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ARTICLE *in* THROMBOSIS RESEARCH · DECEMBER 1980

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THE INHIBITION BY HEPARIN OF THE INTRINSIC PATHWAY ACTIVATION OF FACTOR X IN THE ABSENCE OF ANTITHROMBIN-III*

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(Received 13.5.1980; in revised form 1.10.1980.
Accepted by Editor H.L. Nossel)

ABSTRACT Heparin was found to inhibit the activation of purified factor X by purified factors IXa and VIII (or VIIIa) in the presence of calcium and phospholipid but in the absence of antithrombin-III. In contrast, heparin did not inhibit the activation of factor X by either the extrinsic pathway or by the factor X activator isolated from Russell's Viper Venom (RVV-X). The heparin inhibition of the intrinsic pathway activation of factor X could be totally reversed by polybrene. These results suggest that even in the absence of antithrombin-III, heparin reversibly interferes with the interactions of one or more of factors IXa, VIII (or VIIIa) and X with calcium and phospholipid that result in the activation of factor X.

INTRODUCTION

Heparin was shown to be a potent inhibitor of blood coagulation over forty years ago [1,2]. The major mode of action of heparin as an anticoagulant is the enhancement of the rates of inhibition of activated clotting factors by antithrombin-III [2,3,4,5]. Walker and Esmon [6]

* Abstracts of portions of this work have been published in Blood 54, Suppl. 1, 294a, 1979 and Fed. Proc. 39, 544, 1980. Address correspondence to Dr. F. Ofosu, Canadian Red Cross Blood Transfusion Service, 299 Main Street East, Hamilton, Ontario, Canada L8N 1H8.

Key Words: Factor X, Heparin, Antithrombin-III

recently reported that in addition to its antithrombin-III dependent role, heparin also disrupts the interactions of factor Xa and prothrombin with phospholipid which in the presence of calcium and factor V result in the efficient conversion of prothrombin to thrombin. Brown et al have shown a similar heparin effect in the intrinsic activation of factor X [7]. We report here on studies which confirm an antithrombin-III independent inhibitory effect of heparin on the activation of factor X by factor IXa and VIII (or VIIIa). In this study, we also demonstrate that this effect is reversible by polybrene and that heparin has no inhibitory effect on the activation of factor X by the extrinsic pathway in the absence of antithrombin-III.

MATERIALS AND METHODS

Materials

Porcine mucosal heparin, specific activity 154 USP units/mg, was obtained from Riker Laboratories, Northridge, California; unflavoured amphogel ($Al(OH)_3$) from Wyeth Ltd., Toronto, Ontario; CL-Sepharose 4B from Pharmacia Fine Chemicals, Montreal, Quebec; O-phenanthroline, benzamidine-HCl, soybean trypsin inhibitor (SBTI), bovine serum albumin and epsilon amino caproic acid (EACA) from Sigma Chemical Company, St. Louis, Mo.; plasmas congenitally deficient in factors VIII, IX and XI from George King Biomedical, Kansas City, Mo.; sodium diethylbarbiturate and other reagent grade chemicals from Fisher Scientific, Toronto, Ontario. The factor X activator isolated from Russell's Viper Venom (RVV-X) and partially purified human factor VII (specific activity 4000 u/mg) were generous gifts of Dr. Walter Kisiel, University of Washington, Seattle, Washington. Monospecific antisera to human α_2-macroglobulin, fibrinogen and IgM were obtained from Meloy Laboratories, Inc., Springfield, Va.; and S-2222 (CBzIle-Glu(γ-OR)-Gly-Arg-pNA-HCl) was a product of Kabi Group Inc., Greenwich, Conn.

Purification of human factors VIII, IX, X and XI

Factor VIII was isolated from fresh citrated platelet poor plasma containing heparin (2 units/ml), soybean trypsin inhibitor (0.01 mg/ml), benzamidine-HCl (10 mM), O-phenanthroline (0.1 mM) and epsilon aminocaproic acid (40 mM). The plasma, in 200 ml aliquots, was frozen at -60°C for 1 hour and then thawed at 4°C in circulating water baths. Cryoprecipitates were obtained from the thawed plasma by centrifugation at 7,000 g for 10 minutes, warmed to 37°C and absorbed with $Al(OH)_3$ (10% w/v) for 10 minutes at room temperature. After centrifugation at 20°C for 10 minutes at 15,000 g, the cryoprecipitate from 1 litre of plasma was gel filtered on 2 litres of CL-Sepharose 4B equilibrated with 0.02M sodium diethylbarbiturate, pH 7.35, containing 0.15M NaCl, 40 mM EACA and 0.02% NaN_3 and packed in a K50/100 Pharmacia column. Twenty ml fractions were collected by elution with the equilibrating buffer. Factor VIII in the eluates was located by one-stage factor VIII assays [8] and concentrated by dialysis at 4°C against 50-60% polyethylene glycol (average molecular weight of 20,000 daltons) to a concentration of 0.4 mg/ml. The factor VIII was then dialysed against the eluting buffer and stored at -70°C until use. The factor VIII preparations corrected the prolonged clotting times of severe congenital factor VIII deficient plasma [8], supported platelet aggregation in the presence of ristocetin [9] and reacted with monospecific rabbit antihuman

factor VIII [10]. It failed to react with rabbit antihuman fibrinogen, IgM and α_2-macroglobulin on quantitative immunoelectrophoresis [11].

Factors IX and X were isolated from the human cryosupernatants obtained above using the method of di Scipio et al [12] with the following modifications. The homoarginine- and benzamidine- Sepharose procedures were omitted and were replaced by dextran-sulphate-Sepharose chromatography [13]. Purity of the factors IX and X were ascertained by SDS-polyacrylamide gel electrophoresis [12] and by clotting tests with factors II, VII, VIII, IX and X deficient plasmas [14]. The specific activities of the factors IX and X were 120 u/ml and 90 u/ml respectively.

Purified factor IX was activated with factor XIa [15,16] which was isolated as factor XI from human cryosupernatants [17] and activated with trypsin-Sepharose [18]. Trypsin was coupled to Sepharose 4B by the method of Cautrecasas [19]. The factor IXa was purified in DEAE-Sephadex A-50 [20]. The specific activity of factor IXa was 50 times greater than the factor IX. Two micrograms of the factor IXa did not clot fibrinogen (0.2 mg) after a 24 hour incubation at 37°C.

The effect of heparin on the activation of factor X

The effect of heparin on the activation of factor X was determined by quantitating the generation of factor Xa using three different systems for factor X activation. The rate of factor Xa generation in each system was then determined by measuring the amidolytic activity of factor Xa on the chromogenic substrate S-2222 [21].

(a) Activation of factor X by factors IXa and VIII:

Michaelis buffer, pH 7.4, containing 0.15M NaCl, factor IXa (100-500 ng), factor X (8-80 µg), cephalin (1 µg), calcium chloride (10 µmol) and factor VIII (0.1-1.0 units) were assembled in the stated order at 4°C in a plastic tube (12x75mm) in a final volume of 1.0 ml. The factor X activation mixture was incubated at 37°C and after various incubation periods of 1-20 min, 0.1 ml aliquots of the activation mixture were added to 0.15 ml of 10 mM EDTA in Michaelis buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin and preincubated at 4°C. The concentration of factor Xa produced at each time period was quantitated by adding 0.1 ml of the EDTA containing solution to 4 umol of S-2222 preincubated at 37°C for 5 min in a semi-microcuvette containing 0.9 ml of Michaelis buffer, pH 7.4 and 0.1 mg/ml bovine serum albumin. The amidolysis of S-2222 by factor Xa, measured by Δ OD/min at 405nm, served as the index for factor X activation by the activation system. Factor Xa was obtained by activating factor X with RVV-X and purifying the resulting factor Xa on DEAE-Sephadex A-50 [20]. The addition of 1 pmol factor Xa to 0.4umol of S-2222 (in a total volume of 1 ml in Michaelis buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin) resulted in a Δ OD/min of 0.026 at 37°C.

Factor X activation was also quantitated in a one stage assay. In this assay, Michaelis buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin, factor IXa (100-500 ng), factor X (8-80 µg), cephalin, calcium chloride (10 µmol) and factor VIII (0.1-1.0 u) were assembled in a 12x75mm plastic tube at 4°C. The total volume was 0.9 ml. The mixture was then transferred into a semi-microcuvette containing 0.1 ml of a 4 mM solution of S-2222 and positioned in the cuvette holder of a Gilford Model 250 spectrophotometer at 37°C. After a 1 minute incubation, the amount of factor Xa generated was

quantitated by recording the production of paranitroanaline at 405nm over 20 minutes [21]. In control experiments, the amidolysis of S-2222 was determined subsequent to the omission of either calcium, cephalin, factors IXa, X or VIII.

The effect of heparin on factor X activation in this system was determined by adding heparin to the factor IXa solution prior to the addition of factor X, calcium, cephalin and factor VIII. The final concentration of heparin was varied from 0.01 to 1.0 units/ml. The ratio of factor IXa to factor X varied from 1:20 to over 1:100. Michaelis buffer containing 0.1 mg/ml bovine serum albumin was used as the diluent for factors IXa and factor X.

In some experiments, the factor VIII (1.0 unit) was activated with thrombin (0.01 NIH units/ml) for 5 min at 37°C. The thrombin was then inactivated with 5.0×10^{-5}M dansylarginine N-(3-ethyl-1,5-pentanediyl)-amide (DAPA) kindly provided by Dr. M.E. Nesheim, Mayo Foundation, Rochester, Minnesota. Factor VIII was also activated with 0.2 pmol of factor Xa for 2 and 5 minutes at 37°C in the presence of calcium (10 mM), cephalin and 10^{-5}M DAPA. Factor X activation was then quantitated as above. The incubation mixtures, however, also contaned 1.0×10^{-5}M DAPA.

(b) Activation of factor X by partially purified factor VII and human brain thromboplastin

Factor X was also activated with partially purified factor VII. This preparation which contained 1800 u/ml of factor VII, also contained less than 0.05 units of either prothrombin, factor IX or X per ml. Factor X (2-50 ug) in Michaelis buffer, containing 0.1 mg/ml bovine serum albumin, 0.01-0.1 units of partially purified factor VII, human brain thromboplastin (1.0 u) and Michaelis buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin were incubated at 4°C in a total volume of 0.8 ml. This mixture was then transferred into a semi-microcuvette positioned in the cuvette holder at 37°C and contained 0.1 ml of 0.1M $CaCl_2$, and 0.1 ml of a 4 mM solution of S-2222. After a 1 minute incubation at 37°C, the generation of factor Xa was quantitated by determining the rate of hydrolysis of S-2222 by the factor Xa produced [21]. The effect of 1.0 u/ml heparin on the extrinsic pathway activation of factor X was determined by adding 1 unit of heparin to the factor X, factor VII and thromboplastin mixture in a total volume of 0.8 ml prior to quantitating Xa generation at 37°C in the presence of calcium chloride and S-2222.

(c) Activation of factor X by RVV-X

RVV-X was incubated with purified factor X at weight ratios of 1:50 to 1:250 in a total volume of 0.8 ml and the solution transferred into a semi-microcuvette containing 0.1 ml each of a 4mM S-2222 and 0.1M CaCl at 37°C as above. The factor Xa produced was quantitated after a 1 minute incubation at 37°C. When the amount of factor Xa produced was in excess of the capacity of the detection system, appropriate dilutions of the factor X + RVV-X solutions were made with Michaelis buffer containing 0.1 mg/ml of bovine serum albumin prior to the addition of the diluted solution to the $CaCl_2$ and S-2222 at 37°C. The effect of heparin (0.01, 0.1, 1.0 and 10 units/ml) on the activation of factor X by RVV-X was also investigated by adding heparin to the incubation mixtures.

The effect of heparin on the amidolytic activity of factor Xa

The effect of heparin on the amidolytic activity of factor Xa on S-2222 was investigated by adding purified factor Xa to a 0.4 mM solution of S-2222, buffered with 0.03M Tris-HCl, pH 8.3, containing 0.15M NaCl, at 37°C in a total volume 1 ml and quantitating the Xa by its amidolytic activity on S-2222 [21]. Factor Xa was obtained by activating factor X with RVV-X and purifying the resulting factor Xa on DEAE-Sephadex A-50 [20]. The effect of heparin on the amidolytic activity of factor Xa on S-2222 was determined by adding heparin to the factor Xa solution prior to its addition to S-2222. Heparin was used at final concentrations of 0, 0.01, 0.1, 1.0 and 10.0 units/ml.

Effect of polybrene on the intrinsic pathway activation of factor X and its interaction with heparin

The effect of polybrene on the activation of factor X by factor IXa, factor VIII, calcium and phospholipid was evaluated by quantitating factor Xa generation in the presence of 0.1, 1.0, 5.0, 10 and 20 ug/ml of polybrene. The effect of antithrombin-III and the combined effects of 1.0 unit of heparin and antithrombin-III (20 pmol) and varying concentrations of polybrene on factor X activation were also evaluated. When the effect of polybrene on activation systems containing heparin or heparin and antithrombin-III was evaluated, the activation process was allowed to proceed for 10 minutes in the presence of either heparin or heparin + antithrombin-III before polybrene was added to the incubation mixture.

Detection of Antithrombin-III

Antithrombin-III was isolated from fresh human cryosupernatants using the methods of Miller-Andersson et al [22]. The second order rate constant for the inhibition of human factor Xa by antithrombin-III was determined using the methods of Downing et al [23]. The concentration of factor Xa used was 2.4×10^{-8}M (based on its absorbance of 280 nm [24]). The concentration of antithrombin-III was varied from 3×10^{-10}M to 6×10^{-7}M. The concentration of antithrombin-III was estimated by its optical density at 280 nm [17]. The inhibition studies were conducted at pH 7.4, in Michaelis buffer, containing 0.1 mg/ml bovine serum albumin. Residual factor Xa activity was determined after a 10-fold dilution of the factor Xa + antithrombin-III solution in the buffer containing 0.4 umol/ml of S-2222. The effect of heparin (0.01, 0.1, 1.0 u/ml) on the second order rate constants were also evaluated. In the presence of 1.0 U/ml of heparin 10 ng/ml (10^{-10}M) of antithrombin-III could be detected.

RESULTS

The effect of heparin on the activation of factor X

The amounts of factor X activated by purifed factors VIII and IXa were found to be dependent on the initial concentrations of factor IXa, X and VIII when the concentrations of calcium and cephalin were kept constant. Under the conditions employed, no activation of factor X in the absence of factor VIII or factor IXa could be demonstrated. As shown in figure 1, there was a lag phase of approximately five minutes before a rapid activation of factor X by factors IXa and VIII was observed. Heparin (0.1 u/ml and 1.0 u/ml)

inhibited the activation of factor X by factors IXa and VIII. No inhibition of factor X activation was seen when the concentration of heparin was 0.01 u/ml. Heparin had no effect on the amidolytic activity of factor Xa or on the activation of factor X by either purified RVV-X or the extrinsic pathway.

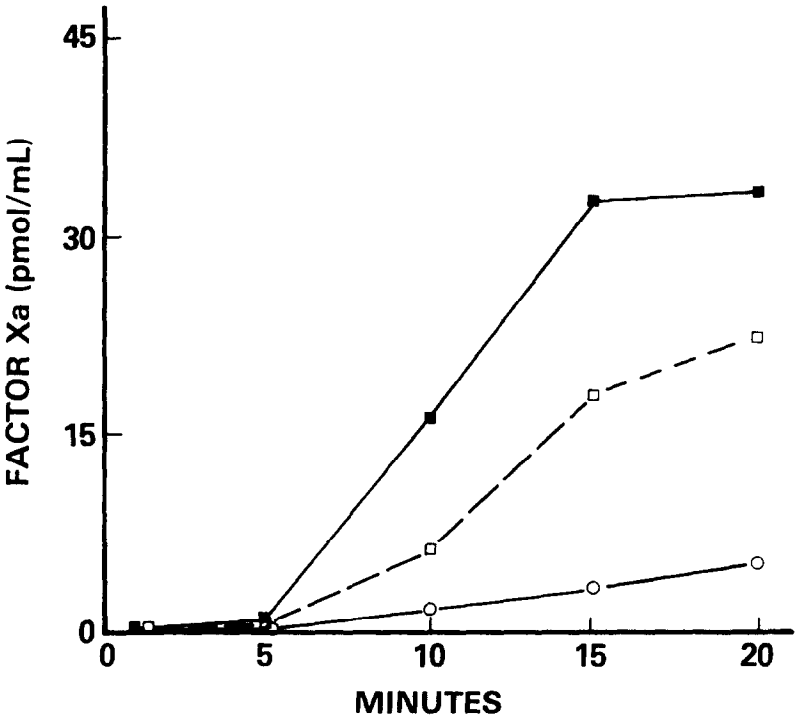

FIG. 1

The effect of heparin on the activation of factor X by factors IXa and VIII in the presence of calcium and cephalin. The concentrations of factors VIII, IXa and X were 1.0 u/ml, 0.1 μg/ml and 20 μg/ml respectively. Symbols: solid squares represent no heparin; open squares 0.1 u/ml heparin; open circles 1.0 u/ml heparin. Details of the conditions employed are specified in the methods. Each data point is the mean of 12-16 determinations.

The activation of factor X by factors IXa and VIII was also quantitated in a one stage assay in which the S-2222 was added to the incubation mixture at the start of the activation. The factor Xa produced was measured continuously during the incubation. While qualitatively similar results were obtained (i.e. 0.1 and 1.0 u/ml heparin inhibited the activation of factor X by factor IXa and VIII) as those obtained in the two stage assay, less factor Xa was generated in the one stage assay. The apparent inhibitory effect of

S-2222 on activation of factor X was more severe when the concentration of factor IXa was less than 100ng/ml. These inhibitory effects were not evaluated further.

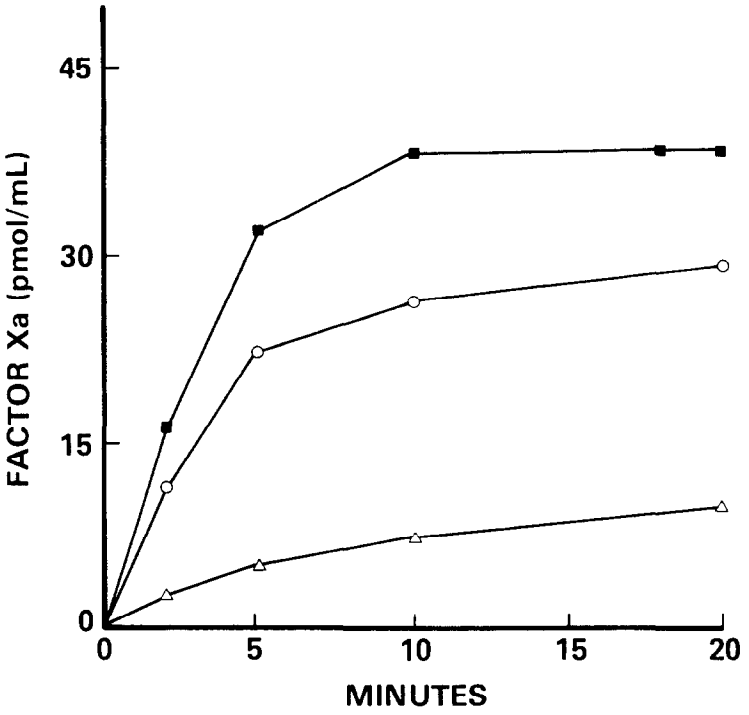

FIG. 2

The effect of heparin on the activation of factor X by factors IXa and thrombin activated factor VIII. The concentrations of factors VIII (before activation with thrombin), IXa and X were 1.0 u/ml, 100 ng/ml and 0.02 mg/ml respectively. Symbols: solid squares represent no heparin; open circles 1.0 u/ml heparin and open triangles 10.0 u/ml of heparin. Each data point is the mean of six determinations

The effects of heparin on the activation of factor X by factors IXa and thrombin activated factor VIII (VIIIa) are shown in figure 2. Prior activation of factor VIII with thrombin eliminated the lag phase associated with the activation of factor X by factors IXa and VIII. Figure 2 also shows that heparin inhibited the activation of factor X by factors IXa and VIIIa. However, the inhibitory effects of heparin were greatly reduced when compared to those seen with unactivated factor VIII. Thus, 0.1 and 0.2 u/ml heparin

had no inhibitory effects on the activation of factor X by factors IXa and thrombin activated VIII. The inhibition obtained with 10.0 u/ml heparin was similar to that obtained with 1.0 u/ml heparin in the presence of unactivated factor VIII (figure 1). Heparin (1.0 u/ml) did not inhibit the activation of factor VIII by thrombin. Prior activation of factor VIII with factor Xa, calcium and phospholipid similarly eliminated the lag phase associated with the intrinsic activation of factor X. Heparin also inhibited the activation of factor X by factor IXa and factor Xa activated factor VIII. In contrast to the results obtained with thrombin, the activation of factor VIII by factor Xa, calcium and cephalin was inhibited by 0.20 and 1.0 u/ml of heparin (data not shown).

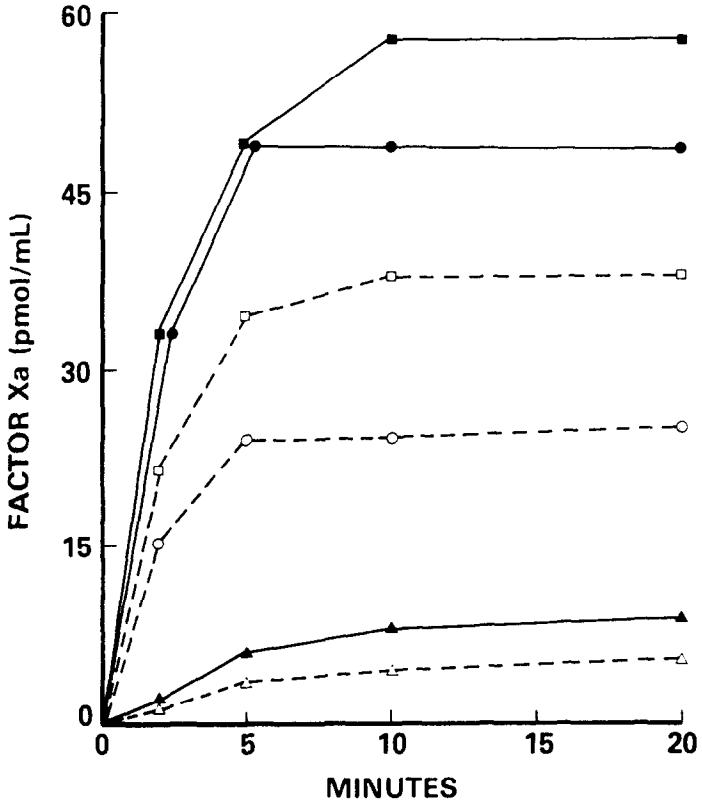

FIG. 3

The effect of heparin on factor Xa generation with varying cephalin concentrations. The concentrations of factor VIII (before activation with thrombin), factor IXa and factor X were kept at 1.0 u/ml, 350 ng/ml and 0.03 mg/ml respectively. Symbols: solid squares represent no heparin and 1 μg/ml cephalin; open squares 1.0 u/ml heparin and 1 μg/ml cephalin; solid circles, no heparin and 2.5 μg/ml cephalin; open circles 1.0 u/ml heparin + 2.5 μg/ml cephalin. The closed traingles represent 0.1 μg/ml cephalin without heparin, while the open triangles represent 0.1 μg/ml cephalin plus 1.0 u/ml heparin.

The activation of factor X was also quantitated in the presence or absence of 1 unit/ml of heparin with varying concentrations of cephalin, factors VIII, VIIIa, IXa and X. The results obtained with varying concentrations of cephalin are shown in figure 3. At all three cephalin concentrations, heparin inhibited to a similar extent the activation of factor X in the absence of antithrombin-III. Similar observations were obtained when the concentrations of factor VIII, VIIIa, IXa and factor X were each varied while the others were kept constant. When the concentration of cephalin exceeded the optimal amount (1 ug/ml), there was an apparent inhibition of factor X activation by the intrinsic pathway. No antithrombin was detected in any of the reagents used (the limit of detection of antithrombin-III was 10^{-10}M).

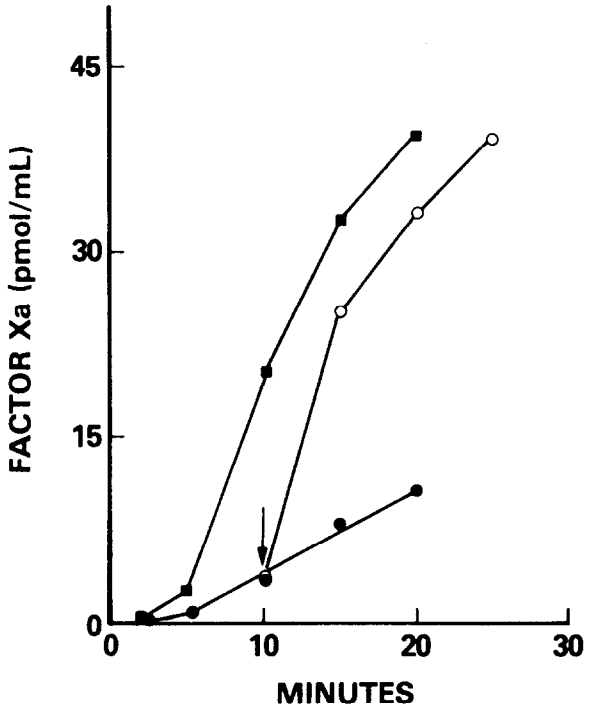

FIG. 4

Reversal of heparin inhibition of factor X activation by polybrene. Each incubation system consisted of 0.5 ug factor IXa, 20 ug factor X and 1.0 u of factor VIII per ml. Polybrene (15 ug/ml) was added at the point indicated by the arrow. Symbols: closed squares represent no heparin; closed circles 1.0 u/ml heparin; open circles 1.0 u/ml heparin after polybrene (15 ug/ml) addition. Each data point is the mean of 8 determinations.

Reversibility of the heparin effects by polybrene

Polybrene completely reversed the antithrombin-III independent effects of heparin on the activation of factor X by the intrinsic pathway. The combined effects of polybrene and heparin are shown in figure 4. Polybrene was found to inhibit the activation of factor X by factors IXa and VIII when tested at concentrations of 5-20 μg/ml. The reversal of the heparin effect by polybrene was observed only at polybrene concentrations of 10-15 μg/ml. Polybrene, however, failed to reverse the inhibition of factor X activation observed in the presence of both heparin and antithrombin-III. These observations are shown in figure 5 which also show that the combined effects of heparin (1.0 u/ml) and antithrombin-III (1.2 μg/ml) resulted in complete inhibition of factor X activation by the intrinsic pathway. Antithrombin-III alone had no inhibitory effect on the activation of factor X by factors IXa and VIII.

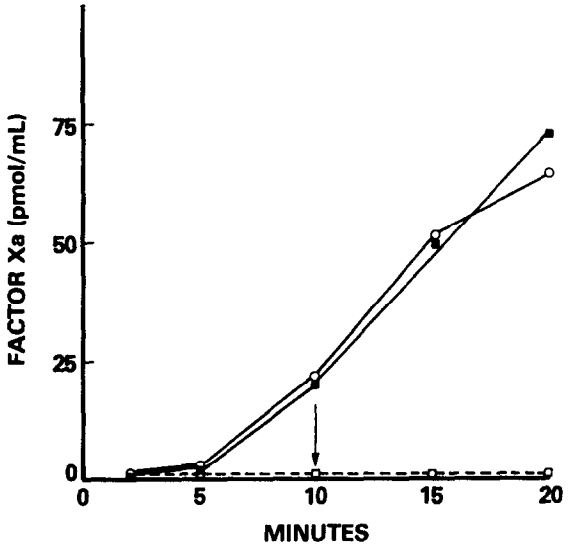

FIG. 5

Irreversible inhibition of factor X activation by heparin and antithrombin-III. Factor IXa (500 ng/ml), factor X (40 μg/ml) and factor VIII (1.0 u/ml). Polybrene (15 μg/ml) was added at the point indicated by the arrow. Symbols: closed squares represent no heparin or antithrombin-III; open circles represent 1.2μg/ml antithrombin -III; open squares - 1.2 μg/ml antithrombin-III + 1.0 u/ml heparin. Each data point is the mean of 16 determinations.

DISCUSSION

The effects of heparin on the activation of factor X by three known pathways for factor X activation were compared. Only the intrinsic pathway for the activation of factor X was inhibited by heparin. The activation of factor X by factors IXa and VIII was associated with a lag phase of approximately five minutes duration under optimal conditions of activation. This lag phase was eliminated by the pre-activation of factor VIII by either thrombin or factor Xa. Similar lag phases have been described by other investigators [24,25,26], and probably represents the activation of factor VIII by the minute amounts of factor Xa [26,27]. Our results confirm the observations of Vehar and Davie [26] that pre-activation of factor VIII by thrombin is not necessary for the rapid activation of factor X after the initial lag phase.

Greater inhibition by heparin was evident when factor X was activated by factors IXa and VIII than when activated by factors IXa and VIIIa. These observations suggest that the antithrombin-III independent inhibitory effects of heparin on factor X activation occurs at two levels: at the first level, heparin reversibly disrupts the binding of the factor X activator complex to phospholipid in a manner analogous to the one suggested by Walker and Esmon [6]. As a consequence of this action of heparin on the assembly of the factor X activator complex, the feedback loop involving the activation of factor VIII by by factor Xa [26] is inhibited by heparin. This latter reaction is also calcium ion and phospholipid dependent [26]. Direct support for this conclusion is our observation that the activation of factor VIII by thrombin is not inhibited by 1.0 u/ml heparin, while the activation of factor VIII by factor Xa, calcium and cephalin is inhibited by heparin.

The differential effects of heparin on the intrinsic and extrinsic pathway activation of factor X may reflect the relative roles of the calcium-dependent binding of factor X to phospholipid in each of these pathways. In conclusion, our observations confirm that heparin, even in the absence of antithrombin-III inhibits the activation of factor X by the intrinsic pathway [7]. In addition, unlike the irreversible inhibition obtained with heparin and antithrombin-III, the inhibitory effect of heparin alone is completely reversible by polybrene. It is likely that this inhibition is analogous to the antithrombin-III-independent inhibition by heparin of prothrombin activation by factor Xa [6], and that the observed inhibition results from the disruption by heparin of the interactions of one or more of factors IXa, VIII (or VIIIa) and X with calcium and phospholipid.

ACKNOWLEDGEMENTS

This work was supported in part by grant #PR646C from the Ontario Ministry of Health and by a grant from the Medical Research Council of Canada. The technical assistance of G. Modi and L. Smith is gratefully acknowledged.

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