Unfractionated heparin inhibits thrombin-catalysed amplification reactions of coagulation more efficiently than those catalysed by Factor Xa

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We have proposed previously that the steps in coagulation most sensitive to inhibition by heparin are the thrombin-dependent amplification reactions, and that prothrombinase is formed in heparinized plasma only after Factor Xa activates Factor VIII and Factor V. These propositions were based on the demonstration that both heparin and Phe-Pro-Arg-CH$_2$Cl completely inhibited ^{125}I-prothrombin activation for up to 60 s when contact-activated plasma (CAP) was replenished with Ca^{2+}. Furthermore, the addition of thrombin to CAP before heparin or Phe-Pro-Arg-CH$_2$Cl completely reversed their inhibitory effects. Additional support for the above hypotheses is provided in this study by demonstrating that, when the activity of thrombin is suppressed by heparin (indirectly) or by Phe-Pro-Arg-CH$_2$Cl (directly), exogenous Factor Xa reverses the ability of these two agents to inhibit prothrombin activation. Prothrombin activation was initiated by adding Factor Xa (1 nM) or thrombin (1 or 10 nM) simultaneously with CaCl$_2$ to CAP. In the absence of heparin or Phe-Pro-Arg-CH$_2$Cl, prothrombin activation was seen 15 s later in either case. Heparin failed to delay, and Phe-Pro-Arg-CH$_2$Cl delayed for 15 s, prothrombin activation in CAP supplemented with Factor Xa. In contrast, heparin and Phe-Pro-Arg-CH$_2$Cl completely inhibited prothrombin activation for at least 45 s in CAP supplemented with 1 nM-thrombin. Heparin failed to delay prothrombin activation in CAP supplemented with 10 nM-thrombin, whereas Phe-Pro-Arg-CH$_2$Cl completely inhibited prothrombin activation in this plasma for 45 s. These results suggest that in CAP: (1) Factor Xa can effectively activate Factor VIII and Factor V when the proteolytic activity of thrombin is suppressed; (2) heparin–antithrombin III is less able to inhibit Factor Xa than thrombin; (3) suppression of the thrombin-dependent amplification reactions is the primary anticoagulant effect of heparin.

INTRODUCTION

There is evidence that the primary effect of heparin on inhibiting intrinsic coagulation is to delay prothrombin activation (Ofosu et al., 1987a; Hemker, 1987). We have proposed that during coagulation the efficient catalytic action of heparin on thrombin inhibition by antithrombin III results in the inhibition of thrombin-mediated activation of Factor VIII and Factor V, thereby delaying prothrombinase formation (Ofosu et al., 1987a). Factor Xa can also activate Factor VIII and Factor V (Smith & Hanahan, 1976; Vehar & Davie, 1980; Foster et al., 1983). Secondly, Factor Xa is less sensitive than thrombin to inhibition by antithrombin III, with and without heparin (Jordan et al., 1980; Ofosu et al., 1984). We have proposed, therefore, that when contact-activated plasma (CAP) contains heparin at concentrations capable of efficiently catalysing thrombin inhibition, prothrombin activation occurs only after the Factor Xa, generated in situ, activates Factor VIII and Factor V (Ofosu et al., 1987a).

To assess the roles of the Factor Xa-dependent and the thrombin-mediated amplification reactions in normalizing prothrombin activation in the presence of heparin, we compared the relative abilities of exogenous Factor Xa and thrombin to reverse the inhibitory effect of heparin on prothrombin activation in CAP. We quantified, by enzyme-linked immunosorbent assays (e.l.i.s.a.s), the concentrations of prothrombin and thrombin–inhibitor complexes after CAP was replenished with Ca^{2+}. Our results are consistent with the hypothesis that the effectiveness of heparin as an anticoagulant results from its ability to inhibit the amplification catalysed by thrombin, thereby delaying prothrombinase formation until Factor Xa-dependent amplification reactions replace those that are usually effected by thrombin.

MATERIALS AND METHODS

Materials

Activated-partial-thromboplastin-time (APTT) reagent was obtained from Organon Teknica, Toronto, Ont., Canada. Phe-Pro-Arg-CH$_2$Cl and dansyl-Glu-Gly-Arg-

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Abbreviations used: CAP, contact-activated plasma; e.l.i.s.a., enzyme-linked immunosorbent assay.
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CH$_2$Cl, chloromethyl ketone inhibitors of thrombin and Factor Xa respectively (Kettner & Shaw, 1979), were obtained from Behring–Calbiochem, San Diego, CA, U.S.A. Rabbit anti-(human prothrombin) and rabbit anti-(human antithrombin III) sera were obtained from Behring, Montreal, Que., Canada, and Wellmark Diagnostics, Guelph, Ont., Canada. S-2238 (D-Phe-Pip-Arg-NH-Np) was obtained from Ingram and Bell, Toronto, Ont., Canada. Goat anti-(rabbit IgG) antibody–alkaline phosphatase conjugate was obtained from Dimension Laboratories, Toronto, Ont., Canada. Arvin was obtained from Connaught Laboratories, Toronto, Ont., Canada. Pig mucosal heparin, with a specific activity of 145 U.S.P. units/mg, was obtained from Organon, Oss, The Netherlands. p-Nitrophenyl phosphate and other reagent-grade chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Dermatan sulphate was obtained from Mediolanum Farmaceutici, Milan, Italy. Procedures for the purification of Factor Xa have previously been described (Ofosu et al., 1981; Modi et al., 1984). Bovine Factor Va was prepared by using the method of Esmon (1979). Human α-thrombin, with a specific activity of 2870 U.S.P. units/mg, was prepared by the method of Fenton et al. (1977). Human antithrombin III and heparin cofactor II were prepared by the methods of Miller-Andersson et al. (1974) and Yamagishi et al. (1984) respectively. Some of the heparin cofactor II used in this study was a gift from Dr. Frank Church, University of N. Carolina, Chapel Hill, NC, U.S.A.

Antisera production and affinity purification of the antibodies

Monospecific sheep antiserum to human prothrombin was produced by the subcutaneous injection of 0.5–1 mg of human prothrombin (in complete Freund's adjuvant) followed 4 weeks later by biweekly injections of 0.2 mg of human prothrombin in 1 ml of 0.15 M-NaCl/10 μM-sodium phosphate buffer, pH 7.4, for a total of 16 weeks. A similar protocol was used to raise sheep antiserum to human heparin cofactor II. The antisera were shown to be monospecific since each formed a single precipitin line with human plasma on immunoelectrophoresis as well as with the immunogen. The IgG fraction from each antiserum was isolated by chromatography on DEAE-Affigel Blue.

To prepare antibodies that reacted with human thrombin and prothrombin but not with prothrombin fragment 1.2, the IgG fraction from sheep antiserum to human prothrombin was fractionated on a thrombin–Sepharose 4B affinity column. The IgG (100 mg in 0.1 M-sodium phosphate buffer, pH 8.0) was filtered on a 15 ml thrombin–Sepharose 4B column containing 40 mg of α-thrombin previously inactivated by the addition of equimolar Phe-Pro-Arg-CH$_2$Cl at 37 °C. The flow-through IgG was discarded; the column was then washed with 4 bed volumes of 0.1 M-sodium phosphate buffer, pH 8.0, and then 2 bed volumes of 2.0 M-NaCl in 0.1 M-sodium phosphate buffer, pH 8.0. The IgG eluted in 2.0 M-NaCl reacted only weakly with prothrombin and failed to react with thrombin on immunoelectrophoresis. The IgG still bound to the affinity column was then eluted with 0.1 M-sodium citrate buffer, pH 3.5, into $\frac{1}{10}$ vol. of 2.0 M-Tris/HCl buffer, pH 8.0, and immediately dialysed overnight against 0.1 M-sodium phosphate buffer, pH 8.0, and stored in 0.1 ml portions at −50 °C until

used. This antibody, called sheep anti-(human thrombin) antibody, reacted on immunoelectrophoresis with both thrombin and prothrombin but not with prothrombin fragment 1.2.

The two rabbit antisera to human prothrombin (5 ml) were also subjected individually to affinity chromatography on a 5 ml column of human prothrombin–Affigel-15 containing 50 mg of human prothrombin bound to the Affigel-15. A washing and elution scheme similar to that described for the isolation of affinity-purified antithrombin antibody was employed for the affinity purification of rabbit anti-(human prothrombin) antibody. One resulting product (Behring) reacted strongly with prothrombin but weakly with thrombin, whereas the second (Wellmark) reacted equally well with both thrombin and prothrombin on immunoelectrophoresis. Neither rabbit anti-(human prothrombin) antibody reacted with human prothrombin fragment 1.2 after affinity purification of prothrombin–Sepharose.

Prothrombin e.l.i.s.a.

To quantify prothrombin in normal plasma or in CAP after CaCl$_2$ had been added, 400 ng of the affinity-purified sheep anti-thrombin antibody (in 0.2 ml of 0.1 M-sodium bicarbonate buffer, pH 9.6) was added to each well of a 96-well poly(vinyl chloride) micro-titre plate and the plate was incubated at 4 °C for 18 h. The unbound antibody was then removed by suction and each well was washed with 0.2 ml of Tween/phosphate buffer (0.05 % Tween 20 in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4). Fatty acid-free bovine serum albumin (0.2 ml of a 1 mg/ml solution in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) was then added to each well and the plate was incubated at 37 °C for 1 h. Each well was then washed four times with 0.2 ml of the Tween/phosphate buffer. Subsequently 100 μl of diluted plasma or diluted CAP that had been replenished with Ca^{2+} was added and the plate was incubated at 37 °C for 1 h. The diluent in each case was 0.05 % Tween 20 in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 1 mg of fatty acid-free bovine serum albumin/ml. A minimum of three dilutions of each test sample and six of the prothrombin standard were used. The initial and final dilutions of unactivated plasma were 1:10000 and 1:200000 respectively, representing prothrombin concentrations varying from approx. 1 to 20 ng/ml. The wells were next washed four times with Tween buffer, after which 0.1 ml of 2 μg/ml solution of the affinity-purified rabbit anti-(human prothrombin) antibody (i.e. the Behring product, which reacted preferentially with prothrombin) was then added to each well and the plate was incubated at 37 °C for 1 h. Each well was then washed four times with the Tween/phosphate buffer, and this was followed by the addition of 0.1 ml of goat anti-(rabbit IgG) antibody–alkaline phosphate (diluted 1:1000 with Tween/phosphate buffer) to each well. After an incubation of 1 h at 37 °C, each well was washed four times with Tween/phosphate buffer. The substrate (0.1 ml of a 1 mg/ml solution of p-nitrophenyl phosphate in 0.5 M-sodium carbonate buffer, pH 10.4, and containing 1 mM-MgCl$_2$) was then added to each well and the plate was incubated for a final 1 h at 37 °C, and the hydrolysis of the substrate obtained was quantified at 405 nm. No cross-reactivity was detected when thrombin (10–50 μg/ml), Factor IX (1–10 μg/ml) or Factor X (10–50 μg/ml) was substituted for prothrombin.

E.l.i.s.a.s for thrombin–antithrombin III and thrombin–heparin cofactor II complexes

The above procedure was adapted to quantify thrombin–antithrombin III and thrombin–heparin cofactor II complexes. Since the sheep anti-(human thrombin) antibody immobilized to the wells of microtitre plates as the
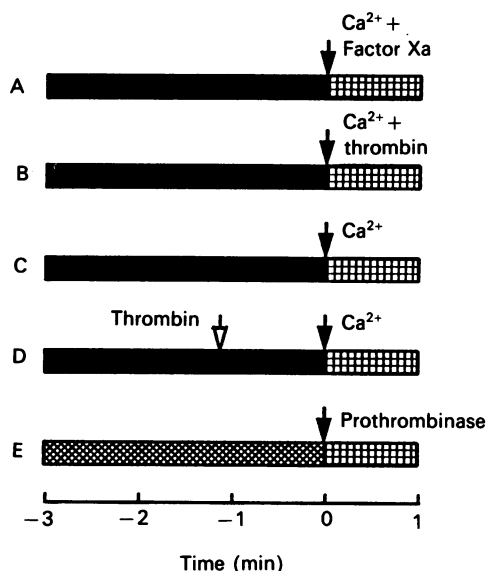

Time (min)

Fig. 1. Summary of the procedures used to initiate prothrombin activation in plasma

In procedures A to D 1 vol. of APTT reagent was added to 2 vol. of defibrinated plasma and the suspension was incubated for 3 min at 37 °C (as indicated by ■). Then 1 vol. of pre-warmed 40 mM-CaCl$_2$ (indicated by ↓ and ▦) was added to initiate prothrombin activation. Timed samples (1 vol.) were then taken into 4 vol. of cold 5 mM-EDTA, which also contained 1 μM-Phe-Pro-Arg-CH$_2$Cl, 1 μM-dansyl-Glu-Gly-Arg-CH$_2$Cl and 1 mg of bovine serum albumin/ml. This step halted further prothrombin activation and thrombin inhibition (Ofosu et al., 1987a). Residual prothrombin and the thrombin–antithrombin III complex formed were then quantified by specific e.l.i.s.a.s. No activation of Factor VIII or Factor V is likely to have taken place in procedures A, B and C before CaCl$_2$ addition. In procedure D some activation of Factor VIII and Factor V by thrombin would have occurred before CaCl$_2$ addition. In procedure A 1 nM-Factor Xa was added with the CaCl$_2$, whereas in procedure B thrombin (1 nM or 10 nM) was added with the CaCl$_2$. In procedure C no coagulant enzyme was added with CaCl$_2$; procedure C thus represented control CAP. In procedure D the thrombin was added for 1 min during contact-activation (indicated by ▽), and before CaCl$_2$. In procedure E 1 vol. of unactivated plasma was incubated at 37 °C for 3 min (▨). Prothrombin activation was initiated by adding an equal volume of prothrombinase to the pre-warmed plasma. Prothrombinase consisted of a suspension of kephalin (containing 6 μg of organic phospholipids from rabbit brain), 1 nM-Factor Xa, 1 nM-Factor Va and 10 mM-CaCl$_2$ (all as final concentrations). Regardless of the stimulus used to initiate prothrombin activation, the final dilution of plasma was 1 in 2. When present in an experiment, heparin was always added with the CaCl$_2$. Phe-Pro-Arg-CH$_2$Cl was also added with the CaCl$_2$, except in procedure B, where the chloromethyl ketone was added to the CAP 15 s before the simultaneous addition of CaCl$_2$ and thrombin.

first antibody in the e.l.i.s.a. for thrombin–antithrombin III complex or thrombin–heparin cofactor II complex reacted equally with thrombin and prothrombin, it was necessary to remove prothrombin from the plasma before quantification of thrombin–inhibitor complexes. Prothrombin was thus precipitated from the plasma by the addition of BaCl$_2$ (40 mM final concentration) followed by centrifugation at 10 000 g for 10 min at 4 °C. Thrombin–antithrombin III and thrombin–heparin cofactor II complexes remained in the supernatant. Failure to precipitate prothrombin with BaCl$_2$ resulted in only a 50 % recovery of thrombin–antithrombin III complex or thrombin–heparin cofactor II complex, each added to plasma to a final concentration of 10 nM.

To quantify thrombin–antithrombin III, the first antibody immobilized was the affinity-purified sheep anti-(human thrombin) antibody. It was used at a concentration of 400 ng/well. The second or detector antibody was rabbit anti-(human antithrombin III) serum diluted 1:1000 with Tween buffer. Thrombin–antithrombin III complex was obtained by adding human α-thrombin to an equimolar concentration of purified human antithrombin III, at 37 °C, in the presence of 0.1 i.u. of heparin. The extent of complex-formation was monitored by subsampling portions into 0.05 mM-S-2238 (Ofosu et al., 1984). For the standard curves, thrombin–antithrombin III complex was added to citrated plasma at six concentrations ranging from 20 to 200 pM either in plasma or in Tween/phosphate buffer containing 10 mg of human albumin/ml. Identity of the colour yields obtained with the standards in both plasma and buffer confirmed the effectiveness of the step to precipitate prothrombin with BaCl$_2$.

To quantify thrombin–heparin cofactor II, the antibody immobilized was affinity-purified rabbit anti-(human thrombin) antibody at 400 ng/well. This antibody was isolated from the Wellmark anti-prothrombin serum by affinity chromatography on thrombin–Sepharose 4B and it reacted equally with thrombin and prothrombin on immunoelectrophoresis. Goat anti-(human heparin cofactor II) antibody was used as the detector antibody at 400 ng/well and rabbit anti-(goat IgG) antibody–alkaline phosphatase conjugate at a dilution of 1:1000 was used as the conjugate. Thrombin–heparin cofactor II complex was obtained by the addition of thrombin (0.2 μM final concentration) to an equimolar concentration of heparin cofactor II in the presence of 150 μg of dermatan sulphate/ml in 0.145 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing 1 mg of bovine serum albumin/ml. Complex-formation was complete within 10 min at 37 °C. The concentrations of thrombin–heparin cofactor II complex used to derive standard curves varied from 0.25 to 2.5 nM. The presence of heparin or dermatan sulphate in the plasma did not influence the sensitivity or specificity of the measurement of prothrombin, thrombin–antithrombin III complex or thrombin– heparin cofactor II complex.

Activation of prothrombin: effects of heparin, Phe-Pro-Arg-CH$_2$Cl and dermatan sulphate

The protocols, shown in summary form in Fig. 1, were used to initiate prothrombin activation in pooled normal human plasma defibrinated with 0.15 unit of Arvin/ml of plasma (Ofosu et al., 1984). Regardless of the stimulus used to activate prothrombin, the plasma was incubated for 3 min at 37 °C before 40 mM-CaCl$_2$ (pre-warmed to

37 °C) was added together with clotting enzyme, co-factors and/or inhibitor under study. Procedures A to D employed CAP, which was obtained when 1 vol. of APTT reagent was added to 2 vol. of defibrinated plasma and the suspension was incubated at 37 °C for 3 min. APTT reagent consisted of a suspension of micronized silicator (as contact activator) and coagulant phospho-lipids from rabbit brain. In experiments that employed exogenous Factor Xa (Fig. 1, procedure A), Factor Xa was introduced simultaneously with the $CaCl_2$ and heparin, since Factor Xa can activate Factor VIII and Factor V only when $CaCl_2$ and coagulant phospholipids are present (Smith & Hanahan, 1976; Foster et al., 1983). As no cofactor is required when thrombin activates Factor V and Factor VIII (Nesheim & Mann, 1979; Vehar & Davie, 1980), thrombin could be added to CAP, either simultaneously with heparin and $CaCl_2$ (Fig. 1, procedure B) or before (Fig. 1, procedure D). Exogenous prothrombinase was used to activate prothrombin in procedure E.

Prothrombin consumption and the subsequent forma-tion of thrombin–antithrombin III and thrombin–heparin cofactor II complexes were quantified by e.l.i.s.a.s described above. The effects of heparin, Phe-Pro-Arg-CH_2Cl or dermatan sulphate on the activation of prothrombin, and on the subsequent formation of the two thrombin–inhibitor complexes, were determined in each case.

RESULTS

Quantification of prothrombin and thrombin–antithrombin III and thrombin–heparin cofactor II complexes in plasma

Prothrombin could be measured in pooled normal plasma diluted with Tween buffer up to 1:200000, corresponding to 10 pM-prothrombin. The sensitivity

and specificity associated with the measurement of thrombin–antithrombin III complex and thrombin–heparin cofactor II complex were 10 and 100 pM respect-ively. No cross-reactivity was seen between thrombin–antithrombin III and thrombin–heparin cofactor II com-plexes. These levels of sensitivity were achieved with affinity-purified antibodies to thrombin, without which the sensitivity decreased by up to 100-fold owing to higher backgrounds associated with non-affinity-purified antibodies. Intra-assay variability associated with measurement of thrombin–inhibitor complexes in un-diluted plasma was approx. 5% at 10 nM and inter-assay variability of standards was between 5 and 10%.

Effects of factor Xa and heparin on prothrombin activation

Prothrombin activation could be demonstrated in control CAP after a lag phase of 30 s (Fig. 2a). At a concentration of 0.66 μg/ml heparin completely sup-pressed prothrombin activation in CAP for 60 s. Pro-longation by heparin of the lag phase associated with prothrombin activation could have resulted from inhibition of Factor Xa generation or inhibition of the amplification reactions of coagulation. Evidence that supports inhibition of the amplification reactions was obtained by observations on the effects of exogenous Factor Xa and exogenous thrombin on prothrombin activation in heparinized plasma.

The effects of 1 nM exogenous Factor Xa on pro-thrombin activation in CAP are summarized in Fig. 2(a). Factor Xa shortened the time required to demonstrate prothrombin activation in CAP to 15 s compared with 45 s in control CAP, suggesting that exogenous Factor Xa significantly shortens the lag phase associated with prothrombinase formation in CAP. The addition of heparin, at the time of replenishment with Ca^{2+}, failed to delay prothrombin activation in CAP supplemented with 1 nM-Factor Xa (Fig. 2a), indicating that heparin was an
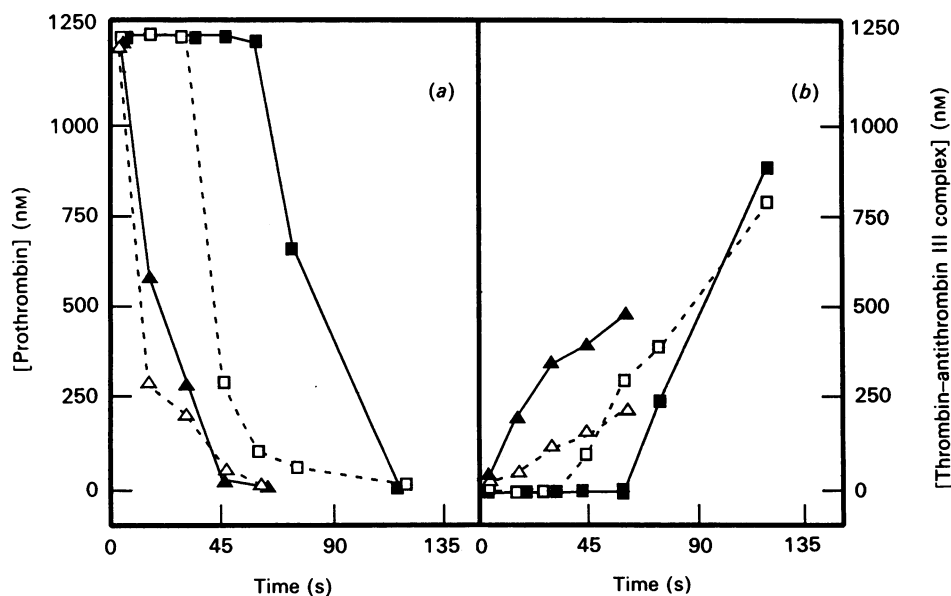

Fig. 2. Quantification by e.l.i.s.a. of the effects of heparin (0.66 μg/ml) on prothrombin activation (a) and the subsequent formation of thrombin–antithrombin III complex (b) in control CAP or CAP supplemented with 1 nM-Factor Xa

See Fig. 1, procedures A and C, for details. □, Control CAP; ■, CAP+heparin; △, CAP+Factor Xa; ▲, CAP+Factor Xa+heparin.

ineffective inhibitor of the actions of Factor Xa in CAP. The major effect of heparin in CAP supplemented with Factor Xa was to accelerate thrombin inhibition by antithrombin III (Fig. 2b).

In contrast with added Factor Xa, exogenous Factor Va failed either to accelerate prothrombin activation or to reverse the inhibitory effect of heparin on prothrombin activation in CAP (results not shown in Fig. 2). This observation suggests that both Factor V and Factor VIII have to be activated in heparinized CAP before efficient prothrombin activation occurs. It is also possible that the amplification reaction most readily inhibited by heparin is the activation of Factor VIII by thrombin. Exogenously added Factor Xa (1 nM) also failed to activate prothrombin in 60 s when it was added to unactivated normal plasma simultaneously with $CaCl_2$ and kephalin (results not shown in Fig. 2). At 45 s, i.e. the time when maximum thrombin activity was detected in control CAP, the concentration of Factor Xa generated in situ was nearly 40 nM (Ofosu et al., 1987a). Failure of 1 nM exogenous Factor Xa to activate prothrombin, 60 s after it was added to unactivated plasma, suggests that some endogenous Factor Xa, generated when $CaCl_2$ was added to CAP supplemented with 1 nM-Factor Xa, was necessary for the rapid activation of prothrombin observed in CAP supplemented with Factor Xa.

Antithrombin III was the major inhibitor of thrombin as less than 5 % of the inactive thrombin was complexed to heparin cofactor II. The concentrations of thrombin–heparin cofactor II complex therefore are not reported in Figs. 2, 3 and 4.

Effects of thrombin and heparin on prothrombin activation

The effects of heparin on prothrombin activation were determined when thrombin was added to CAP with $CaCl_2$, or 1 min before $CaCl_2$. When thrombin (1 or 10 nM) was added with $CaCl_2$, prothrombin activation could be demonstrated 15 s later (Fig. 3a) compared with

45 s in control CAP (Fig. 2a). In contrast with the effects of 1 nM-Factor Xa (Fig. 2a), the simultaneous addition of heparin (0.66 μg/ml), 1 nM-thrombin and $CaCl_2$ resulted in the complete inhibition of prothrombin activation in CAP for 45 s (Fig. 3a). For this reason, no thrombin–antithrombin III was detected until replenishment with Ca^{2+} had proceeded for at least 45 s. Therefore catalysis of the inhibition of exogenous thrombin (1 nM) in heparinized CAP delayed prothrombin activation. The thrombin was added to initiate Factor V and Factor VIII activation. Prothrombin activation was also demonstrable in 15 s when heparinized CAP was supplemented with 10 nM-thrombin (Fig. 3a). The results obtained with 1 nM- and 10 nM-thrombin suggest that, compared with Factor Xa, a higher concentration of thrombin is required to effect activation of Factor V and Factor VIII in CAP when heparin is also present. This is presumably because antithrombin III inactivates thrombin more rapidly than Factor Xa, whether or not heparin is present (Jordan et al., 1980; Ofosu et al., 1984).

Since thrombin-mediated activations of Factor V and Factor VIII do not require a cofactor (Smith & Hanahan, 1976; Nesheim & Mann, 1979), these amplification reactions of coagulation could be initiated during contact activation, and before $CaCl_2$ was added to CAP, as summarized in Fig. 1, procedure D. Prothrombin activation occurred without a lag phase in this plasma (Fig. 4a). Heparin delayed prothrombin activation for 15 s in CAP pretreated with 1 nM-thrombin before replenishment with Ca^{2+}. Heparin, however, failed to delay prothrombin activation in CAP supplemented with 10 nM-thrombin (Fig. 4a). Another major effect of heparin in each case was to accelerate the formation of thrombin–antithrombin III complex (Fig. 4b). The results summarized in Fig. 4 and Fig. 2 imply that, at the concentration used, heparin is less able to inhibit prothrombin activation in CAP after Factor VIII and Factor V have been activated by thrombin or Factor Xa.
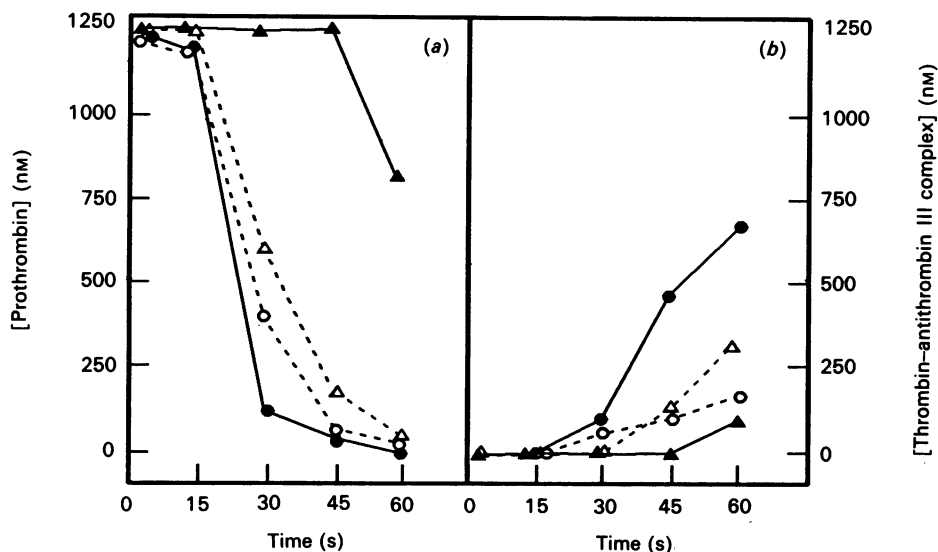

Fig. 3. Quantification by e.l.i.s.a. of the effects of thrombin and heparin on prothrombin activation (a) and the formation of thrombin–antithrombin III complex (b) in CAP

Thrombin, with or without heparin, was added to CAP simultaneously with $CaCl_2$. See Fig. 1, procedure B, for details.
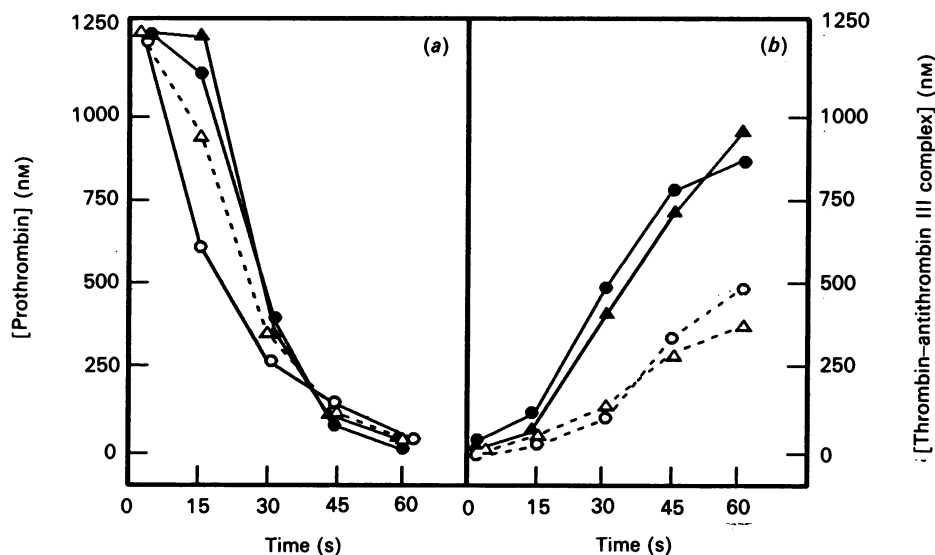△, 1 nM-Thrombin; ▲, 1 nM-thrombin + heparin; ○, 10 nM-thrombin; ●, 10 nM-thrombin + heparin.

Fig. 4. Quantification by e.l.i.s.a. of the effects of thrombin, added to plasma for 1 min during contact activation, on prothrombin activation (a) and the formation of thrombin–antithrombin III complex (b)

$CaCl_2$, with or without heparin, was added to CAP to initiate prothrombin activation. See Fig. 1, procedure D, for details. $\triangle$, 1 nM-Thrombin; $\blacktriangle$, 1 nM-thrombin + heparin; $\bigcirc$, 10 nM-thrombin; $\bullet$, 10 nM-thrombin + heparin.

Activation of prothrombin with exogenous prothrombinase

Prothrombin activation initiated in unactivated plasma with 1 nM exogenous prothrombinase occurred without a lag phase. The effects of heparin on prothrombin activation initiated by exogenous prothrombinase were also determined. Heparin completely inhibited the activation of prothrombin for 15 s. Thereafter only partial inhibition of prothrombin activation was observed (results not shown). These results indicate that prothrombinase generated *in situ* also contributes to the activation of prothrombin achieved with 1 nM exogenous prothrombinase. Catalysis of the inhibition of endogenous thrombin by heparin therefore appears to delay the formation of endogenous prothrombinase that is necessary to activate prothrombin optimally.

Effects of Phe-Pro-Arg-CH_2Cl on prothrombin activation

In order to assess further how inhibition of endogenous thrombin influences prothrombinase formation, the effects of Phe-Pro-Arg-CH_2Cl on prothrombin activation were also quantified and the results are summarized in Fig. 5. Phe-Pro-Arg-CH_2Cl completely inhibited prothrombin activation in CAP that had been replenished with Ca^{2+} for at least 60 s. Phe-Pro-Arg-CH_2Cl completely inhibited prothrombin activation for at least 45 s after $CaCl_2$ and thrombin were added simultaneously to CAP. If CAP was pretreated with 1 nM- or 10 nM-thrombin during contact-activation, and before addition of $CaCl_2$ and the chloromethyl ketone, Phe-Pro-Arg-CH_2Cl failed to delay prothrombin activation (Fig. 5). These findings indicate that, like the effects of heparin, Phe-Pro-Arg-CH_2Cl does not significantly influence prothombin activation in CAP once Factor Va and Factor VIIIa became available. In contrast with its effects when thrombin was added to CAP simultaneously with $CaCl_2$, Phe-Pro-Arg-CH_2Cl delayed prothrombin activation in

CAP supplemented with 1 nM-Factor Xa for only 15 s. Phe-Pro-Arg-CH_2Cl delayed for 15 s prothrombin activation initiated with 1 nM exogenous prothrombinase (results not shown). At a concentration of 1 μM Phe-Pro-Arg-CH_2Cl does not inactivate Factor Xa (Kettner & Shaw, 1979). These results therefore suggest that, when the activity of thrombin is suppressed during intrinsic coagulation, Factor Xa can activate both Factor V and Factor VIII to the extents necessary to facilitate prothrombinase formation.

Effects of dermatan sulphate

At 0.66 μg/ml dermatan sulphate had no effect on prothrombin activation or the formation of thrombin–antithrombin III complex in CAP. Partial inhibition of prothrombin activation was seen with 6.6 μg of dermatan sulphate/ml. Both concentrations of dermatan sulphate catalysed thrombin inhibition by heparin cofactor II. However, antithrombin III remained the major inhibitor of thrombin in CAP that had been replenished with Ca^{2+} and that also contained dermatan sulphate at either concentration (results not shown). In the light of these results, the failure to demonstrate catalysis of the formation of thrombin–heparin cofactor II complex in heparinized plasma (Figs. 2–4) suggests that antithrombin III is the principal inhibitor of thrombin in heparinized plasma.

DISCUSSION

We have demonstrated in this study that prothrombin activation and inhibition of the thrombin generated can be quantified by specific e.l.i.s.a.s. The present results support previous observations made with the use of other methods for prothrombin activation and the subsequent thrombin inhibition (Teitel *et al.*, 1982; Ofosu *et al.*, 1985, 1986, 1987a,b; Hemker, 1987). The present studies suggest that prothrombin activation in CAP is essentially complete within 2 min after $CaCl_2$ is added.
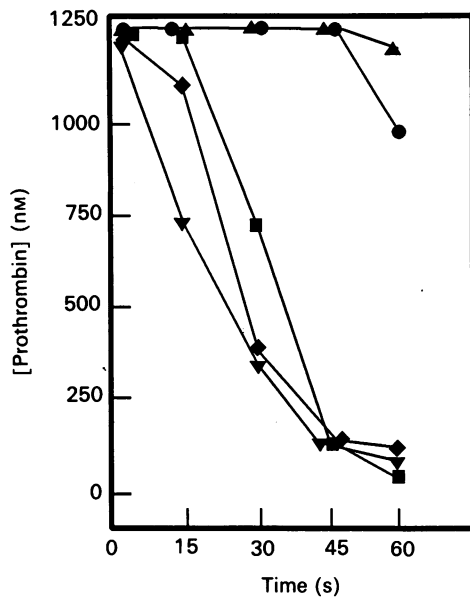
Fig. 5. Quantification by e.l.i.s.a. of the effects of Phe-Pro-Arg-CH₂Cl on prothrombin activation in CAP supplemented with 1 nM-Factor Xa (■) (as in Fig. 1, procedure A), 1 nM-thrombin (▲) and 10 nM-thrombin (●) (as in Fig. 1, procedure B)

In the last two cases Phe-Pro-Arg-CH₂Cl was added to CAP 15 s before the simultaneous addition of CaCl₂ and thrombin. Thrombin, 1 nM (◆) or 10 nM (▼), was added for 1 min during contact activation before CaCl₂ and Phe-Pro-Arg-CH₂Cl were added simultaneously (as in Fig. 1, procedure D). No thrombin–inhibitor complexes were detectable in the presence of Phe-Pro-Arg-CH₂Cl. Note also that Phe-Pro-Arg-CH₂Cl completely inhibited prothrombin activation for at least 60 s (results not shown).

We have also demonstrated in this study that the supplementation of CAP with 1 nM exogenous Factor Xa or exogenous thrombin can accelerate prothrombin activation to approximately the same extent. Unlike the acceleration by Factor Xa, which is only minimally influenced by heparin, the acceleration by thrombin is inhibited considerably. Furthermore, the data suggest that in heparinized plasma Factor Xa is better able than thrombin to activate Factor VIII and Factor V. The data suggest that, once saturating concentrations of both Factor Va and Factor VIIIa become available in CAP, a low concentration of heparin (< 1 μg/ml) does not significantly inhibit prothrombin activation in CAP. They support the proposal by Hemker (1987) that low concentrations of heparin do not inhibit prothrombinase activity.

We had proposed that when plasma contains heparin or Phe-Pro-Arg-CH₂Cl Factor VIII and Factor V are activated by the Factor Xa generated *in situ* (Ofosu *et al.*, 1987*a*). This hypothesis was based on the observation that antithrombin III inactivates thrombin more rapidly than Factor Xa, whether or not heparin is present (Jordan *et al.*, 1980; Ofosu *et al.*, 1984). The present study provides further evidence in support of this hypothesis by demonstrating that exogenous Factor Xa, like thrombin, shortens the lag phase associated with prothrombin activation in CAP from 30 s to less than 15 s (Fig. 2*a*). The observation that heparin fails to delay prothrombin

activation in CAP supplemented with 1 nM-Factor Xa suggests that Factor Xa can activate Factor VIII and Factor V in heparinized CAP when thrombin is depleted by the formation of thrombin–antithrombin III complex. The proposed activation of Factor VIII and Factor V in CAP, obtained by the addition of 1 nM-Factor Xa, appears to be less susceptible to inhibition by heparin than that obtained by the addition of 1 nM-thrombin. The lag phase of prothrombin activation seen in CAP to which Factor Xa and Phe-Pro-Arg-CH₂Cl are added is 15 s or less, compared with 45 s or more for thrombin plus the chloromethyl ketone (Fig. 5). Since no free thrombin activity is detectable in CAP that contains Pro-Phe-Arg-CH₂Cl whereas Factor Xa activity continues to increase in this plasma (Ofosu *et al.*, 1986, 1987*a*), it appears that the Factor Xa present can efficiently by-pass the critical thrombin feedback reactions when the proteolytic activity of thrombin activity is essentially suppressed. It was important to compare the effects of heparin with those of Phe-Pro-Arg-CH₂Cl. Without such a comparison, it would have been difficult to dissociate the effects of heparin due to the catalysis of thrombin inhibition alone from those due to the catalysis of both thrombin and Factor Xa inhibition by antithrombin III.

It is unclear from this present study whether inhibition of Factor VIII activation by thrombin is less or more important than inhibition of Factor V activation for the antigcoagulant action of heparin. We attempted to assess the importance of the inhibition of Factor V activation for the anticoagulant action of heparin and found that exogenous Factor Va did not accelerate prothrombin activation in CAP. Exogenous Factor Va also failed to reverse the inhibition of prothrombin activation in CAP achieved with heparin. Previous studies have demonstrated that heparin inhibits activation of both Factor X and prothrombin in plasma initiated with Factor IXa (Ofosu *et al.*, 1981). At the concentration of heparin used in this study, heparin also inhibits Factor X activation by Factor IXa and Factor VIII, even in the absence of antithrombin III. The same concentration of heparin, however, failed to inhibit Factor X activation initiated with Factor IXa and Factor VIIIa (Ofosu *et al.*, 1980). The results obtained with exogenous Factor Va and Factor IXa provide indirect evidence suggesting that inhibition of Factor VIII activation by heparin is more important than inhibition of Factor V activation for the anticoagulant effect of heparin, as suggested by Hemker (1987).

Results from two previous studies are consistent with this concept. The maximum velocity associated with Factor X activation by intrinsic tenase (i.e. Factor IXa, Factor VIIIa, Ca²⁺ and phospholipid) was reported to be 500 mol of Factor Xa/min per mol of Factor IXa. The catalytic efficiency of this reaction was decreased by five orders of magnitude when Factor VIIIa was omitted (Van Dieijen *et al.*, 1981). The maximum velocity for prothrombin activation by prothrombinase was 1900 mol of thrombin/min per mol of Factor Xa. In contrast with Factor X activation by intrinsic tenase, the catalytic efficiency of prothrombinase was decreased by only two orders of magnitude when Factor Va was omitted (Rosing *et al.*, 1980). If these observations also apply in plasma, then inhibition of Factor VIII activation by heparin would be expected to cause a greater inhibition of prothrombin activation than inhibition of Factor V

activation. Additional studies will be necessary to determine whether Factor VIII activation is more sensitive to inhibition by heparin than is Factor V activation. Our results nonetheless provide evidence for the critical role of thrombin-dependent amplification reactions for normal coagulation. The present findings thus support the concept that the primary action of low concentrations of heparin on inhibiting prothrombin activation is due to its catalytic action on thrombin inhibition, an action that results in the inhibition of thrombin-mediated activation of Factor VIII and Factor V.

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