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The Mode of Action of Heparin in Plasma*

Suzette Béguin, Theo Lindhout, and H. Coenraad Hemker

From the Department of Biochemistry, Biomedical Center, University of Limburg, Maastricht, The Netherlands

Key words

Prothrombin activation – Heparin – Plasma protease inhibitors

Summary

The influence of heparin on prothrombin conversion in plasma was estimated by measuring prothrombin disappearance with the aid of staphylocoagulase or by calculation from the thrombin generation curve, compensating for simultaneous thrombin inactivation by plasma protease inhibitors. In thromboplastin-activated plasma the effect of heparin on prothrombinase (factor X_a-factor V_a-phospholipid) is negligible compared to that on thrombin probably because both the extrinsic factor X activating complex and phospholipid bound factor X_a are insensitive to AT III-heparin action. In contact-activated plasma prothrombinase generation is inhibited by heparin, because heparin lowers the ambient concentrations of thrombin so that the feedback activation of factor VIII by thrombin is diminished. Consequently, the delayed factor X_a generation causes a lag phase in prothrombinase generation. We conclude that heparin in plasma acts by its thrombin scavenging properties. No direct action on prothrombinase or on the factor X activating enzyme needs to be assumed if one takes into account the effect of thrombin depletion on thrombin-mediated feedback reactions.

Introduction

The anticoagulant effect of heparin is considered to be primarily due to the stimulating action of this drug on the inactivation of clotting proteases by antithrombin III (AT III). It has been demonstrated that the activated forms of the factors II, IX, X, XI and XII as well as kallikrein are susceptible to this action (see Ref. 1 for a review). No consensus has as yet been obtained on the question of which of the various possible inactivations are instrumental in causing the effect of heparin on the clotting of plasma.

Data on the decay of activated clotting factors added to plasma and on the influence of heparin on this process are readily and frequently obtained (1). However, for two reasons they cannot be used to judge the situation in clotting plasma. In the first place it has been found that the kinetics of inhibition of thrombin and factor X_a generated endogenously in plasma is different from those of purified proteases added to plasma (2–4).

In the second place, it should be recognized that for coagulation proteases to be generated at a significant rate, multimolecular complexes, consisting of a converting enzyme, a cofactor and a procoagulant surface, have to be formed (5). The converting enzyme, factor X_a for example, in this complex is heavily protected from inhibition by AT III/heparin (6–9).

The question of the mode of action of heparin in plasma has gained importance with the advent of low molecular weight heparins. It has been hypothesized that the antithrombotic properties of a heparin are related to its anti-factor X_a activity, whereas bleeding would correlate to antithrombin action (10). In this context it is tacitly assumed that classical heparin which undoubtedly has antithrombotic properties, does act on factor X_a in vivo. Today, this relationship is seriously questioned (11, 12).

In the present study we report on the effects of heparin on the generation and inhibition of the prothrombin converting, i.e. prothrombinase, activity in clotting plasma. Determination of prothrombinase activity requires the estimation of the rate of prothrombin conversion, which can be obtained by immunological determination of activation peptides (13) or by following the breakdown of labelled prothrombin (14, 15). Both methods are valid but set practical limits to the number of samples that can be handled and therefore do not allow estimation of the precise time course of prothrombinase activity.

Alternatively, one can study factor X activation but this requires the absence of prothrombin because artefacts due to thrombin generation have to be avoided (4). In prothrombin depleted plasma, however, effects of heparin on the thrombin-catalyzed activation of factor V and VIII are missed.

We therefore developed methods based on mathematical analysis of the thrombin generation curve in defibrinated plasma and on measurement of prothrombin consumption with the aid of staphylocoagulase (2). Part of the results obtained have been presented at the XIth ISTH Congress in Brussels 1987 (16).

Materials and Methods

Materials

The chromogenic substrate for thrombin was H. D-Phe-Pip-Arg-pNA (S 2238) from Kabi, Sweden. Kaolin was "Kaolin léger" provided by B. L. B. Laboratoires du Bois de Boulogne (Puteaux, France). The 4th International Standard for Heparin (194 U/mg) was from the National Institute for Biological Standards and Control. The phospholipid vesicle preparation contained 20% phosphatidylserine and 80% phosphatidylcholine and was prepared as described previously (17).

Plasmas

Blood from healthy donors was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution. A first and a second centrifugation were performed at 3,000 × g, at 15° C for 15 min. A third centrifugation was done at 4° C, for 1 hour at 23,000 × g. The platelet free plasma thus obtained was stored at −80° C. It was checked that the clotting factors and the antiproteases were in the normal range (18).

Defibrinated plasma was obtained by mixing an aliquot of plasma with 1:50 volume of a reptilase solution, letting a clot form for 10 min at 37° C and keeping the clotted plasma at 0° C for 10 min. The fibrin formed was discarded by centrifugation (10 min, 5,000 × g, 4° C) or by winding it on a small plastic spatula. The concentrations of factors II, V, VII, VIII, IX, X, XI, and XII did not significantly change by the reptilase treatment as has been shown before (2).

Proteins

Human brain thromboplastin was prepared as described in (19). It was homogenized in a Potter Elvehjem homogeniser for 3 min, centrifugated at 2,000 × g for 15 min and stored at −20° C in 0.1 ml aliquots. Before

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Correspondence to: H. C. Hemker, University of Limburg, Dept. of Biochemistry, P. O. Box 616, 6200 MD Maastricht, The Netherlands

use it was thawed, diluted with 0.05 M Tris-HCl pH 7.35 containing 100 mM of $CaCl_2$, so as to obtain a clotting time of 90 sec when incubated with normal non-defibrinated plasma under the same conditions as used for the thrombin generation experiments. The dilution required was 1:40 for the batch of thromboplastin used here and between 1:30 and 1:50 with other batches. The diluted thromboplastin was incubated at 37° C for 1 hour and then kept at room temperature. In this way it remained stable for at least 4 hours. Reptilase was obtained from Laboratoires Stago (Asnières, France), a solution was obtained according to the instructions of the manufacturer. Soybean trypsin inhibitor (batch no 43 F-800) was obtained from Sigma (St. Louis, USA). Staphylocoagulase, was prepared as described in Hendrix et al. (20) or obtained from Laboratoires Stago (Asnières, France) and prepared according to the instructions of the manufacturer. Human α-thrombin was prepared according to Pletcher and Nelsestuen (21), active site titrated and used for constructing a standard curve. Factors V, V_a and factor X_a were obtained as described by Lindhout et al. (22). Factor VIII was prepared according to Vehar and Davie (23) with the modifications by van Dieijen et al. (24). Factor IX was prepared according to Fujikawa et al. (25) and activated by incubation with purified factor XI_a (26).

Determination of Prothrombinase Activity in Plasma

To 200 µl of defibrinated plasma is added 50 µl of 0.05 M Tris-HCl, 0.1 M NaCl, 0.5 mg/ml ovalbumin, pH 7.35 containing heparin at the desired concentration and incubated for 5 min at 37° C. At zero time, thrombin generation is started by the addition of 50 µl of a solution containing 100 mM of $CaCl_2$ and a trigger of coagulation. For the extrinsic system this is human brain thromboplastin diluted as described above (final dilution 1:240), for the intrinsic system 1 µM phospholipid and 0.25 mg kaolin (final concentrations). When other triggers are used they are indicated in the legends. At intervals a 10 µl aliquot of the mixture is sampled into a disposable plastic cuvette containing 0.465 µl of 0.05 M Tris-HCl, 0.1 M NaCl, 20 mM EDTA, 0.5 mg ovalbumin/ml, pH 7.9 and 25 µl of S 2238 (4 mM) prewarmed for at least 5 min at 37° C. After 120 sec the reaction in the cuvette is stopped by adding 300 µl of concentrated acetic acid. The cuvettes are read at 405 nm in a LKB Ultrospec II spectrophotometer. The amidolytic activities in the plasma samples were calculated from the O.D. readings and expressed as the equivalent concentration of thrombin. The generation of prothrombinase activity was calculated from the amidolytic activity curve with the aid of a computer program as described previously (2). Briefly, the observed amidolytic activity is the sum of thrombin activity and the activity of the α_2M-thrombin complex. The rate of thrombin generation at any moment is the sum of two processes: a) the conversion of prothrombin into thrombin by prothrombinase and b) the decay of thrombin by the action of plasma protease inhibitors. The rate of inhibition of thrombin at any moment can be calculated from the concentration of thrombin at that time and the pseudo first order rate constant of inhibition of thrombin by antithrombin III (k_1) and α_2-macroglobulin (k_2). The rate of prothrombin activation (prothrombinase activity) than can be calculated from the observed rate of generation of amidolytic activity and the rate of thrombin inhibition.
In formula:

$$\int_0^t g(\tau)\,d\tau = A(t) + (k_1 + k_2 - fk_2)e^{-fk_2 t} \int_0^t A(\tau)\,e^{fk_2\tau}\,d\tau \qquad (1)$$

where g(t) = thrombin generation from prothrombin as a function of time, A(τ) = amidolytic activity found at time τ, f = the ratio of the enzymatic activity of 1 mole of α_2M-thrombin over that of 1 mole of free thrombin, k_1 = AT III dependent decay constant of thrombin in plasma, k_2 = α_2M dependent decay constant of thrombin in plasma.

Determination of the Pseudo First Order Rate Constant of Inhibition of Thrombin in Plasma

Defibrinated plasma (200 µl) was incubated with 24 µl of buffer containing 0.05 M Tris-HCl, 0.1 M NaCl, 0.5 mg ovalbumin/ml, pH 7.35. Thrombin generation was started as described in the previous section. Two minutes after the peak activity of thrombin, 10 µl of Soybean Trypsin Inhibitor (SBTI) solution (10 mg/ml) was added, together with 16 µl of buffer that contained the heparin to be tested. SBTI at this concentration was shown to stop completely and instantaneously the activity of prothrombinase and not to influence the activity of thrombin (2). At suitable time intervals after addition of the SBTI solution, 10 µl aliquots of the incubation mixture were added to cuvettes to measure residual thrombin activity as described. The pseudo first order rate constant of inhibition of thrombin generated in plasma was calculated by fitting the data to

$$C_t = C_R + C_o e^{-(k_1 + k_2)t} \qquad (2)$$

where C_t is the thrombin activity at time t, C_R is the steady end-level activity, $C_o + C_R$ is the thrombin activity at the time of SBTI addition, k_1 is the AT III-dependent decay constant of inhibition of thrombin, k_2 is the decay constant of inhibition of thrombin by α_2-macroglobulin (α_2-M). The parameters ($C_o + C_R$) and ($k_1 + k_2$) are calculated by means of a least squares fit of the model to the data, using the Box-Kanemasu modification of Gauss' method (27).

We have shown before that the amounts of thrombin complexed with α_2M and AT III are proportional to the respective rate constants of inhibition of thrombin. The amount of α_2M-thrombin can be determined directly from the residual thrombin activity, C_R, because α_2M-thrombin has under our experimental conditions an amidolytic activity of 56% of that of free thrombin (2). The amount of AT III-thrombin can be calculated from the amount of prothrombin consumed (P) and that is not accounted for by α_2M-thrombin.
Thus,

$$k_2/k_1 = 1.88\,C_R/(P - 1.88C_R) \qquad (3)$$

The rate constants, k_1 and k_2, then can be obtained from equations 2 and 3.

Measurement of Residual Prothrombin Concentration

The residual prothrombin concentration during thrombin generation in plasma was estimated by taking samples (20 µl) at 1 min intervals and incubation with 20 µl of 0.05 M Tris-0.1 M NaCl-0.5 mg/ml ovalbumin, pH 7.9 containing 20 mM EDTA and 2 U/ml of heparin. In this way thrombin in the plasma sample was inactivated almost instantaneously. To a 10 µl portion of this mixture 10 µl of a 2 µM staphylocoagulase solution was added. After 2 min incubation at 37° C the thrombin-like amidolytic activity was measured. This activity (A) gives the amount of prothrombin, because the prothrombin-staphylocoagulase (1:1 mol/mol) complex has the same molar activity towards S 2238 as thrombin and is not inactivated by AT III/heparin (20), plus the amount of α_2M-thrombin already present in the sample. Another portion of the incubation mixture, prior to the addition of staphylocoagulase, was assayed for the amount of α_2M-thrombin (B).

The residual prothrombin concentration at time t was calculated from the difference between the activities prior and after the incubation with staphylocoagulase ($A_t - B_t$). The rate of prothrombin consumption V_t, during thrombin generation in plasma could then be calculated directly from

$$V_t = [(A_{t2} - B_{t2}) - (A_{t1} - B_{t1})]/(t_2 - t_1)$$

Results

In order to investigate the effect of heparin on prothrombinase generation and inactivation in clotting plasma we used two methods: i) an indirect method that calculated the prothrombinase activity from the generation of thrombin like amidolytic activity and ii) a direct measurement of residual prothrombin levels.

In the following section we show that these methods give comparable results. Because the indirect method is more precise and quicker, the remainder of the experiments were performed by this method.

Choice and Evaluation of the Method

Our method of calculating prothrombinase activity from amidolytic activity curves requires pseudo first order reaction

kinetics between thrombin and its plasma inhibitors. This simplifying assumption could be shown to be acceptable within the limits of experimental error in the absence of heparin (2). It is seen in Fig. 1 that the decay of thrombin in the presence of heparin can not be distinguished from a logarithmic approach to a steady end-level, indicating that the process can in good approximation be described as the sum of two pseudo first order reactions, one inactivating thrombin completely (complexing by AT III and thrombin inhibitors of minor importance) and the other yielding a product with a persisting amidolytic activity (α_2-macroglobulin-thrombin complex).

This allowed us to calculate the time course of prothrombinase activity in the presence of heparin. In order to validate the results we compared them to prothrombin consumption velocities as measured directly with the aid of staphylocoagulase as described in Materials and Methods.

This procedure involves calculations on the differences between large values. Even though the standard error of each of the determinations can be reduced to 3–4%, the standard error of the resulting prothrombin disappearance rate was calculated to be between 9 and 15%.

From Fig. 2 it can be seen that at two heparin concentrations the rate of prothrombin activation as calculated from the thrombin generation curve coincided tolerably with the rate of prothrombin activation as determined directly with the aid of staphylocoagulase.

We conclude that our mathematical approach gives an acceptable picture of the rate of prothrombin activation in the absence as well as in the presence of heparin. We cannot exclude the possibility of a slight systematic overestimation of the velocities in the descending limb of the curve. This does not, however, influence the interpretation of the observations.

Effect of Heparin on the Decay of Thrombin Generated in Plasma

In order to compute the prothrombin conversion velocity, i.e. prothrombinase activity, from thrombin generation curves at a series of heparin concentrations it is necessary to determine the breakdown constants of thrombin in plasma in the presence of different concentrations of heparin. This was done as shown in Fig. 1. At all heparin concentrations tested, curves were obtained that showed a logarithmic approach to a final level. Data analysis as described in Materials and Methods resulted in a pseudo first order rate constant of inhibition of thrombin by α_2-macroglobulin of 0.232 ± 0.004 (S.E.M.; $n = 25$) min^{-1}, and was independent of the heparin concentration. The antithrombin III-dependent rate constant of inhibition of thrombin generated in plasma as a function of the heparin concentration is depicted in Fig. 3.

Heparin and the Generation of Prothrombinase Activity in Thromboplastin-Activated Plasma

We determined the course of the generation of thrombin like amidolytic activity in plasma triggered by thromboplastin in the presence of different concentrations of heparin. As expected heparin had a clearcut reducing effect on thrombin yield (Fig. 4). From these curves we calculated the course of thrombin generation (i.e. the activity without the contribution of the α_2M-

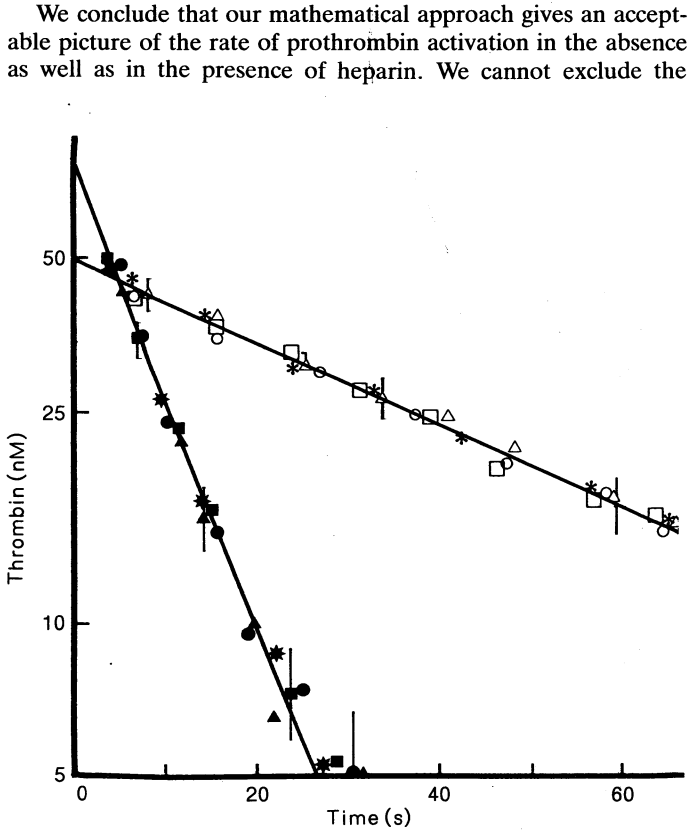

Fig. 1 Inactivation of thrombin, generated in thromboplastin-activated plasma, in the presence and absence of heparin. Defibrinated plasma was incubated with thromboplastin (final dilution 1:240). After 5 min SBTI was added and aliquots were taken to measure amidolytic thrombin activity until a steady end-level, C_R, of residual thrombin activity, i.e. α_2M-thrombin, was obtained (equation 2, Materials and Methods). The vertical axis (log scale) represents the measured thrombin activity minus C_R. The horizontal axis represents the reaction time after the addition of SBTI. Open symbols: no heparin added. Closed symbols: 0.1 U/ml of heparin was added simultaneously with SBTI. The different symbols represent repeated runs of the experiment

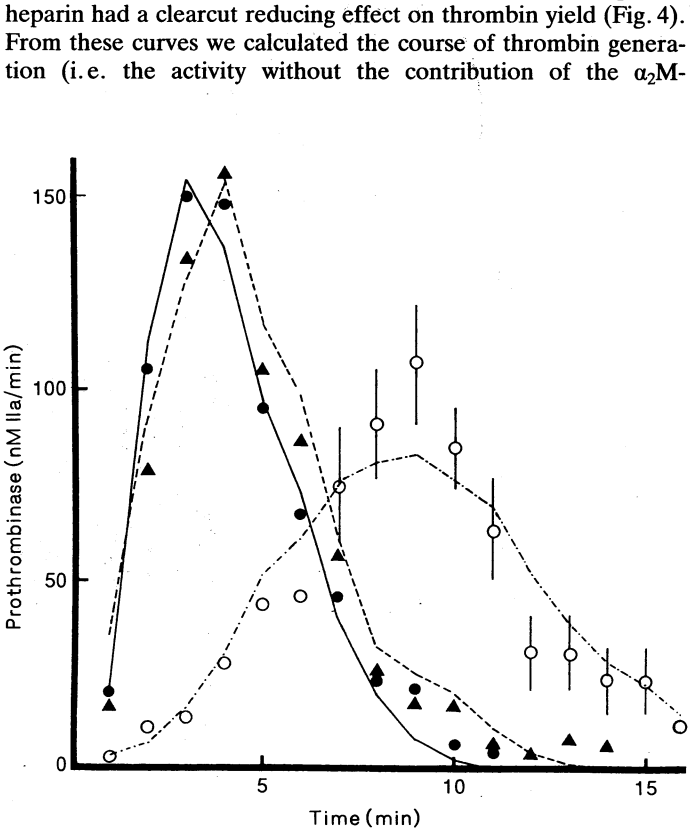

Fig. 2 Generation of prothrombinase activity in thromboplastin-activated plasma. Defibrinated plasma was incubated with thromboplastin (final dilution 1:240) in the absence (●) or presence of 0.02 U/ml (▲) or 0.1 U/ml (○) of heparin as described in Materials and Methods. The symbols represent the rate of prothrombin activation as calculated from residual prothrombin concentrations using staphylocoagulase (see Materials and Methods for further details). The drawn lines join the rates of prothrombin activation calculated from the thrombin generation curves according to equation 1 (Materials and Methods). The error bars are $2 \times$ S.D.

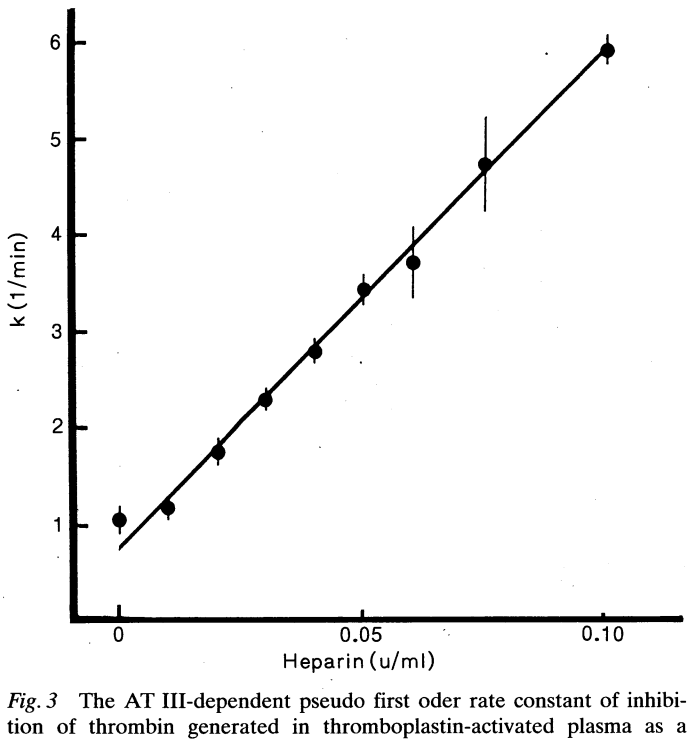

Fig. 3 The AT III-dependent pseudo first oder rate constant of inhibition of thrombin generated in thromboplastin-activated plasma as a function of the heparin concentration. The pseudo first order rate constant, $k_1 + k_2$, was obtained from a plot as shown in Fig. 1. The rate constant of the AT III-dependent inactivation of thrombin, k_1, was obtained by assuming a rate constant of inhibition of thrombin by α_2M, k_2, of 0.232 min^{-1} (see text for further details)

thrombin complex) and the course of the prothrombinase activity curves, using the appropriate rate constant of inhibition endogenously generated thrombin as shown in Fig. 3. The results of these calculations are summarized in Fig. 5.

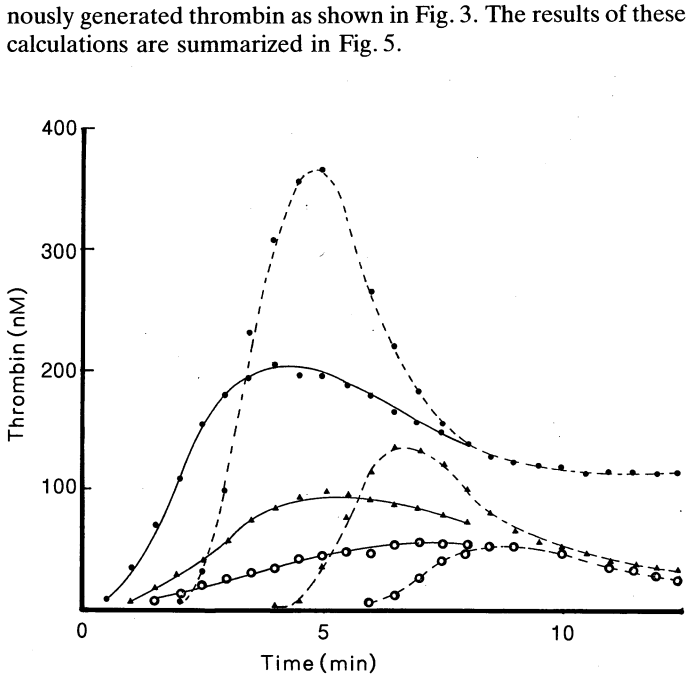

Fig. 4 The effects of heparin on the amidolytic activity generated in plasma via the intrinsic and the extrinsic pathway. The amidolytic activity i.e. the activity of thrombin plus the α_2 macroglobulin-thrombin complex, is expressed as the equivalent amount of thrombin. The curves were obtained as described in Materials and Methods. No heparin: extrinsic (●—●) intrinsic (●---●); 0.05 U/ml heparin: extrinsic (▲—▲) intrinsic (▲---▲); 0.1 U/ml heparin: extrinsic (○—○) intrinsic (○---○)

The most intriguing feature is that the peak activity of prothrombinase generation is hardly inhibited by concentrations of heparin that have an important effect on the thrombin yield. In the concentration range of 0.03–0.04 U/ml, heparin seems even to have a slight enhancing effect on the peak prothrombinase activity. On the other hand the velocity at which the prothrombinase activity builds up is slightly slowed down (results not shown, see ref. 16). At 0.1 U/ml of heparin there is a definite inhibition of both the initial rate of prothrombinase generation and peak prothrombinase activity. The descending slopes of the prothrombinase activity generation curves are the result of prothrombin depletion (2).

Heparin and the Generation of Prothrombinase Activity in Contact-Activated Plasma

After the thromboplastin-induced thrombin generation, we studied the intrinsic pathway as triggered by kaolin in the presence of phospholipid. As shown in Fig. 4, an effect of heparin could be observed, not only on the amount of thrombin generated but also on the lag time of thrombin generation. When we calculated the effect of heparin on the time course of prothrombin activation a significant inhibition of the prothrombinase generation was observed (Fig. 5). In contrast to the situation in thromboplastin-activated plasma, prothrombinase generation via the intrinsic system seems to be inhibited by heparin even at low

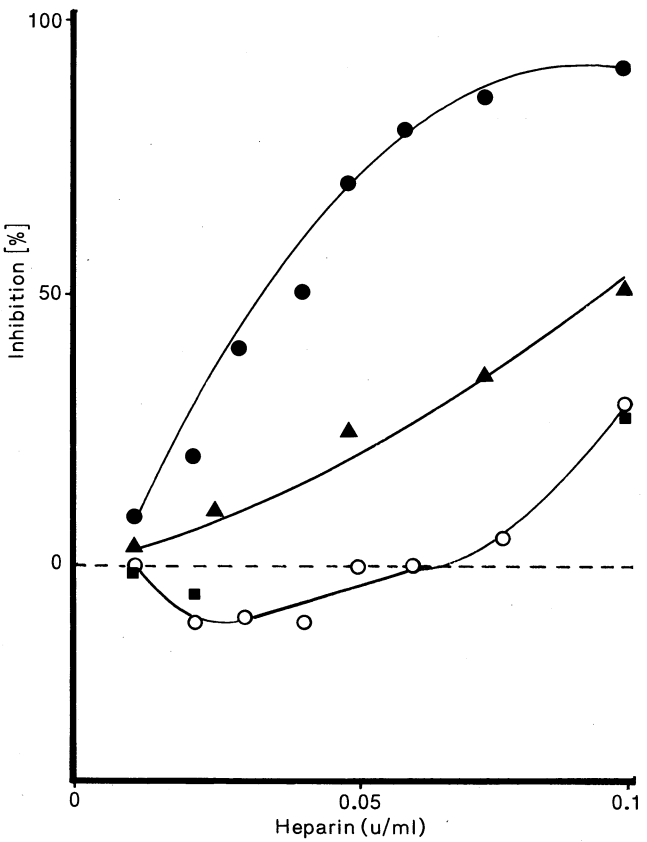

Fig. 5 The inhibition of thrombin generation and prothrombinase generation by heparin. With equation 1 the course of thrombin activity and prothrombinase activity was calculated from data as in Fig. 4. The inhibition of the peak activities relative to an uninhibited control are given. Inhibition of extrinsic thrombin generation (●—●); inhibition of intrinsic prothrombinase generation (▲—▲); inhibition of extrinsic prothrombinase generation. Computed (○—○) and determined by the staphylocoagulase method (■)

concentrations. There is no reason to assume that the prothrombinase itself is different in the two cases, so it is likely that the generation of factor X_a via the intrinsic pathway is inhibited by heparin.

In an attempt to differentiate between an effect on the factor X activating complex itself ($FVIII_a$, FIX_a, phospholipid) and an effect on the generation of the factor X activating complex (factors $VIII_a$ and IX_a generation), we examined the effect of heparin on thrombin generation in plasma activated by a fully assembled factor X converting complex that consisted of the purified factors $VIII_a$ and IX_a, phospholipid and Ca^{2+}. Thrombin generation thus triggered was inhibited by heparin but the lag time had disappeared (data not shown). Like in the case of the extrinsic pathway, the inhibition of thrombin generation must be mainly attributed to the scavenging of thrombin, because when the course of prothrombinase activity was calculated no inhibition was seen at concentrations of up to 0.075 U/ml of heparin and only 35% inhibition was caused by 0.1 U/ml of standard heparin.

From these results we concluded that it was likely that in the case of kaolin activation, the formation of one of the protein constituents of the intrinsic factor X activator is inhibited by heparin. We therefore added one or the other constituent in preactivated form (Fig. 6). When clotting is started by activated factor IX and phospholipid, the inhibition by a heparin concentration of 0.05 U/ml is overwhelming. When activated factor VIII is added to a plasma in which thrombin generation is started by kaolin the inhibition of thrombin formation that was observed is comparable to that seen in the extrinsic system and can be explained by thrombin scavenging. Moreover, no lag phases were observed. When the factor VIII used in this experiment was not preactivated with thrombin, then the inhibition by heparin reappeared, as did the lag phases.

We concluded that the presence of factor $VIII_a$ but not that of factor IX_a prevents the inhibition of intrinsic prothrombinase generation.

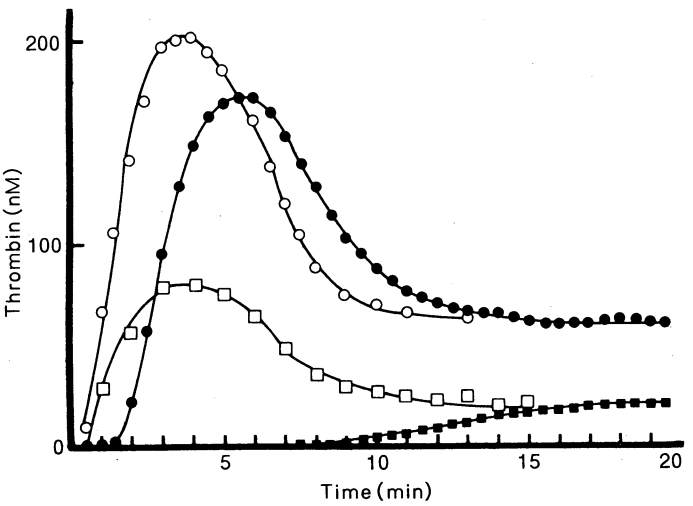

Fig. 6 The effect of heparin on kaolin- and factor IX_a-induced amidolytic activity in plasma. The amidolytic activity, i.e. the activity of thrombin plus the α_2 macroglobulin-thrombin complex, is expressed as the equivalent amount of thrombin. Defibrinated plasma was incubated with kaolin (0.83 mg/ml), calcium (20 mM), phospholipid (1 μM) and factor VIIIa (1 U/ml) in the absence (○) or presence (□) of 0.05 U/ml of heparin, or defibrinated plasma was incubated with 2 nM factor IX_a, 20 mM calcium, 1 μM phospholipid, either in the absence (●) or presence (■) of 0.05 U/ml of heparin

Discussion

Ever since it has been reported that phospholipids and factor V_a can protect factor X_a from the action of AT III (-heparin) it has remained an open question what the importance of this phenomenon is for the coagulation of plasma, especially in the presence of heparin (6–9). It was our purpose to investigate to what extent factor X_a and the factor X activating proteases are inhibited by AT III(-heparin) during thrombin generation triggered via the extrinsic and the intrinsic pathway in the complex medium of whole plasma. This question is relevant to the problem of the mode of action of heparin in vivo. Any action that can be demonstrated not to occur in plasma can hardly be expected to contribute to the antithrombotic effect of heparin.

In order to estimate the rate of prothrombin conversion as a function of time, we used two independent methods i) direct estimation of the level of residual prothrombin in the course of time with the aid of staphylocoagulase and ii) indirect computation of the prothrombinase activity from the experimentally observed rate of thrombin generation corrected for the simultaneous thrombin decay caused by AT III(-heparin) and other protease scavengers (2).

The two methods appear to give similar results both in the absence and in the presence of heparin (Fig. 2). The staphylocoagulase method is relatively cumbersome and, because the velocities are calculated by subtraction of four activities, tends to large experimental errors. We therefore used the indirect method in further experiments.

From Fig. 3 it can be seen that in plasma between 0.005 and 0.010 U/ml of heparin, heparin seems to be neutralized probably by high affinity binding to plasma proteins, other then AT III (28–30). After compensating for this effect we found a linear increase of the pseudo first order rate constant of inhibition of thrombin generated in plasma with the heparin concentration of 8.6 min^{-1} mg^{-1} ml^{-1}. This is considerably lower then was found in purified systems (38.7 min^{-1} mg^{-1} ml^{-1}, data not shown). At least two phenomena may explain this difference: i) Heparin may bind reversibly to various plasma proteins and thus, its effective concentration may be lower than the amount added would predict. ii) Thrombin generated in situ is less vulnerable to AT III than exogenous thrombin is (2), as has also been demonstrated for thrombin formed by prothrombinase from prothrombin in a purified system (31).

From Fig. 4 it can be seen that there are essential differences between the effect of heparin on the intrinsic and the extrinsic system. In the extrinsic system there is no lag phase either in the presence or absence of heparin. In the intrinsic system there is a lag phase, that is significantly increased by heparin.

The peak amount of prothrombinase activity triggered by thromboplastin and Ca^{2+} is not significantly inhibited by 0.075 U/ml of heparin, a concentration that inhibits the net maximal velocity of thrombin formation as much as 85%. This means that the velocity of prothrombin conversion must be the same in the presence and in the absence of heparin so that the inhibition of the net generation of thrombin must be entirely contributed to the increase of thrombin scavenging in the presence of heparin.

Heparin, at concentrations that do not reduce prothrombinase generation induced via the extrinsic pathway, does inhibit the development of prothrombinase activity when thrombin generation is triggered via the intrinsic system (Fig. 5). This must be caused by inhibition of the phospholipid-Ca^{2+}-factor IX_a-factor $VIII_a$ complex itself or by inhibition of the generation of one of its constituents. The generation of factor IX_a has been demonstrated not to be inhibited in plasma (32, 33). Thus, either factor IX_a is scavenged and/or the generation of factor $VIII_a$ is inhibited by heparin. To distinguish between these possibilities we added either one or the other factor in its activated form from zero time, while the other had to be generated during the process. A lag time

is seen only when no factor $VIII_a$ is added to the mixture and then the lag times increase with heparin concentration. The observed lag phases probably represent the time necessary for a sufficient amount of factor VIII to be activated.

Our main conclusion is that the inhibition by heparin of prothrombinase generation is due to inhibition of the feedback activation of factor VIII.

Our observation agree with the fact that the specific thrombin inhibitor D-Phe-Pro-Arg-Chloromethylketone (PPACK) inhibits prothrombin activation (14) and factor X activation via factor IX_a (15) and supports the explanation of this phenomenon by the inhibition of feedback activation.

Our results also agree with the observations of Ofosu et al. (14) who showed that the effective inhibition of the generation of thrombin activity appears to require sulphated polysaccharides which can potentiate the inactivation of thrombin. These authors also report inhibition of prothrombinase by standard heparin in antithrombin III depleted plasma and by heparin with a low affinity to antithrombin III in normal plasma (34). However, these effects are seen at heparin concentrations (0.2–3 U/ml) that are appreciably higher then those used here.

The concentrations of heparin that we studied are relatively low. We do think that they are relevant for the situation in patients that receive heparin because such levels are routinely encountered in low dose heparin treatment.

Our results explain a well known paradox from the clinical laboratory. Although both the thrombin time and the activated partial thromboplastin time (APTT) are sensitive to heparin, the prothrombin time is not. When we carried out experiments with non-defibrinated plasma it appeared that coagulation is always seen at the start of the explosive formation of thrombin. The APTT actually measures the lag time of thrombin formation in the intrinsic system. This lag time is determined by the availability of thrombin for the activation of factor VIII. The APTT, therefore, must be considered to be affected primarily by the action of AT III/heparin on thrombin just like the thrombin time is.

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