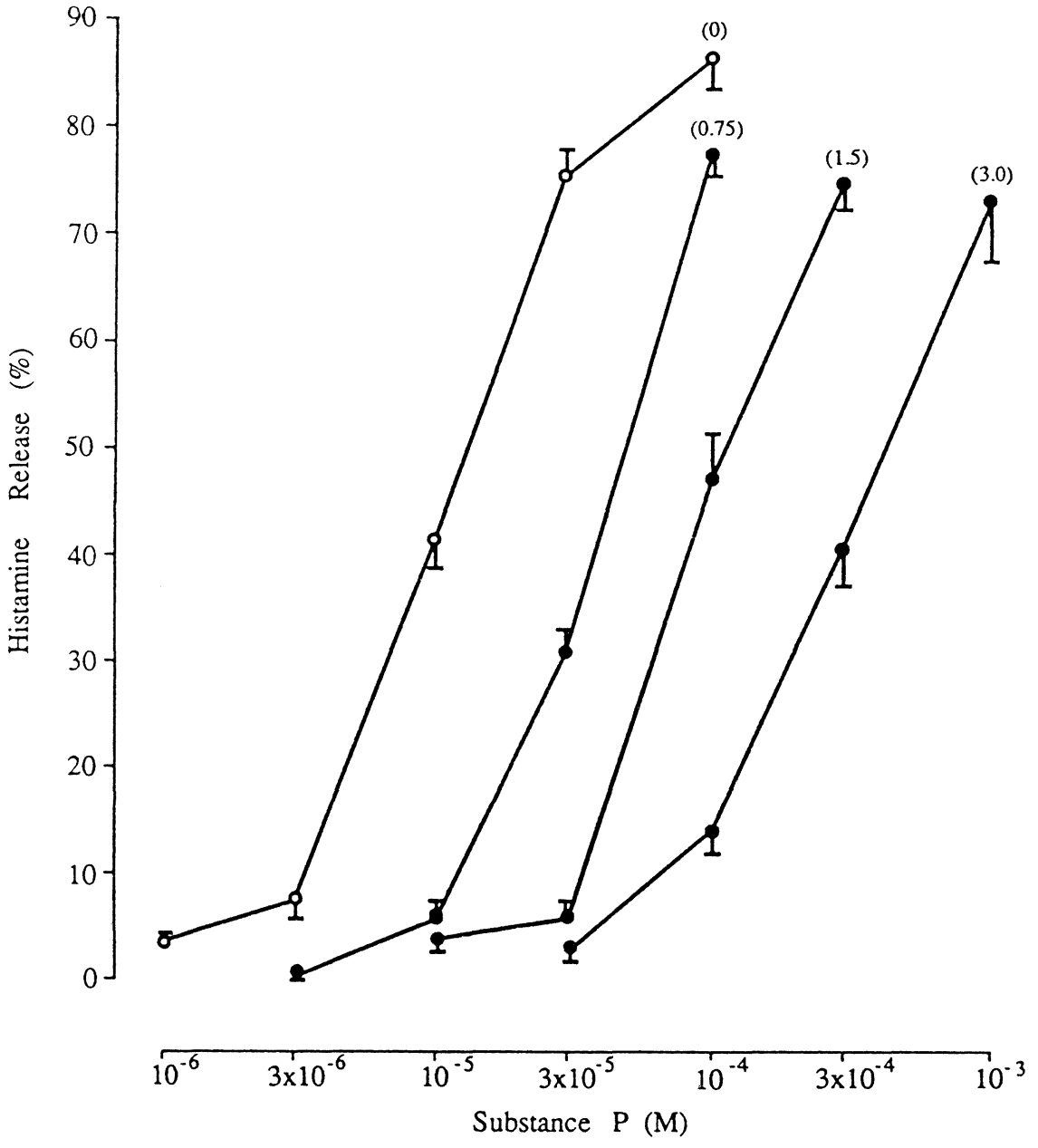


Fig. 6.24c Effects of varying concentrations of BDTA (μg/ml) on the dose response curve of histamine release from rat peritoneal mast cells induced by substance P. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist.

All values ± SEM (n=4)

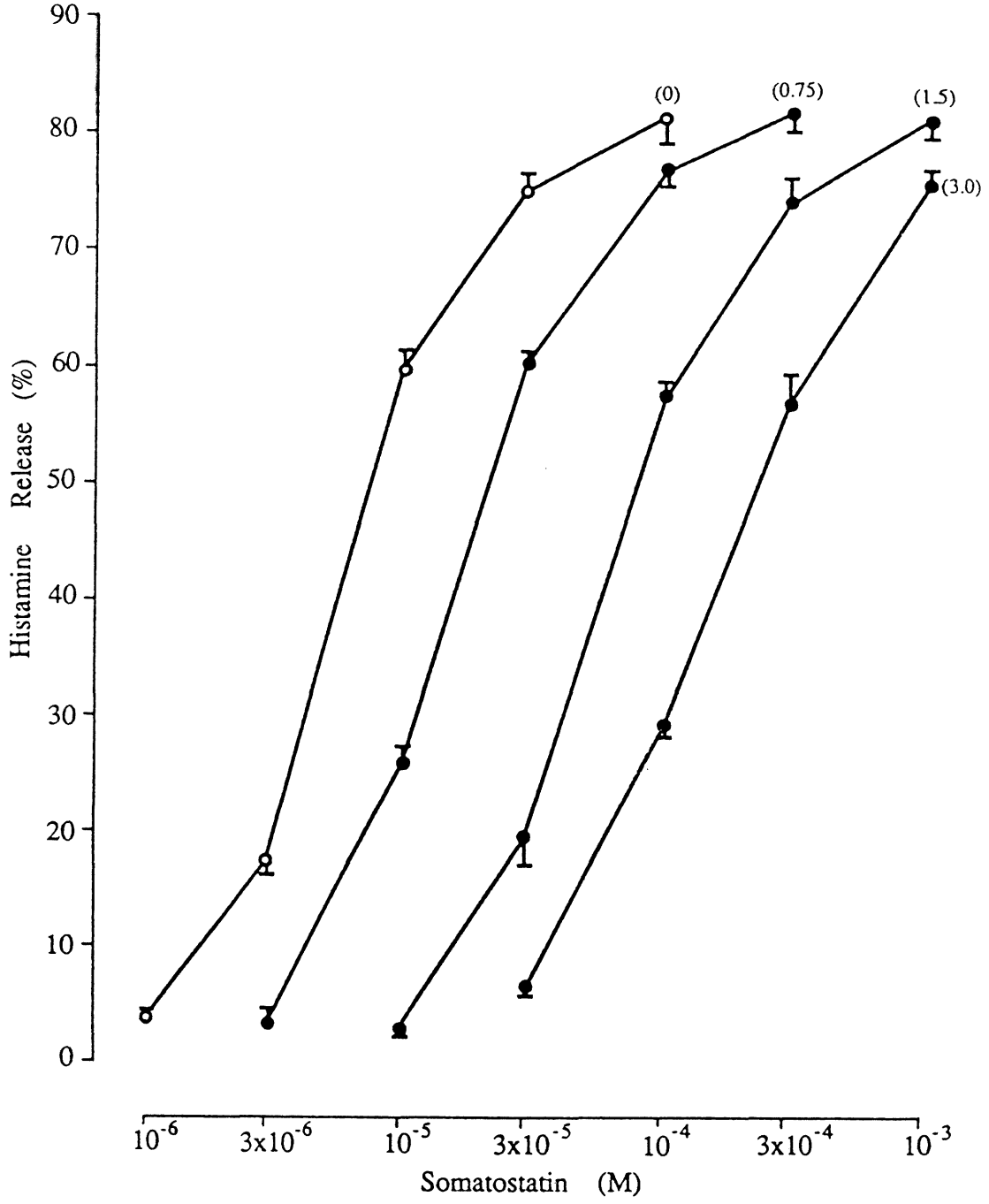


Fig. 6.24d Effects of varying concentrations of BDTA (μg/ml) on the dose response curve of histamine release from rat peritoneal mast cells induced by somatostatin. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist.

All values ± SEM (n=4)

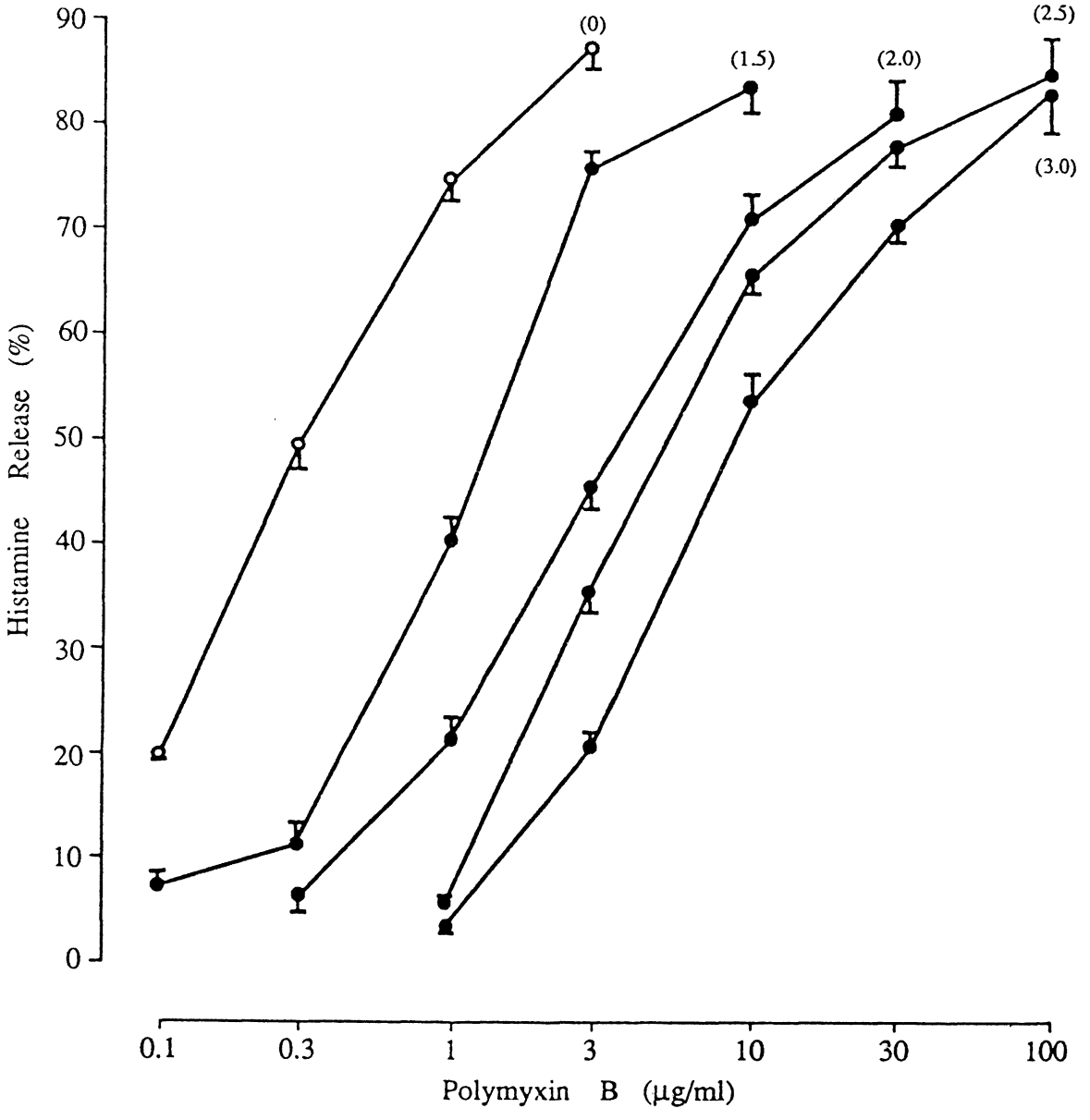


Fig. 6.24e Effects of varying concentrations of BDTA (μg/ml) on the dose response curve of histamine release from rat peritoneal mast cells induced by polymyxin B. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist.

All values ± SEM (n=4)

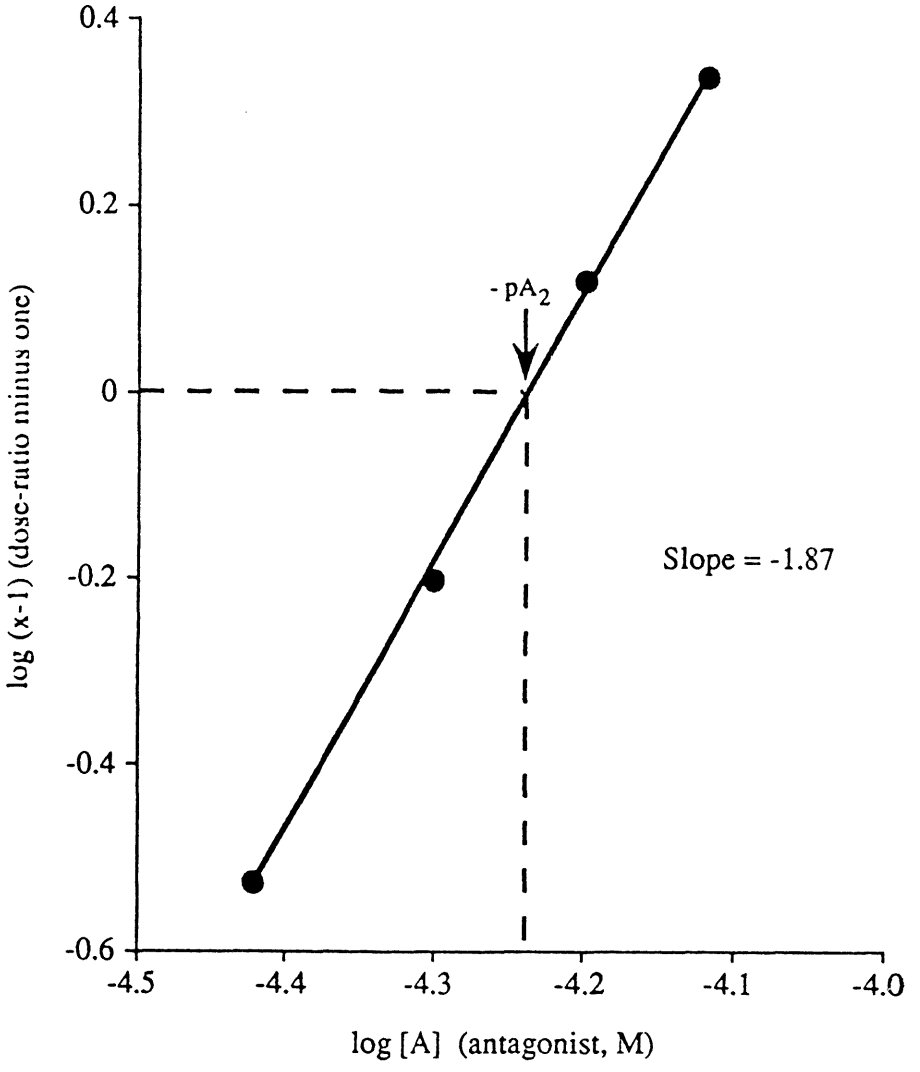


Fig. 6.24f Schild plot for BDTA antagonism of compound 48/80. Points were obtained from Fig 6.24a. pA_2 was evaluated as 4.23

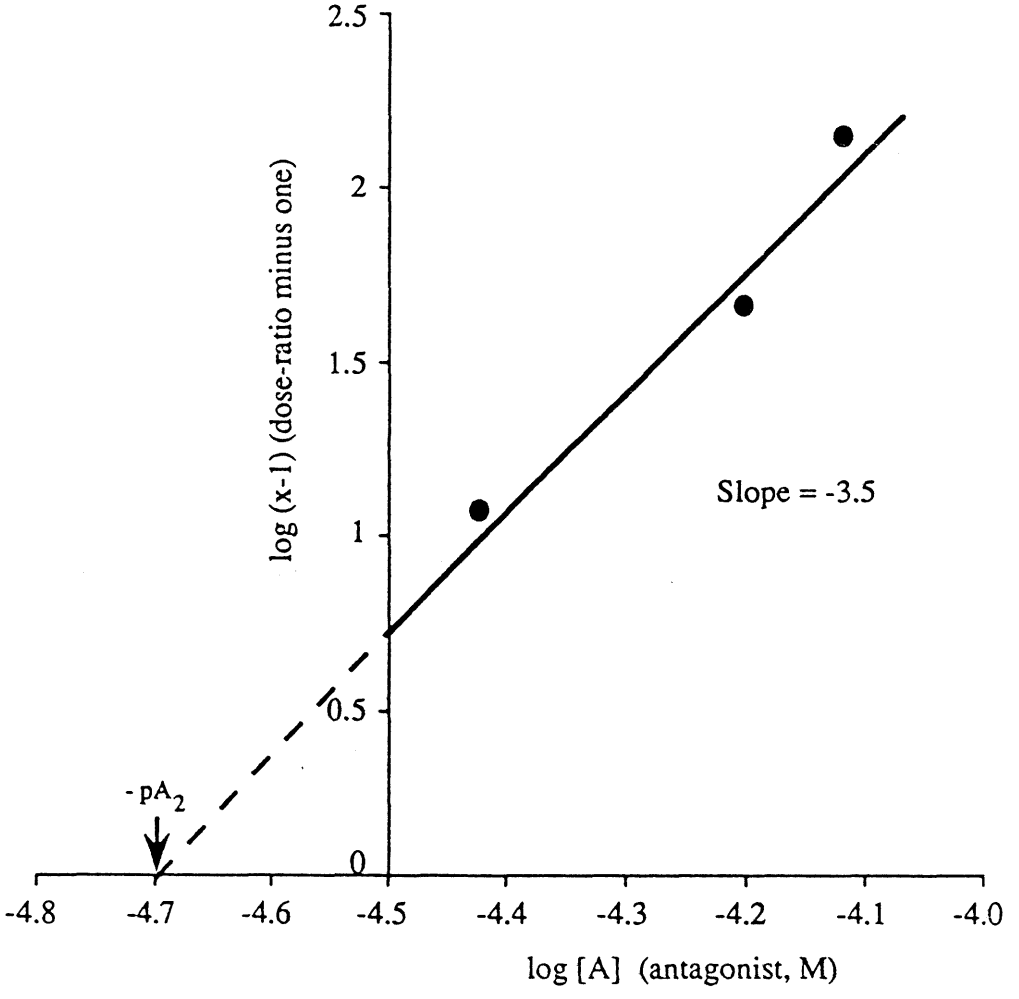


Fig. 6.24g Schild plot for BDTA antagonism of polylysine. Points were obtained from Fig 6.24b. pA_2 was evaluated as 4.69

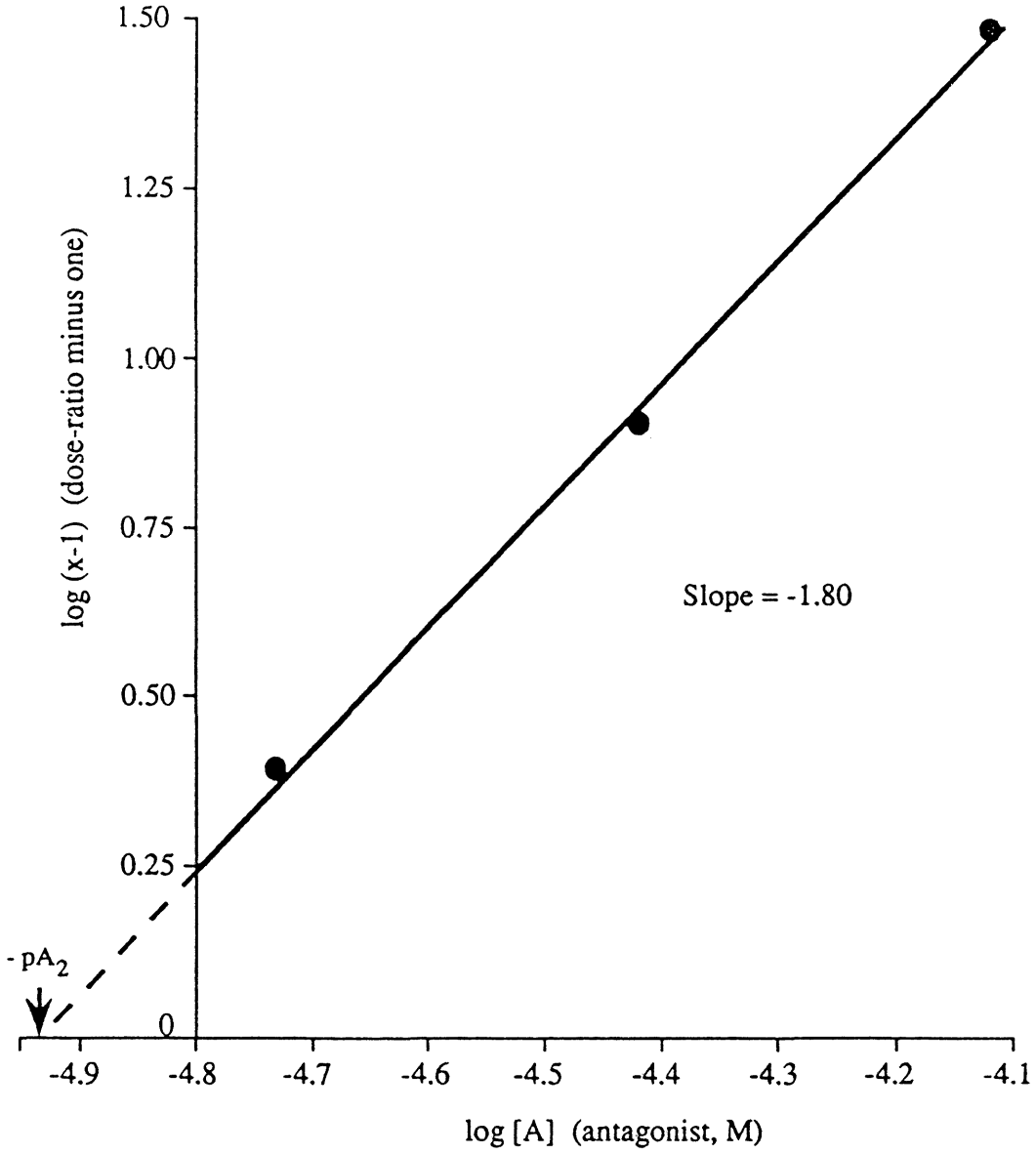


Fig. 6.24h Schild plot for BDTA antagonism of substance P. Points were obtained from Fig 6.24c. pA_2 was evaluated as 4.93

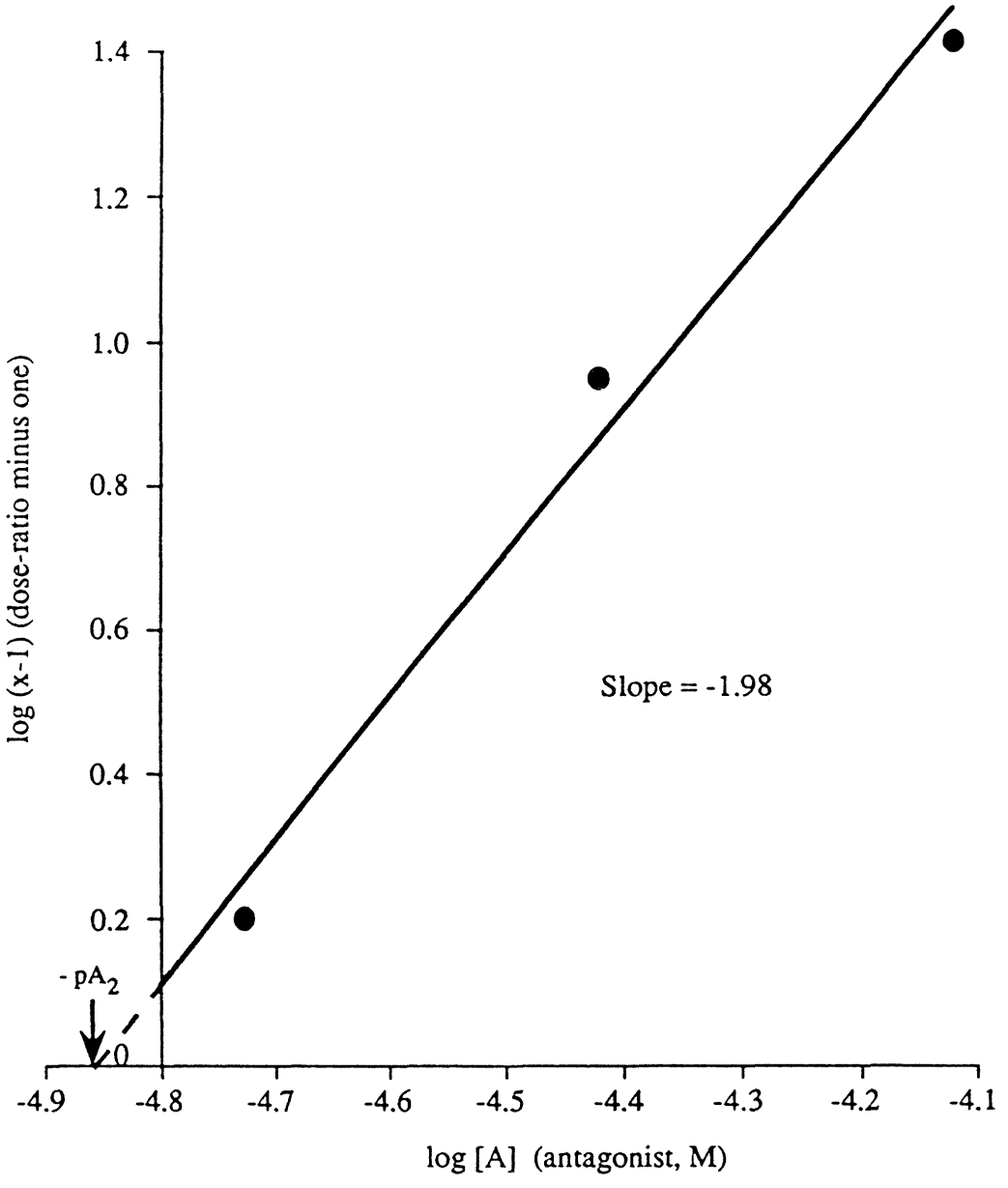


Fig. 6.24i Schild plot for BDTA antagonism of somatostatin. Points were obtained from Fig 6.24d. pA_2 was evaluated as 4.85

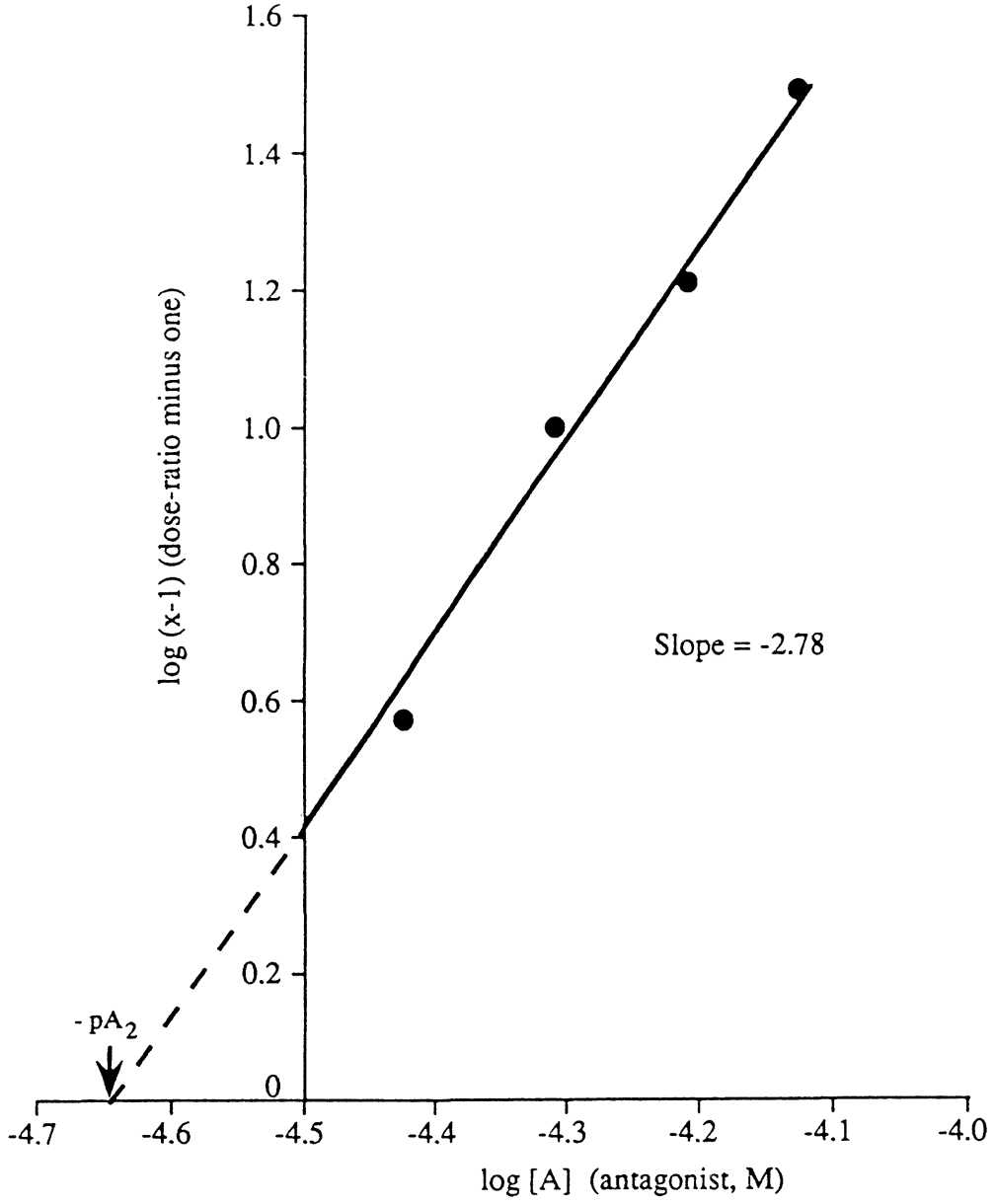


Fig. 6.24j Schild plot for BDTA antagonism of polymyxin B. Points were obtained from Fig 6.24e. pA_2 was evaluated as 4.64

AGONIST	pA_2
Compound 48/80	4.23
Polylysine	4.69
Substance P	4.93
Somatostatin	4.85
Polymyxin B	4.64

Fig. 6.24k Table of pA_2 values calculated for BDTA antagonism of histamine release from rat peritoneal mast cells induced by various polyamines.

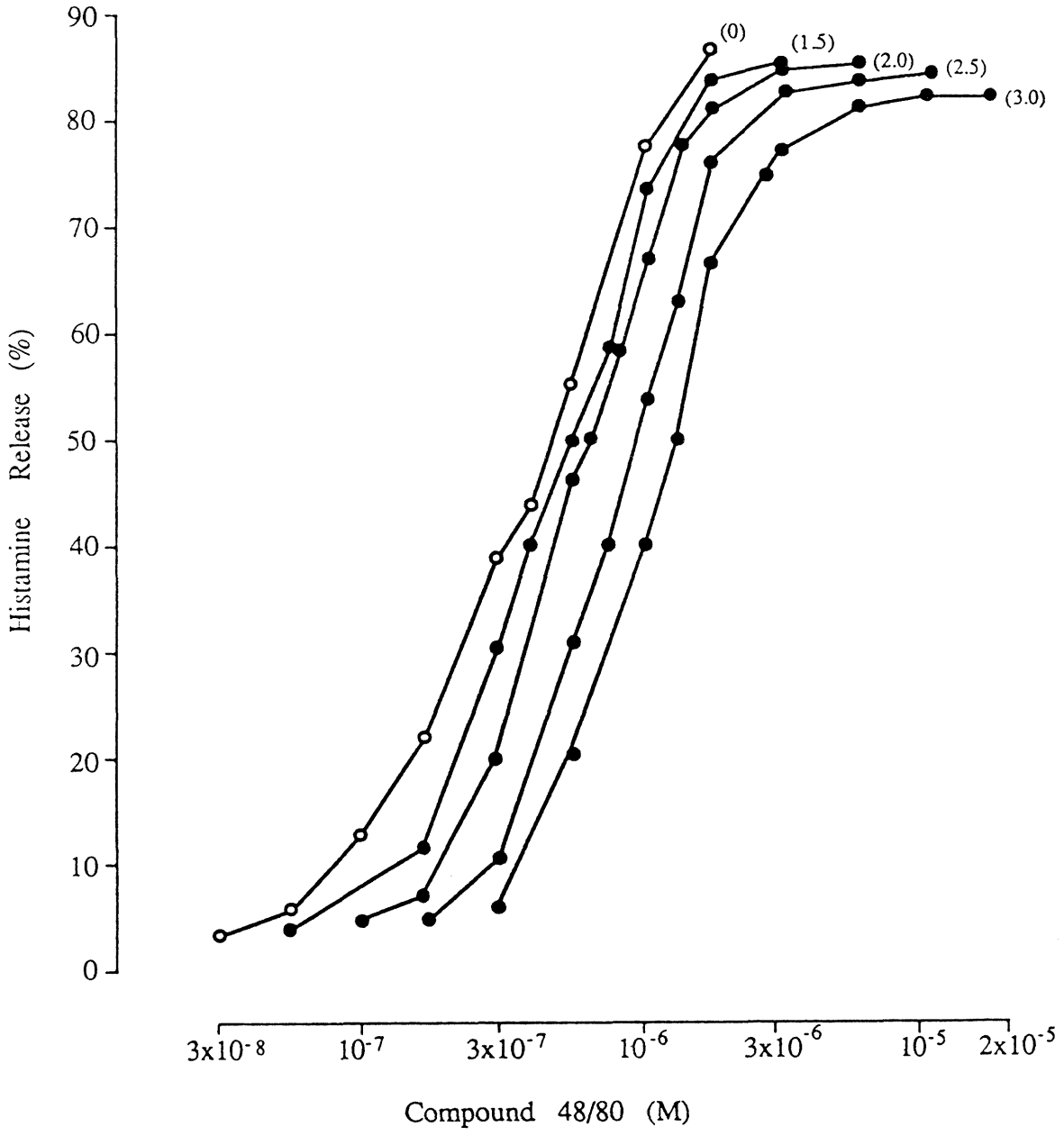


Fig. 6.24l Extended Schild plot for BDTA antagonism of compound 48/80-induced histamine release from rat peritoneal mast cells for use in Lineweaver-Burk analysis. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist. Error bars have been omitted for clarity but SEM did not exceed ± 6.0. (n=8)

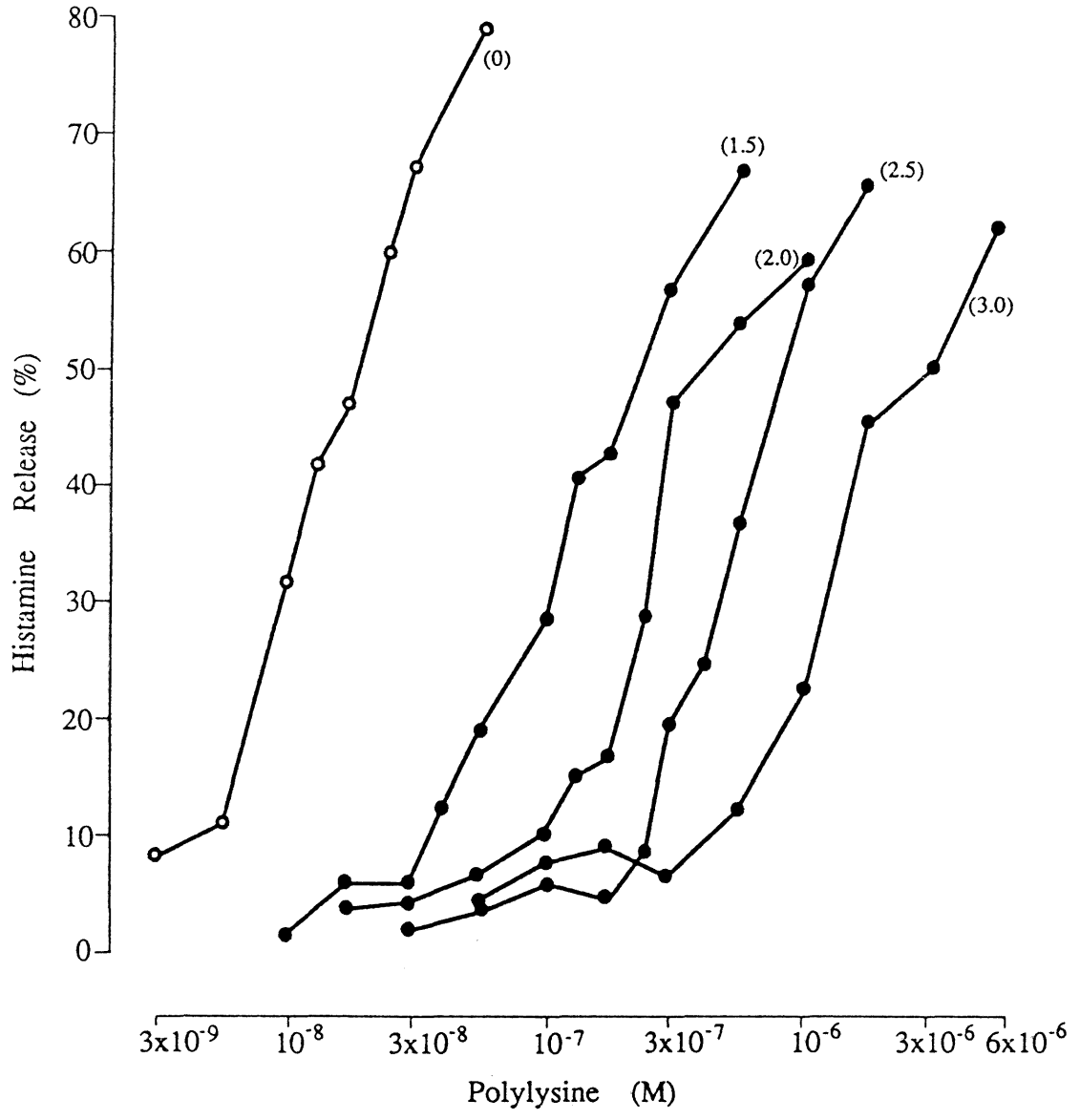


Fig. 6.24m Extended Schild plot for BDTA antagonism of polylysine-induced histamine release from rat peritoneal mast cells for use in Lineweaver-Burk analysis. Open symbols represent the response in the absence of the antagonist and closed symbols represent the response in the presence of the antagonist. Error bars have been omitted for clarity but SEM did not exceed ± 7.7 (n=9)

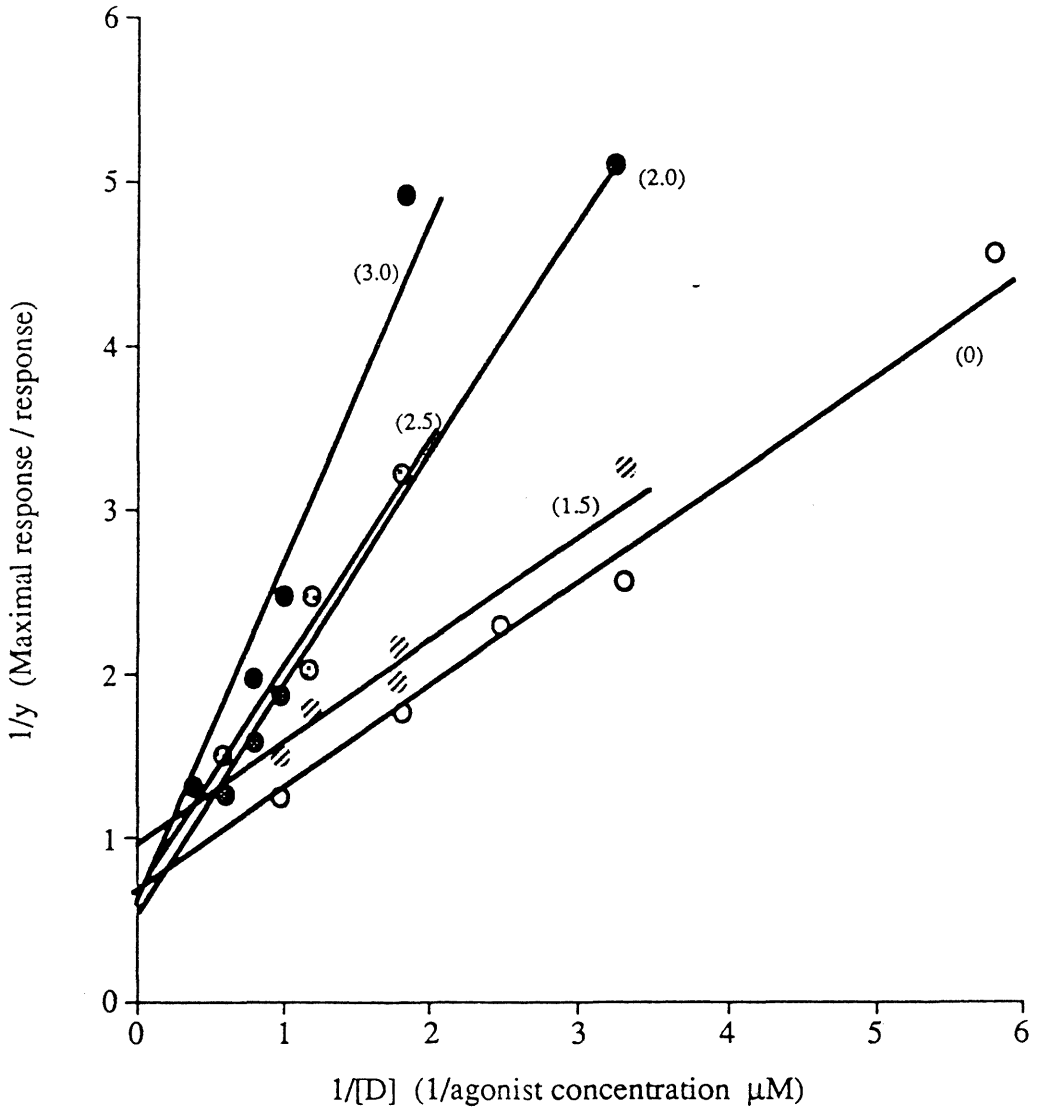


Fig. 6.24n Lineweaver-Burk plot for BDTA antagonism of histamine release from rat peritoneal mast cells induced by compound 48/80. Points were obtained from Fig. 6.24l

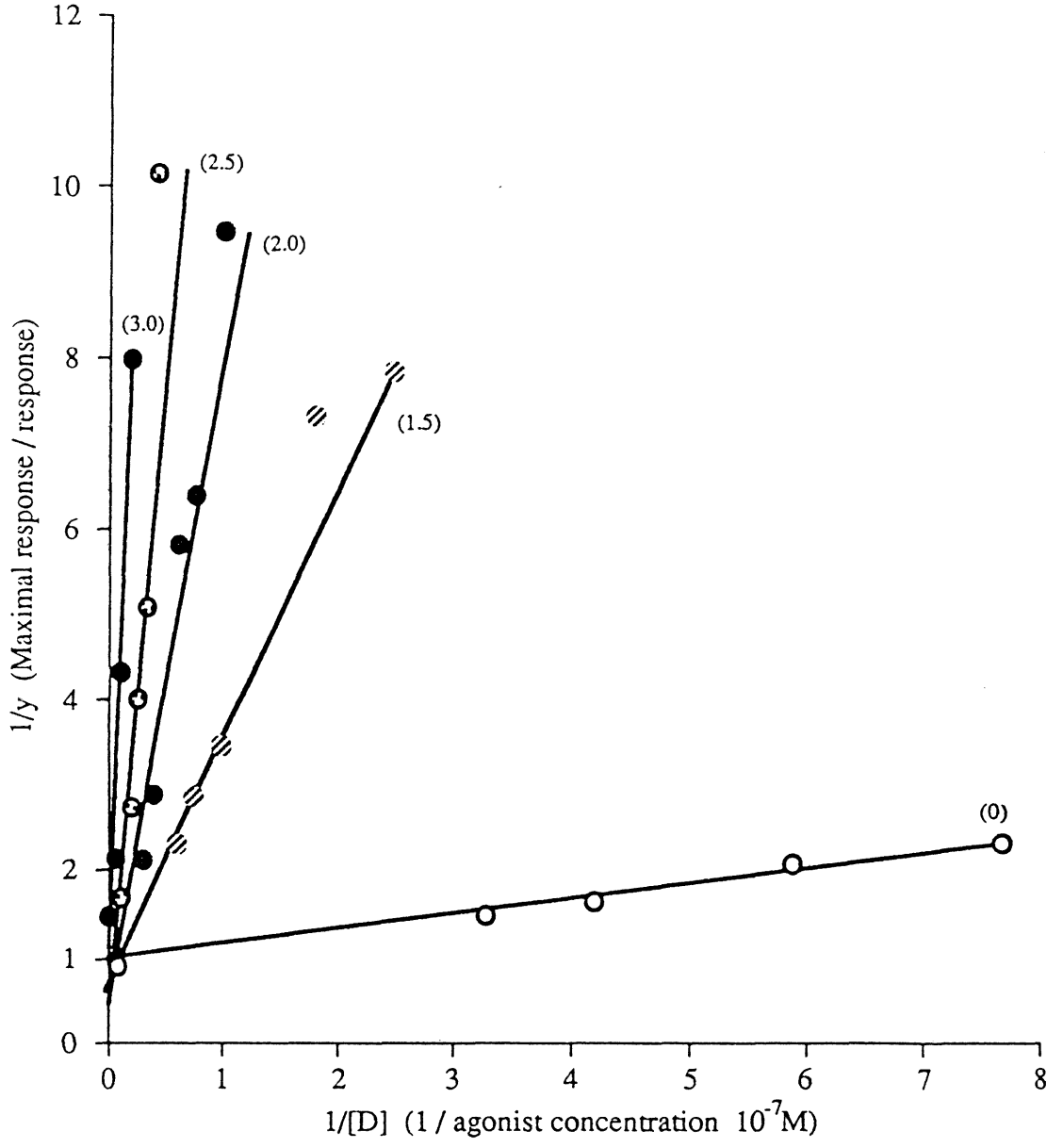


Fig. 6.24o Lineweaver-Burk plot for BDTA antagonism of histamine release from rat peritoneal mast cells induced by polylysine. Points were obtained from Fig. 6.24m.

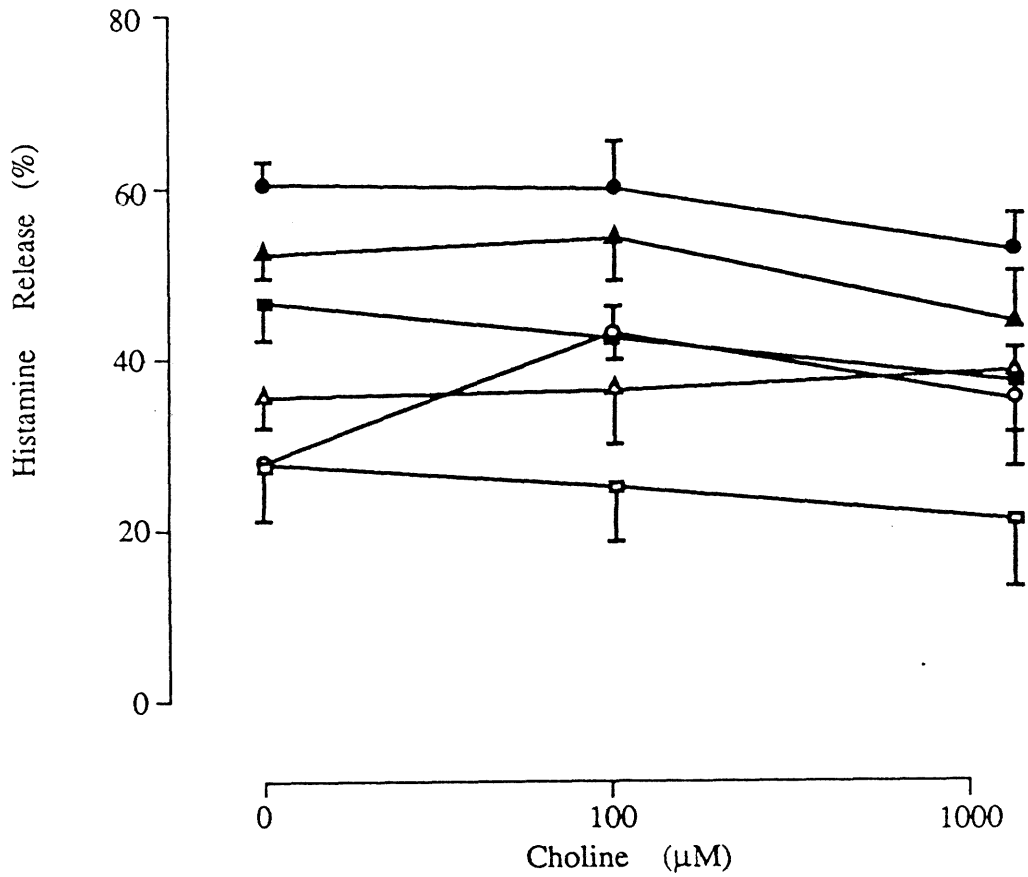


Fig. 6.25a Action of choline on histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, closed triangles) substance P (15μg/ml, closed triangles) compound 48/80 (0.1μg/ml, closed squares) antigen (10WE/ml, open circles) concanavalin A (20μg/ml, open triangles) and anti-IgE (300-fold dilution, open squares)

All values ± SEM (n=4)

induced by somatostatin (7.5μg/ml), compound 48/80 (0.1μg/ml), substance P (15μg/ml), anti-IgE (300-fold dilution), concanavalin A (20μg/ml) or antigen (10WE/ml) (fig.6.25a) Similarly, gallamine, hexamethonium and decamethonium (10^{-6}-10^{-5}M) were without effect on the release induced by somatostatin, compound 48/80 and anti-IgE (Figs.6.25b-d). None of the 'antagonists' tested liberated histamine in their own right.

6.2.6 The effects of BDTA antagonism investigated by fluorescence microscopy

Immunofluorescence studies were conducted on rat peritoneal mast cells treated with somatostatin alone, pretreated with BDTA and then somatostatin, or BDTA and somatostatin simultaneously.

Cells treated with somatostatin in the absence of BDTA exhibited regions of intense staining, consistent with results of earlier experiments (Fig.6.26a). Curiously, cells pretreated with BDTA before the addition of somatostatin seemed to fluoresce to a greater overall extent than those treated with the peptide alone (FIG.6.26b). However, cells treated simultaneously with BDTA and somatostatin exhibited a reduced fluorescence (Fig.6.26c). The above studies were only assessed qualitatively but the apparent trends observed were uniform over the population of cells under examination at any one time.

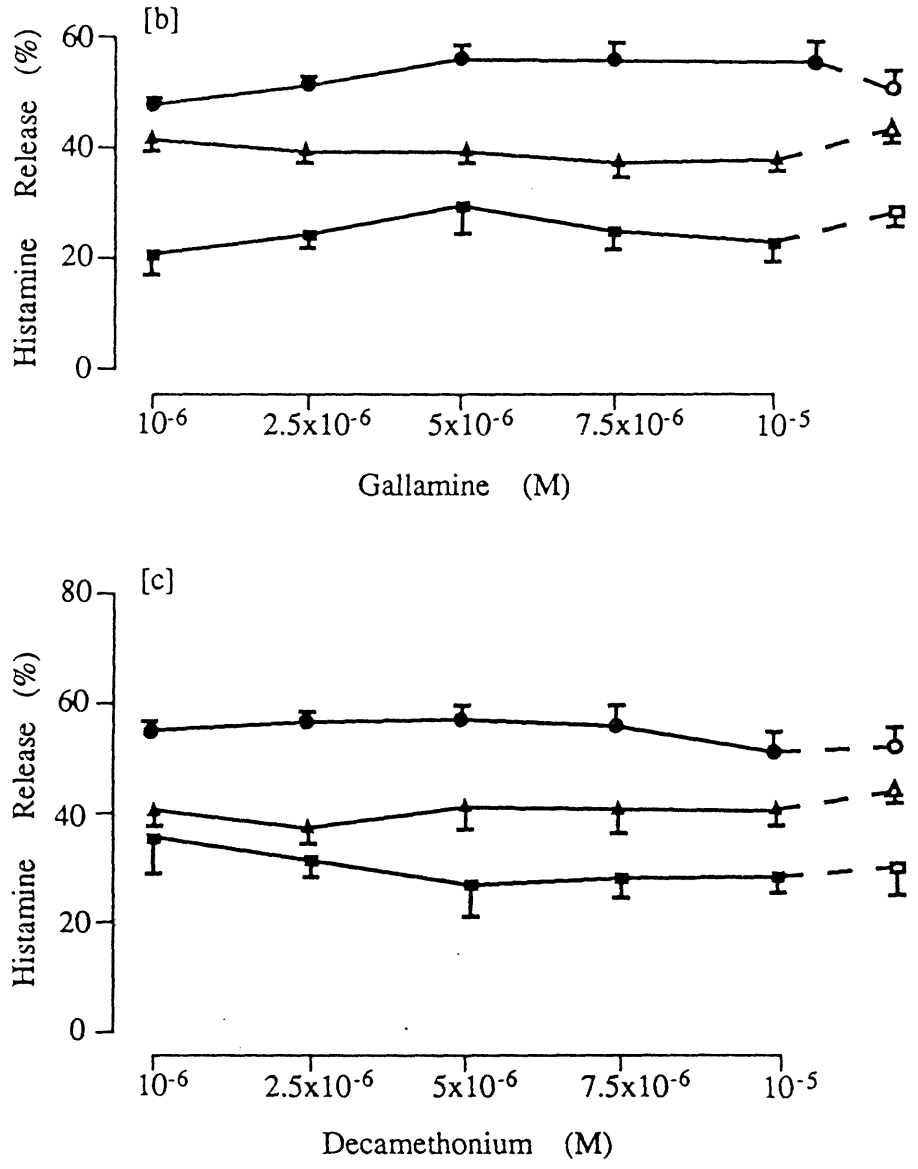


Fig. 6.25b,c Action of [a] gallamine and [b] decamethonium on histamine release from rat peritoneal mast cells stimulated by somatostatin (7.5μg/ml, closed circles) compound 48/80 (0.1μg/ml, closed squares) and anti-IgE (300-fold dilution, closed triangles). Control releases are represented by open symbols.

All values ± SEM (n=4)

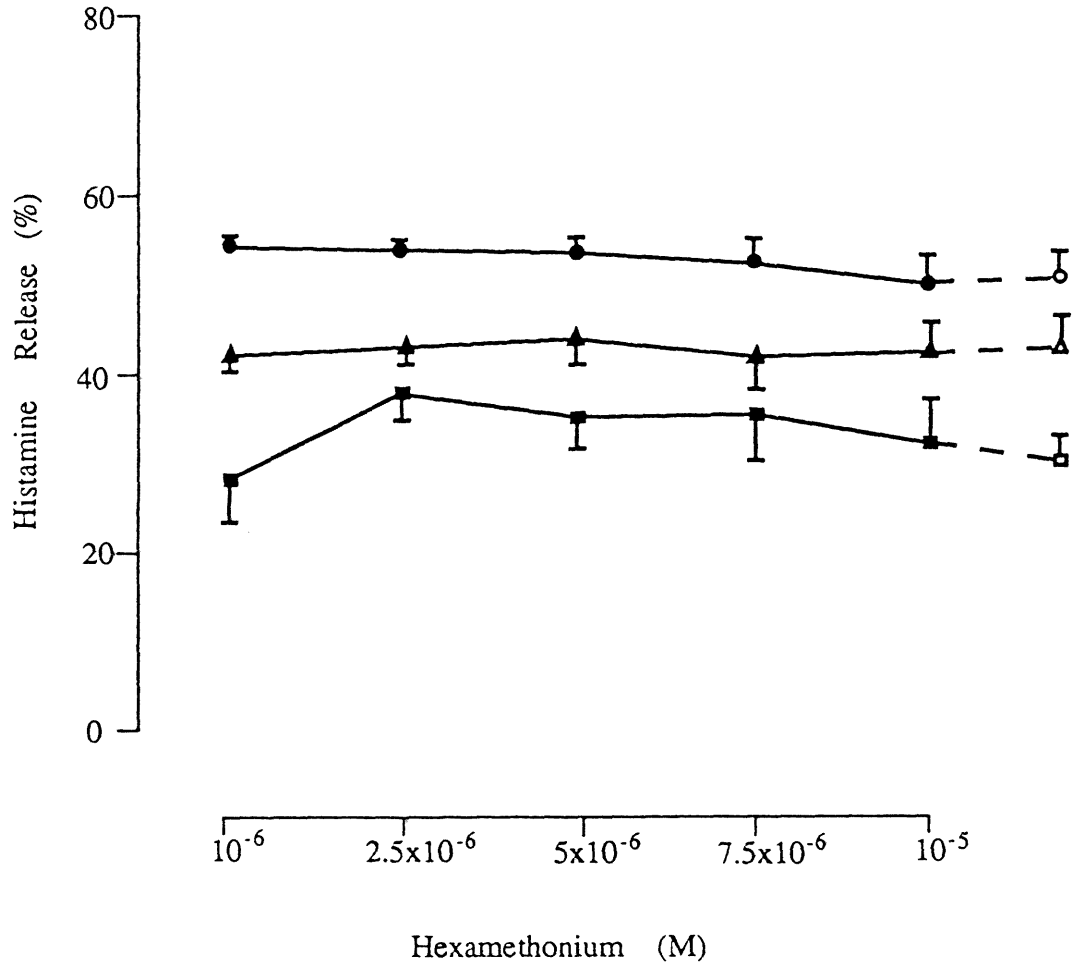


Fig. 6.25d Action of [c] hexamethonium on histamine release from rat peritoneal mast cells stimulated by somatostatin (7.5μg/ml, closed circles) compound 48/80 (0.1μg/ml, closed squares) and anti-IgE (300-fold dilution, closed triangles). Control releases are represented by open symbols.

All values ± SEM (n=4)

Fig. 6.26a Immunofluorescence of rat peritoneal mast cells treated with somatostatin, polyclonal anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[i] phase contrast, magnification x63
[ii] dark field, magnification x63

Fig. 6.26b Immunofluorescence of rat peritoneal mast cells pretreated with BDTA and then somatostatin, polyclonal anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[i] phase contrast, magnification x63
[ii] dark field, magnification x63

Fig. 6.26c Immunofluorescence of rat peritoneal mast cells treated with somatostatin and BDTA, followed by polyclonal anti-somatostatin IgG and FITC-conjugated anti-rabbit IgG.
[i] phase contrast, magnification x63
[ii] dark field, magnification x63

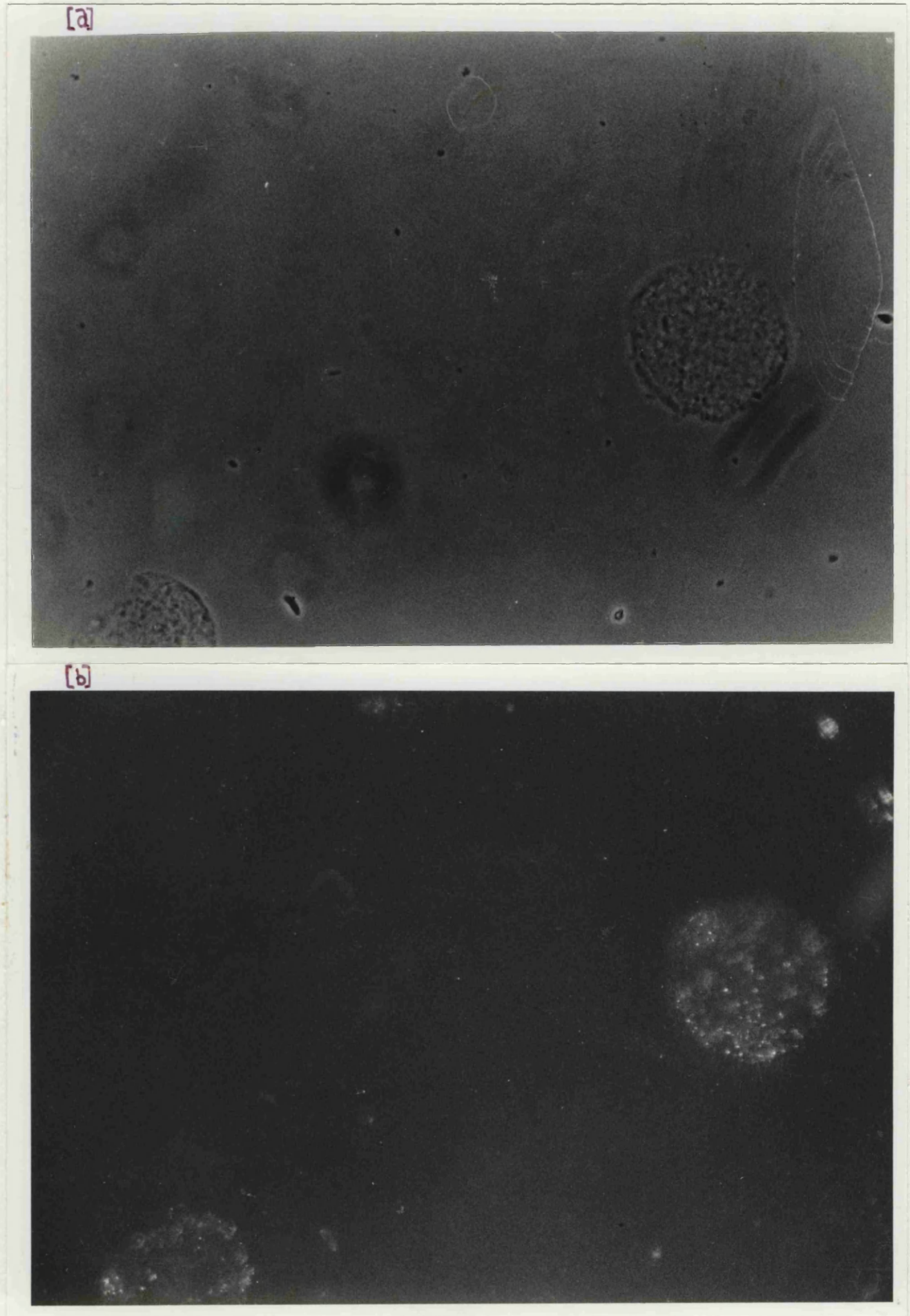


Fig. 6.26a

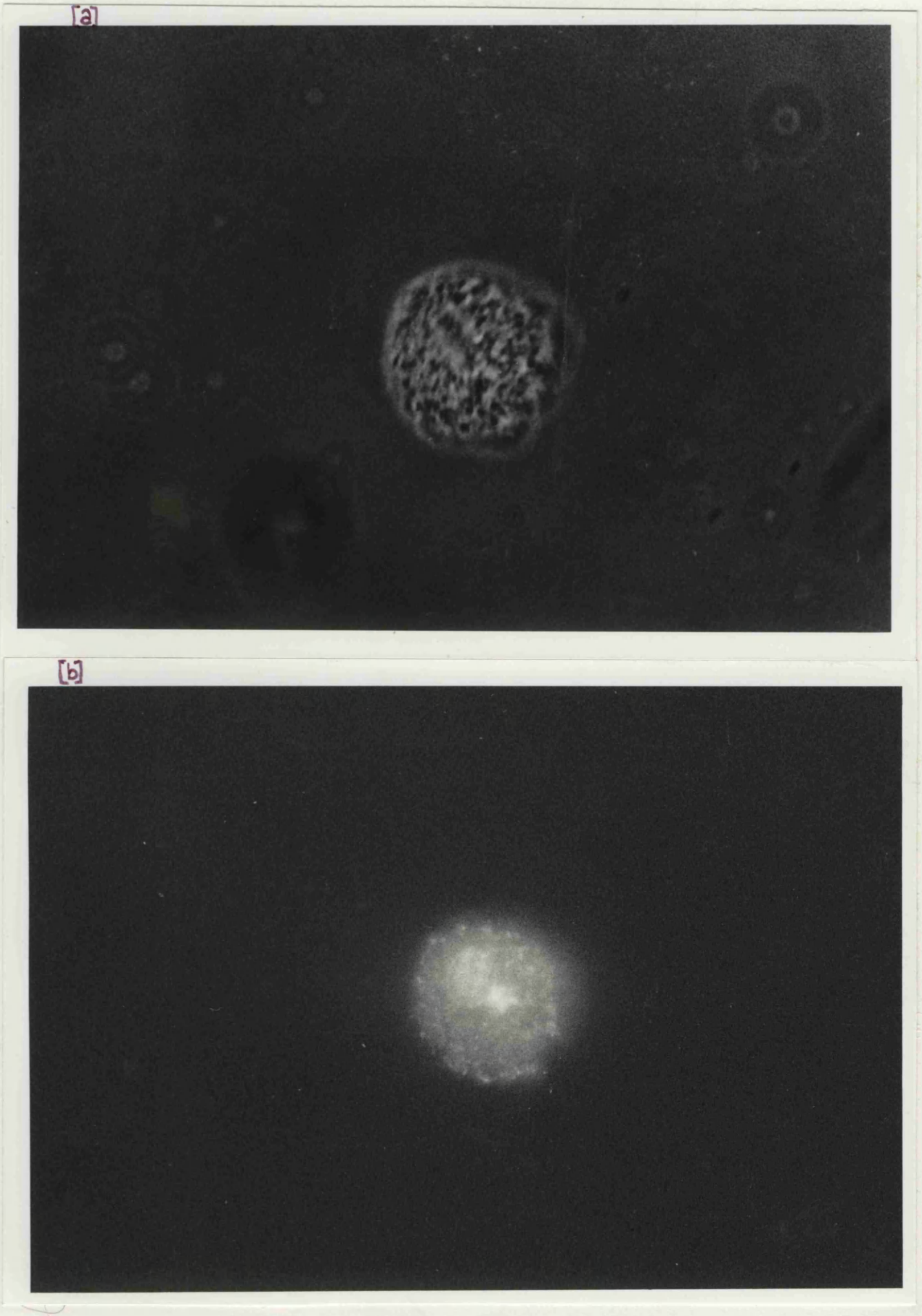


Fig. 6.26b

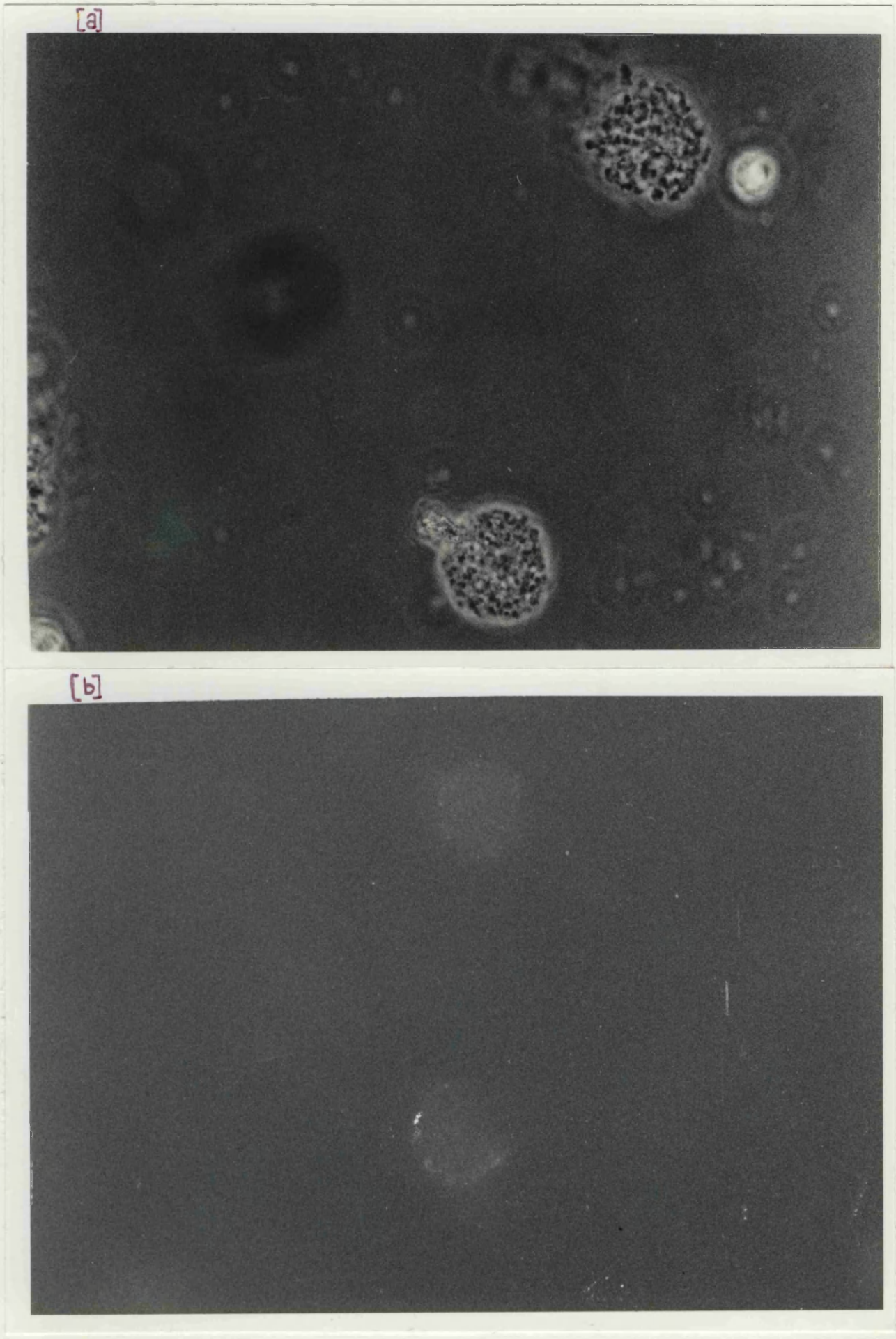


Fig. 6.26c

6.3 *DISCUSSION*

Experiments designed to determine the kinetics of antagonism provided evidence that BDTA selectively prevented polyamine-induced histamine release only when administered simultaneously with the agonist. If cells were preincubated with the antagonist, BDTA also blocked secretion induced by IgE-dependent mechanisms in a time-dependent fashion. The latter effect may reflect a nonspecific membrane or cytotoxic action, since it was partially reversed by elevated, extracellular concentrations of glucose. Moreover, BDTA is clearly able to exert direct effects on the mast cell membrane since high concentrations of the compound induce cytolytic histamine release. In addition, the intercalation of the antagonist into the cell membrane is persistent and resistant to repeated washing. BDTA may thus have two actions: a specific antagonism of polyamine binding sites and a nonspecific membrane interaction. The extent to which these effects are related remains to be clarified.

Read and his coworkers [509] claimed *prima facie* evidence that BDTA competitively antagonized polycation-induced histamine secretion. Specifically, Lineweaver-Burk plots of data from compound 48/80 inhibition provided this evidence. Data presented in this chapter indicate that the polyamines polylysine, polymyxin B, substance P and somatostatin are all surmountably antagonized by BDTA. Schild analysis gives rise to a series of pA_2 values for the above agents, including compound 48/80. The values are similar to each other and fall mid-range. Commonly, two assumptions can be made from these factors. Firstly, the similarity of the values is taken to mean competitive interaction of the agonists and antagonist for the same receptor. Secondly, the mid-range average pA_2 value indicates mid-range antagonist potency since the higher the pA_2 the lower the concentration of antagonist required for the same magnitude of inhibition and the more potent the antagonist. The number of agonists active at this receptor indicates that it is relatively non-specific. Interestingly, there are no available antagonists specific to a single, polyamine mast cell agonist. However, a very recent report [557], published after the completion of the above experiments, states that on consideration of the possibility of accessory binding sites, the finding of equivalent pA_2 values does not necessarily mean action at the same receptor. Conversely, the finding of unequal pA_2 values does not necessarily confirm action at different receptors. If the Schild plot is nonlinear or does not have a slope of negative unity, the calculated pA_2 value does not equal the antagonist-receptor dissociation constant, K_A. The K_A determined in Schild analysis is also known not to equal the true K_A in other situations [558,559]. In the instance of accessory binding sites around the agonist binding site influencing antagonist binding sites, then the interpretation of pA_2 values is clouded.

In the case of BDTA antagonism of polyamines, pA_2 values were essentially equal. However, the slopes of the lines were not equivalent to unity for most of the agonists tested. This would indicate that the calculated pA_2 values are not equatable to the receptor-antagonist dissociation constant. Evidently, competitive inhibition can not be

assured by these data alone. However, further analysis of results using the method described by Lineweaver and Burk does point to a competitive interaction. In this method, the plot of the reciprocal of the response (1/y) against the reciprocal of the agonist concentration (1/[D]) is a straight line, with a slope K_D. The slope becomes steeper in the presence of an antagonist. Lines in the presence and absence of the antagonist intercept on the (1/y)-axis, when 1/[D] = 0, at a point corresponding to the maximal response (1/y = 0.01). The present experimental data could be fitted precisely to plots of this type. In total, it would seem that the issue of whether BDTA antagonism is in fact competitive, remains recondite and somewhat undecided. The evidence would tend to favour this approach but there is also room for doubt.

The structural determinants for inhibitory activity against polycation induced secretion are clearly defined but relatively unspecific. A series of quaternary ammonium salts failed to inhibit histamine secretion induced by polyaminic stimuli. These studies show that a long hydrophobic chain and a region of positive charge are limiting factors for inhibition. The compounds tested failed to inhibit release, probably because of insufficient hydrophobicity. In higher concentrations, some of the agents tested released histamine from certain mastocytes [560].

The ability of BDTA to displace somatostatin from the mast cell membrane could be directly demonstrated by fluorescence microscopy. However, preincubation of the cells with BDTA before the addition of somatostatin caused a dense, overall staining which was greater than that seen on simultaneous administration of both agents. Under these conditions, BDTA might perturb or increase the fluidity of the membrane, thereby both inhibiting exocytosis but facilitating the intercalation of somatostatin.

In conclusion, BDTA can be used as a selective polyamine inhibitor although the experimental conditions must be strictly defined and the precise nature of the antagonism remains to be clarified. Unless these conditions are met, the nonspecific membrane effects of the compound may obscure interpretation of the data.

CHAPTER 7

FURTHER INVESTIGATIONS INTO THE POLYAMINE INDUCTION MECHANISM

7.1 *INTRODUCTION*

Experiments detailed in earlier chapters have provided further insight into the nature of polyamine binding to mast cell membranes. The binding site is not ubiquitous, being confined to particular murine mast cells and those of human skin [554]. A wide range of polycations, including several neuropeptides, activate rat peritoneal mast cells, yet no specific antagonist exists for any one agonist. Only BAC and TPC [528,561] selectively inhibit polyamine-induced histamine release and the antagonism is most likely competitive. There also exists the possibility of a receptor-independent mechanism for these ligands. The nature of the receptor or binding site is still obscure and its actual existence has not been verified. The following series of experiments were designed to investigate the binding and mast cell-activation mechanisms of polyamine stimuli. Several approaches were used based on known steps in the signal transduction sequence.

7.1.1 The role of serine esterases

Amino acid ester substrates and inhibitors of chymotrypsin, including diisopropyl-phosphofluoridate (DFP) and phosphonate esters, have been shown to inhibit immunologic histamine release from rat peritoneal mast cells, human basophil leucocytes and lung fragments of the guinea pig and man [243,246,562,563]. The inhibitor must be present at the time of challenge, indicating that a latent seryl enzyme may be activated in the course of the release process [243,246]. In contrast, DFP has been reported to have no effect on histamine release from rat mast cells stimulated with compound 48/80 [564]. It was then felt to be of interest to examine the effect of a number of inhibitors of seryl enzymes on histamine release induced by polyamines and IgE-directed ligands.

7.1.2 Trypsination of mast cells

Another avenue of investigation involves the use of trypsin. Trypsination of rat mast cells prior to stimulation should inhibit release mediated by a trypsin-sensitive receptor protein. Results from these experiments should help to define the possible involvement of protein receptor sites in polyamine-induced release.

7.1.3 The role of GTP-binding proteins

Recently, considerable interest has focused on the role of GTP-binding proteins in histamine release from mast cells. Two such proteins may be involved, one modulating the receptor mediated activation of phospholipase C and the other acting at a later stage in the exocytotic process [565-567].

Attempts to inhibit GTP-binding proteins with pertussis toxin have yielded some conflicting results but it now appears that the toxin does not affect anti-IgE-induced mediator release

from mast cells but does inhibit secretion induced by compound 48/80 [568]. Pertussis toxin similarly failed to block IgE-mediated release from bone marrow-derived mouse mast cells (BMMC) or human peripheral blood mononuclear cells but did inhibit exocytosis evoked by thrombin and F-met-leu-phe or C5a, respectively [568,569]. These data indicate that the toxin may discriminate between IgE-directed and pharmacologic agonists and it was thus felt to be of interest to examine its effects on polyamine-induced secretion.

7.1.4 The role of sialic acid

N-acetyl neuraminic acid (NANA) is present as the ultimate carbohydrate moiety in cell membrane glycolipids and glycoproteins [570]. This weak acid, which at physiological pH preferentially binds calcium ions [593], is in part responsible for the cell surface negative charge. In many cell systems it is involved in cell to cell interactions and inflammatory cell responses [570-573]. Removal of NANA by the enzyme neuraminidase may be undertaken to study the significance of NANA in the cell membrane.

Bach and Brashler [574] have shown that pretreatment of rat peritoneal mast cells with neuraminidase enhanced antigen-induced histamine release when the enzyme is in sufficiently small amounts. In larger amounts, the enzyme itself caused histamine release from the cells. Similarly, Jensen *et al.* [575] found that treatment of human basophils with neuraminidase also caused histamine release. The release process was independent of extracellular calcium and consequently ascribed to changes in the surface charge and/or changes in the conformation of cell membrane constituents [570,576]. In low concentrations, neuraminidase treatment of basophils increased the maximal release and caused a shift to the left of the dose-response curve upon stimulation with anti-IgE, concanavalin A and A23187. Baxter and Adamik [577] obtained identical results in rat mast cells.

Jensen [575] has also shown that pretreatment with NANA caused the opposite effect to that of pretreatment with neuraminidase, that is it diminished and delayed histamine secretion. The findings may be due to changes in cell surface charge, resulting in an alteration of the binding of stimulators to the cell surface. Coleman made comparable suggestions in rat experiments using charged molecules as liberators of 5-hydroxytryptamine from mast cells [578].

NANA may also be involved in the binding to or exchange of calcium across the membrane of histaminocytes. Thus removal of membrane bound NANA from basophils diminished the concentration of calcium necessary for histamine release. Accordingly, the addition of membrane NANA increased the corresponding calcium requirements [575].

It was then felt to be of considerable interest to examine the effect of neuraminidase on polyamine-induced secretion from rat peritoneal mast cells.

7.2.1 The effects of serine esterase inhibitors on histamine release from rat peritoneal mast cells

Three serine esterase inhibitors were employed in this study. The compounds elicit their inhibitory activity by substrate mimicry. Mast cells were exposed to the secretagogues and esterase inhibitor without preincubation. Stimulation was induced by immunologic and polycationic stimuli, for the purposes of comparison.

7.2.1.1 Action of N-tosyl L-lysine chloromethyl ketone (TLCK)

TLCK (1-30μM) had a negligible inhibitory effect on histamine release stimulated by anti-IgE (300-fold dilution), antigen (10WE/ml), concanavalin A (20μg/ml), somatostatin (7.5μg/ml), compound 48/80 (0.2μg/ml) and substance P (15μg/ml).
Higher concentrations (300μM) of TLCK produced a strong inhibition of release stimulated by both types of agonist. This concentration of TLCK did not increase the spontaneous release of histamine but any potential toxic effects that could result in inhibition, such as blockade of cellular ATP production, were not investigated.

7.2.1.2 Action of N-tosyl L-phenylalanine chloromethyl ketone (TPCK)

Histamine release stimulated by anti-IgE (300-fold dilution), antigen (10WE/ml) and concanavalin A (20μg/ml) was significantly inhibited by TPCK (1-10μM). In contrast, release stimulated by somatostatin (7.5μg/ml), substance P (15μg/ml) and compound 48/80 (0.2μg/ml) was essentially unaffected by TPCK (1-10μM). However, the top concentration (30μM) of the antagonist did produce significant inhibition (Fig.7.212).
As above, TPCK was tested for cytotoxicity and found to have no histamine-releasing properties of its own.

7.2.1.3 Action of phenylmethylsulphonyl fluoride (PMSF)

PMSF produced scattered inhibition of both polyamine and immunologic histamine release with some evidence of a bell-shaped dose-response curve and some potentiation at extremes of concentration (Fig.7.213). Control experiments included in the study show that PMSF did not liberate histamine in its own right, at the concentrations investigated.

7.2.1.4 Action of TPCK in the absence and reintroduction of extracellular calcium

TPCK was the most discriminating of all the serine esterase inhibitors tested, in terms of its ability to distinguish between immunologic ligand and polyamines. Therefore, this agent was selected to examine any possible effects of extracellular calcium on polycation-induced histamine liberation. Experiments were conducted in the absence of calcium throughout or subjected to the reintroduction of the cation with the stimulus. Final calcium concentrations were the same as for ordinary investigations (1mM).

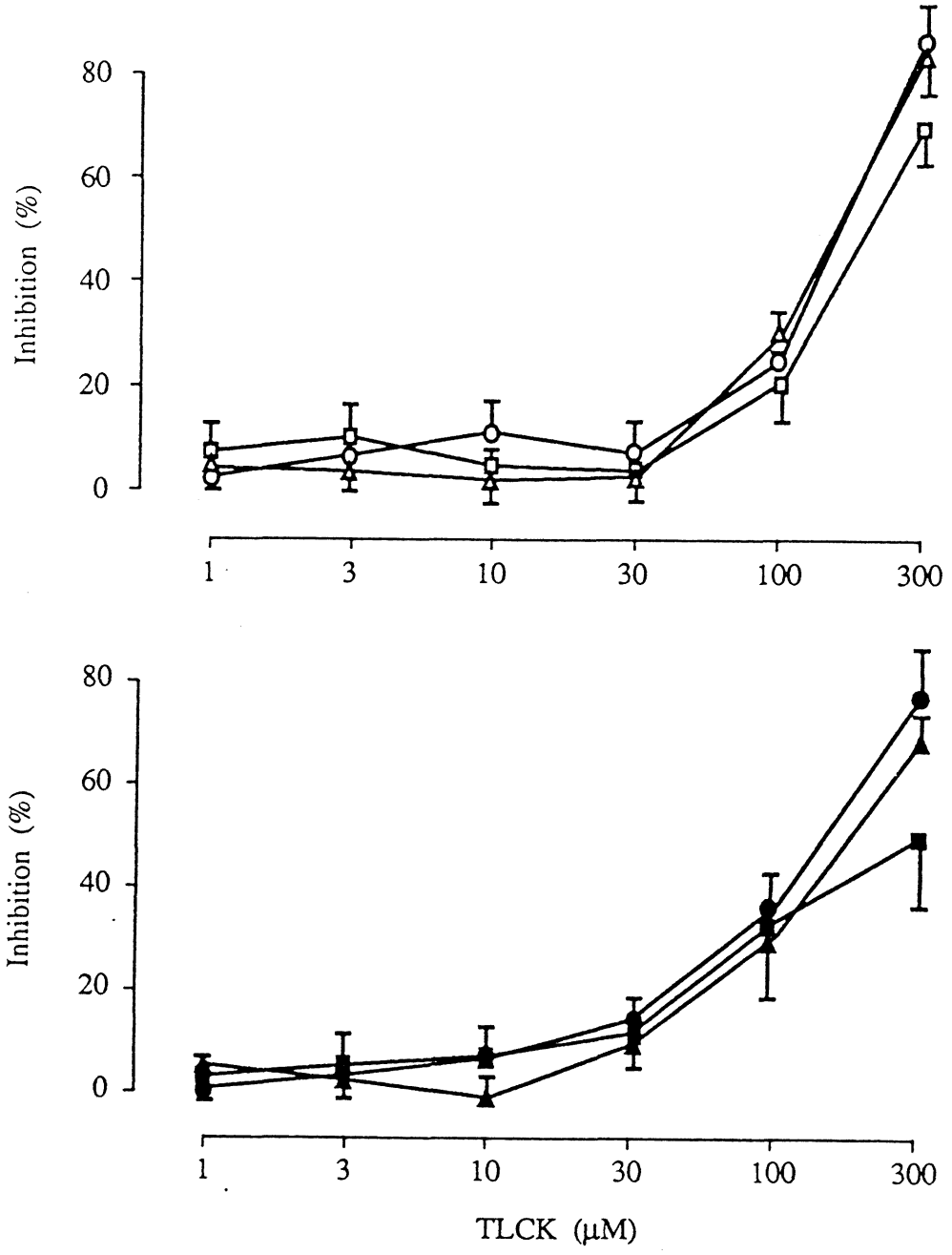


Fig. 7.211 Action of TLCK on histamine release from rat peritoneal mast cells induced by [a] somatostatin (7.5μg/ml, control release 46.3±2.9, open circles) substance P (15μg/ml, control release 42.4±3.1, open triangles) compound 48/80 (0.2μg/ml, control release 46.0±2.7, open squares)
[b] antigen (10WE/ml, control release 33.4±2.4, closed triangles) concancavalin A (20μg/ml, control release 33.4±2.4, closed triangles) anti-IgE (300-fold dilution, control release 38.1±3.6, closed squares). Where error bars have been omitted for clarity, SEM did not exceed ± 3.7. (n=4)

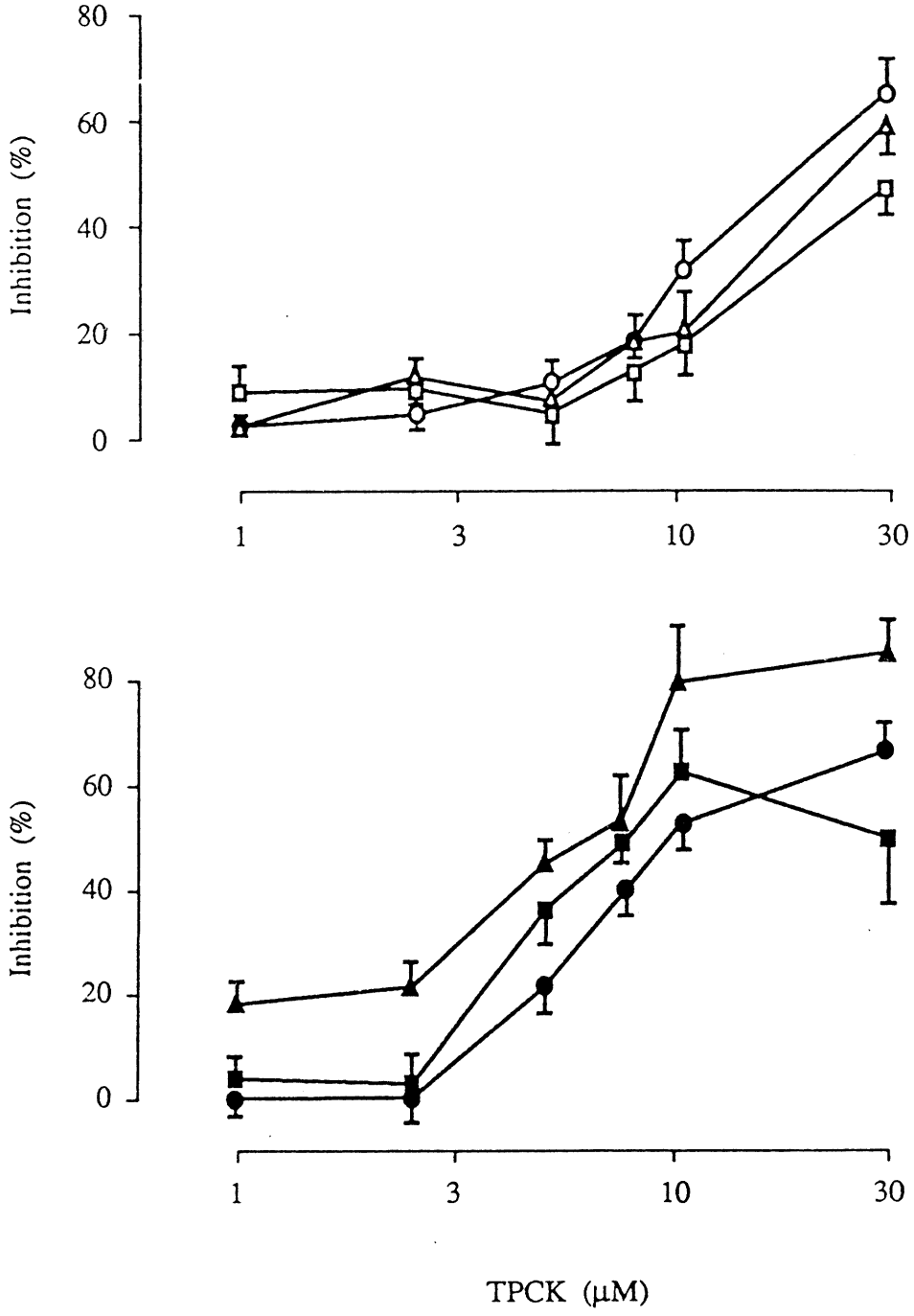


Fig. 7.212 Action of TPCK on histamine release from rat peritoneal mast cells induced by [a] somatostatin (7.5μg/ml, control release 43.6±2.2, open circles) substance P (15μg/ml, control release 45.7±2.7, open triangles) compound 48/80 (0.2μg/ml, control release 41.3±3.1, open squares) [b] antigen (10WE/ml, control release 46.3±1.6, closed triangles) concancavalin A (20μg/ml, control release 47.8±3.4, closed triangles) anti-IgE (300-fold dilution, control release 41.2±2.9, closed squares). Where error bars have been omitted for clarity, SEM did not exceed ± 7.4. (n=4)

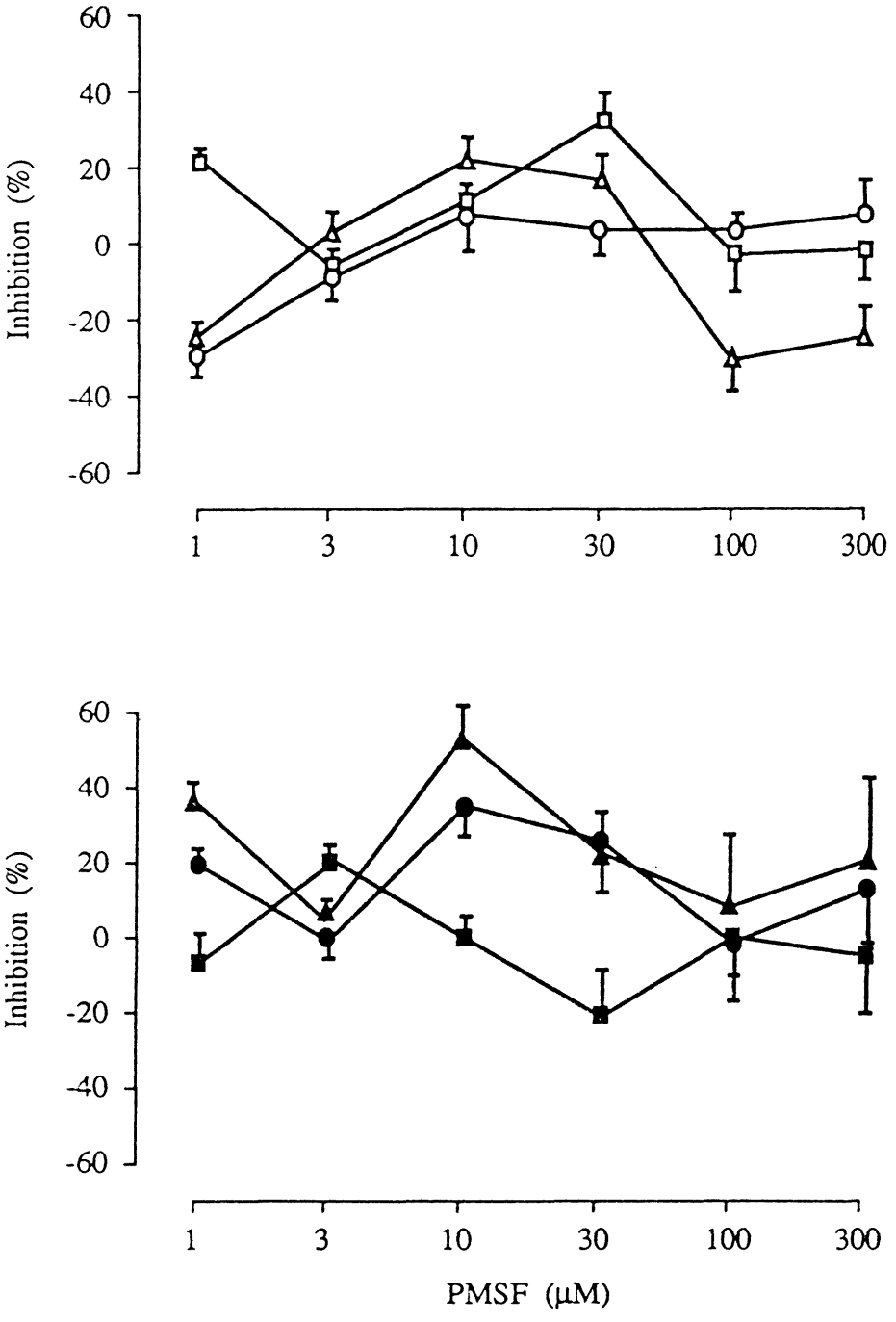


Fig. 7.213 Action of PMSF on histamine release from rat peritoneal mast cells induced by [a] somatostatin (7.5μg/ml, control release 34.4±1.7, open circles) substance P (15μg/ml, control release 30.6±2.2, open triangles) compound 48/80 (0.2μg/ml, control release 39.4±2.4, open squares)
[b] antigen (10WE/ml, control release 43.2±1.6, closed triangles) concancavalin A (20μg/ml, control release 34.1±2.6, closed triangles) anti-IgE (300-fold dilution, control release 34.0±2.5, closed squares).

All values ± SEM (n=4)

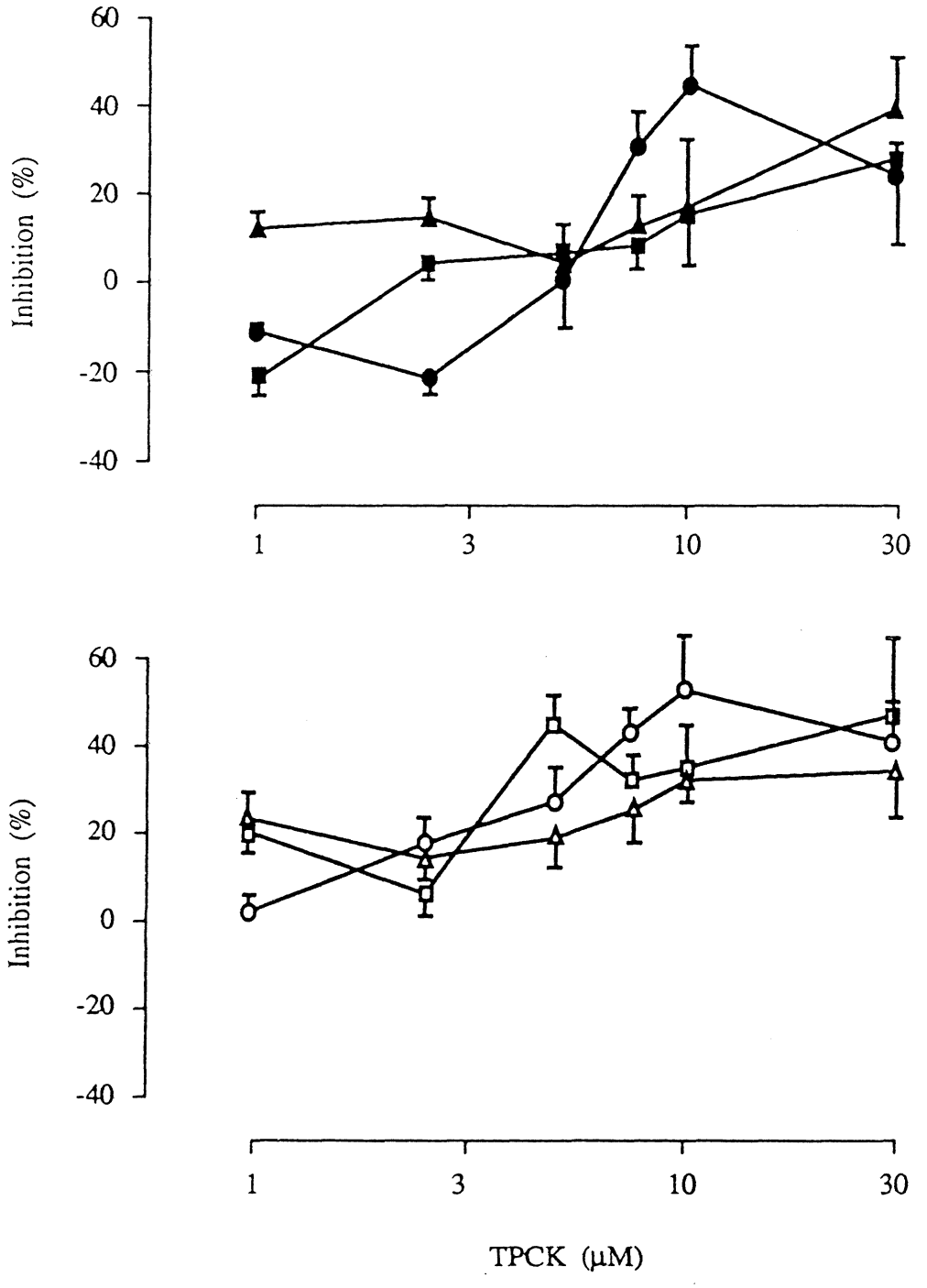


Fig. 7.214 Action of TPCK either in the presence (closed symbols) or absence (open symbols) of extracellular calcium (1mM) on histamine from rat peritoneal mast cells stimulated with somatostatin (7.5μg/ml, control release 46.3±2.9, 47.3±3.1, circles) substance P (15μg/ml, control release 42.4±3.1, 44.4±3.8, triangles) compound 48/80 (0.2μg/ml, control release 46.0±2.7, 43.9±3.3, squares). Control releases are quoted in the presence and absence of calcium respectively.

All values ± SEM (n=4)

TPCK caused a dose-dependent inhibition of polyamine-induced secretion in the absence of calcium (Fig.7.214). Under these conditions, no potentiation of release was observed for any point. This was not the case for studies conducted on cells challenged with the agonist in calcium, where low concentrations of TPCK potentiated the release but the effect was slight.

7.2.2 Effects of trypsinisation of mast cells

The experiments were conducted in two parts, either in the presence or absence of bovine serum albumin (BSA, 0.1%). BSA was included in the incubation medium to overcome possible peptide destruction by any residual trypsin not removed by extensive washing (x3).

i: Absence of BSA

Mast cells were preincubated with trypsin (1-1000μg/ml, 20min), washed and then challenged with a variety of agonists. This treatment produced a substantial inhibition of histamine release produced by somatostatin, substance P, anti-IgE and concanavalin A while that induced by antigen was less affected (Fig.7.22i).

ii: Presence of BSA

BSA (0.1%) was added to the incubation medium with the stimuli to 'mop up' any residual trypsin that may still have remained after several washes. BSA treatment seemed to alter the experimental results tremendously. The extent of inhibition observed was reduced for all the stimuli tested, and the highest concentration of trypsin caused significant potentiation of release evoked by anti-IgE and concanavalin A.

7.2.3 Effect of pertussis toxin

Rat peritoneal mast cells were incubated with pertussis toxin (1-100ng/ml) for a period of two hours before the addition of the agonist.

Pertussis toxin pretreatment had no major effect on the release induced by the immunologic agonists; antigen (10WE/ml), anti-IgE (300-fold dilution) and concanavalin A (20μg/ml). However, in agreement with published reports [568], the toxin was shown to inhibit polyamine induced release in a dose-dependent manner (Fig.7.23).

7.2.4 Effect of neuraminidase

Neuraminidase (from Cl. *perfringens*, 25μU/10^6 cells) was preincubated with rat peritoneal mast cells (60min) in calcium-containing buffer (10ml). Pilot experiments illustrated the necessity for dilute cell suspensions which ensured free access of the enzyme to the cell surfaces. After enzymatic challenge, the cells were washed carefully (x3) and resuspended in calcium-free buffer. The cells were allowed to equilibrate (5min) before the addition of further buffer containing calcium (1mM), EDTA (10mM) or no calcium. After

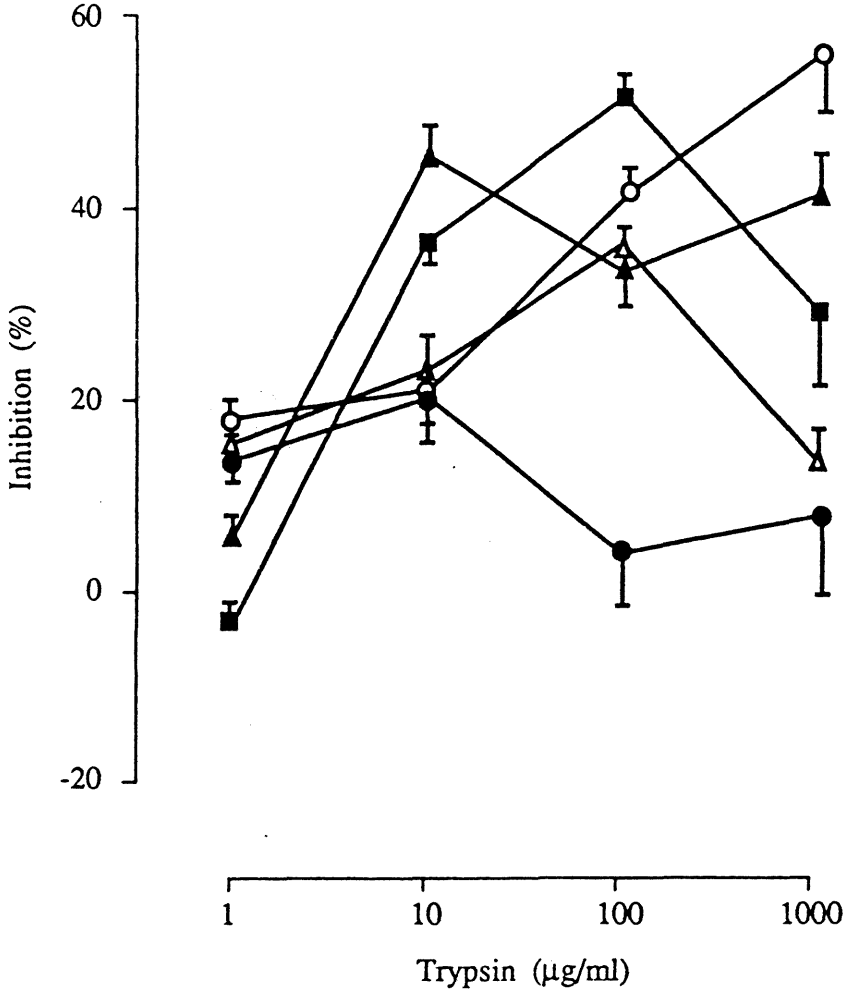


Fig. 7.22i Effect of trypsinisation of rat peritoneal mast cells stimulated with somatostatin (7.5μg/ml, control release 36.7±2.2, open circles) substance P (15μg/ml, control release 38.0±2.9, open triangles) antigen (20WE/ml, control release 36.0±2.3, closed circles) concanavalin A (20μg/ml, control release 29.8±2.7, closed triangles) anti-IgE (300-fold dilution, control release 31.1±4.1, closed squares)

All values ± SEM (n=4)

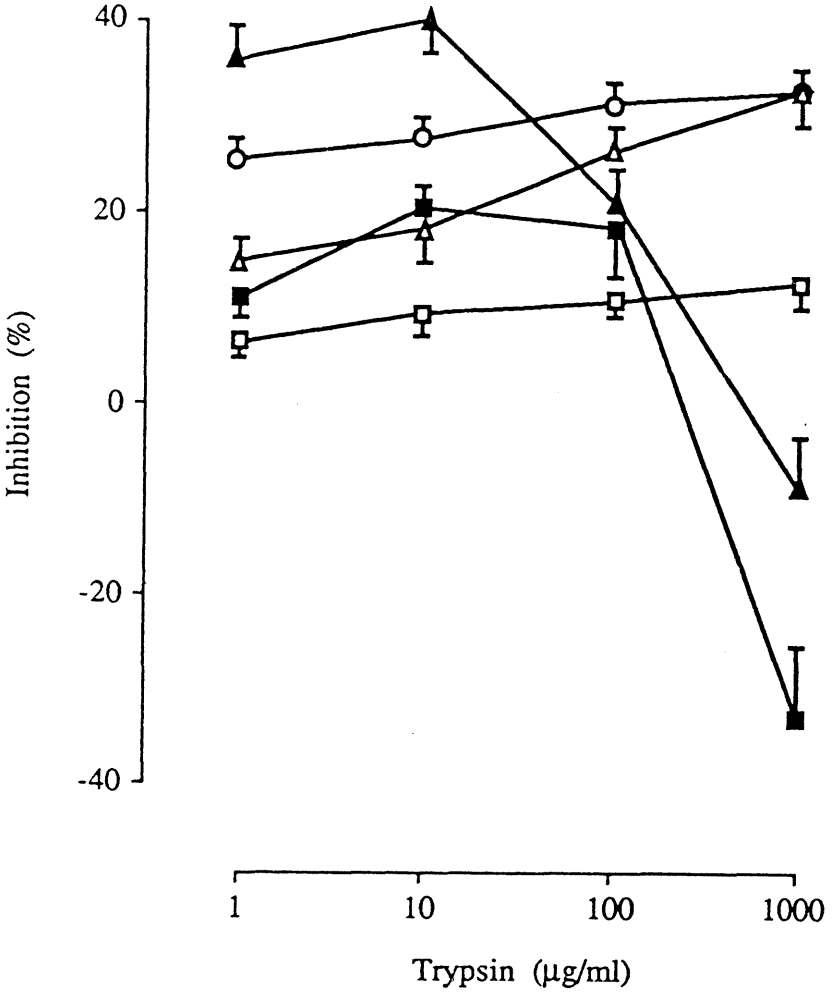


Fig. 7.22ii Effect of trypsinisation of rat peritoneal mast cells, in the presence of BSA (0.1%), stimulated with somatostatin (7.5µg/ml, control release 49.6±1.7, open circles) substance P (15µg/ml, control release 36.6±3.2, open triangles) compound 48/80 (0.2µg/ml, control release 45.0±2.4, open squares) concanavalin A (20µg/ml, control release 32.7±3.2, closed triangles) anti-IgE (300-fold dilution, control release 33.6±3.8, closed squares)

All values ± SEM (n=4)

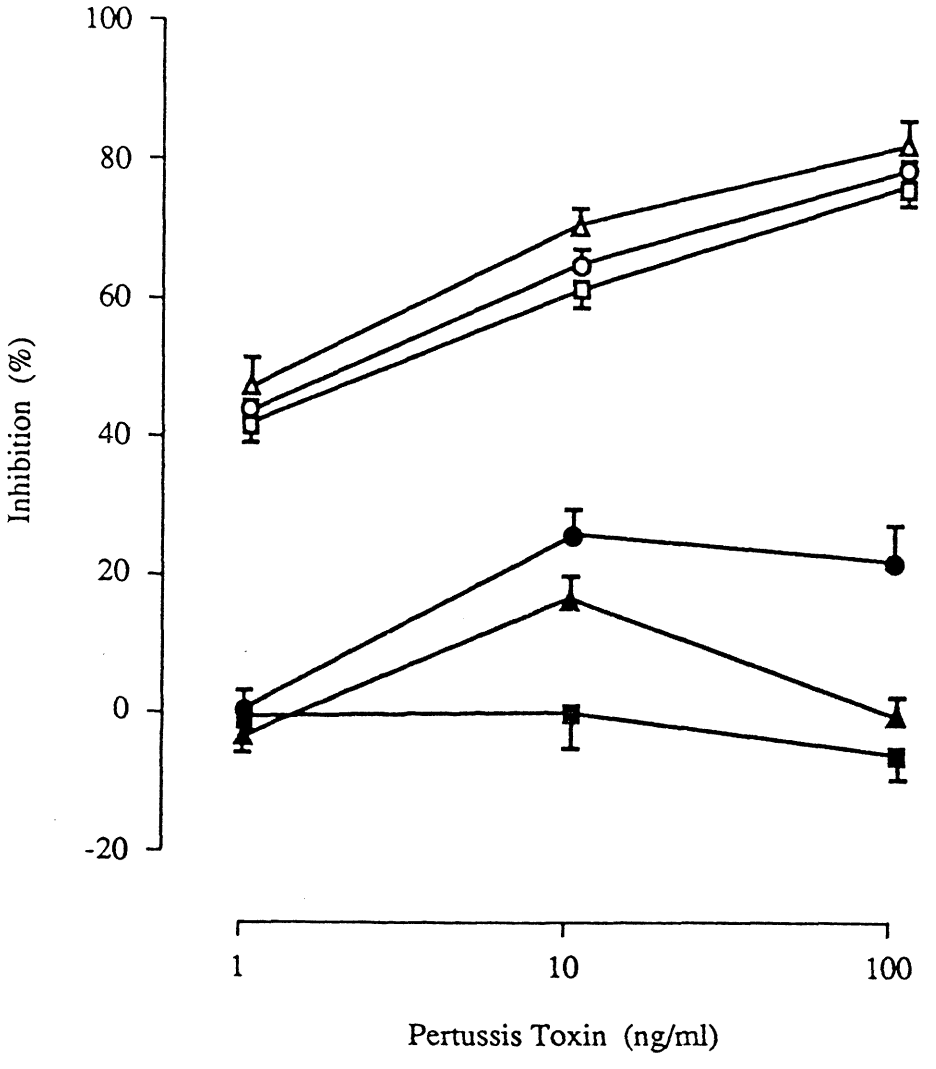


Fig. 7.23 Action of pertussis toxin on histamine release from rat peritoneal mast cells induced by somatostatin (7.5μg/ml, control release 36.9±2.4, open circles) substance P (15μg/ml, control release 38.4±2.0, open triangles) compound 48/80 (0.2μg/ml, control release 32.6±1.9, open squares) antigen (20WE/ml, control release 37.4±4.7, closed circles) concanavalin A (20μg/ml, control release 33.7±4.4, closed triangles) anti-IgE (300-fold dilution, control release 34.4±5.1, closed squares)

All values ± SEM (n=4)

brief preincubation (5min) in the new media, the cells were stimulated with somatostatin (7.5μg/ml), compound 48/80 (0.2μg/ml) or anti-IgE (300-fold dilution).
Neuraminidase treatment seemed partially to antagonise polyamine-induced secretion but to potentiate anti-IgE-induced secretion in the presence of extracellular calcium (Fig.7.24). Polyamine inhibition appeared accentuated on brief preincubation with EDTA and still further in the absence of extracellular calcium. Conversely, brief preincubation in EDTA seemed to ablate anti-IgE-induced potentiation as did the absence of calcium.

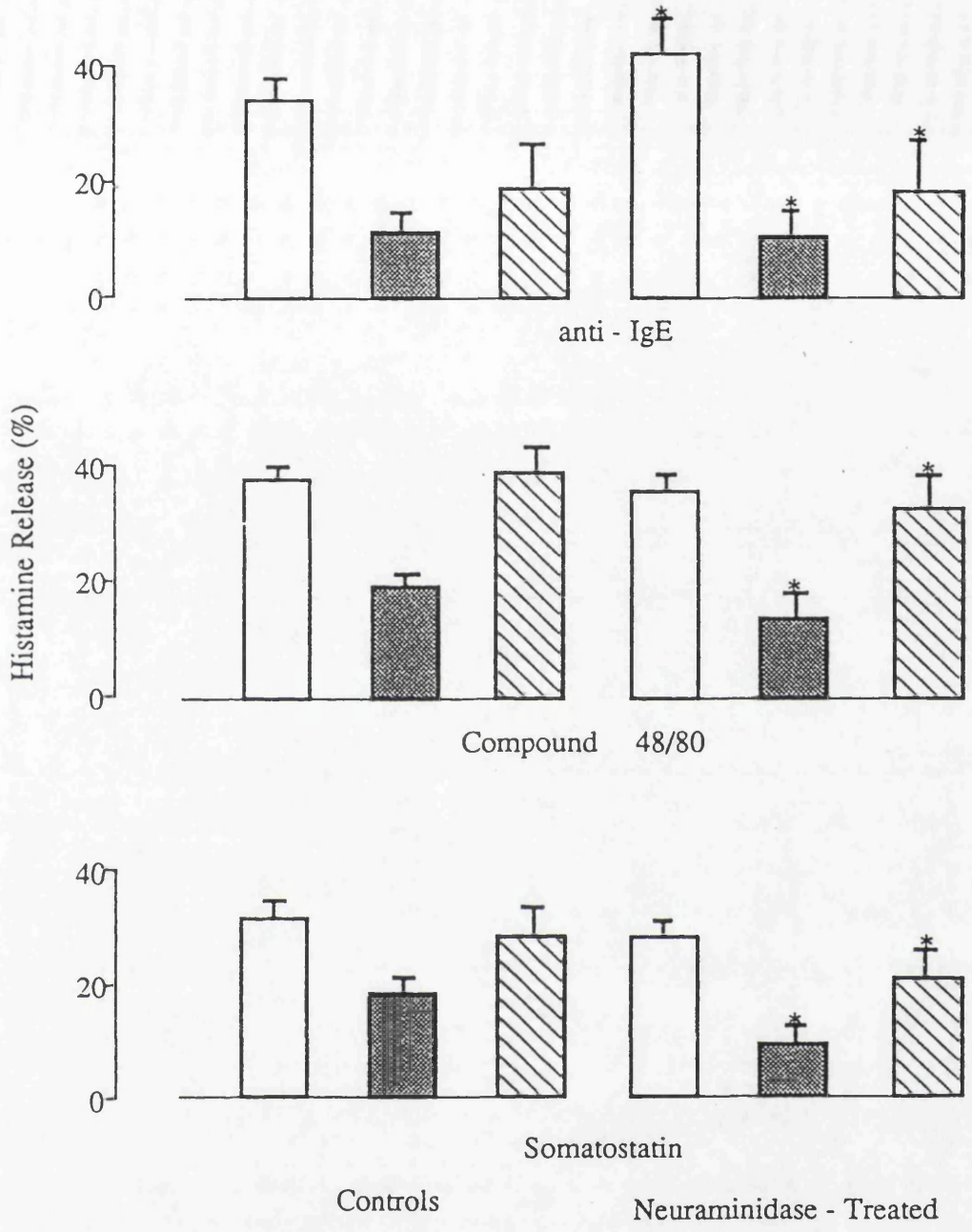


Fig. 7.24 Effect of treatment with neuraminidase (*Cl. Perfringens*, $25\mu U/10^6$ cells) on histamine release from rat peritoneal mast cells stimulated with somatostatin ($7.5\mu g/ml$), compound 48/80 ($0.2\mu g/ml$) and anti-IgE (3000-fold dilution). Experiments were conducted in the presence (open columns) or absence (dotted columns) of extracellular calcium (1mM) or after brief pretreatment with EDTA (5 mins, $10^{-4}M$)

All values ± SEM *=$p<0.05$ (n=4)

Activation of a serine esterase has been implicated as one of the earliest biochemical events following mast cell activation [579]. Accordingly, histamine release from a variety of histaminocytes is blocked by a number of amino acid ester substrates and inhibitors of seryl enzymes [243,580]. In the present study TLCK, an active-site-directed inhibitor of trypsin-like enzymes, was found to have little effect on histamine release from rat mast cells except at very high, and possibly cytotoxic, concentrations. Austen and Brocklehurst [243] have also reported that substrates and inhibitors of trypsin have little or no effect in the rat mast cell. In contrast, the chymotrypsin inhibitor TPCK inhibited polyamine-induced and, rather more effectively, IgE-mediated release from rat mast cells. These results may indicate that a chymotryptic rather than a tryptic enzyme is involved in the release process. The relationship between this enzyme and chymase, the major neutral protease of the rat connective tissue mast cell, remains to be clarified [243].

The inhibition produced by TPCK was independent of extracellular calcium indicating that it did not act by modulating fluxes of the cation across the mast cell membrane and/or that activation of the putative serine esterase did not require external calcium. These findings differ somewhat from those of Kaliner and Austen [579] in human lung who delineated five sequential steps in the release process and concluded that immunologic activation of the esterase in this system was dependent on extracellular calcium. These differences may reflect the cell types or the stimuli involved. Finally, PMSF produced a rather scattered and bell-shaped inhibition of both polyamine and immunologic histamine release. The reasons for this form of the dose-response curve are not clear. In total, the present data then provide further evidence for the involvement of a serine esterase in histamine secretion but do not allow discrimination between the mechanisms involved in IgE-mediated and polyamine-induced activation of the cell.

Treatment of mast cells with trypsin might be expected to cause the digestion of surface membrane proteins. The destruction of receptor proteins might then block receptor-mediated secretion.

In the absence of BSA, trypsinisation inhibited the release induced by concanavalin A, anti-IgE, somatostatin and substance P, while that evoked by antigen and compound 48/80 was less affected. This raised the possibility that residual enzyme might degrade the susceptible ligands, with antigen and compound 48/80 being relatively resistant to this effect. Consistently, addition of BSA to 'mop-up' residual trypsin markedly attenuated the inhibitory action of the enzyme. Indeed, high concentrations of the protease potentiated the release induced by concanavalin A and anti-IgE, possibly indicating a membrane destabilising effect which facilitated exocytosis under these conditions. In total, direct proteolysis of ligands by persistent, residual trypsin, or non-specific membrane effects would seem to preclude the use of the enzyme as a tool to distinguish between receptor-mediated events involved in immunologic and polyamine-induced secretion.

In many systems, the generation of intracellular signals in response to ligand-receptor interactions at the cell surface is controlled by one or more GTP-binding proteins (G-proteins) that are an essential part of the transduction machinery [581-584]. Recent studies have suggested that two G-proteins may, in fact, be involved in the regulation of stimulus-secretion coupling in the mast cell [189,585,586]. One protein (G_P) controls the activation of phospholipase C and hence phosphatidylinositol hydrolysis while the other (G_E) acts synergistically with calcium, possibly through a calcium-binding protein, to induce the final stages of exocytosis.

The effect on histamine release of pertussis toxin, which irreversibly inhibits certain subclasses of G-protein by ADP-ribosylation, has been the subject of conflicting data. Early reports [585] suggested that the toxin inhibited immunologic secretion but more recent studies have not confirmed these results [568]. However, it is clear that histamine release induced by basic agents such as substance P, compound 48/80 and mastoparan can be effectively inhibited by pertussis toxin and it has been suggested that such agents may directly activate G-proteins [569]. Data from the present study, showing potent inhibition by the toxin of release induced by substance P, compound 48/80 and somatostatin with a negligible effect on anti-IgE are entirely in accord with this view. Certainly, pertussis toxin would seem to be a most useful tool in distinguishing between histamine release induced by polyamines and other secretagogues.

The trivial term 'ganglioside' is applied to any glycosphingolipid (GSL) that contains sialic acid, without regard to other structural features. The enzyme sialidase (neuraminidase) cleaves off the ultimate carbohydrate moiety, sialic acid (NANA), from adjacent carbohydrate fractions of GSLs in the cell membrane. It is produced by many bacteria, viruses and mammalian cells [570] and has been widely used in the study of the significance of sialic acid in the cell membrane.

Pretreatment of human basophils with sialidase from *Clostridium perfringens, in vitro*, enhances histamine release induced by various stimulators [575]. The substrate specificity for the various microbial sialidases varies [570], depending on the nature of substrate and the type of linkage between NANA and the neighbouring carbohydrate [570,587]. Jensen *et al.* determined the comparative activity of three sialidases, two bacterial (*Clostridium perfringens, Vibrio cholera*) and one viral (*Influenza A_2 virus*), on human basophil activation [588]. Only sialidase from *Cl. perfringens* released histamine in its own right. Furthermore, secretion does not depend on extracellular calcium and this may or may not be a result of toxicity.

Pretreatment of the cells with any of the sialidases enhanced the maximum response to subsequent stimulation with anti-IgE and also caused a shift to the left of the dose-response curve. However, no differences between treated and control cells were found in relative immunofluorescence using FITC-conjugated anti-IgE, indicating no change in cell bound IgE by neuraminidase treatment.

Bach and Brashler [574] have shown both similarities and discrepancies in the action of various sialidases on rat mast cells. The sialidase from *V. cholera* was found to be equipotent with the purified enzyme from *Cl. perfringens*. Enormous concentrations of sialidase from *Influenza* A_2 *virus* failed to affect the cells. This discrepancy can be ascribed to a different substrate specificity of this enzyme. Bach and Brashler also observed stimulation of IgE-mediated histamine release at low neuraminidase concentrations and inhibition at higher concentrations. The authors noted that low cell density was important to perceive inhibition. The stimulatory concentrations of neuraminidase did not release appreciable histamine by themselves. Dextran-induced secretion was relatively unaffected.

Baxter and Adamik [577] investigated the effects of neuraminidase treatment (from *V. cholera*) on rat peritoneal mast cells. Release induced by concanavalin A, A23187 and antigen was potentiated, whereas that induced by the basic agents protamine, compound 48/80 and polylysine were significantly decreased.

More recently, other authors have reported that neuraminidase treatment (of unspecified origin) inhibited immunologic stimulation and only slightly inhibited that of compound 48/80 [589]. However, high concentrations of the enzyme were investigated and the previous reports have clearly stated the importance of the 'total' amount of enzyme administered to each cell (as opposed to the concentration, w/v) in terms of observed response, a facet which this study appears to have overlooked.

In an alternative approach, it has been shown that incorporation into the cell membrane of exogenous gangliosides rich in NANA inhibits histamine release from human basophil leucocytes and rat mast cells stimulated with anti-IgE, concanavalin A and the ionophore A23187 [590].

In the present study, neuraminidase was found slightly to potentiate immunologic histamine release in the presence of extracellular calcium but to inhibit compound 48/80 and somatostatin, particularly in the absence of the cation. NANA may then have a dual role in histamine secretion. It may bind or sequester extracellular calcium, such that its removal facilitates transmembrane calcium fluxes and enhances release under these conditions [157,591,592]. More interestingly, NANA may also play a vital role in the binding of polyamines to the mast cell membrane, as suggested by Baxter and Adamik [577]. Removal of the carbohydrate would then produce the observed decrease in response to these agents. In total, these data provide an attractive model whereby polyamines intercalate into the cell membrane through their hydrophobic regions and then become anchored in position through ionic interactions with surface NANA residues. However, although this scheme is compelling it lacks direct experimental proof.

CHAPTER 8

CONCLUDING REMARKS

Data presented and discussed in this thesis centres on somatostatin as an example of a polybasic, neuropeptidergic mast cell agonist. In reviewing the previous chapters, two principle concepts seem to emerge, one surrounding the relevance of neurogenic inflammation in the pathogenesis of allergy and the other, the classification of somatostatin as a typical polyamine mast cell agonist and the associated implications for polyamine binding and activation of mast cells.

Antidromic stimulation of peptidergic, afferent nerves results in the liberation of neuropeptides into the extracellular space. Mast cells residing in close proximity to such nerve endings may be exposed to these neuropeptides which have been shown to elicit histamine release from mast cells *in vitro*. These observations constitute the basis of a model for neurogenic inflammation. Obviously, a fundamental prerequisite for this model to flourish, is the successful stimulation of mast cells by the neuropeptides.

Many neuropeptides, including somatostatin, release histamine from rat peritoneal mast cells and human cutaneous mast cells. Studies with somatostatin illustrate that, in addition to the above cell lines, mast cells from the mouse and hamster peritonea are also responsive. Within the rat, mast cells from the peritoneal and pleural cavities are the most responsive, those from the mesentery less so and those from the skin and lung only mildly responsive in comparison. However, somatostatin and its co-agonists fail to provoke significant histamine release from mastocytes situated in other tissues from the rat or, indeed, from other species, including man and the guinea pig.

Under these circumstances, it would be hard to support the widespread application of the neurogenic model to systems other than those in human skin or particular murine mast cells. In fact, the situation in human skin requires some further comment. Intradermal injection of neuropeptides and polyamines in the human produces a flare response which appears to be largely histamine-mediated, indicating that these agents can activate cutaneous mast cells *in vitro* as well as *in vivo*. However, even under the latter conditions, the skin cells are much less reactive than those of the rat; they release limted amounts of histamine and only at high concentrations of agonists. Whether such concentrations can be achieved at any putative neuroeffector mast cell junction remains a matter of pure speculation. In any event, the direct effects of neuropeptides on mucus cells, smooth muscle and the microvasculature would seem to be much more important than mast cell activation.

Perhaps one of the most engaging arguments against a 'universal' neurogenic mechanism is the rather non-specific nature of the putative polyamine receptor on mast cell membranes. For a ubiquitous, somatic mechanism to function, it would be expected that it comprised a sophisticated, specific series of agonists, antagonists and receptors. Clearly,

this is not the case in the instance of polyamine mast cell stimulation. In fact, there is no single, specific antagonist for any one polyaminic stimulus. In depth studies conducted on BDTA antagonism suggested that BDTA competitively antagonised polyamines as a result of, rather than in addition to, its surface active properties. The outcome of pA_2 calculations and Lineweaver-Burk analysis intimated a competitive interaction at the binding site but that the latter was extremely non-specific. Experiments on the basic characteristics of polyamine-induced release defined an induction mechanism quite distinct from that of IgE-receptor dependent ligands. Release is independent of extracellular calcium and exogenous phospholipids, unaffected by desensitisation to antigen and pH, fast (complete within 30s) and inhibited by high concentrations of calcium but not strontium ions. The release is blocked by most inhibitors of IgE-mediated secretion but generally to a lesser extent. The most significant departure from characteristic IgE-mediated secretion is the calcium independence of polyamine-induced secretion. The agonists are presumed to mobilize internal calcium stores via a mechanism unavailable to IgE-directed ligands. The reasons for the particular efficacy of polyamines under the latter conditions remains unknown.

Experiments conducted with neuraminidase suggest that sialic acid-containing GSLs or glycoproteins may represent the binding site for polyaminic stimuli on the mast cell membrane. Histaminocytes which are resistant to polyamine agonism may lack sufficient sialic acid moieties, per carrier molecule, to initiate secretion. It is unlikely that any cell is totally devoid of sialic acid-containing GSLs, since they are centrally implicated in maintaining cell surface charge and may also be involved in transmembrane calcium fluxes. Hence, mastocytes only weakly responsive to polyamine challenge might only possess a small number of appropriate sialic acid-containing elements and/or GSLs or glycoproteins that contain an unsuitable number of sialic acid residues.

To conclude, somatostatin has been shown to be a classic, polybasic mast cell agonist. The polyamines may stimulate certain mast cells by binding to specific sialic acid residues on the membrane. The binding site on responsive mastocytes is ubiquitous for several polyamines but with varying degrees of affinity reflected in varying potencies amongst the agonists. Activation of the mast cell is brought about by the hydrophillic binding and anchorage by hydrophobic binding.

The precise mechanisms of signal transduction remain recondite but may involve pertussis toxin-sensitive G-proteins. Transmembranous calcium fluxes could be modulated by NANA-calcium complexes and secretion may be initiated on polyamine-NANA binding.

Neuropeptide and polyamine activation of mast cells is an exciting area of research and there are almost endless possibilities for further investigations, some of which have been described. The implications of neuropeptides in the expansive field of neuroimmunology are enormous and potentially very exciting, with respect to an increasing number of recognised neuroimmunological disorders such as Alzheimer's disease.

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