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Effects of Calcium and Magnesium on the Anticoagulant Action of Heparin

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The cooperative inhibitory action of heparin and antithrombin III on the thrombin–fibrinogen reaction was neutralized by preincubating these anticoagulant factors in the presence of Ca or Mg, and the effect was larger with Ca than with Mg. However, the neutralizing action of Ca decreased in the presence of Mg. Bindings of poly-L-lysine and antithrombin III to heparin were also inhibited by the addition of Ca and Mg, and Ca had a larger effect than Mg. On the other hand, the binding ability of Ca to heparin was larger than that of Mg, and the coexistence of these metals reduced the binding affinity of each metal.

These data suggest that the neutralizing action of Ca and Mg on the anticoagulant action of heparin and antithrombin III may be related to the ability of these metals to prevent the complex formation of the acid mucopolysaccharide and the thrombin inhibitor by binding to the acid mucopolysaccharide.

Keywords——anticoagulant action; heparin; antithrombin III; calcium; magnesium; neutralizing action

Heparin is an acid mucopolysaccharide containing sulfate and carboxyl groups, and has many clinical uses as an anticoagulant drug. At present, heparin is an indispensable drug for the treatment and prevention of thromboembolism in cardiovascular surgery and in cardiac diagnostics.

However, the control heparin activity in the blood is an important problem during clinical usage in relation to the determination of the effective dose and prevention of hemorrhage.

For this purpose, thrombin clotting time assay (TCT) or activated clotting time assay is widely used at present.[1)] In the TCT system, the clotting time is often unacceptably prolonged even when the level of heparin in the plasma is within the therapeutic range. For improvement of this problem in the TCT system, calcium chloride has been used, but in this case the clotting time is generally shorter than that of the control without addition of calcium. The detailed mechanism of this neutralizing action of calcium against the anticoagulant action of heparin is not yet clear, except that the shortening of the clotting time caused by calcium is independent of generation of additional thrombin *via* the intrinsic pathway.[1a)]

In the present study, the effects of calcium and magnesium on the anticoagulant action of heparin and antithrombin III were examined, and the possible mechanism of the neutralizing action of these metals against the anticoagulant action of heparin and antithrombin III was investigated by determining the effects of these metals on the binding of antithrombin III and poly-L-lysine to heparin, and the binding ability of these metals to heparin.

Materials and Methods

Materials——The sodium salt of pig intestinal mucosa heparin (148 USP units/mg) was purchased from Inorlex. Fibrinogen (87% clottable) and poly-L-lysine (MW; 30000—70000) were obtained from Sigma Chemicals. Thrombin (1000 J. P. units/vial) was obtained from Mochida Pharmaceutical Co., Ltd. Sepharose 4B and Sephadex G-200 were obtained from Pharmacia Fine Chemicals. Diethylaminoethyl (DEAE)-52 was purchased from Whatman. Calcium chloride and magnesium chloride were dissolved in deionized water. Other reagents were dissolved in a buffer (0.05 M Tris-HCl in 0.15 M NaCl, pH 7.4). Final concentrations of thrombin and fibrinogen used in the present study were 40 μg/ml and 2 mg/ml, respectively. The antithrombin III was prepared from defibrinated bovine plasma[2] by affinity chromatography with heparin-Sepharose gel[3,4] according to the method of Damus and Rosenberg,[5] and electrophoretically homogenous inhibitor purified by DEAE-cellulose chromatography and Sephadex G-200 gel chromatography was used.

Effect of Heparin on the Thrombin–Fibrinogen Reaction——A 0.1 ml aliquot of thrombin was added to each of a series of test tube, 1 × 10 cm, containing 0.3 ml of the buffer and 0.1 ml of heparin prepared at various concentrations (1, 5, 10, 30, 50 and 100 μg/ml). The reaction mixtures were incubated for 1, 3 or 5 min at 37 °C, then 0.5 ml of fibrinogen was added to each reaction mixture and the clotting time was observed every 10 s.

Effect of Ca or Mg on the Inhibitory Action of Heparin on Thrombin–Fibrinogen Reaction——This experiment was done in the same way as described above except that 0.1 ml of thrombin was incubated with 0.1 ml of heparin (100 μg/ml) for 1 min at 37 °C in the presence of either Ca or Mg. The final metal concentrations were 0, 0.5, 1.0, 2.5 and 5.0 μmol/ml.

Single or Combined Effects of Ca and Mg on the Inhibitory Action of Heparin and Antithrombin III on Thrombin–Fibrinogen Reaction——These experiments consisted of five reaction systems (RS). In RS-1, 0.1 ml of thrombin was added to a test tube containing 0.4 ml of the buffer described above and the mixture was maintained at 37 °C for 1 min, then 0.5 ml of fibrinogen was added and the clotting time was observed. In RS-2, 0.1 ml of thrombin was added to a test tube containing 0.3 ml of the buffer and 0.1 ml of heparin (10 μg/ml). The reaction mixture was kept at 37 °C for 1 min, then 0.5 ml of fibrinogen was added. In RS-3, 0.1 ml of thrombin was added to a test tube containing 0.3 ml of the buffer and 0.1 ml of antithrombin III (0.8 μg/ml). After incubation of the reaction mixture for 3 min, 0.5 ml of fibrinogen was added. In RS-4, 0.1 ml of heparin was preincubated with 0.1 ml of antithrombin III for 3 min in a test tube containing 0.2 ml of the buffer. Then 0.1 ml of thrombin was added to the reaction mixture. After incubation of the reaction mixture for further 1 min, 0.5 ml of fibrinogen was added. The procedures in RS-5 were the same as in RS-4, except that thrombin and antithrombin III were preincubated for 3 min in the presence of either Ca or Mg. The final metal concentrations are given in Table I. On the other hand, combination effects of Ca and Mg were studied by changing the Mg concentrations under a constant Ca concentration. The final metals concentrations used were 1 μmol/ml for Ca and 0, 0.1, 0.25, 0.50, 1.00, 2.50 and 5.00 μmol/ml for Mg.

Effect of Ca or Mg on the Thrombin–Fibrinogen Reaction——To a test tube containing 0.3 ml of the buffer, 0.1 ml of thrombin and 0.1 ml of $CaCl_2$ or $MgCl_2$ solution at the concentrations indicated in Table I were added. After incubation of the reaction mixture for 1 min at 37 °C, 0.5 ml of fibrinogen was added.

Effects of Ca and Mg on the Binding of Heparin and Antithrombin III——To a series of Pyrex test tube containing 0.4 ml of the buffer and 0.4 ml of heparin-Sepharose gel (settled volume 0.3 ml), 0.1 ml of $CaCl_2$ or $MgCl_2$ (0.29 mM) and 0.1 ml of antithrombin III were added. The reaction mixtures were vigorously shaken at 37 °C for 30 min, then free antithrombin III concentrations in the supernatants were determined according to the method of Lowry *et al.*[6] In the control, 0.1 ml of the buffer was used instead of metal solution.

Effects of Ca and Mg on the Interaction of Poly-L-lysine and Heparin——To a test tube (1.5 × 10 cm) containing 2 ml of $CaCl_2$ or $MgCl_2$ (27 μmol/ml), 2 ml of heparin (0.66 mg/ml) and 2 ml of poly-L-lysine (0.66 mg/ml) were added. The mixture was equilibrated for 30 min at room temperature. In the control, 2 ml of deionized water was used instead of metal solution.

The circular dichroism spectra were recorded by a JASCO J20 spectropolarimeter.

Binding of Ca and Mg to Heparin——This was carried out according to the method of Hughes and Clotz.[7] A solution (10 ml) containing equimolar concentrations of $CaCl_2$ and $MgCl_2$ was added to a test tube (2.5 × 20 cm) and a dialysis tube (24/32 in, 15 cm) containing 10 ml of sodium heparin (2 mg/ml) was suspended in the metal solution. The vessel was sealed with parafilm, then vigorously agitated for 16 h at 37 °C. The metals concentrations in the external solution were analyzed by a Hitachi 170-50A atomic absorption spectrophotometer to estimate the amount of metals bound to heparin. In the control, sodium heparin was dialyzed against $CaCl_2$ or $MgCl_2$. The metal concentrations used were 1.0, 2.0, 3.0 and 4.0 μmol/ml. A similar experiment was conducted by changing the pH of the supporting electrolyte solution from 6.5 to 3.1.

Results

The effect of heparin on the thrombin–fibrinogen reaction was examined. The clotting

time of the control without heparin was 50 s. There was no appreciable effect of heparin on the thrombin–fibrinogen reaction up to a concentration of 0.5 μg/ml.

However, further addition of heparin caused gradual prolongation of the clotting time. On the other hand, the incubation time of heparin and thrombin did not affect the ability of heparin to inhibit the thrombin–fibrinogen reaction.

Table I shows the effect of Ca or Mg on the inhibitory action of heparin and antithrombin III on the thrombin–fibrinogen reaction. The clotting time of the control without the addition of heparin and antithrombin III was 50 s. Addition of heparin (1 μg/ml) caused slight prolongation of the clotting time.

Addition of antithrombin III (0.8 μg/ml) also caused slight prolongation of the clotting time. On the other hand, preincubation of heparin and antithrombin III for 3 min before incubation with thrombin for 1 min considerably prolonged the clotting time.

This indicates that heparin and antithrombin III cooperatively inhibited the thrombin activity. However, such a cooperative inhibitory action of heparin and antithrombin III was decreased when these two inhibitors were preincubated with Ca or Mg. This neutralizing effect increased with increasing concentration of these metals, but Ca was more effective than Mg.

In general, the plasma used for various heparin assays including the TCT system contains inorganic cations such as Na, K and Mg, which may affect the TCT system together with Ca through some unknown mechanism. We selected Mg from among them, since it has a relatively high affinity for heparin,[8] and examined the combination effects of Ca and Mg on

TABLE I. Effects of Calcium and Magnesium on the Inhibitory Action of Heparin and Antithrombin III on the Thrombin–Fibrinogen Reaction

Reaction system (RS)	Metal concentration (μmol/ml)		Clotting time (s)
	Mg	Ca	
RS-1. T+F	0	0	50
RS-2. T+H+F	0	0	60
RS-3. T+AT+F	0	0	70
RS-4. H+AT+T+F	0	0	190
RS-5. Mg or Ca+H+AT+T+F	0.25	0	190
	0.50	0	190
	1.00	0	170
	2.50	0	140
	5.00	0	120
	10.00	0	100
	20.00	0	80
	0	0.25	170
	0	0.50	150
	0	1.00	120
	0	2.50	90
	0	5.00	80
	0	10.00	70
	0	20.00	60

T, thrombin; F, fibrinogen; H, heparin; AT, antithrombin III.

Final concentration: thrombin 40μg/ml, fibrinogen 2 mg/ml, heparin 1μg/ml, antithrombin III 0.8μg/ml.

The detailed experimental procedure is given in the methods section. The results are the means of three observations.

TABLE II. Effects of Calcium and Magnesium on the Inhibitory Action of Heparin on the Thrombin–Fibrinogen Reaction

Reaction components	Metal concentration (μmol/ml)		Clotting time (s)
	Mg	Ca	
Ca or Mg+heparin+thrombin +fibrinogen	0	0	100
	0.5	0	90
	1.0	0	80
	2.5	0	60
	5.0	0	60
	0	0.5	70
	0	1.0	60
	0	2.5	50
	0	5.0	50

Thrombin (40 μg/ml) was incubated with heparin (10 μg/ml) for 1 min at 37 °C in the presence of Ca or Mg at the concentrations indicated. Then fibrinogen was added to the reaction mixture and the clotting time was observed. The data are the means of three observations.

TABLE III. Binding of Antithrombin III and Heparin-Sepharose gel in the Presence or Absence of Calcium or Magnesium

Incubation components	Added antithrombin III	Concentration of Ca or Mg (μmol/ml)	Amount of antithrombin III bound to heparin-Sepharose gel (μg/ml)	
HS+AT+ Ca or Mg	733	0	191	
	1067		277	
	1600		433	
	2167		589	
			Mg addition	Ca addition
	733	29	167	95
	1067		208	116
	1600		308	178
	2167		517	353

HS, heparin-Sepharose gel; AT, antithrombin III.
Heparin-Sepharose gel and antithrombin III were incubated for 30 min at 37 °C in the presence or absence of Ca or Mg. Then antithrombin III concentrations in the supernatants were determined to assess the amount of the protein bound per ml of heparin-Sepharose gel.

the heparin–antithrombin III anticoagulant action in order to gain a better understanding of the neutralizing action of Ca on the TCT system.

When heparin and antithrombin III were preincubated with Ca at a concentration of 1 μmol/ml under the conditions given in Table I, the clotting time (prolonged by these anticoagulant factors) was shortened. However, this action of Ca was decreased in the presence of Mg (below 1 μmol/ml). Further addition of Mg caused shortening of the clotting time again, as occurred with the addition of Ca alone (not shown). This suggests that the level of magnesium in the plasma may be an important factor in assessing the effect of Ca on the TCT system, and these cations may have the same inhibition site for anticoagulant action of heparin.

Mg alone did not have any effect on the thrombin–fibrinogen reaction over a wide

concentration range of this metal. Ca did not affect the reaction up to concentrations of 2.5 μmol/ml, but further addition of this metal caused shortening of the clotting time to some extent (not shown). Table II shows the effects of Ca and Mg on the inhibitory action of heparin on the thrombin–fibrinogen reaction. In this experiment, the final concentration of heparin was 10 μg/ml because lesser concentrations of heparin made it difficult to assess the effects of these cations. The addition of heparin at this concentration caused prolongation of the clotting time, which reached 100 s.

When thrombin and heparin were preincubated for 1 min at 37 °C in the presence of Ca or Mg before addition of fibrinogen the clotting time (prolonged by heparin) was shortened and the effect was larger with Ca than with Mg.

The above data suggest that the neutralizing effect of Ca and Mg on the inhibitory action of heparin and antithrombin III is related to a cooperative inhibitory action of these two anticoagulant factors rather than to an activation of thrombin because both metals could neutralize the inhibitory action of heparin and antithrombin III even at concentrations which had no appreciable effect on the thrombin–fibrinogen reaction. For this reason, the effects of Ca and Mg on the binding of heparin and antithrombin III were examined. The results in Table III indicate that the binding of antithrombin III to heparin was prevented by these metals, and the ability of Ca to prevent the binding was larger than that of Mg.

On the other hand, it is known that lysine residues of antithrombin III participate in the binding of this thrombin inhibitor and heparin.[2] Therefore, the effects of Ca and Mg on the interaction of heparin and poly-L-lysine were investigated. The results are shown in Fig. 1. A mixture of heparin and poly-L-lysine showed two negative CD bands centering at 219 and 205 nm (curve1). The addition of Ca or Mg to the reaction mixture decreased the ellipticities at both wavelengths. The decrease of ellipticity at 219 nm was almost the same with both metals, but Ca caused a greater decrease of the ellipticity at 205 nm (curve 3) than Mg (curve 2). These results indicate that the binding of poly-L-lysine to heparin was reduced in the presence of Ca or Mg.

The results in Fig. 1 also suggest that the ability of Ca and Mg to decrease the inhibitory action of heparin and antithrombin III may be related to the binding of these metals to heparin, because poly-L-lysine undergoes ionic interactions with acid mucopolysaccharides such as chondroitin 6-sulfate, chondroitin 4-sulfate and heparin.[9,10]

Therefore the bindings of Ca and Mg to heparin were studied. Figures 2-(a) and -(b) respectively show the binding of Ca alone and Mg alone with heparin, and Figs. 2-(c) and -(d) respectively show the binding of Ca and Mg in the presence of both metals (concentration ratio was 1 : 1 at all concentration points). Binding of both Ca and Mg to heparin was

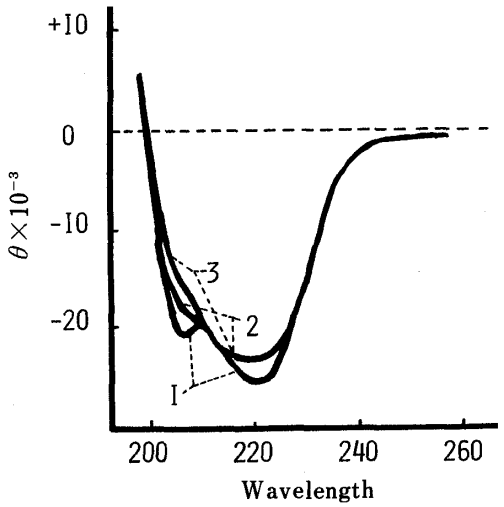

Fig. 1. Effects of Ca and Mg on Heparin–Poly-L-lysine Interaction

θ = molar ellipticity (in degree-cm^2/dmol) of lysine residues (l = 0.5 cm). Concentrations: heparin, 0.22 mg/ml; poly-L-lysine, 0.22 mg/ml; Ca, Mg, 8.7 μmol/ml.
Curve 1: heparin + poly-L-lysine.
Curve 2: heparin + poly-L-lysine + Mg.
Curve 3: heparin + poly-L-lysine + Ca.

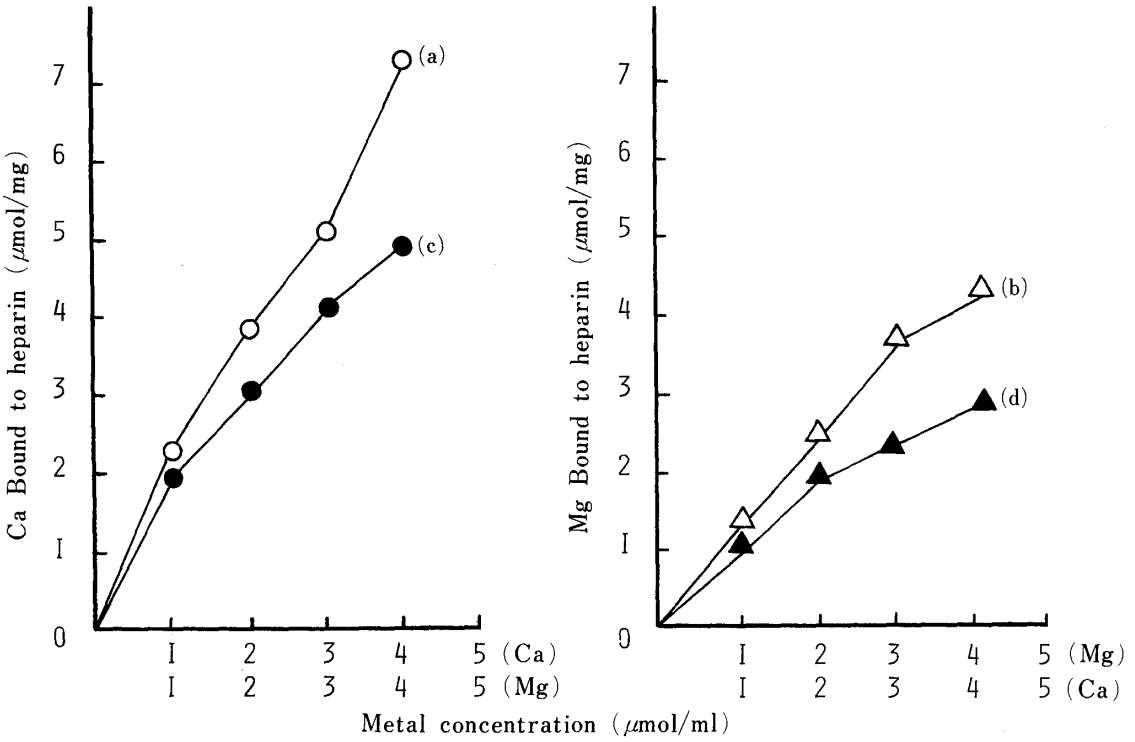

Fig. 2. Binding of Ca and Mg for Heparin

Conditions: pH 6.5, 0.1 M $NaNO_3$.
Curve (a): binding profile of Ca.
Curve (b): binding profile of Mg.
Curve (c): binding profile of Ca in the presence of Mg.
Curve (d): binding profile of Mg in the presence of Ca.

decreased when both metals were present, but Ca had the larger binding ability.

A similar experiment was done at pH 3.1. Carboxyl groups on heparin exist in the protonated form at such an acidic pH.[11] The binding of Ca alone and that of Mg alone to heparin were decreased by changing the pH of the supporting electrolyte solution from 6.5 to 3.1, and this decrease was further enhanced in the presence of both cations (not shown).

Discussion

Anticoagulant action of heparin is mainly due to the ability of this acid mucopolysaccharide to accelerate the rate of thrombin inactivation by antithrombin III, a plasma protease inhibitor, by preferentially binding to the inhibitor with a 1:1 stoichiometry.[2,12] This accelerating effect of heparin on thrombin inactivation by antithrombin III involves a conformational change of the inhibitor molecule which facilitates the binding of thrombin and the protease inhibitor.[13] At present, binding of heparin to antithrombin III is considered to be a key site in the development of anticoagulant action of heparin.

The present study was made to investigate the mechanism of the neutralizing effect of Ca on the anticoagulant action of heparin, as can be observed in the TCT system with thrombin reconstitution by $CaCl_2$, by placing special emphasis on the interaction of heparin and antithrombin III. Thus, the effects of Ca and Mg on the inhibitory action of heparin and antithrombin III on the thrombin–fibrinogen reaction were examined, together with the effects of these metals on the binding of poly-L-lysine and heparin and on the binding of heparin and antithrombin III. The results indicate that the inhibitory action of heparin and antithrombin III on the thrombin–fibrinogen reaction was decreased by the presence of Ca

and Mg, and these cations also reduced the binding of antithrombin III and heparin. The neutralizing action of Ca and Mg is probably independent of thrombin activation by these cations. These results suggest that the neutralizing effect of Ca and Mg on the heparin–antithrombin III anticoagulant action is related to the prevention of complex formation between these anticoagulant factors. The parallelism between the differences in the binding abilities of Ca and Mg for heparin and the differences in inhibition abilities of these cations supports the view that the inhibition of the complex formation between heparin and antithrombin III by Ca and Mg may be related to the binding of these cations to heparin molecule. The binding between heparin and antithrombin III occurs through lysine residues of the protease inhibitor,[2] but the binding site on heparin is not known. Gelman and Blackwell[14] suggested that poly-L-lysine binds to sulfate groups of heparin.

On the other hand, Lindahl *et al.*[15] showed that a unique disaccharide sequence consisting of L-iduronosyl-*N*-sulfo-D-glucosamyl 6-sulfate is involved in the antithrombin III binding region of heparin, and moreover they speculated that L-iduronic acid in this sequence may be essential to the anticoagulant activity of heparin.

Danishefsky[16] also demonstrated that carboxylate anions on the heparin molecule are necessary to allow complete formation of heparin–antithrombin III complex by specifically modifying the charged groups.

These results suggest that both carboxylate and sulfate are necessary for the binding of heparin and antithrombin III.

It is also known that Ca binds to such functional groups on the heparin molecule.[11c,17] In addition, we have some evidence based upon a ^{13}C-nuclear magnetic resonance (^{13}C-NMR) study suggesting that the binding site of Ca and Mg on the heparin molecule may be identical (not shown). Moreover, our present results demonstrate that when Ca and Mg coexisted, the overall neutralizing effect of these cations on the heparin–antithrombin III anticoagulant action was dependent upon the concentration ratio of these cations.

This suggests that the inhibition sites of Ca and Mg on anticoagulant action of heparin and antithrombin III may be identical. Therefore, Ca and Mg may neutralize the anticoagulant action of heparin by binding to carboxylate and sulfate groups of the mucopolysaccharide molecule.

On the other hand, it is known that Ca enhances fibrin monomer polymerization without thrombin activation.[18] This suggests that the neutralizing action of Ca and Mg might not be related to the prevention of the binding of heparin and antithrombin III but to enhanced polymerization of fibrin monomer by these cations. However, this seems unlikely, because anticoagulant action of heparin and antithrombin III was affected at calcium concentrations that had no appreciable effect on fibrin clot formation, and in the case of Mg, only the neutralizing effect was observed. However, higher concentrations of Ca may contribute to the polymerization of soluble fibrin monomer.

As discussed above, the neutralizing action of Ca on the anticoagulant action of heparin observed in the TCT system may be explainable to some extent by prevention of complex formation between heparin and antithrombin III caused by binding of this metal to the acid mucopolysaccharide molecule. However, a direct evidence is still required, and an investigation of the binding site of Ca on heparin seems to be the next step.

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