Cooperative Activation of Human Factor IX by the Human Extrinsic Pathway of Blood Coagulation*

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The activation of human coagulation factor IX by human tissue factor-factor VIIa-PCPS-Ca\textsuperscript{2+} (TF-VIIa-PCPS-Ca\textsuperscript{2+}) and factor Xa-PCPS-Ca\textsuperscript{2+} enzyme complexes was investigated. Reactions were performed in a highly purified system consisting of isolated human plasma proteins and recombinant human tissue factor with synthetic phospholipid vesicles (PCPS: 75% phosphatidylcholine (PC), 25% phosphatidylserine (PS)). Factor IX activation was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, [\textsuperscript{3}H]factor IX activation peptide assay, colorimetric substrate thionbenzyl benzoylarnidyl L-lysinate (Z-Lys-SBzl) hydrolysis, and specific incorporation of a fluorescent peptidyl chloromethyl ketone. Factor IX activation by the TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex was observed to proceed through the obligate non-enzymatic intermediate species factor IXa. The simultaneous activation of human coagulation factors IX and X by the TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex was investigated. When factors IX and X were presented to the TF-VIIa complex, at equal concentrations, it was observed that the rate of factor IX activation remained unchanged while the rate of factor X activation slowed by 45%. When the proteolytic cleavage products of this reaction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it was observed that the intermediate species factor IXα was generated more rapidly when factor X was present in the reaction mixture. When factor IX was treated with factor Xa-PCPS in the presence of Ca\textsuperscript{2+}, it was observed that factor IX was rapidly converted to factor IXα. The activation of factor IXα by the TF-VIIa-PCPS-Ca\textsuperscript{2+} complex was evaluated, and it was observed that factor IXα was activated more rapidly by the TF-VIIa-PCPS-Ca\textsuperscript{2+} complex than was factor IX itself. These data suggest that factors IX and X, when presented to the TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex, are both rapidly activated and that factor Xa, which is generated in the initial stages of the extrinsic pathway, participates in the first proteolytic step in the activation of factor IX, the generation of factor IXα.

Human factor IX (Christmas factor)\textsuperscript{1} is a 57,000-dalton single chain glycoprotein that is essential for normal blood coagulation (1). The biologic importance of factor IX in hemostasis is illustrated by the disease hemophilia B in which patients with congenial defects in factor IX synthesis or function develop a severe bleeding diathesis. Factor IXa participates in the middle phase of the blood coagulation pathway by activating factor X to Xa. Factor XIa in the presence of Ca\textsuperscript{2+} activates factor IX to factor IXα (2). Factor IXa can also be activated by the extrinsic coagulation pathway (tissue factor and factor VIIa) (3). In spite of in vitro evidence demonstrating the importance of factor IX, enigmatic data from in vitro studies have not satisfactorily explained how defects in the function of factor IX, or factor VIII, inhibits the propagation of blood coagulation. It has been shown that the lack of plasma activators of factor IX by the intrinsic pathway (factors XI and XII) results in mild or no bleeding disorders (4). Furthermore, a recent report by Bauer et al. (5) suggested that patients with hereditary factor VII deficiency activated less factor IX in vitro than either normal controls or patients with factor XI deficiency. These reports would suggest that the physiologically important factor IX activation may proceed via the extrinsic pathway. However, when the relative rates of human (6) and bovine (7) factors IX and X activation by the extrinsic pathway were studied, it was observed that factor X was activated between two and seven times more rapidly than bovine factor IX using in vitro activation peptide release assays. From these data it was generally concluded that factor X activation by the extrinsic pathway could rapidly bypass the need for factor IX activation. In contrast to these studies, a recent report by Komiyama et al. (8) has suggested that factor IX may be the preferred substrate for the TF-VIIa enzyme complex in a purified reaction system. Thus, there appears to be little agreement on the physiological role of factor IX activation by either coagulation pathway.

The role of factor Xa in the activation of factor IX is

\textsuperscript{1}The nomenclature used for factor IX and its activation products is that of Davie and co-workers (1, 2): factor IX, single-chain native factor IX (Mr = 57,000); factor IXα, two-chain inactive intermediate consisting of a heavy chain (Mr = 39,000) and a light chain (Mr = 18,000); factor IXα\textsuperscript{2}, two-chain intermediate consisting of a heavy chain (Mr = 28,000) and a heavy light chain (Mr = 29,000); factor IXα, the two-chain enzymatic product of factor IX activation which consists of a heavy chain (Mr = 28,000) and a light chain (Mr = 18,000) after the activation peptide (AP; Mr = 11,000) has been released from the factor IX protein (see Fig. 1).

\textsuperscript{2}The abbreviations used are: TF, tissue factor; PS, 1-palmitoyl-2-oleoyl phosphatidylserine; PC, 1-palmitoyl-2-oleoyl phosphatidylcholine; Z-Lys-SBzl, thiobenzyl benzoylarnidyl L-lysinate; FPR-ck, d-phenylalanl-1-prolyl-L-arginine chloride methyl ketone; Ir-FPR-ck, lissamine rhodamine-labeled FPR-ck; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; VIIa, recombinant human factor VIIa, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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unclear. It has been shown that bovine factor Xa can slowly activate bovine factor IX to IXaβ (2, 9). However, it has been reported that human factor Xa does not activate factor IX to IXaβ (3). This has led to the general conclusion that if factor Xa activates factor IX to IXaβ, this reaction proceeds so slowly that it is of little physiologic significance (2).

During the activation of factor IX, two peptide bonds must be hydrolyzed to generate the active enzyme species IXaβ (Fig. 1) (1, 10). The mechanism of factor IX activation by the TF-VIIa-phospholipid-Ca2+ enzyme complex has been an area of dispute. Zur and Nemerson (11) reported that during the activation of bovine factor IX by the bovine TF-bovine VIIa-mixed brain phospholipid complex that the Arg145-Ala146 and Arg150-Val151 bonds were cleaved so rapidly that no intermediate species was observed upon analysis of the reaction. They concluded that the rate-limiting step in the activation of bovine factor IX was the cleavage of the first peptide bond Arg145-Ala146. Data reported by Bajaj et al. (10) suggested that the activation of human factor IX by human TF-human VIIa-mixed brain phospholipid complex occurs in a two-step process. In their model the first step of factor IX activation occurs with the cleavage of the Arg145-Ala146 peptide bond, which forms the two-chain inactive intermediate IXα. Factor IXα is then cleaved by a second proteolytic step at the Arg150-Val151 peptide bond resulting in the formation of the active enzyme species IXaβ. During this second step, a 35-amino acid activation peptide (AP) is released containing residues 146-180 (Fig. 1). From these two reports, it is unclear whether the difference in activation mechanisms of factor IX can be explained by species differences between the bovine and human molecules or if different methodologies lead to different conclusions about the basic activation mechanisms of factor IX by the extrinsic pathway.

In this report, we clearly establish the order of the proteolytic steps involved in the activation of human factor IX by the human extrinsic pathway (TF-VIIa-PCPS-Ca2+). We have also investigated the relative rates of human factor IX and human factor X activation by the extrinsic pathway when these proteins are presented to the TF-VIIa-PCPS-Ca2+ enzyme complex as simultaneous substrates. Lastly, the role of human factor Xa-PCPS-Ca2+ in the activation of human factor IX was investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**

1-Palmitoyl-2-oleoyl phosphatidylserine (PS), 1-palmitoyl-2-oleoyl phosphatidylcholine (PC), and n-dodecyl octaethylene glycol ether (polyoxyethylene 8 lauryl ether; C12E8) were purchased from Sigma. Phospholipid vesicles (PCPS) composed of 75% PC and 25% PS were prepared as described (12). Thyroxyll benzyloxy-carbonyl-L-lysyl-(Z-Lys-SBzl) was purchased from Peninsula Laboratories. Dithiol-nitrobenzoic acid was purchased from Sigma. Spectrozyme Xa was purchased from American Diagnostica. Sodium [3H]borohydride was purchased from Du Pont-New England Nuclear. D-Phenylalanyl-L-prolyl-1-arginine chloromethyl ketone (PFR-ck) was purchased from Calbiochem. Li-saminase-rodhamine-labeled PFR-ck (Ir-FPR-ck) was prepared as described previously (13). Anti-factor IX-Sepharose was prepared as described previously (14). All other reagents were of analytical grade.

**Proteins**

Human coagulation factors IX and X were isolated from fresh frozen plasma obtained from the Vermont Red Cross using the general methods of Bajaj et al. (15) with modifications described by Krishnaswamy et al. Generally proteins were isolated from fresh frozen plasma by precipitation with barium citrate. The precipitated proteins were subjected to anion-exchange chromatography using DEAE-Sepharose (Pharmacia LKB Biotechnology) and Bio-Gel P-30 (Bio-Rad) columns. The fractions which contained factors IX and X were pooled and subjected to a second chromatographic step on heparin-Sepharose which allowed for distinct resolution of factors IX and X. Factor IX and X were concentrated by ultrafiltration followed by precipitation in 80% saturated (NH4)2SO4. Precipitated proteins were collected by centrifugation and resuspended in 50% (v/v) glycerol and water and stored at -20°C. From seven liters of human fresh frozen plasma, 12 mg of factor IX and 15.6 mg of human factor X were isolated. Factor Xa was prepared by activating factor X with purified Russell's viper venom as described previously (16). Purified human factor VIIa was prepared by the methods of Jenny et al. (14) and was obtained from Haematologic Technologies, Essex Junction, VT. Recombinant human tissue factor (TF) was provided as a gift from Dr. Hisseis of Genentech, Inc. Recombinant human factor VIIa (rVIIa) was provided as a gift from Dr. Ulla Hedner, Novo Pharmaceuticals.

For the proteins used in this study concentration were calculated using the following molecular weights (Mw) and extinction coefficients (ε260): human factor VIIIa/VIIa, 50,000, 1.39 (17); human factor IX, 57,000, 1.33 (18); human factor X, 58,900, 1.16 (18); human factor Xa, 45,000, 1.16 (18). The concentration of recombinant human TF was determined by amino acid analysis provided by Genentech Inc. (19).

**Methods**

**Relipidation of Recombinant Human Tissue Factor—** Human recombinant tissue factor was solubilized in 0.04% (v/v) n-dodecyl octaethylene glycol ether (C12E8, Mw = 539). The solubilized tissue factor was mixed with PCPS and incubated at 37°C for 30 min in 0.02 M Hepes, 0.15 M NaCl, pH 7.4 (HBSS). The TF-n-dodecyl octaethylene glycol ether-PCPS mixture was then diluted to a final concentration of 14 mM tissue factor, 1 nM PCPS, and 1.7 x 10^-5 g/cm3 of H-factor IX. The labeled protein possessed 74% of the biological activity of the nonlabeled control factor IX when tested in coagulation assay generally described by Proctor and Rapaport (21), using factor IX deficient plasma supplied by George King Co. and aPTT reagent provided by Sigma.

**FIG. 1.** Schematic representation of the pathways for factor IX activation. The TF-VIIa enzyme complex catalyzes the cleavage of two peptide bonds in factor IX to yield factor IXaβ. The reaction proceeding via steps 1 and 2 (cleavage at Arg145-Ala146, followed by cleavage at Arg150-Val151) involves factor IXα as an intermediate. The reaction proceeding via steps 3 and 4 involves factor IXαβ as an intermediate. Factor IXαβ was never observed in any factor IX activation reactions where TF-VIIa-PCPS-Ca2+ was used as the activating enzyme complex.
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The progress curve of factor X activation when compared with a observed that n-dodecyl octaethylene glycol ether had no effect on using octyl glucoside followed by extensive dialysis.

were: 500 nM factor x, 1 nM Wa, 14 nM TF, 1 mM PCPs, and 5 mM spectrophotometer. The concentration of components of the reaction change at 405 nm was monitored by a Molecular Devices Vmax spectrophotometer.

Activation of Human Factor IX, Factor X, or Factors IX and X by the TF-VIIa-PCPS-Ca²⁺ Complex—After relipidation of the recombinant tissue factor, natural factor VII/VIIa or rVIIa (5 nM) was added to the reaction mixture and incubated at 37 °C for 15 min. The activation of human factor IX, factor X, or factors IX and X were carried out under the following reaction conditions: tissue factor (14 nM), PCPs (1 mM), and CaCl₂ (5 mM) at 37 °C in 0.02 M Hepes, 0.15 M NaCl, pH 7.4 (HBS). After the TF-VIIa-PCPS-Ca²⁺ enzyme complex was assembled, substrates of factor IX, factor X, or a mixture of factors IX and X (2 μM for each zymogen) were added to the reaction mixture, and the reaction was started. A progress curve of zymogen activation was generated by removing aliquots from the reaction mixture at designated time points and quenching the reaction in 25 mM EDTA (final concentration). The progress of various reactions was monitored by SDS-PAGE, colorimetric substrate hydrolysis, [³H]factor IX activation peptide release assay, and incorporation of Ir-FPR-ck into polypeptides which contain serine protease active sites.

Colorimetric Substrate Assays—Factor IX activation was monitored by two step colorimetric substrate hydrolysis as generally described by Green and Shaw (24). Factor IX was added to the assembled TF-VIIa-PCPS-Ca²⁺ enzyme complex, and reaction subsamples were removed from the activation reaction at designated time points and quenched in EDTA (25 mM) as described above. A 100-μl aliquot from each reaction time point was added to 60 μM thioheisen benzoyloxycarbonyl-L-lysinate (Z-Lys-SBzl) and 60 μM dithiolnitrobenzoic acid dissolved in HBS, pH 7.4. The rate of Z-Lys-SBzl hydrolysis was followed spectrophotometrically by allowing the released benzyl mercaptan to react directly with the dithiolnitrobenzoic. The increase in absorbance at 405 nm was due to the formation of the 3-carboxy-4-nitro-phenoxide ion (E₄₀₀ 13,600 M⁻¹ cm⁻¹), which was monitored continuously in a Molecular Devices Vmax spectrophotometer.

Factor X activation was monitored by chromogenic substrate hydrolysis using the substrate Spectrozyme Xa. Reaction subsamples of factor X activation were removed and quenched in EDTA (25 mM) as described above. Each subsample was mixed with 200 μM Spectrozyme Xa and 100 μM PCPs (1 mM) and kept at 37 °C. The rate of substrate hydrolysis was monitored by an absorbance change at 405 nm in a Molecular Devices Vmax spectrophotometer.

3H-Labeled Factor IX Activation Peptide Release Assay—[³H]Factor IX activation by the TF-VIIa-PCPS-Ca²⁺ enzyme complex was monitored by the method of Zur and Nemerson (11) with the following modifications. [³H]Factor IX (2 μM, pH 7.4) was added to the assembled TF-VIIa-PCPS-Ca²⁺ complex as described above. At designated time points, a 60-μl aliquot of the reaction mixture was removed and immediately treated with an equal volume of 50 mM EDTA. Thirty microliters of the EDTA-treated sample were added to 200 μl of 0.1% bovine serum albumin in 0.02 M Tris, 0.15 M NaCl, pH 7.4 (TBS), and the remaining 90 μl of the EDTA-treated sample from each time point was treated with SDS, 2-mercaptoethanol, and prepared for SDS-PAGE. This mixture was then added to 250 μl of ice-cold 10% trichloroacetic acid and allowed to incubate on ice for 3 min. The sample was then centrifuged for 5 min at 9000 × g in a Beckman Microfuge 151 fixed angle rotor at 22°C. Duplicates of 200-μl aliquots were added to 5 ml of aqueous scintillation fluid and counted for tritium in a liquid scintillation counter. The rate of [³H]activation peptide release, as measured by an increase in trichloroacetic acid-soluble tritium, was compared with the rate of [³H]factor IXa generation as described below. The [³H]factor IXa counts per minute (cpm) in each sample was corrected for the background counts remaining soluble in the trichloroacetic acid supernatant when a plateau was observed in the reaction progress curve. This plateau corresponded well with the complete conversion of [³H]factor IX to [³H]factor IXa as documented by SDS-PAGE analysis of the reaction as described above. Control experiments were performed with no factor IXa and/or tissue factor in the activation reaction mixture to evaluate for possible enzyme contamination of the tissue factor, factor VIII, and factor IX preparations.

Electrophoresis—Factor IX activation was monitored by SDS-PAGE using 10% polyacrylamide gels. Subsamples (50 μl) of activa tion reactions were run on a Microgel 1000 scanning densitometer (TRI Inc.) equipped with a solid-state linear diode array camera digitized images through a photographic lens. Digitized images contain approximately 0.25 × 10⁶ pixels with each pixel measuring 0.16 mm × 0.16 mm. Linearity of the system is 0–2.5 absorbance units. Data were analyzed with a 80826-based computer equipped with a math coprocessor and software which allows for either automatic or manual background subtraction and full editing capability. Data are expressed as integrated volumes for each protein band using the arbitrary density units of the scanning system.

Treatment of Reaction Samples with Ir-FPR-ck Activation reactions of both factors IX and X were monitored by treatment of reaction subsamples with Ir-FPR-ck. Ir-FPR-ck was used to identify polypeptides which contain serine protease active sites. Subsamples from activation reactions (described above) were treated with Ir-FPR-ck as described by Williams et al. (13) with the following modifications. SDS-PAGE-treated samples were incubated with Tris-HCl (50 mM), pH 7.4, and Ir-FPR-ck (30 μM) for 30 min at 37 °C prior to the addition of SDS and 2-mercaptoethanol. Reaction subsamples were then subjected to SDS-PAGE as described above. Protein bands which specifically incorporated Ir-FPR-ck were visualized by excitation with a long-wave UV light box and photographed through a 540-nm cut-off filter. Total protein from each reaction time point was visualized on the gel by staining with Coomassie Brilliant Blue and destaining by diffusion.

Densitometric Analyses—Densitometric analyses of the Coomassie Brilliant Blue-stained SDS-PAGE gel of factor IX and X activation reaction products were performed with a Joyce-Loebl densitometer equipped with a solid-state linear diode array camera to digitize images through a photographic lens. Digitized images contain approximately 0.25 × 10⁶ pixels with each pixel measuring 0.16 mm × 0.16 mm. Linearity of the system is 0–2.5 absorbance units. Data were analyzed with a 80826-based computer equipped with a math coprocessor and software which allows for either automatic or manual background subtraction and full editing capability. Data are expressed as integrated volumes for each protein band using the arbitrary density units of the scanning system.

Activation of Factor IX by Factor Xa—Factor IX was converted to IXa by factor Xa by mixing PCPS (400 μM) with factor Xa (at various concentrations ranging from 80 to 400 nM) and CaCl₂ (5 mM) in HBS, pH 7.4. This mixture was incubated at 37 °C for 5 min prior to the addition of factor IX (2 μM final) to the reaction. Aliquots were removed from the reaction at designated time points, quenched in EDTA (25 mM), and reacted in SDS-PAGE treatment as described above. Protein samples were then subjected to SDS-PAGE analysis as described by Williams et al. (13) with the following modifications. Factor IXa (final concentration) in HBS, pH 7.4, and 0.01 M Tris-HCl, pH 6.8, 10% glycerol, and 2% (v/v) 2-mercaptoethanol, heated for 5 min at 90 °C, and analyzed by SDS-PAGE as described by Laemmli (25). Protein bands were visualized by staining the gels with Coomassie Brilliant Blue and destaining by diffusion.
described above, followed by scanning densitometry of the Coomassie Brilliant Blue-stained gel.

Isoelectric Focusing by Immunoaffinity Chromatography—Factor IX (6 μM) was converted to IXα by factor Xa (100 nM), PCPS (400 μM), and CaCl₂ (5 mM) in HBS, pH 7.4, at 37°C. The reaction was allowed to proceed for 30 min and was stopped by the addition of EDTA (final 25 mM). Two milliliters of the reaction mixture were loaded onto an anti-factor IX-Sepharose antibody column (10-ml bed volume equilibrated in the same buffer at 4°C) and 1-ml fractions were collected. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.05. Proteins that remained bound to the column were eluted with 3 M sodium thiocyanate (NaSCN), pH 7.4, in HBS. The corrected absorbance was measured, the eluted protein pooled, and then dialyzed exhaustively in HBS, pH 7.4, at 4°C. Factor IX was also subjected to immunoaffinity chromatography on the anti-factor IX Sepharose column. Factor IX was eluted from the anti-factor IX Sepharose with 3 M NaSCN. The density of each protein band visualized on the gel was recorded and plotted as a function of total protein detected in each electrophoresis lane.

The Activation of Factor IX by Both the Xa-PCPS-Ca²⁺ and TF-VIIa-PCPS-Ca²⁺ Enzyme Complexes—Factor IX (2 μM) was added to a mixture of factor Xa (400 μM), PCPS (400 μM), and CaCl₂ (5 mM) in HBS at 37°C. At designated time points, after the addition of factor IX to the Xa-PCPS-Ca²⁺ complex, subsamples from the reaction mixture were removed and quenched in 25 mM EDTA. Twenty-four to forty-five seconds after the initiation of the reaction, a mixture of TF (14 nM), factor VIIa (5 nM), PCPS (1 mM final), and CaCl₂ (6 mM final) was added to the reaction. At designated time points after the addition of the TF-VIIa-PCPS-Ca²⁺ complex to the reaction system, aliquots from the reaction were removed and rapidly quenched in 25 mM EDTA. Reaction subsamples were then treated with SDS, 2-mercaptoethanol, and analyzed by SDSPAGE as described above. The Coomassie Brilliant Blue-stained gels were then analyzed by scanning densitometry. The density of each protein band visualized on the gel was recorded and plotted as a function of total protein detected in each electrophoresis lane.

The solutions for IX, IXα, and IXαp as a function of time are given below in Equations 2-4.

\[ \text{IX} \xrightarrow{k_1} \text{IXα} \xrightarrow{k_2} \text{IXαp} \]

\[ \text{IXα} = \frac{[\text{IXα}]_0}{(k_2 - k_1)} \left( e^{-k_2t} - e^{-k_1t} \right) \]

\[ [\text{IXαp}] = [\text{IXα}]_0 \left[ 1 + \frac{1}{k_1 - k_2} \left( k_2 e^{-k_2t} - k_1 e^{-k_1t} \right) \right] \]

where IX, IXα, and IXαp represent the rate constants for independent steps 1 and 2, and IXα, IXαp, and IXαp represent the product of the reaction IXα to form IXαp (see Fig. 1) and where k1 and k2 represent the relative concentration of factor IX, IXα, and IXαp at time t. Rate constants k1 and k2 reported in the results section were determined by fitting data for the formation of factor IXαp to Equation 4 using the IX, concentration of 2 μM. The overall rate of factor IX activation was calculated from the slope of the product factor IXαp formed per unit time as plotted in the results section.

Progress curve fitting for factor X activation was analyzed using to the integrated form of the Michaelis-Menten equation assuming the conversion of a single substrate to product.

**RESULTS**

The Activation of Factor IX by the TF-VIIa-PCPS-Ca²⁺ Enzyme Complex—Purified human factor IX (2 μM final concentration) was added to the TF-VIIa-PCPS-Ca²⁺ enzyme complex as described under “Experimental Procedures.” Factor IX activation by the TF-VIIa-PCPS-Ca²⁺ complex was evaluated by four different methods: SDSPAGE, colorimetric substrate hydrolysis, [³H]factor IX activation peptide assay, and specific incorporation of the fluorescent-labeled Ir-FPR-ck. SDSPAGE analysis of the cleavage products from the activation of the human factor IX activation reaction illustrates that when factor IX is activated by the TF-VIIa-PCPS-Ca²⁺ complex, the reaction proceeds through the intermediate species factor IXα (Fig. 3). The Coomassie Blue-stained gel represented in Fig. 3A was evaluated by scanning densitometry to quantify the factor IX activation reaction sequence and generate a progress curve of factor IX activation.

Relative staining intensities of each protein band were plotted versus reaction time as shown in Fig. 3B. Factor IXα formation as a function of time was fit to Equation 4 as described under “Experimental Procedures” using the IX, concentration of 2 μM, and the rate constants k1 and k2 for the two reactions were determined by nonlinear least squares regression analysis. The values for k1 and k2 were 5.6 × 10⁻⁷ s⁻¹ and 4.7 × 10⁻⁵ s⁻¹, respectively. The overall rate of factor IX conversion to factor IXαp was calculated as the rate of IXαp formed per min/mol of TF-VIIa complex. Using the known dissociation constant of factor VIIa for tissue factor it was calculated that the effective enzyme concentration in our reaction system was 4.5 mM TF-VIIa complex under equilibrium conditions.

From this the overall rate of factor IXαp formation was determined to be 49 mol of factor IX min⁻¹/mmol of VIIa. The activation pathway observed here is consistent with the report of Bajaj et al. (10) in which an intermediate species was observed in the reaction sequence of human factor IX by the proteolytic cleavage of the Arg¹⁴²-Ala¹⁴⁵ bond. This reaction step is then followed by the cleavage of a second peptide bond at residues Arg¹⁸⁰-Val¹⁸¹. This proteolytic step then causes the release of the activation peptide and generates the active enzyme species IXαp (10). The other possible intermediate in the factor IX activation pathway, factor IXα (steps 3 and 4, Fig. 1) was not observed in any activation reactions analyzed in this report suggesting that factor IXα is the sole intermediate in factor IX activation by the TF-VIIa-PCPS-Ca²⁺ complex.

To identify which polypeptide in the factor IX activation sequence contained a serine protease active site, subsamples from the factor IX activation reaction were treated with Ir-FPR-ck. From these experiments it was observed that only the polypeptide which migrated as the heavy chain of activated factor IX (IXαp hc) Mr, = 28,000 specifically incorporated the fluorescent label (Fig. 4). This provides strong evidence that only the heavy chain of activated factor IXαp expresses the serine protease active site. At no time was the IXα heavy chain observed to incorporate Ir-FPR-ck, which is consistent with the conclusion that the IXα species is a non-enzymatic intermediate of the factor IX activation process.

A progress curve of factor IX activation was also generated by removing aliquots from the reaction mixture and assaying each reaction sample for the ability to hydrolyze the thio-
enzyl ester Z-Lys-SBzl. This enzymatic hydrolysis was compared to the rate of factor IXa \( \beta \) hc generation as measured by scanning densitometry of a reduced Coomassie Brilliant Blue-stained gel (Fig. 5). The progress curve from this reaction showed that factor IX was completely converted to IXa after 15 min and that the initial rate of factor IXa \( \beta \) generation under these reaction conditions was 46 mol min \(^{-1}\) mol of VIIa. These data illustrate that enzymatic activity of factor IXa \( \beta \), as reflected by Z-Lys-SBzl hydrolysis, parallels the generation of the polypeptide species which corresponds to the heavy chain of activated factor IX (IXa \( \beta \) hc, \( M_c = 28,000 \)).

The activation of \([\text{H}]\) factor IX by the TF-VIIa-PCPS-Ca\(^{2+}\) complex was investigated. The rate of \([\text{H}]\) factor IX activation was measured using both the activation peptide release assay and scanning densitometry of a Coomassie Brilliant Blue-stained gel, which contained reaction subsamples from a \([\text{H}]\) factor IX activation reaction. These data were compared with the rate of unlabeled factor IX activation as measured by scanning densitometry of a Coomassie Brilliant Blue-stained gel and colorimetric substrate hydrolysis (Fig. 5). Data in this figure illustrate that the rate of \([\text{H}]\) factor IX activation by the TF-VIIa-PCPS-Ca\(^{2+}\) complex was 45% slower than the rate of unlabeled factor IX activation under identical experimental conditions. However, as noted under "Experimental Procedures," the \([\text{H}]\) factor IX that was prepared by the methods of Zur and Nemerson (11) had only 74% of the bioactivity of unlabeled factor IX when tested in factor IX-deficient coagulation assays.

When the rate of \([\text{H}]\) factor IX activation, as measured by release of the \([\text{H}]\) factor IX activation peptide, was compared with the rate of \([\text{H}]\) factor IX activation, as measured by scanning densitometry of Coomassie Brilliant Blue-stained gel, it was observed that the release of the \( \beta \) activation peptide paralleled the appearance of the \([\text{H}]\) factor IXa \( \beta \) heavy chain \( M_c = 28,000 \) (Fig. 5). These data support the work of Zur and Nemerson (11) that the activation peptide assay is useful for evaluating the progress of \([\text{H}]\) factor IX activation. However, the significant loss in bioactivity of the \([\text{H}]\) factor IX after labeling the protein with sodium \([\text{H}]\)
Fig. 5. Comparison of factor IX activation by densitometry of Coomassie Brilliant Blue-stained gel, colorimetric substrate hydrolysis, and [3H]factor IX activation peptide release assay. [3H]factor IX and unlabeled factor IX were activated by the TF-VIIa-PCPS-Ca2+ enzyme complex under identical conditions as described under “Experimental Procedures.” The progress curve of unlabeled factor IX activation was monitored by densitometric analysis of the Coomassie Brilliant Blue-stained gel (O) for factor IXa after 20 min, as described in Fig. 3 and by two-step colorimetric substrate hydrolysis (●). These data show that the increase in enzymatic substrate hydrolysis corresponds well with the appearance of the M1, = 28,000 protein band IXaβ hc as monitored by SDS-PAGE. [3H]factor IX activation was monitored under identical experimental conditions and it was observed that the activation of [3H]factor IX as measured by the activation peptide release peptide assay (△) corresponded well with the rate of [3H]factor IX activation as measured by the appearance of [3H]factor IXaβ hc on a Coomassie Brilliant Blue-stained gel when scanned by densitometry (Δ). It should be noted, however, that the overall rate of [3H]factor IX activation (△ and ○) was 45% slower than the rate of unlabeled factor IX (O and ●).

Borohydride causes the usefulness of this assay for the evaluation of factor IX activation kinetics to be questioned. In control experiments, when no factor VIIa was added to the enzymatic complex (TF-PCPS-Ca2+), no activation of [3H]factor IX was detected by either an increase in soluble [3H]activation peptide in 10% trichloroacetic acid or in reaction samples analyzed by SDS-PAGE over a 120-min reaction period. This illustrates that no contaminating protease was activating factor IX in our TF preparation. Furthermore, when no TF was added to the reaction system (VIIa-PCPS-Ca2+), no activation of [3H]factor IX was observed over a 120-min reaction period. This demonstrates that our preparation of factor VIIa contained no contaminating protease responsible for the activation of factor IX that functioned independently of TF and further illustrates the importance of the entire TF-VIIa-PCPS-Ca2+ complex to be assembled for the rapid activation of factor IX (data not shown).

When factor IX activation reactions were evaluated using rVIIa, no difference in the activation progress curve of factor IX was observed whether purified human factor VII/VIIa or rVIIa was used as the enzymatic cofactor in the above reactions (data not shown). Both the purified human factor VII/VIIa and rVIIa were added to the TF-PCPS-Ca2+ complex 15 min prior to the addition of factor IX. These data suggest that all of the factor VII/VIIa/IX complex was converted to factor VIIICa after the addition of factor IX, when the former is complexed to TF-PCPS-Ca2+.

The Activation of Factor X by the TF-VIIa-PCPS-Ca2+ Complex—The rate of factor X activation by the TF-VIIa-PCPS-Ca2+ complex was investigated. Human factor X was added to the enzyme complex of TF-VIIa as described under “Experimental Procedures.” A progress curve of factor X activation was generated by removing aliquots of the reaction mixture and assaying each reaction sample for the amount of factor Xa generated. Factor Xa generation was monitored by hydrolysis of the chromogenic substrate Spectrozyme Xa and by densitometric analysis of a Coomassie Brilliant Blue-stained gel (Fig. 6). It can be seen from these data that the densitometric analysis of the generation of the Xα and Xaβ bands is consistent with the generation of the active enzyme species as reflected by chromogenic substrate hydrolysis. A progress curve from this reaction showed that factor X was completely converted to factor Xa after 10 min, and the initial rate of factor X conversion to factor Xa was calculated to be 78 mol of factor Xa min−1/mol of factor VIIa.

From these data we have observed that when human factors IX and X were activated independently by the TF-VIIa-PCPS-Ca2+ complex that human factor X was activated nearly two times more rapidly than human factor IX. This is in reasonably good agreement with the report of Morrison and Jesty (6), where it was observed that human factor X was activated about 1.5 times more rapidly than human factor IX in a human plasma system using [3H]-labeled factors IX and X.

The Simultaneous Activation of Human Factors IX and X by the TF-VIIa-PCPS-Ca2+ Enzyme Complex—The activation of human factors IX and X was measured simultaneously when presented to the TF-VIIa-PCPS-Ca2+ enzyme complex. Factor IX activation was measured by densitometric analysis of the Coomassie Brilliant Blue-stained gel shown in Fig. 7. In this reaction system we were unable to use the colorimetric and substrate Z-Lys-SBzl to follow the generation of factor IXaβ because the factor Xa that was simultaneously generated in the reaction mixture could also hydrolyze the factor IX substrate. However, as illustrated by Figs. 2–4, scanning densitometry of Coomassie Brilliant Blue-stained gels is an effective means of following the progress of factor IX activation and corresponds well with an increase of enzymatic activity of factor IXaβ. Factor Xa formation in this complex reaction was measured both by chromogenic substrate hydrolysis and densitometric analysis of the Coomassie Brilliant Blue-stained gel. We were able to use colorimetric substrate hydrolysis to follow factor Xa generation in this reaction because the factor IXaβ that was generated simultaneously in this reaction system was unable to hydrolyze the factor Xa substrate Spectrozyme Xa. When the rates of factor IX and X activation were measured simultaneously, the overall rate of factor IXaβ generation remained unchanged when compared with the rate of factor IXaβ generation measured without factor X present in the reaction system (Fig. 7C). Interestingly, when the rate constants k1 and k2 for factor IX activation in the presence of factor X were determined, it was observed that k1 increased to 15.7 × 10−3 s−1, whereas k2 decreased slightly to 3.2 × 10−3 s−1. The rate of factor Xa formation in the presence of factor IX decreased by 46% to 42 mol min−1 of factor Xa generated/mol of factor VIIa when compared with the rate of Xa generation observed when no factor X was present in the reaction system (Fig. 7D). Analysis of the cleavage products of this complex reaction by SDS-PAGE (7a) suggested that the first bond in the activation of factor IX (Arg45-Ala46) was hydrolyzed at a faster rate than was observed when no factor X was present in the reaction (Fig. 7C). Furthermore the increase in k1 of factor IX activation in the presence of factor X suggested that factor X/Xa in the reaction system may be participating in the first proteolytic step in the activation of factor IX. Thus, the role of factor Xa in the activation process of factor IX was investigated.
This gel illustrates that the Xa-PCPS-Ca²⁺ complex can rapidly convert factor IX to factor IXα in one proteolytic step. It was observed that factor IX generation increased in a dose-dependent manner with increasing concentrations of factor Xa. Furthermore, this reaction was observed to be absolutely phospholipid- and calcium-dependent. These data illustrate that factor Xa can rapidly generate the intermediate species IXα from factor IX by cleavage of the Arg₁⁴⁵-Ala₁⁴⁶ bond. We also observed at high concentration of factor Xa (400 nM) that the second Arg₁⁴⁵-Val₁⁵⁸ bond is cleaved, but at a much slower rate than the first bond cleavage. From these observations we have concluded that factor Xa can rapidly generate the species IXα but is much less efficient in cleaving the second peptide bond (Arg₁⁴⁵-Val₁⁵⁸) and converting factor IXα to IXαβ. It is unlikely that this observation was the result of a contaminating protease from the Russell’s viper venom that was used in the preparation of factor Xa, because in control experiments, when factor Xa was added to the factor IX reaction without PCPS, no proteolysis of factor IX was observed over a 90-min period. Furthermore it has been reported that Russell’s viper venom proteolytically cleaves human factor IX at only the Arg₁⁴⁵-Val₁⁵⁸ peptide bond and generates the product IXαα (1). This product was never observed in any of reactions where human factor Xa was added to the preparation of human factor IX.  

**Isolation of IXα after Activation with Xa**—It was of interest to find out if the factor IXα that was generated by factor Xa could function as an intermediate in the generation of factor IXαβ. In order to investigate this, factor IXα was isolated by immunoaffinity chromatography (Fig. 9). The isolated material, when analyzed by SDS-PAGE under reducing conditions, migrated as a mixture of three protein bands. The two major bands migrated with apparent Mᵣ = 39,000 and Mᵣ = 18,000, which is consistent with the polypeptides IXα hc and IX αc, respectively. The third protein band migrated with an apparent Mᵣ = 28,000 which corresponds to the heavy chain of factor IXαβ (IXαβ hc). At no time was the Mᵣ = 39,000 polypeptide able to incorporate Ir-FPR-ck, suggesting that this polypeptide did not contain a serine protease active site which is also consistent with identification of factor IXα.  

The isolated factor IXα was tested to evaluate if it could function as a substrate for the TF-VIIa-PCPS-Ca²⁺ complex. Isolated IXα was added to the TF-VIIa-PCPS-Ca²⁺ as described under “Experimental Procedures.” Subsamples of the activation mixture were removed from the reaction and analyzed by SDS-PAGE. The Coomassie Brilliant Blue-stained gel was then analyzed by scanning densitometry. From these data, the rate of factor IXαβ generation was determined and plotted in Fig. 10. It was observed that the initial rate of factor IXα conversion to IXαβ was 40% faster than the initial rate of immunoaffinity-isolated factor IX conversion to IXαβ under identical experimental conditions. These data illustrate that the factor IXα species which is generated by proteolytic cleavage by factor Xα can function as a substrate for the TF-VIIa enzyme complex. Furthermore, an increase in the rate of product formation when the reaction is started with factor IXα is consistent with the notion that factor IXα only requires one proteolytic cleavage event (Arg₁⁴⁵-Val₁⁵⁸) to become activated to the IXαβ species, whereas factor IXα requires two proteolytic steps (cleavage of both the Arg₁⁴⁵-Val₁⁵⁸ and Arg₁⁴⁵-Ala₁⁴⁶ peptide bonds) to become activated to factor IXαβ.  

Last, it was of interest to test whether factor Xa-PCPS-Ca²⁺ and TF-VIIa-PCPS-Ca²⁺ could work in concert in the same reaction system in the activation of factor IX. To test this, the activation of factor IX was evaluated by separating the addition of Xa-PCPS-Ca²⁺ from the addition of the TF.
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**Fig. 7. The simultaneous activation of factors IX and X by the TF-VIIa-PCPS-Ca\(^{2+}\) enzyme complex.**

In A, subsamples of an ongoing reaction were withdrawn at designated time points and subjected to analysis by SDS-PAGE in the presence of 2-mercaptoethanol. Components of the reaction were: 2 μM factor IX, 2 μM factor X, 14 nM TF, 5 nM VIIa, 1 mM PCPS, and 5 mM CaCl\(_2\) in HBS, pH 7.4, at 37 °C. Lane 1, molecular weight markers with indicated molecular weights. Lanes 2-11, samples obtained by quenching each time point in 25 mM EDTA at the designated intervals 0, 0.5, 1, 2, 4, 8, 12, 16, 30, and 60 min after the simultaneous addition of factors IX and X to the TF-VIIa-PCPS-Ca\(^{2+}\) enzyme complex. The indicated bands in A are identified as factors IX, X, IXα hc, Xα, IXα B hc, IXα C, and X C, respectively. In B, a subsample from each reaction time point was treated with Ir-FPR-ck as described above. Each reaction sample was then subjected to SDS-PAGE analysis. Prior to staining the gel with Coomassie Brilliant Blue, the polypeptides which specifically incorporated the Ir-FPR-ck were visualized by excitation with a long-wave UV light box and photographed through a 540-nm cut-off filter. Bands which incorporated Ir-FPR-ck are identified with arrows and correspond with the polypeptide bands noted on the Coomassie Brilliant Blue-stained gel in A. C illustrates the relative concentrations of factor IX, factor IXα, and factor IXα when factor X is and is not present in the reaction mixture. The relative amount of factor IX activation was determined by densitometric analysis of the Coomassie Brilliant Blue-stained gel. Staining intensities were assigned arbitrary density units as described under “Experimental Procedures.” The activation of factor IX by the TF-VIIa-PCPS-Ca\(^{2+}\) enzyme complex, when no factor X is present in the reaction, is depicted by a solid line with open symbols where factor IX is represented with open squares, factor IXα open triangles, and factor IXα open circles. Factor IX activation when an equal concentration of factor X is present in the reaction mixture is depicted by a dotted line with filled symbols where factor IX is represented with filled squares, factor IXα filled triangles, and factor IXα filled circles. D illustrates the rate of factor X activation when an equal amount of factor IX is and is not present in the reaction mixture. Factor X activation was evaluated by scanning densitometry of a Coomassie Brilliant Blue-stained gel shown in Fig. 6 (no factor IX present in the reactor) and A (equal molar amounts of factor IX in the activation reaction). The relative concentrations of factor X activation when no factor IX was present in the reaction system are depicted by a solid line and open symbols where factor X is represented by open squares and factor Xα by open circles. The relative concentrations of factor X activation when factor IX was present in the reaction system are depicted by a dotted line with closed symbols where factor X is represented by closed squares and factor Xα by closed circles.

**VIIa-PCPS-Ca\(^{2+}\)** as shown in Fig. 11. In this experiment factor IX was first added to a mixture of factor Xa-PCPS-Ca\(^{2+}\), as described under “Experimental Procedures,” and the rapid generation of factor IXα was observed. Fifteen seconds prior to the removal of the 30-min time point of this reaction, TF-VIIa-PCPS-Ca\(^{2+}\) was added to the reaction system and the rapid conversion of the IXα species to IXαP was observed. These data clearly show that factor IXα generated by treatment with factor Xa is able to function as an efficient substrate for the TF-VIIa-PCPS-Ca\(^{2+}\) enzyme complex, further
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FIG. 8. The activation of factor IX by factor Xa-PCPS-Ca\textsuperscript{2+}. A, SDS-PAGE analysis of a progress curve of factor IX activation by factor Xa-PCPS-Ca\textsuperscript{2+}. Subsamples of an ongoing reaction were withdrawn and subjected to analysis after treatment with SDS and reduction of disulfide bonds with 2-mercaptoethanol. The concentration of components in the reaction were 2 \mu M factor IX, 80 \mu M factor Xa, 400 \mu M PCPS, and 5 mM CaCl\textsubscript{2} in HBS buffer, pH 7.4, at 37 °C. Lane 1, molecular weight markers with known molecular weights. Lanes 2-11, samples obtained from an ongoing reaction by quenching subsamples of the reaction at 0, 0.5, 1, 2, 5, 10, 20, 30, 60, and 90 min after the addition of factor IX to the Xa-PCPS-Ca\textsuperscript{2+} enzyme complex. Protein bands are identified as factor IX, factor IX\textsubscript{hc}, factor IXa\textsubscript{p}, and factor IX\textsubscript{lc}, respectively. B illustrates the relative concentration of factor IX, factor IX\textsubscript{hc}, and factor IX\textsubscript{lc} as determined by densitometric analysis of the Coomassie Brilliant Blue-stained gel shown in A. The relative concentration of factor IX is shown by \textit{open squares}; factor IX\textsubscript{hc}, \textit{open triangles}; and factor IX\textsubscript{lc}, \textit{open diamonds}.

suggesting that human factors Xa and VIIa may work together in the generation of human factor IX\textsubscript{a,b}.

DISCUSSION

In this report we have evaluated the activation of human factor IX by the human TF-VIIa-PCPS-Ca\textsuperscript{2+} complex. Data presented here illustrate that human factor IX, when activated in an exclusively human reaction system consisting of recombinant human tissue factor, purified human factor VIIa, and synthetic phospholipid vesicles (PCPS) in the presence of calcium, takes place in a two step process (Fig. 1). The first step of factor IX activation is the cleavage of the Arg\textsuperscript{392}-Ala\textsuperscript{396} peptide bond with the generation of the intermediated species IX\textsubscript{a} (step 1). This step is followed by the subsequent cleavage of the second peptide bond Arg\textsuperscript{383}-Val\textsuperscript{387} and the generation of the active enzyme IXa\textsubscript{b} (step 2). This two-step reaction mechanism is identical to the mechanism of bovine factor IX activation by bovine factor XIa that has been reported by Di Scipio et al. (1). This mechanism is also consistent with the reports of Bajaj and co-workers (10) for the activation of

FIG. 9. The isolation of factor IX\textsubscript{p} by immunoadfinity chromatography after treatment of factor IX by the Xa-PCPS-Ca\textsuperscript{2+} enzyme complex. As described under "Experimental Procedures" 6 \mu M factor IX was treated with 100 \mu M factor Xa, 400 \mu M PCPS, and 5 mM CaCl\textsubscript{2} for 30 min prior to stopping the reaction with 25 mM EDTA. After the reaction period, 2 ml of the mixture were applied to a column of anti-factor IX-Sepharose at 4 °C. The material which bound to the anti-factor IX-Sepharose was eluted with 3 M NaSCN in HBS, pH 7.4, (starting at fraction 25). The gel depicted in an inset of this figure illustrates the isolation of factor IX\textsubscript{p} at various steps in the process outlined above. Lanes 1 and 5, molecular weight markers with designated molecular weights. Lane 2, the starting material factor IX. Lane 3, a subsample of the material removed from the reaction at 30 min. Lane 4, the isolated factor IX\textsubscript{p} after chromatography on anti-factor IX-Sepharose (fractions 27-33).

FIG. 10. The activation of factor IX\textsubscript{a} to factor IXa\textsubscript{b} by the TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex. The isolated factor IX\textsubscript{a} illustrated in Fig. 9 was activated by the TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex. Components of the reaction were 1.4 \mu M factor IX\textsubscript{a} or factor IX, 5 mM factor VIIa, 14 mM TF, 1 mM PCPS, and 5 mM CaCl\textsubscript{2} in HBS buffer, pH 7.4, at 37 °C. Subsamples of an ongoing reaction were withdrawn and quenched in 25 mM EDTA at time 0, 0.5, 1, 2, 4, 8, and 12 min after the addition of factor IX\textsubscript{a} or factor IX to the established TF-VIIa-PCPS-Ca\textsuperscript{2+} enzyme complex and subjected to analysis on SDS-PAGE (10% polyacrylamide gels) under reducing conditions. After staining with Coomassie Brilliant Blue, the gels were scanned and the amount of factor IX\textsubscript{a} or factor IX which was converted to factor IXa\textsubscript{b} was plotted as a function of reaction time. The conversion of factor IX\textsubscript{a} to IXa\textsubscript{b} is designated by \textit{filled circles}, whereas the conversion of factor IX to IXa\textsubscript{b} is designated by \textit{open circles}.

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human factor IX by the extrinsic pathway complex. These data do not agree with the reports of Zur and Nemerson (11) who stated that no reaction intermediate was observed when bovine factor IX was treated with a mixture of bovine factor VIIa and bovine tissue factor.

The clear establishment of this intermediate species in the activation of factor IX is important for a proper mechanistic evaluation of the factor IX activation. A large body of literature exists on factor IX activation using the [3H]factor IX activation peptide release assay as described by Zur and Nemerson (11). Although this assay has proven useful for following the release of the activation peptide from the 3H-labeled factor IX molecule, data presented in this report illustrate that this assay does not detect potentially important proteolytic events in the activation of factor IX and provides only a quantitative approximation of the kinetics of the factor IX to factor IXaβ reaction. This point is illustrated by both the reactions of factor IX with factor Xa-PCPS-Ca\(^{2+}\) and the simultaneous activation of factors IX and X by the TF-VIIa-PCPS-Ca\(^{2+}\) complex. In both of these reactions the rapid generation of factor IXa was observed. Using the activation peptide release assay, these reaction steps proceed completely unnoticed because they take place before the release of the factor IX activation peptide from the factor IX molecule. These data illustrate the need to study all of the proteolytic steps involved in a reaction process. Furthermore, when factor IX is treated with [3H]factor borohydride using commonly reported methods (11), we routinely lose >25% of the bioactivity of factor IX. This observation is consistent with other reports (10) where it was also observed that biological activity of the 3H-labeled factor IX was only 82% of unlabeled factor IX. When the activation of 3H-labeled factor IX was compared with the activation of unlabeled factor IX by the TF-VIIa-PCPS-Ca\(^{2+}\) complex, it was observed that 3H-labeled factor IX was activated 45% slower than unlabeled factor IX (Fig. 5). These observations demonstrate the need to use caution in the analysis of kinetic data generated by the [3H]factor activation peptide release assay in the activation of factor IX.

The relative importance of human factor IX and factor X activation by the extrinsic pathway is unknown. In this report we have evaluated the activation of human factors IX and X by the human TF-VIIa-PCPS-Ca\(^{2+}\) complex. Initially, we expected to observe that human factors IX and X would function as competitive substrates for the TF-VIIa-PCPS-Ca\(^{2+}\) enzyme complex in view of the report by Jesty and Silverberg (7). However when this hypothesis was tested under the experimental conditions described in this report, it was surprising to observe that the rate of factor IX activation in the presence of factor X did not change significantly while the rate of factor X activation in the presence of factor IX decreased by 46%. Furthermore, when the proteolytic steps involved in the activation of factor IX were analyzed by SDS-PAGE, it appeared that the conversion of factor IX to IXa (cleavage of Arg\(^{45}\)-Ala\(^{46}\) peptide bond) occurred faster when factor Xa was present in the reaction mixture. From these data we hypothesized a reaction model in which the factor Xa, which was generated simultaneously in the reaction, could play a role in the first proteolytic step in the activation of factor IX.

When the role of human factor Xa-PCPS-Ca\(^{2+}\) in the activation of human factor IX was investigated, it was observed that human factor Xa-PCPS-Ca\(^{2+}\) was able to rapidly cleave factor IX to the intermediate species factor IXα. The proteolysis of factor IX by factor Xa has been an area of significant confusion. Lindquist et al. (2) and Kalousek et al. (9) have reported that bovine factor Xa could activate bovine factor IX to factor IXαβ. However, Osterud and Rapaport (3) have stated that when human factor IX was reacted with human factor Xa that no activation of factor IX was observed. In this report we have clearly observed the generation of the
IXα species after treatment of factor IX with factor Xa-PCPS-Ca²⁺. Furthermore, we have observed that both PCPS and Ca²⁺ are essential for this reaction to proceed. We have been unable to perform a complete kinetic study of the factor Xa cleavage of factor IX because factor IXα is a nonenzymatic intermediate in the reaction process and its formation is undetectable by activation peptide release assays, clotting studies, or chromogenic substrate hydrolysis. Thus, we have limited the analysis of this reaction to concentrations of factor IXα which can be visualized and quantitated in a Coomassie Brilliant Blue gel system. However, because the activation of factor IX takes place in a two-step process, with the first step being the generation of factor IXα, the generation of the factor IXα species by factor Xa is potentially a significant step in the activation of factor IX.

To test if factor IXα, which was generated by treatment of factor IX with Xa-PCPS-Ca²⁺ could function as a substrate for the TF-VIIa-PCPS-Ca²⁺ complex, factor IXα was isolated by immunoaffinity chromatography. As Fig. 10 illustrates, the isolated factor IXα can function as a substrate for the TF-VIIa-PCPS-Ca²⁺ enzyme complex. Furthermore, as Fig. 11 illustrates, the factor IXα generated by Xa-PCPS-Ca²⁺ was able to act directly as a substrate for the TF-VIIa-PCPS-Ca²⁺ complex in the generation of factor IXαβ. These data suggest that TF-VIIa-PCPS-Ca²⁺ and Xa-PCPS-Ca²⁺ work in concert to produce the proteolytic cleavage of factor IX, the generation of factor IXα.

We propose a model of human factor IX activation by the extrinsic pathway where in step 1 factor IX is converted to IXα by either TF-VIIa-PCPS-Ca²⁺ or Xa-PCPS-Ca²⁺ by the proteolytic cleavage of the Arg³⁴-Ala³⁵ peptide bond. In step 2 the factor IXα is converted to IXαβ primarily by the TF-VIIa-PCPS-Ca²⁺ complex (Fig. 1). These data do not rule out the possibility of a large enzymatic complex consisting of TF-VIIa-PCPS-Ca²⁺ being a rapid activator of factor IX. We hypothesize that factor Xa rapidly generates factor IXα, whereas factor IXα takes the generated factor IXα and rapidly converts it to factor IXαβ. In this model, the TF-VIIa-Xa-PCPS-Ca²⁺ complex provides a pivotal point in the regulation of blood coagulation. As Rapaport and others (28, 29) have shown, extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI) is an inhibitor of both factor IXα in solution and the assembled TF-VIIa-Xa-PCPS-Ca²⁺ complex. In our model, the rapid activation of factor IX by this enzyme complex provides a reasonable pathway around the extrinsic pathway inhibition by LACI and provides a direct link between intrinsic and extrinsic coagulation pathways.

The role of factor IX in the propagation of blood coagulation has remained puzzling. It is obvious that factor IXα is essential for normal hemostasis in vivo as illustrated by the diseases hemophilia A and B. However, current in vitro reaction models have not satisfactorily described how the lack of factor IX inhibits the propagation of the blood coagulation reaction. We suggest a model (Fig. 12) that may add insight into the important role that factor IX plays in blood coagulation. In this model, TF-VIIa initially generates a small amount of both factors IXα and Xa. The factor Xa that is generated then works in concert with the TF-VIIa complex to rapidly convert factor IX to IXα and then to IXαβ. Ultimately the factor IXαβ, when complexed with factor VIIIa, propagates the activation of factor X to factor Xa. This notion is supported by published kinetic constants shown in Fig. 12, where the activation of factor X by the VIIIa-IXαβ enzyme complex is at least 50-fold more efficient (kcat/Km) than the activation of factor X by the TF-VIIa enzyme complex. This model provides a reasonable activation mechanism for factor IX in blood and may explain the role of human factor IX in the propagation of the coagulation cascade as illustrated by the disease hemophilia B.

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