Proteolytic Activation of Human Factors IX and X by Recombinant Human Factor VIIa: Effects of Calcium, Phospholipids, and Tissue Factor†

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ABSTRACT: Previous studies indicated that factor VIIa, in complex with tissue factor, readily activates either factor X or factor IX in the presence of calcium ions. In order to assess the relative physiological importance of the activation of factor IX versus the activation of factor X by recombinant factor VIIa, we have obtained steady-state kinetic parameters for the factor VIIa catalyzed activation of factor IX and factor X under a variety of cofactor conditions that include calcium alone, calcium and phospholipids, calcium, phospholipids, and tissue factor apoprotein, and calcium and cell-surface tissue factor. Calcium alone stimulated the activation of factors IX and X by factor VIIa maximally at 1 and 2.5 mM, respectively. In the presence of 25 μM phospholipids, maximal rates of factor IX and factor X activation were achieved at 2.5–5 mM calcium. With calcium alone, or with phospholipid and calcium, the initial rates of factor IX activation by factor VIIa were significantly higher than that observed for factor X. Kinetic studies revealed that the K_m for the factor VIIa catalyzed activation of factor IX was essentially constant in the presence of 5 mM calcium and 1–500 μM phospholipid, whereas the K_m for factor X activation varied with phospholipid concentration, reaching a minimum at 7–20 μM phospholipid. At all concentrations of added phospholipid, the k_{cat}/K_m ratio for the activation of factor IX by factor VIIa appeared to be considerably greater than that observed for the activation of factor X. In the presence of 5 mM calcium and varying concentrations of factor VIIa–relipidated tissue factor apoprotein complex (9–600 pM), k_{cat}/K_m values for factor IX activation were 2–9-fold greater than that obtained for factor X activation. In addition, in the presence of 5 mM calcium, factor IX activation by a complex of factor VIIa and endothelial cell tissue factor was approximately 3-fold more efficient than factor X activation. In contrast, factor VIIa bound to cell-surface tissue factor on monolayers of a human bladder carcinoma cell line activated factor X 4-fold more efficiently than factor IX in the presence of 5 mM calcium. These results indicate that factor IX appears to be the preferred substrate for factor VIIa under a variety of cofactor conditions and suggest that the activation of factor IX by factor VIIa may be an important reaction in normal hemostasis.

Factor VII is a single-chain, vitamin K dependent glycoprotein (M_r ~50 000) synthesized in the liver and secreted into the blood where it circulates as a zymogen to a serine protease, factor VIIa. Although the precise mechanism whereby factor VII is activated in vivo remains largely unknown, single-chain factor VII is converted to two-chain factor VIIa in the test tube by factor Xa (Radcliffe & Nemerson, 1976; Bajaj et al., 1981; Wildgoose & Kisiel, 1989), factor XIIa (Kisiel et al., 1977; Radcliffe et al., 1977), factor IXa (Seligsohn et al., 1979; Wildgoose & Kisiel, 1989), and thrombin (Radcliffe & Nemerson, 1976; Broze & Majerus, 1980) through the cleavage of a single internal peptide bond located at Arg_{152}–Ile_{153} (Hagen et al., 1986). In the presence of cell-surface tissue factor and calcium ions, factor VIIa rapidly activates either factor X or factor IX by limited proteolysis (Nemerson & Gentry, 1986). The proteolytic activity of the factor VIIa–tissue factor complex toward factor X is regulated in blood by a novel extrinsic pathway inhibitor (M_r ~40 000) that recognizes a ternary complex of factor VIIa–tissue factor–factor Xa and inhibits further activation of factor X and/or factor IX (Sanders et al., 1985; Broze & Miletich, 1987).

For several decades, it was widely accepted that complex formation between factor VIIa and tissue factor was an obligatory step for the expression of factor VIIa proteolytic activity. However, several recent studies demonstrated the tissue factor independent activation of factor X by factor VIIa in the presence of calcium ions alone or calcium and phospholipid vesicles (Telgt et al., 1989; Rao & Rapaport, 1990; Bom & Bertina, 1990). Telgt et al. (1989) reported that recombinant human factor VIIa activated human factor X in the presence of phospholipid vesicles and calcium ions at a rate approximately 0.04% of that observed with tissue factor. This finding was later confirmed by Rao and Rapaport (1990) and presumably accounted for the decreased APTT routinely observed in plasma samples of patients infused with recombinant factor VIIa. In a more detailed kinetic analysis, Bom and Bertina (1990) examined the relative contributions of calcium, phospholipids, and tissue factor apoprotein in the activation of human factor X by human factor VIIa. In these studies, calcium ions (6 mM) alone stimulated this reaction 10-fold by decreasing the apparent K_m for factor X. The addition of phospholipid increased the V_{max} 2-fold with an apparent 150-fold decrease in the K_m. The co-presence of tissue factor apoprotein, phospholipid, and calcium ions increased the V_{max} 41 000-fold with a corresponding 15 million-fold increase in the k_{cat} of the reaction. These data, using human reagents, largely corroborate earlier kinetic analyses by Nemerson and co-wokers using purified bovine reagents (Silverberg et al., 1977).

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In the present study, we report on the effects of calcium, phospholipid, and tissue factor (fluid phase and solid phase) on the kinetic parameters of the activation of human factors IX and X by recombinant factor VIIa in an effort to assess the relative physiological importance of the activation of factor IX versus the activation of factor X. Our results indicate that, under a variety of experimental conditions, factor IX appears to be the preferred substrate for factor VIIa, and suggest that the activation of factor IX by the extrinsic pathway may be a significant reaction in normal hemostasis.

Materials and Methods

Materials. Bovine serum albumin (fatty acid free), porcine mucosal heparin, phosphatidyl-L-serine (bovine brain), phosphatidylcholine (type V-E), and human fibronectin were obtained from Sigma Chemical Co., St. Louis, MO. Octyl β-glucoside was a product of Pierce Chemical Co., Rockford, IL. Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck)[1] was obtained from Calbiochem, San Diego, CA. Bz-Ile-Glu-Arg-*p*-nitroanilide (S-2222) was purchased from Helena Laboratories, Beaumont, TX. Tissue culture flasks and 24-well plates were purchased from Corning. Minimum essential medium (Eagle's), medium 199 (M-199), and nonessential amino acids were purchased from Mediatech. Fetal bovine serum, endothelial cell growth supplement, *Escherichia coli* lipopolysaccharide endotoxin (LPS) (serotype 026:B6), and collagenase (CLS 2) were obtained from Hyclone Laboratories (Logan, UT), Collaborative Corp. (Bedford, MA), Difco Laboratories (Detroit, MI), and Worthington Biochemical Corp. (Freehold, NJ), respectively. Factor IX deficient plasma and a calcium-dependent anti-human factor IX monoclonal antibody (A-7) were kindly provided by Dr. Kenneth Smith, University of New Mexico. Affinity-purified biotinylated rabbit anti-human antithrombin III polyclonal antibody was prepared as previously described (Smith, 1988). Ninety-six-well microtitration plates were obtained from Dynatech Laboratories, Chantilly, VA. Peroxidase-conjugate streptavidin was purchased from Bethesda Research Laboratories, Gaithenberg, MD. All other reagents were of the highest purity commercially available.

Proteins. Human plasma factor IX, factor X, and antithrombin III were purified to homogeneity essentially as described (Kondo & Kisiel, 1987). Factor IX was treated with 50 μM DEGRck at room temperature for 30 min to inactivate trace amounts of contaminating factor IXa followed by extensive dialysis. Recombinant human factor VIIa was purified from baby hamster kidney cell culture medium as previously described (Thim et al., 1988). Recombinant human tissue factor apoprotein, produced in *Escherichia coli* and purified to homogeneity by immunoaffinity chromatography (Paborsky et al., 1989), was kindly provided by Dr. Gordon Vehar, Genentech, Inc., South San Francisco, CA. Human brain tissue factor apoprotein was purified to homogeneity from acetone brain powder and relipidated essentially as described (Pedersen et al., 1990). Since tissue factor apoprotein is randomly oriented when inserted into phospholipid vesicles (Bach et al., 1986), the effective tissue factor apoprotein concentration was assumed to be 50% of the total tissue factor apoprotein concentration in relipidated samples. Antibodies against human brain tissue factor apoprotein were produced in rabbits, and IgG was purified from antisera by protein A–Sepharose affinity chromatography essentially as described for the preparation of antirecombinant human tissue factor apoprotein IgG (Sakai et al., 1989).

Preparation of Various Phospholipid Vesicles. Small, unilamellar phospholipid vesicles were prepared by ultrasonic irradiation of aqueous phospholipid dispersions followed by ultracentrifugation essentially as described by Barenholz et al. (1977). Phosphatidylcholine/phosphatidylserine (70:30 mol/mol) vesicles were prepared by mixing the appropriate amount of phosphatidylcholine and phosphatidylserine in a heavy-wall glass tube followed by drying under a stream of nitrogen. The phospholipids were then dispersed in 4 mL of TBS (total phospholipid concentration ~8 mM) and sonicated intermittently at 0 °C for 60 s followed by a 60-s cooling period for a total sonication time of 15 min using a Kontes microultrasonic cell disrupter (Power Setting 5). Following sonication, the vesicle dispersion was centrifuged at 4 °C for 60 min at 140000*g* (type 65 fixed-angle rotor) in a Beckman L2-65B ultracentrifuge to remove probe particles and multilamellar liposomes. In addition to defined PC/PS preparations, small unilamellar vesicles of mixed brain phospholipids (cephalin) were prepared essentially by the procedure described above. In these experiments, 25 g of human brain acetone powder (Quick, 1959) was extracted with 100 mL of chloroform at 25 °C for 4 h and the suspension filtered. The filtrate was evaporated to dryness and the phospholipid residue uniformally dispersed in 100 mL of TBS. This stock cephalin solution (~50 mM phospholipid) was diluted with TBS to approximately 10 mM phospholipid and subjected to ultrasonic irradiation and ultracentrifugation to obtain small, unilamellar phospholipid vesicles (region III). Phospholipid concentrations in the supernatant unilamellar phospholipids vesicles were determined according to Chen (1956), and vesicles preparations were used within 24 h after preparation (Lentz et al., 1987). For human brain cephalin, the following composition was assumed: phosphatidylcholine, 30.3%; phosphatidylethanolamine, 36.2%; phosphatidylinositol, 2.6%; phosphatidylserine, 17.7%; and sphingomyelin, 13.2% (White, 1973).

Cell Culture. The human bladder carcinoma J82 cell line (ATCC: HTB-1) was obtained from American Type Culture Collection, Rockville, MD. J82 cells were grown in T-75 flasks containing minimum essential medium (Eagle's) supplemented with 10% fetal bovine serum, nonessential amino acids mixture, and penicillin–streptomycin, as previously described (Sakai et al., 1989). Tumor cells were cultured at 37 °C in an atmosphere containing 6% CO_2 and 98% relative humidity. Prior to subculturing, the cells were removed from T-75 flasks using trypsin solution, centrifuged (1000*g*, 10 min), resuspended in fresh serum-containing medium, and reseeded in 24-well tissue culture plates. Assays were performed within 24 h after reaching confluency. Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cord veins within 3 h after delivery as described previously (Noguchi et al., 1989). HUVEC were grown to confluence in M-199 supplemented with 20% fetal bovine serum, 90 μg/mL porcine mucosal heparin, 100 μg/mL ECGS, penicillin–streptomycin in a T-75 flask coated with human fibronectin (1 μg/cm^2). HUVEC were perturbed with LPS (100 μg/mL) at 37 °C for 6 h as described previously (Noguchi et al., 1989) in order to express cell-surface tissue factor activity.

Analysis of Factor IX and Factor X Activation in the Presence and Absence of Calcium and Phospholipids. Re-

[1] Abbreviations: HUVEC, human umbilical vein endothelial cells; EDTA, ethylenediaminetetraacetic acid; TBS/BSA, Tris-buffered saline/0.1% bovine serum albumin; J82, human bladder carcinoma cell line; DEGRck, dansyl-Glu-Gly-Arg chloromethyl ketone; PS, phosphatidylserine; PC, phosphatidylcholine.

combinant factor VIIa (100 nM) was incubated separately in 300-μL snap-cap polypropylene centrifuge tubes with factor IX (600 nM) or factor X (1300 nM) and varying concentrations of calcium chloride (0–5 mM) in a total volume of 100 μL of TBS/BSA. The reaction was initiated by the addition of either factor IX or factor X to the incubation mixture. Following 6 h of incubation at 37 °C, 4 μL of 0.5 M EDTA was added to stop the reaction, and the incubation mixtures were kept in an ice water bath until assay. To measure the effect of calcium concentration in the presence of phospholipids, factor VIIa (60 nM) was incubated with either factor IX (500 nM) or factor X (500 nM) in the presence of 25 μM PC/PS and various concentrations of calcium (0–5 mM) in a total volume of 100 μL of TBS/BSA. Following a 1-h incubation at 37 °C, the reaction was stopped by the addition of 0.5 M EDTA (4 μL). Factor IXa coagulant activity generated in reaction mixtures was determined in a Becton–Dickinson fibrometer using nonactivated factor IX deficient plasma essentially as described (Griffith et al., 1985). Factor IXa concentrations were interpolated from a log–log plot relating clotting times and factor IXa concentrations that was linear in the range 1–100 ng/mL factor IXa. Factor Xa was assayed by using the factor Xa specific chromogenic substrate S-2222 essentially as described (Sakai et al., 1989). Factor Xa standard curves were linear between 5 and 300 ng of factor Xa/mL.

On the basis of the above results, steady-state kinetic studies were performed to determine the kinetic parameters for the factor VIIa mediated activation of factor IX and factor X in the presence of either calcium alone or a mixture of calcium and phospholipids. In calcium incubation mixtures, factor VIIa (100 nM) was incubated at 37 °C with varying concentrations of either factor IX (125–4000 nM) or factor X (125–4000 nM), 2.5 mM $CaCl_2$, and TBS/BSA in a total volume of 100 μL. At selected intervals, the reaction was stopped by the addition of EDTA and factor Xa or factor IXa assayed as described above. In incubation mixtures containing calcium and phospholipids (small unilamellar vesicles), factor VIIa (20 nM) was incubated at 37 °C with varying concentrations of either factor IX (5–100 nM) or factor X (100–10000nM), 5 mM $CaCl_2$, phospholipids (cephalin or PC/PS, 70:30), and TBS/BSA in a total volume of 100 μL. Kinetic parameters were obtained at several phospholipid concentrations ranging between 0.3 and 500 μM for cephalin vesicles and between 1.4 and 564 μM PC/PS vesicles. In all cases, initial velocity conditions were used where less than 10% of the substrate was converted to product in the time interval examined.

Analysis of Factor IX and Factor X Activation by Factor VIIa and Relipidated Human Tissue Factor Apoprotein. In these experiments, stoichiometric amounts of relipidated human brain tissue factor apoprotein were added to varying concentrations of factor VIIa (9–600 pM) in 1 mL of TBS/BSA/5 mM $CaCl_2$, and the mixture was incubated at 37 °C for 20 min in order to establish a factor VIIa–tissue factor complex. Factor IX (3–600 nM) or factor X (6.25–1000 nM) was then added to these reaction mixtures, and the initial rate of factor IXa or factor Xa formation was determined as described above.

Analysis of Factor IX and Factor X Activation by Cell-Bound Factor VIIa. The initial rate of factor IX or factor X activation by cell-bound factor VIIa was performed by using confluent monolayers of either a human bladder carcinoma cell line (J82) or human umbilical vein endothelial cells (HUVEC) following incubation with lipopolysaccharide endotoxin (LPS). The J82 cell line constitutively synthesizes tissue factor apoprotein which forms a cell-surface receptor for factor VII/VIIa (Fair & MacDonald, 1987; Sakai et al., 1989; Drake et al., 1989). In contrast, human endothelial cells do not synthesize tissue factor, but can be induced to express cell-surface tissue factor activity following stimulation with LPS (Lyberg et al., 1983). In these experiments, 24-well plates were initially seeded with either J82 cells or HUVEC and allowed to grow to confluence. Confluent cultures were washed once with buffer A [10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, and 11 mM glucose] supplemented with 10 mM EDTA and subsequently washed 3 times with buffer A. In J82 cell studies, duplicate wells were incubated with factor VIIa (10 nM) in buffer A supplemented with 0.5% BSA and 5 mM $CaCl_2$ (buffer A^+) for 2 h at 37 °C with constant oscillation on an orbit shaker. After this period, the plates were rapidly washed 6 times with buffer A^+ to remove unbound factor VIIa. Each well was then treated with 500 μL of varying concentrations of either factor IX (25–800 nM) or factor X (50–800 nM) dissolved in buffer A^+. After a predetermined incubation time at 37 °C, 20 μL of 0.5 M EDTA was added to stop the reaction and factor Xa or factor IXa in the supernatant assayed. Preliminary time course studies indicated that the rates of factor X and factor IX activation by J82 cell-bound factor VIIa were linear between zero time and 10 min at the high and low substrate concentrations used. Thus, in factor X incubation mixtures, the reaction was stopped at 2 min, whereas in factor IX incubation mixtures the reaction was stopped at 4 min in order to accumulate sufficient factor IXa for assay. In J82 cell studies, factor Xa was assayed by the S-2222 chromogenic assay while factor IXa concentration was determined by a specific enzyme-linked immunosorbent assay (Smith, 1988) in order to avoid spurious results in the clotting assay caused by variable amounts of EDTA-induced J82 cell detachment from the 24-well plate. In the latter assay, 10 μL of cell supernatant was mixed with 90 μL of 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1 mM benzamidine, and 10 μg/mL carrier factor IX and incubated at 4 °C overnight in a 96-well microtitration plate precoated with anti-human factor IX monoclonal antibody (A-7). After the plate was washed with 20 mM Tris-HCl (pH 7.2)/0.15 M NaCl/5 mM $CaCl_2$/0.05% Tween 20, 50 μL of antithrombin III (100 ng) and 50 μL of heparin (0.2 unit), both dissolved in TBS/BSA/5 mM $CaCl_2$, were added to each well. Following a 2-h incubation at 37 °C, the plates were washed, reacted with biotinylated rabbit anti-human antithrombin III IgG, and subsequently incubated with peroxidase-conjugated streptavidin for 2 h at 37 °C. Peroxidase activity was detected with *o*-phenylenediamine–H_2O_2. Factor IXa concentration was interpolated from a linear standard curve relating A_{492} versus factor IXa concentration (10–500 ng of factor IXa/mL). In studies using perturbed HUVEC, duplicate wells were incubated with factor VIIa (20 nM) in buffer A^+ for 2 h at 37 °C on an orbit shaker. The plates were washed 6 times with buffer A^+, and each well was then treated with 500 μL of varying concentrations of either factor IX (3–200 nM) or factor X (50–800 nM) dissolved in buffer A^+. After a 4-min incubation at 37 °C, 4 μL of 0.5 M EDTA was added to each well to stop the reaction, and factor IXa and factor Xa were assayed in the supernatant by either a clotting assay (factor IXa) or a chromogenic assay (factor Xa). As with the J82 cell studies, a 4-min incubation time was found to be in the linear portion of the time course curve for the high and low substrate concentrations of both factor IX and factor X (data not shown).

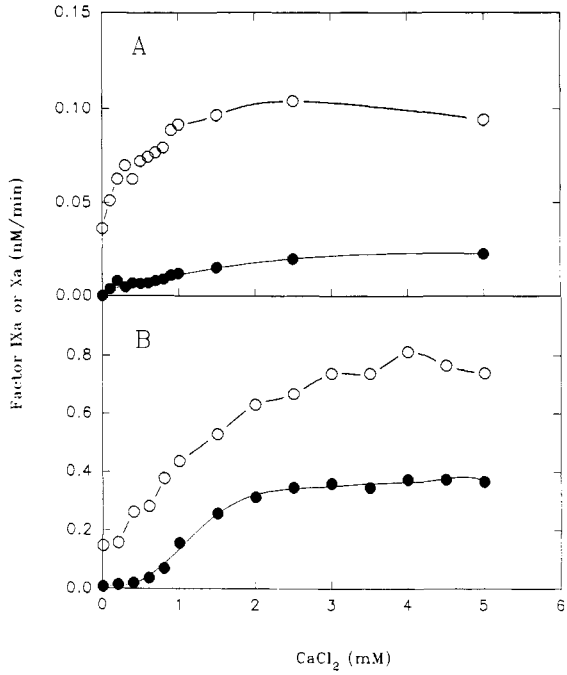

FIGURE 1: Effect of calcium concentration on the activation of factors IX and X by factor VIIa. Prior to the reaction, factor IX, factor X, Factor VIIa, and BSA were each treated with 10 mM EDTA to remove endogenous calcium followed by extensive dialysis against TBS. Factor IXa (○) and factor Xa (●) were assayed as described under Materials and Methods. (A) In the absence of phospholipid; (B) in the presence of 25 μM PC/PS (70:30) small unilamellar vesicles.

Kinetic parameters for the activation of factors IX and X by factor VIIa were also determined by using sonicated J82 cells as the source of tissue factor. In these experiments, J82 cells (10^7 cells) were suspended in 1 mL of buffer A and sonicated for six 10-s intervals. An aliquot (10 μL) of the sonicated cell suspension was mixed with 10 nM factor VIIa and various concentrations of factor IX or factor X (13–800 nM) in a total volume of 100 μL of buffer A^+. Following a 2-min incubation at 37 °C, EDTA was added to stop the reaction, and factor IXa and factor Xa were assayed by the enzyme-linked immunosorbent assay and the S-2222 chromogenic assay, respectively. Inasmuch as tissue factor apoprotein concentrations were not assessed in the sonicated J82 mixtures, V_{max} values were obtained in place of k_{cat} values.

RESULTS

Effect of Calcium and Phospholipid Concentration on the Activation of Factors IX and X by Factor VIIa. To elucidate the role of calcium ions in the factor VIIa mediated activation of factors IX and X, we initially examined the rate of each activation reaction as a function of calcium ion concentration in the absence of additional cofactors. As shown in Figure 1A, factor IX was activated by recombinant factor VIIa at an appreciable rate in the absence of calcium. Factor IX activation rates progressively increased from 0 to 1 mM added calcium, and remained relatively constant from 1 to 5 mM calcium. In contrast to factor IX, no activation of factor X was detected in the absence of calcium ions. Factor X was, however, activated by factor VIIa as calcium was added to the reaction mixture and appeared to reach a plateau rate at ~2.5 mM calcium (Figure 1). At 2.5 mM $CaCl_2$, the apparent K_m and k_{cat} values for the activation of factor IX by factor VIIa were obtained from a linear Hanes–Woolf plot and were 2.6 μM and 9.1×10^{-4} min^{-1}, respectively. Under comparable conditions the K_m and k_{cat} values for the activation of factor X by factor VIIa were 2.1 μM and 1.1×10^{-4} min^{-1},

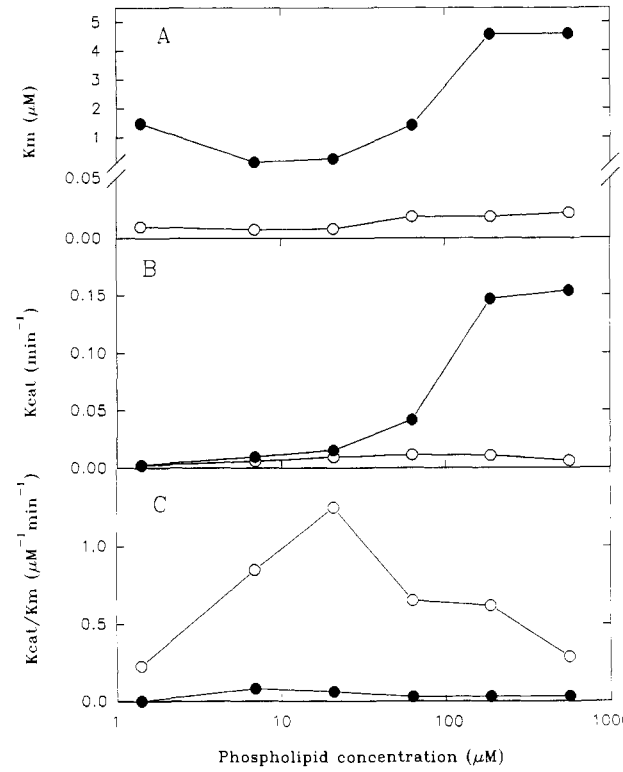

FIGURE 2: Effect of phospholipid (PC/PS) concentration on the activation of factors IX and X by factor VIIa in the presence of 5 mM calcium. Factor IX (5–100 nM) or factor X (100–10 000 nM) was activated by factor VIIa (20 nM) at 37 °C for 4 h with 1.4 μM PC/PS, for 90 min with 6.9 μM PC/PS, for 60 min with 20.8 and 62.8 μM PC/PS, or for 30 min with 188 and 564 μM PC/PS. Factor IXa (○) and factor Xa (●) were assayed as described under Materials and Methods. (A) K_m; (B) k_{cat}; (C) k_{cat}/K_m.

respectively. We next examined the effect of calcium concentration on the factor VIIa mediated activation of factors IX and X in the presence of 25 μM PC/PS. Concurrent studies indicated that this concentration of phospholipid was optimal for each activation reaction (see below). As shown in Figure 1B, factor IX and factor X were each activated at a greater rate in the presence of calcium and phospholipid in comparison to calcium alone. Maximal rates of factor X and factor IX activation occurred in the range 2.5–5 mM calcium and 4–5 mM calcium, respectively. At each calcium concentration studied, the rate of factor IX activation by factor VIIa was approximately 2-fold greater than that observed for factor X activation.

Effect of Phospholipid Concentration on the Activation of Factors IX and X by Factor VIIa in the Absence of Tissue Factor Apoprotein. In these experiments, the kinetic parameters for the activation of factors IX and X were obtained by Hanes–Woolf analysis at several concentrations of added small, unilamellar phospholipid vesicles and 5 mM calcium. In addition, two different phospholipid vesicle preparations were examined in these experiments. In one set of kinetic studies, a defined mixture of phosphatidylcholine and phosphatidylserine (70:30 PC/PS) small unilamellar vesicles were employed, while in another set of kinetic studies, small unilamellar vesicle preparations of mixed brain phospholipids (cephalin) were examined. Figure 2 illustrates the results obtained with PC/PS vesicles. At phospholipid concentrations <20 μM, a slight substrate inhibition was observed in Lineweaver–Burk plots presumably due to competition between factor VIIa and substrate (factor X or factor IX) for a limited number of binding sites on the PC/PS vesicles. However, no evidence for substrate inhibition was observed at phospholipid con-

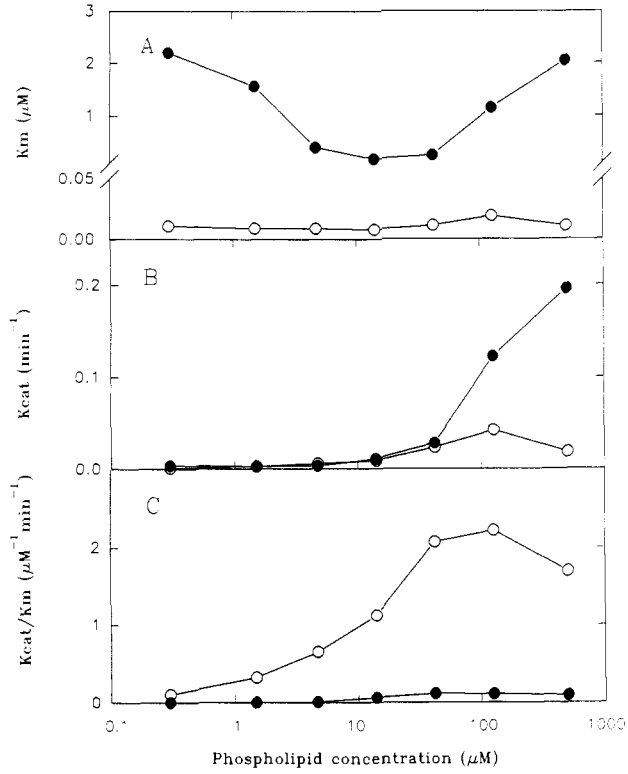

FIGURE 3: Effect of phospholipid (cephalin) concentration on the activation of factors IX and X by factor VIIa in the presence of 5 mM calcium. Factor IX (5–100 nM) or factor X (100–10 000 nM) was activated by factor VIIa (20 nM) at 37 °C for 4 h with 0.3 μM cephalin, for 90 min with 1.5 μM cephalin, for 60 min with 4.7 and 14.1 μM cephalin, and for 15 min with 504 μM cephalin. Factor IXa (○) and factor Xa (●) were assayed as described under Materials and Methods. (A) K_m; (B) k_{cat}; (C) k_{cat}/K_m.

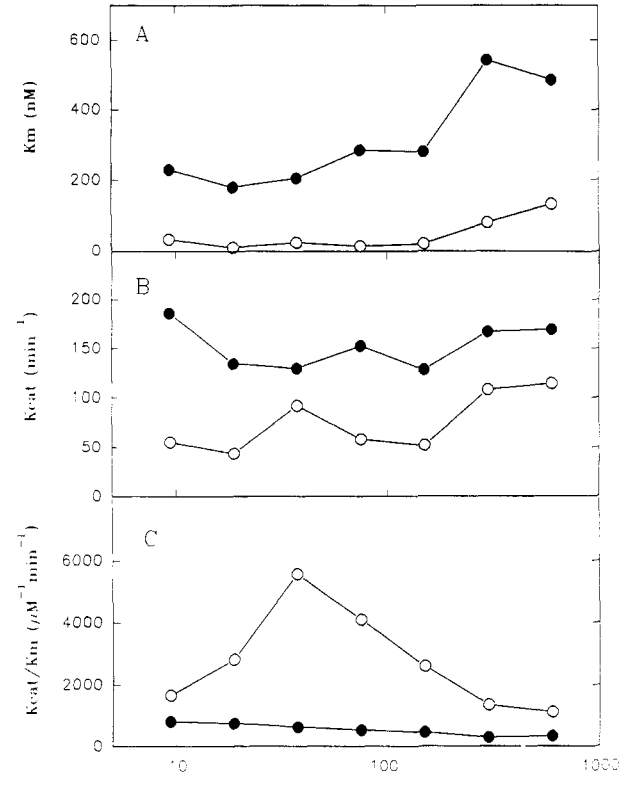

FIGURE 4: Effect of factor VIIa–tissue factor complex concentration on the activation of factors IX and X. Factor IX (3–600 nM) or factor X (6–1000 nM) was activated by factor VIIa–relipidated tissue factor apoprotein complex (9–600 pM) at 37 °C. Factor IXa (○) and factor Xa (●) were assayed as described under Materials and Methods. (A) K_m; (B) k_{cat}; (C) k_{cat}/K_m.

centrations >20 μM. The K_m values for the activation of factor IX by factor VIIa were relatively constant over a wide range of added PC/PS (Figure 2A). In contrast, the K_m value for factor X varied as a function of added PC/PS and was minimal at 7–20 μM phospholipids. k_{cat} values for the activation of factor IX reached a maximum between 20 and 200 μM PC/PS and declined slightly at 500 μM PC/PS. K_{cat} values for the activation of factor X progressively increased between 10 and 500 μM PC/PS (Figure 2B) and declined at higher PC/PS concentrations (data not shown). When k_{cat}/K_m ratios were plotted as a function of PC/PS concentration (Figure 2C), maximal values for each reaction were observed at ~20 μM PC/PS. At all concentrations of added PC/PS, the k_{cat}/K_m ratio for the activation of factor IX by factor VIIa appeared to be ~20-fold higher than that observed for factor X (Figure 2C). Comparable results were obtained when mixed brain phospholipid vesicles were used as the source of phospholipid, although higher concentrations of mixed brain phospholipids were required to obtain the same K_m and k_{cat} values seen with defined PC/PS mixtures (Figure 3A–C).

Effect of Relipidated Tissue Factor Apoprotein on the Activation of Factors IX and X by Factor VIIa. In these experiments, the kinetic parameters (K_m and k_{cat}) for the activation of factors IX and X by factor VIIa were determined at several concentrations of human brain tissue factor apoprotein (9–600 pM) in complex with stoichiometric amounts of factor VIIa in the presence of 5 mM calcium. The contribution of phospholipid to the activation rate in this system, in comparison to the rate-enhancing effect of tissue factor apoprotein, was determined in separate experiments and found to be negligible (0.1%). In the presence of 9–600 pM factor VIIa–tissue factor apoprotein complex, the K_m for factor IX activation was consistently lower than that observed for factor X (Figure 4A). Furthermore, between 9 and 150 pM factor VIIa–tissue factor apoprotein complex, the K_m for factor IX activation was below the plasma concentration of factor IX (~80 nM), while at all factor VIIa–tissue factor apoprotein complex concentrations, the K_m for factor X activation was either at or greater than 200 nM, values well above the factor X plasma concentration (~125 nM). In contrast, the k_{cat} values for factor X activation at each factor VIIa–tissue factor concentration were greater than that observed for factor IX activation (Figure 4B). Plotting k_{cat}/K_m ratios as a function of factor VIIa–tissue factor apoprotein complex concentration revealed that the catalytic efficiency for the activation of factor IX was consistently higher than that observed for factor X at all activator concentrations studied, reaching a maximum at ~38 pM tissue factor apoprotein and progressively declining at higher concentrations of factor VIIa–tissue factor (Figure 4C). At 38 pM tissue factor concentration, the k_{cat}/K_m ratio was ~9-fold higher for factor IX than for factor X activation. In comparison to factor IX activation, the k_{cat}/K_m values for factor X activation were relatively constant in the range of factor VIIa–tissue factor concentrations employed in this study (Figure 4C).

Activation of Factors IX and X by Cell-Bound Factor VIIa. Recent work by Rapaport and co-workers (Almus et al., 1989) demonstrated that tissue factor expressed on thrombin-stimulated human umbilical vein endothelial cells, or purified tissue factor apoprotein reconstituted into mixed phospholipid vesicles, in complex with factor VIIa, activated factor X severalfold faster than factor IX. However, K_m values for these substrates were not provided in that report. To ascertain the preferred substrate for factor VIIa–tissue factor on cell sur-

Table I: Kinetic Parameters of Factor IX and Factor X Activation by Factor VIIa in the Presence of Various Cofactors

		factor IX			factor X		
cofactor	concn	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
calcium	2.5 mM	2.61	0.0009	0.0003	2.10	0.0001	0.00005
PC/PS (7:3)	1.4 μM	0.009	0.0022	0.23	1.48	0.0025	0.0017
	21 μM	0.008	0.0097	1.25	0.25	0.0156	0.062
	188 μM	0.017	0.011	0.62	4.60	0.147	0.032
relipidated tissue factor	9.4 pM	0.032	55.1	1722	0.23	186	885
	37.5 pM	0.016	91.9	5560	0.205	130	634
	300 pM	0.082	108	1317	0.542	167	308
LPS-perturbed HUVEC		0.024	32.4	1349	0.24	103	428
J82 cells (intact)		0.25	6.8	28	0.39	40	103
J82 cells (sonicated)		0.038	55.4[a]	1.45[b]	0.160	87[a]	0.54[b]

[a] V_{max} (nM/min). [b] V_{max}/K_m (min^{-1}).

faces, experiments were performed to obtain kinetic parameters for factor IX and factor X activation by cell-bound factor VIIa. Previous studies by us (Sakai et al., 1989, 1990), as well as other laboratories (Fair & MacDonald, 1987; Drake et al., 1989), demonstrated that the human bladder carcinoma cell line J82 expresses abundant functional cell-surface tissue factor. In addition, lipopolysaccharide endotoxin (LPS) readily induces de novo synthesis and cell-surface expression of tissue factor in human endothelial cells (Lyberg et al., 1983; Noguchi et al., 1989). Accordingly, in separate experiments, we determined kinetic constants for the activation of factors IX and X on monolayers of J82 cells of LPS-stimulated human umbilical vein endothelial cells, each previously saturated with factor VIIa in the presence of 5 mM calcium. In control experiments, the activation of factors IX and X by cell-bound factor VIIa on LPS-stimulated endothelial cells was shown to be completely dependent on cell-surface tissue factor as pretreatment of the cells with anti-tissue factor apoprotein IgG inhibited these reactions in a dose-dependent manner (Figure 5). Previous studies demonstrated that the activation of factor X by J82 cell-bound factor VIIa was completely blocked by anti-tissue factor IgG (Sakai et al., 1989). Kinetic parameters for the activation of factor IX and factor X by endothelial cell-bound factor VIIa were obtained from linear Hanes–Woolf plots. The apparent K_m values were determined to be 24.24 ± 18.4 nM factor IX and 240.7 ± 73.4 nM factor X. Assuming 5×10^4 tissue factor apoprotein molecules per stimulated endothelial cell, and a 50% factor VIIa internalization rate after 2 h of incubation at 37 °C (Noguchi et al., 1989), a factor VIIa–tissue factor concentration of 18 pM was used to calculate apparent k_{cat} values of 32.39 ± 13.67 and 103.2 ± 18.8 min^{-1} for factor IX and factor X activation, respectively. The calculated k_{cat}/K_m value for factor IX activation (1349 μM^{-1} min^{-1}) was approximately 3-fold greater than that calculated for factor X activation (428 μM^{-1} min^{-1}). In contrast to stimulated endothelial cells, factor VIIa bound to monolayers of a human bladder carcinoma cell line activated factor X more efficiently than factor IX. K_m values for factor IX and factor X were 248 ± 50 and 391 ± 48 nM, respectively. Assuming 3×10^5 tissue factor molecules per J82 cell and no factor VIIa internalization (Sakai et al., 1989), a factor VIIa–tissue factor complex concentration of 300 pM was used to obtain k_{cat} values of 6.83 ± 0.42 and 40.2 ± 1.53 s^{-1} for factor IX and factor X activation, respectively. Thus, k_{cat}/K_m values for the activation of factor X by J82 cell-bound factor VIIa (103 μM^{-1} min^{-1}) were approximately 4-fold greater than the k_{cat}/K_m value for factor IX activation (28 μM^{-1} min^{-1}). Lysis of J82 cells by sonication decreased the apparent K_m for factor IX and factor X activation by factor VIIa approximately 7-fold and 2-fold, respectively. At a fixed factor VIIa concentration (10 nM), lysed J82 cells activated factor X at a

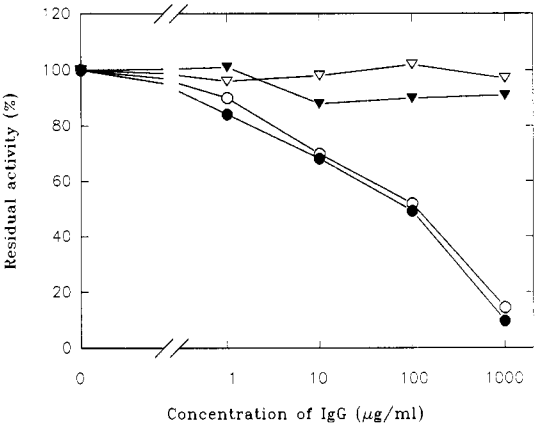

FIGURE 5: Effect on polyclonal anti-human brain tissue factor apoprotein IgG on the activation of factors IX and X by factor VIIa and LPS-stimulated human umbilical vein endothelial cells. Stimulated endothelial cells were pretreated with various concentrations of either anti-tissue factor apoprotein IgG (○, ●) or preimmune rabbit IgG (△, ▲) for 1 h at 37 °C. Factor VIIa (20 nM) was offered to the cells and incubated at 37 °C for 2 h, the cells were washed, and factor IX (○, ▽) and factor Xa (●, ▼) were assayed as described under Materials and Methods.

faster rate than that observed for factor IX (V_{max} = 87 nM Xa/min vs. 55 nM IXa/min). However, V_{max}/K_m ratio values indicated that factor IX was the kinetically preferred substrate for factor VIIa using sonicated J82 cells as the source of tissue factor.

Table I summarizes the kinetic data obtained at selected concentrations of phospholipid and calcium, relipidated tissue factor and calcium and cell-surface tissue factor and calcium. The relipidated tissue factor concentrations presented in Table I were chosen for comparison with concentrations of cell-surface tissue factor on endothelial cells (18 pM) and J82 cells (300 pM). The activation of factor IX was augmented maximally ~49 000-fold by tissue factor apoprotein (300 pM relipidated tissue factor vs 1.4 μM phospholipids) while the activation of factor X was enhanced ~74 000-fold by tissue factor apoprotein (9.4 pM relipidated tissue factor vs 1.4 μM phospholipids). Factor VIIa bound to endotoxin-stimulated endothelial cells activated factor IX more efficiently than factor X, consistent with kinetic data obtained with relipidated tissue factor in fluid phase. In contrast, kinetic data obtained with J82 cells as a source of functional tissue factor were not consistent with those obtained in systems containing relipidated tissue factor apoprotein at comparable concentrations, i.e., 300 pM tissue factor apoprotein. On J82 cell monolayers, k_{cat}/K_m values indicated that the preferred substrate for factor VIIa–tissue factor was factor X. However, when sonicated J82 cells were used as the source of tissue factor in the presence of calcium ions, V_{max}/K_m ratios suggested that factor IX was the

preferred substrate for factor VIIa–tissue factor (Table I).

Discussion

Several earlier studies have demonstrated that blood coagulation factor VIIa, in the presence of tissue factor and calcium, activates both factor IX and factor X by limited proteolysis. The aim of the present work was to gain further insight into the relative physiological importance of each factor VIIa catalyzed reaction through a comparative kinetic analysis of these reactions under a variety of cofactor conditions. In this study, we have separately examined the activation of factors IX and X by recombinant human factor VIIa as a function of calcium concentration, various phospholipid (purified PC/PS vesicles and mixed brain phospholipid vesicles) concentrations at 5 mM calcium, relipidated human brain tissue factor apoprotein/5 mM calcium, and cell-surface tissue factor/5 mM calcium. Assuming that the highest k_{cat}/K_m value reflects the preferred substrate for factor VIIa (Segel, 1976), our data provide the first kinetic evidence which suggests that the preferred physiological substrate for factor VIIa–tissue factor is factor IX. Initial experiments demonstrated that factors IX and X were each activated by factor VIIa in the absence of phospholipids or tissue factor at physiological levels of calcium (1–2 mM). In the presence of 2.5 mM calcium, factor VIIa activated factor IX approximately 10-fold more efficiently than factor X. In addition, the activation of factors IX and X by factor VIIa progressively increased with increasing calcium concentration (0–3 mM) in the presence of 25 μM PC/PS vesicles. As with calcium alone, the rate of factor IX activation by factor VIIa in the presence of a fixed phospholipid concentration was significantly greater than that observed for factor X at all calcium concentrations studied (0–5 mM). When the phospholipid concentration was varied at a fixed calcium concentration (5 mM), the K_m for factor IX decreased approximately 300-fold and remained essentially constant, while the K_m for factor X varied and exhibited a minimum value between 7 and 20 μM PC/PS. The latter findings do not agree with those recently published for the activation of factor X by factor VIIa in the presence of phospholipids (PC/PS; 50:50) and 10 mM calcium (Bom & Bertina, 1990). In that study, K_m values progressively increased between 10 and 100 μM phospholipids and ranged from 59 to 290 nM factor X, values significantly lower than those observed under our conditions (0.12–4.6 μM factor X). The reason(s) for this discrepancy is (are) unknown, but may be related to the phospholipid composition and calcium concentration used. Consistent with the data of Bom and Bertina (1990), we also observed a progressive increase in the k_{cat} for factor X activation by factor VIIa/calcium as phospholipid was varied from 1 to 200 μM. Plotting k_{cat}/K_m values as a function of phospholipid concentration revealed that factor IX was the preferred substrate for factor VIIa at all phospholipid concentrations examined. Similar results were obtained with mixed brain phospholipid vesicles, although higher final concentrations of phospholipids were required to achieve the same effect as seen with defined concentrations of PC/PS vesicles. Presumably, this reflects a lower concentration of phosphatidylserine in brain phospholipids (White, 1973) relative to that used in PC/PS (70:30) vesicles.

In the presence of calcium ions, relipidated tissue factor apoprotein enhanced the rate of factor IX activation 5000–10 000-fold, and factor X activation 1000–10 000-fold, over that observed with 2–200 μM phospholipid (PC/PS) in the presence of calcium. In these studies, equimolar concentrations of factor VIIa and tissue factor apoprotein were employed between 9 and 600 pM factor VIIa–tissue factor. At all concentrations of factor VIIa–tissue factor, k_{cat} values for factor X activation were significantly higher than that observed for factor IX activation. However, at each factor VIIa–tissue factor concentration, k_{cat}/K_m values for factor IX activation were greater than that computed for factor X activation. These results using a soluble tissue factor particle were also observed with a complex of factor VIIa and LPS-stimulated endothelial cell-surface tissue factor, inasmuch as the k_{cat}/K_m ratio for factor IX activation was ~3-fold greater than the k_{cat}/K_m ratio obtained for factor X activation. In contrast, a complex of factor VIIa and J82 cell-surface tissue factor exhibited a kinetic preference for factor X activation using intact J82 monolayers. Following J82 cell lysis, however, factor VIIa exhibited a kinetic preference for factor IX. The mechanism(s) responsible for the change in kinetic preference in intact J82 cells vs lysed J82 cells is (are) unknown. Drake and co-workers have previously demonstrated that virtually all J82 cell tissue factor apoprotein antigen is located on the cell surface with minor amounts of intracellular tissue factor apoprotein in a perinuclear granular distribution (Drake et al., 1989). Lysis of J82 cells greatly increased the apparent tissue factor activity (Sakai et al., 1989; Drake et al., 1989) which was taken as presumptive evidence for the release of phosphatidylserine from the inner leaflet of the lipid bilayer membrane and the formation of a relipidated tissue factor particle functionally distinct from that found on the intact cell surface (Drake et al., 1989). Conceivably, the composition of J82 cell-surface phospholipids surrounding the tissue factor apoprotein is distinct from that surrounding tissue factor apoprotein on endotoxin-stimulated unbilical vein endothelial cells, and, accordingly, these cell-surface phospholipids may play an important role in the kinetic specificity of the factor VIIa–tissue factor complex.

Our data appear relevant not only insofar as elucidating mechanisms of normal hemostasis but also in providing insight as to why hemophiliacs fail to adequately initiate and propagate blood coagulation through the classical extrinsic pathway. In this regard, it is noteworthy that, with the exception of the intact J82 cells, the K_m values for factor IX were significantly less than its concentration in plasma (~80 nM) at all phospholipid and tissue factor concentrations examined in this study, while K_m values for factor X were greatly above factor X plasma levels (~125 nM). Our data would argue that the activation of factor IX by factor VIIa–tissue factor occurs at an appreciable rate in normal individuals. Factor IXa, in turn, activates factor X in the presence of its cofactor, factor VIIIa, and an appropriate phospholipid membrane, and competes with factor VIIa–tissue factor for available substrate. In the absence of functional factor IX (or factor VIII), considerably less factor Xa is formed by factor VIIa–tissue factor than in the presence of these two factors (Osterud & Rapaport, 1980; Marlar et al., 1983; Stern et al., 1985). Furthermore, we have shown in separate experiments using purified proteins that recombinant human extrinsic pathway inhibitor, at its plasma concentration, inhibits the factor VIIa–tissue factor mediated activation of factor X ~90% in the absence of factors IX and VIII (Pedersen et al., 1990). However, in the presence of plasma levels of factor IX and factor VIII, approximately 10-fold higher levels of extrinsic pathway inhibitor are required to achieve this effect in the test tube (Pedersen et al., 1990). Thus, our present kinetic data, coupled with the inability of plasma levels of extrinsic pathway inhibitor to completely block factor VIIa–tissue factor activity in the presence of factors IX and VIII, strongly support the existence of an alternative, if not essential, extrinsic pathway of blood

coagulation that requires the participation of functional factor IX and factor VIII.

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Registry No. Ca, 7440-70-2; factor VIIa, 65312-43-8; factor IX, 9001-28-9; factor X, 9001-29-0; tissue thromboplastin, 9035-58-9.

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