Activation of Factor X by Factors IXa and VIII; A Specific Assay for Factor IXa in the Presence of Thrombin-activated Factor VIII

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We studied the activation of factor X by the intrinsic pathway of blood coagulation using a new assay of factor X activation. When factor X, tritiated in its sialic acid residues is activated, activation can be measured by the release of tritiated activation peptide, and the initial rate of activation can be determined under varying conditions. In the presence of phospholipid and calcium ions, factor IXa activated factor X slowly without factor VIII, and this activation was blocked by a specific factor IX inhibitor. These data provide strong evidence that factor IXa is the enzyme responsible for factor X activation by the intrinsic pathway. The role of factor VIII was also investigated. Factor VIII could be reproducibly thrombin activated and then stabilized by the addition of 2 mM benzamidine hydrochloride; this suggests that inactivation is due to proteolysis. Neither unactivated nor thrombin-activated factor VIII produced factor X activation without factor IXa. With a constant level of factor IXa, factor X activation was directly proportional to the level of activated factor VIII. With a constant level of activated factor VIII, factor X activation was proportional to the factor IXa concentration. This observation was exploited to develop a specific, sensitive assay for factor IXa.

The activation of factor X by the intrinsic pathway of blood coagulation involves the interaction of factors IXa and VIII, calcium ions, and phospholipid. It is possible that these reactants form an activating complex, since antibodies to either factor IX or factor VIII could block factor X activation. Since factor IX is highly homologous with other zymogens of serine proteases involved in blood coagulation, it is hypothesized that factor IXa is the enzyme responsible for factor X activation and factor VIII is a cofactor to factor IXa. The activity of factor VIII in this reaction is markedly enhanced by prior incubation with thrombin.

Direct proof that factor IXa alone activates factor X has been lacking. Most studies have assessed factor X activation by coagulation assays, which cannot be made completely specific and may be variably influenced by one or more feedback reactions; two such studies suggested that factor IXa activates factor X. A recent study utilizing a chromogenic substrate assay of factor Xa also suggested that factor IXa alone activates factor X and that factor VIII is a cofactor. This approach is limited by the requirement for functional factor
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Xa, so that potential factor Xa feedback on factors VIII, IX, or X cannot be inhibited.

Previous studies have shown that the activation of factor X in the presence of factor IXa and factor VIII is identical to factor X activation by factor VIIa, with cleavage of the heavy chain at Arg111-Ile and release of a 9000-dalton activation peptide.13 In order to study factor X activation by factors IXa and VIII, we modified a method first developed in this laboratory for studying the isolated step of factor X activation catalyzed by factor VIIa and tissue factor.14 Purified bovine factor X was radiolabeled at its sialic acid residues using the van Lenten-Ashwell technique of oxidation with periodate followed by reduction with tritiated sodium borohydride.15 This tritiated factor X retains normal activatability as judged by isotope dilution experiments.14 Most of the sialic acid in factor X (six to eight residues) is on the amino-terminal peptide of the heavy chain, which is released during activation of factor X. Only one or two residues are attached to a carboxyl-terminal peptide, which is slowly released autocatalytically by factor Xa.16 These peptides are soluble in 5% trichloroacetic acid (TCA), whereas factors Xa and X are not.14 The increase of TCA-soluble tritiated peptide over time parallels the appearance of factor Xa by polyacrylamide gel electrophoresis (PAGE) and by clotting assay.14

If the progress curve of release of tritiated activation peptide versus time is linear, then the slope of the progress curve provides a direct measurement of the initial velocity ($v_0$) of factor X activation. Since factor Xa feedback on factor X, with release of tritiated carboxy- and amino-terminal peptides from the heavy chain, would obscure the rate of the forward reaction of factor X activation, an inhibitor of factor Xa can be included in the activation mixture. We adapted this method for the specific assay of factor IXa and evaluated the role of factor IXa, factor VIII, and thrombin-activated factor VIII (VIIIa) in factor X activation.

MATERIALS AND METHODS

Materials. Tritiated sodium borohydride (10 Ci/mmol) was purchased from Amersham/Searle, DEAE-Sephadex A-50 and Sepharose 4B from Pharmacia, Biogel A-15m from BioRad, Rabbit brain cephalin, Li-heparin, sucrose, Russell viper venom, bovine serum albumin (BSA-fatty acid-free), sodium lauryl sulfate, "Trizma" base, and guanidine hydrochloride from Sigma, and alumina C'y gel from Calbiochem. Acrylamide, N,N'-methylenebisacrylamide, and benzamidine hydrochloride were purchased from Aldrich and "Scintrex" scintillant and polyethylene glycol (PEG) 6000 from Baker. Other chemicals were reagent grade products of Sigma, Baker, or Fisher. Factor VIII concentrate (Hemofil) was kindly supplied by Hyland laboratories. Thrombofax was purchased from Ortho Diagnostics.

Coagulation assays. Factor Xa was measured by the method of Jobin and Esnouf17 with factor VII- and factor X-deficient plasma. Factors VIII, IX, and XI were measured by a one-stage modified partial thromboplastin time test18 with human factor VIII-, factor IX-, or factor XI-deficient plasma. Assays for factor VIII were performed by subsampling from a thrombin-factor VIII mixture directly into factor VIII-deficient plasma that had been incubated with kaolin-Thrombofax for 7.5 min, followed by the immediate addition of CaCl₂ (8 mM final concentration). When a dilution of the factor VIII was necessary, it was made in 15 sec or less and immediately added to preactivated factor VIII-deficient plasma as above. Thus in every case the factor VIII assay was completed within 1.5 min of subsampling from an activation mixture. Antithrombin III was assayed as heparin cofactor activity by the following method: 0.3 ml of test sample diluted in 0.1 M NaCl-0.05 M Tris-Cl, pH 7.5 (TBS)-0.1% BSA-0.6% PEG 6000, with Li-heparin 1.50 U/ml, was brought to 37°C and 10 μl topical thrombin, 15 U/ml in TBS-0.1%
BSA-0.6% PEG 6000, was added. Then, 30 sec later, 0.1 ml warmed fibrinogen, 12 mg/ml in TBS, was added, and the clotting time was determined. Pooled citrated platelet-poor plasma from ten normal volunteers was stored in aliquots at −70°C, and a fresh aliquot was thawed and assayed for a standard curve for each of the coagulation assays above, with the normal level of each factor defined as 1 U/ml. Thrombin was measured by the method of Fenton and Fasce19 and expressed as NIH U/ml, with NIH thrombin lot J as standard. The coagulant activity of Russell viper venom, or purified coagulant protein, was measured by the method of Williams and Ensou20 except that factor VIII-deficient plasma was used in order to make the assay insensitive to factor IXa. Factor IXa was measured by a modification of the assay of Schiffman et al.21 A 0.1-ml sample in TBS-0.1% BSA was brought to 37°C in a polystyrene tube, and 0.1 ml prewarmed Thrombofax and 0.1 ml hemophilic factor IX-deficient plasma (kept at 4°C) were added. After 1 min at 37°C, 0.1 ml prewarmed 40 mM CaCl₂ was added and the clotting time determined. The assay was calibrated with purified factor IXa as the reference. All clotting assays were performed by hand by the tilt-tube method except for the thrombin and antithrombin assays, for which a Fibrometer (BBL) was used. Antithrombin III was also measured immunologically (Partigen plate, Behring Diagnostics).

**Purification of proteins. Analysis.** The purity of protein preparations was assessed by sodium dodecyl sulfate (SDS) PAGE according to the method of Weber and Osborn.22 Bovine factor X was purified by the method of Jesty et al.16 purified factor X showed a single band on SDS-PAGE and had a specific activity of 95 U/mg. Purified factor X was radiolabeled with tritium at sialic acid residues by the method of van Lenten and Ashwell15 to a specific activity of 26,800 cpm/μg as described by Silverberg et al.14 Tritiated factor X was stored in 50% glycerol at −20°C.

Factor IX was purified from a barium citrate eluate of bovine plasma (New England Enzyme Center, Tufts University, Boston) by DEAE-Sephadex chromatography, benzamidine-agarose chromatography, and heparin-agarose chromatography,23,24 and a final step of preparative PAGE (Prep Disc, Miles Labs). A 3.0-ml sample (40 mg protein from the factor IX peak of the heparin-agarose step, in 10% (w/v) sucrose, in tank buffer) was placed on a 6.25% acrylamide, 0.16% bisacrylamide gel (1.6 × 7.0 cm), run at a constant current of 8 mA for 12 hr, and eluted at a rate of 0.8 ml/min. The eluting buffer was 0.50 M Tris-HCl pH 8.9, and the tank buffer was 0.02 M Tris-0.19 M glycine pH 8.9. The final preparation showed a single band on SDS-PAGE with an apparent molecular weight of 56,000 daltons and had a specific activity of 128 U/mg, which represented a 9000-fold purification from plasma.

Purified factor IX was activated in two ways, (1) by contact factor and (2) by solid-phase purified coagulant protein (CP) from Russell viper venom. Contact factor was prepared according to the method of Nossel.25 Factor IX (1 mg/ml) was activated by incubation with 0.2 vol contact factor in TBS, 10 mM CaCl₂, at 37°C for 1.5 hr. Approximately 5%-10% of the factor IX was activated as judged by the appearance of a band at 46,000 daltons on unreduced gels by SDS-PAGE, as well as two new bands at 27,000 and 17,000 daltons on reduced gels. Varying the amount of contact factor and incubation time did not improve the yield of factor IXa; a plateau of factor IXa activity was reached at 60-90 min in each activation. Coagulant protein was purified from Russell viper venom (RVV) by the method of Jesty et al.16 Purified CP was coupled to Sepharose 4B by the method of Cuatrecasas.26 CP, 6.0 mg in 0.1 M NaHCO₃, pH 8.25, was stirred with 5.0 ml of activated Sepharose 4B for 20 hr at 4°C and then washed in 4 M guanidine-HCl and stored in TBS; 95% of the CP was coupled to Sepharose as judged by A₃₈₀ of the eluate and washes. Purified factor IX (4.0 ml of 1.34 mg/ml in TBS) was incubated with CP-Sepharose in 10 mM CaCl₂ at 37°C for 1.5 hr x three incubations (0.05, 0.3, and 2.0 ml of CP-Sepharose, respectively). The serial incubations were necessary to achieve >80% activation of factor IX as judged by SDS-PAGE and clotting assay, which showed a plateau of factor IX activation at 60-90 min in each incubation. Factor IXa showed no change in molecular weight compared to factor IX (56,000 daltons) by SDS-PAGE. Upon reduction, factor IXa showed a doublet by SDS-PAGE at 28,000-30,000 daltons; this finding is consistent with the report of Lindquist et al. that the primary form of factor IXa produced by RVV is composed of two chains of approximately equal molecular weight with no loss of activation peptide.27

Factor IXa prepared by either method was purified by gradient chromatography on DEAE-Sephadex (2.0 x 20 cm) with 0.1-0.5 M NaCl, 0.05 M Tris, pH 8.0 at 40 ml/hr and 4°C. Fractions containing factor IXa were pooled and concentrated by ultrafiltration (Diaflo PM-10,
Amicon) dialyzed against TBS overnight and stored in aliquots at -70°C. No loss of factor IXa procoagulant activity was detected when aliquots were thawed and refrozen as many as six times. Factor IXa (364 µg/ml) contained no measurable thrombin and 0.1 ng/ml of factor Xa by clotting assay.

Purified human thrombin (2914 NIH units/mg, 97% α-thrombin) was a gift of Dr. John Fenton. Human factor VIII was purified from Hyland Method IV concentrate (Hemofil) by absorption with 0.1 vol Al(OH)₃ for 10 min at 20°C and centrifugation at 5000 g for 10 min followed by chromatography at 20°C on Biogel A-15m (4.0 x 80 cm) in TBS, 0.25% e-amino-caproic acid and 0.05% sodium azide at a flow rate of 50 ml/hr. The V⁰ factor VIII peak was localized by clotting assay, pooled, stabilized with the addition of 0.2% BSA, and concentrated fivefold by dialysis against 20%, PEG 6000, and then stored in aliquots at -70°C. Purified factor VIII had a specific activity of 50 U/mg and contained no other detectable clotting factors; it could be activated greater than 20-fold with thrombin. The possible presence of some PEG 6000 in the purified factor VIII after dialysis was not excluded but was probably insignificant, since the concentrated factor VIII showed an appropriate four- to fivefold increase after concentration in clotting activity and ability to support ³H-factor X activation. In later experiments, partially purified human factor VIII was also used [Al(OH)₃-absorbed Hemofil]. Bovine factor VIII was partially purified from oxalated bovine plasma by cryoprecipitation, absorption with 0.1 vol Al(OH)₃ at 20°C for 10 min and defibrination with ancorod 1.0 U/ml (Venacil, kindly supplied by Abbott Labs) for 1 hr.²⁸ No change in the factor VIII procoagulant activity or antigen of the cryoprecipitate occurred following absorption and defibrination. All factor VIII preparations were stored in aliquots in plastic tubes at -70°C. No or minimal loss of factor VIII activity (<5%) was detected after one thawing, and thawed factor VIII was stable at 4°C for at least 8 hr.

Hemophilic factor IX-deficient plasma with a specific, high-titer factor IX inhibitor was clotted with purified human thrombin (1 U/ml) at 37°C for 1 hr, and the serum was obtained by centrifugation. To remove antithrombin III, 1.5 ml serum was chromatographed on heparin-Sepharose (0.5 x 10 cm) in TBS without Ca²⁺ at a flow rate of 5 ml/hr, and the breakthrough fractions containing protein were pooled. Normal human plasma was treated in the same way as a control. Both sera showed <3% of the normal level of antithrombin III activity and antigen following chromatography. The sera were then absorbed with 0.1 vol Al(OH)₃ for 10 min at 20°C followed by centrifugation at 5000 g for 10 min. The sera were then heated to 56°C for 30 min and stored at -20°C. The factor IX inhibitor titer of the treated serum was 25 U/ml (that is, a serum dilution of 1:25 inhibited 90% of the factor IX in pooled normal plasma).

Protein concentrations were measured by absorbance at 280 nm using the following values for Aₐₙₐ: 9.6 for factor X,²⁹ 9.4 for factor Xa,³⁰ 14.9 for factor IX and (assumed) for factor IXa activated by RVV-CP, 14.3 for factor IXa activated by contact factor,³¹ and 10.0 (assumed) for RVV coagulant protein and for factor VIII. Molar concentrations were determined using a molecular weight of 56,000 daltons for factors X, IX, and IXa (by RVV-CP) and 46,000 daltons for factor IXa (by contact factor).

Factor X activation assay. All assays were performed in polystyrene tubes in a 37°C water-bath. Small volumes (1-20 µl) of factor IXa, ³H-factor X, cephalin, and Ca²⁺ were added to 0.3-0.6 ml TBS-1% BSA or factor VIII in TBS-1% BSA to achieve appropriate final concentrations. One vial of cephalin was reconstituted as a concentrated suspension with 1.0 ml of TBS in order to permit use of small volumes. A ratio of 40 µl of this concentrated cephalin to 1.0 ml of total volume was found to be optimal. Some experiments were performed in 2 mM benzamidine-HCl. The final concentration of factor VIII after all the reactants were added was lowered by only 10%-15% of the initial concentration, which facilitated attempts to study ³H-factor X activation at high factor VIII concentrations. A single stock solution of 1 M CaCl₂ was used throughout the experiments; titration of CaCl₂ in the assay in the presence of factor IXa and thrombin-activated factor VIII showed the optimal range to be approximately 6-11 mM. Therefore all experiments were performed at 7-10 mM CaCl₂.

Initially, a 3-4-min preincubation of factor VIII, factor IXa, Ca²⁺, and cephalin was used with a final addition of ³H-factor X. This did not appear to enhance ³H-factor X activation compared to experiments in which no preincubation was used and Ca²⁺ was the final addition. Therefore the latter method was adopted as the standard technique. The addition of Ca²⁺ was considered the zero timepoint. Serial timed 50-µl subsamples (or 20 µl in some experiments) were withdrawn and the reaction was stopped by adding each subsample to a microcentrifuge tube con-
taining 0.1 ml TBS-0.1% BSA and 0.1 ml 50 mM EDTA pH 7.5 on ice. At the end of the time course, 0.1 ml 15% TCA at 4°C was added to each tube, which was vortexed and then centrifuged at 10,000 g for 4 min in a Beckman Microfuge B. A subsample taken for total counts was not centrifuged. The supernatants were removed immediately and duplicate 0.1-ml aliquots of each supernatant were counted in 4 ml of toluene-based scintillant in a Searle Delta 300 liquid scintillation counter or, in one series of experiments, a Beckman LS-3155T Counter. A 50-μl subsample was taken before the addition of Ca^{2+} (or immediately after the addition of ^3H-factor X by the prior method) in order to determine the baseline cpm of tritium attributable to the small fraction of factor X soluble in TCA at a final concentration of 4.3% or 4.7%. The baseline count was <3% of the total counts in all cases, just as in 5% TCA; the baseline was subtracted from the experimental timed points. The subsample for total counts was used to calculate the ^3H-factor X concentration.

The raw data in cpm were converted to nmoles/ml of factor X activated and plotted arithmetically versus the time of subsampling from the activation mixture. If the plot was linear, the v₀ of factor X activation was expressed as the slope of this plot, which was calculated by the least-squares regression method. A slope of 1.0 (Figs. 6-9) equals a v₀ of factor X activation of 14 nmoles/min.

RESULTS

When factor IXa was incubated with ^3H-factor X in the presence of cephalin and 10 mM CaCl₂, factor X activation began after a lag of 5–10 min and displayed a steadily increasing rate. This nonlinearity was probably due to feedback autocatalysis of factor X by the product factor Xa. In order to determine if factor IXa alone could activate factor X, an inhibitor of factor Xa was included in subsequent experiments. Preliminary experiments with varying levels of soybean trypsin inhibitor, antithrombin III, and benzamidine hydrochloride showed that 2 mM benzamidine hydrochloride (BZA) was most feasible, since it substantially inhibited factor Xa without abolishing factor IXa activity. This is similar to the finding that BZA inhibited factor Xa more effectively than factor VIIa.¹⁴

Figure 1 shows the results of four experiments performed in 2 mM BZA. When ^3H-factor X was incubated with cephalin and 10 mM CaCl₂, no tritiated peptide release was observed over 1 hr (Fig. 1D), whereas in the presence of

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**Fig. 1. Activation of factor X (nmoles/ml) by factor IXa.** A (×): factor IXa (RVV-activated, 0.1 μM), ^3H-factor X (1.25 μM), cephalin, 10 mM CaCl₂, and 2 mM BZA in TBS. B (○): same as A with treated normal serum added. C (●): same as A with treated anti-factor IX serum added. D (•): same as A with omission of factor IXa.
factor IXa a linear rate of tritiated peptide release was observed after an initial lag of 5 min. (Fig. 1A). It is highly likely that this peptide release represented factor X activation due to the action of factor IXa, since the addition of a partially purified human factor IX inhibitor prevented the activation (Fig. 1C). In contrast, the activation of factor X in the presence of factor IXa was not prevented by a similarly prepared fraction of normal human serum (Fig. 1B); the partial inhibition of factor IXa by this serum fraction is probably due to residual antithrombin III. These data support the concept that factor IXa can activate factor X proteolytically in the absence of factor VIII.

The role of factor VIII in factor X activation was studied in incubation mixtures of factor VIII, 3H-factor X, cephalin, and CaCl2 with and without factor IXa. Purified human factor VIII enhanced factor X activation in the presence of factor IXa and shortened the initial lag to 1-2 min; the same qualitative effect was observed with either contact factor-activated or RVV-activated factor IX (Fig. 2). When factor X activation was followed for incubation periods of >5 min, a nonlinear, increasing rate of activation was observed in some experiments. Since no inhibitor of factor Xa was included in these experiments, this increasing rate may represent factor Xa feedback on factor X or factor VIII. When factor IXa was omitted, no factor X activation was observed by factor VIII over 4 hr (Fig. 2C).

The amount of factor X activation in the presence of unactivated factor VIII and factor IXa was small and not consistently reproducible. Since it has been suggested that thrombin activation of factor VIII is essential for its function, subsequent experiments were designed to evaluate the ability of factor VIII to support the activation of 3H-factor X. Purified human factor VIII was activated with purified human thrombin (0.01 U/ml) for 4 min at 37°C, with a subsample taken at 3 min for factor VIII assay. At 4 min, factor IXa, cephalin, CaCl2, and 3H-factor X were added to the activated factor VIII and 3H-factor X activation monitored (Fig. 3A). Considerably more 3H-factor X activation was observed with factor VIII than in the control experiment with unactivated factor VIII, and the initial lag was shortened to 30 sec or less (Fig. 3A). Thrombin alone, without factor VIII, produced no factor X activation. Repetitive experiments confirmed this qualitative observation but showed considerable variation in the rate of 3H-factor X activation.

Since this variation was most likely due to differences in the degree of activation of factor VIII in each experiment, a method was developed to activate factor VIII more reproducibly and stabilize factor VIII. A stock dilution of purified thrombin (9 U/ml in TBS-1% BSA) was kept at -70°C in aliquots; a
Fig. 3. Effect of thrombin-activated factor VIII on activation of factor X (nmol/mL). (A) 
$^{3}$H-factor X (1.4 μM), RVV-activated factor IX (36 nM), cephalin, and 10 mM CaCl$_2$ in 
TBS with (x) thrombin-activated purified factor VIII (1 U/mL prior to activation, >20 U/mL 
after activation) or (o) unactivated factor VIII (1 U/mL). (B) $^{3}$H-factor X (1.8 μM), RVV-
activated factor IX (54 nM), cephalin, 10 mM CaCl$_2$, and 2 mM BZA in TBS with (x) thrombin-
activated partially purified factor VIII (5 U/mL prior to activation, at least 50 U/mL after 
avtivation) or with (o) unactivated factor VIII (5 U/mL). Thrombin-activated factor VIII (x) 
produced no factor X activation in absence of factor IXa, with all other reactants same as with 
(o) symbols.

fresh aliquot was thawed just before use and diluted in TBS–1% BSA in a polystyrene tube on ice. Partially purified human factor VIII could be reproducibly activated 10-20-fold at 4-5 min incubation with 0.01 U/mL of thrombin at 37°C and stabilized by the addition of 2 mM BZA (final concentration) after 4.5 min incubation (Fig. 4). This stabilization was also observed, but to a lesser degree, at higher thrombin concentrations. Partially purified factor VIII, in 2 mM BZA supported $^{3}$H-factor X activation in the presence of factor IXa similarly to purified factor VIII, with an initial lag of 30-60 sec (Fig. 3B), whereas unactivated factor VIII in 2 mM BZA showed little or no ability to support $^{3}$H-factor X activation over 10 min (Fig. 3B). Factor VIII, in 2 mM

Fig. 4. Activation of factor VIII by thrombin (0.01 U/mL added at time zero) at 37°C. After 4 min incubation, activated factor VIII was split into two aliquots and BZA added to one (x) at a final concentration of 2 mM. Subsequent factor VIII assays showed stabilization of factor VIII, in BZA compared to aliquot without BZA (x). Thrombin at this concentration does not affect the factor VIII assay.
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Fig. 5. Effect on factor X activation (nmoles/ml) of varying factor VIII, 3H-factor X (1.8 μM), RVV-activated factor IX (89 nM), cephalin, 10 mM CaCl₂, and 2 mM BZA in TBS with varying factor VIII concentrations (○, □, △), with unactivated factor VIII (1 U/ml) (●), or with no factor VIII (△).

BZA without factor IXa did not support 3H-factor X activation (Fig. 3B). This method for activating and stabilizing factor VIII, markedly decreased day-to-day variation in the amount of 3H-factor X activation observed relative to the initial factor VIII concentration. When a single batch of factor VIII was activated and varying aliquots tritiated with a constant level of factor IXa, the amount of 3H-factor X activation was directly proportional to the factor VIII concentration (Fig. 5). In the absence of factor VIII, factor IXa activated 3H-factor X very slowly (Fig. 1A). The greatest potentiation of v₀ observed in the presence of factor VIII, was 550-fold. Unactivated factor VIII showed minimal 3H-factor X activation after 10 min incubation in 2 mM BZA (Fig. 5).

When the factor IXa concentration was varied with a constant level of factor VIII, the amount of 3H-factor X activation observed was directly proportional to the factor IXa concentration (Fig. 6A). The secondary plot of v₀ versus factor IXa concentration was linear (Fig. 6B); thus in the presence of a constant amount of factor VIII, this method provides an assay for factor IXa. The reproducibility of the assay using factor IXa activated by RVV as the standard was tested by six independent assays at a factor IXa concentration of 5 μg/ml; the mean ± 1 SD of the v₀ was 1.8 ± 0.2 (Fig. 6B). The range of sensitivity of the assay in 2 mM BZA was 1–10 μg/ml.

The specificity of the assay was compared to the clotting assay for factor IXa in the following manner: Purified factor Xa and thrombin were added separately at various concentrations to factor IXa and both the clotting assay and 3H-factor X activation assay were performed on the mixtures and on the same concentration of factor IXa alone. The range of the clotting assay was 0.1–1.5 μg/ml. At a factor IXa concentration of 0.2 μg/ml, as little as 1 ng/ml of thrombin or 0.1 ng/ml of factor Xa completely invalidated the factor IXa clotting assay by increasing the apparent factor IXa activity 7-fold and 13-fold, respectively. In contrast, equivalent amounts of thrombin (10 ng/ml) or factor Xa (1 ng/ml) added to 2 μg/ml factor IXa did not affect the factor IXa assay by the 3H-factor X activation method.

The specificity of the latter assay has great potential advantage for measuring factor IXa in samples that contain factor Xa or thrombin. However, the lack of sensitivity of the assay to levels of factor IXa below 1 μg/ml may be a disadvantage. Therefore a modification was developed permitting the omission of BZA and increasing the sensitivity of the assay tenfold, to a range equivalent to the clotting assay. Bovine factor VIII, is known to be more stable than human factor VIII, and therefore it was used instead. Bovine factor VIII, factor IXa, 3H-factor X, and cephalin were combined in TBS at 37°C, and a mixture of thrombin-CaCl₂ (0.01 U/ml and 10 mM final concentrations respectively) was
added to start the activation of factor VIII. $^3$H-factor X activation became apparent by 60 sec using this method, and the progress curve was linear for 4–5 min followed by a gradual drop in rate. When factor IXa was kept constant the $v_0$ (the slope of the linear 4-min plot) was a linear function of the factor VIII concentration (Fig. 7). It is evident that we were unable to saturate a small amount of factor IXa (0.16 μg/ml) with increasingly high levels of factor VIII, (up to 270 U/ml). When thrombin was omitted, minimal $^3$H-factor X activation was observed. In other words, $^3$H-factor X activation is dependent on the presence of factor VIII, and is directly proportional to the concentration of factor VIII. In the absence of factor IXa, bovine factor VIII, did not activate $^3$H-factor X. Thus the results for bovine and human factor VIII are similar.

When a constant amount of bovine factor VIII was incubated with varying amounts of factor IXa, the $v_0$ of $^3$H-factor X activation was directly proportional to the factor IXa concentration over a range of 0.1–1.0 μg/ml (Fig. 8). The reproducibility of this method for assaying factor IXa was somewhat better.
Fig. 7. Effect of varying bovine factor VIII, on $v_0$ (slope of factor X activation in presence of constant levels of RVV-activated factor IX (0.16 μg/ml or 3 nM), $^3$H-factor X (1.8 μM), cephalin, and 7 mM CaCl₂ in TBS without BZA.

than the assay using human factor VIII, in 2 mM BZA (Fig. 6); at 0.16 μg/ml factor IXa, the mean ± 1 SD of $v_0$ was 0.43 ± 0.01, and at 0.31 μg/ml it was 0.79 ± 0.04 ($n = 6$). However, the specificity of the assay was less than that of the assay using human factor VIII, in BZA, since the presence of small amounts of thrombin affected the assay by changing the level of factor VIII. Figure 9 shows the effect on the factor IXa assay of small changes in the thrombin concentration and the initial factor VIII concentration. The addition of a trace amount of factor Xa to factor IXa (0.1 ng/ml to 0.1 μg/ml) did not change the factor IXa assay using bovine factor VIII, without BZA, whereas the clotting assay of factor IXa was invalidated by this level of factor Xa. Thus this $^3$H-factor X activation method provides an assay of factor IXa that is more specific than the clotting assay with an equivalent range of sensitivity.

Fig. 8. (A) Effect of varying RVV-activated factor IX on activation of factor X (nmoles/ml). $^3$H-factor X (1.8 μM), bovine factor VIII, (100 U/ml), cephalin, and 7 mM CaCl₂ in TBS without BZA were kept constant with varying factor IXa concentrations of 0.16 μg/ml or 3 nM (*), 0.31 μg/ml (•), or 0.62 μg/ml (x). (B) Secondary plot of factor IXa concentration versus $v_0$ (slope) of factor X activation. Conditions of activation same as in A. Bars at 0.16 and 0.31 μg/ml, mean ± 1 SD of six determinations.
DISCUSSION

The mechanism of activation of factor X by the intrinsic pathway of blood coagulation is not well understood. Previous studies have suggested that an activator complex is formed composed of factor IXa, factor VIII, phospholipid, and Ca²⁺. One theory of the role of these reactants holds that factor IXa is a serine protease, since it is highly homologous to the other serine proteases involved in blood coagulation, and that factor VIII must serve as a cofactor to factor IXa. If this were so, one would expect that factor IXa alone could cleave factor X, albeit perhaps slowly. Previous studies suggested this but were limited by the techniques used to assess factor X activation. Using our newly developed ³H-factor X activation assay we showed that RVV-activated factor IX can activate factor X very slowly in the presence of cephalin, calcium ions, and a factor Xa inhibitor. The amount of activation seen within a 1-hr incubation was considerably less than 1% of the factor X and would not be detected by SDS-PAGE. The fact that this activation was blocked by a specific factor IX inhibitor supports the conclusion that the factor X activation observed is the specific effect of factor IXa and not of some undetected contaminating enzyme. This evidence confirms that RVV-activated factor IX is a proteolytic enzyme, as was expected from the structure of factor IX. RVV-activated factor IX possesses the same heavy chain with the active serine site as factor XIa-activated factor IX but has half the specific coagulant activity of the latter. Thus it is probable that these two forms of factor IXa possess the same enzymatic activity toward factor X, although the quantitative kinetic parameters may differ.

The role of factor VIII in factor X activation is controversial. Most previous evidence points to its being a cofactor to factor IXa, but one recent study suggested that it may be a serine protease itself. However, neither this nor other studies were able to show that factor VIII or factor VIIIᵢᵢ in the absence of factor IXa, could activate factor X. We also were unable to demonstrate any factor X activation by factor VIII or factor VIIIᵢᵢ. Whereas unactivated factor
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VIII only weakly potentiated factor X activation by factor IXa after an initial lag, factor VIII, markedly enhanced factor X activation in the presence of factor IXa as much as 550-fold. These data are consistent with the concept that factor VIII is a cofactor to factor IXa and that the cofactor function of factor VIII is greatly improved by thrombin activation. Whether or not native, unactivated factor VIII can support factor X activation was not resolved by our data. The fact that we were unable to demonstrate saturation may simply reflect a requirement for higher levels of factor VIII, than we were able to generate in vitro. In the similar case of factor X activation by the extrinsic pathway it was also difficult to saturate factor VIIa with tissue factor. Once saturation is achieved, this method could be used to quantify the kinetics of factor X activation by the intrinsic pathway, as was recently reported for the extrinsic pathway.

The reason for the initial lag prior to factor X activation is unclear. When unactivated factor VIII is used, the lag was 1–2 min in the absence of BZA and more than 10 min in the presence of BZA. This suggests that some initial activation of factor VIII may occur during the lag and is a prerequisite for subsequent factor X activation to occur. The prolongation of the lag by BZA could be explained by an inhibition of the activation of factor VIII. The enzyme responsible for the activation of factor VIII could be a small amount of factor Xa produced by factor IXa, that is, a feedback of the product. When factor VIII was pre-activated, the lag in factor X activation was 30 sec or less, which may be explained as a real lag required for the reactants in this lipid-dependent activation to reach a steady state.

We showed that 3H-factor X could be used to assay factor IXa and that this assay in the presence of BZA is more specific than a factor IXa clotting assay, which is invalidated by the presence of thrombin or factor Xa. This method also has potential as an assay for factor VIII and factor VIII, but it requires refinement. The observation that benzamidine stabilizes factor VIII, supports the hypothesis that inactivation of factor VIII in the presence of thrombin is due to proteolysis by thrombin. It is uncertain whether or not factor VIII, is produced in vivo and whether or not it is a stable species. Our data suggest that factor IXa may not be saturated by factor VIII (or factor VIII, in vivo, since we were unable to saturate a factor IXa concentration equal to 2% of the plasma factor IX level with levels of factor VIII activity unlikely to be achieved in vivo. If this hypothesis is true, then the activation of factor X by the intrinsic pathway may be a rate-limiting step in coagulation in vivo.

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