

Effect of heparin and low molecular weight heparins on thrombin-induced blood platelet activation in the absence of antithrombin III

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EFFECT OF HEPARIN AND LOW MOLECULAR WEIGHT HEPARINS ON THROMBIN-INDUCED
BLOOD PLATELET ACTIVATION IN THE ABSENCE OF ANTITHROMBIN III

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ABSTRACT

We have investigated the antithrombin III independent effect of crude heparin, two heparin fractions and a heparinoid on in vitro thrombin-induced platelet activation. Thrombin-induced platelet factor Va generation and thrombin plus collagen-induced platelet prothrombin converting activity were tested. Crude heparin was a more potent inhibitor of these reactions than the fractions or the heparinoid. The inhibitory action of the heparins was found to be the result of a direct effect on thrombin and not of an effect either on platelet activation functions or on the assembly or functioning of the prothrombinase complex. Probably this heparin inhibition is due to the masking of secondary macromolecular substrate binding sites on the thrombin molecule. We found no correlation between IC_{50} values and the antithrombin III-dependent antithrombin specific activities of the heparins. This supports the notion that heparin properties other than their affinity for antithrombin III may contribute to the action of this drug in blood coagulation.

INTRODUCTION

Heparin is an effective antithrombotic agent, widely used in the treatment and prophylaxis of venous thrombosis and thromboembolism. However, its clinical use is limited by its major side effect, bleeding. For many years, it was generally assumed that both the antithrombotic and hemorrhagic properties of heparin are related to its ability to augment the reactions between activated coagulation factors and antithrombin III. More recently, numerous comparative studies on the hemorrhagic, antithrombotic and anticoagulant effects of heparinoids and low molecular weight heparin fractions, prepared from crude heparin by a variety of methods, indicate that:

- 1) low molecular weight heparins with an anti-factor Xa to anti-thrombin

Key words : Thrombin, heparin, LMW heparin, platelet coagulant activities.

ratio, larger than that of crude heparin, have similar antithrombotic activities but produce less hemorrhage (1), 2) The anti-factor Xa activity of heparin is not related to its antithrombotic activity (2,3), 3) The excess low affinity material present in heparin with a low affinity to antithrombin III increases hemorrhage, without having either in vivo antithrombotic or ex vivo anticoagulant activity (4) and 4) Dermatan sulfate, known to potentiate only the activity of heparin cofactor II on thrombin, has an antithrombotic activity equal to that of crude heparin but leaves hemostasis largely unaffected (5).

These studies illustrate the difficulty in attempting to correlate in vitro assays and in vivo blood changes. It can be questioned whether one has to search for other, more specific markers of the anti-thrombin effect of heparin, that could be either an antithrombin III-dependent or independent one. In this context it is important to note that coagulation and platelet reactions are strongly coupled by a positive feedback mechanism because thrombin-induced platelet activation generates procoagulant activities. This thrombin-dependent mechanism leads to an important amplification of thrombin generation and so of both hemostatic plug and thrombus formation.

The present study was undertaken to determine to what extent heparin-(fractions) inhibit the formation of thrombin-induced platelet procoagulant activities and to investigate whether heparin(fractions) exert such an anti-thrombin activity through a mechanism that is independent of anti-thrombin III.

MATERIAL AND METHODS

S 2238 (H-D-Phe-Pip-Arg-pNa) was purchased from AB Kabi Diagnostica, Stockholm, Sweden. Fatty acid free human serum albumin (HSA), Russell's Viper Venom and Echis Carinatus venom were obtained from Sigma. Horse tendon collagen was obtained from Hormon-Chemie München, Germany. It was added in the non-fibrillar form dissolved in acidic dilution buffer obtained from Hormon-Chemie. Reaction tubes were 2 ml plastic flat-bottom tubes obtained from Sterilin Ltd (Teddington, England). Teflon-coated magnetic spinning bars 7x2 mm were from Bel-Art Products, USA. Phosphatidylcholine (18:1 cis/18:1 cis phosphatidylcholine) was from Sigma, USA. All reagents used were of the highest grade commercially available.

Preparation of Phospholipids and Phospholipid Vesicles. Phosphatidylserine (18:1 cis/18:1 cis) was prepared from phosphatidylcholine by enzymatic synthesis according to the method of Comfurius and Zwaal (6). Single bilayer vesicle solutions were prepared as previously described (7). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (8).

Proteins. Bovine prothrombin, factor X, factor Xa, factor V and factor Va were purified according to established procedures (9). Human and bovine α -thrombin were prepared as described by Pletcher et al. (10). The specific clotting activity was 2800 NIH U/mg for human thrombin and 2200 NIH U/mg for bovine thrombin. The protein preparations were stored at -70°C after dialysis against 50 mM Tris/HCl 175 mM NaCl at pH 7.9.

Protein Concentrations. Concentrations of thrombin and factor Xa were determined by active site titration with p-nitrophenyl-p-guanidinobenzoate hydrochloride (11). Prothrombin concentrations were determined after

complete activation with E.carinatus venom followed by active site titration. Similarly, factor X concentrations were obtained by active site titration after complete activation with factor X - converting protein from Russell's viper venom (11). Factor Va concentrations were determined by kinetic analysis (9).

Heparin and Heparin Fractions. Crude porcine intestinal mucosal heparin (MW 1500-30000, mean MW 15000, anti Xa and anti IIa activity 175 U/mg) was obtained from Organon Laboratories (Oss, the Netherlands). The following low molecular weight compounds were prepared from standard heparin by Choay Institute (Paris): CY 216, a calcium salt was obtained by ethanol extraction (mean MW 4500, USP activity 50 U/mg, anti Xa activity 200 U/mg, anti IIa activity 10-20 U/mg) and CY 222, a sodium salt was obtained by nitrous acid depolymerization (mean MW 2500, USP activity 25 U/mg, anti Xa activity 250 U/mg, anti IIa activity 5-15 U/mg). Org 10172, a sulphated mucopolysaccharide was isolated by Organon Laboratories (Oss, the Netherlands) from porcine intestinal mucosa (mean MW 6400, USP activity 2.94 U/mg, anti Xa activity 8 U/mg, anti IIa activity 0.32 U/mg).

Isolation of Human Platelets. Blood was drawn by venepuncture from healthy male volunteers who had not taken any medication for at least one week. As anticoagulant, one volume of acid citrate/dextrose (0.18 M glucose/0.08 M sodium citrate/0.052 M citric acid) was added to 5 volumes of whole blood. Platelet-rich plasma was obtained after centrifuging at 220xg for 15 min. Platelets were isolated by centrifuging at 600xg for 20 min and the pellet was resuspended in a Hepes buffer containing 136 mM NaCl, 2.68 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 5 mM glucose and 0.4% HSA, pH 6.7. The platelets were washed twice in this buffer by centrifuging 350xg, 15 min. Before centrifuging acid citrate/dextrose was added to the suspension (1 vol. to 14 vol.). Finally the platelets were resuspended in the Hepes buffer at pH 7.5 containing 0.1% HSA. The entire procedure for platelet preparation took about 2h and was carried out at room temperature (22 °C). Platelet concentration was determined with a Coulter counter. Platelets were kept at room temperature and no loss of platelet functions was found for at least 15h.

Determination of Platelet Factor V Release and Activation. 292 µl of platelet suspension (5.5×10^6 /ml) were incubated with 13 µl of 75 mM CaCl₂ and stirred (350 revolutions/min) for 3 min at 37 °C followed by the addition of thrombin, resulting in a final volume of 350 µl. Subsequently, aliquots (usually 20 µl) were taken and incubated with factor Xa (1.3×10^{-11} M), phospholipid₅ containing 20% phosphatidylserine and 80% phosphatidylcholine (1.0×10^{-5} M) and CaCl₂ (10 mM) in a buffer containing 50 mM Tris, 175 mM NaCl and 0.5 mg/ml HSA, pH 6.7 in a final volume of 0.225 ml for 5 min at 37 °C in a plastic cuvette. The reaction was initiated by the addition of 25 µl of prothrombin (2.0×10^{-6} M). After 2 min, 0.65 ml of the same buffer containing 20 mM EDTA and 0.24 µmol S2238 was added to the reaction mixture. The amount of thrombin formed was measured by the change in absorbance recorded on an Aminco DW2 spectrophotometer operating in the dual wave-length mode ($\lambda = 405$ nm and $\lambda_r = 500$ nm) thermostated at 37 °C. Standard curves were constructed by assaying dilutions of a bovine factor Va preparation whose concentration was determined utilizing active site titrated factor Xa (9). The assay had a functional range from 2×10^{-14} M to 1×10^{-12} M of factor Va.

Platelet Beta-Thromboglobulin (BTG) Release. To a reaction tube containing 292 µl of a platelet suspension (5×10^6 /ml), 13 µl of 75 mM CaCl₂ was added. The tube content was stirred for 5 min at 37 °C and platelet activation was

started by addition of a platelet activator (thrombin or collagen) resulting in a final volume of 350 μ l. After a variable time period at 37 $^{\circ}$ C, the release reaction was stopped by adding to the platelet suspension 56 μ l Hepes buffer containing 135 mM formaldehyde, 100 mM EDTA, and bringing it to a melting ice-bath (12). Platelets were centrifuged for 1 min in an Eppendorf centrifuge 5412, 200 μ l of the supernatant was carefully removed, to which 50 μ l of a 2 M Tris solution was added to neutralize the excess of formaldehyde. The sample was diluted 1:3 in Hepes buffer pH 7.5 containing 20 % of horse serum and the amount of BTG was measured using the BTG radioimmunoassay kit from Amersham International Limited according to the instruction of the manufacturer.

Determination of Platelet Prothrombin Converting Activity (PPCA). 292 μ l of platelet suspension (5×10^6 /ml) were incubated with 13 μ l of 75 mM CaCl_2 and stirred for 3 min at 37 $^{\circ}$ C. Platelet stimulation was started by adding thrombin or collagen plus thrombin resulting in a final volume of 325 μ l and a CaCl_2 concentration of 3 mM. After a variable time of stirring at 37 $^{\circ}$ C (i.e. platelet trigger time) the PPCA was determined as follows. 50 μ l factor Va (5nM) and 50 μ l factor Xa (10 nM) were added to the triggered platelet suspension, to allow equilibration with the platelets. After 2 min period of preincubation, prothrombin activation was started with the addition of 75 μ l of a prepared mixture containing 6.6 μ M prothrombin and 27 mM CaCl_2 in 50 mM Tris, 175 M NaCl and 0.5 mg/ml HSA, pH 7.9, to result in a final volume of 0.5 ml. The final concentrations of the reactants were: 0.5 nM factor Xa, 1 nM factor Va, 1 μ M prothrombin and 6 mM CaCl_2 . After 30 and 60 s aliquots (usually 10 μ l) were taken from the reaction mixture to determine the amount of thrombin formed.

Analysis of the Data. We assumed that thrombin forms a complex with heparin and that such a complex has no enzymatic activity. The reversible association between heparin and thrombin is expressed as:

$$K_d = \frac{[H][IIa]}{[H \cdot IIa]} \quad (1)$$

where [H] is the free heparin concentration and [IIa] is uncomplexed thrombin. Because heparin is present in excess over thrombin, [H] can be equaled to the heparin concentration added. Since the initial rate of factor Va generation (v) is directly proportional to the uncomplexed thrombin concentration:

$$v = k[IIa] = k([IIa]_0 - [H \cdot IIa]) \quad (2)$$

where $[IIa]_0$ is the initial thrombin concentration. From (1) and (2) we find:

$$v/(v_0 - v) = K_d \cdot (1/[H]) \quad (3)$$

where $v_0 = k[IIa]_0$ is the initial rate of factor Va generation in the absence of heparin. A plot of $v/(v_0 - v)$ versus the reciprocal heparin concentration gives a straight line with slope K_d . Since K_d is the amount of heparin required to bind half the thrombin, the K_d value can also be expressed as IC_{50} , the amount of heparin causing 50 % inhibition.

RESULTS

Inhibition of Platelet Factor V Release and Activation by Heparin and Heparin Fractions. Factor V is released from the α -granules as a profactor and activated after the release reaction when thrombin is present (13,14). We determined that our assay is virtually a quantitative assessment of the presence of factor Va, rather than factor V activity, i.e. under the assay conditions factor V activity is 1.3 % of factor Va activity (data not shown). The initial rate of factor Va activity generation after triggering the platelets with thrombin, appeared to be proportional with the thrombin concentration up to 1.0 nM. The maximal amount of factor Va that could be generated was about 1 ng/ 1.6×10^6 platelets (Figure 1). This is similar to the amount of platelet factor V Tracy et al. (15) reported for Triton-lyzed platelets.

To study the effects of heparin and heparin fractions on the initial rate of platelet factor Va generation, 4.6×10^6 platelets/ml were stimulated with 0.5 nM thrombin and the amount of factor Va formed was determined by assaying samples taken 3 min after initiating the release. It is important to note that under these conditions the rate of factor Va generation is proportional to the functional thrombin concentration (Figure 1).

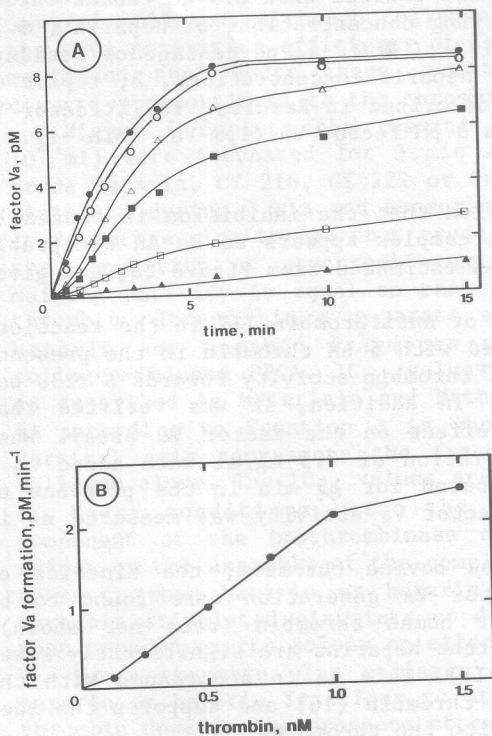


FIG. 1

Effect of varying thrombin concentrations on the time course of factor Va generation. A, platelets (4.6×10^6 /ml) were incubated at 37°C with varying thrombin concentrations (\blacktriangle , 0.125 nM; \square , 0.25 nM; \blacksquare , 0.5 nM; \triangle , 0.75 nM; \circ , 1 nM; \bullet , 1.5 nM). B, replot of the initial rate of factor Va formation as a function of thrombin concentration.

The influence of varying amounts of crude heparin, CY 216, CY 222 and Org 10172 on the initial rate of factor Va generation is shown in Figure 2A. It is apparent that crude heparin is the most potent inhibitor. When the data from Figure 2A were replotted according to Equation 3 (see Materials and Methods) straight lines were obtained going through the

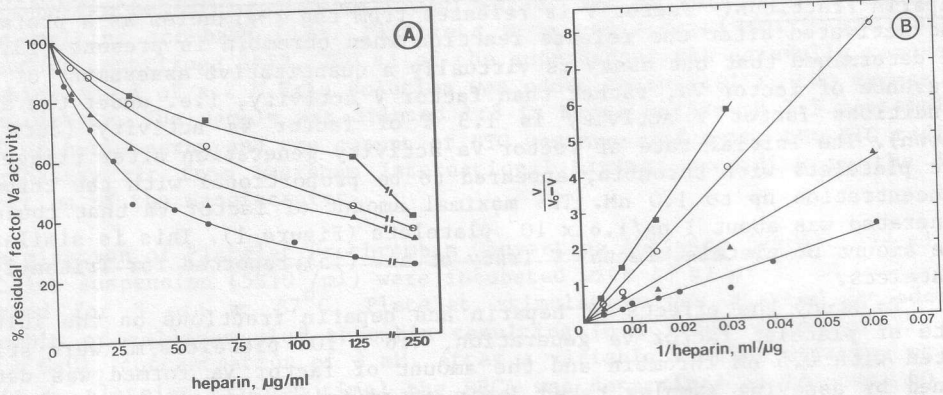


FIG. 2

Effect of heparin (fractions) on thrombin induced platelet factor Va generation. Platelets ($4.6 \times 10^6/\text{ml}$) were stirred with 0.5 nM human thrombin for 3 min, in the presence of varying concentrations of heparin (\bullet), CY 216 (\circ), CY 222 (\blacktriangle), or Org 10172 (\blacksquare). A, percentage of residual factor Va activity as a function of heparin concentrations. B, replot of the data according to Equation 3 as described in Results. 100 % factor Va activity in the absence of heparin was 3 pM factor Va ($1 \text{ pm Va. min}^{-1}$).

origin (Figure 2B). Thus, our assumption that the inhibition is caused by the formation of the heparin-thrombin complex appears to be an acceptable minimal hypothesis. The IC_{50} values, as estimated from Figure 2B, are given in Table I.

We could exclude the involvement of antithrombin III in the reaction. When platelets ($10^7/\text{ml}$) were stimulated with 5 nM thrombin in the presence of crude heparin ($10 \mu\text{g}/\text{ml}$) no loss of thrombin activity towards S 2238 was observed over a 20 min time period. In addition, it was verified that heparin or heparin fractions had no effect on the factor Va assay. When heparin(fractions) at a final concentration of $0.5 \text{ mg}/\text{ml}$ were added to a platelet suspension ($4.6 \times 10^6/\text{ml}$) incubated for 15 min in the presence of 0.5 nM thrombin, the same level of factor Va activity was measured as in the absence of heparin.

If human thrombin was replaced by bovine thrombin, the kinetics of bovine thrombin-induced platelet factor Va generation were found to be identical to those reported here for human thrombin (data not shown). However, the IC_{50} values for the different heparins are significantly lower than those for human thrombin (Table II). This is in accordance with the higher affinity of heparin for bovine thrombin (16) and supports the idea that the heparins interact primarily with the thrombin.

Effect of Heparin on Platelet Beta-Thromboglobulin Release. In order to investigate if any interaction between platelets and heparin, might result in an impaired release reaction, we studied the release of another platelet alpha-granule protein, BTG. Platelets ($4.6 \times 10^7/\text{ml}$) were incubated with $10 \mu\text{g}$ collagen/ml or with 0.5 nM thrombin either in the absence or presence of crude heparin ($0.1 \text{ mg}/\text{ml}$). When platelets were stimulated by collagen,

heparin had no effect on the kinetics of BTG release, whereas heparin (100 $\mu\text{g/ml}$) diminished BTG release to less than 10% of the control value if thrombin was used to stimulate the platelets.

Effect of Heparin and Heparin Fragments on the Generation of Platelet Activity in Prothrombin Activation. Platelet prothrombin converting activity (PPCA) was measured under conditions where added factor Va exceeded greatly the amount of factor Va that was generated from platelets, so as to make the test independent of factor V release. By omitting phospholipids from our assay, the rate of thrombin formation becomes directly proportional to the amount of procoagulant surface that was generated upon platelet stimulation. It has been shown that platelets activated by a combination of collagen plus thrombin form a more suitable catalytic surface for the prothrombinase complex than platelets treated with thrombin alone (17). Therefore, we investigated the effect of heparins on human thrombin plus collagen-induced PPCA. Conditions had to be found where PPCA generation is linear in time and directly proportional to the thrombin concentration. The generation of a procoagulant surface was followed in time for platelets ($4.6 \times 10^6/\text{ml}$) stimulated with thrombin (0.3 nM) plus collagen (5 $\mu\text{g/ml}$) (Figure 3). The dependency of platelet activation on the thrombin concentration is shown in Figure 4, either in the absence or presence of 5 $\mu\text{g/ml}$ collagen. As a standard procedure to study the effect of heparin, platelets were stimulated for 3 min with 0.3 nM thrombin plus 5 $\mu\text{g/ml}$ collagen. Although collagen had to be added in order to potentiate the PPCA generating efficiency of low thrombin concentrations, it has to be mentioned that under these conditions the rate of PPCA generation is directly proportional to the thrombin concentration (Figure 4).

Figure 5 depicts the results of experiments where platelets ($4.6 \times 10^6/\text{ml}$) were incubated for 3 min at 37°C with varying concentrations of crude heparin, CY 216, CY 222 or Org 10172, followed by the stimulation of platelets during 3 min and measurement of prothrombin converting activity. It is interesting to see that crude heparin is not able to block completely the generation of prothrombin converting activity. Apparently, the remaining activity is equal to that generated by collagen in the absence of thrombin. Indeed, in a separate experiment we found that neither crude heparin nor heparin fragments were able to block the formation of collagen-induced PPCA. IC_{50} values were determined by analyzing the data as described in Materials and Methods. The replot of the data from Figure 5A according to Equation 3 is shown in Figure 5B. The intercept on the vertical axis represents the fraction of the PPCA activity generated by collagen alone. The IC_{50} values are listed in Table I.

Under conditions where the procoagulant surface is the limiting component of the prothrombinase complex, heparin might have as well an effect on the assembly of the complex and so affecting the PPCA assay (17). However, when heparin or heparin fractions were added to collagen plus thrombin stimulated platelets no decrease in PPCA was observed, indicating that heparin exerts solely an effect on the generation of PPCA (Table III).

Human thrombin plus collagen acting on human platelets requires more heparin or heparin fractions to attain 50% inhibition of PPCA than bovine thrombin does in the presence of collagen (Table II).

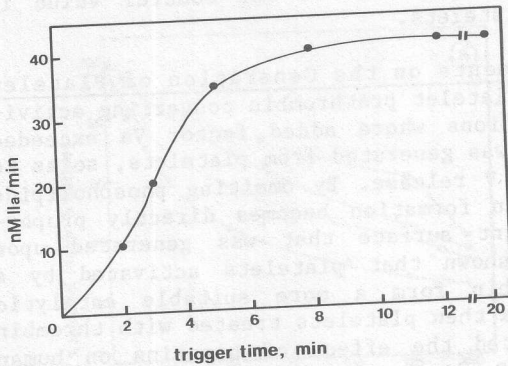


FIG. 3

Time course of appearance of PPCA. Platelets (4.6×10^6 /ml) were incubated at 37°C with 0.3 nM human thrombin and $5 \text{ }\mu\text{g/ml}$ collagen. At different trigger times, coagulation factors Xa, Va and prothrombin were added and thrombin formation was assayed as described in Material and Methods.

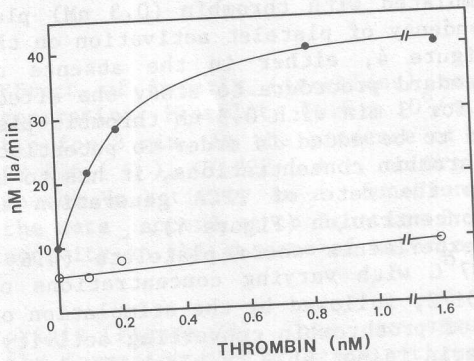


FIG. 4

PPCA generation as a function of human thrombin concentrations. Platelets (4.6×10^6 /ml) were triggered at 37°C for 5 min with varying human thrombin concentrations in the absence (○) or in the presence (●) of $5 \text{ }\mu\text{g/ml}$ collagen. Thrombin formation was assayed as described in Material and Methods.

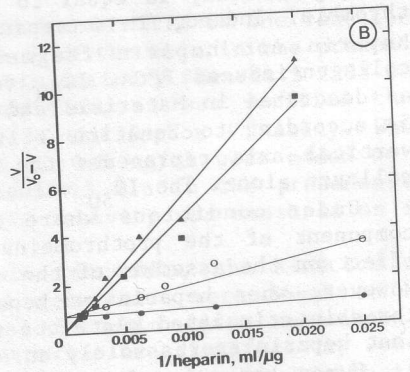
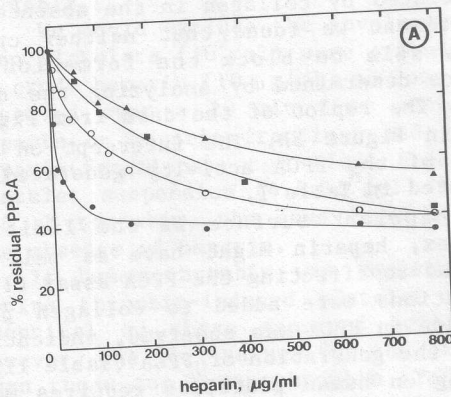


FIG. 5

Effect of heparin (fractions) on thrombin plus collagen-induced PPCA. Platelets (4.6×10^6 /ml) were triggered 3 min with 0.3 nM human thrombin and $5 \text{ }\mu\text{g/ml}$ collagen in the presence of varying concentrations of heparin (●), CY 216 (○), CY 222 (▲), or Org 10172 (■). A, percentage of residual PPCA as a function of heparin concentrations. B, replot of the data as described in Materials and Methods. 100 % PPCA in the absence of heparin was 24 nM Ila/min .

TABLE I

Effect of Heparin Fractions on Human Thrombin-Induced Factor Va Generation and Human Thrombin Plus Collagen-Induced PPCA

Compound	Factor Va generation IC ₅₀ (μg/ml)	PPCA IC ₅₀ (μg/ml)
Heparin	40	10
CY 216	130	100
CY 222	70	540
Org 10172	180	440

TABLE II

Effect of Heparin Fractions on Bovine Thrombin-Induced Factor Va Generation and Bovine Thrombin Plus Collagen-Induced PPCA

Compound	Factor Va generation IC ₅₀ (μg/ml)	PPCA IC ₅₀ (μg/ml)
Heparin	0.5	3.4
CY 216	3.3	3.8
CY 222	1.8	90.0
Org 10172	15.0	125.0

TABLE III

Effect of Heparin Addition on the Thrombin Plus Collagen-Induced Procoagulant Prothrombin Converting Activity

Compound	Rate of thrombin formation (nM/min)	PPCA (%)
None	24.0	100
Heparin (640 μg/ml)	24.0	100
CY 216 (1000 μg/ml)	22.4	92
CY 222 (1000 μg/ml)	23.4	96
Org 10172 (1000 μg/ml)	26.3	108

DISCUSSION

The heparin preparations that are in general use at this moment are extremely complex mixtures with components that differ with respect to molecular weight, chemical composition, secondary structures as well as antithrombin III affinity and other biological properties. It is not surprising that, besides the well known antithrombin III potentiating effect many other inhibitory actions on the haemostatic system can be found. Crude heparin shows a marked anticoagulant and antithrombotic, but also a hemorrhage-inducing effect. It was generally assumed that these three effects were intimately linked. More recently it has been shown that these three biological functions of crude heparin can to a large extent be dissociated (1-5). This suggests that different fractions of crude heparin can have different effects as to anticoagulation, antithrombotic action and the induction of hemorrhages. This is extremely interesting and of great practical interest because, where antithrombotic action is pursued and hemorrhage must be avoided, the therapeutic ratio between the two actions is always estimated on basis of anticoagulant action.

However, the relationship between *in vitro* kinetics as assessed by clotting assays and the antithrombotic action of heparin is far from being understood. Although binding to antithrombin III plays an imported role in the antithrombotic action of heparin, low-affinity heparin seems to contribute to the anti-thrombotic action of heparin (19).

Although platelets are essential to the process of blood coagulation, no data are available on the effects of heparin or heparin fractions on the generation of platelet procoagulant activities. The present study was undertaken to assess the effects of crude heparin, different heparin fractions and a heparinoid on thrombin-induced platelet factor Va generation and thrombin plus collagen-induced platelet prothrombin converting activity (PPCA).

Heparin IC_{50} values were determined under conditions where both initial rates of platelet factor Va and platelet PCA generation, were directly proportional to the thrombin concentration. The assumption was made that thrombin, when complexed with heparin, has no activity towards macromolecular substrates. Our experimental data appear to be consistent with that model (Figures 2D and 5B).

An interesting observation was the absence of effect of heparin (fractions) on the assembly and functioning of the prothrombinase complex at the outer membrane of activated platelets (Table III). This seems to be in contrast with the findings of Walker et al. (18). They reported that heparin inhibited prothrombin activation by preventing the binding of activated clotting factors as well as prothrombin to a phospholipid surface. When we performed equilibrium binding studies according to Van de Waart et al. (20) we found that crude heparin (100 μ g/ml) did not change the affinity of factor Va, factor Xa and prothrombin to phospholipid as well as total phospholipids binding capacity (to be published).

Thrombin-induced platelet factor Va generation is the result of release of the non-activated factor and subsequently activation by thrombin. Because our factor Va assay is insensitive for factor V the inhibitory action of heparin on the release reaction could not be separated from its action on thrombin-catalyzed factor V activation. However, we demonstrated that heparin did inhibit the thrombin-induced beta-thromboglobulin release, whereas heparin did not inhibit the collagen-induced beta-thromboglobulin release. This supports the notion that heparin exerts its effect through a heparin-thrombin rather than through a heparin-platelet interaction.

As to the IC_{50} values measured for the different heparins in the two thrombin-induced platelet reactions, it is obvious that for crude heparin relatively low IC_{50} values were found, compared to CY 216, CY 222 and Org 10172. Therefore, on a weight basis ($\mu\text{g/ml}$) crude heparin is a more potent inhibitor of both factor Va and PCA generation. The inhibitory capacity of a heparin fraction on thrombin induced reactions of platelet activation is not always in accordance with its antithrombin III-dependent antithrombin specific activity (anti-IIa U/mg). Moreover when IC_{50} values are given in anti-IIa U/ml, no correlation is found. This strongly suggests that, in studying the inhibitory action of heparin on thrombin, an antithrombin III-dependent effect can be distinguished from an independent one. As yet, we can only speculate about the occurrence in different heparin molecular species, of such differential antithrombin actions. It would be interesting to establish whether or not low-affinity heparins have antithrombin activities in platelet reactions as studied in the present report.

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