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Inhibitors of Tryptase as Mast Cell Stabilising Agents in the Human Airways. Effects of Tryptase and Other Agonists of PAR2 on Histamine Release.

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Running title: Actions of Tryptase and PAR2 Agonists on Lung Mast Cells.

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Abbreviations: BAPNA, *N*-benzoyl-D,L-arginine-*p*-nitroanilide; HBSS, HEPES balanced salt solution; NA, nitroanilide; MES, 2-(*N*-morpholino)ethane-sulphonic acid; PAR2, proteinase activated receptor 2.

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Abstract

Tryptase, the major secretory product of human mast cells, is emerging as new target for therapeutic intervention in allergic airways disease. We have investigated the ability of tryptase and inhibitors of tryptase to modulate histamine release from human lung mast cells, and have examined the potential contribution of proteinase activated receptor 2 (PAR2). The tryptase inhibitor APC366 was highly effective at inhibiting histamine release stimulated by anti-IgE antibody or calcium ionophore from enzymatically dispersed human lung cells. A concentration of APC366 as low as 10 μM was able to inhibit anti-IgE-dependent histamine release by some 50%. Addition of leupeptin or the tryptic substrate BAPNA also inhibited IgE-dependent histamine release. Purified tryptase in the presence of heparin stimulated a small but significant release of histamine from lung cells, suggesting that tryptase may provide an amplification signal from activated cells that may be susceptible to proteinase inhibitors. Trypsin was also able to induce histamine release apparently by a catalytic mechanism. Moreover, pre-treatment of cells with metabolic inhibitors or with pertussis toxin reduced responses, indicating a non-cytoxic pertussis toxin-sensitive G protein-mediated signaling process. Addition to cells of the PAR2 agonists SLIGKV-NH_2 or tc-LIGRLO-NH_2 or appropriate control peptides were without effect on histamine release; and PAR2 was not detected by immunohistochemistry in tissue mast cells. The potent actions of tryptase inhibitors as mast cell stabilising agents could be of value in the treatment of allergic inflammation of the respiratory tract, possibly by targetting the non-PAR2 mediated actions of tryptase.

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Mast cell activation is prominent in allergic airways disease. The mast cell has been implicated as an initial effector cell, and also as a key cellular participant in later processes of acute inflammation, and in tissue remodelling (Church et al., 1997). Several drugs used to treat allergic inflammation of the lower airways (such as salmeterol and salbutamol) or upper airways (terfenadine and cetirizine) possess mast cell stabilising activity (Okayama & Church, 1992, 1994; Butchers et al., 1991; Naclerio et al., 1990); and more recently the potential for mast cells to play a critical role in allergic inflammation has been highlighted by reports that omalizumab, a humanised antibody specific for IgE may be efficacious in the treatment of asthma and other allergic conditions (D'Amato, 2003). The major secretory product of human mast cells is the serine proteinase tryptase (Walls, 2000). This enzyme is emerging as a major mediator of allergic disease and as a promising target for therapeutic intervention. Tryptase inhibitors have been reported to be particularly potent as mast cell stabilising compounds, though their effects on mast cells of the lung have not been examined.

The ability of tryptase to stimulate mast cell degranulation first became apparent in studies involving transfer of this proteinase to laboratory animals. Microvascular leakage provoked by injection of human tryptase into guinea pig skin was found to be blocked by antihistamine pretreatment of the animals, and addition of human tryptase to guinea pig lung and skin fragments elicited histamine release (He and Walls 1997). Subsequently it has been established that tryptase can stimulate histamine release from enzymatically dispersed human tonsil and synovial mast cells, but not from human skin;

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and inhibitors of tryptase have been found to inhibit both IgE and non-IgE dependent histamine release from all three of these sources of mast cells (He et al 1998; 2001).

Administration of inhibitors of tryptase to both sheep and guinea pig models of allergic airways disease has been reported to reduce allergen-induced early increases in specific lung resistance (Clark et al., 1995, Wright et al., 1999), consistent with inhibition of mast cell activation. Also reduced in these animal models were the late phase increases in lung resistance, and airways hyperresponsiveness. In a clinical trial with the tryptase inhibitor APC366, significant reductions in allergen-induced late phase responses were observed in subjects with mild to moderate asthma (Krishna et al., 2001). In that study there was a trend for the early phase reaction to be reduced, though it did not reach significance.

Tryptase can interact with various cell types in addition to mast cells, and induce profound alterations in cell behaviour. Thus, tryptase can stimulate the accumulation and activation of eosinophils and neutrophils both *in vivo* and *in vitro* (He et al., 1997; Walls et al., 1995), induce the release of inflammatory cytokines from epithelial and endothelial cells (Cairns and Walls, 1996; Compton et al., 1998), act as a growth factor for epithelial cells (Cairns and Walls, 1996), fibroblasts (Cairns and Walls, 1997), and airway smooth muscle cells (Berger et al., 2001) and provoke the release of collagen and collagenase from fibroblasts (Cairns and Walls, 1997). The precise mechanisms remain unclear, but the finding that tryptase may activate proteinase activated receptor 2 (PAR2) (Molino et al., 1997; Schechter et al., 1998), has raised the possibility that at

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least some of the actions of tryptase on cellular targets may be mediated through this receptor.

PAR2 has been identified on various cell types present in the human lung, and the activation of this G protein coupled receptor has been associated with increases in microvascular permeability, cell accumulation and cytokine release in various experimental models (Lan et al., 2002). Functional PAR2 has been demonstrated on human endothelial cells (Mirza et al., 1996), epithelial cells (Böhm et al., 1996), airway (Berger et al., 2001) and vascular smooth muscle cells (Molino et al., 1998) and on neutrophils (Howells et al., 1997) and eosinophils (Temkin et al., 2002). The immunohistochemical detection of PAR2 in mast cells has been reported in some human tissues (D'Andrea et al., 2000), but a subsequent study failed to find evidence of functional PAR2 in rat peritoneal mast cells (Stenton et al 2002). The potential of human mast cells to respond to agonists of PAR2 remains to be determined.

In the present studies, we have investigated the ability of tryptase and inhibitors of tryptase to modulate histamine release from human lung mast cells, and we have examined the potential role of PAR2 in mast cell activation.

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Materials and Methods

Materials

The following compounds were purchased from Sigma (Poole, Dorset, UK): leupeptin, benzamidine, *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA), *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide (NA), *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-NA, porcine heparin glycosaminoglycan, histamine dihydrochloride, collagenase (type I), hyaluronidase (type I), BSA (fraction V), penicillin and streptomycin, MEM containing 25 mM HEPES, heparin agarose, calcium ionophore A23187, tris-base, 2-(*N*-morpholino)ethane-sulphonic acid (MES), antimycin A, 2-deoxy-D-glucose, Extravidin® staining kits, 3-amino-9-ethylcarbazole, Mayer's haematoxylin. 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); CNBr-activated Sepharose 4B was from Pharmacia (Milton Keynes, UK); FCS was from Gibco (Paisley, Renfrewshire, UK); phthaldialdehyde was obtained from Fluka (Gillingham, Dorset, UK); Coomassie protein assay reagents from Pierce (Rockford, IL,USA); the silver staining kit from Bio-Rad (Hemel Hempstead, UK), glycol methacrylate (GMA; JB4 resin) from Park Scientific (UK); 3,3- diammobenzadine (DAB) was from Biogenex (San Ramon, CA, USA). APC366 was a kind gift from Celera Corporation (South San Francisco, CA, USA). Peptides SLIGKV-NH_2, VKGILS-NH_2, LSIGKV-NH_2, TNRSSKGRSLIGKVC-NH_2, GPNSKGRSLIGRLDTP-YGGC-NH_2, trans-cinnamoyl-LIGRLO-NH_2 (tc-LIGRLO-NH_2) and trans-cinnamoyl-OLRGIL-NH_2 (tc-OLRGIL-NH_2)

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were synthesised as carboxyamide products by solid phase methods at the Peptide Synthesis Facility, University of Calgary, Canada.

Preparation of Tryptase

Tryptase was purified from human lung tissue by high salt extraction, heparin agarose and immunoaffinity chromatography procedures with monoclonal antibody AA5 against tryptase as described previously (He et al., 1997). The purified tryptase was then concentrated in C-30 Centricon centrifugal concentrators (Amicon, Stonehouse, Glos., UK) and stored at -80°C until use. Tryptic activity was determined with the chromogenic substrate BAPNA, protein concentration by the Coomasie protein assay with BSA as standard, as described previously (He et al., 1997). Tryptase concentration was expressed in terms of μM of tetrameric enzyme as determined by protein concentration. On SDS-PAGE with silver staining, and Western blotting with specific monoclonal antibody AA5 (Walls et al., 1990), tryptase appeared as a single diffuse band with a molecular weight of approximately 32 kDa (corresponding to the disassociated subunits of tetramer). The specific activity of the tryptase used in these studies was 1.84 U/mg, where one unit of enzyme was taken as the amount that catalyzed the cleavage of 1 μmol of BAPNA per minute at 25°C. The preparation had no detectable chymotryptic or elastolytic activity (as determined using the substrates *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-NA or *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-NA, respectively; He et al., 1997) and endotoxin levels were very low, being less than 49 pg/mg tryptase (38 pg/ml).

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Preparation of Compounds

As tryptase is enzymatically unstable in physiological solutions, considerable care was taken in its preparation. Purified tryptase stored in high salt buffer in the presence or absence of heparin was diluted immediately prior to challenging the cells, first with sterile distilled water, adjusting the NaCl concentration to 0.15 M, and then with HEPES balanced salt solution (HBSS) to obtain the required tryptase concentration.

Mast Cell Challenge and Analysis of Histamine Release

Macroscopically normal lung tissue was collected at bronchial resection from patients with lung cancer. The procedure for mast cell dispersion was similar to that described previously with human tonsil tissues (He et al., 1998). Briefly, tissue was chopped finely with scissors into fragments of 0.5-2.0 mm^3, and incubated with 1.5 mg/ml collagenase and 0.75 mg/ml hyaluronidase in MEM containing 2% FCS (1 g lung/10 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), and were maintained in MEM (containing 10% FCS, 200 U/ml penicillin, 200 µg/ml streptomycin) on a roller overnight at room temperature. Mast cell numbers were determined by light microscopy after staining with Kimura staining solution, and represented 2.3 to 4.5% of nucleated cells in suspensions.

Prior to challenge with stimulus the cells were washed with HBSS (pH 7.4) without added calcium or magnesium (500g, 10 min, 25°C; Okayama et al., 1994), and then

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resuspended in HBSS with 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Aliquots of 100 µl containing 4-6 $\times 10^3$ mast cells were added to a 50 µl aliquot of purified tryptase, PAR2 agonist, control secretagogue or inhibitor in complete HBSS and incubated for 15 to 60 min at 37°C. The reaction was terminated by the addition of 150 µl ice cold HBSS and the tubes centrifuged immediately (500g, 10 min, 4°C). All experiments were performed in duplicate. For the measurement of total histamine concentration the suspension in some tubes was boiled for 6 min. Supernatants were stored at -20°C until histamine concentrations were determined.

A glass fibre-based, fluorometric assay was employed to determine histamine levels in supernatants, as previously described (He et al., 1998). Histamine bound to a glass-fibre matrix (Lundbeck Diagnostics, Copenhagen, Denmark) was detected by addition of o-phthaldialdehyde (OPT) and the colour change measured using a spectrophotofluorometer (Perkin-Elmer LS 2, Denmark). 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 were incubated with a range of concentrations of anti-IgE or calcium ionophore A23187, and net histamine release calculated. Maximal non-cytotoxic release of histamine was observed with 1% anti-IgE or with 1 µM calcium ionophore A23187, where cytotoxic mechanisms were assessed by comparing responses with cells preincubated with antimycin A and 2-deoxy-D-glucose

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(data not shown). Both 1% anti-IgE and 1 μM calcium ionophore were selected as positive controls in all experiments involving mast cell challenge.

Immunohistochemistry

Bronchial biopsy tissue was collected from six subjects with mild asthma and embedded in GMA resin. The subjects (aged 35 to 57) had normal lung function, and were receiving no treatment apart from β-adrenoceptor agonists. In addition, human lung tissue (containing large airway and tissue parenchyma) was obtained at surgical resection from two subjects and tonsil tissue (obtained at tonsillectomy) from five subjects. The study was approved by the Southampton and Southwest Hampshire Local Research Ethics Committee. Sections (2 μm) were incubated with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min, washed with Tris-buffered saline (pH 7.6) and non-specific protein binding sites blocked with 10% FCS and 5% bovine serum albumin in RPMI 1640 culture medium. Sequential sections were incubated overnight at room temperature with monoclonal antibody P2A specific for a peptide sequence of human PAR2 (diluted 1/400; Aslam et al., 2002), at 4^{o}C rabbit antiserum (B5) specific for a peptide sequence of rat PAR2 (diluted 1/1000, Kong et al., 1997), or monoclonal antibody AA1 specific for tryptase (1/50; Walls et al., 1990). In control studies the PAR2-specific antibodies were pre-adsorbed with 10 μM of the peptide immunogens, ^{30}TNRSSKGR↓SLIGK^{42}VC (↓ tryptic cleavage site) and ^{30}GPNSKGR↓SLIGRLDT^{46}P-YGGC (YGGC per conjugation) for P2A and B5, respectively. Biotinylated secondary antibodies were applied for 2 hr at room temperature, and after washing, streptavidin-

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biotin-peroxidase complexes were applied for a further 2 hr at room temperature. Immunostaining was developed with diaminobenzidine and the sections counterstained with Mayer's haematoxylin. Areas of tissue stained were measured using a computerised image analysis system (Zeiss, Eching, Germany).

Statistical Analysis

All statistical analyses were performed using StatView software (Version 4.02, Abacus Concepts, Berkeley, California). Data are shown as the mean ± SEM for the number experiments (n) indicated and the paired Student's t test was applied to evaluate two independent samples. In all analyses $P < 0.05$ was taken as significant.

Results

Inhibition of Histamine Release by Inhibitors of Tryptase

A concentration-dependent inhibition of anti-IgE or calcium ionophore induced histamine release was observed when dispersed lung cells were incubated with the tryptase inhibitor APC366 (from 10 to 300 μM) at 37°C for a period of 30 min (Fig. 1). With a 30 min preincubation period, significant inhibition of histamine release was achieved with as little as 10 μM APC366, and up to 60% inhibition of IgE-dependent histamine release was achieved with 300 μM APC366. Significant concentration-dependent inhibition was observed also when APC366 was added 5 min prior to the

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challenge, or at the same time as the anti-IgE or calcium ionophore stimulus (data not shown). There was a trend for the degree of inhibition of IgE-dependent histamine release to be related to the period the compound was incubated with cells (Fig. 2), but this pattern was not observed with calcium ionophore induced histamine release (data not shown). APC366 by itself at concentrations of up to 300 μM did not stimulate significant histamine release from mast cells.

Leupeptin, a broad-spectrum serine proteinase inhibitor, inhibited IgE-dependent histamine release by 35 ± 9.0% (mean ± SE, n = 6, P = 0.0218) at a concentration of 10 μg/ml following a 30 min preincubation with cells. With a shorter preincubation period of 5 min, however, this leupeptin concentration did not have consistent effects on anti-IgE-induced histamine release from lung cells (data not shown). Benzamidine, a less potent inhibitor of tryptase, failed to inhibit IgE-dependent lung mast cell activation at concentrations of 10 and 100 μg/ml, while at higher concentrations it induced histamine release when added alone to cells (data not shown).

Like APC366, the tryptic substrate BAPNA also inhibited IgE-dependent histamine release in a concentration-dependent manner following 30 min preincubation with cells (Fig. 3). However, histamine release was not inhibited consistently when this compound was preincubated with cells for 5 min or added to cells at the same time as the anti-IgE (data not shown). Leupeptin, benzamidine and BAPNA had no significant effect on calcium ionophore induced histamine release from lung mast cells with preincubation periods of up to 30 min at the concentration tested (data not shown).

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Tryptase in the presence of heparin (added to stabilise enzymatic activity) stimulated a small but significant release of histamine from dispersed lung cells over the concentration range of 1.0 to 100 μM (Fig. 4A). In the absence of heparin, however, there was negligible histamine release (data not shown). Greater histamine release was elicited with anti-IgE or calcium ionophore at the optimal non-cytotoxic doses employed (Fig 4B). Histamine release provoked by 100 μM tryptase represented about 25% of that elicited by 1% anti-IgE (a concentration provoking maximal release). Addition of trypsin to cells also stimulated a concentration-dependent release of histamine from lung mast cells at concentrations from 1.0 to 100 μM (Fig. 4A), with maximal release of histamine representing about 40% of that provoked by 1% anti-IgE (Fig. 4B).

Histamine release induced by tryptase (100 μM) was inhibited by leupeptin (10 μg/ml) by 68 ± 8.0% (n = 6, $P = 0.046$), while histamine release provoked by trypsin (10 μM) was inhibited by SBTI (10 μg/ml) by 61 ± 6.8 % (n = 8, $P = 0.018$), suggesting that an intact catalytic site was required for the actions of these two proteinases on mast cells. Preincubation of cells with the metabolic inhibitors 2-deoxy-D-glucose (10 mM) and antimycin A (1.0 μM) for 40 min at 37 ^{o}C abolished histamine release in response to trypsin completely (data not shown), indicating that the action of this proteinase on cells involved a non-cytoxic process. Treatment with pertussis toxin (0.1 or 1 μg/ml) for 4h

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at 37 ^{o}C before challenge with trypsin, also resulted in a complete inhibition of histamine release, suggesting singalling via a pertussis toxin-sensitive G protein.

Effects of Tryptase and Heparin on IgE-Dependent Mast Cell Activation

Preincubation of dispersed lung mast cells with either 3.0 or 30 µM tryptase (concentrations which are capable of activating mast cells), in the presence of heparin for 5 or 30 min lead to significant inhibition of the subsequent anti-IgE-induced histamine release (Fig. 5). This was not observed in the absence of heparin. With 0.3 µM tryptase there was no significant inhibition of IgE-dependant histamine release. Adding tryptase at concentrations from 0.3 to 30 µM (either in the presence or absence of heparin) at the same time that anti-IgE was added to cells did not alter the extent of histamine release induced by anti-IgE (data not shown).

When various concentrations of heparin (0.3 to 30 µg/ml) were added simultaneously with anti-IgE to cells, IgE-dependent histamine release was inhibited by some 30 to 50% (Fig. 6). However, when cells were preincubated with heparin for 5 or 30 min prior to addition of anti-IgE, heparin had less influence on anti-IgE induced histamine release (data not shown). Under the same conditions, neither heparin (up to 30 µg/ml) nor tryptase (up to 100 µM) had any significant effect on calcium ionophore-induced histamine release from dispersed lung mast cells. Heparin by itself (at 30 µg/ml) had no effect on histamine release from dispersed lung cells (Fig. 4B).

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Effects of Peptide Agonists of PAR2 on Histamine Release

The PAR2 agonist peptides SLIGKV-NH_2 and tc-LIGRLO- NH_2 failed to stimulate histamine release from dispersed lung cells following incubation with cells for 20 min (Table 1), or for 60 min (data not shown). Similarly, non PAR2-activating peptides of similar amino-acid composition (VKGILS-NH_2, LSIGKV-NH_2 and tc-OLRGIL-NH_2) did not provoke histamine release when added for the same periods. We considered the possibility that the peptide agonists could be degraded by proteinases released from the cells and included the proteinase inhibitor amastatin at concentrations of 0.1 μM, 1.0 μM and 10.0 μM (added either simultaneously with the peptides or added to cells for 30 min prior to addition of the peptides). However, addition of amastatin was without effect at any of the concentrations employed (n = 4 separate experiments, data not shown).

Immunohistochemical Identification of PAR2

In bronchial biopsy tissues, PAR2 immunostaining as detected with monoclonal antibody P2A was found to be present predominantly on the epithelium, with little or no staining of cells in the underlying tissue layer. Mast cells identified with tryptase-specific antibody AA1 were present throughout the subepithelial tissue, but absent from the epithelium, and numbers ranged from 10 to 45 (median 30) per tissue (corresponding to 6.5 to 16.7 (median 14.8) mast cells/mm^2 subepithelial tissue). No PAR2 staining was detected on a total of 162 mast cells examined in the bronchial biopsy tissue (illustrated Fig 7A, B). Similarly, no PAR2 immunostaining was found using antiserum B5 on a

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total of 39 mast cells identified in resected lung tissue (8 and 31 in each of the two tissues, representing 8.4 and 16.3 mast cells/mm^2; Fig. 7C, D) or in 879 mast cells in tonsil tissue (90 to 259 mast cells (median 178) per tissue; with 84 to 136 (median 93.7) cells per mm^2).

Discussion

These studies indicate that inhibitors of mast cell tryptase may have potent actions as stabilising agents for human lung mast cells. In that tryptase was found to be able to modulate the release of histamine from lung mast cells, the mechanism may depend, at least in part, on inhibition of this proteinase following release from mast cells. However, the lack of responsiveness of lung mast cells to certain other agonists of PAR2, and the failure to detect this receptor on mast cells by immunohistochemistry must call into question a role for PAR2 in mediating the actions of tryptase on mast cells in the human airways.

The tryptase inhibitor APC366 inhibited IgE-dependent histamine release by some 50% at a concentration as low as 10μM, and by about 60% at 300 μM. This degree of inhibition has been noted when this tryptase inhibitor has been studied with mast cells of certain other human tissues (He et al., 1998; He et al., 2001), but it is high when compared with that for other anti-allergic drugs with mast-cell stabilising properties. Thus, in similar models of lung mast cell activation with dispersed cells, sodium cromoglycate has been reported to inhibit IgE dependent histamine release by 20% (at

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1000 µM), lodoxamide by 20% (100 µM), salbutamol by 40% (10 µM), ketotifen by 11% (10 µM), terfenadine by 15% (10 µM) and ceterizine by 25% (100 µM) (Church and Hiroi, 1987; Okayama and Church, 1992; Okayama et al., 1994).

Inhibition of IgE-dependent histamine release from lung cells, similar to that with APC366, was observed when lung cells were incubated with the substrate BAPNA. This further supports the idea that APC366 acts by inhibiting tryptic activity. The possibility of effects on proteases other than tryptase cannot be excluded, but some degree of inhibition was seen also with the proteinase inhibitor leupeptin. The apparent absence of an effect of BAPNA or leupeptin on calcium ionophore-induced histamine release could be related to these compounds being less effective, to the calcium ionophore providing a supramaximal signal, or perhaps to differences in underlying cell signalling processes.with each of these stimuli. Benzamidine, which was the least effective as an inhibitor of tryptase, did not significantly alter cell responsiveness to either IgE- or non-IgE-dependent stimulation, and the potential for cytotoxic actions precluded the use of higher concentrations in the present study.

As tryptase was able to elicit significant histamine release from lung cells, it is possible that the proteinase inhibitors may act in part by inhibiting the ability of tryptase released from mast cells to stimulate further mast cell degranulation. Such a mechanism could underlie the ability of APC366 to inhibit histamine release triggered by both anti-IgE and the calcium ionophore. Relatively high concentrations of tryptase were required to stimulate histamine release, but with quantities of some 10 to 35 pg tryptase present in a

human mast cell (Schwartz et al 1987), the levels in the vicinity of a degranulating mast cell are also likely to be very high. The degree of histamine release stimulated by tryptase was, however, quite small and maximal histamine release stimulated by tryptase represented just some 25% of that induced by optimal concentrations of anti-IgE antibody. This proportion is lower than that found previously with tonsil (approximately 70%) or synovial mast cells (50%), though in studies with skin tissues no significant release of histamine was stimulated by addition of exogenous tryptase (He et al 1998, 2001).

The stabilisation of tryptase activity by heparin appeared to be necessary for tryptase to stimulate histamine release from lung mast cells. However, heparin was itself able to inhibit the activation of lung mast cells, at least with anti-IgE as the stimulus. This is consistent with a previous report by Ahmed et al. (1993) who found that addition of heparin to human uterine and rat peritoneal mast cells reduced the degree of IgE-dependent histamine release. Heparin may have mutually antagonistic roles in inhibiting histamine release and in allowing tryptase to act as a stimulus, and this makes it more difficult to assess the contribution of tryptase as an amplification signal. Moreover, addition of tryptase at concentrations capable of eliciting histamine release, was found to reduce histamine release in response to subsequent addition of anti-IgE. It is not clear whether this is a consequence of mast cell unresponsiveness being induced by the initial stimulus, as has been reported with other secretagogues (Rubinchik et al 1998), or to the actions of the heparin added with the tryptase. The mast cell stabilising properties of

inhibitors of tryptase could be related in part to the inhibition of tryptase secreted following degranulation, but other mechanisms could be important.

There was a trend for APC366 to be more effective in stabilising lung mast cells when the cells were preincubated with this inhibitor prior to challenge rather than added at the same time as the stimulus. The time dependency of this inhibitor has been noted previously with substrate cleavage (McEuen et al 1996) and with studies with skin, tonsil and synovial cells *in vitro* (He et al 1998, 2001), as well as when administered in a sheep model of allergic airways disease (Clark et al 1995). Leupeptin and even BAPNA also appeared to be more effective at inhibiting histamine release when cells were preincubated with these compounds. The extent to which APC366 and the other inhibitors may actually enter mast cells remains to be determined, though there would be parallels with previous observations that human mast cells may take up lactoferrin (a destabiliser of the tryptase-heparin complex; He et al 2003), and rat peritoneal mast cells can ingest soybean trypsin inhibitor and $F(ab')_2$ fragments of a chymase specific antibody (Kido et al 1988). If the substrate whose cleavage is inhibited is in an intracellular or a pericellular location, then one might expect that uptake of the inhibitor by the cells could increase its effectiveness.

Trypsin, like tryptase, was able to stimulate histamine release from human lung mast cells, and the actions of both proteinases were reduced by addition of proteinase inhibitors. Trypsin and tryptase are both potentially able to activate PAR2; and support for involvement of a G protein coupled receptor was provided by observation of an

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inhibitory action for pertussis toxin on trypsin-induced histamine release. On the other hand, the PAR2 peptide agonists SLIGKV-NH_2 and tc-LIGRLO-NH_2 failed to stimulate histamine release even in the presence of amastatin. Moreover, although human mast cells have been reported to express immunoreactive PAR2 (D'Andrea at al., 2000), we were unable to find evidence for this in the present studies. Using either a specific monoclonal antibody or rabbit antiserum against PAR2 in immunohistochemistry, this receptor was not detected on any of the several hundred mast cells examined, including those from asthmatic subjects.

The presence of functional PAR2 on human mast cells has not previously been examined, though it has been reported that the peptide agonist SLIGRL-NH_2 (based on the sequence of the tethered ligand of rat PAR2) fails to stimulate the release of histamine (Nishikawa et al., 2000) or β-hexosaminidase (Stenton et al., 2002) from rat peritoneal mast cells. Stenton and colleagues did observe β-hexosaminidase release from rat peritoneal mast cells in response to tc-LIGRLO-NH_2, but the absence of responsiveness of those cells to trypsin or tryptase as well as to SLIGRL-NH_2 would argue against involvement of a PAR2-mediated process. Mast cells from different sources and different species exhibit a considerable degree of functional heterogeneity (Church et al., 1997) and a role for PAR2 in mast cell degranulation cannot be excluded. However, the present studies suggest that tryptase induced histamine release from human lung mast cells is not a consequence of PAR2 activation.

The potential of tryptase inhibitors to act as potent mast cell stabilising agents would make them particularly suitable as a novel treatment for bronchial asthma, and other inflammatory conditions of the airways. The underlying mechanism may be related in part to the actions of tryptase on mast cells, but there is little evidence for the involvement of PAR2 mediated processes.

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Footnotes

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Legends

Fig. 1. Inhibitory actions of APC366 on histamine release induced from dispersed lung cells by anti-IgE (-o-) or calcium ionophore A23187 (-□-). Cells were preincubated with APC366 for 30 min at 37°C before challenge. Data are presented as mean ± SE for six to eight separate experiments. * $P < 0.05$ compared with the responses with uninhibited controls. A mean net histamine release (± SE) of 17 ± 2.5% was elicited with anti-IgE, and 45 ± 6.1% with calcium ionophore.

Fig. 2. Inhibition of IgE-dependant histamine release from dispersed human lung mast cells by APC366 (300 μM). Cells were preincubated with APC366 for periods of 0, 5 or 30 min at 37°C before challenge. Values shown are the mean ± SE for five to nine separate experiments. * $P < 0.05$ compared with the responses with the uninhibited controls. There was a mean net histamine release (± SE) of 16 ± 3.9, 20 ± 5.5 or 17 ± 5.1% in response to anti-IgE with preincubation periods of 0, 5 or 30 min, respectively.

Fig. 3. Inhibitory actions of the substrate BAPNA on anti-IgE induced histamine release from dispersed human lung cells. Cells were preincubated with BAPNA for 30 min at 37°C before challenge. Values shown are the mean ± SE for six separate experiments. * $P < 0.05$ compared with the responses with the uninhibited controls. There was a mean net histamine release (± SE) of 9.0 ± 2.9% in response to anti-IgE.

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Fig. 4. (A) Histamine release from lung cells induced by tryptase in the presence of 30 ug/ml heparin (-o-) or with trypsin (-□-). Data are presented as mean ± SE for five to ten experiments. (B) Mean net histamine release (± SE) with anti-IgE (20 separate experiments), calcium ionophore A23187 (20 experiments) and 30 µg/ml heparin (6 experiments) is shown. Cells were incubated with the stimulus for 15 min at 37°C. Mean spontaneous histamine release (± SE) was 8.6 ± 1.2%. * $p < 0.05$. .

Fig. 5. Inhibitory actions of tryptase on anti-IgE induced histamine release from dispersed lung cells. Cells were preincubated with 0, 0.3, 3 or 30 µM tryptase in the presence of 30 µg/ml heparin for 5 or 30 min prior to addition of the stimulus. Values shown are mean ± SE for five to seven experiments. * $P < 0.05$ compared with the responses with the uninhibited controls. There was a mean net histamine release (± SE) of 24 ± 10% with a preincubation period of 5 min, and 17 ± 8.2% with a 30 min preincubation period.

Fig. 6. Inhibitory actions of heparin on anti-IgE induced histamine release from dispersed human lung cells. Heparin (range from 0.3 to 30 µg/ml) and anti-IgE were added to the cells at the same time, and incubated with the cells for 15 min. Values shown are the mean ± SE for four separate experiments. * $P < 0.05$ compared with the responses with the uninhibited controls. The mean net histamine release (± SE) in response to anti-IgE alone was 12 ± 1.7%.

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Fig. 7. Mast cells (arrowed) identified in resected human lung (A) and bronchial biopsy tissue (C), with monoclonal antibody AA1, and adjacent sections immunostained for PAR-2 with monoclonal antibody P2A (B) and antiserum B5 (D).

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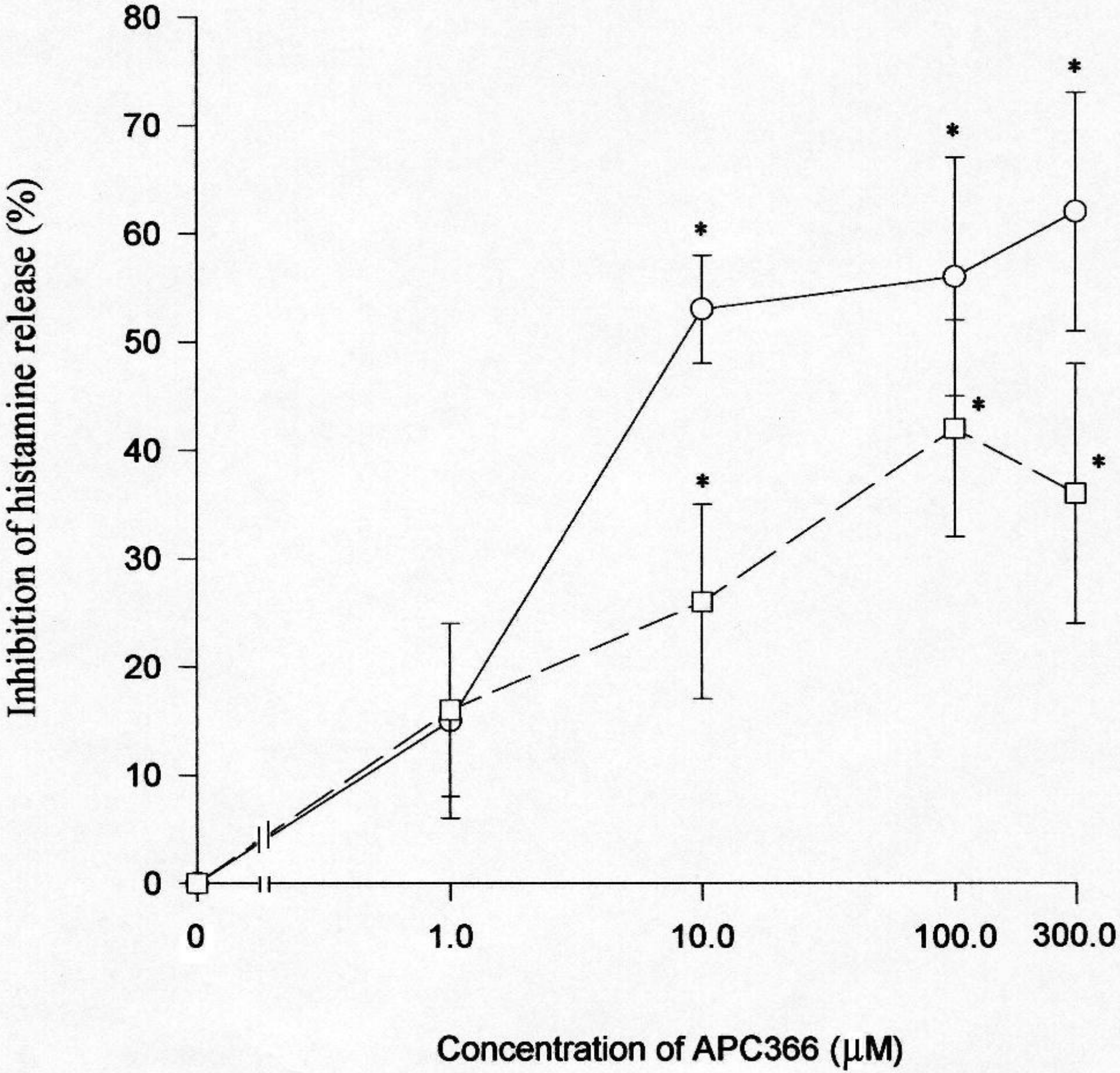
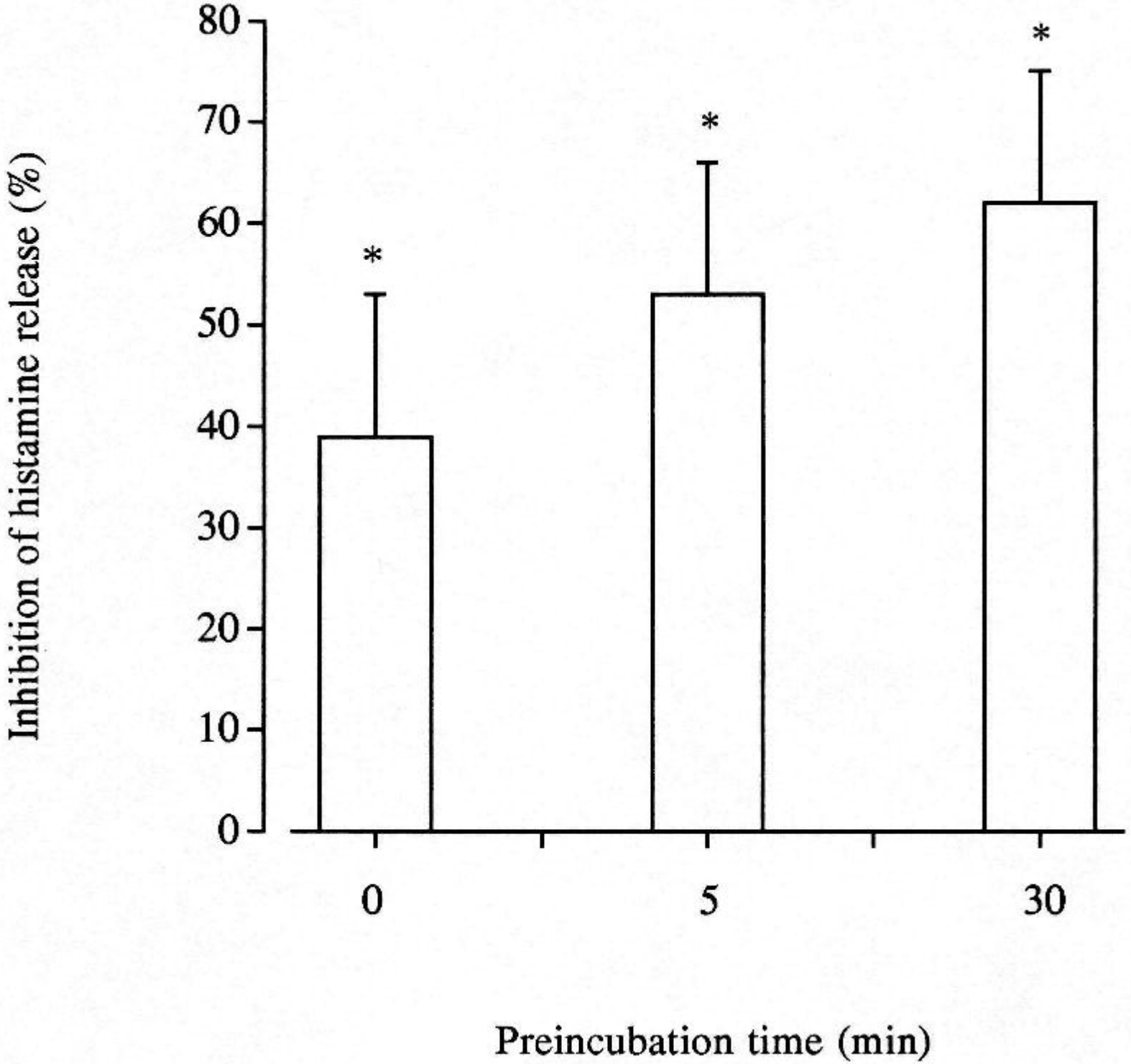
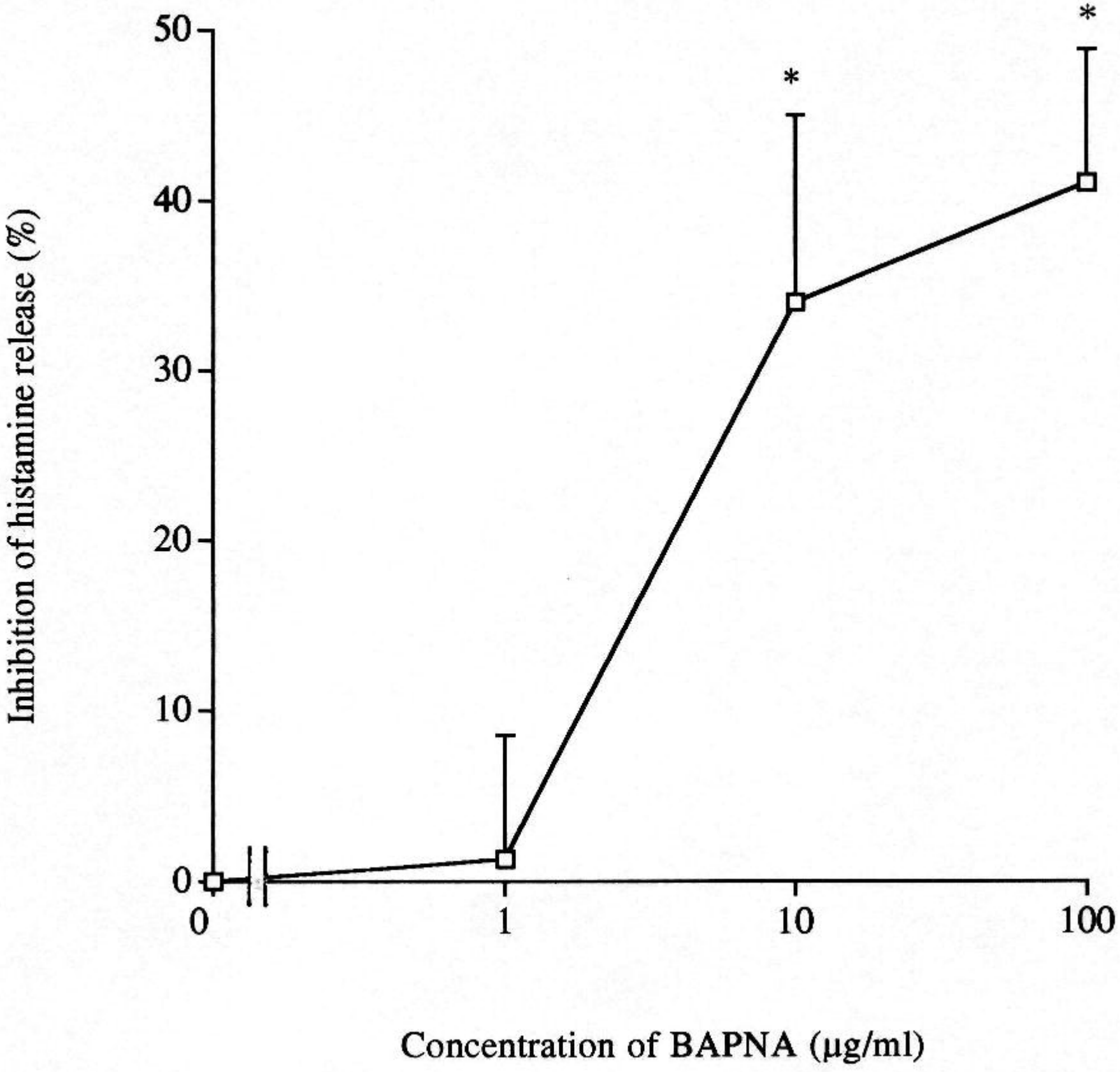
TABLE 1. Effect of peptide agonists of PAR2 and control peptides on histamine release from human lung mast cells. The values are mean ± SE for six to eight separate experiments. Cells were incubated with each concentration of the compound for 15 min at 37°C. Spontaneous histamine release from these cells were 9.1 ± 1.7%.

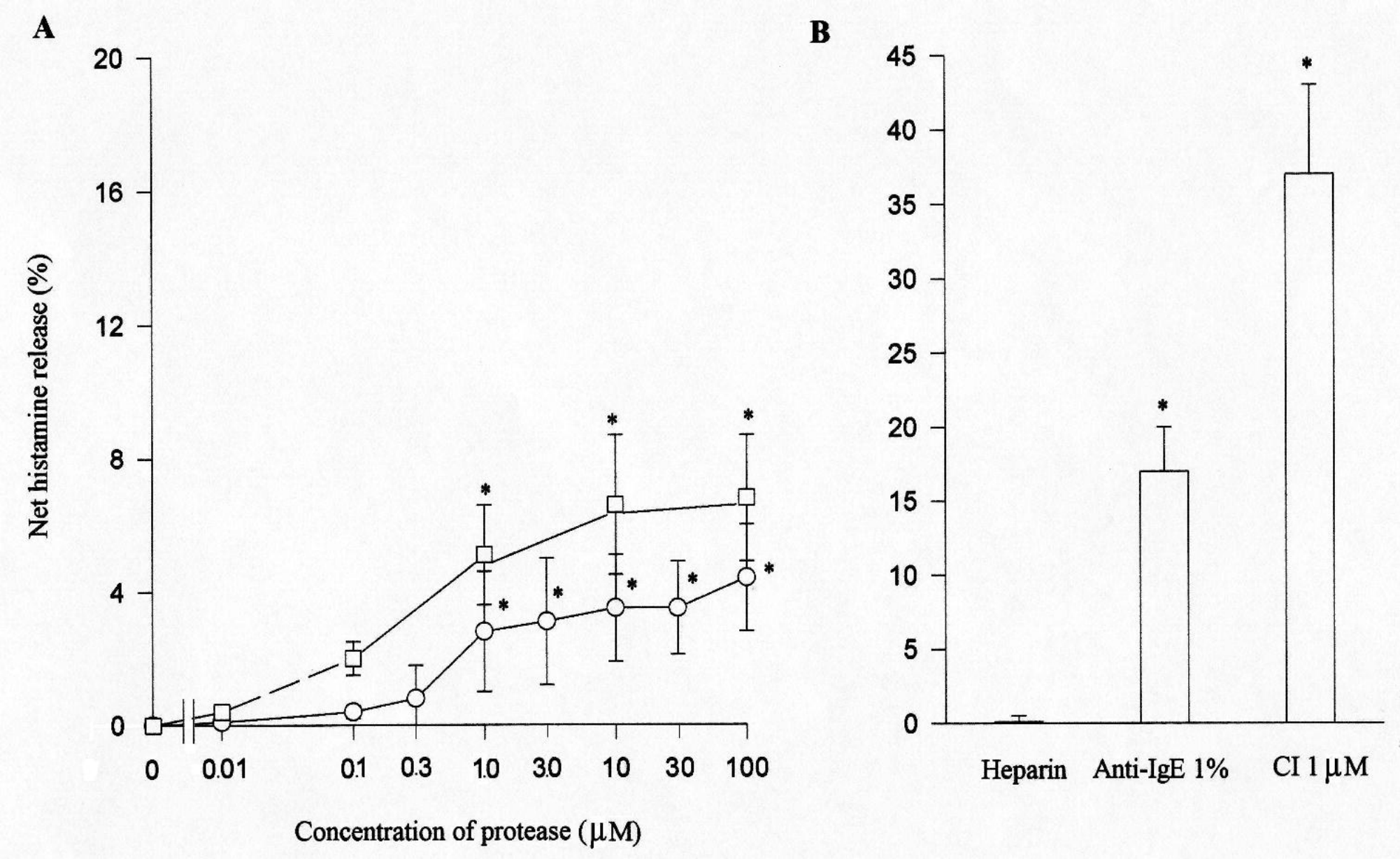
Compounds	Concentration	Net histamine release (%)
SLIGKV-NH_2	1.0 μM	1.0 ± 0.5
	10 μM	0.3 ± 0.9
	100 μM	0 ± 0.5
VKGILS- NH_2	10 μM	0.4 ± 0.6
LSIGKV-NH_2	10 μM	2.2 ± 1.5
tc-LIGRLO-NH_2	1.0 μM	2.7 ± 1.6
	10 μM	3.0 ± 0.9
	100 μM	1.1 ± 0.8
	300 μM	2.7 ± 1.6
tc-OLRGIL-NH_2	1.0 μM	1.2 ± 1.5
	10 μM	0.7 ± 1.0
	100 μM	2.2 ± 0.9
	300 μM	1.5 ± 1.2
Anti-IgE	1%	18 ± 3.8[a]
Calcium ionophone	1.0 μM	60 ± 8.5[a]

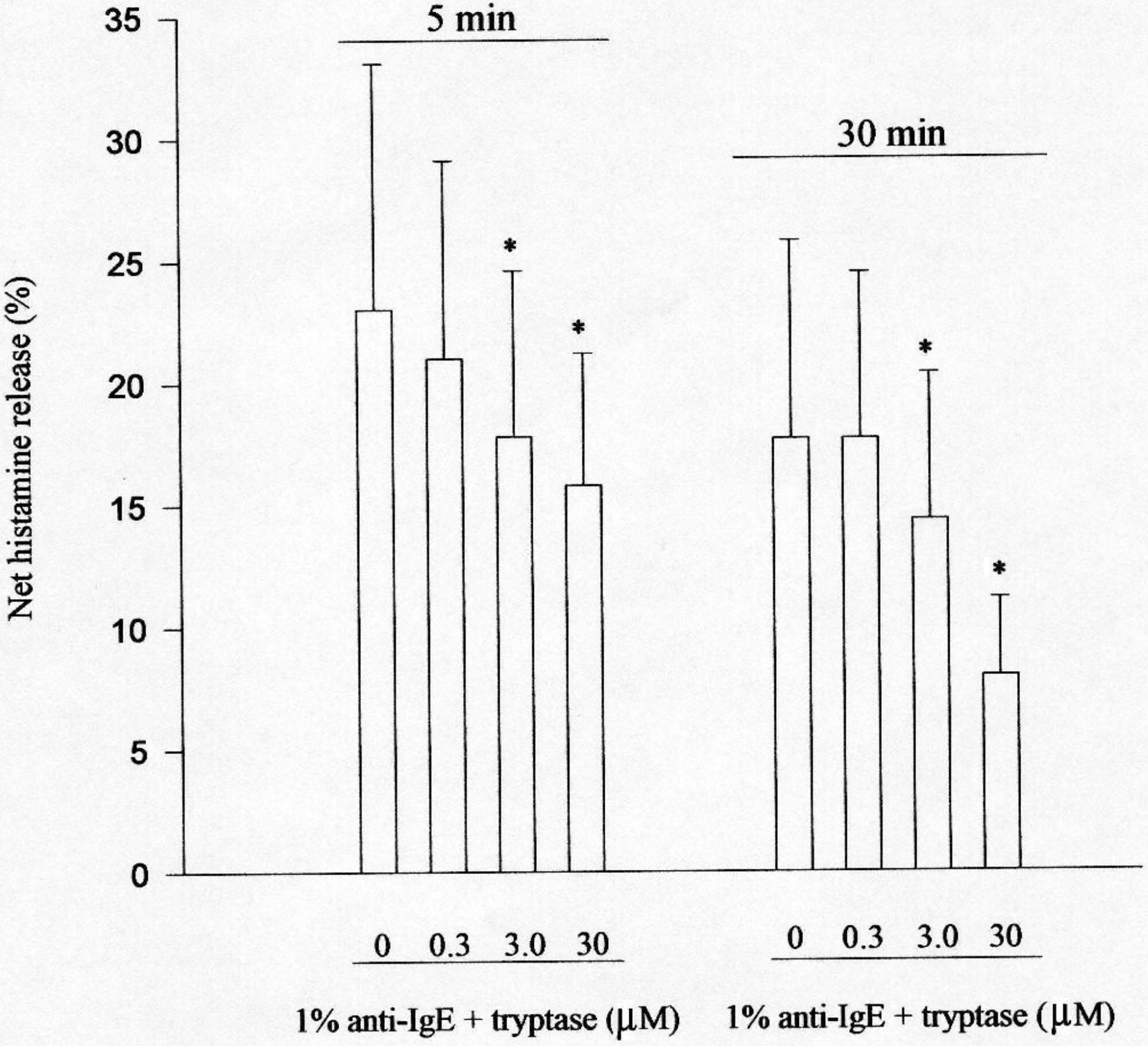
[a] $P < 0.05$ compared with buffer alone control (Student's t test).

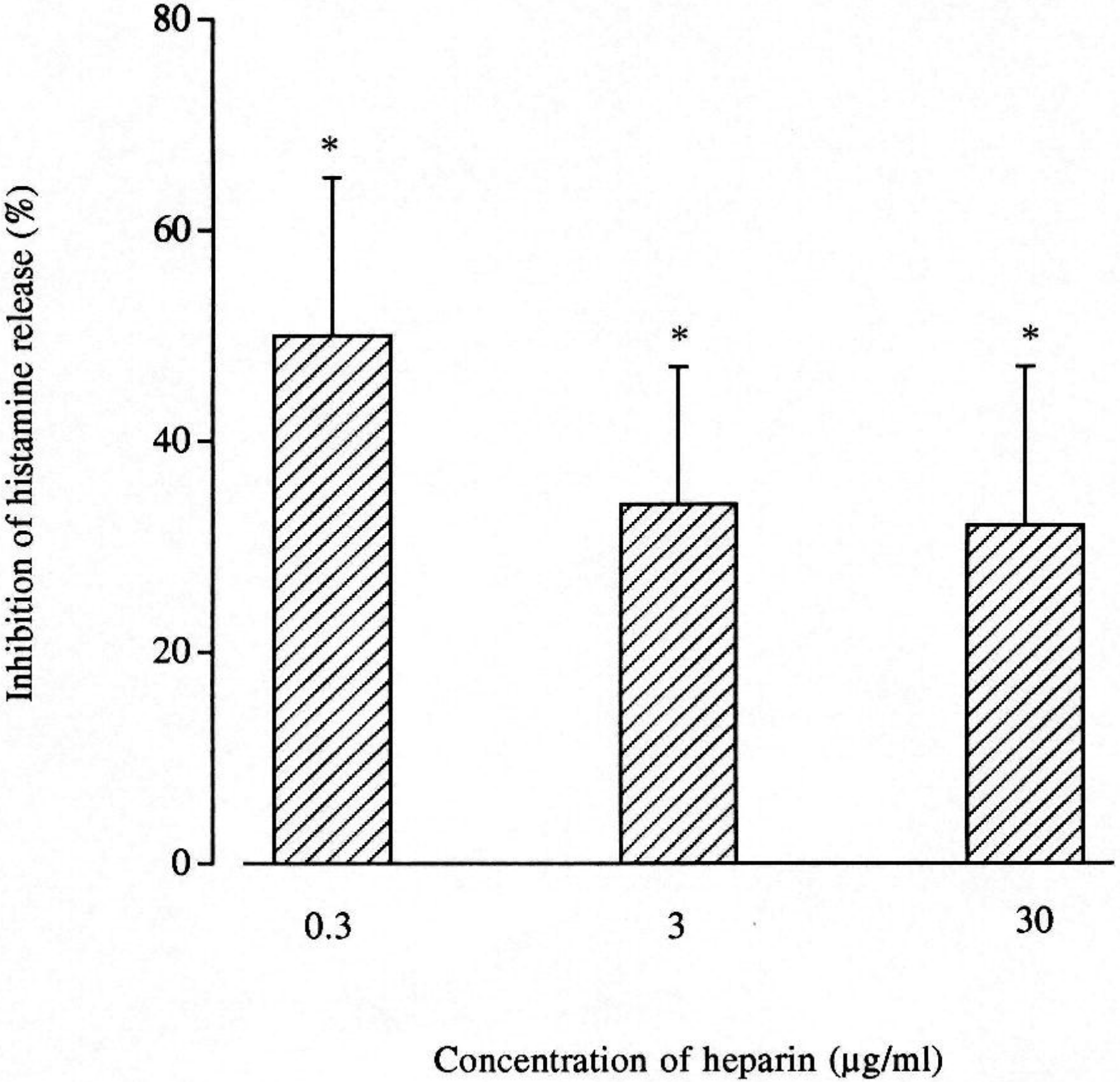

Inhibition of histamine release (%)
80
70
60
50
40
30
20
10
0
0
1.0
10.0
100.0
300.0
Concentration of APC366 (μM)
*
*
*
*
*
*


Inhibition of histamine release (%)
80
70
60
50
40
30
20
10
0
*
*
*
0
5
30
Preincubation time (min)


50
40
30
20
10
0
Inhibition of histamine release (%)
*
*
0
1
10
100
Concentration of BAPNA (μg/ml)


A
B
Net histamine release (%)
20
16
12
8
4
0
0
0.01
0.1
0.3
1.0
3.0
10
30
100
Concentration of protease (μM)
45
40
35
30
25
20
15
10
5
0
Heparin
Anti-IgE 1%
CI 1 μM


5 min
30 min
Net histamine release (%)
35
30
25
20
15
10
5
0
*
*
*
*
0 0.3 3.0 30
0 0.3 3.0 30
1% anti-IgE + tryptase (μM)
1% anti-IgE + tryptase (μM)


80
60
40
20
0
Inhibition of histamine release (%)
*
*
*
0.3
3
30
Concentration of heparin (μg/ml)

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