Heparin Antagonists Are Potent Inhibitors of Mast Cell Tryptase†

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ABSTRACT: Tryptase may be a key mediator in mast cell-mediated inflammatory reactions. When mast cells are activated, they release large amounts of these tetrameric trypsin-like serine proteases. Tryptase is present in a macromolecular complex with heparin proteoglycan where the interaction with heparin is known to be essential for maintaining enzymatic activity. Recent investigations have shown that tryptase has potent proinflammatory activity, and inhibitors of tryptase have been shown to modulate allergic reactions in vivo. Many of the tryptase inhibitors investigated previously are directed against the active site. In the present study we have investigated an alternative approach for tryptase regulation. We show that the heparin antagonists Polybrene and protamine are potent inhibitors of both human lung tryptase and of recombinant mouse tryptase (mouse mast cell protease 6). Protamine inhibited tryptase in a competitive manner whereas Polybrene showed noncompetitive inhibition kinetics. Treatment of tetrameric, active tryptase with Polybrene caused dissociation into monomers, accompanied by complete loss of enzymatic activity. The present report thus suggests that heparin antagonists potentially may be used in treatment of mast cell-mediated diseases such as asthma.

Mast cells are important effector cells in various inflammatory conditions. In particular, mast cells are implicated in allergic reactions. Cross-linking of IgE molecules bound to the high-affinity IgE receptor FCϵRI on the mast cell surface causes release of the secretory granules. These granules contain a variety of compounds, collectively termed mast cell mediators, which can both initiate and modulate an inflammatory response (*1*). In addition to histamine, the secretory granules contain various proteases as well as heparin proteoglycan and cytokines. The mast cell proteases are divided into chymases (serine proteases with chymotrypsin-like substrate specificities), tryptases (tetrameric serine proteases with trypsin-like substrate specificities), and carboxypeptidase A (*2*, *3*). Heparin proteoglycan consists of a protein core to which highly sulfated, negatively charged heparin glycosaminoglycan chains are attached (*4*). By virtue of its high negative charge, heparin proteoglycan binds to the various positively charged compounds within the granules, including tryptase, chymase, and histamine, thereby neutralizing their charge and facilitating their packaging. The role of heparin in the packaging of mast cell mediators was recently clearly demonstrated using a mouse strain with a defect in its heparin biosynthesis. These mice were essentially devoid of mast cell proteases and contained drastically reduced levels of histamine as compared with normal controls (*5*, *6*).

Heparin is important not only for the packaging of the mast cell proteases but also in the regulation of their activity. Several previous studies have shown that heparin is an essential stabilizing factor for mast cell tryptase (*7*−*9*). Further, in a recent study we showed that heparin is necessary for the formation of the active tryptase tetramers (*10*). In addition, heparin has been shown to regulate the activity of the other class of mast cell serine proteases, the chymases (*11*, *12*).

The role of the various mast cell proteases in mast cell-mediated disease is not fully understood. However, several recent investigations have implicated mast cell proteases, in particular the tryptases, as proinflammatory agents (*13*−*16*). The mechanism by which the tryptases act is not clear, although a plausible pathway for initiation of inflammation by tryptase involves cleavage of protease-activated receptor 2 (PAR-2), present on the surface of, e.g., nerve cells (*17*).

The identification of tryptase as a proinflammatory substance has resulted in the search for tryptase inhibitors and an assessment of whether tryptase inhibitors can modulate mast cell-mediated inflammatory reactions (*17*−*24*). One such tryptase inhibitor, APC-366, has gained a large pharmacological interest due to its effect on late-phase allergic reactions in sheep (*18*, *25*). APC-366 is an active site-directed competitive inhibitor. In this investigation we have explored another possible mode for tryptase inhibition. Considering the importance of heparin for maintaining tryptase activity, we investigated whether polycationic heparin antagonists can act as tryptase inhibitors. Our results show that both protamine and Polybrene, two polycationic compounds that are commonly used for neutralization of

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heparin therapy during surgery, are powerful tryptase inhibitors.

MATERIALS AND METHODS

Reagents. Polybrene (hexadimethrine bromide; 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was obtained from Janssen Chimica (Beerse, Belgium). Protamine (molecular mass ~4500), purified from salmon, was from Sigma. Human lactoferrin purified from milk was purchased from Calbiochem (La Jolla, CA). APC-366 [*N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride] was a kind gift from AstraZeneca AB (Lund, Sweden). *N*-*p*-Tosyl-Gly-Pro-Lys-pNA was purchased from Sigma. 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 from Chromogenix (Mölndal, Sweden). Pig mucosal heparin was a gift from Ulf Lindahl (Uppsala University, Sweden). Bovine α-thrombin was a gift from Ingemar Björk (Swedish University of Agricultural Sciences, Uppsala, Sweden). Rat chymase (RMCP-1) was purified from peritoneal mast cells as described previously (*26*). Human lung tryptase was from Calbiochem (San Diego, CA). Enterokinase was from Boehringer Mannheim (Mannheim, Germany).

Recombinant mouse tryptase, mouse mast cell protease 6 (mMCP-6),[1] was expressed in human 293 cells, purified, and activated as previously described (*10*). Briefly, the 293 cells were transfected with pCEP-Pu2 vector containing an insert coding for mMCP-6. The vector contained the BM40 signal peptide, ensuring secretion of the fusion protein into the culture medium. Further, the construction contained an N-terminal 6× His tag followed by an enterokinase site (EK; replacing the natural activation peptide of mMCP-6) and the mMCP-6 sequence. The 6×His-EK-mMCP-6 fusion protein secreted into the culture medium was purified on Ni−NTA−agarose (QIAGEN GmbH, Hilden, Germany) according to instructions from the manufacturer. Subsequently, 6×His-EK-mMCP-6 protein was digested with enterokinase at a 6×His-EK-mMCP-6:EK ratio of 500:1 (by mass) at 37 °C for ~20 h. Enterokinase cleavage was performed at pH 6.0 since mMCP-6 was recently shown to require acidic pH for activity (*10*). Enterokinase cleavage thus releases the mature mMCP-6 which, in the absence of heparin, is essentially without enzymatic activity (*10*). Active mMCP-6 was obtained by adding heparin at a 5−10-fold (by mass) excess over the tryptase.

Enzymatic Assays. Enzymatic assays were performed in 96-well microtiter plates. In standard incubations, 50 ng of mMCP-6 (4 nM) together with 250 ng of heparin was added to the wells in a total volume of 100 μL of PBS, pH 6.0. Human lung tryptase (25 ng/sample; 2 nM) was generally assayed without addition of heparin. However, the human tryptase preparation contains heparin as an additive at a tryptase:heparin ratio (by mass) of ~2.5. In some experiments, heparin was added at both higher and lower concentrations than during standard conditions. Inhibitors, in 10 μL of PBS (pH 6.0), were added followed by 40 min−4 h incubation at room temperature. Next, 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. Thrombin (25 ng/sample; 7 nM; final volume 100 μL of PBS, pH 7.4) was assayed with the chromogenic substrate S-2238, and mast cell chymase (25 ng/sample; 10 nM; final volume 100 μL of PBS, pH 7.4) was assayed with S-2586 (*27*). For K_m and k_{cat} determinations, substrate concentrations ranging from 0.09 to 3.6 mM were used. Although mMCP-6 generally was assayed with S-2288, the K_m and k_{cat} determinations for this enzyme were performed using *N*-*p*-tosyl-Gly-Pro-Lys-pNA. The reason for this was that high concentrations of S-2288 caused marked substrate inhibition of mMCP-6 (but not of human lung tryptase). High concentrations of *N*-*p*-tosyl-Gly-Pro-Lys-pNA did not cause substrate inhibition of mMCP-6. Absorbance changes were generally followed over 1 h, with absorbance readings performed every minute. Initial reaction velocities (obtained within 5 min) were used for calculations of enzymatic activity. The data obtained were used for calculations of kinetic parameters after nonlinear regression analysis.

Inhibition constants, K_i, and IC_{50} values were determined by incubating increasing concentrations of inhibitor, in at least 10-fold molar excess over the enzyme, with protease for 40 min−4 h (depending on the time needed to obtain full inhibitory potential). Residual enzyme activity was monitored as for standard assays, with a fixed substrate concentration (Figure 1). The ratio of inhibited over uninhibited rates of substrate hydrolysis was plotted versus the inhibitor concentration, and the apparent inhibition constants, $K_{i,app}$, were determined by nonlinear regression analysis of the data to the equation: $v_0/v_i = 1 + [I]/K_{i,app}$, where v_0 and v_i are the uninhibited and inhibited reaction rates, respectively (*29*). IC_{50} values were calculated in an analogous fashion where $K_{i,app}$ in the latter equation was substituted for IC_{50}. K_i values were obtained after correction for substrate competition according to $K_i = K_{i,app}/(1 + [S]/K_m)$ (*28*) by using the following K_m values: human tryptase, $K_m = 0.23$ mM (S-2288; see Table 3); thrombin, $K_m = 1.5$ μM [S-2238 (*29*)]; rat chymase, $K_m = 1.6$ mM [S-2586 (*27*)]. The molar concentration of Polybrene in the experiments is approximate, since this polycation is heterogeneous with molecular weights ranging from 5000−10 000. A mean molecular weight of 7500 was estimated for calculation of molar Polybrene concentrations. The determined IC_{50} value for Polybrene is therefore approximate.

To assess whether protamine is a substrate for tryptase, 10 μg of protamine was incubated (at room temperature) with 0.1 μg of either human lung tryptase or mMCP-6 in a total volume of 100 μL of PBS, pH 6.0. At various time points (up to 48 h), incubations were stopped by adding 50 μL of SDS−PAGE sample buffer. Samples were analyzed by electrophoresis on 4−17% Tris-Tricine polyacrylamide gels followed by staining with Coomassie.

Size Exclusion Gel Chromatography. Size exclusion gel chromatography was performed in a FPLC system (Amersham Pharmacia Biotech) using a Superdex 200 column (10 × 300 mm). The column was equilibrated with PBS (pH 6.0 or 7.4) and was run at a flow rate of 0.5 mL/min. The column was calibrated with the following gel filtration standards (Sigma): carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa),

[1] Abbreviations: PBS, phosphate-buffered saline; EK, enterokinase site; mMCP, mouse mast cell protease.

α-amylase (200 kDa), and blue dextran (2000 kDa; v_0 marker). Recombinant mMCP-6 (11 μg) was analyzed either alone or after preincubation (30 min) with 55 μg of heparin (in PBS, pH 6.0; total volume 200 μL). To study the effect of heparin antagonists on tetramer integrity, 220 μg of either Polybrene or protamine (in 114 μL of H_2O) was added to the mMCP-6/heparin complex followed by immediate analysis on the Superdex 200 column. Fractions (0.5 mL) were collected. Samples (200 μL) from each fraction were analyzed for tryptase activity after addition of 20 μL of S-2288 (2 mM in H_2O).

RESULTS

Inhibition of Tryptase by Heparin Antagonists. In this study we tested the effect of protamine and Polybrene, two known heparin antagonists, for their ability to function as tryptase inhibitors. In addition, we compared the potency of these heparin antagonists with those of APC-366 and lactoferrin, which are previously characterized tryptase inhibitors. As targets for inhibition, we used both human lung tryptase and recombinant mouse tryptase, mMCP-6.

Protamine is an Arg-rich polycationic protein involved in DNA binding in sperms (*30*). It is used clinically as a heparin antagonist to reverse the effect of injected heparin during, e.g., surgery (*31*). Protamine was a strong inhibitor of both human (Figure 1A) and mouse (Figure 1B) tryptase. Complete inhibition of mouse tryptase was achieved at ∼1 μM protamine whereas a small portion of the human tryptase preparation appeared to be resistant to inhibition. An IC_{50} value of 65 nM was determined for the inhibition of human tryptase by protamine (Table 1).

Polybrene is a nonprotein polycationic heparin antagonist (M_r 5000−10 000) that is used clinically in the reversal of heparin therapy as well as in gene transfer (*32*). Polybrene proved to be a very potent inhibitor of both human (Figure 1A) and mouse (Figure 1B) tryptase, with an IC_{50} value of ∼3.6 nM for human tryptase (Table 1).

Lactoferrin (*27*) and myeloperoxidase (*22*) have previously been reported to inhibit human tryptase (*24*). Apparently, inhibition of human tryptase by these proteins involved displacement of glycosaminoglycans bound to tryptase and dissociation of tryptase tetramers. However, in this study lactoferrin did not appear to be a potent inhibitor of either heparin-stabilized human lung tryptase (Figure 1A) or mMCP-6 (Figure 1B). Even at the highest concentration of lactoferrin tested (24 μM), only moderate inhibition of mMCP-6 was observed, and no inhibition of the human tryptase was observed at concentrations up to 11 μM. In fact, low concentrations of lactoferrin actually showed some stimulatory effect on tryptase activity (Figure 1).

APC-366 is a low molecular weight active site-directed, competitive tryptase inhibitor (*18*, *21*, *25*). The efficacy of this drug was compared with those of the heparin antagonists. From Figure 1A it is apparent that the heparin antagonists Polybrene and protamine are more potent inhibitors of human tryptase than APC-366. Curiously, APC-366 requires a long period of time to achieve its full inhibitory capacity on human tryptase. After 40 min incubation of APC-366 with human tryptase, only very weak inhibition is observed (Figure 1A and Table 1), with a K_i value as high as 250 μM (Table 2). However, after ∼4 h of incubation the inhibitory potential was increased drastically (Figure 1A), with a K_i value of 530 nM (Table 2). This effect has been observed previously, and the suggested explanation is that the hydroxynaphthoyl group in APC-366 is either slowly oxidized to a quinone or tautomerized to the keto form, which may lead to a closer

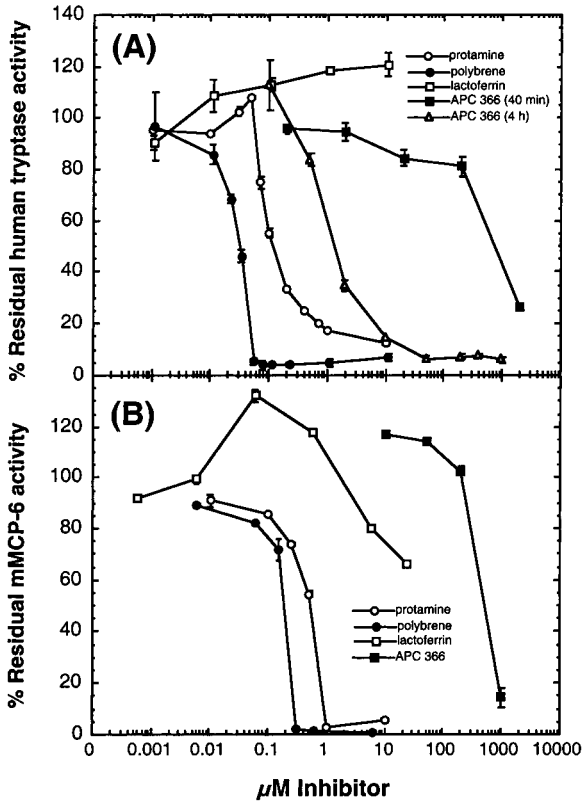

FIGURE 1: Inhibition of human lung tryptase and mMCP-6 by heparin antagonists. Human lung tryptase (A) or mMCP-6 (B) was incubated with the heparin antagonists protamine (○), Polybrene (●), or lactoferrin (□) as well as APC-366 (■, △) at the concentrations indicated. Human lung tryptase was incubated with protamine for 40 min, lactoferrin for 40 min, Polybrene for 3 h, and APC-366 for either 40 min (■) or 4 h (△). mMCP-6 was incubated with protamine (○), Polybrene (●), lactoferrin (□), and APC-366 (■) for 40 min. Subsequently, residual tryptase activities were determined after addition of the chromogenic substrate S-2288. The results shown represent the mean of triplicate determinations ± SD (error bars are frequently hidden by the symbols).

Table 1: IC_{50} Values for the Inhibition of Human Lung Tryptase[a]

inhibitor	IC_{50} (nM)
polybrene	∼3.6 ± 0.8[b]
protamine	65 ± 17
APC-366 (4 h incubation)	1400 ± 240

[a] Calculated values are expressed ± SD of the linear regression. [b] Since Polybrene is heterogeneous with regard to molecular weight, the calculated IC_{50} value is approximate (see Materials and Methods).

Table 2: K_i Values for the Inhibition of Various Proteases by APC-366[a]

protease	$K_{i,app}$ (μM)	K_i (μM)
human lung tryptase (40 min incubation)	640 ± 220	250
human lung tryptase (4 h incubation)	1.4 ± 0.24	0.53
bovine α-thrombin	3.3 ± 0.64	0.015
rat chymase (40 min incubation)	110 ± 8.6	100
rat chymase (4 h incubation)	103 ± 22	94

[a] Calculated values are expressed ± SD of the linear regression.

fit with the tryptase active site (*25*). In contrast, APC-366 was a very weak inhibitor of mouse tryptase after both 40 min incubation (Figure 1B) and 4 h of incubation (not shown). APC-366 inhibited bovine α-thrombin with a K_i value of 15 nM (Table 2). Unexpectedly, APC-366 also inhibited the mast cell chymase RMCP-1, although with a relatively high K_i value (110 μM; Table 2). The presence of APC-366 resulted in increased K_m of RMCP-1 for the chromogenic substrate S-2586 but no effect on the k_{cat} value, indicating competitive inhibition (not shown). In contrast to the human tryptase, the potency of inhibition of the chymase by APC-366 was similar at 40 min of incubation and after 4 h (Table 2).

Mechanism for Tryptase Inhibition by Heparin Antagonists. K_m and k_{cat} values of human and mouse tryptases were determined in the absence and presence of heparin antagonists. In the presence of protamine, the K_m values of both human lung tryptase and mMCP-6 for the chromogenic substrates used were markedly enhanced, without any obvious effect on the k_{cat}. Thus, the inhibition by protamine displays clear competitive kinetics. In contrast, treatment of both tryptases with Polybrene did not affect the K_m values but instead resulted in decreased k_{cat}, indicating noncompetitive kinetics (Table 3).

Heparin Dependence of Tryptase Inhibition. Inhibition of human tryptase and mMCP-6 by heparin antagonists was tested at various heparin concentrations. Human lung tryptase was routinely stabilized by the presence of heparin at a tryptase:heparin ratio (by mass) of 2.5. When increasing amounts of heparin were present, corresponding increased amounts of both Polybrene (Figure 2A) and protamine (Figure 2B) were required for inhibition of human tryptase. Similarly, increasing amounts of heparin present together with mMCP-6 resulted in higher amounts of heparin antagonist required for enzyme inhibition (Figure 2C). In contrast, tryptase inhibition by APC-366 did not appear to be sensitive to the amount of heparin present (Figure 2D).

Reversibility of Tryptase Inhibition. Experiments were conducted to investigate whether tryptase can be reactivated following inactivation by heparin antagonists. Human lung tryptase and mMCP-6 were incubated with either Polybrene or protamine. After 15 min, excess amounts of heparin were added, and after an additional 30 min incubation tryptase activity was measured. The inhibition of human lung tryptase by Polybrene appeared to be irreversible, since excess heparin failed to reactivate the enzyme. The inhibition of mMCP-6 by Polybrene appeared to be partly reversible. In contrast, addition of excess heparin to protamine-inactivated human lung tryptase or mMCP-6 resulted in reactivation to approximately the same level as for uninhibited tryptase (Figure 3). Thus, protamine appears to be a reversible inhibitor of both tryptases.

Since protamine is rich in Arg residues and tryptase (similar to all trypsin-like proteases) is known to cleave peptide bonds on the C-terminal side of Arg or Lys residues, the possibility that protamine also is a substrate for tryptase was investigated. However, incubation of protamine with either human lung tryptase or mMCP-6 (see Materials and Methods) did not result in degradation of protamine (results not shown).

Rate of Tryptase Inhibition. Residual tryptase activity after addition of heparin antagonists was monitored over a 3 h period (Figure 4). In the absence of inhibitor, human lung tryptase was fairly stable during the incubation time with

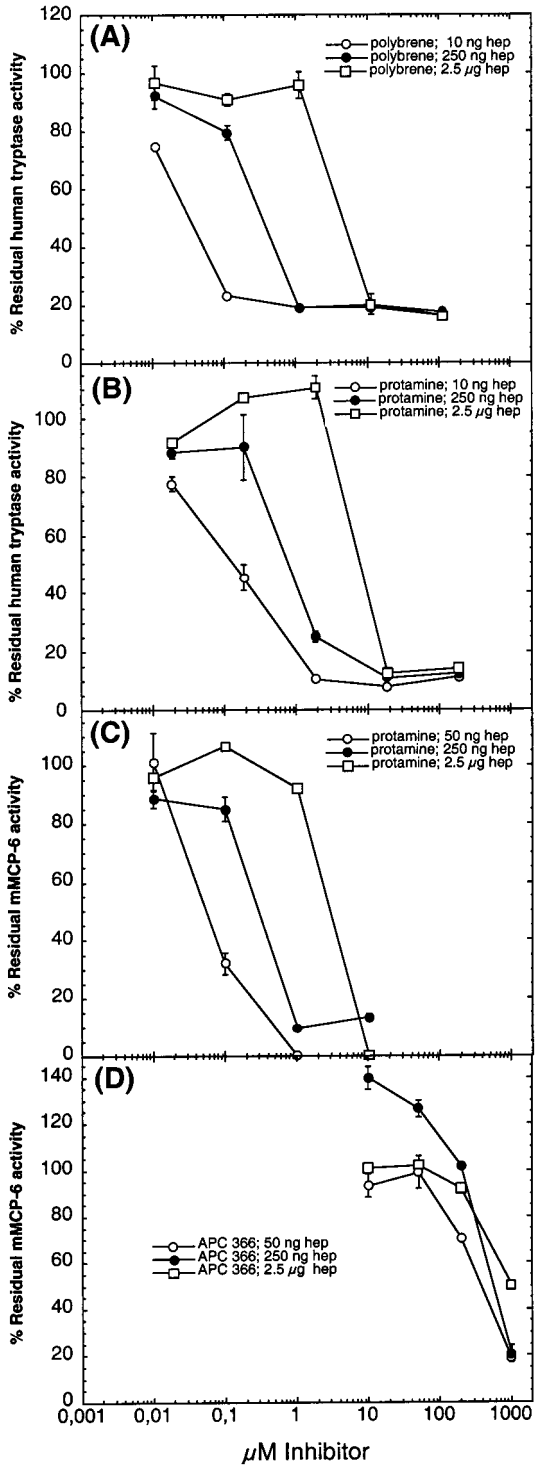

FIGURE 2: Heparin dependence of the inhibition of human lung tryptase and mMCP-6 by protamine and Polybrene. (A, B) Human lung tryptase was stabilized by the presence of either 10 ng (○), 250 ng (●), or 2.5 μg (□) of heparin (hep). Polybrene (A) or protamine (B) was added at the concentrations indicated. After 1 h, residual tryptase activity was determined with the chromogenic substrate S-2288. (C, D) mMCP-6 was stabilized by the presence of either 50 ng (○), 250 ng (●), or 2.5 μg (□) of heparin. Protamine (C) or APC-366 (B) was added at the concentrations indicated. After 1 h, residual tryptase activity was determined with the chromogenic substrate S-2288. The results shown represent the mean of triplicate determinations ± SD (error bars are frequently hidden by the symbols).

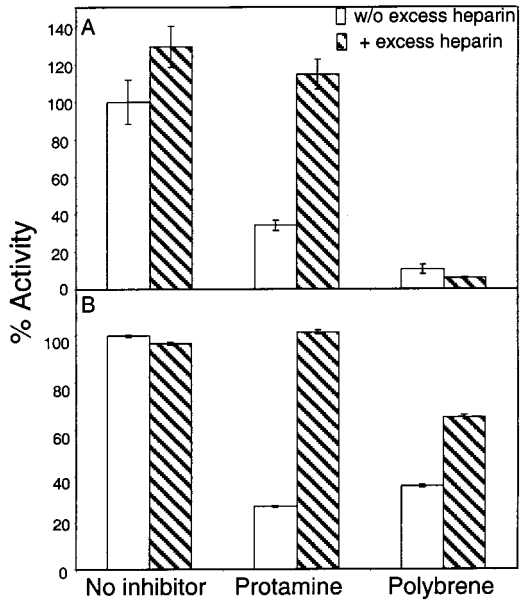

FIGURE 3: Reversibility of tryptase inhibition. (A) Human lung tryptase (25 ng) or (B) mMCP-6 (100 ng) in 100 μL of PBS (pH 6.0) was incubated alone or in the presence of either Polybrene (1 μg) or protamine (2 μg). After 15 min, excess heparin (50 μg in 5 μL of H_2O; hatched bars) or 5 μL of H_2O (open bars) was added, and after an additional 30 min of incubation, tryptase activity was assayed with S-2288. Calculated values are expressed ± SD (n = 3).

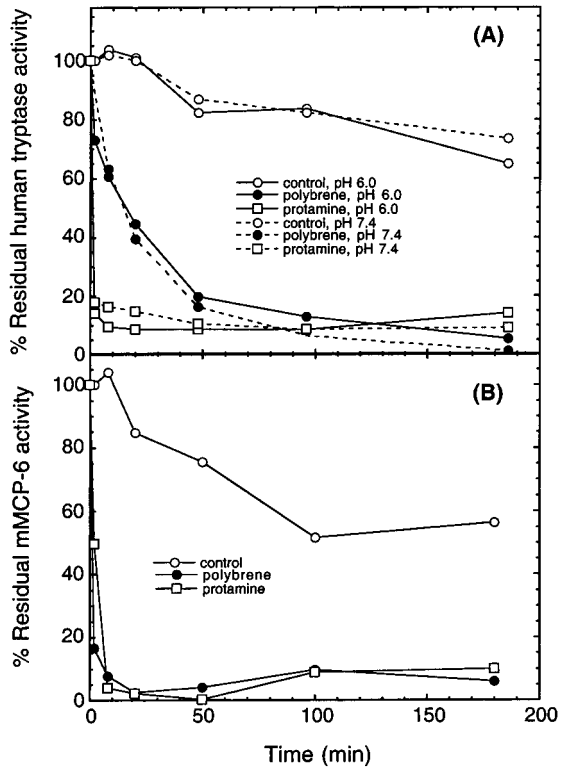

FIGURE 4: Velocity of tryptase inhibition by heparin antagonists. Human lung tryptase (25 ng; A) or mMCP-6 (50 ng; B) was incubated either alone (○) or in the presence of 10 μg of protamine (□) or 10 μg of Polybrene (●). Incubation of human tryptase was carried out either at pH 7.4 (dashed lines) or at pH 6.0 (solid lines). After the various times indicated, residual tryptase activity was determined with the chromogenic substrate S-2288.

only a moderate (~30%) loss of activity toward S-2288 after 3 h of incubation at either pH 6.0 or pH 7.4 (Figure 4A). Protamine caused a rapid inactivation, with essentially

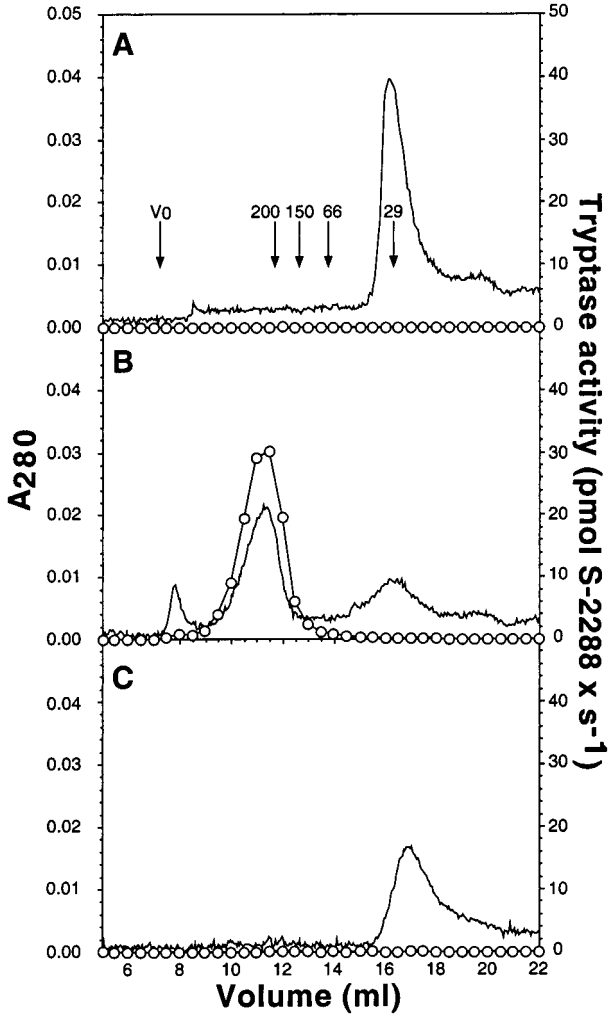

FIGURE 5: Effect of heparin antagonists on tryptase tetramers. Recombinant mMCP-6 (11 μg in 200 μL of PBS, pH 6.0) was analyzed on a Superdex 200 column eluted with PBS, pH 6.0. Tryptase samples were analyzed without addition of heparin (A) or after preincubation (30 min) with 55 μg of heparin (B). In addition, mMCP-6 samples were analyzed after 30 min preincubation with heparin followed by addition of 220 μg of Polybrene (C). The sample was injected immediately after the addition of Polybrene. Absorbance at 280 nm (—) was recorded. Fractions (0.5 mL) were assayed for mMCP-6 activity with the chromogenic substrate S-2288 (○). Arrows indicate the elution positions of various molecular weight markers (see Materials and Methods).

complete effect reached within 1 min. In contrast, the inhibition of human tryptase by Polybrene was much slower, with complete effect reached after ~3 h of incubation. Mouse tryptase was inhibited very rapidly by both protamine and Polybrene, with essentially complete inhibition achieved within 1 min (Figure 4B).

Effect of Heparin Antagonists on Tryptase Tetramers. In the absence of heparin, recombinant mMCP-6 was present in monomeric, inactive form as shown by FPLC analysis on a Superdex 200 column (Figure 5A). After addition of heparin, a major peak of ~200 kDa was observed, consistent with tetramer formation (Figure 5B). The ~200 kDa material showed enzymatic activity toward S-2288. After addition of Polybrene (Figure 5C) or protamine (results not shown), the tetramer peak was abolished, accompanied by formation of enzymatically inactive mMCP-6 monomers. Active human lung tryptase was eluted from the Superdex 200 column at a position similar to that of the mMCP-6 tetramer. Treatment

of human lung tryptase with either Polybrene or protamine abolished the tetramer peak and resulted in formation of components eluting at a position similar to that of the mMCP-6 monomers (results not shown).

DISCUSSION

Tryptase inhibitors are currently attracting an increased interest as pharmacological agents in the treatment of allergic disease. The background is that several studies have implicated tryptase as a potent inflammatory stimulus. Mouse tryptase mMCP-6 (*3*) as well as human tryptase (*13*) has been found to induce neutrophil recruitment in the peritoneum of mice. Further, injection of tryptase into the skin of sheep (*33*) and guinea pigs (*13, 14*) results in the recruitment of inflammatory cells and in microvascular leakage. Studies in a sheep model have indicated that inhalation of tryptase causes bronchoconstriction via a histamine-dependent pathway (*34*), and tryptase has been demonstrated to mediate hyperresponsiveness in isolated guinea pig bronchi (*35*). Moreover, it has been shown that tryptase inhibitors such as APC-366 (*18*) and AMG-126737 (*19*) can reduce both acute and late-phase airway responses in allergic sheep, thus further suggesting a role for tryptase in allergic conditions.

APC-366 is a relatively selective tryptase inhibitor, although inhibition of a variety of other trypsin-like serine proteases, e.g., thrombin, has been observed (*18*). In addition, we show here that APC-366 may also be an inhibitor of chymotrypsin-like serine proteases, e.g., mast cell chymase. Thus, the reported in vivo effects of APC-366 may be related not only to tryptase but also to inhibition of various other proteases. The inhibition of a chymotrypsin-like enzyme by APC-366 may seem surprising, considering that APC-366 is thought to bind to trypsin-like proteases through interaction of its arginyl side chain with the S(1) pocket Asp^{189}, whereas chymotrypsin-like proteases generally show a preference for cleaving peptide chains on the C-terminal side of bulky, aromatic side chains. However, APC-366 contains an aromatic 1-hydroxy-2-naphthoyl group that possibly may fit into the active site of chymase.

APC-366 is an active site-directed inhibitor. Here we have investigated an alternative route for tryptase inhibition. Since tryptases are highly dependent on heparin for activity, we reasoned that heparin antagonists may be inhibitory for tryptase. As heparin antagonists we used protamine, an arginine-rich polycationic DNA binding protein, and Polybrene (hexadimethrine bromide), a nonprotein polycation. Our results indicate that both Polybrene and protamine were highly efficient tryptase inhibitors. Comparison of these heparin antagonists with APC-366 showed that both Polybrene and protamine were much more effective than APC-366 as tryptase inhibitors. For example, Polybrene and protamine inhibited human tryptase with IC_{50} values that were ~20−400-fold lower than that for APC-366. APC-366 appears to be a very slow tryptase inhibitor, with full inhibitory potential reached only after several hours of incubation with tryptase. After 40 min of incubation, the K_i value is in the millimolar range for both mouse and human tryptase (Table 2) whereas an inhibition constant of 530 nM was observed after 4 h of incubation with human tryptase. In contrast, the inhibition of mouse tryptase was not potentiated after a prolonged period of incubation. The reason for this difference in susceptibility to inhibition by APC-366 between human and mouse tryptase is not known but may reflect subtle structural differences in the active sites of these proteases. Inhibition of tryptase by the heparin antagonists Polybrene and protamine was more rapid than the inhibition by APC-366. Inhibition of both human and mouse tryptase by protamine and inhibition of mouse tryptase by Polybrene were essentially completed within 1 min, whereas the inhibition of human tryptase by Polybrene required a prolonged incubation time to reach full effect (Figure 4).

Table 3: Kinetic Constants of Human Lung Tryptase and mMCP-6 for Chromogenic Substrates in the Absence and Presence of Heparin Antagonists[a]

	K_m (mM)	k_{cat} (s^{-1})
human lung tryptase		
without inhibitor	0.46 ± 0.038	14 ± 0.40
+100 ng of protamine	1.7 ± 0.038	16 ± 2.0
+200 ng of protamine	8.2 ± 0.069	36 ± 2.3
+400 ng of protamine	7.4 ± 0.11	40 ± 5.0
+800 ng of protamine	6.7 ± 0.034	26 ± 10
+60 ng of Polybrene	0.40 ± 0.029	17 ± 0.45
+80 ng of Polybrene	0.43 ± 0.018	9.9 ± 0.16
+100 ng of Polybrene	0.44 ± 0.051	8.0 ± 0.34
+150 ng of Polybrene	0.40 ± 0.020	5.0 ± 0.09
mMCP-6		
without inhibitor	0.27 ± 0.046	4.3 ± 0.27
+1 μg of protamine	0.41 ± 0.018	5.8 ± 0.1
+1.5 μg of protamine	0.53 ± 0.091	5.1 ± 0.42
+2 μg of protamine	0.64 ± 0.149	3.9 ± 0.48
+600 ng of Polybrene	0.20 ± 0.032	3.6 ± 0.16
+800 ng of Polybrene	0.21 ± 0.043	2.6 ± 0.17
+1000 ng of Polybrene	0.21 ± 0.034	1.5 ± 0.08
+1200 ng of Polybrene	0.19 ± 0.023	0.74 ± 0.03

[a] Human lung tryptase (25 ng) was assayed with S-2288 whereas mMCP-6 (100 ng) was assayed with *N*-*p*-tosyl-Gly-Pro-Lys-pNA (see Materials and Methods). Calculated values are expressed ± SD of the linear regression.

The exact mechanism by which the investigated heparin antagonists inhibit the tryptases is not entirely clear. The most likely mechanism would be that these polycationic compounds compete with tryptase for binding to heparin. After dissociation of tryptase from heparin the tryptase tetramer may be destabilized, dissociate into monomers, and lose its enzymatic activity (*8*, *9*). The finding that the heparin antagonists caused monomerization of tryptase (Figure 5) thus clearly supports such a mechanism. This type of mechanism would most probably be reflected by noncompetitive inhibition kinetics. Indeed, the inhibition of tryptase by Polybrene is clearly noncompetitive (Table 3). In contrast, the inhibition of tryptase by protamine showed, somewhat unexpectedly, clear competitive kinetics (Table 3). Tryptases, like all trypsin-like serine proteases, show substrate specificities with preference for peptides with Arg or Lys at the P1 position. Since protamine is rich in Arg residues, it is possible that some of the Arg side chains may interact with the active site of tryptase, thereby resulting in competitive inhibition.

The noncompetitive model of inhibition, with the heparin antagonists competing with tryptase for binding to heparin, implies that the potency of inhibition would be reduced at higher heparin concentrations. Indeed, when increasing amounts of heparin were present together with the tryptases, a corresponding increase in the amount of heparin antagonists

needed for inhibition of both human and mouse tryptase was observed (Figure 2). It is noted that also the inhibition of tryptase by protamine, which showed clear competitive kinetics (Table 3), was sensitive to the heparin concentration (Figure 2). A possible explanation for this observation would be that protamine is able to bind to free heparin but is relatively unable to compete with tryptase for binding to the tryptase-associated heparin. Thus, at low heparin concentrations, most of the heparin would be bound to tryptase and thereby not accessible to protamine which instead may be free to interact with the active site. At higher heparin concentrations, the excess heparin that is not occupied in binding to tryptase will bind to protamine, thereby preventing this heparin-associated protamine from interacting with the active site. When sufficient amounts of protamine are added, all of the excess heparin will be associated with protamine, and the remaining free protamine may thus interact with the active site.

An earlier study has indicated that lactoferrin, a heparin binding protein (*27*), reduces late-phase allergic reactions in sheep (*24*). Since lactoferrin proved to be an inhibitor of tryptase in vitro, it was suggested that tryptase may also be the target for lactoferrin when administered in vivo. However, it should be noted that lactoferrin is also an inhibitor of heparin-dependent chymase activity (*27*). In our hands, lactoferrin is only moderately inhibitory for heparin-activated mouse tryptase mMCP-6 and does not inhibit heparin-stabilized human tryptase. Control experiments showed that the same lactoferrin preparation showed similar inhibition of the heparin-dependent activities of mast cell chymase as reported previously (*27*), thus ruling out that the lactoferrin preparation was defective (results not shown).

The results presented here may encourage the assessment of whether heparin antagonists may function as modulators of allergic reactions. Both protamine and Polybrene proved to be highly efficient tryptase inhibitors. Further, the heparin antagonists inhibited tryptase very rapidly, with the exception of the inhibition of human tryptase by Polybrene (Figure 4). The heparin antagonists may therefore be suitable candidates for treatment of mast cell-mediated disease, e.g., asthma. However, it should be noted that, in rare cases, protamine has been shown to give rise to adverse reactions involving generation of anti-protamine IgE antibodies and associated allergic reactions (*36*, *37*). These observations may thus question the relevance of using protamine in treatment of allergic reactions. However, it is important to note that the observed side effects of protamine have been obtained after injection of the compound into the circulation during reversal of heparin therapy. For treatment of, e.g., asthma, the compound would be inhaled into the lung, and it is not certain that protamine will cause side effects under such conditions. In contrast, fewer side effects have been reported for Polybrene (*38*), which therefore may be a more suitable compound for assessment for antiinflammatory properties in vivo. Further, it is possible that other polycationic compounds may be developed in the future that can be used as tryptase-inhibiting and antiinflammatory agents.

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