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POTENTIATION BY CALCIUM IONS OF THE ANTITHROMBIN III INHIBITION OF THROMBIN

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Calcium ions potentiated heparin-modulated antithrombin III inhibition of amidolysis catalysed by thrombin. Potentiation by calcium ions of heparin-independent antithrombin III inhibition of thrombin activity appeared to contribute to this effect. These results suggest a complex modulatory role for calcium ions in proteinase-catalysed reactions influenced by anti-proteinases and glycosaminoglycans.

INTRODUCTION

Interactions between the plasma proteinase inhibitor antithrombin III, the intrinsic coagulation pathway proteinase thrombin (EC3.4.21.5.) and the clinically-administered glycosaminoglycan preparation heparin are the best explored of several glycosaminoglycan-modulated proteinase-antiproteinase systems, but remain incompletely understood (1). Such interactions are influenced by the nature of their ionic environment (2,3). Calcium ions, by reversibly binding to multi-anionic centres of glyco-conjugates and proteins, may be particularly capable of triggering changes in macromolecular conformation and influencing biological activity (4,5).

The present report investigates the effect of calcium ions on heparin-potentiated and heparin-independent antithrombin III inhibition of thrombin-catalysed amidolysis.

MATERIALS AND METHODS

Materials:

Heparin, (Grade 1, from porcine intestinal mucosa; Lot No. 46C-0035; activity stated by supplier as 170 U.S.P. units/mg), was supplied by Sigma Chemical Co. Ltd., Poole, Dorset, U.K., and dissolved in buffer (50 mM Tris, 110 mM NaCl, pH 7.2 at 18°C)immediately before use.

Bovine topical thrombin, (Lot No. YE 584), was supplied by Parke, Davis and Co., Pontypool, Gwent, U.K., dissolved as suggested by the supplier, stored at 100 units/ml at -20°C, and further diluted in 0.9% (w/v) NaCl immediately before use.

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Human antithrombin III. Most experiments involved antithrombin III supplied by KabiVitrum Ltd., Ealing, U.K., (Lot No. 1101 57030; activity stated by supplier as approximately 5 units/mg protein), which was dissolved at an appropriate concentration in 0.9% (w/v) NaCl immediately before use. Some experiments involved samples of antithrombin III generously supplied by the National Institute for Biological Standards and Control, London, U.K., (samples 75/564, 77/626 and 78/558; activities stated by the supplier as approximately 4.0, 4.5 and 4.7 units/mg protein respectively). Samples were dissolved as suggested by the supplier, and diluted in 0.9% (w/v) NaCl to an appropriate concentration immediately before use. All antithrombin III samples used were prepared by heparin affinity chromatography (6).

S-2238 (D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride, a chromogenic substrate of thrombin) was supplied by KabiVitrum Ltd., dissolved at 1 mg/ml in distilled water, and stored at 4°C until used.

Polybrene (Lot. No. 37C-0261) from Sigma Chemical Co. Ltd., was dissolved in distilled water at an appropriate concentration immediately before use.

Calcium chloride dihydrate ($CaCl_2$, Analar grade) was from BDH Chemicals Ltd., Poole, Dorset, U.K., and was dissolved in distilled water.

Measurement of thrombin inhibition:

Antithrombin III (0.7 units/ml), thrombin (6 or 12 units/ml), heparin (0.67, 0.33 or 0.13 µg/ml), and either water or $CaCl_2$ (1-15 mM) were preincubated in a total volume of 120 µl for various times at 18°C. In some experiments, polybrene (10 µg/ml) was included. The concentrations of Tris (pH 7.2 at 18°C) and NaCl in this reaction mix were 8.33 mM and 96 mM respectively. The calcium concentration of the reaction mix lacking $CaCl_2$ was estimated, using a Pye Unicam SP900 flame photometer, as 0.23 mM. Much of this 'endogenous' calcium originated in the thrombin preparation used.

Following preincubation, a 60 µl sample of the reaction mix was added to 740 µl of a solution of S-2238 (0.125 mg/ml) in 150 mM Tris buffered to pH 8 at 37°C contained within a spectrophotometer cell, and the rate of thrombin-catalysed amidolysis at 37°C estimated by measuring the increase in absorbance of the solution at 405 nm using a Pye Unicam SP800 spectrophotometer fitted with an electrically-controlled SP870 constant temperature cell housing.

RESULTS

Effect of $CaCl_2$ on thrombin inactivation: Figure 1 (A-C) shows the effect on thrombin inactivation of preincubating a mixture composed of thrombin, antithrombin III and heparin at each of three concentrations, with various concentrations of $CaCl_2$. At concentrations of $CaCl_2$ between 1 and 5 mM, the rate of proteinase inactivation increased in proportion to the $CaCl_2$ concentration. Concentrations of $CaCl_2$ between 5 and 15 mM did not further increase the rate of inactivation. Preincubation of thrombin with heparin in the absence of antithrombin III, or with heparin and 15 mM $CaCl_2$ in the absence of antithrombin III, did not lead to proteinase inactivation (Fig. 1 A-C).

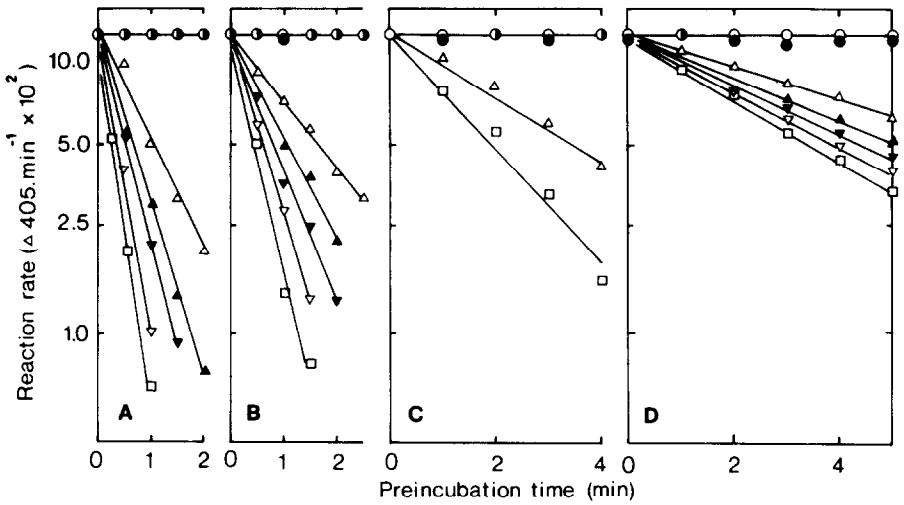

Figure 1 The effect of $CaCl_2$ on thrombin inactivation.

Combinations of thrombin (12 units/ml) with antithrombin III, heparin, and $CaCl_2$ were incubated for the indicated times before measurement of residual thrombin activity.

A. 0.67 µg/ml heparin; B. 0.33 µg/ml heparin; C. 0.13 µg/ml heparin; D. no heparin.

$CaCl_2$ concentration: (□) 5-15 mM; (▽) 4 mM; (▼) 2 mM; (▲) 1 mM; (△) no $CaCl_2$

Controls: (○) no antithrombin III or $CaCl_2$; (●) no antithrombin III; 15 mM $CaCl_2$.

Figure 1(D) shows that over a similar concentration range, (1-5 mM), $CaCl_2$ potentiated antithrombin III inactivation of thrombin in the absence of heparin. Preincubation of thrombin and 15 mM $CaCl_2$ in the absence of antithrombin III did not affect proteinase activity (Fig. 1D).

Effect of polybrene on thrombin inactivation: Figure 2 (A) shows that preincubation of a mixture of thrombin, antithrombin III and heparin with polybrene decreased the rate of thrombin inactivation to that observed when thrombin was incubated with antithrombin III alone (Fig. 2B). Preincubation of polybrene with thrombin and heparin in the absence of antithrombin III did not affect proteinase activity (Fig. 2A).

Figure 2(B) shows that preincubation with polybrene did not alter the rate of thrombin inactivation by antithrombin III in the absence of added heparin. Preincubation of polybrene with thrombin alone did not affect proteinase activity (Fig. 2B).

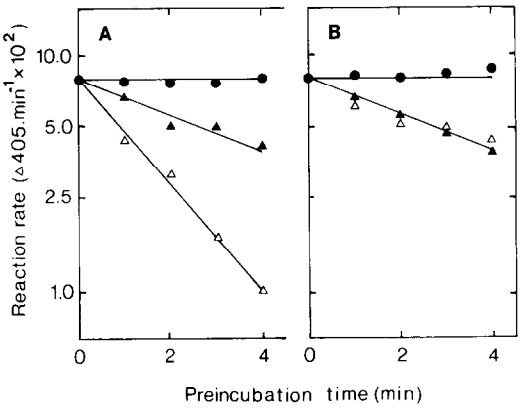

Figure 2. The effect of polybrene on thrombin inactivation.

Combinations of thrombin (6 units/ml) with antithrombin III, heparin and polybrene were incubated for the indicated times before measurement of residual thrombin activity. A. 0.33 µg/ml heparin; B. no heparin. (▲) antithrombin III, polybrene; (△) antithrombin III, no polybrene (●) no antithrombin III, polybrene.

Effect of $CaCl_2$ and of polybrene on thrombin inactivation by other antithrombin preparations: Table 1 summarises the results of experiments similar to those reported above, in which other antithrombin III preparations were used. In each case, the presence of $CaCl_2$ accelerated inactivation of thrombin by antithrombin III in the presence or absence of added heparin. The presence of polybrene did not alter the rate of antithrombin III inactivation of thrombin in the absence of added heparin.

In none of the experiments reported did amidolysis occur with any combination of components in a reaction mix which lacked thrombin.

DISCUSSION

Physico-chemical measurements and model building studies in this laboratory suggest that calcium binding locks specific heparin tetrasaccharide units into a particular rigid conformation (7), and we postulated that this binding may enhance the ability of heparin to potentiate antithrombin III inhibition of serine proteinases (8,9).

Data recorded in this communication accord with this possibility, by indicating an acceleration by $CaCl_2$ of heparin-modulated antithrombin III inhibition of thrombin. A similar potentiation by calcium ions has been

TABLE 1

Thrombin inhibition by other antithrombin III preparations

Reaction Mix	Rate of Thrombin-catalysed Reaction*		
	75/564	77/626	78/558
antithrombin III	46	50	58
antithrombin III; heparin	39	43	45
antithrombin III; $CaCl_2$	18	23	26
antithrombin III; heparin; $CaCl_2$	6	6	4
polybrene	108	108	108
antithrombin III; polybrene	40	50	55
antithrombin III; heparin; polybrene	70	77	80

Final concentrations: antithrombin III (0.7 units/ml); heparin (0.33µg/ml); $CaCl_2$ (5 mM); polybrene (10 µg/ml). All mixes contained thrombin (12 units/ml). Heparin-containing mixes were preincubated for 5 min., heparin-free mixes for 1.5 min.

*as a percentage of that rate recorded with corresponding reaction mix containing thrombin and substrate but no further additions.

reported elsewhere (2), but in this previous paper, it is suggested that the effect on thrombin inactivation is heparin-dependent. Modulation of heparin conformation and activity seem unlikely, however, to be the only way in which calcium ions might affect the inactivation process, and data reported here indeed suggest that $CaCl_2$ is able to potentiate antithrombin III inactivation of thrombin in the absence of heparin.

The antithrombin III preparations that were used in our study were prepared by heparin-affinity chromatography, and might be contaminated by traces of heparin. Similar results were recorded with four separate antithrombin III preparations, however, and the inhibitory activity of the preparations towards thrombin was not affected by polybrene under conditions in which the antithrombin III-enhancing effects of exogenous heparin were abolished. Most of the previous experiments involved thrombin-catalysed fibrinogen degradation (2). In this system, $CaCl_2$ slightly delayed thrombin inactivation by antithrombin III in the absence of heparin. We studied thrombin-catalysed amidolysis of a chromogenic substrate in order to avoid the complicating effects of calcium ions on fibrinogen structure (10). When,

in the previous study (2), a chromogenic substrate was used, $CaCl_2$ elicited a small increase in the rate of heparin-independent antithrombin III inactivation of thrombin consistent with our results.

The plasma concentration of calcium, although probably subject to transient local changes (11), is relatively high (1-3 mM)(12). Changing plasma calcium concentrations are therefore unlikely to switch on or off antithrombin III inhibition of thrombin. However, calcium ions appear to play a complex role in the interactions involving thrombin, heparin, antithrombin III and fibrinogen. These roles may include the maintenance of heparin and fibrinogen in particular, functionally essential conformations (7,10), and, as suggested here, the modulation of antithrombin III-thrombin interactions.

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