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Polycationic peptides as inhibitors of mast cell serine proteases

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Abstract

When mast cells are activated, e.g. during allergic responses, they secrete the serine proteases chymase and tryptase, which both are complex-bound to heparin proteoglycan *in vivo*. Previous reports have demonstrated potent pro-inflammatory effects of both tryptase and chymase in different animal models, suggesting that these serine proteases may be relevant targets for therapeutic intervention. Recent investigations have shown that heparin-binding compounds can cause tryptase inhibition and it has been suggested that the inhibitory activity of such compounds is due to interference with the binding of heparin to tryptase. Here we tested various polycationic peptides for their ability to inhibit heparin-free human recombinant βI-tryptase. We demonstrate powerful direct inhibition of tryptase (IC_{50} values ∼ 1–100 nM) by poly-Arg and poly-Lys of different molecular weights. Poly-Arg and poly-Lys showed predominantely competitive inhibition kinetics, although decreases in the k_{cat} values for the chromogenic substrate S-2288 were also observed. Peptides built up from heparin-binding motifs were also inhibitors of tryptase, albeit of lower efficiency than poly-Arg/Lys. Tryptase inhibition was strongly dependent on the size of the polycationic peptides. The various polycationic peptides were also inhibitory for heparin-dependent activities of chymase. The tryptase inhibition caused by the polycationic peptides could be reversed by adding heparin. After heparin-induced rescue of tryptase activity, the major part of the tryptase activity was sensitive to inhibition by bovine pancreatic trypsin inhibitor, whereas tryptase before addition of polycationic peptide was completely resistant. Taken together, our findings indicate that polycationic peptides can be used as powerful agents for combined inhibition of mast cell tryptase and chymase.

Keywords: Tryptase; Chymase; Heparin; Polycations; Mast cell

1. Introduction

Mast cells are powerful effector cells, well known in particular for their harmful effects in allergic responses [1]. However, several investigations have suggested an involvement of mast cells in many other types of pathophysiological conditions, e.g. wound repair [2], rheumatoid arthritis [3], cancer [4] and HIV infection [5]. It is also well established that mast cells participate in the defence against bacterial infections [6]. Mast cells synthesize and store in their secretory granules a large number of inflammatory mediators, including histamine, cytokines, heparin proteoglycan and various proteases. The mast cell proteases are of three classes—tryptases, chymases and carboxypeptidase A [7], all of which are dependent on proteoglycans for storage in the mast cell granules [8]. In humans only one gene has been identified for carboxypeptidase A and for chymase, respectively. In contrast, several human tryptase genes have been identified: α, βI, βII, βIII, γ, as well as a number of pseudogenes [9]. Of these, the α-tryptases are considered as being proteolytically inactive [10] and are continuously secreted by the mast cells, whereas the β-tryptases are active and constitute the main form of tryptase stored in the mast cell granules. Thus, β-tryptase is the main form of tryptase that is secreted as a consequence of mast cell activation. Tryptase, in its active form, is tetrameric and has been shown to be resistant to most macromolecular protease inhibitors [11]. Crystallization of human β-tryptase confirmed the tetrameric organization of tryptase and showed, furthermore, that all of the active sites were faced towards a narrow central pore, well explaining its resistance to large protease

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; PBS, phosphate buffered saline; rMCP, rat mast cell protease.

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inhibitors that for steric reasons are unable to enter the central cavity of tryptase [12].

Previous investigations have demonstrated that tryptase has potent pro-inflammatory properties. It has been demonstrated, in a sheep model for allergic reactions, that inhalation of β-tryptase causes bronchoconstriction *via* histamine release [13]. Further, it was shown that β-tryptase induces immediate cutaneous reactions in allergic sheep [14]. It has also been shown that human tryptase stimulates microvascular leakage [15] as well as recruitment of neutrophils and eosinophils [16] in guinea pigs. Further, it has been demonstrated that endogenous tryptase has pro-inflammatory properties in mice [17,18]. Although the pro-inflammatory properties of tryptase are well established it is still not clear how tryptase induces inflammation, i.e. its physiological target protein(s) or peptide(s) has not been identified. Potential targets include the peptides vasoactive intestinal peptide [19], calcitonin gene-related peptide [20] as well as protease activated receptor 2 [21], fibrinogen [22], and urokinase plasminogen activator [23].

The well known pro-inflammatory properties of tryptase have evoked an interest for tryptase inhibitors, with the potential of using such compounds as anti-inflammatory drugs. In a previous report it was demonstrated that APC 366, a monovalent inhibitor that was reported to be selective for tryptase, blocked bronchoconstriction in the sheep model for allergy [24]. Later, it was shown that APC 366 also reduces the acute airway responses to allergen in allergic pigs [25].

It has been known for a long time that tryptase is stabilized by its physiological ligand, heparin [11]. Thus, it is not surprising that various compounds that are known to interact with heparin, e.g. lactoferrin [26], myeloperoxidase [27], Polybrene [28] and protamine [28] cause tryptase inhibition. One of these compounds, lactoferrin, has also been shown to block late phase bronchoconstriction in allergic sheep [26]. The general interpretation of these findings is that the heparin-binding compounds compete with tryptase for binding to heparin and that tryptase without access to heparin is de-stabilized, followed by monomerization and loss of activity. We have also shown that rodent chymases are strongly influenced by heparin, where the heparin moiety of the chymase/heparin complexes capture heparin-binding substrates, present them to chymase, and thus enhance the rate of substrate cleavage dramatically [29,30].

In this investigation the aim was to identify optimal polycationic compounds that can be used for inhibition of mast cell tryptase and chymase. Previous comparisons of various heparin-binding proteins have identified two different heparin-bindings motifs, XBBXBX and XBBBXXBX, where X are hydrophobic or uncharged amino acids and B are basic amino acids [31,32]. To test if peptides that contain these motifs are inhibitory for the mast cell serine proteases, peptides containing 1–4 repeats of the respective heparin-binding motif were synthesized and their ability to inhibit human recombinant βI-tryptase and rodent chymases were tested. Peptides that were polymers of Lys and Arg, respectively, were also tested for inhibitory activity.

2. Materials and methods

2.1. Reagents

Recombinant human βI-tryptase (rh-βI-tryptase) was purchased from Promega. The tryptase used in this study is reported by the manufacturer to be free from heparin. However, since heparin is used during the expression procedure [33], we wanted to test if any residual heparin could be present in the rh-βI-tryptase preparation. 10 μg of rh-βI-tryptase was therefore subjected to analysis by the carbazole method [34], which detects the presence of uronic acids. This analysis confirmed that the tryptase preparation was completely negative for heparin or at least below the level of detection; the assay would detect heparin at an amount down to ~1 μg, i.e. the tryptase contains less than 0.1 μg heparin/μg tryptase (results not shown). The rat chymase, rat mast cell protease 1 (rMCP-1), was purified from peritoneal mast cells as described previously [35]. The chromogenic substrates S-2288 (H-D-Ile-Pro-Arg-pNA), S-2238 (H-D-Phe-Pip-Arg-pNA) and S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) were purchased from Chromogenix (Mölndal, Sweden). Poly-Lys with M_r of ~3800, ~13,400 and ~27,400 and poly-Arg with M_r of ~13,000 were from Sigma–Aldrich. The average molecular weights of the poly-amino acids are based on measurements with viscosity methods.

Protamine (M_r ~4500) purified from salmon was from Sigma. Polybrene (hexadimethrine bromide; 1,5-dimethyl-1,5-diaziundecamethylene polymethobromide) was obtained from Janssen Chimica. Porcine mucosal heparin (M_r ~15,000) was a kind gift from Ulf Lindahl (Uppsala University). Bovine α-thrombin was donated by Ingemar Björk (Swedish University of Agricultural Sciences). Bovine pancreatic trypsin inhibitor (BPTI) was from Roche. All other chemicals used were of p.a. grade.

Peptides were synthesized on solid phase according to Hirata *et al.* [36]. In brief, an automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc-procedure. The final deprotected peptides were purified by binary semipreparative HPLC using an Econosil C-18 column (10 mm, 22.5 mm × 250 mm). Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV-Vis detector coupled to an Ultrasphere C-18 column (5 μm, 4.5 mm × 150 mm). The HPLC column eluates were monitored by their absorbance at 220 nm. The molecular weight and purity of synthesized peptides were checked by MALDI-TOF

mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu).

2.2. Cells

Peritoneal cells (~2–3% mast cells, the rest being ~50% macrophages and ~50% lymphocytes) from female C57BL/6 mice (~6 months of age) were collected by peritoneal lavages with 15 mL of cold phosphate-buffered saline (PBS), pH 7.4. Cells were centrifuged (300 *g*; 4°; 10 min) and were resuspended and cultured in serum-free medium H4281 (Sigma–Aldrich). The serum-free medium was supplemented with penicillin (100 IU/mL; GibcoBRL), streptomycin (100 mg/mL; Life Technologies Inc.) and L-glutamine (10 mM). The cells were distributed in 24-well plates (Nunc; $\sim 0.5 \times 10^6$ cells in 0.5 mL per well) and incubated at 37° in a humidified atmosphere of 5% CO_2.

2.3. Enzymatic assays

All enzymatic assays were performed at room temperature. Enzymatic assays were performed in 96-well microtiter plates. In standard incubations, 10 ng of rh-βI-tryptase was added to the wells in a total volume of 100 μL of PBS (pH 7.4)/0.1% Triton X-100. Inhibitors in 10 μL of PBS (pH 7.4)/0.1% Triton X-100, were added followed by 10 min incubation. Thereafter, 20 μL of a 2 mM solution (in H_2O) of S-2288 was added, and increased absorbance at 405 nm was monitored with a Titertek Multiscan spectrophotometer. IC_{50} values were determined by incubating increasing concentrations of inhibitor with protease for 10 min. Residual enzymatic activities were monitored as for standard assays. The ratio of inhibited over uninhibited rates was plotted against the inhibitor concentration, and IC_{50} values were determined by nonlinear regression analysis.

rMCP-1 (12.5 ng per sample in PBS, pH 7.4, 0.1% Triton X-100) was assayed with S-2586 [35]. IC_{50} values were determined as for rh-βI-tryptase, with the difference that the values were corrected for the non heparin-dependent activity and therefore the IC_{50} values expresses the concentration needed for a 50% reduction of the heparin stimulated activity. Heparin induces a ~100–200% increase in the activity of rMCP-1 towards S-2586, in agreement with previous observations [29]. In the absence of heparin, the various polycations caused only moderate reductions, up to 20%, of rMCP-1 activity towards S-2586 (not shown).

For K_m and k_{cat} determinations, substrate concentrations ranging from 0.03 to 3.6 mM (S-2288) and 0.06 to 3.6 mM (S-2586) were used. Absorbance changes were followed over 30 min with readings every 30 s. Initial reaction velocities (obtained within 5 min in all of the analyses) were used to calculate the enzymatic activity. The data obtained were used for calculations of kinetic parameters after nonlinear regression analysis.

Molar concentrations of the poly-Arg and poly-Lys peptides are, as well as for heparin and Polybrene, approximate since these molecules are heterogeneous in size and therefore an average M_r is used for the calculations. Incubation of rh-βI-tryptase (50 ng) with poly-Arg (100 μg) or poly-Lys (100 μg) for 24 hr did not lead to any detectable degradation of these compounds, as judged by SDS–PAGE separation followed by staining with Coomassie.

2.4. Thrombin inactivation

Inhibitors at different concentrations (duplicates) were added to over-night cultures of peritoneal cells (see Section 2.2), and incubated for 30 min before 1 μg of thrombin was added to the cells. Media samples (50 μL) were collected at different time points and stored at −20°. Residual thrombin activities in these samples were assayed using S-2238: 10 μL of sample was mixed with 190 μL of PBS (pH 7.4) followed by the addition of 20 μL S-2238 solution (4 mM in H_2O) and recording of absorbance as already described.

2.5. Inhibition rate

The rh-βI-tryptase or rMCP-1 was incubated together with various polycationic peptides as during standard assays. After various incubation times, S-2288 (rh-βI-tryptase) or S-2586 (rMCP-1) was added and residual activities were measured as above.

2.6. Reversibility of tryptase inhibition

The rh-βI-tryptase (10 ng in 100 μL PBS, pH 7.4, 0.1% Triton X-100) was incubated with polycationic compounds for either 15 min or 2 hr. Next, heparin (50 μg; 5 μL of a 10 μg/μL solution in H_2O) was added. After 5 min, samples were treated with BPTI (1 μg; 5 μL of a 0.2 mg/μL solution in PBS) or buffer without BPTI. After an additional 5 min, residual activity was measured with S-2288.

3. Results

3.1. Potency of polycationic peptides in inhibition of rh-βI-tryptase and rMCP-1

IC_{50} values for the inhibition of rh-βI-tryptase and heparin-stimulated activity (see Section 2) of rMCP-1 are listed in Table 2. Generally, the peptides containing repeats of heparin-binding motifs (see Table 1) appeared to have somewhat stronger effects on rMCP-1 than on rh-βI-tryptase. The peptides containing only one repeat of the respective heparin-binding motifs did not show any inhibitory effect at concentrations up to 25 μM. However, clear inhibition of both rh-βI-tryptase and rMCP-1 was seen when the number of heparin-binding motifs were increased. The

Table 1
Sequence of polycationic peptides used in this study

Peptide	Sequence	K_D (nM)
M0192	Ac AKKARA-NH_2	NI
PS0191D	Ac $(AKKARA)_4$-NH_2	55
PS0177G	Ac $(ARKKAAKA)_2$-NH_2	1070
PS0178C	Ac $(ARKKAAKA)_3$-NH_2	30
PS0178D	Ac $(ARKKAAKA)_4$-NH_2	7

Affinities for heparin are taken from Pimenta *et al.* [42]. NI, no interaction detected.

strongest inhibitory effect of any of these peptides was seen for PS0178D, which inhibited rMCP-1 with an IC_{50} value in the nM range, although it showed only weak inhibition of rh-βI-tryptase. Interestingly, this peptide carries the highest net charge out of the tested heparin-binding motif containing peptides (Table 2). It should be noted, however, that all of the peptides containing heparin-binding motifs were relatively weak inhibitors for both rh-βI-tryptase and rMCP-1.

Various homopolymers of Arg and Lys were strongly inhibitory for both rh-βI-tryptase and rMCP-1, with IC_{50} values in the low nM range. The inhibition of the mast cell serine proteases showed a strong dependence on size. Poly-Lys of M_r ~27,400 inhibited tryptase with an IC_{50} value as low as 1.5 nM and poly-Lys of half this size (M_r ~13,400) displayed a similar IC_{50} value (0.85 nM). Decreasing the size of the poly-Lys to M_r ~3800 led to a ~100-fold increase in the IC_{50} value (95 nM). The inhibition of rMCP-1 by the differentially sized poly-lysines showed a similar dependence on size. Poly-Arg inhibited rh-βI-tryptase and rMCP-1 with IC_{50} values that were in the low nanomolar range (Table 2). It is important to note that the polycations inhibited tryptase that is not stabilized by heparin (see Section 2). Thus, the polycations are directly inhibitory for tryptase.

3.2. Inhibition kinetics

The influence of the various polycationic peptides on kinetic parameters of tryptase and rMCP-1 was studied (Table 3). Incubation of tryptase with poly-Arg (M_r ~13,000) at 5–100 nM led to a gradual increase (up to ~30-fold) in the K_m values for S-2288, indicating competitive inhibition kinetics. At these concentrations of poly-Arg, there was only a slight effect on the k_{cat} values. Likewise, the heparin-binding peptide PS0178C induced an increased K_m for S-2288 with no apparent effect on the k_{cat} value. The differently sized poly-Lys peptides all induced marked increases (up to ~20-fold) in the K_m values of tryptase for S-2288. However, the poly-lysines also consistently affected the k_{cat} values negatively. Thus, the inhibition of tryptase by poly-lysines appears to show predominantly competitive inhibition kinetics, but also a component of noncompetitive inhibition kinetics. Similarly, the heparin-binding peptide PS0191D produced both increased K_m values as well as decreased k_{cat} values of tryptase for S-2288. All of the polycationic peptides tested in this study appeared to inhibit rMCP-1 with predominately competitive inhibition kinetics.

3.3. Thrombin inactivation

We have previously shown that thrombin is inactivated/degraded in cultures of mouse peritoneal cells, and that the thrombin inactivation is catalyzed by mast cell chymase in a strongly heparin-dependent manner [30]. To test if the polycationic peptides used in this study were able to inhibit thrombin inactivation, the influence of the peptides on thrombin inactivation in peritoneal cell cultures was tested (Fig. 1). It is clear from Fig. 1 that poly-Arg (M_r ~13,000) at 25 nM inhibits the thrombin inactivation in these cultures completely and that inhibition is also observed for poly-Lys (M_r ~13,400) at 25 nM. Poly-Arg at lower concentrations did not inhibit thrombin inactivation and other polycationic peptides did not show any inhibitory activity at the concentrations tested.

3.4. Inhibition velocity

Polycationic inhibitors were added to rh-βI-tryptase and rMCP-1 and residual enzymatic activities were measured at different time points. In the absence of inhibitors the rMCP-1 activity towards S-2586 was quite stable throughout the measurement (Fig. 2A). The inhibitors were able to

Table 2
IC_{50} values for the inhibition of rh-βI-tryptase and heparin-stimulated activity of rat chymase (rMCP-1) by polycationic peptides

Peptide	M_r	Charge	IC_{50}; rh-βI-tryptase (nM)	IC_{50}; rMCP-1 (nM)
Poly-Arg	~13000	~75	2.9 ± 0.4	52 ± 25
Poly-Lys	~27400	~187	1.5 ± 0.3	6 ± 2
Poly-Lys	~13400	~92	0.85 ± 0.38	16 ± 2
Poly-Lys	~3800	~26	95 ± 32	460 ± 130
M0192	720	3	>25000	>25000
PS0191D	2921	12	2100 ± 1000	1300 ± 560
PS0177G	2013	8	>25000	>25000
PS0178C	2983	12	7300 ± 2400	1600 ± 800
PS0178D	3952	16	4200 ± 680	220 ± 190

Results are expressed as mean of triplicate determinations ± SD of the linear regression.

Table 3
Kinetic parameters for the inhibition of recombinant human β-tryptase and rat chymase (rMCP-1) by polycationic peptides

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
rh-βI-tryptase			
Control	0.017 ± 0.004	8.7 ± 1.8	400
Poly-Lys 13400 (5 nM)	0.026 ± 0.0062	8.2 ± 0.79	310
Poly-Lys 13400 (10 nM)	0.022 ± 0.0034	5.4 ± 0.33	250
Poly-Lys 13400 (20 nM)	0.036 ± 0.0043	4.5 ± 0.25	120
Poly-Lys 13400 (50 nM)	0.091 ± 0.0064	3.2 ± 1.4	35
Poly-Lys 13400 (100 nM)	0.12 ± 0.077	2.0 ± 0.64	17
PS0191D (2 μM)	0.038 ± 0.0044	12 ± 0.52	320
PS0191D (10 μM)	0.089 ± 0.064	1.9 ± 0.66	21
PS0191D (50 μM)	0.083 ± 0.072	1.3 ± 0.54	16
PS0178C (2 μM)	0.025 ± 0.0044	10 ± 0.91	410
PS0178C (5 μM)	0.14 ± 0.046	11 ± 2.1	80
PS0178C (7.5 μM)	0.11 ± 0.033	4.8 ± 0.78	46
PS0178C (20 μM)	0.86 ± 0.11	10 ± 0.25	12
Poly-Arg 13000 (5 nM)	0.018 ± 0.0007	11 ± 0.13	600
Poly-Arg 13000 (10 nM)	0.036 ± 0.0032	9.8 ± 0.32	270
Poly-Arg 13000 (50 nM)	0.26 ± 0.34	6.3 ± 2.8	24
Poly-Arg 13000 (100 nM)	0.60 ± 0.33	6.3 ± 2.8	11
rMCP-1			
Control	0.064 ± 0.013	5.2 ± 0.69	82
Poly-Lys 3800 (500 nM)	2.0 ± 0.4	13 ± 2.2	6.7
Poly-Lys 13400 (10 nM)	0.12 ± 0.011	5.9 ± 0.25	51
Poly-Lys 13400 (20 nM)	0.24 ± 0.046	6.1 ± 0.67	26
Poly-Lys 13400 (50 nM)	0.73 ± 0.30	7.4 ± 2.3	10
Poly-Lys 13400 (100 nM)	1.1 ± 0.19	7.3 ± 0.92	6.5
Poly-Lys 27400 (5 nM)	1.6 ± 0.48	12 ± 2.6	7.4
PS0191D (7.5 μM)	1.2 ± 0.10	6.8 ± 0.44	5.7
Poly-Arg 13000 (20 nM)	0.23 ± 0.12	4.9 ± 1.4	21
Poly-Arg 13000 (50 nM)	0.44 ± 0.15	5.5 ± 1.3	13
Poly-Arg 13000 (75 nM)	0.59 ± 0.29	6.2 ± 2.2	10
Poly-Arg 13000 (100 nM)	0.67 ± 0.18	8.2 ± 1.6	12

Results are expressed as mean of triplicate determinations ± SD of the linear regression.

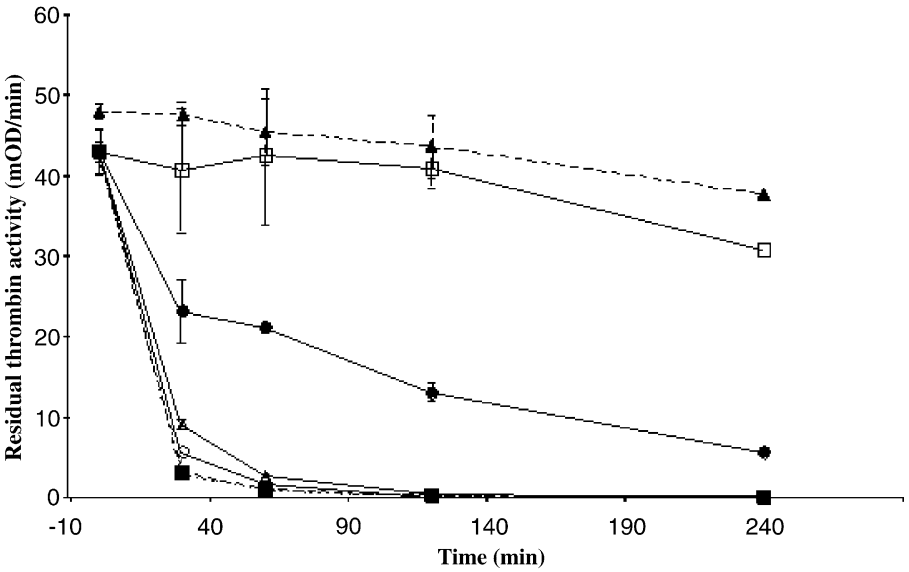

Fig. 1. Inhibition of chymase-catalyzed thrombin inactivation by polycationic peptides. Peritoneal cells from C57BL/6 female mice were cultured ~18 hr. Polycationic peptides were added at different concentrations: poly-Arg (5 nM; open triangles), poly-Arg (25 nM; filled triangles), poly-Lys (M_r ~13,400; 5 nM; open circles), poly-Lys (M_r ~13,400; 25 nM; filled circles) or PS0191D (25 μM; filled squares). Cell control (open squares, dashed line), cells w/o polycation added (symbols partly invisible due to overlay with PS0191D). Medium control (open squares, filled line), thrombin incubated with cell culture medium (w/o cells). 30 min after addition of polycation to the cells, 1 mg thrombin was added. Media samples (50 mL) were taken at the indicated time-points and were analyzed for residual thrombin-like activity using the chromogenic substrate S-2238. Results are expressed as the mean of duplicate determinations ± range of variation.

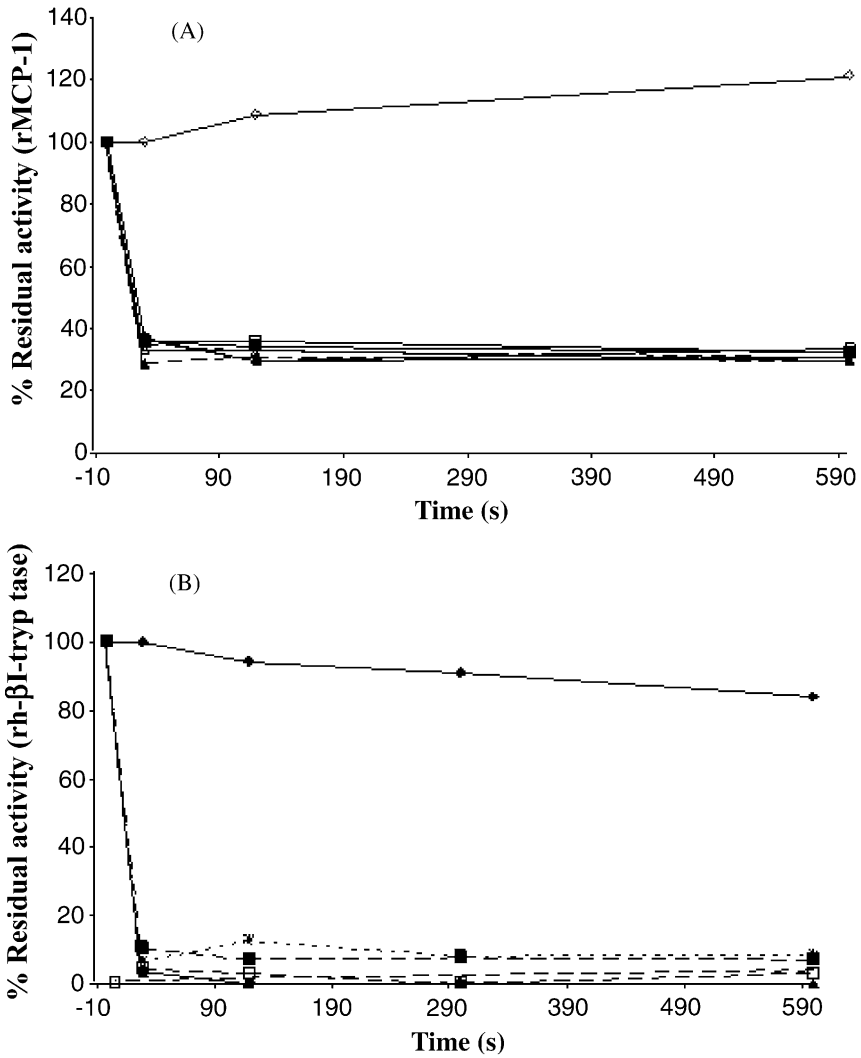

Fig. 2. Inhibition velocity for rMCP-1 and rh-βI-tryptase. (A) 12.5 μg rMCP-1 was incubated for 10 min with 10 μg of heparin (in 100 μL PBS, 0.1% Triton X-100). Polycations (in 10 μL PBS, 0.1% Triton X-100) were added as follows: poly-Arg (1 μM final concentration; open squares), poly-Lys (M_r ~27,400; 1 μM final concentration; open triangles), poly-Lys (M_r ~13,400; 1 μM final concentration; filled triangles), poly-Lys (M_r ~3800; 1 μM final concentration; open circles), PS0191D (25 μM final concentration; crosses), protamine (1 μM final concentration; filled circles), Polybrene (1 μM final concentration; filled squares). Control, rMCP-1 w/o added polycation (open diamonds). At the time points indicated, residual rMCP-1 activity was analyzed with S-2586. (B) 10 ng of rh-βI-tryptase (in 100 μL PBS, 0.1% Triton X-100) was incubated alone (filled diamonds) or with either of the polycations: poly-Arg (M_r ~13,000), poly-Lys (M_r ~13,400), protamine, Polybrene or PS0191D (concentrations of polycations and symbols were the same as in (A)). At the time points indicated, residual rh-βI-tryptase activities were assayed with S-2288.

reduce the activity to ~30% of the initial activity within 30 s from their addition. The residual activity obtained in the presence of the different polycations thus corresponds to the activity of rMCP-1 towards S-2586 before addition of heparin, i.e. the polycations are able to block the stimulatory effect of heparin on rMCP-1-catalyzed hydrolysis of S-2586. rh-βI-tryptase activity was slightly more unstable in comparison to rMCP-1 (Fig. 2B), in agreement with the known instability of this enzyme in the absence of stabilizing agents such as heparin [37]. After 30 min incubation without the presence of any inhibitor, a ~20% loss of activity was observed. The inhibitors poly-Lys (M_r ~13,400), poly-Arg and protamine caused essentially complete inhibition of rh-βI-tryptase within 30 s, whereas a small (~10%) residual activity was present after the additions of Polybrene or PS0191D.

3.5. Reversibility of tryptase inhibition

To test if the tryptase inhibition was reversible, rh-βI-tryptase was treated with different polycationic peptides, followed by the addition of excess heparin (Fig. 3). Addition of poly-Arg, poly-Lys (M_r ~27,400), poly-Lys (M_r ~13,400) or protamine at the indicated concentrations resulted in complete inhibition of rh-βI-tryptase activity (bars not visible in figure), whereas poly-Lys (M_r ~3800) addition gave ~65% inhibition. When heparin was added to rh-βI-tryptase 15 min after poly-Arg treatment of the

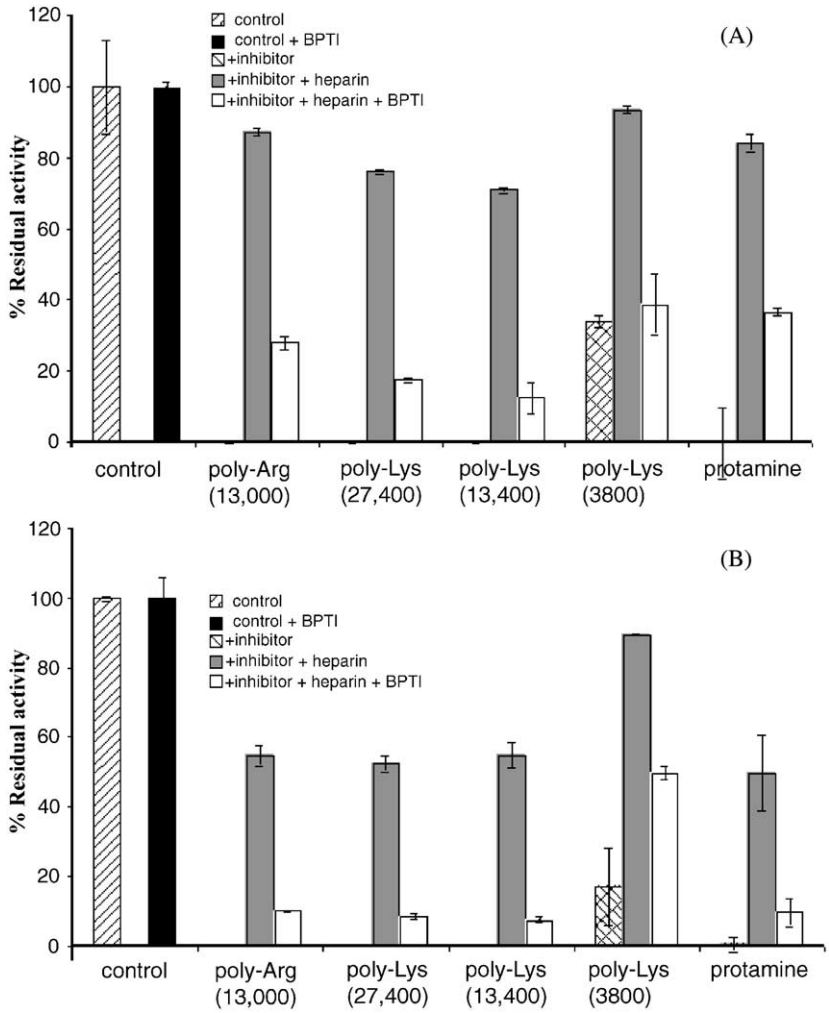

Fig. 3. Reversibility of tryptase inhibition. rh-βI-tryptase (10 ng in 100 μL PBS, 0.1% Triton X-100) was incubated for 15 min (A) or 2 hr (B) with poly-Arg (M_r ~13,000; 0.1 μM final concentration), poly-Lys (M_r ~27,400; 0.1 μM final concentration), poly-Lys (M_r ~13,400; 0.1 μM final concentration), poly-Lys (M_r ~3800; 0.1 μM final concentration) or protamine (0.1 μM final concentration); total volume 110 μL. Control, rh-βI-tryptase w/o polycation. Next, an excess of heparin (50 μg; in 5 μL of H_2O) was added to indicated samples. After an additional 5 min of incubation, BPTI (1 μg) was added to a set of samples. As a control, BPTI was added to rh-βI-tryptase that had not been treated with polycation/heparin (filled bars). Finally, 20 μL of 2 mM S-2288 was added and residual rh-βI-tryptase activities were measured. A ~50% decrease in activity of control rh-βI-tryptase (w/o polycation) was observed after the 2 hr incubation time. Results are expressed as mean of triplicate determinations ± SD.

sample, almost full (~90%) recovery of tryptase activity was observed (Fig. 3A), but when heparin was added 2 hr after poly-Arg only ~55% of the activity was restored (Fig. 3B). Similar results were obtained with protamine (84% recovery after 15 min; 37% after 2 hr). Samples treated with poly-Lys with M_r ~13,400 or ~27,400 showed a somewhat lower degree of heparin-mediated recovery after 15 min but showed similar degrees of reactivation after 2 hr of polycation treatment as for poly-Arg treated rh-βI-tryptase (Fig. 3A and B).

Tetrameric tryptase is known to be resistant to most macromolecular protease inhibitors. To investigate whether polycation-treated tryptase retains such properties after heparin-mediated rescue we treated control as well as polycation-treated/heparin-rescued rh-βI-tryptase with bovine pancreatic trypsin inhibitor (BPTI). Untreated rh-βI-tryptase was, as expected, totally resistant to this protease inhibitor (Fig. 3B). In contrast, ~80% of the activity recovered after treatment with poly-Arg and subsequent rescue with heparin after 15 min was inhibited by BPTI. The degree of inhibition by BPTI was similar when samples had been treated with poly-Arg for 2 hr before heparin rescue (Fig. 3B). Similar findings were seen for rh-βI-tryptase that had been treated with poly-Lys of different molecular sizes as well as for protamine-treated rh-βI-tryptase (Fig. 3A and B).

4. Discussion

In the present report we show that various polycationic peptides are inhibitory for human mast cell βI-tryptase.

Further, we show that similar polycationic peptides also are inhibitory for the rodent chymase, rMCP-1, in complex with heparin. It is important to note that the polycations do not influence the actual active site of rMCP-1 [38]. Instead, they bind to the heparin part of the heparin/rMCP-1 complex and thus block the ability of the heparin chains to capture heparin-binding substrates from the solution. We have previously shown that the association of rMCP-1 (or its mouse orthologue mMCP-4) with heparin strongly potentiates the ability of the rodent chymases to degrade a variety of heparin-binding proteins, e.g. thrombin [29], plasmin [30] and fibronectin [39]. Polycationic substances could thus provide a powerful tool to inhibit heparin-dependent activities of certain chymases *in vivo*, without affecting its non heparin-dependent activities. For example, we showed previously that binding of non heparin-binding plasma protease inhibitors, e.g. α_2-macroglobulin, α_1-antichymotrypsin and α_1-protease inhibitor, is decreased by the association of chymase with heparin proteoglycan [40]. Treatment of native chymase/heparin complexes with polycations would accordingly enhance the rate of binding of the plasma protease inhibitors to chymase, which could lead to rapid and complete destruction of the chymase active site. Although a number of studies have established a profound influence of heparin on substrate cleaving properties of rodent chymases (see above), it is important to stress that it remains to be shown that heparin has a similar effect on human chymase.

Tryptase inhibition by the polycations appears to function according to an entirely separate mechanism. Several previous reports have shown that various substances that are able to bind to heparin are inhibitory for tryptase (see Section 1). Since tryptase is dependent on heparin for stabilization it has been assumed that the inhibitory mechanism of these compounds involves binding to heparin and interference with its stabilizing action on tryptase. This mechanistic model would imply that heparin-binding substances are only inhibitory for tryptase if heparin is present. Here we show, on the contrary, that the polycationic peptides are powerful inhibitors for tryptase that is free from heparin. We note, however, that heparin is present in the culture medium during the expression of the recombinant tryptase but is later removed during the purification procedure [33]. Possibly, heparin has thereby been involved in the initial tetramerization/activation of the recombinant tryptase (see [18,41]), but is no longer mandatory for activity when the enzyme is stored at high NaCl concentration and acidic pH [33]. The exact mechanism by which the polycations inhibit rh-βI-tryptase is not clear. However, the predominantely competitive inhibition kinetics for poly-Arg indicates that this compound interacts directly with the active site of tryptase. In order for the polycations to gain access to the active sites they would have to enter the narrow central pore of the tryptase tetramer. This may not be an easy task for a heavily charged substance. However, it is recognized that the surface of the central pore carries a marked negative charge [12] and it is thus possible that the entrance of polycations to the central cavity is facilitated by electrostatic interactions with the inner surface of the tryptase tetramer.

The inhibition of rh-βI-tryptase by the various polycationic peptides could be reversed by adding excess heparin subsequent to the polycation treatment. The most likely explanation for this reversal is that heparin binds to the polycations and thus prevents them from interacting with tryptase. Interestingly, it appears as treatment of rh-βI-tryptase with the polycations followed by rescue by addition of heparin alters the macromolecular arrangement of rh-βI-tryptase. Before addition of polycations, rh-βI-tryptase is completely resistant to BPTI, as expected since macromolecular protease inhibitors are known to be prevented from entering the central pore of the tryptase tetramer [12]. However, after rescue of polycation-inhibited tryptase with heparin, a large part of the activity is sensitive to BPTI. This observation clearly indicates that the rescued tryptase has a more open arrangement where access to BPTI is permitted.

Out of the tested compounds it is clear that the various polymers of Arg and Lys were by far the most efficient inhibitors for both rh-βI-tryptase and rMCP-1. It is clear that all of the various peptides containing varying numbers of heparin-binding motifs were relatively weak inhibitors of the proteases. This may be unexpected considering the high affinity for heparin of some of these compounds [42]. However, in our suggested mechanism for tryptase inhibition the peptides do not have to interact with heparin to cause tryptase inhibition and it can therefore not be predicted that the potency of a polycationic substance to inhibit tryptase is a function of its affinity for heparin. For inhibition of heparin-dependent activities of chymases, on the other hand, heparin affinity should be a major criterion for potent inhibitory action. In agreement with such a notion, we note that the heparin-binding motif containing peptides are generally more potent inhibitors of rMCP-1 than for rh-βI-tryptase.

We show here that both poly-Lys and poly-Arg are extremely potent inhibitors of the mast cell serine proteases, with IC_{50} values in the low nanomolar range. These findings thus raise the possibility of using such (or similar) compounds in the treatment of pathological conditions where mast cell serine proteases may be involved (see Section 1). Importantly, the polycationic peptides will inhibit both tryptase and heparin-stimulated activities of certain chymases, although the inhibitory mechanisms for these subclasses of proteases may differ significantly (see above). The fact that the most potent polycations are those of the highest molecular weight may, however, be a distinct disadvantage for use in therapy, and may possibly prevent their usage at least for administration within the circulation system. On the other hand, the previous successful use of a macromolecular compound (lactoferrin; [26]) to block bronchoconstriction in allergic sheep indicates that it may be possible to use high molecular weight substances

for treatment of allergic conditions, provided that the regimen for administration of the compound is relevant.

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