Mast cell chymase in complex with heparin proteoglycan is regulated by protamine

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Abstract Protamines are polycationic proteins that are widely used for neutralisation of the anticoagulant action of heparin. However, several reports have shown adverse, mast cell-dependent reactions to protamine. The exact mechanism by which protamine causes these adverse effects is not clear. In the present study, the possibility that protamine may influence mast cell chymase function was investigated. Mast cell chymase is in vivo recovered in a macromolecular complex with heparin proteoglycan, and this interaction is essential for expression of optimal enzymatic activity. Protamine was shown to strongly reduce the activity of mast cell chymase by a mechanism that involved displacement of the chymase from heparin proteoglycan.

Key words: Protamine; Mast cell; Chymase; Rat mast cell protease 1; Heparin proteoglycan

1. Introduction

Protamines are small polycationic proteins that have widespread clinical use in the reversal of heparin anticoagulation. In addition, protamines are often added to insulin preparations in order to improve the pharmacological properties of the hormone. A number of reports have shown that protamine can cause adverse reactions. These range from mild reactions such as rash and urticaria to more severe conditions including anaphylaxis, bronchospasm and cardiovascular collapse [1]. The mechanism(s) by which protamine causes these adverse reactions is not clear. Several reports suggest that elevated serum levels of IgE or IgG to protamine increase the risk of obtaining adverse reactions to the protein [2,3]. Other reports have shown that protamine can interact directly with mast cells (MC), causing non-immunological histamine release [4,5]. In addition, inhibition of plasma carboxypeptidase N [6] and activation of the complement system [7,8] by protamine have been reported.

The importance of MC in various hypersensitivity reactions is well established [9]. When MC are activated they release different inflammatory mediators such as histamine, cytokines, heparin proteoglycan (PG), carboxypeptidase and serine proteases [10,11]. The serine proteases are either tryptases (trypsin-like substrate specificities) or chymases (chymotrypsin-like substrate specificities). Although the exact physiological function(s) of the MC chymases is not known, different identified substrates for these enzymes [12–15] indicate the potential involvement in a variety of processes related to the inflammatory response. The chymases are in vivo strongly bound to heparin PG [16–19] and the association with heparin is necessary for the chymases to express optimal activity towards various substrates [13–15,20]. Since protamine is known to bind strongly to heparin I hypothesised that protamine, by interfering with the binding of chymase to heparin PG, could influence the activity of the protease. Indeed, in the present report it is shown that protamine strongly reduces the activity of MC chymase. Possibly, this observation can be related to the various adverse reactions caused by protamine.

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Abbreviations: MC, mast cell; PBS, phosphate-buffered saline; PG, proteoglycan; RMCP-, rat mast cell protease.

2. Materials and methods

Rat mast cell protease 1 (RMCP-1) (EC 3.4.21.39) and heparin PG were purified from rat peritoneal MC by a combination of anion exchange chromatography on DEAE-Sephacel and HPLC on a Superdex 75 column as described [18]. Heparin from pig intestinal mucosa was a gift from Ulf Lindahl, Uppsala University, Dept. of Medical and Physiological Chemistry, Uppsala, Sweden. The chromogenic peptide substrates S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) and S-2238 (H-D-Phe-Pip-Arg-pNA) were from Chromogenix (Mölndal, Sweden). Protamine isolated from Salmon was purchased from Sigma. Bovine α-thrombin was a gift from I. Björk, Dept. of Veterinary Medical Chemistry, Uppsala, Sweden. DEAE-cellulose (DEAE-Sephacel) was purchased from Pharmacia (Uppsala, Sweden).

2.1. Enzymatic assays

RMCP-1 activities were measured in 96-well microtiter plates. RMCP-1 (25–50 ng) was diluted with phosphate-buffered saline (PBS)/0.1% Triton X-100 to a final volume of 200 μl. In standard conditions, enzyme activity was detected after addition of 20 μl of a solution (4 mM in H_2O) of the chromogenic chymotrypsin substrate S-2586, and monitoring of the absorbance at 405 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories). Alternatively, when thrombin was used as substrate for RMCP-1, 10 ng of thrombin was added to wells containing RMCP-1, followed by an additional 15 min incubation time and monitoring of residual thrombin activity after addition of 20 μl of a solution (2.5 mM in H_2O) of the chromogenic thrombin substrate S-2238. In experiments where the effect of various heparins on RMCP-1 activity was studied, the polysaccharides were added 15 min prior to addition of the chymase substrate. In standard incubations a 2.5:1 ratio (w/w) of RMCP-1/heparin PG was used. This protein/PG ratio was chosen to give optimal potentiation of RMCP-1 activity (see inset in Fig. 2A). The effect of protamine on chymase activity was determined after the addition of increasing amounts of protamine to RMCP-1/heparin mixtures or to free RMCP-1, followed by an additional incubation time of 15 min and monitoring of residual enzymatic activities as above. The K_m and k_{cat} values for RMCP-1-catalysed hydrolysis of S-2586 were determined from initial rate measurements, followed by non-linear regression analysis.

Peritoneal MC were purified and cultured as described [18,21]. In experiments where the thrombin-inactivating activities of intact MC were determined, 1×10^4 purified MC (in 10 μl) were incubated in 400 μl of cell culture medium. Thrombin (100 ng) was added and samples (20 μl) from the media were collected at 10, 20, 30, 60 and 90 min. The samples were mixed with 200 μl of PBS and were analysed for residual thrombin activities. The effect of protamine on thrombin inactivation was studied after preincubating the cells for 15 min

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with protamine before addition of thrombin. The S-2586-cleaving activities of intact MC were determined after mixing of 1×10^4 MC (in 10 μl) with 100 μl PBS in 96-well microtiter plates. 20 μl of an S-2586 solution (4 mM in H_2O) was added followed by monitoring of the absorbance at 405 nm. The effect of protamine was determined after preincubation (15 min) of the MC with the compound followed by the addition of S-2586. Cellular extracts were obtained after solubilisation of 1×10^6 MC with 5 ml of PBS/0.5% Triton X-100.

2.'. DEAE-cellulose precipitation assay

50 ng of RMCP-1 was incubated in the absence or presence of 20 ng of heparin PG, and various amounts of protamine. Incubations were performed in 1.5 ml Eppendorf tubes in a volume of 600 μl of PBS/0.1% Triton X-100. After 20 min, 400 μl of a 1:1 mixture of DEAE-Sephacel and PBS/0.1% Triton X-100, or 400 μl of PBS/0.% Triton X-100 was added followed by gentle mixing of the tubes (end over end) for 30 min. The gels were allowed to settle and 200 μl of the resulting supernatants were transferred to 96-well plates, followed by determination of residual RMCP-1 activity using the chromogenic substrate S-2586 (see above). In some experiments 1 μg of heparin PG had been added to the wells before transferring of the supernatants.

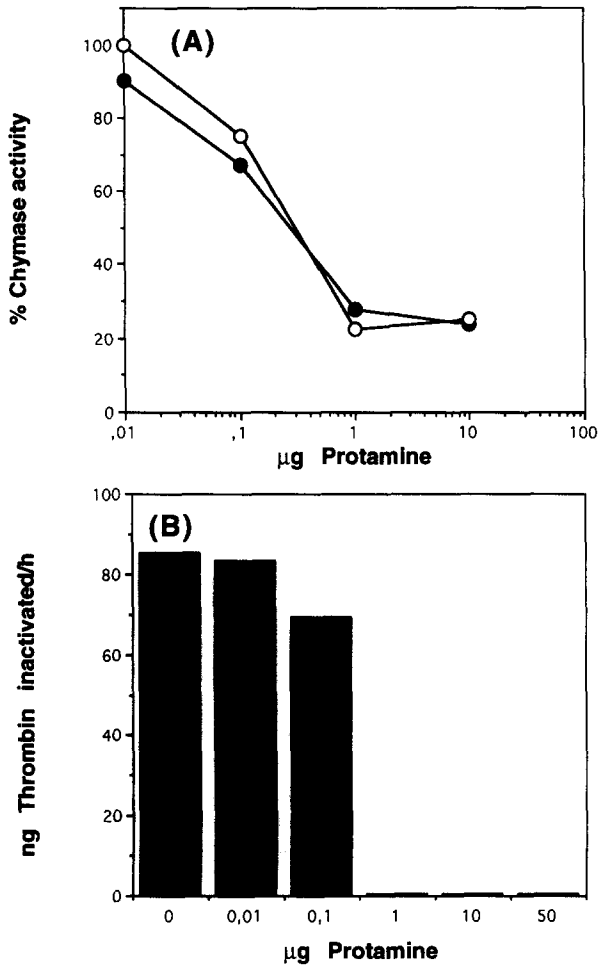

Fig. 1. Regulation of chymase activity expressed by intact MC and MC extracts. (A) Intact MC (1×10^4; ●) or MC extracts (5 μl, corresponding to 1×10^3 cells; ○) were preincubated with the amounts of protamine indicated. After 15 min, residual RMCP-1 activities towards S-2586 were determined. Results are expressed as % of the activity compared with chymase activity in the absence of protamine. (B) Protamine at the amounts indicated was added to 1×10^4 MC. After 15 min, 100 ng of thrombin was added followed by monitoring of thrombin inactivation. No detectable inactivation of thrombin was observed in the absence of MC.

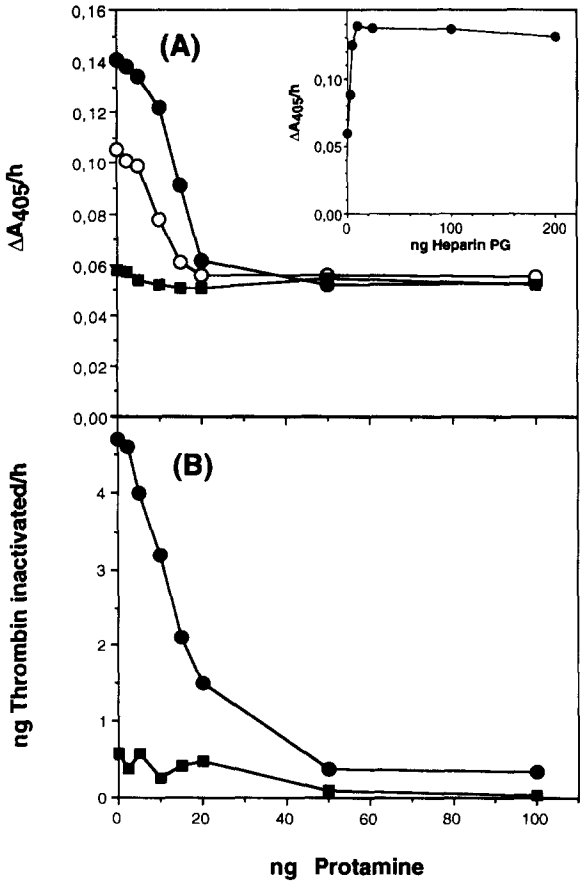

Fig. 2. Regulation of RMCP-1 by protamine. RMCP-1 (25 ng) was incubated in the presence of 10 ng of heparin PG (●), 10 ng of pig mucosal heparin (○) or without heparin (■). After 15 min, protamine at the amounts indicated was added. (A) After an additional 15 min, residual RMCP-1 activities towards S-2586 were measured. (B) After 15 min, 10 ng of thrombin was added followed by the determination of thrombin-inactivating activity. The inset in (A) shows the potentiation of RMCP-1 (25 ng) by heparin PG.

3. Results

We have previously shown that purified rat peritoneal MC express chymase activity that is bound to the cell surface, and that the surface-associated chymase is a potent inactivator of thrombin [18]. The inactivation of thrombin involves degradation of the coagulation enzyme [22]. Experiments were performed to investigate if protamine could influence the chymase activity expressed on intact MC, using both the chromogenic peptide substrate S-2586 and thrombin as substrates for the chymase. The results showed that protamine decreased chymase activity towards both S-2586 (Fig. 1A) and thrombin (Fig. 1B). In addition, protamine decreased chymase activity present in cellular extracts (Fig. 1A). When thrombin was used as substrate, 100% inhibition of chymase activity was readily achieved whereas ~25% of the activity towards S-2586 (both on intact MC and in MC extracts) was resistant to regulation by protamine. Maximal reduction of chymase activity was obtained at 1 μg of added protamine, both when thrombin and S-2586 were employed as substrates for the chymase.

RMCP-1 is the major chymase expressed by rat peritoneal MC [23], and experiments were performed to determine whether protamine could regulate the activity of purified

RMCP-1. *In vivo*, RMCP-1 is recovered in a tight macromolecular complex with heparin PG and we have previously shown that heparin strongly potentiates the activity of the enzyme [18,20]. It is thus appropriate to use the RMCP-1/heparin PG complexes, rather than the free chymase, as targets for regulation by protamine. The amount of heparin PG added to RMCP-1 was chosen to give optimal stimulation of enzyme activity (see inset in Fig. 2A). Protamine did not show any significant inhibition of S-2586 hydrolysis by free RMCP-1, whereas the activity of RMCP-1 present in complex with either heparin PG or pig mucosal heparin ('commercial heparin') was reduced by protamine, both using thrombin and S-2586 as RMCP-1 substrates (Fig. 2). Again, protamine gave essentially complete inhibition of the thrombin-inactivating activity of RMCP-1 whereas a portion (~37%) of the activity against S-2586 was resistant to regulation by protamine. The level of RMCP-1 activity towards S-2586 that was resistant to regulation was similar to the amount of activity expressed by the free chymase, indicating that protamine acts by displacing RMCP-1 from heparin PG. When increased amounts of heparin PG was added to RMCP-1 the effect of protamine was partially blocked, with greater amounts of protamine being required to obtain reduction of the chymase activity (Fig. 3). The time coarse for the regulation showed that the process was very rapid, with maximal decrease in activity being achieved within 1 min (Fig. 4).

To obtain direct evidence for displacement of RMCP-1 from heparin PG by protamine, an assay involving precipitation of RMCP-1/heparin PG complexes by an anion exchange resin, DEAE-cellulose, was developed. RMCP-1 was incubated either alone, together with heparin PG or in the presence of both heparin PG and protamine. DEAE-cellulose was added to the incubation mixtures followed by the determination of residual RMCP-1 activities in the resulting supernatants. In the absence of heparin PG no significant precipitation of RMCP-1 activity by DEAE-cellulose was observed (Fig. 5). When heparin PG was added to RMCP-1, as expected (see inset in Fig. 2A), a ~3-fold increase in enzymatic

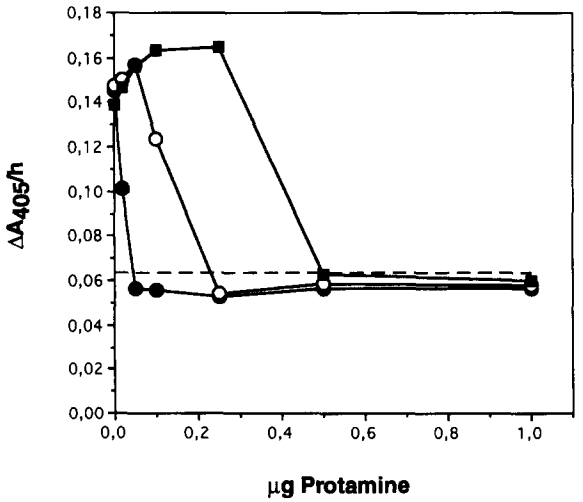

Fig. 3. Effect of heparin PG on the regulation of RMCP-1 by protamine. RMCP-1 (25 ng) was incubated in the presence of 10 ng (●), 50 ng (○) or 200 ng (■) of heparin PG. After 15 min, protamine at the concentrations indicated was added and after an additional incubation time of 15 min, residual RMCP-1 activities towards S-2586 were measured. The broken line represents RMCP-1 activity in the absence of both heparin PG and protamine.

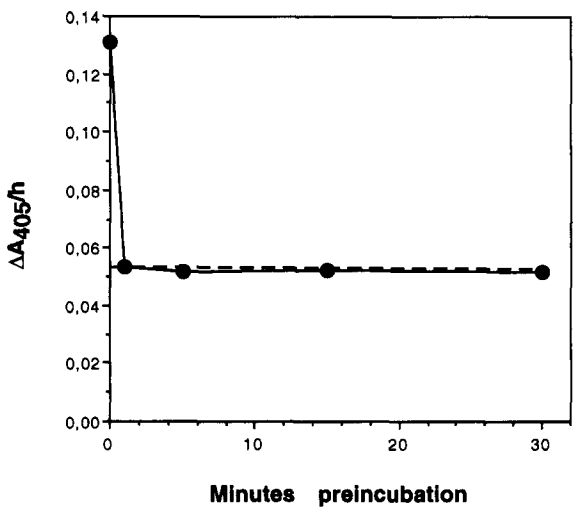

Fig. 4. Time course for the regulation of RMCP-1 by protamine. RMCP-1 (25 ng) was incubated together with 10 ng of heparin PG. After 15 min, protamine (50 ng) was added and after the times indicated residual RMCP-1 activity towards S-2586 was determined. The broken line represents RMCP-1 activity in the absence of both heparin PG and protamine.

activity was observed, and the majority of this activity was precipitated following the addition of DEAE-cellulose. These results thus indicate that heparin PG is bound to the DEAE-cellulose and that the observed reduction of RMCP-1 activity in the supernatants is due to binding of the chymase to the DEAE-cellulose-associated heparin PG. Addition of protamine to the RMCP-1/heparin PG complexes resulted in increased recovery of RMCP-1 activity in the supernatants obtained after DEAE-cellulose precipitation, indicating displacement of RMCP-1 from the PG (Fig. 5). The maximal amount of RMCP-1 activity that could be recovered in the supernatants after addition of protamine corresponded to the level of activity displayed by the free chymase. If the chymase activity obtained in the supernatants after addition of protamine is due to free RMCP-1, it should be possible to increase its activity by addition of heparin PG. Addition of excess amounts of heparin PG to the RMCP-1 recovered after displacement by protamine resulted in ~2.7-fold enhancement of chymase activity, strongly indicating that the recovered RMCP-1 was indeed present in a free form (Fig. 5). In contrast, RMCP-1 that had been previously incubated with heparin PG in the absence of protamine was, as expected (see inset in Fig. 2A), not stimulated further by addition of excess amounts of heparin PG (Fig. 5).

The K_m and k_{cat} values of RMCP-1 for S-2586 were determined for the free chymase, chymase in complex with heparin PG and for RMCP-1/heparin PG complexes in the presence of protamine (Table 1). Heparin PG induced a ~5-fold decrease in the K_m of RMCP-1 for S-2586, whereas no significant effect on the k_{cat} value was observed. When protamine was added to the RMCP-1/heparin PG complexes the K_m value was increased back to approximately the same level as that displayed by the free chymase, without any significant effect on the k_{cat} value.

4. Discussion

The interaction of MC chymases with heparin PG influ-

Table 1
Kinetic constants of RMCP-1 for S-2586 in the presence of heparin PG and protamine[a]

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
w/o heparin PG	1.6 ± 0.2	9.5 ± 0.4	5.9×10^3
+10 ng heparin PG	0.31 ± 0.03	7.8 ± 0.2	25×10^3
+10 ng heparin PG, +50 ng protamine	2.0 ± 0.2	10.4 ± 0.5	5.2×10^3

[a]25 ng of RMCP-1. For further experimental details see section 2.

ences the proteases in several ways. Firstly, heparin potentiates the activity of the enzymes by a mechanism that appears to involve a reduction of the K_m of the proteases for their substrates [20]. Secondly, heparin PG-bound chymase tends to remain associated with the MC surface after cellular degranulation [17,18]. Thirdly, we have recently shown that RMCP-1 bound to heparin PG is largely resistant to inhibition by a variety of plasma protease inhibitors such as α_1-antitrypsin, α-antichymotrypsin and α_2-macroglobulin [20]. Since RMCP-1 associated with heparin PG appears to be resistant to inhibition by conventional protease inhibitors I hypothesised that heparin antagonists, by interfering with the binding of chymase to PG, could act as regulators of the MC proteases. Indeed, in the present study it is shown, to my knowledge for the first time, that protamine, a heparin antagonist, strongly decreases the activity of MC chymase present in complex with heparin PG. The mechanism of regulation is shown to involve displacement of the chymase from its physiological ligand, heparin PG. The physiological inhibitors of MC chymases have not yet been identified. However, considering the resistance of these enzymes to inhibition by conventional protease inhibitors, a regulation pathway involving heparin antagonists such as protamine-like proteins, vitronectin [24] or lactoferrin (G. Pejler, unpublished observation) may be of physiological significance.

It is important to emphasise that the dissociation of RMCP-1 from heparin PG caused by protamine does not result in destruction of the active site of the chymase. Although free RMCP-1 is essentially completely devoid of activity when thrombin is used as substrate, it still expresses significant activity towards S-2586. In addition, reconstitution of the chymase with optimal amounts of heparin PG results in an increase in activity back to the same level as that of the original RMCP-1/heparin PG complex. The fate of the free chymases released after dissociation from the PG is uncertain. Clearly, the dissociation process would facilitate diffusion of the chymases away from the MC surface and allow the chymases to exert their action (although being less active) at other sites in the body. Alternatively, released chymases may be rapidly neutralised by various protease inhibitors.

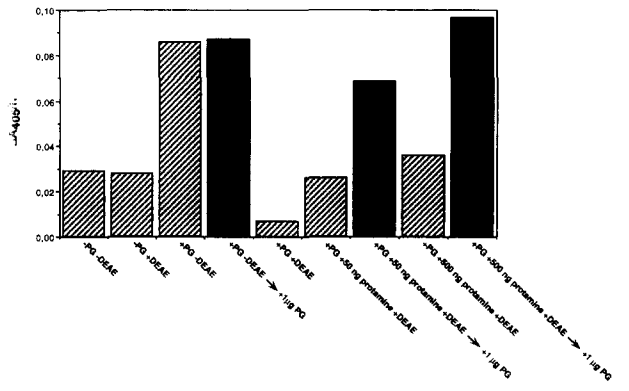

Fig. 5. Precipitation of RMCP-1/heparin PG with DEAE-cellulose. RMCP-1 (50 ng) was incubated alone (−PG) or together with 20 ng of heparin PG (+PG) and protamine as indicated. After 20 min, 400 µl of a 1:1 mixture of DEAE-Sephacel and PBS/0.1% Triton X-100 (+DEAE), or 400 µl of PBS/0.1% Triton (−DEAE) was added, followed by the determination of residual RMCP-1 activity present in the resulting supernatants (hatched bars). 1 µg of heparin PG was added as indicated (→+1 µg PG; closed bars) to RMCP-1 that had been previously incubated with 20 ng of heparin PG, or to supernatants from tubes where RMCP-1 had first been incubated together with 20 ng heparin PG and protamine followed by precipitation with DEAE-Sephacel.

Due to the evolution of new techniques in surgery the use of protamine in the reversal of heparin anticoagulation has increased over the last decade. Along with the increased use of protamine, increased numbers of adverse reactions associated with i.v. protamine administration have been reported. In order to improve the strategy for protamine treatment it is necessary to understand the mechanisms by which protamine causes these adverse reactions. It is well established that IgE and IgG to protamine constitute major risk factors for developing adverse reactions to protamine [1]. However, the identification of several apparently immunoglobulin-independent effects of protamine [4–6,8] indicates that the mechanism of action may be complex. Since protamine is an established heparin antagonist in vivo it is plausible that the compound will interact with endogenous chymase/heparin PG complexes, thereby decreasing the heparin-dependent activities displayed by the proteases. When predicting the potential in vivo effects of chymase regulation by protamine it is therefore necessary to consider those activities of MC chymases that are influenced by heparin. For instance, the effect of protamine on the capacity of RMCP-1 to inactivate thrombin may indeed be relevant in relation to the regulation of the inflammatory response. Extravascular activation of the coagulation cascade, accompanied by thrombin formation, is a common feature of various inflammatory reactions, including immediate hypersensitivity [25]. Thrombin has, in addition to its procoagulant activities, a number of properties of potential importance in inflammation, e.g. chemotactic activity [26], mitogenic activity [27] and capacity to induce MC degranulation [28]. Thus, the blocking of chymase-mediated thrombin inactivation may increase the pro-inflammatory effects of generated thrombin, thereby contributing to the adverse effects observed for protamine. Further, it has previously been shown that MC chymase has the ability to degrade anaphylatoxin C3a, by a mechanism that is dependent on heparin PG [13]. Dissociation of the chymase from heparin PG by protamine would consequently result in a reduced capacity of the MC protease to inactivate C3a, and would lead to accumulation of the anaphylatoxin in the tissue. Clearly, subsequent MC and basophil activation caused by C3a would contribute to the clinical symptoms observed in patients that show adverse reactions to protamine.

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