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A Model for the Tissue Factor Pathway to Thrombin

I. AN EMPIRICAL STUDY*

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The activation of prothrombin, factor V, factor VIII, factor IX, and factor X by the tissue factor-factor VIIa complex, *in vitro*, in a system in which each precursor protein was present at plasma concentration, was evaluated using a combination of activity assays, immunoblots, active-site blots, and autoradiography. The thrombin generation curves observed were distinctly nonlinear and typically displayed a time lag in which little or no thrombin was observed. This was followed by an almost linear propagation phase of thrombin formation. The lag was a function of tissue factor/factor VIIa concentration and represented primarily the interval of factor V and factor VIII activation. The postlag propagation phase of thrombin generation was nearly independent of the initial activator (factor VIIa or tissue factor) concentration over a 10^3-fold range in factor VIIa-tissue factor concentration. Maximum thrombin generation rates were observed when less than 1% of the factor IX and X present was activated but when nearly 100% activation of the cofactors, factor V and factor VIII, was achieved. Analyses of the activation pattern of factor V indicated that the cofactor is activated by both factor Xa and thrombin which are formed at low levels during the lag phase of the reaction. When the initial reaction mixture contained factor Va instead of factor V, the lag was substantially reduced. When factor V was deleted from the reaction mixture, no thrombin formation was observed. When either factor VIII or factor IX was deleted from the reaction system, the propagation phase of thrombin formation (at 5 pM tissue factor-factor VIIa complex) was only one-third that observed for reactions which contained factor VIII and factor IX. The addition of factor XI to the experimental system increased the rate of thrombin formation by 15% during the propagation phase but had no effect upon the lag phase of the reaction. Our data suggest that normal hemostasis may be initiated by the factor VIIa-tissue factor complex and support the concept of multiple feedback reactions which amplify and propagate the hemostatic response.

The blood coagulation process maintains the integrity of the mammalian circulatory system in response to vascular damage. The hemostatic response involves a complex series of events that require the interaction of blood and vascular cellular elements and blood plasma proteins. The regulation of these cellular and molecular events determines one of three outcomes: hemorrhage, controlled hemostasis, or thrombosis. The coagulation response has been characterized as a "cascade" or "waterfall" of enzymatic reactions, which convert a group of plasma proenzymes to their active enzyme forms. These sequential enzymatic reactions lead to the formation of the proteolytic enzyme α-thrombin, which converts fibrinogen to fibrin by limited proteolysis (1, 2). Although this cascade/waterfall model of sequential reactions has provided enormous insights into the general processes of hemostatic reactions *in vitro*, it has not satisfactorily explained the dynamic regulation of blood coagulation reactions that occur *in vivo*, which requires localized reactions initiated at the point of vascular damage.

In vitro, plasma coagulation may be initiated by either the "intrinsic" or "extrinsic" pathway. The intrinsic or (contact) pathway of coagulation consists of a group of plasma proteins which are activated by interaction with exogenous negatively charged surfaces such as glass or kaolin. This pathway has been described as a series of enzymatic reactions which starts with the formation of factor XIIa. The reactions of the intrinsic pathway have been well characterized *in vitro*; however, the physiologic activator of factor XII has not been identified. Furthermore, while individuals have been identified with deficiencies of prekallikrein (3), high molecular weight kininogen (4), and factor XII (5), none of these individuals have been reported to develop significant bleeding diathesis or require prophylactic therapy prior to or during hemostatic challenge. Thus, the biological relevance of the contact pathway in the initiation of the physiologic hemostatic response has become suspect (6). However, individuals have been identified with factor XI deficiencies and, although rarely requiring prophylactic replacement therapy, do have significant episodes of bleeding complications associated with surgical challenge (7, 8). From these observations, it has been concluded that factor XI plays some role in the hemostatic response. Broze and co-workers (9) have reported that factor XI is activated by α-thrombin. These investigators have proposed that factor XIa is formed by positive feedback from thrombin activation. In their model, the activation of factor XIa then functions as an important propagating event following the initial activation of thrombin via the extrinsic pathway. This conclusion however has been challenged (10, 11).

The extrinsic pathway is characterized as the interactions of plasma-derived elements with tissue factor from "outside" the vasculature (or more appropriately outside plasma) (12, 13). The enzymatic complex consisting of tissue factor and factor VIIa activates factors IX and X by limited proteolysis forming the enzymatic product factor IXaβ and factor Xaα, respectively (14). Once formed, factor IXaβ and factor Xaα bind with their respective cofactor proteins, factors VIIIa and Va and form the appropriate enzyme complexes. The extrinsic pathway converges with the intrinsic pathway at the level of factor Xa

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TABLE I
Characteristics of human proteins necessary for blood clotting
Numbers in parentheses refer to reference citations in text.

Protein	Molecular weight	$E^{1\%}_{280\ nm}$	Plasma concentration
Prothrombin	72,000 (23)	13.8 (24)	1.4 μM (24)
Factor V	330,000 (25)	9.6 (25)	20 nM (26)
Factor VIII	280,000 (27)	12.0 (28)	0.7 nM (28)
Factor VII	50,000 (29)	13.9 (29)	10 nM (30)
Factor IX	57,000 (31)	13.3 (30)	90 nM (32)
Factor X	58,900 (33)	11.6 (30)	170 nM (30)
Factor XI	160,000 (34)	13.4 (34)	31.2 nM (35)

formation and ultimately leads to the generation of thrombin via the *prothrombinase* complex. The formation of factor Xa then designates the "common pathway" where both intrinsic and extrinsic pathways converge.

Current literature supports the notion that the physiologic hemostatic response is initiated by the extrinsic pathway (9, 12). However, a significant paradox appears to exist; the "extrinsic pathway" route of activation of factor X would seem to make the formation of factor Xa by the factor VIIIa-factor IXa complex superfluous. However, patients who have deficiencies in the X-chromosome-linked factor VIII and factor IX proteins exhibit the hemorrhagic bleeding tendencies hemophilia A and B (15). Patients who exhibit deficiencies in factors II (15), V (16), VII (16), and X (17), although rare due to the autosomal recessive nature of the genes required for these proteins, have bleeding episodes of equivalent magnitude to those more commonly described for the classic hemophilias. From these *in vivo* observations, it is clear that blood clotting factors II, V, VII, VIII, IX, and X are all essential for normal hemostasis.

The research described here attempts to reconstruct the tissue factor pathway to thrombin and evaluates the activation of factors II, V, VIII, IX, and X, at their physiologic concentrations in plasma, when the reaction is initiated by the enzymatic complex of tissue factor and factor VIIa.

EXPERIMENTAL PROCEDURES

Reagents—1-Palmitoyl-2-oleoyl phosphatidylserine (PS),[1] 1-palmitoyl-2-oleoyl phosphatidylcholine (PC), Hepes, and $C_{12}E_8$ (octaethylene glycol dodecyl ether (polyoxyethylene-8-lauryl ether)) were purchased from Sigma. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPR-ck) and octyl-β-D-glucopyranoside (octyl glucoside) were purchased from Calbiochem. Biotin-labeled FPR-chloroketone (bc-FPR-ck) was prepared according to the methods of Williams *et al.* (18) and provided as a gift from Haematologic Technologies Inc. Hirulog-1 was provided as a gift from Dr. John Maraganore (Biogen). Spectrozyme TH and Spectrozyme Xa were purchased from American Diagnostica, Inc. All other reagents were of analytical grade.

Human coagulation factors IX, X, and prothrombin were isolated from fresh frozen plasma obtained from the Vermont Red Cross using the general methods of Bajaj *et al.* (19). Human factor X was passed through an anti-factor VII immunoaffinity column prior to use. Human factor V was isolated by using the methods of Nesheim *et al.* (20). Recombinant human factor VIII was provided as a gift from Dr. Shu-Len Liu, Hyland Division, Baxter Healthcare Corp. Recombinant human tissue factor (TF) was provided as gifts from Dr. Hissids, Genentech Inc. and Dr. Shu-Len Liu, Hyland Division, Baxter Healthcare Corp. Human factor Xa was prepared by activating factor X with purified Russell's viper venom as described previously (21). Recombinant human coagulation factor VIIa was purchased from NOVO Pharmaceuticals (22). Human factor Va and factor XI were gifts from Haematologic Technologies. Protein concentrations were calculated using the molecular weights (M_r) and extinction coefficients ($E^{0.1\%}_{280}$) listed in Table I as reported (23–35).

Prior to use, the zymogens (prothrombin, factor IX, and factor X) were individually treated with 10 μM FPR-ck for 20 min to inhibit any contaminating proteases from the zymogen preparations. Following treatment, free FPR-ck was removed from each protein preparation by filtration using a Centricon-30 filter (Amicon) by washing each protein solution using a 10× volume of HBS buffer.

For certain experiments in these studies, radiolabeling of factor VIII was required. Solutions containing 50 μg of factor VIII were radioiodinated using IODOGEN (Pierce) (36). 200 μl of IODOGEN (1 mg/ml) was dried on the wall of a 1.5-ml Eppendorf tube and purged with N_2 for 5 min. This was followed by the addition of 10 μl of 1 M Tris, pH 7.4, and the protein to be radiolabeled in the IODOGEN-treated tube. The protein, Tris, and IODOGEN mixture was incubated for 1 min followed by the addition of 0.35 to 0.55 mCi of Na[^{125}I]iodide (Amersham). This mixture was incubated in the dark for 10 min at room temperature. ^{125}I-Labeled protein was separated from free [^{125}I]iodide using an Exocellulose GF-5 gel filtration column (Pierce) which had be pretreated with 0.1% w/v bovine serum albumin, 0.01 M Hepes, 0.15 M NaCl, pH 7.4. Recovered yields of radiolabeled protein were greater than 97% precipitable in 10% trichloroacetic acid. Activity recovery was 33%.

Two methods of reconstitution of tissue factor-membrane were employed. Human recombinant tissue factor (50 pM to 5 nM) was relipidated into 200 μM PCPS vesicles by incubation at 37 °C for 30 min in the presence of 2 mM $CaCl_2$, 20 mM Hepes, 150 mM NaCl (37), pH 7.4. Alternatively, recombinant human tissue factor was solubilized in 0.04% $C_{12}E_8$ mixed with PCPS vesicles and incubated at 37 °C for 30 min in 0.02 M Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4 (HBS). The TF/$C_{12}E_8$/PCPS mixture was then diluted to a final concentration of 10^{-9}–10^{-12} M tissue factor, 10^{-4}–10^{-3} M PCPS, and 1.7×10^{-5}% $C_{12}E_8$ as described previously (14). Both methods of reconstitution gave equivalent results.

Coagulation Factor Activation Experiments—Reactions were carried out by the addition of zymogens (prothrombin, factor IX, and factor X) and cofactors (factors V and factor VIII) to a mixture containing tissue factor, factor VIIa, PCPS vesicles, and Ca^{2+}. All experiments were performed in 20 mM Hepes, 150 mM NaCl, pH 7.4 at 37 °C.

Three solutions were prepared as follows. (*a*) Relipidated tissue factor-factor VIIa-PCPS and Ca^{2+} were mixed in the reaction tube (total volume 1 ml) and mixed for 15 min to allow the tissue factor-factor VIIa-PCPS-Ca^{2+} complex formation to reach equilibrium. The factor VIIa and tissue factor concentrations were varied (5 pM to 10 nM) and (50 pM to 5 nM), respectively. (*b*) A pro-cofactor solution (500 μl) containing factor V (80 nM) and factor VIII (2.8 nM) was prepared. (*c*) A zymogen solution (500 μl) containing prothrombin (5.6 μM), factor IX (360 nM), and factor X (680 nM) was also prepared. The solutions were prepared in 20 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4.

These latter two solutions, *b* and *c*, were individually mixed and then rapidly added to the tube containing factor VIIa-tissue factor complex to initiate activation. The final concentrations in the ultimate reaction mixture were the following: prothrombin, 1.4 μM; factor VIII, 0.7 nM; factor V, 20 nM; factor IX, 90 nM; factor X, 170 nM; and factor XI (if present), 31 nM. PCPS and $CaCl_2$ were 200 μM and 2 or 5 mM, respectively. The entire reaction system was mixed and aliquots were removed at timed intervals. Each aliquot removed was quenched in an equal volume of 25 or 50 mM EDTA. Each 200-μl quenched sample was then quickly divided into 4 aliquots: 1) 25 μl, assay for thrombin formation; 2) 25 μl, assay for factor Xa formation; 3) 50 μl was mixed with 0.1 mM bc-FPR-ck for active site blotting; and 4) 100 μl was mixed with 25 μl of a solution which contained 4% SDS (w/v), 0.01 M Tris-HCl, pH 6.8, 20% glycerol (v/v) for SDS-PAGE followed by immunoblotting for each antigen in the reaction system or autoradiography.

Reaction Product Analyses—The reaction products derived from prothrombin, factor V, factor VIII, factor IX, and factor X activation by the tissue factor-factor VIIa complex were evaluated by activity assays for thrombin and factor Xa formation, active site blot analysis of reaction products following SDS-PAGE (for factor IXa, factor Xa, and thrombin), and immunoblot/autoradiographic analyses of the proteolytic products formed from the activation of each protein in the reaction following SDS-PAGE.

Assays for thrombin activity were performed using the substrate Spectrozyme TH. Quenched samples from each reaction time point were added, in duplicate, to a 96 well assay plate (Corning, Corning, NY) and the activity assays were started by the addition of 200 μl of 0.2 mM Spectrozyme TH to each well. The rate of substrate hydrolysis was monitored by the absorbance change per unit time at 405 nm in a Molecular Devices V_{max} spectrophotometer. The concentration of thrombin formed in the reaction per unit time was then directly determined using a standard curve of substrate hydrolysis with known concentrations of thrombin.

[1] The abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; FPR-ck, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; bc-FPR-ck, biotin-labeled FPR-ck; TF, tissue factor; PAGE, polyacrylamide gel electrophoresis.

The activity assays for factor Xa were performed using the substrate Spectrozyme factor Xa. Control experiments demonstrated that α-thrombin, which was also formed in the experimental system, also hydrolyzed the synthetic substrate Spectrozyme Xa. Thus, to determine the active concentration of factor Xa in each reaction sample, 35 μM recombinant Hirulog-1 was added to each reaction sample as a specific thrombin inhibitor. Control experiments demonstrated that the addition of Hirulog-1 to each reaction sample inhibited all of the α-thrombin activity but had no inhibitory effect on factor Xa activity. The samples were added in duplicate to a 96 well assay plate (Corning, Corning, NY) and activity assays were started by the addition of 200 μl of 0.2 mM Spectrozyme Xa. The rate of substrate hydrolysis was monitored by the absorbance change per unit time at 405 nm in a Molecular Devices V_{max} spectrophotometer. The concentration of factor Xa formed in the reaction per unit time was then directly determined using the standard curve of substrate hydrolysis with known concentrations of factor Xa.

To identify protein bands which expressed a serine protease active site during the activation process of the reaction described above, reaction aliquots were treated with 0.1 mM bc-FPR-ck for 30 min at room temperature. Following treatment, samples were subjected to SDS-PAGE under both reducing and non-reducing conditions using 4–12% polyacrylamide gels as generally described by Laemmli (38). Following SDS-PAGE, total protein from each gel was transferred to nitrocellulose membranes for immunoblot analysis using general techniques described by Towbin *et al.* (39). Membranes were blocked with TBS, 0.05% Tween-20 for 1h at 25 °C and then probed with Avidin DH and biotinylated horseradish peroxidase H (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) according to the manufacturers specification. Bound Avidin-biotin horseradish peroxidase H complexes were detected using the chemiluminescent substrate (Luminol) according to manufacturers specifications (ECL Western blotting detection system, Amersham, Arlington Hts., IL). Light emitted from the hydrolysis of the added Luminol substrate exposed the provided x-omat film in 2 to 5 minutes.

Immunoblotting for specific reaction products was performed upon EDTA quenched samples from each activation time point. The samples were further treated by the addition of an equal volume of a solution which contained 4% SDS (w/v), 20% glycerol (v/v), and 0.1 M Tris, pH 6.8. Samples were subjected to SDS- PAGE analyses under both reducing and non-reducing conditions using 4 to 12% polyacrylamide gels as generally described by Laemmli (38). Following SDS-PAGE, total protein from each gel was transferred to nitrocellulose membranes for immunoblot analysis using general techniques described by Towbin *et al.* (39). Nonspecific binding of nitrocellulose membranes was blocked by the addition of 5% nonfat dry milk (Carnation Co., Los Angeles, CA) (w/v) in TBS which contained 0.05% Tween-20 (v/v) (TBS/Tween) for 1 hr at 25 °C. Nitrocellulose membranes were washed 3 times with TBS/Tween and then probed individually for each antigen using specific monoclonal or polyclonal antibodies (diluted in TBS/Tween) to each antigen in the reaction. The following antibodies were used to detect each of the following antigens: total prothrombin antigen was detected using 10 μg/ml of a polyclonal burro anti-human prethrombin 1 (Grettle) antibody. Total factor V antigen was detected using 130 μg/ml of a polyclonal horse anti-human factor V antibody. Factor IX antigen was detected using 10 μg/ml of a mouse anti-human factor IX antibody. Factor X was detected using a mixture of two mouse anti-human factor X antibodies (10 μg/ml for each antibody), where one antibody was specifically directed against the heavy chain of factor X and one antibody was specifically directed against the light chain of factor X. To detect one of the specific antigens listed above, the specific antibody directed against that antigen was incubated with the blocked nitrocellulose membranes for 1 hr at room temperature. The bound antibody was then detected using either a conjugated goat anti-mouse-horseradish peroxidase IgG or a conjugated goat anti-horse-horseradish peroxidase IgG (Southern Biotechnology Associates, Inc.) which was diluted 1:5000 in TBS/Tween. The bound secondary antibody was detected using the chemiluminescent substrate (Luminol) as described above. Following the identification of one of the antigens from a specific immunoblot, nitrocellulose membranes were washed with 0.5 M NaCl, 0.1 M Glycine, pH 2.8 for 2 hrs to remove bound antibodies from the particular antigen on the nitrocellulose membranes. Following this washing step, the nitrocellulose membranes were reprobed for a second antigen by reblocking the nitrocellulose membranes with 5% nonfat dry milk and then adding a second antibody to probe for a different antigen on the same nitrocellulose membrane. Using this method, the same nitrocellulose membrane could be reprobed for prothrombin, factor V, factor IX, and factor X from the same reaction mixture.

For certain experiments designed to evaluate the proteolytic products formed by the activation of factor VIII, immunoblot analysis was not sufficiently sensitive to identify the proteolytic fragments of factor VIIIa (at 0.7 nM) which were formed in the reaction mixture. When radiolabeled factor VIII was used in place of unlabeled factor VIII, the concentration of factor VIII in the reaction system was increased to maintain factor VIII activity at a constant level (equivalent to 0.7 nM activity). Labeling of factor VIII reduced the specific activity to 33% that of unmodified factor VIII. Therefore, when radiolabeled factor VIII was used, the total factor VIII protein concentration was raised to 2.1 nM to maintain the level of activity of unlabeled factor VIII. Control experiments were conducted to examine the influence of this increased factor VIII (inactive) protein concentration upon the generation of thrombin. The alteration had no effect on the thrombin activity progress curve nor did the progress curve differ from that observed with 0.7 nM unlabeled factor VIII. The proteolytic products of factor VIII activation in this reaction mixture were then identified by SDS-PAGE using a 4–12% polyacrylamide gel. The gel was dried and exposed to Kodak x-ray film for 48 h at −70 °C.

Quantitative analyses of the immunoblots and autoradiograms were performed with several different scanners. One scanner was the Microscan 1000 scanning densitometer (TRI Inc.) equipped with a solid-state linear diode array camera to digitize images through a photographic lens. 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. Another scanner used was the Shimadzu dual-wavelength thin layer Chromatoscan Model CS-930 equipped with a data recorder DR-2. Another scanner used was a noncommercial instrument that utilized Bioscan Optimas software and was equipped with a Javelin JE 7442 camera and frame grabber image technologies vision plus FC-AT. Data are expressed as integrated volumes for each protein band using the arbitrary density units of the scanning system.

RESULTS

The Vitamin K-dependent Protein Reaction Pathway—The activation of the procoagulant proteins identified as components of the vitamin K-dependent procoagulant reactions of normal hemostasis *in vivo* were studied when the reaction is started with various concentrations of tissue factor or factor VIIa. The experimental protocol developed allowed for the reaction to be initiated by mixing prothrombin, factor V, factor VIII, factor IX, and factor X, at their reported plasma concentrations, with varying amounts of tissue factor-factor VIIa.

For initial experiments, factor VIIa was added at the physiological factor VII concentration (10 nM), and tissue factor and PCPS were at 5 nM and 200 μM, respectively. In this reaction system, the tissue factor-factor VIIa complex concentration was at one-half physiological saturation or 5 nM. The reaction progress was followed using a combination of antigen immunoblotting, enzymatic activity assays, active site blotting, and autoradiography to describe the progress curves for the proteins. The initial rate of thrombin formation was calculated at >70 nM/s. At this high tissue factor and factor VIIa concentrations, no lag in the progress of thrombin formation was observed in the reaction (Fig. 1), and prothrombin was quantitatively converted to α-thrombin during the first 40 s of the reaction. In contrast, factor Xa and factor IXa were formed nearly quantitatively only at ~400 s into the reaction time course (Fig. 1). Maximum thrombin generation rates were achieved when far less than 10% of the potential factor Xa or factor IXa concentrations were formed. Immunoblot and active site analyses of reaction products were consistent with expectations from previously published studies of the independent reactions involving the activation of prothrombin, factor V, factor VIII, factor IX, and factor X. No major unexpected products were observed for the combined reaction system. In separate experiments, we established that the factor VIIa-tissue factor complex alone does not cleave (activate) either factor VIII or factor V. Thus, the reaction products observed were consistent with the expected reactions.

The factor VIIa-tissue factor complex concentrations were

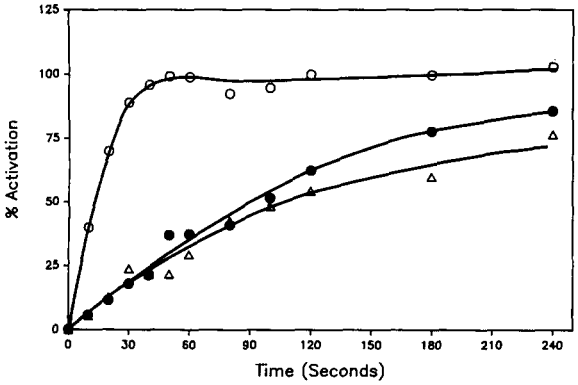

FIG. 1. **Activation of human factors II, IX, and X.** Experiments were initiated by 5 nM tissue factor, 10 nM recombinant VIIa. In the reaction illustrated, protein concentrations used were: 1 μM prothrombin, 20 nM factor V, 0.35 nM factor recombinant VIII, 80 nM factor IX, and 150 nM factor X in the presence of 200 μM PCPS vesicles. Data represent normalized values from immunoblot density scans and spectrozyme TH activity assays. *Open circles* represent activation of prothrombin to thrombin measured by activity assay. 100% represents full α-thrombin activation according to a standard curve, *open triangles* represent activation of factor X to factor Xa measured by immunoblot density units. 100% equals full factor Xa activation and total disappearance of the heavy chain of factor X; *filled circles* represent activation of factor IX to factor IXa measured by immunoblot density units. 100% equals full factor IXa activation and total disappearance of the heavy chain of factor IX.

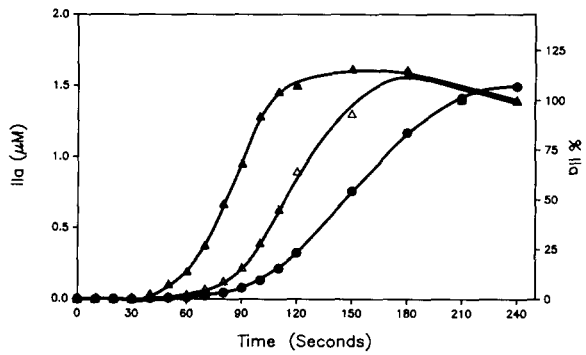

FIG. 2. **Thrombin generation at low tissue factor-factor VIIa concentration.** Thrombin generation over time was measured by using 0.2 mM Spectrozyme TH. Zymogens (II, IX, and X) and cofactors (V and VIII) were used at their plasma concentrations (Table I). Human recombinant factor VIIa was added (100 pM, 50 pM, and 5 pM) into the reaction system at a constant tissue factor concentration, 1 nM. *Filled triangles* represent the data when the experiment was initiated with 100 pM factor VIIa, *open triangles* represent 50 pM factor VIIa, whereas *filled circles* show the results obtained when the reaction was initiated with 5 pM factor VIIa.

varied over a significant range of concentrations from 5 nM to 5 pM. Representative data at the lower concentrations of factor VIIa are presented in Fig. 2. These data show the progress curves for the activation of prothrombin, in terms of thrombin formation at 100, 50, and 5 pM VIIa, when tissue factor is maintained at 1 nM. At these lower concentrations, the progress curves observed for the generation of thrombin activity can be divided into two phases: a significant lag or "initiation phase," in which no significant thrombin concentrations are seen, this is followed by a "propagation phase" in which prothrombin is quantitatively converted to thrombin. An intermediate maximum in the thrombin generation time course curve can be seen (see for example the points at 150 to 180 s (at 100 pM factor VIIa) and the point at 180 s (at 50 pM factor VIIa) of the reaction). This "bump" represents the formation of meizothrombin, the intermediate in the progression of prothrombin activation to α-thrombin. Meizothrombin has slightly higher activity (110–115%) toward Spectrozyme TH than does α-thrombin under our experimental assay conditions (40, 41). The activity of this intermediate in the prothrombin activation process is expressed and then eliminated in the formation of α-thrombin, causing the "maximum" in activity toward Spectrozyme TH. Under all conditions studied for the complete reaction mixture, prothrombin was quantitatively converted to α-thrombin.

A qualitative examination of Fig. 2 illustrates that the initiation phase of the reaction is primarily responsive to the concentration of factor VIIa-tissue factor complex used to start the reaction. During the propagation phase, the rate of thrombin generation is nearly independent of the initiating factor VIIa-tissue factor concentration. The maximum propagation phase velocity of thrombin activation observed at 5 pM factor VIIa-tissue factor in Fig. 2 is approximately 14 nM s^{-1} while at 100 pM complex, the maximum rate of thrombin expression is 30 nM s^{-1}. Thus, a 20-fold change in activator concentration produces just a 2-fold change in the maximum propagation phase rate of thrombin generation once the initiation phase has been passed. This is seen even more dramatically in a comparison of Fig. 1 with Fig. 2. For Fig. 1 at 5 nM factor VIIa-tissue factor complex, no initiation phase is observed, and the propagation phase rate of thrombin generation is ~70 nM s^{-1}. Thus, over a 1000-fold range of factor VIIa-tissue factor concentration, the propagation phase rate changes by only approximately 5-fold. In contrast, the initiation phase interval changes from 0 to 100 s over this range of factor VIIa-tissue factor complex concentration. Similar results are seen when factor VIIa and PCPS are held constant and tissue factor concentrations are varied.

The reaction progress was evaluated by a combination of techniques which allowed selective evaluation and quantitation of the major protein and enzyme species present in the reaction. Representative illustrations of these data are found in Figs. 3, 4, and 5 which illustrate the activation of prothrombin (Fig. 3), factor V (Fig. 4), and factor VIII (Fig. 5) in reactions initiated with 5 nM tissue factor-factor VIIa complex. The legend to each figure identifies the molecular fragments associated with the reaction process. As shown in Figs. 3–5, prothrombin, factor V, and factor VIII were quantitatively converted to their activated peptide products during the initial 30–40 s of the reaction as assessed by immunoblotting experiments and autoradiography. In contrast, under conditions in which active site blots using bc-FPR-ck revealed complete α-thrombin generation, by 40–50 s (Fig. 3, *A* and *B*, *lanes 5–7*) visible amounts of bc-FPR-factor Xa are not observed until 600 s (Fig. 3*B*, *bands b* and *c*, *lanes 15* and *16*). These data illustrate that the conversion of all the available factor X and factor IX to their active species is not required for quantitative α-thrombin formation (*i.e.* the conversion of 1.4 μM prothrombin to 1.4 μM α-thrombin).

Representative quantitative data obtained from immunoblot gel scanning data from an experiment at 5 pM factor VIIa-50 pM tissue factor are represented in Fig. 6. The time course of the loss of intact factor V and factor VIII which are associated with their expression as active cofactors factor Va and factor VIIIa are clearly major events associated with the initiation phase which precedes maximum thrombin generation. The limited amounts of factor Xa and factor IXa generated (Fig. 6) illustrates that the maximum rate of thrombin generation is obtained under conditions representing less than 1% of the potential concentration of the activated enzyme factor IXa and factor Xa based upon the respective plasma zymogen concentrations. It should be noted further that the formation rate of factor Xa is almost linear with time. These data indicate that

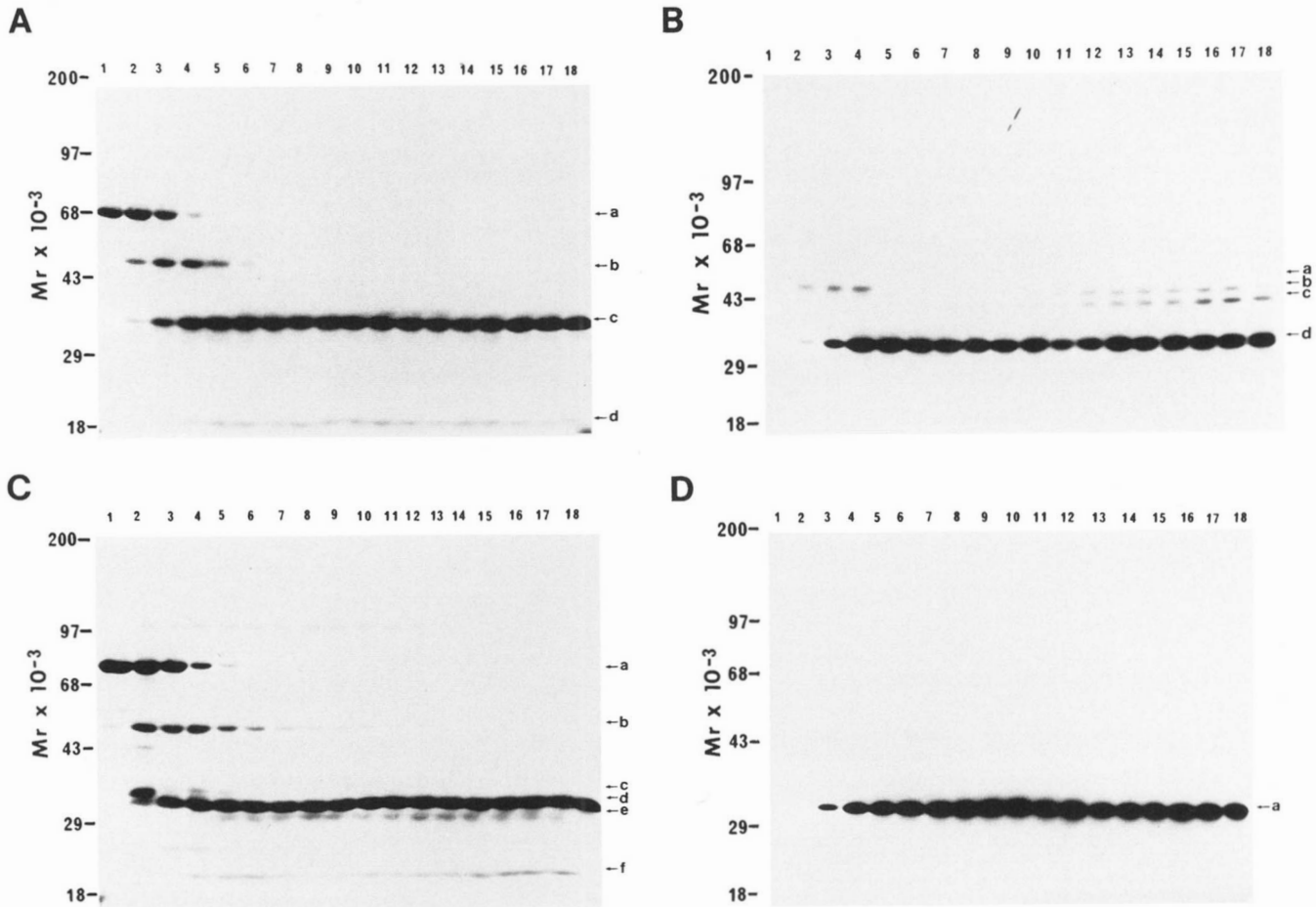

FIG. 3. **Prothrombin activation.** Analysis of prothrombin (1 μM) activation in the presence of factors V (20 nM), VIII (0.35 nM), IX (80 nM), and X (150 nM) by tissue factor (5 nM)-factor VIIa (10 nM). *Panel A* represents an immunoblot of the reaction components after SDS-PAGE using a polyclonal anti-human prethrombin 1 antibody. *Lanes 1–18* of the gel represent reaction time points of 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 180, 240, 360, 480, 600, 900, 1200, and 1800 s after initiation of the reaction with tissue factor-factor VIIa. *Panel B* represents an active site immunoblot of the same reaction time points after each sample was treated with bc-FPR-ck for 30 min prior to SDS-PAGE. *Panel C* represents an analysis of the same reaction samples as *panel A* under reducing conditions (2% β-mercaptoethanol). *Panel D* represents an analysis of the same reaction samples as *panel B* under reducing conditions (2% β-mercaptoethanol). The electrophoretic migrations of the fragments are as follows: *panel A*: *a*, prothrombin and/or meizothrombin; *b*, prothrombin des-fragment 1 and/or meizothrombin des-fragment 1; *c*, α-thrombin; and *d*, prothrombin fragment 1; *panel B*: *a*, meizothrombin des-fragment 1; *b*, factor Xaα; *c*, factor Xaβ; *d*, α-thrombin; *panel C*: *a*, prothrombin; *b*, prethrombin 1; *d*, B chain of thrombin; *f*, prothrombin fragment 1; *panel D*: *a*, B chain of thrombin.

under the experimental conditions used, once a steady state is achieved, the concentrations of the sum of factor X activating systems must be nearly unchanged. This result is not predicted by a cascade theory. These data imply that if the factor VIIIa-factor IXa complex is playing a role in the reaction, the concentration of the catalyst is governed by formation-degradation processes which provide a near steady state level of the catalyst. For all factor VIIa-tissue factor concentrations studied, prothrombin was activated in the reaction system to yield quantitative levels of α-thrombin, and the maximum rate of thrombin production was reached at a time point consistent with the point at which factor V and factor VIII are nearly fully cleaved to products associated with activation (>80%) and able to participate in their responsive reaction complexes. Thus, it appears that a major controlling event leading to the burst-like, explosive generation of thrombin activity is associated with the cofactor activation events.

Role and Activation of Factor V in the Reaction Progress—In order to explore this cofactor activation role, experiments were conducted in which the initial reaction system contained factor Va rather than factor V. Fig. 7 represents the thrombin generation progress curves observed under three sets of conditions: when the reaction mixture initially contains factor V (*squares*), when the reaction mixture initially contains factor Va (*circles*), and when no factor V or Va is added (*triangles*). When no factor V or factor Va is present in the reaction system, no thrombin is produced, attesting to the importance of the *prothrombinase* complex to the generation of thrombin. When the reaction is initiated with factor Va present rather than factor V, there is a significant decrease in the initiation phase time interval of the reaction, although a lag is still present. The experiment shows the importance of factor V activation to the generation of thrombin, and that the activation of this cofactor is a major, but not the only important step leading to the generation of maximum *prothrombinase* activity. Other events which must be clearly contributing to the lag are the activation of factor VIII to factor VIIIa and the generation of small but significant amounts of factor Xa and factor IXa.

When thrombin alone is responsible for the cleavage of factor V, the activation has been shown to proceed through the rapid appearance of the cofactor heavy chain (105,000), followed by the later appearance of the light chain (74,000) (42–44). Factor Xa/PCPS has been shown to cleave human factor V at Arg^{709} generating the heavy chain (105,000) and the intermediate (220,000), which is the light chain precursor. Thus, no factor Va light chain is observed when the human plasma cofactor is activated by factor Xa alone (44). The factor V activation cleavage pattern, observed in Fig. 4, does not coincide with activa-

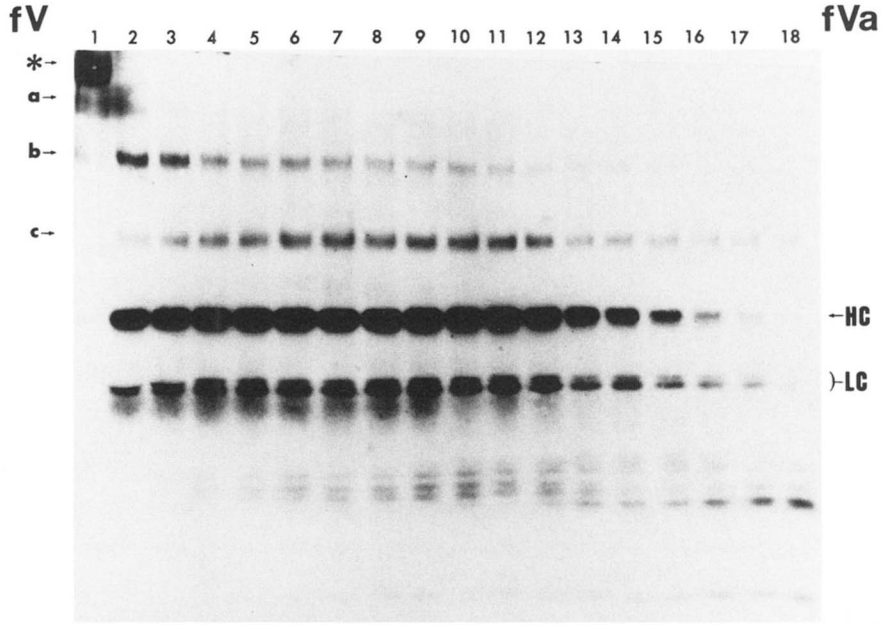

FIG. 4. **Factor V activation.** The figure represents an immunoblot of the reaction components after SDS-PAGE under reducing conditions (2% β-mercaptoethanol) using a polyclonal anti-human factor V antibody. The reaction was carried out in the presence of prothrombin (1 μM), factors IX (80 nM), X (150 nM), V (20 nM), and VIII (0.35 nM). *Lanes 1–18* represent reaction time points 0, 10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 180, 240, 360, 480, 600, 900, 1200, and 1800 s after initiation of the reaction with tissue factor (5 nM)/factor VIIa (10 nM). *Asterisk* represents the M_r = 330,000 single chain species; *a* represents the M_r = 280,000 fragment; *b* represents the M_r = 220,000 species; *c* represents the M_r = 150,000 species; *HC* represents the heavy chain species at 105,000; and *LC* represents the light chain doublet 74,000/72,000.

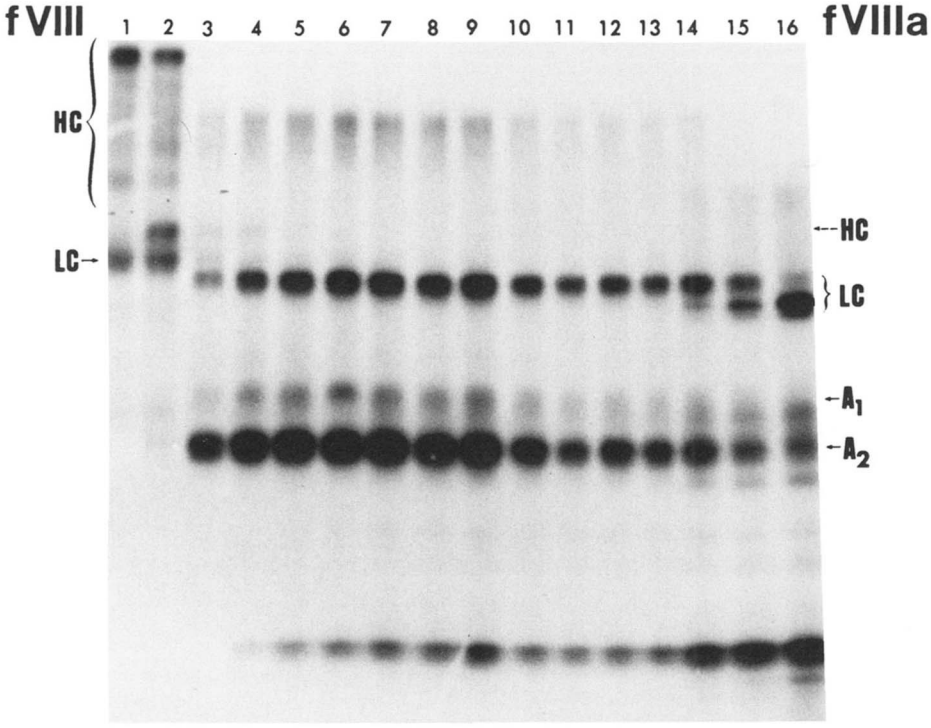

FIG. 5. **Activation of factor VIII.** All components were at plasma concentrations in the presence of radiolabeled factor VIII as described under "Experimental Procedures" (see Table I). The SDS-PAGE gel was performed in the presence of 2% β-mercaptoethanol. *Lanes 1–18* represent 30,000 cpm/well at time intervals; 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 600, 900, and 1500 s after initiation of the reaction with tissue factor (50 pM)/factor VIIa (5 pM). The gel was dried and the factor VIII/factor VIIIa components were visualized after autoradiography. *HC*, M_r = 200,000–90,000 fragments; *LC*, M_r = 80,000 before activation and M_r = 74,000 after activation; A_1 and A_2 depict fragments deriving from the heavy chain of the cofactor after cleavage by thrombin at Arg^{372}.

tion of factor V by either thrombin or factor Xa acting alone; it is rather similar to the activation cleavage pattern observed in human platelet-rich plasma (43). In order to elucidate the mechanism by which factor V is activated in the presence of all coagulation factors, an experiment was performed in which factor V (20 nM), in the presence of PCPS vesicles (200 nM), was activated by a mixture of thrombin and factor Xa. The concentration of thrombin was chosen to be 4 nM to approximate the concentration found at the beginning of the propagation phase in the complete reaction system discussed earlier. Since no factor Xa could be detected in this phase, and since the sensitivity of our detection methods is 1 nM or greater, the concentration of factor Xa was arbitrarily chosen to be 500 pM. The data of this experiment mimic the data of Fig. 4. By 10 s there is appearance of heavy and light chains of the cofactor. These results in this representative system of the initiation phase are consistent with the conclusion that factor V is activated by both thrombin and factor Xa, during the initiation phase to rapidly provide maximum active cofactor concentration.

Role of Factor VIIIa and Factor IXa in Factor X Activation—Activation experiments with factor VIIIa were not attempted because of the instability of factor VIIIa, especially at the physiologic (0.7 nM) concentrations used. It is well established that factor VIIIa once produced is unstable and rapidly loses activity, probably as a result of two processes: (*a*) dissociation of the A_2 subunit (45–47) and (*b*) proteolysis by thrombin, factor Xa, and factor IXa (48–52). Therefore, quantitative experiments which would proceed with full knowledge of the initial factor VIIIa concentration present were not possible. The contributions of factor VIIIa and factor IXa to the reaction scheme were assessed in experiments in which either factor VIII or factor IX was deleted from the initial conditions of the experi-

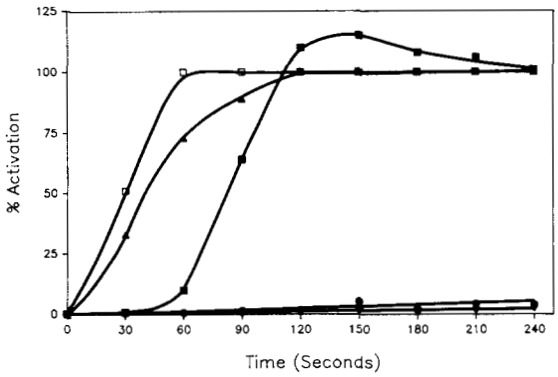

FIG. 6. **Activation of factor V, factor VIII, prothrombin, factor IX, and factor X *versus* time.** Experiments were performed at initiating concentrations of tissue factor and recombinant VIIa, 50 pM and 5 pM, respectively. Data were normalized to 100% by appearance of activation products in conjunction with the disappearance of single chain species, by immunoblot or autoradiograph scanning densitometry or by activity assay. The figure displays the percent activation as a function of time. The *open squares* represent activation of single chain factor V to factor Va as measured by immunoblot scanning densitometry (100% factor Va representing the total disappearance of single chain factor V and the appearance of activation products. The *filled triangles* depict activation of recombinant factor VIII to factor VIIIa as measured by autoradiograph scanning densitometry (100% factor VIIIa representing the total disappearance of the heavy chain of factor VIII. However, the disappearance of the M_r = 200,000/90,000 heavy chain(s) of factor VIII does not necessarily indicate that factor VIIIa retains full cofactor activity.) The *filled squares* show the conversion of prothrombin to thrombin as measured by chromogenic assay (Spectrozyme TH). The data were normalized using a thrombin standard curve and plateau of initial rates. Values greater than 100% represent the effects of meizothrombin present prior to the complete activation to α-thrombin. The *filled circles* depict activation of factor IX to factor IXaβ measured by immunoblot scanning densitometry and the disappearance of factor IXα heavy chain. The *filled inverse triangles* show the activation of factor X to factor Xa as measured by immunoblot scanning densitometry and the loss of factor X heavy chain.

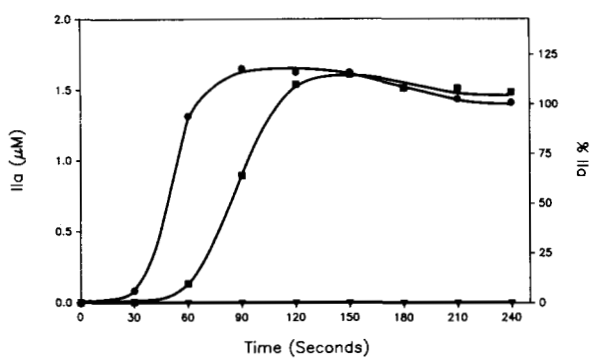

FIG. 7. **The consequence of preactivating factor V.** Thrombin generation was measured by a chromogenic assay with 0.2 mM Spectrozyme TH. Experiments were performed at initiating concentrations of TF and VIIa, of 50 pM and 5 pM, respectively, and at plasma concentrations of the zymogens (II, IX, X) and cofactors (V, VIII) (Table I). The *filled circles* represent the reaction as it proceeds when human factor Va (20 nM) is added in place of human factor V in the reaction. The *filled squares* represent the control reaction (all components as listed above). The *filled inverse triangles* represent the reaction as it proceeds in the absence of human factor V or Va. The same tracing was also seen when human factor X was left out of the reaction. In the absence of either factor V or factor X there was no measurable thrombin after 1500 s.

mental system. An illustration of this sort of experiment is presented in Fig. 8, *panels A* and *B*. In Fig. 8*A*, curves for thrombin generation are presented for experiments in which factor VIII was either present (*squares*) or deleted (*triangles*) from the reaction mixture (thrombin formation was initiated with 5 pM factor VIIa and 50 pM tissue factor). In contrast to

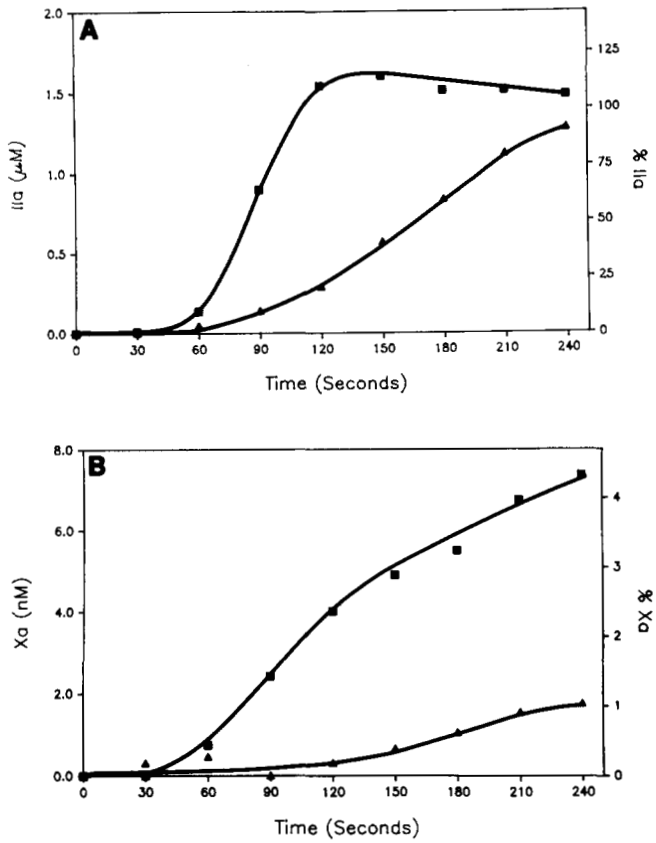

FIG. 8. *A*, the influence of factor VIII on thrombin generation. Thrombin was measured by activity assay (0.2 mM Spectrozyme TH, read for 5 min at 405 nm). Experiments were performed at initiating concentrations of tissue factor and factor VIIa at 50 pM and 5 pM, respectively. Zymogens (II, IX, and X) and cofactors (V and VIII) were used at plasma concentrations (Table I). The *filled squares* represent the control reaction (all components as listed above), whereas the *filled triangles* depict the reaction as it proceeds in the absence of factor VIII. *B*, the influence of factor VIII on factor Xa generation. Factor Xa generation was measured by activity assay (0.2 mM Spectrozyme fXa) over time (read for 20 min at 405 nM) in the presence of 35 μM Hirulog-1. Experiments were performed as in *A*. The *filled squares* represent the control reaction. The *filled triangles* represent the reaction in the absence of factor VIII.

experiments in which factor V was deleted, thrombin is still produced. The deletion of factor VIII from the reaction system does not significantly influence the initiation phase but has a profound effect on the propagation phase at this low concentration of factor VIIa-tissue factor. A comparison with the factor Xa generation rate, presented in Fig. 8*B*, illustrates the profound influence of the factor VIIIa-factor IXa complex upon the generation of factor Xa at low factor VIIa-tissue factor concentration when physiologic concentrations of the other relevant proteins are present in the reaction mixture. In the absence of factor VIIIa, factor Xa generation must occur exclusively through the factor VIIa-tissue factor complex pathway. Under these circumstances (*closed triangles*), a slow generation of factor Xa is seen. In contrast, factor Xa generation (*closed squares*) when factor VIII is present in the initial condition proceeds with a lag which corresponds to the interval of factor VIII activation (data not shown, see Fig. 6), followed by an increased rate of factor Xa activation. A comparison of *A* and *B* of Fig. 8 reveals that for the complete reaction system, maximum thrombin generation rates are achieved in 60–90 s. The extent of factor X activation observed during this interval corresponds to less than 1% of the initial factor X being converted to factor Xa. In contrast, when factor VIII was deleted, 240 s were required to produce 1% of the possible factor Xa. Similar

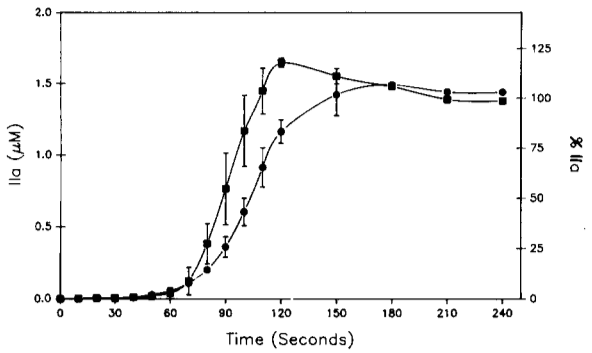

FIG. 9. **Effect of factor XI on thrombin generation.** Thrombin generation was measured by chromogenic assay (0.2 mM Spectrozyme TH) over time (read for 5 min at 405 nm). Experiments were performed at initiating tissue factor and factor VIIa concentrations, of 50 pM and 5 pM, respectively. Zymogens (II, IX, and X) and cofactors (V and VIII) were used at their plasma concentrations (Table I). The *filled circles* represent the control reaction (all components as listed above). The *filled squares* depict thrombin generation when all components as above plus 31 nM XI are added to the cofactor mixture (factor V and factor VIII) prior to the immediate addition to the VIIa-TF-PCPS-Ca^{2+} solution. The *error bars* represent the average determination from three different experiments.

experiments were conducted with factor IX deleted from the reaction system. The results of these experiments mirrored those of the factor VIII deletion experiments. These data illustrate an *in vitro,* kinetic regulatory process consistent with the expectations of the pathologies for the conditions of factor VIII and factor IX deficiency. The data in Fig. 8*B* also illustrate that, after the initial acceleration of factor X activation, between 60 and 120 s, the subsequent rise in factor Xa level is almost linear. Since the increase in rate of factor Xa generation is certainly due to the presence of the factor VIIIa-factor IXa complex, it must be concluded that inactivation events ultimately regulate the extent of the participation of the intrinsic *tenase* complex to factor X generation.

As expected from examination of Fig. 8*B*, the presence of factor VIII has little or no effect upon thrombin generation at the highest concentrations of factor VIIa-tissue factor (5 nM) used (Fig. 1). The data of Fig. 8 permit an estimation of the formation of the *prothrombinase* complex by two pathways, *i.e.* the factor VIIa-tissue factor and the factor VIIIa-factor IXa complex under the specified experimental conditions. It is clear from these data that while the factor VIIa-tissue factor complex has the capacity to initiate the reaction system when present at low concentrations, under these conditions, the major pathway of factor Xa generation must occur via the factor VIIIa-factor IXa complex (12, 53–55).

Influence of Factor XI on the Reaction Progress—It has been hypothesized that factor XI plays a major role in the propagation steps of blood clotting by acting as a feedback activator following its activation to factor XIa by α-thrombin (9). In this scenario, factor IX activation and propagation of the coagulation reaction is principally mediated by factor XI activation. The reconstructed reaction system was studied under conditions in which factor XI was present at plasma concentrations, or absent. The comparative experiments are presented in Fig. 9. The presence of factor XI in the initial mixture has virtually no effect on the initiation phase represented by the time lag prior to the propagation phase of thrombin production. However, as can been seen in Fig. 9, the presence of factor XI increases the rate of thrombin generation in a reproducible and significant degree. These data serve to illustrate the accessory role of factor XI anticipated from the pathology of factor XI deficiency. Factor XI thus appears as an accessory accelerator of the coagulation system, but not an essential activator in the generation of thrombin. Its contribution to thrombin generation is probably most essential under circumstances of pathology when maximum achievable thrombin generation rates are required.

DISCUSSION

A description of the pathology of bleeding disorders requires, at a minimum, the inclusion of the vessel wall, platelets, fibrinogen, prothrombin, factor X, factor IX, factor VII, factor V, factor VIII, and factor XIII in any model of the hemostatic system (56). Anecdotal clinical experience with individuals who have significant hemorrhagic risk suggests that a role also exists for factor XI (8).

It is likely that little or no α-thrombin or factor Xa circulates in the blood vessels of normal individuals, on account of the efficiency of antithrombin III neutralization of these enzymes. Factor VII circulates as the single chain zymogen form, but small amounts of the two-chain enzyme, factor VIIa, appear to escape inhibition and are present in blood (54, 55, 57–59). The competence of factor VIIa to hydrolyze any substrate in the absence of tissue factor is diminishingly small (53, 54). Thus, the factor VIIa active site is incomplete prior to its complexation with tissue factor. Following complexation with tissue factor, the factor VIIa k_{cat} increases substantially. Because of its catalytic insufficiency, factor VIIa is only poorly inhibited, if at all, by antithrombin III in the absence of tissue factor and heparin (58, 60–62). The other known inhibitor of factor VIIa, the tissue factor pathway inhibitor (63), can only inhibit factor VIIa when factor Xa is bound to that inhibitor. Thus, a small amount of factor VIIa, 1–2%, escapes inhibition and is constitutively present in blood.

Most reports suggest that tissue factor is abundant in extravascular tissue and also present in cryptic states within the vasculature (64–67). Tissue factor, present in endothelial cells, and monocytes can be made available by activation of these cells with cytokines (66, 67). In addition, all of the blood coagulation vitamin K-dependent enzyme complexes require the equivalent of an anionic phospholipid membrane for expressing their activities (reviewed in Ref. 68). This surface is either provided by cellular activation, mechanical cell damage, or both (69–71). In view of the above, many investigators believe that the coagulation process is initiated by the presentation of tissue factor (9, 12, 13) either because of a perforating injury or because of cytokine activation. Tissue factor forms a complex with plasma factor VIIa and can initiate activation (12, 53, 54). Because of feedback activation, the extent to which the factor VIIa-tissue factor complex is available ultimately resides in the amount of tissue factor and membrane presented. The tissue factor-factor VIIa complex activates factor IX and factor X. The factor Xa expressed can also participate in factor IX activation by the conversion of factor IX to factor IXα, the intermediate in both tissue factor-factor VIIa complex and factor XIa activation of factor IX (14, 72). Through processes of feed-forward and feed-back reactions, the pro-cofactors factor V and factor VIII are proteolytically converted by factor Xa and thrombin to their active species and participate in the formation of subsequent coagulation factor complexes. The result of the process is that α-thrombin is produced in explosive fashion by a collection of membrane-bound enzymatic complexes and the intensity of the response is dependent upon the amount of tissue factor and the amount of procoagulant membrane available.

The present investigation establishes a model system to study the kinetics of coagulation reactions when the zymogens and pro-cofactors required for the reaction are at or near their physiologic concentrations. The progress of the reactions is monitored by a composite of enzyme assays, immunoblots, active site blots, and autoradiography. The data obtained permits

the reconstruction of intermediate/product concentrations at various points during the reaction. The results, viewed in terms of α-thrombin generation, have unusual characteristics of product formation in which threshold levels of reactants are initially reached and ultimately lead to explosive generation of α-thrombin activity. The duration of the threshold process (initiation phase) is associated with factor VIIa-tissue factor concentrations; however, the ultimate kinetics of α-thrombin generation in the propagation phase of the reaction, is mostly dependent upon the fixed proenzyme, membrane, and pro-cofactor reagents initially present in the reaction system. During the initiation phase, seemingly trivial amounts of factor IXa and Xa ($\leq$1% of the available factor X and factor IX) are generated and nearly quantitative activation of factor V and factor VIII occurs. These pro-cofactor activation reactions appear to be the major triggers for the propagation phase of maximum rates of thrombin generation. When reactions are initiated in the presence of factor Va, the initiation phase is substantially diminished consistent with a contribution of the activation of factor V to duration of the initiation phase.

It is most interesting to note that the thrombin generation rate observed in the propagation phase of the reaction varies by only 5-fold over a 1,000-fold change in the concentration of factor VIIa-tissue factor complex. The maximum rate of thrombin generation is thus largely independent of the initiating concentration of tissue factor-factor VIIa complex. The tissue factor-factor VIIa complex provides the "spark" for the ignition of the reaction; the propagation is largely controlled by the invariant cofactor-zymogen concentrations.

The roles of the factor VIIIa-IXa complex *versus* that of the factor VIIa-tissue factor complex in the activation of factor Xa and ultimately of *prothrombinase* activity is illustrated in reactions performed at low tissue factor-factor VIIa concentrations. At high concentrations of factor VIIa-tissue factor complex (5 nM), little dependence on factor VIIIa in the reaction system is observed (Fig. 1). Our data are in agreement with clinical data which show that recombinant human factor VIIa infused to patients with Hemophilia A at supraphysiological concentrations can overcome their requirement for factor VIII (73, 74). In contrast, when activation is initiated by low levels of the factor VIIa-tissue factor complex (5–10 pM), the factor VIIIa-factor IXa complex is a major contributor to factor X activation. However, only 10% of the potential factor IXa-factor VIIIa complex is ever available. Thus, only 70 pM factor IXa-factor VIIIa complex is available for the completion of the reactions (*i.e.* the conversion of 1.4 μM prothrombin to 1.4 μM thrombin), and less than 1% of the potentially available factor IXa is actually used as a catalyst to generate the 2–3 nM factor Xa expressed during the entire process of quantitative prothrombin activation.

The K_d for the factor VIIIa subunit dissociation is 0.27 μM (47), and the maximum active factor VIIIa circulating exists at concentrations far below the K_d for the subunit association (~0.7 nM). Thus, the concentration of active factor VIIIa on the membrane surface, in the vicinity of factor IXa and factor X, becomes critical. Once factor VIIIa is formed, it complexes with factor IXa to activate factor X. However, the active factor VIIIa is also rapidly inactivated, probably as a result of two processes: the slow dissociation of the A2 subunit from the A1/light chain dimer, and the cleavage by factor IXa, factor Xa, and thrombin (45–47, 51, 52). Under these reaction conditions, there is generation of 500 nM thrombin after only 90 s. This extremely high thrombin concentration may contribute to the factor VIIIa inactivation process.

Our data suggest: 1) that the expression or exposure of tissue factor locally at the site of the vascular injury is the rate-limiting step of the reaction which is largely a function of the initiation phase; 2) only a small fraction of the available factor IX and factor X are formed by the factor VIIa-tissue factor complex during normal blood clotting; 3) high concentrations of plasma factor IX and factor X are necessary to satisfy multiple equilibria for the formation of the appropriate enzyme and enzyme-substrate complexes; 4) the pro-cofactor activation processes most likely occur by virtue of thrombin and factor Xa-catalyzed activation of factor V and factor VIII; 5) no unexplained major products derived from the zymogens or pro-cofactors in the reaction systems were observed. Thus, it is unlikely that any alterations in mechanisms derived from preceding experiments developed using either isolated coagulation enzyme complexes and substrates or limited mixtures of substrates need to be invoked to explain the reaction system. Left unanswered are the roles of the constitutive (antithrombin III, tissue factor pathway inhibitor) and dynamic (protein C-thrombomodulin) inhibitor systems, fibrinogen and von Willebrand factor.

Acknowledgment—We thank Natalie Coe for technical assistance.

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