Activation of Human Factor VII in Plasma and in Purified Systems

ROLES OF ACTIVATED FACTOR IX, KALLIKREIN, AND ACTIVATED FACTOR XII

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ABSTRACT Factor VII can be activated, to a molecule giving shorter clotting times with tissue factor, by incubating plasma with kaolin or by clotting plasma. The mechanisms of activation differ. With kaolin, activated Factor XII (XII_a) was the apparent principal activator. Thus, Factor VII was not activated in Factor XII-deficient plasma, was partially activated in prekallikrein and high-molecular weight kininogen (HMW kininogen)-deficient plasmas, but was activated in other deficient plasmas. After clotting, activated Factor IX (IX_a) was the apparent principal activator. Thus, Factor VII was not activated in Factor XII-, HMW kininogen-, XI-, and IX-deficient plasmas, but was activated in Factor VIII-, X-, and V-deficient plasmas. In further studies, purified small-fragment Factor XII_a (β-XII_a), kallikrein, and Factor IX_a were added to partially purified Factor VII and to plasma. High concentrations of β-XII_a activated Factor VII in a purified system; much lower concentrations of β-XII_a activated Factor VII in normal plasma but not in prekallikrein or HWM kininogen-deficient plasmas. Kallikrein alone failed to activate partially purified Factor VII but did so when purified Factor IX was added. Kallikrein also activated Factor VII in normal, Factor XII-, and Factor IX-deficient plasmas. Purified Factor IX_a activated partially purified Factor VII and had no additional indirect activating effect in the presence of plasma. These results demonstrate that both Factor XII_a and Factor IX_a directly activate human Factor VII, whereas kallikrein, through generation of Factor XII_a and Factor IX_a, functions as an indirect activator of Factor VII.

INTRODUCTION

Factor VII activity, as measured in one-stage Factor VII clotting assays (1), increases several fold when blood is exposed to a surface such as glass or kaolin (1), or when blood is allowed to clot (2). Factor VII activity also increases when plasma from women taking oral contraceptives is stored at 4°C (3). The increased Factor VII activity is thought to stem from an accelerated initial rate of reaction between Factor VII, tissue factor, and calcium ions, which leads to an increased rate of accumulation of Factor X_a and, subsequently, thrombin in the clotting mixture of one-stage Factor VII assays (4). In purified bovine preparations, the increased Factor VII activity has been shown to be associated with conversion of a native single-chain Factor VII molecule into a two-chain molecule (5, 6), and, therefore, may be viewed as an activation of Factor VII.

We report here the results of a systematic evaluation of the mechanism of activation of Factor VII after surface contact and after clotting. Data were obtained from whole plasma systems and from mixtures containing purified clotting factors. The findings establish that the enzymes involved in the activation of Factor VII after surface contact differ from the enzymes involved in the activation of Factor VII after clotting and

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clarify the roles of activated Factor XII, kallikrein, and Factor IX_a in each circumstance.

METHODS

Reagents

Tissue factor was a saline extract of human brain tissue (7) centrifuged twice at 12,000 *g* to remove large particulate material. It clotted recalcified normal plasma in 17 s. A reference standard plasma assumed to contain 1 U/ml activity of all clotting factors was prepared by pooling plasma from 12 healthy males and was stored at −20°C. Severe hereditary clotting factor-deficient plasmas were obtained from patients seen in this laboratory, from a commercial source (George King, Bio-Medical, Overland Park, Kans.) or through the courtesy of Dr. C. Hougie, University of California, San Diego, and of Dr. C. Kasper and Dr. S. Schiffman, University of Southern California, Los Angeles.

All chemicals used were of the best grade available from commercial sources. Tris-buffered saline (TBS)[1] was a solution containing Tris 0.05 M and NaCl 0.15 M, pH 7.5 TBS containing bovine serum albumin (TBS-BSA) was made by adding BSA (Sigma Chemical Co., St. Louis, Mo.) to TBS in a final concentration of 1 mg/ml.

Assays of Factor VII

A clotting assay for Factor VII was carried out 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 × 75-mm glass tube for 3 min at 37°C. Then, 25 μl of test materials and 50 μl of 35 mM calcium chloride were added and the clotting time noted. A 1:10 dilution in TBS was used for plasma or serum samples with an expected normal or low Factor VII level. A 1:100 to 1:300 dilution in TBS was used for plasma or serum samples with an expected high Factor VII activity. When partially purified Factor VII was assayed, it was diluted in TBS-BSA. Clotting times were converted to units per milliliter from a dilution curve prepared with 1:10–1:80 dilutions in TBS of the reference standard plasma.

A coupled amidolytic assay for Factor VII, developed in this laboratory, is described in detail elsewhere (4). It is carried out in two steps. In the first, the test material containing Factor VII is incubated at 37°C with tissue factor, calcium ions, and purified Factor X. The generation of Factor X_a is stopped at 3 min by adding Na_2 EDTA and placing the mixture in ice. In the second step the Factor X_a activity is measured by adding a subsample of the incubation mixture to an aliquot of the specific chromogenic substrate (S-2222) (Bz-Ile-Gly-Glu-Arg-*p*-nitroanilide) and by determining the initial rate of its cleavage in a spectrophotometer. In contrast to the clotting assay, the coupled amidolytic assay measures total Factor VII level independent of the activity state of Factor VII (4). Therefore, an increase in Factor VII activity in the clotting assay but not in the coupled amidolytic assay is taken as evidence for activation of Factor VII.

Purified human clotting factors

Factor IX. Factor IX was purified by a modification of a published method (8) and as described in detail elsewhere (9). It involved $BaSO_4$ absorption, batch chromatography on DE-11 Whatman cellulose (Whatman Inc., Clifton, N. J.), preparatory polyacrylamide gel electrophoresis, heparin-agarose chromatography, and concentration on a small DEAE cellulose column. Preparations contained 5–20 U/ml of Factor IX as measured in a clotting assay (10) and by electroimmunoassay (11) with a 207 clotting U/mg protein sp act (9). Before storage, preparations yielded comparable values by the two assay techniques. After storage at −20°C in 0.05 M Tris-HCl, 0.375 M NaCl, pH 7.5, purified preparations gave higher values by electroimmunoassay than in the clotting assay. For example, the stored preparation used for one series of experiments had a final concentration of 1.68 U/ml by electroimmunoassay and 0.85 U/ml in the clotting assay. When stored preparations were incubated for 20 min with Factor XI_a and calcium ions, the amount of Factor IX_a generated, as measured by a coupled amidolytic assay (12) was that expected for the level of Factor IX as measured by electroimmunoassay. The lower values obtained in the one-stage clotting assay could stem from aggregation of purified Factor IX on storage,[2] with resultant slowing of its initial rate of activation by Factor XI_a. Because in the experiments described here Factor IX was incubated over hours with Factor XI_a or kallikrein, we have expressed Factor IX concentration in units per milliliter as measured by electroimmunoassay. Labeling of purified Factor IX with ^{125}I was carried out by the chloramine T method (13) and as described in detail elsewhere (9). The incubation time with chloramine T was 2.5 min at room temperature.

Factor IX_a. Factor IX_a was prepared, as described (12), by incubating purified Factor IX with Factor XI_a and $CaCl_2$, and separating the Factor IX_a from the Factor XI_a with DEAE-cellulose chromatography. Factor IX_a concentration was measured by a coupled amidolytic technique (12) and by electroimmunoassay (11). Higher values were obtained in the electroimmunoassay than in the coupled amidolytic assay, e.g., in one preparation 4.04 U/ml by electroimmunoassay and 0.88 U/ml by the coupled amidolytic assay. Because we do not know under what circumstances the functional activity of stored Factor IX_a preparations may increase in clotting mixtures to approach levels expected from electroimmunoassay, we have expressed Factor IX_a concentration in electroimmunoassay units. But we have also given its concentration as measured in the coupled amidolytic assay.

Factor XII. Factor XII was purified and labeled with ^{125}I as described (14, 15).

Factor XII_a small fragment. The 28,000-mol wt form of activated Factor XII that contains the active site of the serine protease is defined as β-XII_a (16). β-XII_a was generated by activating the purified single chain form of Factor XII and was isolated from the activation mixture by ion exchange chromatography.[3] The purified β-XII_a stock solution contained 350 μg/ml in 0.005 M sodium acetate, 0.15 M NaCl, pH 5.3. When 50 μl of a 1:1000 dilution of this preparation was incubated with 50 μl of a preparation of purified prekallikrein (0.7 U/ml) and 100 μl of TBS-BSA for 20 min at 37°C, 20% of the prekallikrein was converted to kallikrein. The kallikrein was measured in an amidolytic assay with the tripeptide substrate Bz-Pro-Phe-Arg-paranitroanilide (17).

Kallikrein. Human plasma kallikrein was prepared and assayed as described elsewhere (17).

Factor XI_a. Factor XI_a was prepared as described (12).

Factor VII. Factor VII was partially purified by a modification of a described method (12). The preparation contained 14.8 U/ml Factor VII in the clotting assay and 16 U/ml in the

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; HMW kininogen, high-molecular weight kininogen; SDS sodium dodecyl sulfate; TBS, Tris-buffered saline.

[2] Østerud, B. Unpublished data.

[3] Griffin, J. H., and G. Beretta, Manuscript in preparation.

coupled amidolytic assay described above. The preparation also contained 0.5 U/ml prothrombin activity as determined in a clotting assay (18). It contained no detectable Factor IX or Factor X activity. When the preparation was labeled with ^{125}I by the chloramine T method, as described above for Factor IX, and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (19), the radioactivity profile of the gel gave two peaks. The partially purified Factor VII was stored at 4°C in TBS-BSA.

Monospecific antibodies to human clotting factors

Anti-Factor IX antibody. An antibody against Factor IX was prepared in rabbits, with purified Factor IX as the antigen. The antiserum was absorbed with $BaSO_4$ and heated for 30 min at 56°C. On double immunodiffusion the antiserum gave a single precipitation line of identity with purified Factor IX and normal plasma. One part of the antiserum neutralized the clotting activity of Factor IX present in 8 parts of normal plasma as tested in a Factor IX clotting assay (10). The antiserum did not neutralize Factor VII activity, as tested in the Factor VII clotting assay.

Anti-Factor XI antiserum. An antiserum against Factor XI was prepared in goats injected with purified Factor XI (20). The antiserum was absorbed with $BaSO_4$ and heated for 30 min at 56°C. On double immunodiffusion the antiserum gave a single line of identity with purified Factor XI and normal plasma. One part of the antiserum neutralized the clotting activity of 100 parts of normal plasma as measured in a one-stage clotting assay for Factor XI (20).

Anti-Factor X antiserum. An antiserum against Factor X was prepared with purified Factor X in a goat (12). The immunoglobulin fraction from the goat serum was separated by 40% $(NH_4)_2$ SO_4 precipitation and DEAE-cellulose chromatography (21). The antiserum gave a single precipitation line of identity on double immunodiffusion with purified Factor X and normal plasma. One part of the antiserum solution neutralized the Factor X clotting activity of about 320 parts of normal plasma as measured in a clotting assay for Factor X (22). The antiserum also neutralized the clotting activity of Factor X_a. Thus, after 100 μl of a purified Factor X preparation containing 2.0 U/ml was incubated with Russell's viper venom and calcium ions for 120 min and then incubated with 10 μl of the antiserum for ≅10 min, an aliquot of the mixture failed to clot Factor X-deficient plasma (control clotting time with buffer in place of antibody, 51 s).

Antiprothrombin antiserum. An antiserum against prothrombin was prepared in a goat, with purified prothrombin kindly provided by Dr. Bruce and Dr. Barbara Furie of the Tufts Medical School, Boston, Mass. The goat immunoglobulin fraction was separated from serum by DEAE-Sephadex column chromatography, 50% ammonium sulfate precipitation, and concanavalin A affinity column chromatography. The gamma globulin fraction gave a single precipitation line of identity on double immunodiffusion against purified prothrombin and normal plasma. Mixing equal parts of this preparation with the partially purified Factor VII preparation completely neutralized the prothrombin activity of the latter as measured in a one-stage assay for prothrombin (18). The gamma globulin fraction also inhibited the ability of purified human thrombin to clot fibrinogen in normal plasma.

Antiprekallikrein antiserum. An antiprekallikrein antiserum was prepared with purified prekallikrein in a goat (23). The gamma globulin fraction containing the antiprekallikrein antibody was separated from the goat serum by a procedure that involved kaolin absorption, incubation at 56°C for 30 min, DEAE-cellulose batch chromatography, 50% ammonium sulfate precipitation, and dialysis into 0.01 M Tris, 0.4 M NaCl pH 7.5. 1 part of the gamma globulin fraction neutralized 0.65 parts of a purified kallikrein solution containing 1 U/ml as measured in an amidolytic assay (17). The gamma globulin fraction gave a single line of identity with purified prekallikrein and normal plasma on double immunodiffusion.

Antiplasminogen antiserum. An antiserum against plasminogen was prepared with purified plasminogen in a goat (24). One part of the gamma globulin fraction neutralized >95% of the plasminogen activity present in 2 parts of normal plasma as measured on fibrin plates after the addition of streptokinase.

Control normal goat gamma globulin. The gamma globulin fraction from the serum of a normal goat was prepared by the technique used to isolate the gamma globulin fraction of the antiprothrombin antibody.

Measurement of cleavage of ^{125}I-Factor IX and ^{125}I-Factor XII

Activation of Factor IX and of Factor XII is associated with limited proteolysis of the native single polypeptide chain zymogens to yield products giving characteristic patterns of the polypeptide fragments on reduced SDS-polyacrylamide gel electrophoresis (9, 15). When either purified ^{125}I-Factor IX or ^{125}I-Factor XII is added to a test mixture, activation of that particular clotting factor can be evaluated from the radioactivity profile of a SDS-polyacrylamide gel electrophoresis gel by relating the integrated radioactivity of the cleavage products to the sum of integrated radioactivities of the native uncleaved factor and the cleavage products.

The cleavage of Factor IX was measured after the clotting of citrated plasma in 12 × 75-mm glass tubes and incubation of the clots for 2.5 h at 37°C. 15 μl of 2-U/ml purified ^{125}I-Factor IX were added to 200-μl plasma samples before the addition of 100 μl of 35 mM $CaCl_2$. 2.5 h after clotting the clots were removed and 25 μl of 0.3 M Na_2 EDTA was added to the serum to prevent calcium-dependent aggregation of Factor IX (9). 4.5 μl of serum was then transferred to 1.5 ml snap cap polypropylene centrifuge tubes to which 50 μl 3% SDS and 10 μl mercaptoethanol were added. The mixtures were then boiled for 5 min and subjected to polyacrylamide gel electrophoresis in the presence of SDS on 10% gels (6 mm × 8 cm) (19). After electrophoresis, the radioactivity profile was obtained by counting gel slices (1.3 mm) in a Searle gamma spectrometer (Searle Diagnostics, Inc., Des Plaines, Ill.) and plotting on paper radioactivity against the slice number. The precent cleavage of ^{125}I-Factor IX was determined as described above.

In experiments on contact activation of plasma by kaolin, 1 μl of a 60-μg/ml preparation of ^{125}I-Factor XII was added to 200 μl of plasma in snap cap polypropylene tubes. 20 μl of 20-mg/ml kaolin was added and the mixture incubated for 4.5 h at 4°C. A 4.5-μl sample of the kaolin-plasma suspension was removed and added to 50 μl of 3% SDS and 10 μl of mercaptoethanol in a polypropylene tube. The mixture was then boiled for 5 min and centrifuged for 5 min at 8,000 *g*. The supernate was removed and treated as described above. Under these conditions >90% of the ^{125}I-Factor XII is removed from the kaolin.

RESULTS

Activation of Factor VII in plasmas incubated with kaolin. Factor VII activity in plasma from healthy subjects and from patients with different hereditary

clotting factor deficiencies was measured in the clotting assay and in the coupled amidolytic assay before and after incubation of the plasma samples with kaolin. The incubation was carried out by adding 20 μl of a 20-mg/ml kaolin suspension to 200 μl of citrated plasma and allowing the mixture to stand, with occasional shaking, at 4°C for 4.5 h. The kaolin was then removed by centrifugation. As shown in Table I, exposure to kaolin increased the Factor VII activity of normal plasma as measured in the clotting assay but not as measured in the coupled amidolytic assay. This evidence of Factor VII activation was found in all of the deficient plasmas except Factor XII-deficient plasma. However, in high-molecular weight (HMW) kininogen-deficient plasma and in prekallikrein-deficient plasma the extent of activation of Factor VII appeared to be less than in normal plasma.

Purified ^{125}I-Factor XII was added to plasma from three additional healthy subjects. Incubation of these plasma samples with kaolin at 4°C resulted in a 55–63% cleavage of the ^{125}I-Factor XII, and was associated with a 4.5- to 7-fold increase in Factor VII activity in the clotting assay.

Incubation of normal plasma with kaolin for 4.5 h was also carried out at 37°C. A four- to fivefold increase in the clotting activity of Factor VII was observed.

Activation of Factor VII induced by clotting of plasma. Factor VII activity was measured in the clotting assay and the coupled amidolytic assay before and after recalcification of plasmas and incubation of the clots. 200 μl of normal plasma or a clotting factor-deficient plasma in a 12 × 75-mm glass tube was clotted by adding 100 μl of 35 mM $CaCl_2$ at 37°C. 2.5 h after clotting, the clot was removed and the serum assayed for Factor VII activity. The data from these experiments are summarized in Table II. As in the experiments with kaolin, normal plasma showed a marked rise in Factor VII activity in the clotting assay, whereas Factor XII-deficient plasma showed no rise in Factor VII activity. However, in contrast to the kaolin experiments, plasmas deficient in HMW kininogen, Factor XI, and Factor IX exhibited only a slight increase in Factor VII activity. Interestingly, activation of Factor VII in prekallikrein-deficient plasma did not appear to differ from activation of Factor VII in normal plasma. Factor VIII-deficient plasma and Factor IX-deficient plasma differed markedly from each other. Clotting of Factor VIII-deficient plasma was associated with a substantial mean rise of Factor VII activity in the clotting assay (from 0.95±0.17 to 4.39±0.88 U/ml), whereas clotting of Factor IX-deficient plasma was associated with only a small mean rise of Factor VII activity in the clotting assay (from 0.75±0.35 to 1.30±0.53 U/ml). The normal activation of Factor VII after clotting Factor X-deficient plasma is also noteworthy.

Because these data suggested activation of Factor VII by Factor IX_a, trace amounts of purified ^{125}I-Factor IX were added to normal plasma to measure the extent of generation of Factor IX_a when the plasma was clotted and incubated under our experimental conditions. A representative experiment is shown in Fig. 1. In experiments with plasma from six subjects 40–46% of the Factor IX was cleaved after 2.5 h in the serum. This was associated with a 4.5- to 5-fold increase in Factor VII activity measured in the clotting assay.

TABLE I

Factor VII Activity of Normal and Clotting Factor-deficient Plasmas before and after Incubation with Kaolin for 4.5 h at 4°C

Type of plasma*		Clotting assay		Amidolytic assay	
		0 time	4.5 h	0 time	4.5 h
		U/ml			
Normal	(6)	0.99	4.63	0.96	0.79
XIIdp	(2)	0.80	0.78	1.19	0.89
HMWKdp	(1)	1.25	2.75	1.20	1.27
PKdp	(2)	1.30	2.99	1.58	1.58
XIdp	(3)	0.80	4.53	1.19	1.06
IXdp	(5)	0.58	3.88	0.53	0.56
VIIIdp	(5)	0.90	8.33	1.12	1.21
Xdp	(2)	0.70	2.94	1.15	1.01
Vdp	(1)	0.83	6.40	1.28	0.93
VIIdp	(1)	0.02	0.06	0.01	0.01

* The Roman numerals denote the clotting factors; dp, deficient plasma; HMWK, HMW kininogen; PK, prekallikrein. The numbers in parentheses indicate the number of subjects from whom plasma was obtained. The mean activities listed are the averages of these numbers of patients.

TABLE II

Factor VII Activity of Normal and Clotting Factor-deficient Plasmas before Clotting of the Serum 2.5 h after Clotting at 37°C

Type of plasma*		Clotting assay		Amidolytic assay	
		0 time	2.5 h	0 time	2.5 h
		U/ml			
Normal	(5)	1.23	5.74	1.43	1.50
XIIdp	(2)	0.84	0.97	1.08	0.65
HMWKdp	(2)	1.11	1.45	1.45	1.30
PKdp	(2)	1.24	4.88	1.42	1.12
XIdp	(7)	0.94	1.46	1.14	1.24
IXdp	(11)	0.75	1.30	1.01	1.05
VIIIdp	(10)	0.95	4.39	1.26	1.50
Xdp	(2)	0.71	3.60	0.81	0.84
Vdp	(1)	1.05	5.55	0.86	1.00
VIIdp	(1)	0.03	0.15	0.05	0.04

* See footnote to Table I.

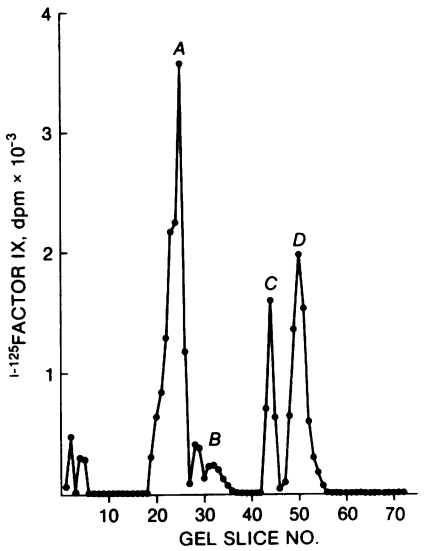

FIGURE 1 Radioactivity profile of ^{125}I-Factor IX on reduced SDS polyacrylamide gel from serum obtained 2.5 h after clotting plasma by recalcification in a glass tube at 37°C. The sharp peak on the left (A) represents native Factor IX. The smaller peaks to the right are as follows: B, an intermediate of activation; C the heavy chain of Factor IX_a; and D, the light chain of Factor IX_a. These smaller peaks account for 46% of the total radioactivity.

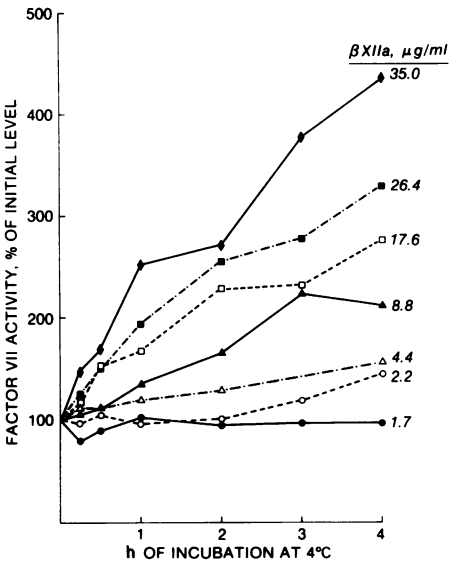

FIGURE 2 Effect of purified β-XII_a on the activity of partially purified Factor VII. Partially purified Factor VII in a final concentration of from 0.65 to 0.74 U/ml was incubated in a plastic tube at 4°C for 4 h with purified β-XII_a in the final concentrations (μg/ml) shown in the figure. At the times shown, subsamples were removed and tested for Factor VII clotting activity.

To bypass generation of Factor XII_a, experiments were carried out in which the clotting of normal and hereditary clotting factor-deficient plasmas was initiated with purified Factor XI_a. In a plastic tube, 100 μl of plasma was clotted by the addition of 10 μl of purified Factor XI_a (final concentration 2.5 U/ml) and 50 μl of 35 mM $CaCl_2$. After 1 h incubation at 37°C, the clots were removed and the sera tested for Factor VII activity in the clotting assay. The following increases in Factor VII activity were observed: for normal plasma, 6.3-fold; for Factor XII-deficient plasma, 10.9-fold; for Factor VIII-deficient plasma, 8.6-fold. However, for Factor IX-deficient plasma, less than a 1.4-fold increase was measured in Factor VII clotting activity.

Effect of purified β-XII_a upon the activity of partially purified Factor VII and Factor VII in plasma. Increasing concentrations of purified β-XII_a were incubated at 4°C for 4 h with partially purified Factor VII at a final concentration of from 0.65 to 1.5 U/ml. Subsamples of these incubation mixtures were removed at intervals and assayed for Factor VII activity in the clotting assay. The results are shown in Fig. 2. At a final concentration of 1.7 μg/ml, β-XII_a had no measurable effect upon the activity of partially purified Factor VII. Because the normal mean plasma Factor XII concentration is 29 μg/ml and the molecular weight of native Factor XII is 80,000 and of β-XII_a is 28,000 (16), it is calculated that 1.7 μg/ml of β-XII_a would be generated by the activation of 16% of the Factor XII in normal plasma.

However, at higher concentrations, β-XII_a activated partially purified Factor VII. Thus, at a final concentration of 8.8 μg/ml of β-XII_a, which would be equivalent to 90% cleavage of Factor XII in plasma, an ≅twofold activation of partially purified Factor VII was observed. A concentration of 35 μg/ml, which corresponds to four times the maximum concentration of β-XII_a attainable in plasma, was required to produce the degree of activation observed when normal plasma was incubated with kaolin under similar conditions (Table I).

Purified β-XII_a was much more effective in activating Factor VII in plasma than in activating partially purified Factor VII. When β-XII_a at a final concentration of 1.7 μg/ml was incubated (plastic tube, 4°C, 4 h) with either normal plasma or with Factor VII-deficient plasma that was reconstituted with partially purified Factor VII a 4- to 4.5-fold increase in Factor VII activity was found (Fig. 3). However, Factor VII was not activated when the same experiment was carried out with HMW kininogen-deficient plasma or prekallikrein-deficient plasma. A delayed and partial activation was seen when Factor XII-deficient plasma was used. When Factor XI-deficient plasma or Factor IX-deficient plasma was used the extent of activation approached that found with normal plasma, with a resultant ≅threefold activation of Factor VII.

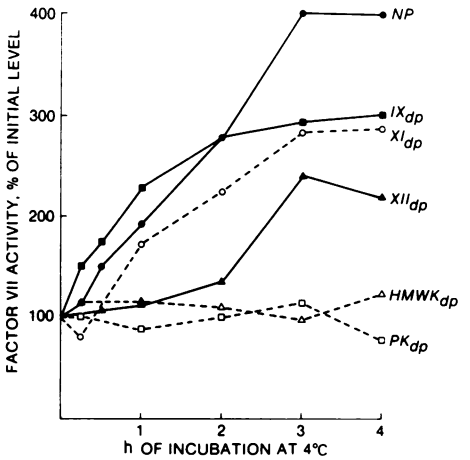

FIGURE 3 Effect of purified β-XII_a on the Factor VII activity of normal plasma and different hereditary deficient plasmas (dp). In all experiments the final concentration of β-XII_a was 1.7 μg/ml. Initial Factor VII levels in these plasmas varied from 0.47 to 1.25 U/ml. The line marked IX_{dp} is the mean of results obtained with plasma from four severe hemophilia B patients and the lines marked XI_{dp}, XII_{dp}, HMWK(HMW kininogen)$_{dp}$, and PK(prekallikrein)$_{dp}$ represent the data for plasma from single patients with each of these hereditary deficiencies.

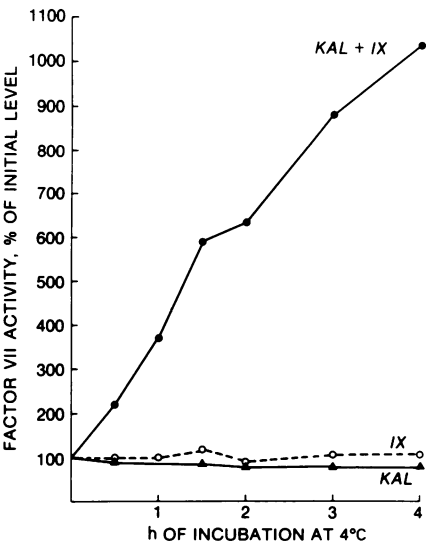

FIGURE 4 Effect of purified plasma kallikrein (KAL) on the activity of partially purified Factor VII in the presence of purified Factor IX. In these experiments the final concentration of kallikrein was 1.9 U/ml, the final concentration of Factor VII was 2.5 to 3.3 U/ml, and the final concentration of Factor IX was 2.5 U/ml. The two lower lines, labeled IX and KAL, represent experiments in which only Factor IX was added to Factor VII (○), or only kallikrein was added to Factor VII (▲). In these experiments TBA-BSA replaced the material omitted.

Effect of purified plasma kallikrein upon factor VII activity. Factor VII activity failed to increase when purified kallikrein at a final concentration of either 1.0 or 5.7 U/ml was incubated at 4°C for 4 h with partially purified Factor VII. However, Factor VII activity increased fourfold when purified kallikrein at a final concentration of 1.0 U/ml was incubated in a plastic tube at 4°C for 4 h with a mixture of partially purified Factor VII and Factor VII-deficient plasma. Factor VII activity increased similarly when kallikrein was incubated with normal plasma, hereditary Factor XII-deficient plasma, or hereditary Factor IX-deficient plasma.

Because kallikrein reportedly activates plasminogen (24) and plasmin activates Factor VII (25), Factor VII activity was also measured after adding kallikrein to Factor XII-deficient plasma that had been incubated with an antiserum to plasminogen. One part of Factor XII-deficient plasma was incubated for 10 min at 37°C with 0.7 parts of either an antiplasminogen antiserum or a control normal goat gamma globulin fraction. Then kallikrein was added at a final concentration of 2.3 U/ml and the mixtures were incubated for 4 h at 4°C. In both incubation mixtures Factor VII activity increased by 3.8-fold.

Because kallikrein also activates Factor IX (9), we next carried out experiments in which purified kallikrein (final concentration 1.9 U/ml) and purified Factor IX (final concentration 2.5 U/ml) were incubated at 4°C with partially purified Factor VII (final concentration 2.5 U/ml). In control experiments either kallikrein or Factor IX was replaced with TBS-BSA. As shown in Fig. 4, Factor VII activity increased by about 10-fold in the incubation mixture containing both kallikrein and Factor IX. When trace amounts of ^{125}I-Factor IX were added to an incubation mixture of Factor VII, kallikrein and unlabeled Factor IX, 74% of the labeled Factor IX was cleaved as judged on SDS gels.

Antiserum against either prekallikrein or Factor IX abolished the activation of Factor VII in reaction mixtures kept at 4°C for 3 h that contained the following final concentrations of reactants: Factor VII, either 1.2 or 2.5 U/ml; Factor IX, 2.5 U/ml; kallikrein, 1.9 U/ml; and either antiprekallikrein or anti-Factor IX antiserum at a 5:8 dilution. In contrast, an antiserum to Factor XI did not prevent activation of Factor VII. For example, a 12-fold activation of Factor VII was found in a reaction mixture kept at 4°C for 4 h that contained the following final concentrations of reactants: Factor VII, 0.6 U/ml; Factor IX, 2.5 U/ml; kallikrein, 1.9 U/ml; and anti-Factor XI antiserum at a 1:40 dilution. This provided evidence that trace contamination of the kallikrein preparation with Factor XI_a was not responsible for the activation of Factor IX.

Effect of purified Factor IX_a upon the activity of partially purified Factor VII and Factor VII in plasma. Purified Factor IX_a and calcium ions were added in a

plastic tube to normal plasma, Factor VIII-deficient plasma, and Factor X-deficient plasma. The incubation mixtures contained 15 μl of purified Factor IX_a at a final concentration of 1.8 U/ml by the coupled amidolytic assay and 4.5 U/ml by the electroimmunoassay, 50 μl of 35 mM $CaCl_2$ and 100 μl of plasma. After 1 h at 37°C clots were removed and the serum assayed for Factor VII activity in the clotting assay. The differences between the Factor VII activity in the original plasma and in the serum were as follows: with normal plasma, a sixfold increase in activity; with Factor VIII-deficient plasma, a sevenfold increase in activity; with Factor X-deficient plasma, an eightfold increase in activity. Increased Factor VII activity was also found when lower final concentrations of Factor IX_a were used. For example, when the final concentration of Factor IX_a in the mixture was 0.4 U/ml by the amidolytic assay and 1 U/ml by the electroimmunoassay, the following increases were observed: with normal plasma, a threefold increase; with Factor VIII-deficient plasma, a fivefold increase; and with Factor X-deficient plasma, a 4.5-fold increase.

To determine whether Factor IX_a directly activates Factor VII in plasma or whether it might also lead indirectly to Factor VII activation through a plasma intermediate, an experiment was designed employing antibodies against Factor IX. 10 μl of a Factor IX_a preparation containing 3.9 U/ml by electroimmunoassay and 0.85 U/ml by coupled amidolytic assay, was incubated with 20 μl of Factor VII-deficient plasma for 3.5 h at 4°C. Then, either 10 μl of anti-Factor IX antiserum (an amount that inhibited all detectable Factor IX activity) or 10 μl of adsorbed normal rabbit serum was added and the mixture was incubated for 10 min at 37°C. At this point, 10 μl of a 4.0 U/ml partially purified Factor VII was added to the mixtures, which were further incubated at 4°C to allow generation of increased Factor VII activity. Subsamples were removed at various times for measurement of Factor VII clotting activity. Factor VII activity steadily rose in the control incubation mixture containing nonimmune serum and showed a fourfold increase in Factor VII activity after 4 h of incubation. Factor VII activity failed to rise in the incubation mixture containing the Factor IX antiserum. Thus, it appeared that Factor IX_a did not activate Factor VII in plasma through the generation of an intermediate.

Further evidence for a direct activation of Factor VII by Factor IX_a was obtained from experiments in which increasing concentrations of purified native Factor IX were incubated at 37°C for 20 min with purified Factor XI_a and calcium ions to generate Factor IX_a. Then, partially purified Factor VII was added to the incubation mixtures, which were incubated further at 4°C. Subsamples over time were assayed for Factor VII activity in the clotting assay. Activation of increasing physiologic amounts of purified native Factor IX resulted in increasing activation of partially purified Factor VII (Fig. 5). The data plotted in the bottom line and the top line of Fig. 5 also show that Factor XI_a by itself had no measureable effect upon Factor VII activity. These experiments were also performed at an incubation temperature of 37°C with similar results.

Additional direct evidence for the activation of Factor VII by Factor IX_a was obtained in experiments in which preparations of purified Factor IX_a that had been stored frozen were incubated with partially purified Factor VII in the presence of calcium ions. Again, increasing concentrations of Factor IX_a yielded increasing increments of Factor VII activity. For example, when partially purified Factor VII at a final concentration of 0.5–0.6 U/ml was incubated at 4°C with two concentrations of Factor IX_a and 5 mM $CaCl_2$, the following increases in Factor VII clotting activity were found after 4 h: for the mixture containing a final Factor IX_a concentration of 0.22 U/ml in the coupled amido-

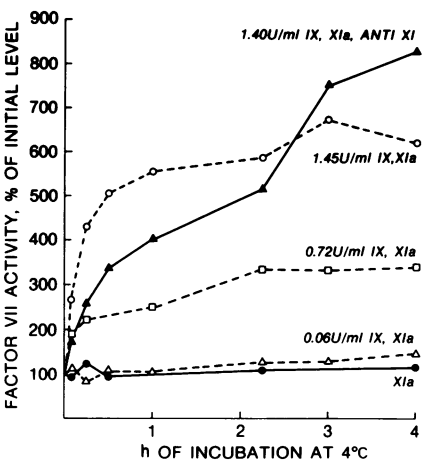

FIGURE 5 Evidence that Factor IX_a, freshly generated from purified Factor IX by purified Factor XI_a and calcium ions, activates partially purified Factor VII. Purified Factor IX (25 μl) at the final concentrations shown in the figure, was incubated for 20 min at 37°C with $CaCl_2$ (3 μl) at a final concentration of 5.3 mM and Factor XI_a (1 μl) at a final concentration of 1.3 U/ml. Then, Factor VII (10 μl) at a final concentration of 0.6–1.0 U/ml was added and the mixtures were incubated at 4°C. Subsamples were removed at the times shown for measurement of Factor VII clotting activity. (The Factor IX_a concentrations, after adding the Factor VII and assuming full conversion of Factor IX to Factor IX_a equaled 0.75 of the concentrations of Factor IX given in the figure). The bottom line labeled XI_a (●) represents a control experiment in which TBS-BSA replaced Factor IX. Another control experiment is shown (▲), in which an anti-Factor XI antiserum at a final dilution of 1:30 was added after the first incubation and allowed to react for 10 min at 37°C before the subsequent addition of the Factor VII.

lytic assay and 1.0 U/ml in the electroimmunoassay, a 2.3-fold increase in Factor VII activity; for the mixture containing a final Factor IX_a concentration of 0.40 U/ml in the coupled amidolytic assay and 1.8 U/ml in the electroimmunoassay, a fourfold increase in Factor VII activity. Similar experiments were repeated in the presence of increasing concentrations of an antiserum to Factor IX. Mixtures of purified Factor IX_a (final concentration 1.7 U/ml in the coupled amidolytic assay, 4.3 U/ml by electroimmunoassay), and partially purified Factor VII (final concentration 1.6–2.3 U/ml) were incubated at 37°C for 3 h with different concentrations of anti-Factor IX antiserum. The antiserum at a final dilution of 1:60 in the reaction mixture abolished the three- to fourfold rise in Factor VII activity found in incubation mixtures with more diluted antiserum or without antiserum.

Finally, because bovine Factor X_a and thrombin have been shown to activate bovine Factor VII (26), the possible influence of trace contamination of incubation mixtures with these factors was studied. A specific antiserum against Factor X at a final dilution sufficient to neutralize 10 U/ml of Factor X or a specific antiserum against prothrombin at a final dilution sufficient to neutralize 1.5 times the prothrombin concentration in the partially purified Factor VII preparation was added to incubation mixtures of partially purified Factor VII and purified Factor IX_a. The marked increases in Factor VII activity in mixtures containing these antibodies did not differ from the marked increases in Factor VII activity found in control mixtures containing a normal goat gamma globulin fraction. Thus, activation of Factor VII in these experiments seems a result of Factor IX_a and not caused by possible traces of Factor X_a or thrombin in the incubation mixtures.

DISCUSSION

The principal activator of Factor VII when plasma is exposed to kaolin at 4° or 37°C differs from the principal activator of Factor VII when plasma is recalcified and incubated in a glass tube at 37°C.

The pattern of activation of Factor VII found when clotting factor-deficient plasmas were incubated with kaolin (Table I) suggests that Factor XII_a functions as the major activator of Factor VII in this experimental circumstance. The data are consistent with recent reports that activated forms of Factor XII activate purified bovine Factor VII (5, 6) and with a new understanding of the participation of HMW kininogen and kallikrein in the generation of Factor XII_a on a surface (17). The normal activation of Factor VII in Factor IX-deficient plasma indicates that Factor IX_a is not required to activate Factor VII in plasma incubated with kaolin.

A different pattern of activation was found when clotting factor-deficient plasmas were clotted, and the incubated sera were tested for Factor VII activity (Table II). Factor VII was activated in Factor VIII-deficient plasma and in Factor X-deficient plasma, but not in Factor IX-deficient plasma. This suggests to us that Factor IX_a was the principal activator of Factor VII under these experimental conditions. The activation of Factor VII found after clotting of Factor X-deficient plasma confirms earlier observations of Hougie (27) and of Johnston and Hjort (2).

The experiments in which purified β-XII_a was incubated with partially purified Factor VII (Fig. 2) confirm and extend an earlier report of Laake and Østerud (25) that the small fragment forms of human Factor XII_a function as a weak activator of partially purified human Factor VII. The degree of activation that we found was much less than the 40-fold activation of purified bovine Factor VII obtained by Radcliffe et al. (5) with a purified preparation containing multiple forms of human Factor XII_a, and much less than the 10- to 30-fold activation of purified bovine Factor VII obtained by Kisiel et al. (6) with a purified bovine α-XII_a (28). Despite quantitative differences, studies with purified proteins suggest that both β-XII_a and α-XII_a can activate Factor VII directly.

The generation of kallikrein is associated with the activation of Factor VII in plasma stored in the cold (29) and in plasma incubated with kaolin (30). Our data (Figs. 2 and 3) demonstrate that a low concentration of β-XII_a unable to activate partially purified Factor VII was able to activate Factor VII in plasma. Because β-XII_a is a prekallikrein activator (16, 31), the ability of a low concentration of β-XII_a to activate Factor VII in plasma is thought to result from the generation of kallikrein.

Nevertheless, neither Laake and Østerud (25) nor Saito and Ratnoff (30) could demonstrate that purified kallikrein directly activated purified Factor VII. In our experiments, purified kallikrein also failed to activate partially purified Factor VII. However, when Factor IX was added to reaction mixtures, Factor VII activity increased impressively (Fig. 4). Furthermore, Factor VII activity increased when kallikrein was added either to Factor XII-deficient plasma or to Factor IX-deficient plasma. Our data support the hypothesis that kallikrein can indirectly activate Factor VII in plasma through the generation of at least two materials, Factor XII_a and Factor IX_a.

Shanberge and Matsuoka (32) first suggested that Factor IX_a could activate Factor VII, and Laake and Østerud (25) provided preliminary evidence in partially purified systems for a direct effect of Factor IX_a upon Factor VII. The results present here provide extensive and conclusive evidence that Factor IX_a directly activates Factor VII. Moreover, as the experi-

ments with recalcified plasma demonstrated, Factor IX_a can activate Factor VII at Factor IX_a concentrations achievable in serum. It is noteworthy that phospholipid was not added to reaction mixtures and that the recalcification experiments were carried out with platelet-poor plasma. The apparent lack of a requirement for added phospholipid is consistent with an early observation (2) that Factor VII was activated after clotting of blood from patients with extreme thrombocytopenia. It should also be pointed out that in some of our experiments with antisera, Factor VII was activated by Factor IX_a in the absence of added calcium ions.

Experiments in which monospecific antibodies to clotting factors were added to incubation mixtures provided further evidence that Factor IX_a directly activates Factor VII. Adding antibodies to prothrombin (a major contaminant of our partially purified Factor VII) or antibodies to Factor X to incubation mixtures of purified Factor IX_a and partially purified Factor VII did not impede the activation of Factor VII, whereas adding antibodies to Factor IX abolished the activation of Factor VII. This would seem to eliminate the possibility that contaminating thrombin or Factor X_a, rather than Factor IX_a, could have been responsible for the activation of Factor VII in such incubation mixtures. An antiserum to Factor IX was also used to rule out the possibility that Factor IX_a might activate Factor VII in plasma not only by a direct mechanism but also by an indirect mechanism. Thus, Factor VII was not activated when Factor IX_a was incubated with Factor VII-deficient plasma, to allow for generation of any possible intermediate, and then the Factor IX_a was neutralized by antibodies to Factor IX before the addition of Factor VII to the mixture.

It is also important to point out that Factor IX_a activated Factor VII at 37°C as well as at 4°C. Activation at 37°C was demonstrated both in the experiments with recalcified plasma and also in incubation mixtures of purified Factor IX and partially purified Factor VII. Thus, the potential importance of this pathway of Factor VII activation is not necessarily limited to the phenomenon of cold-activation of plasma Factor VII (33).

A schematic diagram that summarizes our conclusions from the data is presented in Fig. 6. It shows two pathways for the activation of Factor VII, one through Factor XII_a and the other through Factor IX_a. Kallikrein does not activate Factor VII directly and is depicted as activating Factor VII indirectly through both pathways. Apparently, when plasma is exposed to maximum surface contact through incubation with kaolin, sufficient Factor XII_a is generated to cause extensive activation of Factor VII independent of the Factor IX_a pathway. When plasma is clotted and the serum incubated under the conditions used here, Factor IX_a appears to function as a principal activator of Factor VII.

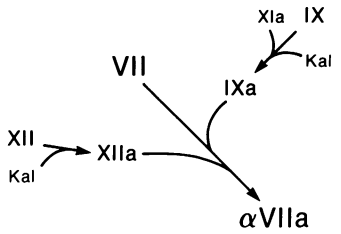

FIGURE 6 Schematic diagram summarizing the two pathways of activation of Factor VII for which evidence has been presented in this report. Roman numerals denote clotting factors. VII_a is the activated two-chain form of Factor VII. Kal, plasma kallikrein. Other agents that have been reported to activate Factor VII, such as plasmin, Factor X_a and thrombin, are not included in the diagram.

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