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Effect of a Chymotrypsin-like Inhibitor, TPCK, on Histamine Release from Cultured Human Mast Cells

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Abstract

The involvement of endogenous proteases in the secretory process from human mast cells remains to be clarified.

A chymotrypsin-like protease inhibitor, *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK), blocked both FcεRI- and A23187-mediated histamine release from cultured human mast cells at concentrations above 1 μM. At 10 μM, the concentration that completely inhibited FcεRI-mediated histamine release, TPCK did not inhibit the chymase activity of the lysate or that in intact cells. The addition of TPCK to cells 30 min before challenge did not affect FcεRI- or A23187-mediated Ca^{2+} mobilization.

These findings suggest that a TPCK-sensitive molecule distinct from chymase is involved in a late stage of the process of histamine release from mast cells in man.

The roles of endogenous proteases in the process of mast-cell degranulation have been exclusively studied in rats. The chymotrypsin-like serine protease, chymase, from rat peritoneal mast cells causes non-cytotoxic mast-cell degranulation in-vitro (Schick & Austen 1986, 1990), and chymotrypsin inhibitors (Kido et al 1985a, b; Urata & Siraganian 1985; Kato et al 1988; Emadi-Khiav & Pearce 1994), substrates (Kido et al 1985b; Kato et al 1988), and antibodies against chymase (Kido et al 1985a, 1988) inhibit histamine release from rat mast cells. In man also the serine proteases, tryptase (Schwartz et al 1981, 1985; Smith et al 1984) and chymase (Schechter et al 1983, 1986), have been identified as the principal protein components of mast-cell granules. However, the content and characteristics of these proteases differ from those in rodents (Schwartz et al 1987) and the roles of these proteases in the degranulation of mast cells in man are less well understood. Some studies using protease inhibitors suggest that chymase might be an important factor in the process of histamine release from pulmonary (Hultsch et al 1988) and tonsilar mast cells (Dietze et al 1990) in man.

We recently established pure human mast cells from cord blood cells in the presence of stem cell factor and interleukin-6 (Nakahata et al 1995; Saito et al 1995; Yanagida et al 1995). These mast cells are functionally mature, and stained with anti-tryptase and anti-chymase antibodies at ratios of 100% and 20–40%, respectively (Nakahata et al 1995; Saito et al 1995; Yanagida et al 1995). These cultures have enabled precise studies of human mast-cell biology.

This study was undertaken to evaluate the effects of several protease inhibitors on histamine release from human mast cells and also to characterize the inhibitor-sensitive molecule which is critical in the secretory process. Here we report that the chymotrypsin-like protease inhibitor, TPCK (Schoellmann & Shaw 1962) effectively blocks both FcεRI- and A23187-mediated histamine release from cultured human mast cells and that a TPCK-sensitive element distinct from chymase is involved in the process of histamine release from mast cells in man.

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Materials and Methods

TPCK, *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), chymostatin, leupeptin, phenylmethanesulphonyl fluoride, aprotinin, soybean trypsin inhibitor, *N*-succinyl-L-ala-ala-pro-phe-*p*-nitroanilide and benzoyl-DL-arg-p-nitroanilide were from Sigma (St Louis, MO) and calcium ionophore A23187 was from Calbiochem (San Diego, CA).

Cell culture

The reported procedures (Nakahata et al 1995; Saito et al 1995; Yanagida et al 1995) were modified. Mononuclear cells were obtained from umbilical cord blood and cultured in α-minimum essential medium (Gibco, Grand Island, NY) containing 20% (v/v) foetal calf serum (HyClone, Logan, UT), 10 μg mL^{-1} deoxyadenosine, deoxyguanosine, deoxycytidine, adenosine, guanosine, cytidine, thymidine and uridine (Gibco) in the presence of 100 ng mL^{-1} stem-cell factor and 10 ng mL^{-1} interleukin-6 (Kirin Brewery, Gunma, Japan). Mast cells ($\geq$ 12 weeks) of 100% purity were used for the experiments.

Histamine release

The cells were sensitized with 2 μg mL^{-1} human IgE (a gift from Dr Kimishige Ishizaka, La Jolla, CA) at 37°C overnight for FcεRI-mediated histamine release. After three washes, they

were suspended at 1×10^5 cells mL^{-1} in Tyrode-Hepes buffer (pH 7·4) containing 137 mM NaCl, 2·7 mM KCl, 1·6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM $NaHCO_3$, 5·5 mM glucose, 0·4 mM NaH_2PO_4, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES) and 0·05% gelatin. The cells were incubated with protease inhibitors at 37°C for various periods, then challenged at 37°C with 4 μg mL^{-1} anti-IgE antibodies (Chemicon, Temecula, CA) for 30 min or 0·1 μg mL^{-1} A23187 for 10 min. The histamine content of the supernatant and that remaining in the cell pellets were determined by an automated fluorimetric procedure (Siraganian 1974). The ratio (%) of inhibition of histamine release was calculated using the formula: $100 \times [1 - (\%$ inhibited release $- \%$ spontaneous release)/(% control release $- \%$ spontaneous release)].

Enzymatic efficacy of protease inhibitors

Mast-cell lysate was prepared using a detergent and a hypotonic buffer as described elsewhere (Schwartz et al 1987). Chymase and tryptase activity were measured by the cleavage of 0·2 mM *N*-succinyl-L-ala-ala-pro-phe-*p*-nitroanilide in reaction buffer containing 2 M NaCl, 0·1 M Tris-HCl (pH 7·8), 15 μM aprotinin (Schechter et al 1983; Schwartz et al 1987; Bastos et al 1995) or cleavage of 0·4 mM benzoyl-DL-arg-*p*-nitroanilide in reaction buffer containing 2 M NaCl, 0·1 M Tris-HCl (pH 7·8), 10 μg mL^{-1} soybean trypsin inhibitor (Lavens et al 1993; Tanaka et al 1983), respectively. The level of aprotinin in the assay is enough to inhibit > 95% of the chymotryptic activity (predominantly cathepsin G) from 10^6 neutrophil equivalents and can be used to distinguish between human chymase and cathepsin G (Schechter et al 1983; Schwartz et al 1987). Lysate (25 μL; 2×10^6 mast cell equivalents mL^{-1}) was incubated with various concentrations of each inhibitor in an equal volume at 37°C for 30 min, then 100 μL of the substrate was added to the mixture. After an incubation at 37°C for 2·5 h the absorbance was measured at 405 nm.

Ca^{2+} mobilization

The intracellular Ca^{2+} concentration was measured as described elsewhere (Magistris et al 1992; Takel & Endo 1994). The cells (5×10^6 cells mL^{-1}) were incubated at 37°C for 45 min with 5 μM fura-2, AM (Microprobe, Funakoshi, Tokyo, Japan) in Tyrode-Hepes buffer. After washing with Hanks balanced salt solution, samples of the cell suspension (2×10^6 cells mL^{-1}) were placed in a cuvette, incubated with or without inhibitors at 37°C for 30 min, then challenged with anti-IgE antibodies or A23187. Fluorescence was recorded by use of a fluorimeter (CAF-100 type, Spectroscopic Co., Tokyo, Japan) with excitation and emission wavelengths of 340 and 380 nm, respectively.

Results

We tested the effects of five low-molecular-weight protease inhibitors upon histamine release from cultured human mast cells. The chymotrypsin inhibitor TPCK at concentrations above 1 μM inhibited both FcεRI- and A23187-mediated histamine release in a dose-dependent manner (Fig. 1) whereas the chymotrypsin inhibitor chymostatin, the trypsin inhibitor leupeptin, and the trypsin-chymotrypsin inhibitor phenylmethylsulphonyl fluoride (PMSF) at concentrations of 0·01 ~ 100 μM did not inhibit histamine release ($n \geq 4$, data not shown). The trypsin inhibitor TLCK at 100 μM occasionally exerted a slight effect on FcεRI-mediated histamine release (% inhibition was approximately 15% in two out of five experiments).

The enzymatic efficacy of these inhibitors for chymase and tryptase is shown in Figs 2a and 2b, respectively. Chymostatin inhibited chymase activity substantially ($IC50 \approx 0{\cdot}5$ μM), whereas TPCK and PMSF partially inhibited the activity at concentrations above 33·3 μM and neither TLCK nor leupeptin had any effect. Tryptase activity was slightly inhibited by leupeptin and TLCK, but not by TPCK, chymostatin, or PMSF. For further clarification of the efficacy of TPCK for chymase in intact cells, we examined chymase activity in mast cells after treatment of the cells with TPCK. After treatment with TPCK at 1, 10, and 100 μM at 37°C for 30 min the percentages of chymase activity in the lysates were 104.1 ± 2.1, 103.0 ± 5.1, and $82{\cdot}0 \pm 1{\cdot}9$%, respectively (one representative of three independent experiments performed in triplicate).

The kinetics of the TPCK-induced inhibition of FcεRI- and A23187-mediated histamine release is shown in Figs 3a and 3b, respectively. Both types of release were effectively blocked by TPCK when added 10 or 30 min before challenge. Adding TPCK at the same time as the challenge completely inhibited FcεRI-mediated release but only partially inhibited that mediated by A23187. The addition of TPCK 5 min after challenge still inhibited the FcεRI-mediated release to some extent, whereas it had no effect on that mediated by A23187.

We tested the effects of TPCK upon Ca^{2+} mobilization during histamine release, because the increase in intracellular Ca^{2+} is a critical event among the biochemical changes

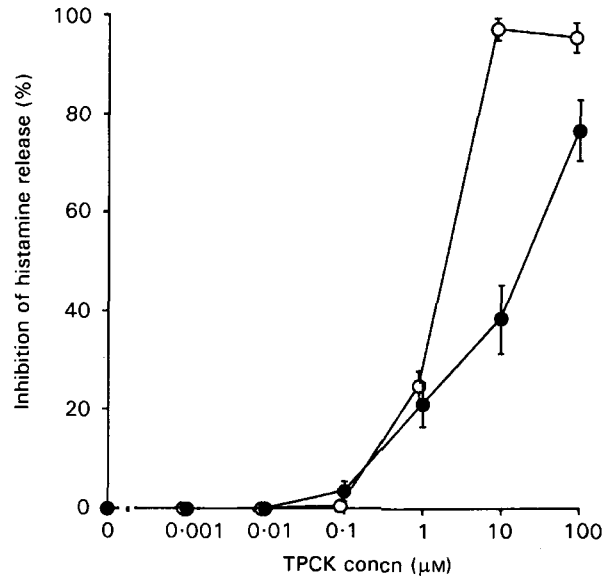

FIG. 1. Dose-dependent inhibitory effects of TPCK on FcεRI- and A23187-mediated histamine release from mast cells. Cells sensitized or not with IgE were incubated with various concentrations of TPCK for 30 min, then challenged with 4 μg mL^{-1} anti-IgE antibodies for 30 min (○) or 0·1 μg mL^{-1} A23187 for 10 min (●). Each point represents the mean of results from four experiments and vertical bars indicate s.d. Spontaneous release was 4.3 ± 1.3 and 3.3 ± 1.7, and control release was $37{\cdot}5 \pm 3{\cdot}1$ and $66{\cdot}5 \pm 4{\cdot}7$% in the studies of FcεRI- and A23187-mediated histamine release, respectively.

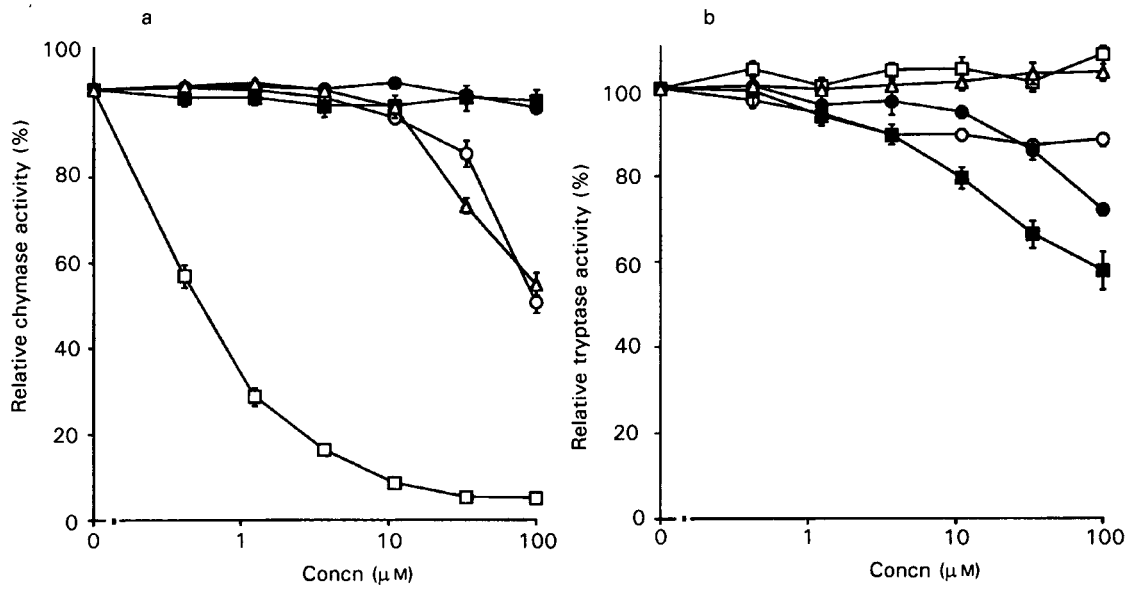

FIG. 2. Enzymatic inhibition of chymase (a) and tryptase (b) by protease inhibitors. Mast cell lysate was incubated with various concentrations of TPCK (○), TLCK (●), chymostatin (□), leupeptin (■), or phenylmethanesulphonyl fluoride (△) at 37°C for 30 min, and the mixture was incubated with N-succinyl-L-ala-ala-pro-phe-*p*-nitroanilide or benzoyl-DL-arg-*p*-nitroanilide at 37°C for 2.5 h. Absorbance at 405 nm was taken as a measure of the activity of chymase or tryptase and is indicated as a percentage of control absorbance. Each point represents the mean of results from triplicate experiments and vertical bars indicate s.d. The results are representative of one of three independent experiments.

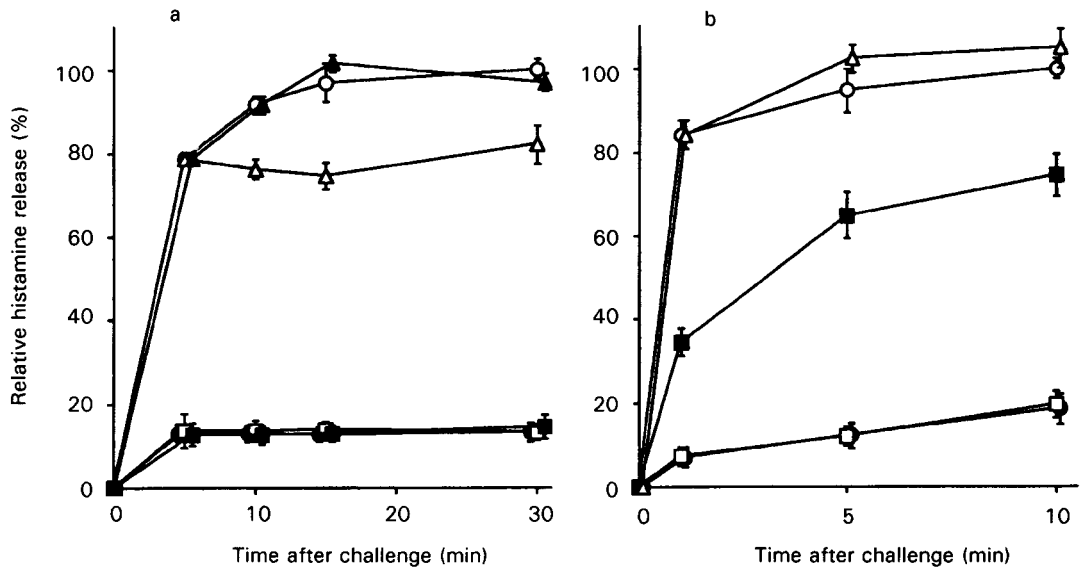

FIG. 3. Kinetics of TPCK (100 μM)-induced inhibition of (a) FcεRI- and (b) A23187-mediated histamine release. Cells sensitized or not with IgE were incubated with TPCK for 0, 10 or 30 min then challenged with anti-IgE antibodies or with A23187. Otherwise, TPCK was added to the cells 5 or 10 min after the challenge. The percentage of FcεRI- and A23187-mediated histamine release was measured at various times after challenge and is indicated as a percentage of the final control release. Each point represents the mean of results from triplicate experiments and vertical bars indicate s.d. The final spontaneous release was 4.1 ± 1.7 and $3.7 \pm 1.2\%$, and control release was 38.1 ± 3.5 and $65.9 \pm 4.3\%$ after the FcεRI- and A23187-mediated challenge, respectively. The results are representative of one of three independent experiments. ●, TPCK addition at − 30 min; □, at − 10 min; ■, at 0 min; △, at 5 min; ▲, at 10 min; ○, no addition.

involved in the triggering of histamine release. TPCK at 100 μM had no effect on FcεRI-mediated Ca^{2+} mobilization after incubation for 30 min (Fig. 4. Similar results were also obtained for A23187-mediated release (data not shown).

Discussion

This is the first report that the chymotrypsin-like protease inhibitor TPCK, an alkylating agent that specifically and irreversibly reacts with histidine residues in the active centre of proteases (Schoellmann & Shaw 1962), effectively blocked FcεRI-mediated histamine release from human mast cells. TPCK at a concentration of 10 μM (that completely blocked FcεRI-mediated histamine release) did not inhibit the chymase activity of the lysate or that in intact cells. These findings suggest that a TPCK-sensitive molecule distinct from chymase is involved in the process of histamine release. In addition, the TPCK-induced inhibition could not be caused by its cyto-

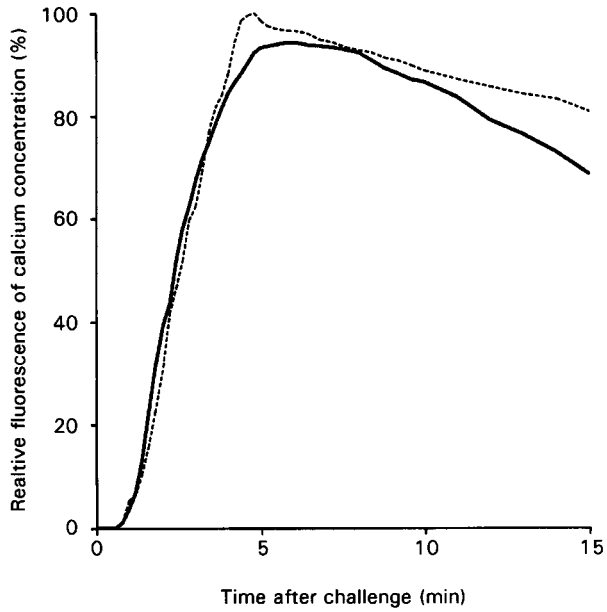

FIG. 4. Effects of TPCK on FcεRI-mediated Ca^{2+} mobilization. Cells sensitized with IgE were incubated with fura-2, AM for 45 min, then incubated with or without TPCK at 100 μM for 30 min. The cells were challenged with 4 μg mL^{-1} anti-IgE antibodies at 0 min. Fluorescence was continuously monitored and is indicated as a percentage of the peak value of the control. Dotted line, no treatment; solid line, treatment.

toxicity, because the concentrations of all inhibitors tested were not toxic, as estimated by trypan blue exclusion (data not shown).

Chymostatin, a potent chymase inhibitor in man (Powers et al 1985), did not, at concentrations of 0·01 ~ 100 μM, inhibit histamine release from cultured human mast cells even though it has been shown to inhibit histamine release from rat mast cells (Kido et al 1985a, b; Kato et al 1988). There are two possible explanations. The first is that chymase is not essential in the process of histamine release from mast cells in man, the second is that chymostatin could not reach a target molecule, presumably chymase, because of lack of penetration. TLCK slightly inhibited FcεRI-mediated histamine release only at high concentrations. The TLCK-hydrochloride used in this study is hydrophilic and might not penetrate sufficiently; its structure and mechanism of action (Shaw et al 1965) have some similarities with those of TPCK (Schoellmann & Shaw 1962). TLCK might block histamine release if it can penetrate the cell membrane in sufficient quantities.

Cross-linking of FcεRI on mast cells induces tyrosine phosphorylation (Kawakami et al 1992), the activation of phospholipase C (Ishizaka & Ishizaka 1984), an increase in intracellular Ca^{2+} (White et al 1984), activation of protein kinase C (White et al 1985) and histamine release. We investigated where the TPCK-sensitive element is involved in the pathway leading to histamine release. In a preliminary study, the tyrosine phosphorylation of total cell lysate and mitogen-activated protein kinase was examined by anti-phosphotyrosine immunoblotting after sensitized cells were incubated with or without TPCK, then challenged with anti-IgE antibodies. There was, however, no significant difference between mast cells incubated with or without TPCK beforehand (data not shown) which suggests that TPCK does not affect tyrosine phosphorylation mainly induced by cross-linking of FcεRI.

As TPCK did not affect the FcεRI- or A23187-mediated Ca^{2+} mobilization, it is supposed that the TPCK-sensitive factor is involved in later stages than Ca^{2+} mobilization. This notion agrees well with the findings that it blocked the histamine release induced by A23187, which evokes Ca^{2+} influx directly by by-passing the initial membrane events (Diamant & Patkar 1975), and its addition at 5 min after challenge partially inhibited FcεRI-mediated histamine release. These results are, however, in contrast with the findings that in rat basophilic leukaemia cells, both FcεRI- and A23187-mediated Ca^{2+} influx was blocked by TPCK (Urata & Siraganian 1985). Judging from this discrepancy, there might be some differences between the mechanisms of mast-cell degranulation in man and rat.

According to a previous report, TPCK inhibits protein kinase C in-vitro (Solomon et al 1985). It is not likely that TPCK inhibits histamine release through protein kinase C inhibition, however, because its activity as a protein kinase C inhibitor is too low (IC50 ≈ 8 mM) in comparison with its effect on FcεRI-mediated histamine release (IC50 ≈ 1·2 μM).

TPCK inhibits a variety of biological events, such as interleukin-2 synthesis in Jurkat T cells (Auberger et al 1989), interleukin-1-induced activation of nuclear factor κB in HeLa cells (Guesdon et al 1995), and tumour necrosis factor-induced apoptosis of leukaemia cell line (Higuchi et al 1995). The mechanisms of these effects have not yet been determined, however. Future studies on the characterization of the TPCK-sensitive element might provide further insight into the mechanisms of histamine release from mast cells in man.

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