Activation of Human Factor VII by Activated Factors IX and X

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Factor VII clotting activity increases about five-fold when blood is clotted in glass. Prior studies suggested that this results from activation induced by activated factor IX (IXa). However, in purified systems containing phospholipid and calcium, activated factor X (Xa) is known to activate factor VII rapidly. Therefore, we studied activation of factor VII by IXa and Xa in systems using purified human factors. Concentrations of IXa and Xa were calculated from total activated protein concentrations rather than from active site concentrations. In the presence of phospholipid and calcium, both IXa and Xa activated factor VII 25-fold; however, Xa was roughly 800 times more efficient than IXa. Without added phospholipid, activation of factor VII by both Xa and IXa was markedly slowed, and Xa was roughly 20 times more efficient than IXa. When both phospholipid and calcium were omitted, activation of factor VII by either enzyme was negligible. Adding normal prothrombin, but not decarboxylated prothrombin, substantially slowed activation of factor VII by both Xa and IXa. Adding thrombin-activated factor VIII and antithrombin-III did not change rates of factor VII activation by either enzyme. These results from purified systems do not provide an explanation for the prior data from plasma systems.

Clotting Factor Assays

Assays of Factor VII and VIIa

Factor VII clotting activity (VIIc) was measured by incubating 25 μl of hereditary factor-VII-deficient plasma, 50 μl of tissue factor, and 25 μl of adsorbed bovine plasma in a 12 x 75 mm glass tube for 3 min at 37°C. Then 25 μl of test material and 50 μl of 35 mM CaCl2 were added and the clotting time noted. Test samples were diluted in 0.05M Tris, 0.15M NaCl containing 1 mg/ml of bovine serum albumin, pH 7.5 (TBS-BSA). A 1:10 dilution was used for samples with low or normal factor VII activity and a 1:100 or greater dilution in TBS-BSA was used for samples with a high factor VII activity. Clotting times were converted to units per milliliter from a dilution curve prepared with 1:10 to 1:80 dilutions in TBS-BSA of the reference standard plasma.

Factor VII coupled amidolytic activity (VIIam) was measured by an assay described in detail earlier. The assay is carried out in two steps: in the first, the test material is incubated at 37°C with tissue factor, calcium ions, and purified factor X. The generation of Factor Xa, which is dependent on the factor VII content of the test material, is stopped at 3 min by adding Na2EDTA and placing the mixture in ice. In the second step, the factor Xa activity is measured by adding a sub-sample of the incubation mixture to an aliquot of S-2222 and determining the initial rate of its hydrolysis spectrophotometrically (ΔA405).

In contrast to the clotting assay, the coupled amidolytic assay measures total factor VII level independent of the activity state of factor VII. Therefore, an increase in the ratio of factor VII activity, as measured in the clotting assay, to factor VII activity, as measured in the coupled amidolytic assay (VIIc/VIIam), represents evidence for activation of factor VII. Purified native factor VII should give a

MATERIALS AND METHODS

Reagents

Tissue factor was a saline extract of human brain tissue. A reference standard plasma, assumed to contain 1 U/ml activity of all clotting factors, was prepared by pooling plasma from 14 healthy males and was stored at -70°C. Hereditary factor-deficient plasmas were obtained from patients seen in this laboratory, or from George King Biomedical, Overland, Kans. Rabbit brain cephalin and insolubilized trypsin were products of Sigma, St. Louis, Mo. Human antithrombin-III and the specific chromogenic substrate Bz-Ile-Gly-Glu-Arg-p-nitroanilide (S-2222) were obtained from Kabi. Human factor VIII concentrate (Koate), was purchased from Cutter Laboratories (Berkeley, Calif.). Automated aPTT reagent was obtained from General Diagnostics, Raritan, N.J. All chemicals were of the best grade available from commercial sources.

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ratio of 1. Full activation of human factor VII results in a 20–25-fold increase in the VIIc/VIIam ratio.

**Assays of Factors IX, IXα, XI, and Xla**

A clotting assay for factor IX was carried out by incubating 50 µl of hereditary factor IX-deficient plasma, 50 µl of automated aPTT reagent, and 50 µl of a test sample in a 12 × 75 mm glass tube for 5 min at 37°C. Then, 50 µl of 25 mM CaCl₂ was added and the clotting time noted. Clotting times were converted to units per milliliter by comparison to a dilution curve prepared with 1:10 to 1:80 dilutions in TBS-BSA of the reference standard plasma. Test samples of purified factor IX were diluted from 1:100 to 1:10,000 in TBS-BSA to obtain clotting times in the midrange of the standard curve.

Factor IXa activity was measured in the same test system. Several different dilutions of a test sample were assayed to prepare a dilution curve for the sample. Its factor IXa activity, in units per milliliter was then derived from that portion of the dilution curve for the sample that was parallel to the factor IX assay reference curve prepared with dilutions of our standard reference plasma.

Assays for factors XI and Xla were performed as described for factors IX and IXa, except that hereditary factor XI-deficient plasma was substituted for hereditary factor IX-deficient plasma.

**Assays of Factors X, Xa, and Prothrombin**

Factor X activity was measured according to Bachmann et al. A mixture of equal parts of bentonite-adsorbed human plasma and barium-adsorbed ox plasma was used as a factor X-deficient substrate plasma. Factor Xa activity was determined as for factor X, except that Russell's viper venom was omitted from the test system. Prothrombin was measured by a modification of the method of Hjort et al., in which tissue factor replaced the Russell's viper venom cephalin reagent.

**Purified Human Clotting Factors**

**Factor VII**

Factor VII was purified by a procedure involving adsorption from citrated human plasma onto barium chloride, elution with ammonium sulfate, chromatography on DEAE Sephadex, and preparative polyacrylamide gel electrophoresis. Details are provided elsewhere. Four liters of human plasma yielded about 300 µg of protein containing approximately 500 U of factor VII activity as measured in the coupled amidolytic assay. Preparations were greater than 90% pure as judged by polyacrylamide gel electrophoresis. Different preparations had VIIc/VIIam ratios of from 1.5 to 3.5, which represents approximately 2%–10% contamination of native factor VII with factor VIIa.

**Prothrombin, Factor IX, and Factor Xa**

Prothrombin, factor IX, and factor X were purified as described in detail elsewhere. The initial steps, up to and including DEAE-Sephadex column chromatography, were the same as described for factor VII. DEAE-Sephadex fractions, which contained prothrombin, factor IX, and factor X, were separated from each other and purified utilizing a simplified technique involving heparin-agarose column chromatography in (sodium) citrate buffer, pH 7.5. Preparations were greater than 95% pure as judged by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Specific activities were as follows: prothrombin, 6 ± 0.3 U/mg; factor IX, 200 ± 20 U/mg; factor X, 100 ± 10 U/mg.

**Factor XI**

Human factor XI was purified from barium chloride-adsorbed citrated human plasma by a modification of the method of Kurachi and Davie. This technique involves chromatography on heparin agarose, CM sephadex, and heparin agarose again. Our procedure differed from that published in that the last step of purification, benzamidine-agarose column chromatography, was omitted. The preparation was concentrated by ultrafiltration using an Amicon PM-30 membrane and stored frozen. The specific activity of the factor XI preparation was 220 U/mg protein.

**Activated Clotting Factors**

**Factor IXa**

Insolubilized factor Xa, for use in activating factor IX, was prepared by incubating 0.9 ml of factor XI, 1.1 mg/ml in TBS, pH 8.0, with 0.1 ml of insolubilized trypsin. After about 30 min the insolubilized trypsin was removed by centrifugation. Approximately 50% of the factor XI was converted to factor Xa as judged by SDS gel electrophoretic analysis. Factor Xa was insolubilized on CNBr-activated Sepharose 4B, essentially as described for Russell's viper venom.

Factor IXa was prepared by incubating 0.4 ml of factor IX, 0.5 mg/ml in TBS, pH 7.5, with 0.1 ml of insolubilized factor Xa and 0.05 ml of CaCl₂ to give a final concentration of 6 mM. In preliminary experiments, factor IX was completely activated within 10 min, as evaluated by SDS gel electrophoretic analysis. No loss of factor IXa clotting activity was found on incubation for up to 30 min. Therefore, a 20-min incubation time was chosen, following which the insolubilized factor Xa was removed by centrifugation. Factor IXa consistently yielded a specific activity of 600 ± 40 U/mg of factor IX protein. Fresh factor IXa was made for each experiment. Active site concentration of the enzyme preparations was not determined.

**Factor Xa**

Factor Xa was prepared by activating purified factor X with the factor X-activating protein of Russell's viper venom, which had been insolubilized on CNBr-activated Sepharose 4B. The procedure has been described in detail earlier. Activation of factor X was complete as judged by SDS gel electrophoresis. The factor Xa, which had a specific activity of 1350 U/mg protein, was stored in 50% glycerol at −20°C. Active site concentration of the factor Xa preparation was not determined.

**Preparation of Decarboxylated Prothrombin**

Fully decarboxylated prothrombin was prepared by heating native prothrombin that had been lyophilized from ammonium bicarbonate buffer. The preparation was heated at 110°C for 12 hr in vacuo. The decarboxylated protein had less than 0.5 gamma carboxyglutamic acid residues per molecule and less than 5% of the physiologic activity of native prothrombin. On prolonged incubation with factor Xa, decarboxylated prothrombin yielded thrombin of similar specific activity to that obtained on activation of native prothrombin. Decarboxylated prothrombin had the same molecular weight (70,000) as native prothrombin by SDS polyacrylamide gel electrophoresis and yielded a line of identity with normal prothrombin on double immunodiffusion plates using a goat anti-human prothrombin antiserum.

**Determination of Protein Concentrations**

Protein concentrations were determined spectrophotometrically using E₂₈₀ of 13.2 for factor IX and factor IXa, 11.6 for factor X and factor Xa, and 13.8 for prothrombin. The concentration of factor VII protein in our preparations was not high enough to measure accurately by absorbance; its concentration was determined from its activity in the coupled amidolytic assay and the assumption of a value of 1800 U/mg protein.
Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was performed according to the method of Laemmli\textsuperscript{13} utilizing 12% acrylamide gels. The protein standards used to determine apparent molecular weight have been described elsewhere.\textsuperscript{3}

Antibodies to Clotting Factors

An antiserum against factor X was prepared in a rabbit using purified human factor X.\textsuperscript{2} The immunoglobulin fraction from the rabbit serum was separated by ammonium sulfate precipitation and DEAE cellulose batch chromatography. The antiserum was then adsorbed with insolubilized human factor IX. One part antiserum solution neutralized the factor X clotting activity of about 80 parts of normal plasma and had no effect on factor IX clotting activity.

An antiserum against factor IX was prepared in a rabbit using purified factor IX.\textsuperscript{2} The immunoglobulin fraction was separated by ammonium sulfate precipitation and DEAE cellulose chromatography. One part of the antiserum neutralized the factor IX clotting activity of about 60 parts of normal plasma and did not inhibit factor X clotting activity.

Activation of Factor VII

Purified Systems

Purified factor VII, stored in 10 mM benzamidine, was made 1 mg/ml in BSA and dialyzed into TBS-BSA, pH 7.5, buffer to remove the benzamidine. Reaction mixtures of 0.1–0.2 ml total volume were made using this diazlyzed factor VII as the substrate and different concentrations of purified factor IXa or purified factor Xa as the enzyme. As described below, the other reagents of the reaction mixture varied in different experiments and included: calcium, phospholipid, specific antiserum, prothrombin, factor VIII, and anti-thrombin-III. Reactions were carried out at 37°C. At intervals, aliquots of 10 μl were removed from the mixtures and added to 90 μl of TBS-BSA buffer containing 6 mM EDTA. The subsamples were then diluted further in TBS-BSA and assayed for factor VII activity in both the clotting and amidolytic assays. Results were expressed as the VIIc/VIIam ratio.

Plasma Systems

Activation of factor VII during clotting of normal plasma was studied by allowing 2.5 ml of fresh whole blood to clot in 12 × 75 mm glass tubes. After 2.5 hr of incubation at 37°C, the factor VII activity of the resultant sera was measured in the clotting and amidolytic assays. The baseline factor VII activity of the plasma was determined from a specimen of whole blood drawn into citrate anticoagulant (9:1 v/v). Activation of factor VII during clotting of factor IX-deficient plasma was studied by measuring factor VII activity in the clotting and amidolytic assays, before and 2.5 hr after stored platelet-poor factor IX-deficient plasma was recalcified in glass tubes. In some experiments, purified factor IXa or purified factor Xa was added, with phospholipid and calcium, to normal serum or factor IX-deficient serum 2.5 hr after clotting to determine whether these materials could induce further activation of the factor VII in the sera.

**RESULTS**

Comparison of Activation of Factor VII by Factor IXa and Factor Xa in the Presence of Added Phospholipid and Calcium

The ability of purified factor Xa and purified factor IXa to activate purified factor VII in the presence of phospholipid and calcium is illustrated in Fig. 1. Factor Xa, final concentration 2 nM, fully activated factor VII, final concentration 50 nM (2.5 μg/ml) in approximately 1 min. When the concentration of factor Xa was reduced to 0.5 nM, the initial rate of activation was decreased and full activation required 6 min.

Factor IXa was also capable of fully activating factor VII, but much higher enzyme concentrations were needed for rapid activation. At a final concentration of 50 nM, only about one-half of the factor VII was activated at 4 min. When the final concentration of factor IXa was increased to 400 nM, a rate of activation was obtained similar to that obtained with 0.5 nM factor Xa. These findings were consistently observed in repeated experiments utilizing different factor VII and factor IXa preparations.

In three experiments, the reaction mixtures of factor VII, calcium, phospholipid, and either factor IXa or factor Xa were allowed to stand overnight and then assayed again for factor VII clotting and coupled amidolytic activity. Full factor VII activity was retained, i.e., no evidence was found for degradation.

[Fig. 1. A comparison of the relative rates of activation of factor VII by factor IXa and factor Xa in the presence of phospholipid and calcium ions. In all experiments, final total molar concentrations were: factor VII, 50 nM (2.5 μg/ml); calcium, 6 mM; phospholipid, a 1/4 dilution of the stock cephalin suspension. Concentrations of factor Xa and IXa are depicted in the figure. Xa, 2 nM (O——O); Xa, 0.5 nM (A——A); IXa, 400 nM (■——■); IXa, 50 nM (□——□).]
of the activated molecule under these experimental conditions.

Because much higher concentrations of factor IXa than factor Xa were needed to activate factor VII at a comparable rate, the question arose of possible contamination of our factor IXa preparations with factor Xa. Therefore, experiments were carried out in which factor IXa and factor Xa preparations were incubated with antisera against factor IX or antisera against factor X for 30 min at 37°C prior to their addition to mixtures of factor VII, phospholipid, and calcium. Treatment of factor Xa preparations with antiserum to factor X prevented the subsequent activation of factor VII. In contrast, treatment of factor IXa preparations with antiserum to factor X had no effect on the rate or extent of activation induced by the factor IXa. Similarly, antiserum to factor IX blocked activation induced by factor IXa preparations, but had no effect on activation induced by factor Xa preparations. Thus, the ability of the factor IXa preparations to activate factor VII was not the result of their contamination with trace amounts of factor Xa.

Activation of Factor VII by Factor Xa and Factor IXa in the Absence of Added Phospholipid

When phospholipid was not added to the activation mixtures, neither factor Xa nor factor IXa could activate factor VII rapidly. Results of a typical experiment are shown in Fig. 2. In this figure, incubation time is plotted in hours rather than in minutes. The initial rates of activation of factor VII obtained with 20 nM factor Xa and 400 nM factor IXa were approximately equal. Full activation required 3–4 hr. With lesser concentrations of enzymes, 2 nM factor Xa and 50 nM factor IXa, less than a twofold increase in the VIIc/VIIam ratio was found at 3 hr. In additional experiments in which both phospholipid and calcium were left out of incubation mixtures, incubation of 50 nM factor VII with 20 nM factor Xa produced only a twofold rise in the VIIc/VIIam ratio after 3 hr. Incubation with 400 nM factor IXa resulted in no significant change in the baseline VIIc/VIIam ratio.

Effect of Prothrombin on the Activation of Factor VII by Factor Xa and Factor IXa

Prothrombin is a competing substrate for factor Xa in plasma. Therefore, purified prothrombin was added to reaction mixtures of factor VII, phospholipid, calcium, and factor Xa. Final substrate concentrations in these mixtures approximated plasma concentrations: for factor VII, 0.5 µg/ml (10 nM); for prothrombin, 150 µg/ml. The addition of prothrombin substantially slowed the rate of activation. As shown in Fig. 3, 0.5 nM factor Xa fully activated 10 nM factor VII in approximately 6 min in the absence of prothrombin. When prothrombin was present, factor VII was only about 50% activated at 6 min. Full activation required 60 min.

Experiments were also carried out in which prothrombin was added to reaction mixtures of factor VII, phospholipid, calcium, and factor IXa. Although not a competing substrate for factor IXa in plasma, prothrombin also substantially slowed the activation of factor VII by factor IXa in these reaction mixtures. As shown in Fig. 4, 10 nM factor IXa substantially activated 10 nM factor VII in 4 min in the absence of prothrombin. When prothrombin was present, factor VII was less than 50% activated at 10 min. Full activation required 30 min.

Additional experiments were carried out in which decarboxylated prothrombin was substituted for native prothrombin (see Figs. 3 and 4). Decarboxylated prothrombin had no apparent effect on either the rate or extent of activation of factor VII by factor Xa or factor IXa.

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**Fig. 2.** A comparison of the relative rates of activation of factor VII by factor IXa and factor Xa in the presence of calcium, but without phospholipid. In all experiments, final total molar concentrations were: factor VII, 50 nM (2.5 µg/ml); calcium, 6 mM. Concentrations of factors Xa and IXa are depicted in the figure. Xa, 20 nM (■); Xa, 2 nM (○); IXa, 400 nM (■); IXa, 50 nM (○).
Fig. 3. A comparison of the relative rates of activation of factor VII by factor Xa in the presence of calcium, phospholipid, and either prothrombin or fully decarboxylated prothrombin. Final total molar concentrations were: factor VII, 0.5 µg/ml (10 nM); factor Xa, 0.025 µg/ml (0.5 nM); prothrombin or fully decarboxylated prothrombin, 150 µg/ml (2100 nM); calcium, 6 mM; phospholipid, a 1/4 dilution of the stock cephalin suspension. Xa alone (--;--); Xa + prothrombin (--;—); Xa + decarboxylated prothrombin (--;—).

Lack of Effect of Activated Factor VIII or Antithrombin III on Activation of Factor VII

Since thrombin-activated factor VIII, in the presence of phospholipid and calcium, strikingly accelerates the activation of factor X by factor IXa, it seemed important to investigate the effect of adding thrombin-activated factor VIII on the rate of activation of factor VII by factor IXa. Human factor VIII, in a concentration calculated to yield the activated product of 1 U/ml of native factor VIII in the final incubation mixture, was mixed with 0.02 U/ml of thrombin for 1 min. The thrombin-activated factor VIII was then added to reaction mixtures containing factor VII, final concentration 10 nM, factor IXa, final concentration 10 nM, calcium, and phospholipid. The rate of factor VII activation in such mixtures containing thrombin-activated factor VIII did not differ from the rate of activation in control mixtures in which either buffer or thrombin was substituted for the thrombin-activated factor VIII.

Because antithrombin III (AT-III) can inhibit the activities of factor IXa and factor Xa in plasma, additional experiments were carried out in which AT-III was added to reaction mixtures. Reagents were added in the following order: thrombin-activated factor VIII, AT-III (or control buffer), factor VII, phospholipid, calcium, and either factor IXa or factor Xa. Final concentrations in these mixtures were: AT-III 1 U/ml, factor IXa 10 nM, factor Xa 0.5 nM. The presence of AT-III had no effect on the rate of activation of factor VII by either enzyme.

Effect on Factor VII Activity of Adding Factor IXa or Factor Xa to Serum

Venous blood was drawn from a normal donor. One portion was added to citrate anticoagulant in a plastic
tube for measurement of the factor VIIc/factor VIIam ratio in the plasma. A second portion was clotted in a glass tube and allowed to incubate for 2.5 hr at 37°C. The VIIc/VIIam ratio of the resultant serum was then measured. In four experiments, the VIIc/VIIam ratio of normal plasma varied between 1.0 and 1.1. The VIIc/VIIam of the normal serum varied between 4.9 and 5.4.

On two occasions, serum separated 2.5 hr after clotting was incubated with calcium, phospholipid, and either purified factor IXa or purified factor Xa. VIIc/VIIam activity in the serum was measured in subsamples taken over 60 min. Factor Xa was added in a final concentration of 5 nM, that is, in a concentration 10 times that needed in our purified reaction mixtures to give a VIIc/VIIam ratio greater than 20 within 6 min (see Fig. 3). As can be seen in Fig. 5, this concentration of factor Xa, in the presence of added phospholipid and calcium, failed to increase further the VIIc/VIIam ratio of the serum.

Factor IXa was added to the serum in a final concentration of 40 nM, which was 4 times that needed to activate factor VII fully within 4 min in purified systems (see Fig. 4). On both occasions, this concentration of factor IXa, in the presence of added phospholipid and calcium, produced an approximate twofold rise in the VIIc/VIIam ratio over 60 min (see Fig. 5). The VIIc/VIIam ratio did not rise further when the incubation time was prolonged to 120 min.

The VIIc/VIIam ratio was also determined in citrated platelet-poor factor IX-deficient plasma, and in serum obtained 2.5 hr after recalifying the plasma in glass tubes at 37°C. The VIIc/VIIam of the serum was 2.5 in one experiment and 2.8 in a second experiment, low values consistent with prior results. When either factor IXa, final concentration 40 nM, or factor Xa, final concentration 5 nM, was added to the factor-IX-deficient serum, along with calcium and phospholipid, the VIIc/VIIam ratio failed to increase (see Fig. 5).

**DISCUSSION**

The experiments described above were designed to compare the relative abilities of purified factor IXa and purified factor Xa to activate purified human factor VII. It was known from earlier work, initially in purified bovine\(^3\)\(^4\) and later in purified human systems,\(^5\)\(^6\) that factor Xa functions as a potent activator of factor VII in the presence of phospholipid and calcium. In 1974, Laake and Østergard\(^3\) provided preliminary data suggesting that factor IXa could also activate factor VII. Later, Seligsohn and coworkers\(^3\) reported more definite data showing that purified factor IXa slowly activated partially purified factor VII over 4 hr in reaction mixtures containing calcium but no added phospholipid. The present data extend these observations, establishing that factor IXa can fully activate factor VII within minutes in the presence of both calcium and phospholipid.

Although both factor IXa and factor Xa fully activated factor VII, the two enzymes differed strikingly in their relative efficiencies. At the two factor VII substrate concentrations used—10 nM, which approximates plasma concentration, and 50 nM—factor Xa was found to be 800 times more efficient than factor IXa. Limited availability of purified human factor VII prevented our carrying out the more extensive kinetic studies required to obtain the kinetic constants for the two reactions.

It should be noted that the factor IXa and factor Xa

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**Fig. 5.** VIIc/VIIam activity after the addition of factor IXa or factor Xa, phospholipid, and calcium to serum produced by clotting either normal blood or factor IX-deficient plasma. Sera were incubated 2.5 hr at 37°C after clotting, prior to the addition of other reagents. Final total molar concentrations were: factor IXa, 40 nM (2 μg/ml); factor Xa, 5 nM (0.25 μg/ml); calcium, 6 mM; phospholipid, a 1:4 dilution of the stock cephalin suspension. Normal serum + IXa (A-A); normal serum + Xa (B-B); factor-IX-deficient serum + IXa (A-A); factor-IX-deficient serum + Xa (B-B).
concentrations were determined from total activated protein concentrations rather than from active site titrations. We do not believe that this resulted in substantial error for two reasons. First, the factor IXa and factor Xa preparations were demonstrated, by reduced SDS polyacrylamide gel electrophoresis, to be cleaved fully to their activated molecular forms. Second, the specific clotting activity, 1350 U/mg, of the stored factor Xa preparation used for all experiments agreed with earlier data for factor Xa specific activity, and the factor IXa preparations freshly made for each experiment consistently yielded values for specific activity of between 559 and 650 U/mg. Nevertheless, the possibility cannot be completely ruled out that the stored factor Xa preparation and the multiple freshly made factor IXa preparations used for these experiments contained amounts of enzymatically inactive molecules that affected to an unknown extent the calculation of relative efficiencies.

The present data from purified systems agree with Østerud’s recent observation that factor Xa is much more potent than factor IXa in activating the factor VII of recalcified prothrombin-deficient plasma containing activated platelets as a source of phospholipid. Both sets of observations contrast with earlier results from this laboratory in which factor IXa appeared to be the principal activator of factor VII when platelet-poor, specific clotting-factor-deficient plasmas were clotted and incubated without added phospholipid. The differing data could be reconciled if reducing the phospholipid concentration of reaction mixtures conferred a selective advantage upon factor IXa, allowing it then to function more efficiently than factor Xa as an activator of factor VII. However, omitting phospholipid from our purified reaction mixtures markedly slowed the rates of activation of factor VII by both factor Xa and factor IXa (see Fig. 2). Although activation by factor Xa was reduced more than activation by factor IXa, factor Xa retained an approximate 20-fold higher relative efficiency than factor IXa. Therefore, the low phospholipid content of the platelet-poor plasmas used in the earlier experiments does not explain why factor IXa, rather than factor Xa, appeared to be the principal activator of factor VII in those experiments.

If prothrombin, a substrate for factor Xa but not for factor IXa, selectively inhibited activation of factor VII by factor Xa, then the present data from purified systems and the prior data from plasma systems, including the data from prothrombin-deficient plasma, might also be reconciled. However, when we added prothrombin to purified reaction mixtures, activation of factor VII by both factor Xa and factor IXa was slowed. A selective effect on activation by factor Xa could not be demonstrated.

Decarboxylated prothrombin, in contrast to native prothrombin, had no effect on reaction rates of mixtures of factor VII, calcium, phospholipid, and either factor Xa or factor IXa. Since the γ-carboxylglutamic acid residues are required to bind calcium and phospholipid to prothrombin, these data suggest that the inhibition observed with native prothrombin involved the binding of calcium and phospholipid to prothrombin. In plasma, prothrombin exists in 200-fold molar excess over factor VII, and during physiologic hemostasis, availability of phospholipid may dampen activation of factor VII by factors IXa and Xa.

In further experiments, we added other materials from plasma to purified reaction mixtures. Thrombin-activated factor VIII, which strikingly accelerates the activation of factor X by factor IXa, was without effect on the activation of factor VII by factor IXa. Physiologic concentrations of antithrombin III failed to slow activation of factor VII by either factor Xa or factor IXa. This could conceivably reflect an effect of the phospholipid in the reaction mixtures, since phospholipid has been shown to protect factor Xa from inactivation by antithrombin III.

Whereas activation of factor VII in purified systems results in a 20–25-fold increase in the VIIc/VIIam ratio, clotting of blood or plasma and incubation of the serum for 2.5 hr results in an approximate 5-fold increase in the VIIc/VIIam ratio. This difference could be accounted for if factor VII were completely activated during clotting and then partially inactivated as the serum was incubated. In purified bovine systems, it has been shown that incubation of factor VII with factor Xa, calcium, and phospholipid leads first to activation and then to inactivation of factor VII. In earlier work from this laboratory, prolonged incubation of purified human factor VII with factor Xa, calcium, and phospholipid was associated, in some but not all experiments, with a partial loss of factor VII activity. However, on the three occasions in the present study in which purified human factor VII was incubated overnight with either factor IXa or factor Xa, phospholipid, and calcium, no loss of factor VII activity was observed. VIIc/VIIam ratios were not measured serially during clotting and incubation of serum, because of difficulty in measuring factor VII clotting activity accurately in early samples containing factor Xa and thrombin.

A lower VIIc/VIIam ratio in serum than in a purified system would also be found if factor VII were only partially activated during clotting and incubation of serum. We attempted to demonstrate residual, activatable factor VII in serum by adding to it calcium, phospholipid, and either purified factor Xa or purified factor IXa. Adding factor Xa failed to increase the VIIc/VIIam ratio of serum prepared from either fresh
normal blood or from stored factor IX-deficient plasma. Adding factor IXa resulted in a further gradual twofold increase in the VIIc/VIIa ratio in serum prepared from normal blood but not in serum prepared from factor IX-deficient plasma. Although the data for factor IXa are difficult to explain, the data as a whole do not support the hypothesis that serum contains substantial amounts of residual, readily activatable, native factor VII. However, a definitive study appears to us to require the development of techniques to measure the molecular changes in radiolabeled factor VII added to blood before clotting and incubation of the serum.

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