British Journal of Pharmacology (1998) 123, 1267–1273

Role of serine esterases in mast cell activation

B. Emadi Khiav & [1]F.L. Pearce

Department of Chemistry, University College London, 20 Gordon Street, London WC1H OAJ

1 A variety of chymotryptic substrates and inhibitors prevented the release of histamine and prostaglandin D_2 from rat peritoneal mast cells stimulated with anti-IgE but not the calcium ionophore A23187 or a variety of polyamines.

2 The activity of the compounds was strikingly increased in cells reversibly permeabilized with ATP, indicating the importance of their effective incorporation into the cytosol.

3 The compounds produced a comparable inhibition of immunological, but not pharmacological, histamine release from human mast cells and basophils.

4 Treatment of rat mast cells with anti-IgE led to a marked increase in the total chymotryptic activity expressed by the cells.

5 Immunological, but not pharmacological, stimulation of permeabilized rat mast cells loaded with a fluorescent chymotryptic substrate led to a pronounced and rapid increase in fluorescence, indicating activation of the enzyme and hydrolysis of the substrate. These changes were attenuated by chymotryptic inhibitors.

6 In total, these data provide compelling evidence for the direct involvement of a serine protease in IgE-mediated histamine release from mast cells.

Keywords: Histamine; mast cells; serine esterase; signal transduction

Introduction

The release of chemical mediators from tissue mast cells and circulating basophil leukocytes has been centrally implicated in a diversity of allergic and inflammatory disorders. These mediators are typified by the autacoid histamine and lipid products of the oxidative metabolism of arachidonic acid. In addition, the mast cell has been widely used as a model system to study the detailed biochemical events involved in stimulus-secretion coupling.

The pathophysiological stimulus for mast cell activation is provided by the cross-linking of high affinity receptors ($Fc_{\varepsilon}RI$) for antibody IgE on the plasma membrane (Ishizaka, 1982; Sagi-Eisenberg, 1993). This induces a series of biochemical changes, including the stimulation of phosphatidylinositol (PI) metabolism which results in the generation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacyclglycerol (DAG). IP_3 acts to mobilize intracellular stores of calcium ions and DAG activates protein kinase C, both of which are thought to be essential steps in the secretory process. However, the exact sequence of events following receptor bridging is not clear and the precise steps involved may be different for immunological stimuli and agents which act independently of IgE antibody (Ishizaka, 1982; Sagi-Eisenberg, 1993). Moreover, it has become clear in recent years that mast cells from different locations are functionally heterogeneous and the detailed mechanisms involved in signal transduction may vary from one histaminocyte to another (Barrett & Pearce, 1991; 1993).

One of the earliest events in stimulus-secretion coupling in the mast cell appears to involve activation of an endogenous serine esterase. This hypothesis was first proposed more than 35 years ago when it was demonstrated that histamine release from fragments of guinea-pig lung could be inhibited by a range of protease inhibitors and substrates (Austen & Brocklehurst, 1961; Becker & Austen, 1964). Similar findings were subsequently obtained for rat peritoneal mast cells, human lung fragments and basophil leucocytes (Becker & Austen, 1966; Kaliner & Austen, 1973; Prozansky & Patterson, 1975). In each case, the inhibitors were only effective if present at the time of cell stimulation, suggesting that the enzyme normally occurs in the form of a zymogen which is converted to the active entity following IgE receptor cross-linking. The use of defined inhibitors and substrates suggested that the enzyme had chymotryptic specificity.

Despite the above very early observations, the nature of the putative esterase is still obscure and evidence for its existence remains indirect. We have thus now investigated this hypothesis further and have examined the effect of chymotryptic inhibitors and substrates on a range of histaminocytes. In addition, we have obtained direct evidence for the activation of the enzyme by monitoring hydrolysis of an entrapped fluorescent substrate following immunological stimulation of rat permeabilized mast cells. Such stimulation also leads to an increase in the total chymotryptic activity expressed by the cells.

Methods

Isolation of histaminocytes

Male Sprague Dawley rats (200–300 g) were used throughout the present study and were sensitized by immunization with the nematode parasite *Nippostrongylus brasiliensis* (White & Pearce, 1982). Peritoneal washings were obtained by direct lavage and mast cells purified to greater than 95% homogeneity by density gradient centrifugation over Percoll (Mackay & Pearce, 1992). Cell numbers were quantified after staining by the method of Kimura *et al.* (1973). Macroscopically normal human tissue was recovered following surgery for bronchial carcinoma or for circumcision. Lung parenchyma was dissected free of major airways and blood vessels and adult foreskin was cleared of underlying fat.

[1] Author for correspondence.

Fragments of tissue (1 mm^2) were digested (2×90 min at 37°C or longer if, after this time, substantial tissue remained undisrupted) with collagenase (160 units ml^{-1}) and hyaluronidase (500 units ml^{-1}, skin only) in HEPES-Tyrode buffer (pH = 7.4) composed of (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.0, NaH_2PO_4 0.4, glucose 5.6, HEPES 10 and additionally containing bovine serum albumin (1 mg ml^{-1}). Cells were then recovered as previously described (Ali & Pearce, 1985).

Human basophil leucocytes were obtained by dextran sedimentation of peripheral blood recovered by venipuncture (Lichtenstein & Osler, 1964).

Measurement of mediator release

In simple release experiments, aliquots (450 μl) of cells were allowed to equilibrate (37°C, 5 min) in HEPES-Tyrode buffer in a metabolic shaker with gentle mechanical agitation. A solution (50 μl; $10 \times$ final concentration) of the releasing agent was then added, secretion allowed to proceed for a further 10 min (mast cells) or 20 min (basophils) and the reaction terminated by the addition of ice-cold Tyrode solution (1 ml). Cells and supernatants were recovered by centrifugation (4°C, 2 min, 150 *g*). The cell pellets were resuspended in Tyrode solution (1.5 ml) and allowed to stand in a boiling water bath to release residual histamine. Individual supernatants were treated similarly. Histamine was then determined spectrofluorometrically with an automated system (Technicon Autoanalyser II) as previously described (Atkinson *et al.*, 1979). Histamine release was expressed as a percentage of the total cellular content of the amine and calculated as the ratio ($100 \times$ (histamine in supernatant)/(histamine in supernatant + residual histamine in cells)). All values were corrected for the spontaneous release (ca. 5%) occurring in the absence of any stimulus. In experiments with inhibitors, cells were preincubated (10 min) with the agents before challenge with the appropriate secretagogue. Results were conveniently expressed in terms of the percentage inhibition of the control release and thus given as the ratio ($100 \times$ (percentage histamine release in absence of drug) − (percentage histamine release in presence of drug)/(percentage histamine release in absence of drug)). Prostaglandin D_2 (PGD_2) was measured by radioimmunoassay by use of a commercially available kit (Amersham).

Permeabilization with ATP and fluorescence measurements

In some experiments, compounds were directly introduced in the cytosol of rat mast cells by reversible permeabilization with ATP according to the method of Bennet *et al.* (1981). Aliquots (150 μl) of purified cells (5×10^5 ml^{-1}) in calcium- and magnesium-free Tyrode solution were incubated (37°C, 10 min) with the inhibitors (250 μl), treated with ATP (50 μl to a final concentration of 5 μM, 10 min, 37°C), resealed by the addition of excess magnesium (50 μl to a final concentration of 2 mM), washed twice in complete Tyrode solution and challenged as before. This treatment did not *per se* alter the histamine content of the cells nor their responses to any of the secretory stimuli used in the present study. In all experiments with inhibitors, the responses were compared to the corresponding control secretions in permeabilized or naive cells as appropriate.

The above procedure was also used to incorporate into the cells the fluorescent chymotryptic substrate *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (S-Pep-MCA). Purified mast cells were incubated (20 min, 37°C) with the substrate (100 μM) and any appropriate inhibitor, permeabilized by addition of ATP as described above, resealed and washed twice. To avoid interference with granule-associated neutral proteases, they were then treated (20 min, 37°C) with the metabolic blocker antimycin A (1 μM). Parallel experiments showed that secretion was totally abolished under these conditions. Cells (2×10^5 ml^{-1}) preloaded with the substrate were then incubated in a thermostatted (37°C) quartz cuvette (5 mm path length) of a Perkin Elmer LS 5B luminescence spectrometer fitted with an electronic stirrer (Model 1100, Rank Brothers, Cambridge). The sample was illuminated at an excitation wavelength of 380 nm and emitted light was recorded at 460 nm. Secretagogues were added in a minimum volume as indicated.

Measurement of total chymotryptic activity

Total chymotryptic activity was recovered from the cells according to the method of Harvima *et al.* (1988). Briefly, aliquots of purified cells (5×10^5 cells in 250 μl of solution) were frozen and thawed five times in buffer of high ionic strength (0.1 M Na_2HPO_4/NaH_2PO_4, 2 M KCl, pH = 7.0) and then stirred overnight at 4°C. The extract was clarified by

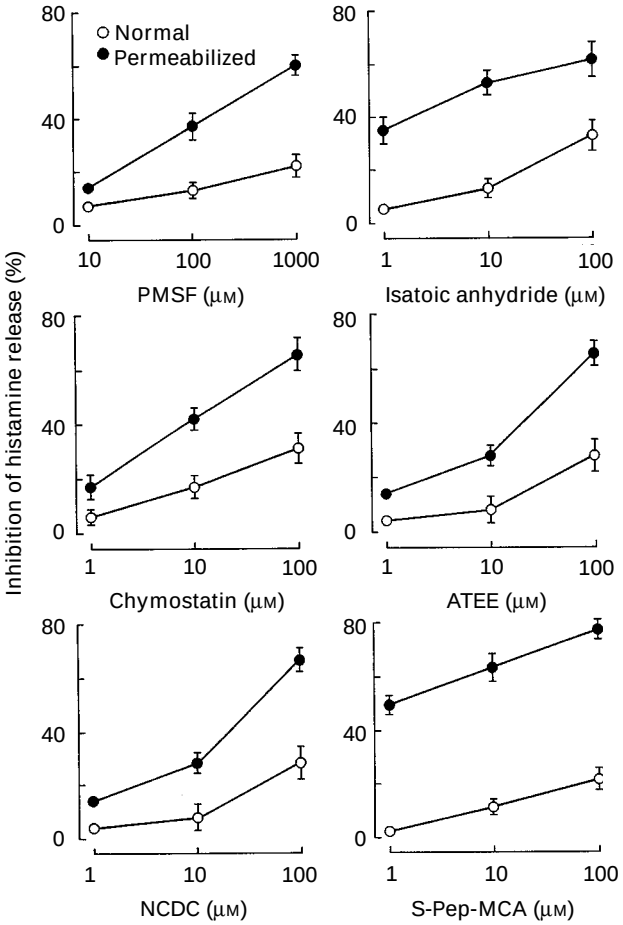

Figure 1 The effect of chymotryptic inhibitors and substrates on histamine release from normal and permeabilized rat peritoneal mast cells stimulated with anti-IgE (100 fold dilution). Cells were incubated (10 min, 37°C) with the agents or the latter were introduced into the cytosol by reversible permeabilization with ATP. Control release of histamine was typically 35–45%. Secretion was unaffected by treatment of the cells with ATP alone but, in each case, data are given as the percentage inhibition of the control release from normal or permeabilized cells as appropriate. Values are means and vertical lines s.e.mean for 4 experiments. For abbreviations and experimental details, see text.

centrifugation and activity was measured by use of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate and expressed as units (μmol substrate hydrolyzed min^{-1}) per 10^6 mast cells (Dawson *et al.*, 1986).

Reagents

N-Acetyl-L-tyrosine ethyl ester (ATEE), adenosine 5′-triphosphate (ATP), anti-human IgE (Dako), anti-rat IgE (ICN), antimycin A, *N*-α-benzoyl-L-arginine-7-amido-4-methylcoumarin, *N*-α-benzoyl-L-tyrosine ethyl ester (BTEE), calcium ionophore A23187 (Calbiochem), chymostatin, collagenase (Type 1A), compound 48/80, concanavalin A, dextran (RMM 70,000), 3,4-dichloroisocoumarin, formyl-met-leu-phe (FMLP), hyaluronidase (Type I-S), isatoic anhydride, leupeptin, lysophosphatidylserine, mastoparan (Neosystem), nerve growth factor (NGF), 2-nitro-4-carboxyphenyl-*N*, *N*-diphenylcarbamate (NCDC), phenylboronic acid, phenylmethylsulphonyl fluoride (PMSF), phorbol 12-myristate-13-acetate (PMA), substance P (Neosystem), *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (S-Pep-MCA), *N*-tosyl-L-lysine chloromethylketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethylketone (TPCK) were purchased from Sigma unless otherwise stated.

Statistics

All values are given as means ± s.e.mean for the number (n) of experiments stated. Statistical analysis was carried out by use of Student's t test and values of $P < 0.05$ were considered to be significant.

Results

Inhibition of mediator release from rat mast cells

The general serine esterase inhibitor PMSF (10 – 1000 μM), the suicide inactivator isatoic anhydride, the microbial product chymostatin, and the chymotryptic substrates ATEE, NCDC, and S-Pep MCA (all at 1 – 100 μM) produced a modest inhibition of histamine release from rat peritoneal mast cells stimulated with anti-rat IgE (100 fold dilution). However, the potency of the compounds was strikingly increased when they were directly introduced into the cells by permeabilization with ATP (Figure 1). In contrast, maximal concentrations of the drugs had a limited or negligible effect on both permeabilized and naive mast cells treated with the calcium ionophore A23187 (0.5 μM), compound 48/80 (0.5 μg ml^{-1}), mastoparan (5 μM) or substance P (5 μM) (Table 1).

In a further series of experiments with permeabilized cells, the above inhibitors again effectively attenuated histamine release induced by anti-IgE, as did the suicide inhibitor 3,4-dichloroisocoumarin, the transition state analogue phenylboronic acid and the active site directed ligand TPCK (Table 2). The compounds also showed considerable activity against the secretion induced by the lectin concanavalin A (5 μg ml^{-1}) but had a variable and reduced effect against

Table 1 Inhibition of histamine release from normal and permeabilized peritoneal mast cells of the rat treated with various stimuli

	Inhibition (%)									
	Anti-IgE		*Calcium ionophore*		*Compound 48/80*		*Mastoparan*		*Substance P*	
Inhibitor	*Normal*	*Permeabilized*	*Normal*	*Permeabilized*	*Normal*	*Permeabilized*	*Normal*	*Permeabilized*	*Normal*	*Permeabilized*
PMSF	22.0 ± 2.0	61.0 ± 3.0	8.0 ± 2.3	11.5 ± 6.5	5.0 ± 3.0	−1.0 ± 2.0	6.0 ± 2.8	8.5 ± 5.5	4.3 ± 2.3	6.7 ± 5.5
Isatoic anhydride	31.0 ± 3.0	64.0 ± 4.0	3.0 ± 2.3	10.7 ± 5.0	9.0 ± 2.0	9.0 ± 3.0	1.0 ± 3.0	7.6 ± 4.8	4.0 ± 2.3	9.7 ± 5.6
Chymostatin	31.0 ± 2.1	62.0 ± 1.4	5.2 ± 3.0	11.5 ± 7.0	3.5 ± 1.7	3.8 ± 2.2	3.2 ± 2.8	5.7 ± 1.4	0.0 ± 2.5	1.5 ± 4.0
ATEE	28.0 ± 2.0	66.0 ± 3.0	0.0 ± 0.5	3.5 ± 4.0	3.0 ± 2.3	0.0 ± 1.0	2.0 ± 0.5	6.0 ± 3.0	0.5 ± 1.5	3.8 ± 3.6
NCDC	30.0 ± 4.0	56.0 ± 4.0	0.0 ± 0.5	10.0 ± 2.5	1.0 ± 3.0	1.0 ± 2.0	−4.0 ± 1.8	6.3 ± 4.4	3.8 ± 2.5	6.0 ± 4.5
S-Pep-MCA	21.0 ± 1.0	78.0 ± 3.0	5.0 ± 1.8	4.5 ± 1.0	3.0 ± 2.0	5.0 ± 1.0	ND	ND	ND	ND

Normal and permeabilized mast cells of the rat were treated with the inhibitors shown (100 μM except PMSF, 1 mM) and stimulated with the various agonists. Control releases of histamine were balanced so as to be approximately equal for each secretagogue and were typically 35 – 45%. Secretion was unaffected by treatment of the cells with ATP alone but, in each case, data are given as the percentage inhibition of the control release from normal or permeabilized cells as appropriate. Values are means ± s.e.mean for 4 experiments. ND = not determined. For abbreviations and experimental details, see text.

Table 2 Inhibition of histamine release from rat permeabilized peritoneal mast cells treated with various stimuli

	Inhibition (%)			
Inhibitor	*Anti-IgE*	*Concanavalin A*	*Dextran*	*NGF*
PMSF	57.3 ± 4.0	40.3 ± 6.3	−5.6 ± 6.5	−0.5 ± 3.0
Isatoic anhydride	72.7 ± 7.0	52.4 ± 8.9	30.6 ± 9.5	34.0 ± 8.0
Chymostatin	72.7 ± 7.0	52.4 ± 8.9	30.6 ± 9.5	21.6 ± 6.7
ATEE	59.2 ± 8.5	42.3 ± 8.7	15.8 ± 9.3	4.0 ± 8.0
NCDC	62.5 ± 5.0	54.8 ± 8.9	17.2 ± 9.5	10.0 ± 5.9
S-Pep-MCA	78.0 ± 3.0	ND	ND	ND
3,4-Dichloro-iosocoumarin	59.7 ± 6.5	51.8 ± 8.9	17.3 ± 9.8	21.5 ± 8.0
Phenylboronic acid	48.7 ± 3.0	41.0 ± 7.0	9.4 ± 8.5	26.5 ± 11.0
TPCK	85.0 ± 1.8	56.0 ± 4.1	ND	ND

Rat permeabilized mast cells were treated with the inhibitors shown (100 μM except for PMSF, 1 mM) and stimulated with the various agonists. Lysophosphatidylserine (1 μg ml^{-1}) was added to enhance the response to dextran and NGF. Control releases of histamine were balanced so as to be approximately equal for each secretagogue and were typically 25 – 35%. Values are means ± s.e.mean for 4 – 6 experiments. ND = not determined. For abbreviations and experimental details, see text.

dextran (5 μg ml^{-1}) and NGF (10 ng ml^{-1}). Lysophosphatidylserine (1 μg ml^{-1}) was added to potentiate the response to the latter two ligands. The tryptic inhibitors TLCK ($\leqslant$100 μM) and leupeptin ($\leqslant$1 μM) had little or no effect (maximum inhibition $\leqslant$15%) against any of the agonists tested in either permeabilized or normal cells (data not shown).

In addition to the release of histamine PMSF, isatoic anhydride, ATEE and chymostatin also prevented the generation of PGD_2 from rat mast cells treated with anti-IgE. The effect was again greater in permeabilized cells (Table 3). In contrast, these agents had no effect on the modest production of the prostanoid (4.1$\pm$2.8 ng/10^6 cells) induced by compound 48/80 (0.25 μg ml^{-1}, data not shown).

Inhibition of histamine release from human basophils and mast cells

ATEE, chymostatin, isatoic anhydride and phenylboronic acid all produced a pronounced inhibition of histamine release from human basophils and pulmonary and cutaneous mast cells stimulated with anti-human IgE (Table 4). The compounds were ineffective, or much less active, against secretion induced by the calcium ionophore A23187, particularly in the case of the mast cells. The drugs were also weakly active against basophils stimulated with F-MLP and PMA, and cutaneous mast cells treated with substance P.

Table 3 Inhibition of the release of histamine and PGD_2 from rat normal and permeabilized peritoneal mast cells treated with anti-rat IgE

	Inhibition (%)			
	Histamine		*PGD_2*	
Inhibitor	*Normal*	*Permeabilized*	*Normal*	*Permeabilized*
PMSF	43.4$\pm$5.4	84.3$\pm$1.9	45.0$\pm$6.3	77.5$\pm$3.1
Isatoic anhydride	36.7$\pm$3.0	66.4$\pm$1.9	56.0$\pm$3.3	88.5$\pm$3.6
ATEE	44.7$\pm$5.3	55.0$\pm$4.3	51.4$\pm$4.8	93.9$\pm$1.0
Chymostatin	44.7$\pm$5.3	67.7$\pm$1.2	52.3$\pm$3.9	84.6$\pm$1.3

Normal and permeabilized mast cells of the rat were treated with the inhibitors shown (100 μM except for PMSF, 1 mM) and stimulated with anti-IgE (200 fold dilution). The control release was 29.7$\pm$2.1% histamine and 18.5$\pm$4.9 ng PGD_2/10^6 cells. Values are means$\pm$s.e.mean for 3 experiments.

Total chymotryptic activity expressed by rat peritoneal mast cells

Extracts of rat mast cells showed a high basal level of chymotryptic activity, as demonstrated by the hydrolysis of BTEE. However, activation of the cells with anti-IgE led to a pronounced and significant increase in the total activity recovered from the cells (Figure 2).

Activation of a chymotryptic enzyme in rat mast cells as evidenced by hydrolysis of an entrapped fluorescent substrate

In a further series of experiments, permeabilized mast cells were loaded with the fluorescent chymotryptic substrate S-Pep-MCA. Cells were also pretreated with the metabolic inhibitor antimycin A to prevent exocytosis and possible interference from granule-associated neutral proteases. Under these conditions, simulation of the cells with anti-rat IgE led to a pronounced and rapid increase in fluorescence, indicating hydrolysis of the substrate (Figure 3a). Centrifugation of the preparations showed that all of the fluorescence was associated with the cells and none was released into the supernatant (data not shown). The increase in fluorescence was independent of the presence of extracellular calcium ions, indicating that it was not an epiphenomenon of ion fluxes. No changes were observed on activation of cells preloaded under identical conditions with the fluorescent tryptic substrate *N*-α-benzoyl-L-arginine-7-amido-4-methylcoumarin (data not shown), showing the specificity of the effect for a chymotryptic protease.

In contrast to the response to IgE cross-linking, activation of the cells with compound 48/80 produced no change in fluorescence (Figure 3b). Thereafter, the cells still responded to anti-IgE, although the rise in fluorescence occurred rather more slowly than in naive cells. Similarly, stimulation with the calcium ionophore A23187 (0.5 μM), substance P (50 μM), or mastoparan (5 μM) produced no increase in fluorescence (data

Table 4 Inhibition of histamine release from human basophils, lung and skin mast cells

			Inhibition (%)		
Inhibitor	*Anti-IgE*	*Ionophore*	*Substance P*	*FMLP*	*PMA*
Basophils					
ATEE	35.2$\pm$7.0	12.9$\pm$3.6	ND	12.9$\pm$5.2	6.0$\pm$1.5
Chymostatin	36.5$\pm$5.2	13.0$\pm$3.0	ND	7.8$\pm$6.0	14.0$\pm$5.0
Isatoic anhydride	31.0$\pm$5.9	4.5$\pm$2.5	ND	2.3$\pm$2.5	3.0$\pm$1.0
Phenylboronoic acid	35.0$\pm$3.1	21.0$\pm$4.0	ND	5.4$\pm$3.8	12.0$\pm$3.3
Lung mast cells					
ATEE	61.4$\pm$5.1	2.8$\pm$1.9	ND	ND	ND
Chymostatin	58.2$\pm$3.3	6.8$\pm$2.8	ND	ND	ND
Isatoic anhydride	62.1$\pm$2.6	1.5$\pm$1.8	ND	ND	ND
Phenylboronic acid	32.4$\pm$5.6	1.4$\pm$1.6	ND	ND	ND
Skin mast cells					
ATEE	48.3$\pm$4.4	7.9$\pm$2.8	4.6$\pm$2.5	ND	ND
Chymostatin	41.5$\pm$3.8	12.5$\pm$4.2	4.0$\pm$1.6	ND	ND
Isatoic anhydride	53.0$\pm$4.6	7.4$\pm$2.1	6.9$\pm$1.9	ND	ND
Phenylboronic acid	49.5$\pm$4.0	1.2$\pm$0.5	3.0$\pm$2.0	ND	ND

Cells were treated with the inhibitors shown (100 μM) and stimulated with anti-IgE (300 fold dilution for basophils and 100 fold dilution for mast cells), calcium ionophore A23187 (0.5 μM and 3 μM, respectively), substance P (50 μM), FMLP (1 μM) and PMA (1 ng ml^{-1}). Control release of histamine was balanced so as to be approximately equal for each secretagogue and was typically 30–45% (basophils), 25–30% (lung mast cells) and 15–35% (skin mast cells). Values are means$\pm$s.e.mean for 4–8 experiments. ND = not determined. For abbreviations and experimental details, see text.

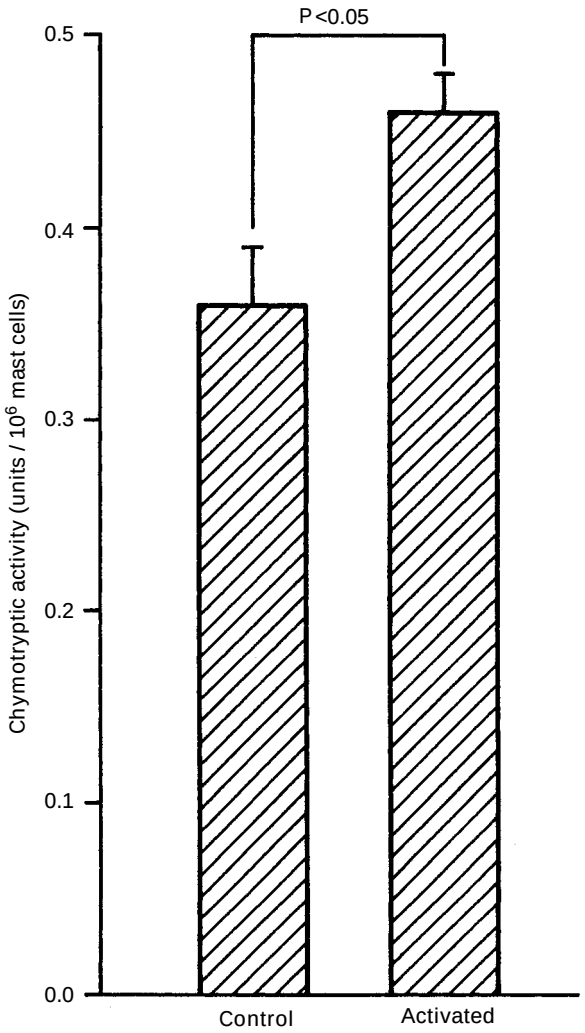

Figure 2 Total chymotryptic activity expressed by rat peritoneal mast cells. Cells were incubated in buffer or activated by anti-IgE (200 fold dilution). They were then disrupted by freeze-thawing (5 cycles) and enzyme activity extracted with buffer of high ionic strength. Chymotryptic activity was measured with BTEE as substrate and expressed as units (μmol substrate hydrolyzed min^{-1}) per 10^6 mast cells. Values are given as means $\pm$ s.e.mean for 4 experiments. For abbreviations and experimental details, see text.

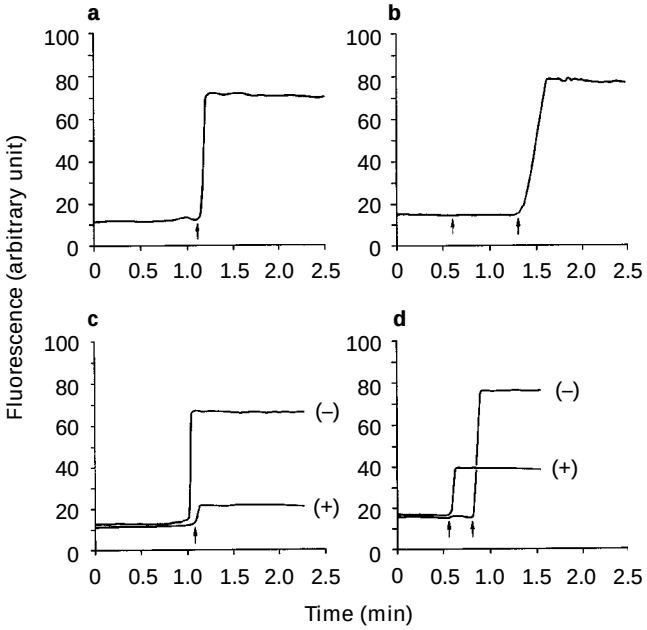

Figure 3 Fluorescence changes in permeabilized rat peritoneal mast cells containing the chymotryptic substrate S-Pep-MCA. Secretagogues were added in a minimum volume at the points indicated. These comprised (a) anti-rat IgE (250 fold dilution), (b) compound 48/80 (1 μg ml^{-1}) followed by anti-IgE, and anti IgE in the presence (+) and absence (−) of (c) isatoic anhydride (100 μM) and (d) TPCK (100 μM). Traces are representative of at least 4 similar recordings in each case. For abbreviations and experimental details, see text.

not shown). The fluorescence changes evoked by immunological activation were markedly reduced by isatoic anhydride (Figure 3c), TPCK (Figure 3d) and PMSF (data not shown). Consistently, the analogous trypsin inhibitor (TLCK (100 μM) had no effect (data not shown).

Discussion

The present study has confirmed and extended previous work which showed that a number of chymotryptic inhibitors and substrates inhibited the immunological release of histamine from rat peritoneal mast cells. We used a variety of compounds of widely diverse structural type, including peptide and non-peptide substrates, the general serine esterase inhibitor PMSF, the active site directed ligand TPCK, the microbial product chymostatin, the transition state analogue phenylboronic acid and the suicide inhibitors isatoic anhydride and dichloroisocoumarin. The latter agents are of particular interest as they are chemically stable entities which are coverted to reactive species only after binding to, and by the direct action of, the target enzyme (Moorman & Abeles, 1982; Harper *et al.*, 1985). In total, the activity of this wide spectrum of compounds provides strong evidence for the involvement of a chymotryptic protease in the release process.

The activities of all of the test compounds were strikingly increased when they were directly introduced into the target cells by reversible permeabilization with ATP. This is an extremely useful tool and it has been widely used to incorporate a number of probes into mast cells and thus to explore some of the biochemical events involved in stimulus secretion coupling in this cell type (Gomperts, 1983; Howell *et al.*, 1989). The increased activity seen in the present study indicates the importance of the effective incorporation of the test compounds into the cell cytosol.

In contrast to their activity against secretion induced by anti-IgE, none of the test compounds was effective against naive or permeabilized cells stimulated with compound 48/80, mastoparan and substance P. These polyamines are thought to bypass many of the biochemical events following IgE receptor cross-linking, and directly to activate one or more G-proteins involved in the secretory process (Mousli *et al.*, 1990). As such, they may circumvent that step which involves the serine protease.

The various drugs also significantly attenuated histamine release induced by the lectin concanavalin A but had a variable and reduced effect against dextran and NGF. Concanavalin A is thought to cross-link IgE molecules by binding to sugar moieties on the immunoglobulin, and may thus mimic the effects of antigen and anti-IgE, while dextran binds to glucose receptors and NGF to its unique receptor on the mast cell membrane. However, the secretion induced by the latter ligands closely resembles that evoked immunologically (Truneh & Pearce, 1981; Leoutsakos *et al.*, 1984; Pearce &

Thompson, 1986) and the present data suggest that the involvement of the serine protease may be confined to the release process triggered by IgE cross-linking or related stimuli.

In contrast to the above, the tryptic inhibitors TLCK and leupeptin had no effect against any of the agonists tested in either permeabilized or normal cells. This complements much comprehensive, earlier data showing that the effect is specific for a chymotryptic protease (Austen & Brocklehurst, 1961; Becker & Austen, 1964; 1966).

Chymotryptic inhibitors also prevented the generation of PGD_2 from rat mast cells activated with anti-IgE. The effect is then not confined to exocytosis and the release of preformed, granule-associated amines but extends to the production of newly synthesized lipid mediators.

ATEE, chymostatin, isatoic anhydride and phenylboronic acid all produced a pronounced inhibition of immunological histamine release from human pulmonary and cutaneous mast cells and, to a rather lesser extent, basophil leucocytes. Again, the compounds were weakly active or ineffective against non-IgE-mediated stimuli. Unfortunately, human histaminocytes cannot be permeabilized with ATP and these experiments could only be carried out on naive cells but they show, as a minimum, that the putative enzyme is also involved in signal transduction in these cell types. These data may be important given the known heterogeneity of mast cells from different sources (Barrett & Pearce, 1991; 1993) and imply that activation of a chymotryptic protease may be a universal event in stimulus-secretion coupling in all histaminocytes.

Extracts of rat mast cells, prepared by repeated freeze-thawing in buffer of high ionic strength (Harvima *et al*., 1988), showed a high basal level of chymotryptic activity presumably due to the presence of granule-associated chymase. However, stimulation of the cells with anti-IgE led to a significant increase of ca. 30%, in the total activity recovered from the cells. This provides direct evidence for the activation of a zymogen following IgE cross-linking.

Further direct evidence for the involvement of a chymotrypsin-like enzyme in immunological activation of rat mast cells was provided with the fluorescent substrate S-Pep-MCA. This compound was entrapped in cells permeabilized with ATP. Stimulation with anti-rat IgE led to a pronounced and rapid increase in fluorescence, indicating activation of the enzyme and hydrolysis of the substrate. No such changes were seen in cells treated with calcium ionophore or polyamines. Following the latter treatment, cells still responded to anti-IgE, although the rise in fluorescence occurred more slowly than in naive cells. The significance of this observation remains unclear. Consistent with the given hypothesis, the fluorescence changes evoked by immunological activation were markedly attenuated by chymotryptic inhibitors previously shown to block histamine release. Again, tryptic inhibitors were ineffective in this system and no fluorescence changes were seen with tryptic substrates.

In total, this study provides the most direct evidence to date for the involvement of a serine esterase in immunological, but not pharmacological, activation of mast cells. Also, such a protease may be generally involved in the function of a range of immune and inflammatory cells, and chymotryptic inhibitors block the movement and chemotaxis of polymorphonuclear leucocytes, neutrophils and B-lymphocytes (Ward, 1967; Becker, 1971; Becker & Unanue, 1976) and suppress complement-dependent erythrophagocytosis (Pearlman *et al*., 1969) and antibody-dependent cell-mediated cytotoxicity (Trinchieri & De Marchi, 1976). Identification of the enzyme may then provide a new therapeutic target for the modulation of the function of mast cells and other inflammatory cells.

This work was supported by grants from the Wellcome Trust.

References

ALI, H. & PEARCE, F.L. (1985). Isolation and properties of cardiac and other mast cells from the rat and guinea pig. *Agents Actions,* **16,** 138 – 140.

ATKINSON, G., ENNIS, M. & PEARCE, F.L. (1979). The effect of alkaline earth cations on the release of histamine from rat peritoneal mast cells treated with compound 48/80 and peptide 401. *Br. J. Pharmacol.,* **65,** 395 – 402.

AUSTEN, K.F. & BROCKLEHURST, W.E. (1961). Anaphylaxis in chopped guinea pig lung. I. Effect of peptidase substrates and inhibitors. *J. Exp. Med.,* **113,** 521 – 539.

BARRETT, K.E. & PEARCE, F.L. (1991). Heterogeneity of mast cells. *Hdbk. Exp. Pharmacol.,* **97,** 93 – 117.

BARRETT, K.E. & PEARCE, F.L. (1993). Mast cell heterogeneity. In *Immunopharmacology of Mast Cells and Basophils*. ed. Foreman, J.C., pp 29 – 42. London: Academic Press.

BECKER, E.L. (1971). Phosphonate inhibition of the accumulation and retention of K^+ by rabbit neutrophils in relation to chemotaxis. *J. Immunol.,* **106,** 689 – 697.

BECKER, E.L. & AUSTEN, K.F. (1964). A comparison of the specificity of inhibition by phosphonate esters of the first component of complement and the antigen-induced release of histamine from guinea pig lung. *J. Exp. Med.,* **120,** 491 – 506.

BECKER, E.L. & AUSTEN, K.F. (1966). Mechanisms of immunologic injury of rat peritoneal mast cells. I. The effect of phosphonate inhibitors on the homocytotropic antibody-mediated histamine release and the first component of rat complement. *J. Exp. Med.,* **124,** 379 – 395.

BECKER, E.L. & UNANUE, E.M. (1976). The requirement for esterase activation in the anti-immunoglobulin-triggered movement of B lymphocytes. *J. Immunol.,* **117,** 27 – 32.

BENNETT, J.P., COCKROFT, S. & GOMPERTS, B.D. (1981). Rat mast cells permeabilized with ATP secrete histamine in response to calcium ions buffered in the micromolar range. *J. Physiol.,* **317,** 335 – 345.

DAWSON, R.M.C., ELLIOTT, D.C., ELLIOTT, W.H. & JONES, K.M. (1986). *Data for Biochemical Research,* Oxford: Clarendon Press.

GOMPERTS, B.D. (1983). Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. *Nature,* **306,** 64 – 66.

HARPER, J.W., HEMMI, K. & POWERS, J.C. (1985). Reaction of serine proteases with substituted isocoumarins: discovery of 3,4-dichloroisocoumarin, a new general mechanism based serine protease inhibitor. *Biochemistry,* **24,** 1831 – 1841.

HARVIMA, I.T., HARVIMA, R.J., ELORANTA, T.O. & FRAKI, J.E. (1988). The allosteric effect of salt on human mast cell tryptase. *Biochem. Biophys. Acta,* **956,** 133 – 139.

HOWELL, T.W., KRAMER, I.M. & GOMPERTS, B.D. (1989). Protein phosphorylation and the dependence on Ca^{2+} and GTP-γ-S for exocytosis from permeabilised mast cells. *Cell. Signalling,* **1,** 157 – 163.

ISHIZAKA, T. (1982). Biochemical analysis of triggering signals induced by bridging of IgE receptors. *Fedn. Proc.,* **14,** 17 – 21.

KALINER, M. & AUSTEN, K.F. (1973). A sequence of biochemical events in the antigen-induced release of chemical mediators from sensitized human lung tissue. *J. Exp. Med.,* **138,** 1077 – 1094.

KIMURA, I., MORITANI, Y. & TANIZAKI, Y. (1973). Basophils in bronchial asthma with reference to reagin-type allergy. *Clin. Allergy,* **3,** 195 – 202.

LEOUTSAKOS, A., PEARCE, F.L. & THOMPSON, H.L. (1984). On the mechanism of dextran-induced histamine secretion from rat peritoneal mast cells. *Br. J. Pharmacol.,* **83,** 382P.

LICHTENSTEIN, L.M. & OSLER, A.G. (1964). Studies on the mechanisms of hypersensitivity phenomena. *J. Exp. Med.,* **120,** 507–530.

MACKAY, G.A. & PEARCE, F.L. (1992). A comparison of rat peritoneal mast cells purified using Percoll and Path-O-Cyte 4. *Agents Actions, Special Conference Issue,* C315–C317.

MOORMAN, A.R. & ABELES, R.H. (1982). A new class of serine protease inactivators based on isatoic anhydride. *J. Am. Chem. Soc.,* **194,** 6785–6786.

MOUSLI, M., BRONNER, C., BOCKAERT, J., ROUOT, B. & LANDRY, Y. (1990). Interaction of substance P, compound 48/80 and mastoparan with the α-subunit C-terminus of G protein. *Immunol. Letts.,* **25,** 355–358.

PEARCE, F.L. & THOMPSON, H.L. (1986). Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. *J. Physiol.,* **372,** 379–393.

PEARLMAN, D.S., WARD, P.A. & BECKER, E.L. (1969). The requirement of serine esterase function in complement-dependent erythrophagocytosis. *J. Exp. Med.,* **130,** 745–764.

PROZANSKY, J.J. & PATTERSON, R. (1975). The diisopropylfluorophosphate inhibitable step in antigen-induced histamine release from human leukocytes. *J. Immunol.,* **114,** 939–943.

SAGI-EISENBERG, R. (1993). Signal-transmission pathways in mast cell exocytosis. In *Immunopharmacology of Mast Cells and Basophils*. ed. Foreman, J.C., pp 71–88. London: Academic Press.

TRINCHIERI, G. & DE MARCHI, M. (1976). Antibody-dependent cell-mediated cytotoxicity in humans. III. Effect of protease inhibitors and substrates. *J. Immunol.,* **116,** 855–891.

TRUNEH, A. & PEARCE, F.L. (1981). Characteristics of and calcium requirements for histamine release from rat peritoneal mast cells treated with concanavalin A. *Int. Archs. Allergy Appl. Immunol.,* **66,** 68–75.

WARD, P.A. (1967). Mechanisms of the inhibition of chemotaxis by phosphonate esters. *J. Exp. Med.,* **125,** 1001–1020.

WHITE, J.R. & PEARCE, F.L. (1982). Characteristics of histamine secretion from rat peritoneal mast cells sensitized to the nematode *Nippostrongylus brasiliensis*. *Immunology,* **46,** 353–359.

(Received September 9, 1997
Revised November 5, 1997
Accepted December 9, 1997)