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The activation of synovial mast cells: modulation of histamine release by tryptase and chymase and their inhibitors

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Abstract

Mast cells have been implicated as having pivotal roles in arthritis, but little is known of the processes leading to the activation of synovial mast cells or their potential for pharmacological control. We have investigated the ability of tryptase and chymase, and inhibitors of these major mast cell proteases to modulate the activation of mast cells from human synovial tissue. The tryptase inhibitor drug *N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride (APC366) inhibited immunoglobulin E (IgE)-dependent histamine release in a dose-dependent manner, with about 70% inhibition being achieved at a dose of 300 μM. Histamine release stimulated by calcium ionophore A23187 was also inhibited by this compound. The chymase inhibitor chymostatin inhibited IgE-dependent histamine release by approximately 60% at 1 μg/ml. Tryptase at concentrations of 3.0 μg/ml and greater stimulated histamine release from synovial cells, which was dependent on catalytic activity, whereas chymase had little effect on these cells. The activation of mast cells by tryptase may represent an amplification process in the synovium. The mast cell stabilising properties of inhibitors of tryptase and chymase could be of therapeutic value in arthritis.

Keywords: Chymase; Mast cell; Proteinase inhibitor; Synovium; Tryptase

1. Introduction

Mast cells have the potential to play a key role in the pathogenesis of arthritis. These cells are present in markedly increased numbers in the synovial tissues of patients with rheumatoid (Wynne-Roberts et al., 1978; Crisp et al., 1984) and osteoarthritis (Godfrey et al., 1984; Buckley et al., 1998). The detection of the mast cell products tryptase and histamine in the synovial fluid from these and other categories of arthritis (Buckley et al., 1997; Renoux et al., 1996) suggests that mast cell activation occurs in the joints. Immunocytochemical evidence for mast cell degranulation has been provided by detection of extracellular tryptase, particularly at sites of cartilage erosion (Tetlow and Woolley, 1995). However, the mechanisms of mast cell activation in the joint, and the potential for pharmacological control have received relatively little attention.

Mast cells can release a range of potent mediators of inflammation and tissue remodeling. These include proteases, histamine, heparin, eicosanoids and an array of cytokines, though the proteases represent the most abundant secretory products of human mast cells. Prominent among the proteases is the unique tryptic enzyme tryptase, which is present in the secretory granules of almost all synovial mast cells, and the chymotrypsin-like chymase, which is restricted to a subpopulation which comprises some 30% of synovial mast cells in rheumatoid and osteoarthritis and approximately 60% of mast cells in the non-diseased joint (Buckley et al., 1998; Gotis-Graham and McNeil, 1997).

Both tryptase and chymase have been implicated in the induction of microvascular leakage (He and Walls, 1997, 1998a) and cell accumulation (He et al., 1997; He and Walls, 1998b), as well as in processes of matrix breakdown and fibrosis (reviewed in Walls, 2000). The ability of tryptase to stimulate the activation of certain populations of human and guinea pig mast cells has prompted the suggestion that the secretion of this enzyme may provide an amplification mechanism in inflammatory disease (He and Walls, 1997; He et al., 1998). Stimuli for the activation of synovial mast cells include antibody specific for immunoglobulin E (IgE) (Kopicky-Burd et al., 1988; Kiener et al., 1998), calcium ionophore A23187 (Verbsky

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et al., 1996), stem cell factor (De Paulis et al., 1996; Kiener et al., 1998), anaphylatoxin C5a (Kiener et al., 1998), compound 48/80, and substance P (De Paulis et al., 1996). The extent to which mast cell proteases may induce activation has not been investigated.

Perhaps reflecting a role for proteases in mast cell activation, it has been found that inhibitors of tryptase and chymase can be effective as stabilisers of mast cells from human skin, tonsil and lung tissue (He et al., 1998, 1999). Certain other compounds, including sodium cromoglycate and theophylline which have stabilising properties on mast cells from these tissues have proved particularly ineffective for synovial mast cells (Kopicky-Burd et al., 1988; De Paulis et al., 1997). In the present study, we have investigated the actions of mast cell tryptase and chymase on mast cells of the human joint, and have examined the ability of inhibitors of these mast cell proteases to modulate their activation.

2. Materials and methods

2.1. Materials

The following compounds were purchased from Sigma (Poole, Dorset, UK): leupeptin, benzamidine, soybean trypsin inhibitor, chymostatin, aprotinin, *N*-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA), *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, porcine heparin glycosaminoglycan, histamine dihydrochloride, collagenase (type I), hyaluronidase (type I), bovine serum albumin (fraction V), penicillin and streptomycin, Minimum Essential Medium (MEM) containing 25 mM *N*-2-hydroxylethylpiperazine-*N*′-2-ethane sulphonic acid (HEPES), heparin agarose, S-200 Sephacryl agarose, calcium ionophore A23187, tris-base, 2-(*N*-morpholino)ethane-sulphonic acid (MES), antimycin A, 2-deoxy-D-glucose. Goat anti-human IgE (inactivated) was from Serotec (Kidlington, Oxford, UK). HEPES and all other chemicals were of analytical grade and were purchased from BDH (Poole, Dorset, UK). Cyanogen bromide (CNBr)-activated Sepharose 4B was from Pharmacia (Milton Keynes, UK). Foetal calf serum was from Gibco (Paisley, Renfrewshire, UK). Phthaldialdehyde was obtained from Fluka (Gillingham, Dorset, UK); Coomassie protein assay reagent from Pierce (Rockford, IL, USA); Silver staining kit from Bio-Rad (Hemel Hempstead, UK). *N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride (APC366) from Axys Pharmaceutical (South San Francisco, USA).

2.2. Dispersion of mast cells

Human synovium was obtained from patients with osteoarthritis at total knee replacement. The mast cell dispersion procedure employed was similar to that described previously with human skin (He et al., 1998). After removing fat, cartilage and fibrous materials, tissue was chopped finely with scissors into fragments of 0.5–2.0 mm^3, and then incubated with 1.5 mg/ml collagenase and 0.75 mg/ml hyaluronidase in MEM containing 2% foetal calf serum (Gibco; 1 g synovium/15 ml buffer) for 70 min at 37°C. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100 μm diameter), washed and maintained in MEM (containing 10% foetal calf serum, 200 U/ml penicillin, 200 μg/ml streptomycin) on a roller overnight at room temperature. Mast cell purity as determined by light microscopy after staining by the procedure of Kimura et al. (1973) ranged from 3.2% to 6.8%.

2.3. Mast cell challenge

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with $CaCl_2$ and $MgCl_2$ (complete HBSS), and 100 μl aliquots containing $4–6 \times 10^3$ mast cells were added to 50-μl purified tryptase, chymase, control secretagogue or inhibitor in complete HBSS and incubated for 15 min at 37°C. The reaction was terminated by the addition of 150 μl ice cold incomplete HBSS and the tubes centrifuged immediately (500 g, 10 min, 4°C). All experiments were performed in duplicate. For the measurement of total histamine concentration, in certain tubes the suspension was boiled for 6 min. Supernatants were stored at −20°C until histamine concentrations were determined (in duplicate for each tube) using a glass fibre-based, fluorometric assay (He et al., 1998). Histamine release was expressed as a percentage of total cellular histamine levels, and corrected for the spontaneous release measured in tubes in which cells had been incubated with the HBSS diluent alone.

In preliminary experiments, dispersed mast cell preparations from synovial tissue were incubated with a range of concentrations of anti-IgE or calcium ionophore A23187, and net histamine release calculated. Optimal non-cytotoxic histamine release from synovial cells was observed with 1% anti-IgE or with 1 μM calcium ionophore (data not shown). Cytotoxicity was assessed by determining the ability of the metabolic inhibitors 2-deoxy-D-glucose and antimycin A to inhibit histamine release. Both 1% anti-IgE and 1 μM calcium ionophore were selected as positive controls in all experiments involving mast cell challenge.

2.4. Inhibition of histamine release

Inhibitors of tryptase investigated were APC366, benzamidine and leupeptin and the tryptic substrate BAPNA was also examined. Chymostatin and soybean trypsin inhibitors were employed as inhibitors of chymase, and aprotinin, an inhibitor of cathepsin G was also tested. Data were expressed as the percentage inhibition of histamine release,

taking into account histamine release in the presence and absence of the inhibitor and in the absence of the stimulus.

2.5. Purification of mast cell proteases

Tryptase was isolated from human lung tissue obtained post mortem, employing high salt extraction, heparin agarose chromatography and immunoaffinity chromatography with tryptase specific monoclonal antibody AA5, as described previously (He et al., 1997). Chymase was purified from human skin using a protocol involving high salt extraction, heparin agarose chromatography and S-200 Sephacryl gel filtration (He and Walls, 1998a). Enzymatic activity was determined spectrophotometrically (410 nm), measuring for tryptase, the rate of hydrolysis of 20 mM BAPNA in 0.1 M Tris–HCl, 1 M glycerol, pH 8.0 containing 1 mg/ml bovine serum albumin at 25°C (Smith et al., 1984); and for chymase, the cleavage of 0.7 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide in 1.5 M NaCl, 0.3 M Tris, pH 8.0 (McEuen et al., 1995). Purity was evaluated using sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS-PAGE) with silver staining and the identity of the protein band confirmed by Western blotting with monoclonal antibody AA5 against human tryptase (Walls et al., 1990) or with rabbit antiserum to human chymase (McEuen et al., 1995). In order to test the possible presence of contaminating endotoxin in the final preparations of tryptase, the Toxicolor system was employed according to the manufacturer's protocol.

2.6. Preparation of compounds

As purified preparations of tryptase (Schwartz and Bradford, 1986) and chymase (McEuen et al., 1995) are both enzymatically unstable in physiological solutions, great care was taken in their preparation. The purified proteases stored in a 10-mM MES buffer containing 1 M NaCl (pH 6.8) were diluted immediately prior to addition to the cells, first with sterile distilled water to adjust the NaCl concentration to 0.15 M, and then with normal saline to obtain the required concentration. Where added, leupeptin or the buffer alone was incubated with tryptase for 30 min on ice before adding to the cells. APC366 was disolved in dimethyl sulphoxide (DMSO), and diluted to desired concentration with complete HBSS. The top final concentration of DMSO used was 0.05%, a concentration which is unlikely to alter mast cell responsiveness (Benyon et al., 1987).

2.7. Statistical analysis

Statistical analysis was performed using StatView software (Version 4.02, Abacus Concepts, Berkeley, CA). Data are shown as the mean ± S.E.M. for the number of experiments indicated. Where analysis of variance indicated significant differences between groups, for the preplanned comparisons of interest, paired Student's *t*-test was applied. For all analyses, $P < 0.05$ was taken as significant.

3. Results

3.1. Inhibition of histamine release by proteinase inhibitors

When synovial cells were preincubated for 30 min with the tryptase inhibitor APC366, a dose-dependent inhibition

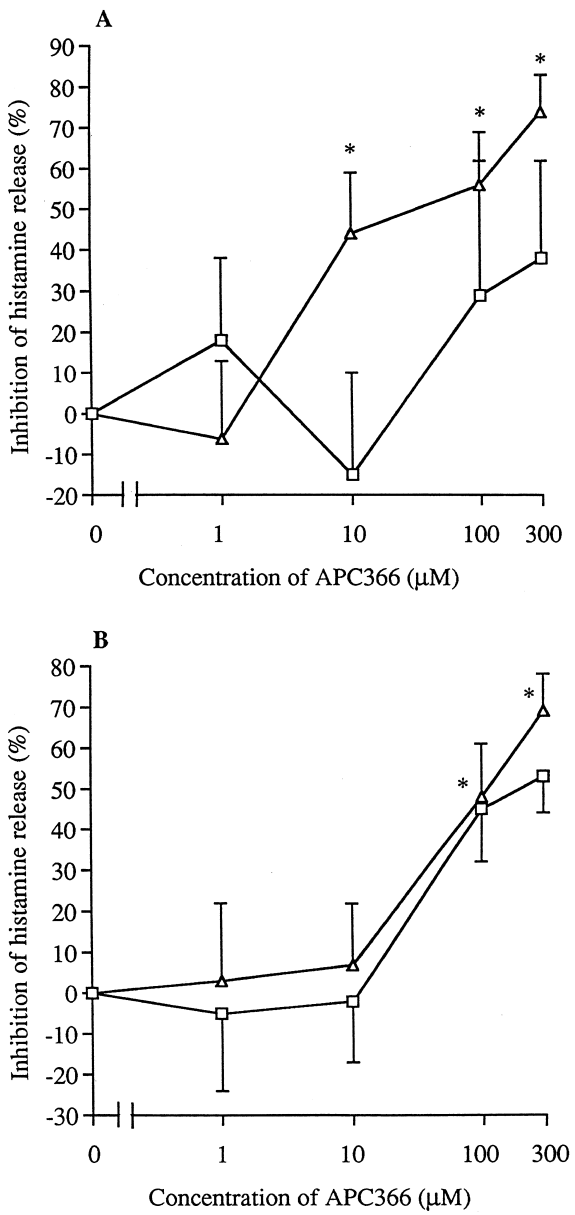

Fig. 1. Inhibitory actions of APC366 on (A) anti-IgE and (B) calcium ionophore A23187 induced histamine release from dispersed synovial cells. Various concentrations of APC366 were added to cells at the same time as the stimulus (–□–) or for 30 min before challenge (–△–). Data are presented as mean ± S.E.M. for five to seven separate experiments. * $P < 0.05$ compared with the responses with uninhibited controls. Net histamine release induced by anti-IgE was 19 ± 11% and by calcium ionophore A23187, 44 ± 13%.

Table 1
The effects of proteinase inhibitors (PI) or substrate on IgE-dependent histamine release from synovial cells

PI/subtrate (μg/ml)		Percentage inhibition of histamine release		
		0 min	5 min	30 min
Leupeptin	30	34 ± 30	−2.7 ± 28	−13 ± 17
Benzamidine	30	52 ± 29	nd	55 ± 8.5
BAPNA	100	8.7 ± 37	30 ± 17	−6.7 ± 30
Chymostatin	1.0	47 ± 30	38 ± 21	69 ± 12[a]
SBTI	30	−30 ± 31	−5.4 ± 2.8	−14 ± 21
Aprotinin	30	33 ± 29	−8.3 ± 42	−0.4 ± 32

Cells were preincubated with either proteinase inhibitor, BAPNA or diluent alone for 0, 5 or 30 min at 37°C before challenge with anti-IgE. Values shown are mean ± S.E.M. for four to seven separate experiments.
[a] $P < 0.05$ compared with the uninhibited control.

of IgE-dependent histamine release was observed (Fig. 1A). As little as 10 μM APC366 could reduce the extent of histamine release by some 40% and approximately 70% inhibition of histamine release was achieved with 300 μM APC366. The actions of APC366 appeared to be time-dependent. There was a trend for APC366 to inhibit histamine release when added at the same time as the anti-IgE (Fig. 1A) or when there was a 5-min preincubation period (data not shown), but this was not significant. Calcium ionophore-induced histamine release was also inhibited in a dose-dependent manner by APC366 (Fig. 1B). There was more than 60% inhibition when cells were preincubated for 30 min, though once again significant inhibition of histamine release was not quite achieved with preincubation periods of 0 or 5 min. At all concentrations tested, APC366 by itself did not induce histamine release from mast cells.

IgE-dependent histamine release could be inhibited by more than 60% with the broad-spectrum chymase inhibitor chymostatin at a concentration of 1 μg/ml following 30 min preincubation with cells (Table 1). However, significant inhibition was not achieved with shorter incubation periods, and chymostatin itself at 10 μg/ml induced significant histamine release following a 20-min (21 ± 7.1%, $n = 5$) or 45-min (26 ± 11%, $n = 5$) incubation with cells; and this prevented higher doses being studied. There was a trend for the broad-spectrum tryptase inhibitor, benzamidine to inhibit IgE-dependent histamine release though this did not reach significance, and leupeptin, soybean trypsin inhibitor and aprotinin (all up to 30 μg/ml) as well as the tryptic substrate BAPNA (up to 100 μg/ml) failed to alter the extent of IgE-dependent histamine release from cells with preincubation periods of up to 30 min (Table 1). None of these inhibitors by itself at the concentrations tested above could induce histamine release from dispersed synovial mast cells following up to 45 min incubation (data not shown). Chymostatin (up to 10 μg/ml), leupeptin, soybean trypsin inhibitor and aprotinin (all up to 30 μg/ml) as well as the tryptic substrate BAPNA (up to 10 μg/ml) did not alter the degree of histamine release induced by calcium ionophore from synovial mast cells (data not shown).

3.2. Mast cell proteases

On SDS-PAGE with silver staining and Western blotting, the purified tryptase appeared as a single diffuse band

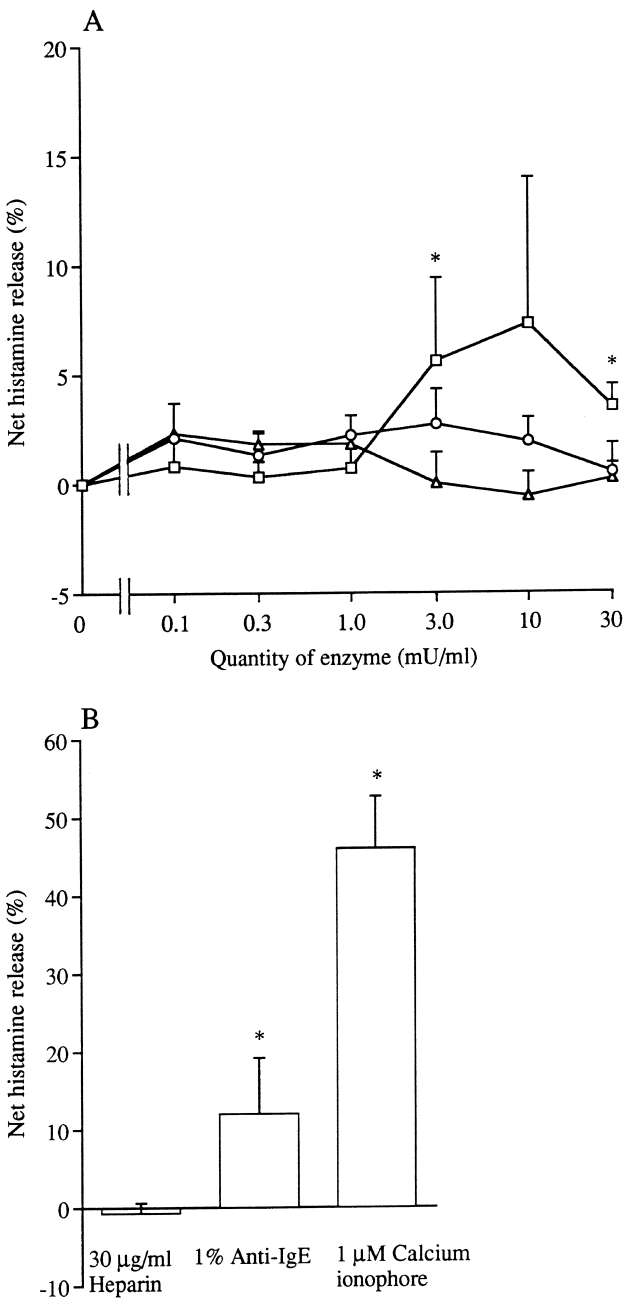

Fig. 2. (A) Histamine release from synovial cells incubated with tryptase (–□–), tryptase with heparin (–○–) or chymase (–△–). The quantity of protease is expressed in terms of enzymatic activity. The mean ± S.E.M. are presented for five to seven experiments. (B) Mean net histamine release (± S.E.M.) is shown with anti-IgE ($n = 7$), calcium ionophore A23187 ($n = 7$) and heparin ($n = 6$). * $P < 0.05$ compared with the baseline values (paired Student's *t*-test).

with an apparent molecular weight of approximately 32 kDa (representing disassociated monomers), and the chymase as a band of 30 kDa. Only very low endotoxin levels were detected, with less than 49 pg/mg tryptase and 38 pg/mg chymase. There was a specific activity of 1.84 U/mg protein for tryptase (where one unit (U) of enzyme was taken as the amount that catalyzed the cleavage of 1 μmol of BAPNA per minute at 25°C) and 4.9 U/mg for chymase (where 1 U of enzyme represents that required to hydrolyse 1 μmol of *N*-suc-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide per min at 25°C.

3.3. Effect of tryptase and chymase on mast cell activation

Addition of tryptase to synovial mast cells provoked significant histamine release at concentrations of 3.0 and 30 mU/ml (Fig. 2A). The proportion of total histamine release in response to tryptase represented about a half of that elicited with anti-IgE, while calcium ionophore was appreciably more potent than either of these two more physiological stimuli (Fig. 2B). There was no significant correlation between the extent of histamine release induced by tryptase and that induced by either anti-IgE or calcium ionophore (Spearman's coefficient of rank correlation). Prior addition of heparin to tryptase (1:1, w/w) appeared to abolish histamine release induced by this enzyme (Fig. 2A). The actions of tryptase on mast cells were inhibited by the inhibitor leupeptin, and by heat inactivation of the enzyme (Table 2). Chymase at concentrations up to 30 mU/ml failed to stimulate histamine release from synovial mast cells (Fig. 2A). Spontaneous histamine release from these cells, determined in the absence of any stimulus was 8.9 ± 0.9% (mean ± S.E.M.).

In order to investigate the potential for tryptase or chymase to modulate mast cell responsiveness to anti-IgE, cells were preincubated with tryptase in the presence or absence of heparin, with heparin alone or with chymase for 5 or 30 min at 37°C before challenge with anti-IgE. The concentrations of tryptase used were below those capable of inducing histamine release (0.1 and 1 mU/ml) and the chymase concentrations employed were 0.1 and 1 mU/ml. The degree of IgE-dependent histamine release was unaffected by these treatments ($n = 3$ to 5, data not shown).

Table 2
The effect of leupeptin or heat treatment on the ability of tryptase (30 mU/ml) to cleave the substrate BAPNA and to induce histamine release from dispersed synovial cells

Treatment	% Inhibition of BAPNA cleavage	% Inhibition of histamine release
Leupeptin 10 μg/ml	100	76 ± 17[a]
Heat inactivation	100	73 ± 21[a]

Values shown are mean ± S.E.M. for four separate experiments. Tryptase was preincubated with leupeptin for 30 min on ice or heated at 56°C for 2 h.

[a] $P < 0.05$ compared with the response with the uninhibited controls.

4. Discussion

We have provided evidence that mast cells of the human synovium, like those of the human tonsil (He et al., 1998) and from certain guinea pig tissues (He and Walls, 1997) may be activated by the major mast cell product tryptase. The extent to which this may represent an amplification mechanism in inflammatory conditions of the joint remains to be established, but our studies do highlight the potential for proteolytic processes in cell activation to be exploited therapeutically. The tryptase inhibitor APC366 proved particularly effective as a stabiliser of synovial mast cells.

Our observation that the tryptase inhibitor drug APC366 could inhibit histamine release from synovial mast cells by more than 60% contrasts with the lack of efficacy of various other classes of pharmacological agents. The list of compounds that has been reported to have little or no effect on IgE-dependent histamine release from this source of mast cells includes sodium cromoglycate, the non-steroidal antiinflammatory drugs (NSAIDs), indomethacin, aspirin and piroxicam, and the immunosuppressive agents cyclosporin H, rapamycin and methotrexate (Kopicky-Burd et al., 1988; De Paulis et al., 1997). Moreover, synovial mast cells are unresponsive to theophylline except at very high doses (1 or 10 mM) (Kopicky-Burd et al., 1988) despite the efficacy of this adrenergic agonist with various other sources of human mast cells (reviewed by Church et al., 1995). Other compounds reported to have some stabilising properties for this source of mast cells include nimesulide and diclofenac, cyclosporin A and FK506, a carbocyclic lactone–lactane (De Paulis et al., 1997), and gold sodium thiomalate (Kopicky-Burd et al., 1988).

We have found previously that APC366 over the concentrations tested can inhibit IgE-dependent histamine release from mast cells of human skin (by up to 80%) and from tonsil (30%) (He et al., 1998). As in that earlier study, a time-dependent effect for APC366 was seen with the length of the preincubation period with cells appearing to be related to the degree of mast cell stabilisation. In the present study we found that APC366 was also able to inhibit histamine release stimulated by calcium ionophore, with dose-dependent inhibition of up to 70% achieved. A similar effect has been observed also with skin mast cells (He et al., 1998). The apparent ability of this tryptase inhibitor to affect calcium flux suggests that it has the potential to inhibit histamine release induced by non-IgE-dependent stimuli. APC366 has been reported to have efficacy in both sheep and human models of bronchial asthma (Clark et al., 1995; Krishna et al., 1998). It will be important for tryptase inhibitors to be tested in models of inflammatory joint disease.

Significant inhibition of IgE-dependent histamine release was achieved with chymostatin, suggesting that a chymotryptic activity may also be involved in IgE-dependent histamine release from synovial mast cells. Both

chymostatin and a selective chymase inhibitor Z-Ile-Glu-Pro-Phe-CO_2Me have been found to inhibit histamine release from human skin mast cells (He et al., 1999). It is possible that the chymotryptic activity involved is chymase but if this is the case, then the lack of effect of exogenous chymase on histamine release would argue that the substrate is intracellular or exposed to the enzyme only once the process of degranulation has commenced. Various other protease inhibitors, including leupeptin, benzamidine and soybean trypsin inhibitor and the substrate BAPNA proved ineffective as mast cell stabilisers. This may be attributed to insufficient concentrations of these compounds being employed, but we were constrained by their cytotoxicity at higher concentrations.

The ability of tryptase to activate synovial mast cells could at least in part underlie the mast cell stabilising activity of APC366, though with this and with the other inhibitors the possibility cannot be excluded of effects on other enzymes or processes. We have found previously that human tryptase can provoke histamine release from guinea pig lung and skin mast cells (He and Walls, 1997), and from human tonsil, but not from skin mast cells (He et al., 1998). The histamine release stimulated by tryptase was somewhat lower than that induced from synovial cells by anti-IgE antibody, but this could nonetheless provide an amplification process in inflammatory conditions of the joint. Tryptase levels of more than 30 μg/ml have been detected in synovial fluid (Buckley et al., 1997), but this is likely to represent a considerable dilution of concentrations in areas of mast cell degranulation in the synovial tissues. Mast cells are frequently sited in close proximity to each other in the joints (Buckley et al., 1998), and one would anticipate that there will be high local concentrations of tryptase in the vicinity of degranulating mast cells. In the present studies significant histamine release was triggered by tryptase at a dose of some 3 mU/ml, a concentration that is likely to be achieved in vivo.

Tryptase-induced histamine release was dependent on an intact catalytic site, being inhibited by the protease inhibitor leupeptin and by heat inactivating the enzyme. This is consistent with our previous observations with tonsil mast cells for which tryptase-induced mast cell degranulation could also be inhibited by APC366 (He et al., 1997). The finding that tryptase-induced histamine release was abolished when this protease was added in the presence of heparin may seem paradoxical given the stabilising actions of heparin on tryptase (Schwartz and Bradford, 1986). However, the same effect has been observed previously with tonsil mast cells (He et al., 1998) and it is likely to be related to the ability of heparin to inhibit mast cell degranulation (Ahmed et al., 1993).

Inhibitors of mast cell proteases could provide a new therapeutic approach in joint disease. It will be interesting to know to what extent they could control the release of mediators other than histamine from mast cells. Secretory leukoprotease inhibitor, an inhibitor of both tryptase (Robinson et al., 1996) and chymase (Walter et al., 1996) has been found to be able to suppress inflammation and joint damage in a rat model of arthritis (Song et al., 1999). Secretory leukoprotease inhibitor, and the synthetic tryptase inhibitors APC366 and 1,5-bis-{4-[(3-carbamimidoyl-benzenesulfonylamino)-methyl]-phenoxy}-pentane (AMG-126737), have all been demonstrated to protect against early and late phase allergen-induced airway responses in sheep (Clark et al., 1995) and guinea pig (Havill et al., 1997; Wright et al., 1999) models of allergic inflammation; while, recently, a synthetic inhibitor of chymase has been found to be beneficial in a murine model of atopic dermatitis (Imada et al., 2000). The ability of inhibitors of tryptase and chymase to inhibit the activation of synovial mast cells could be of value as a new anti-inflammatory strategy in arthritic conditions.

Acknowledgements

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