Zymogen Factor IX Potentiates Factor IXa-Catalyzed Factor X Activation†

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Received February 2, 2000; Revised Manuscript Received May 18, 2000

ABSTRACT: Intrinsic factor X activation is accelerated $>10^7$-fold by assembly of the entire complex on the activated platelet surface. We have now observed that increasing the concentration of zymogen factor IX to physiologic levels (~100 nM) potentiates factor IXa-catalyzed activation of factor X on both activated platelets and on negatively charged phospholipid vesicles. In the presence and absence of factor VIIIa, factor IX (100 nM) lowered the $K_{d,appFIXa}$ ~4-fold on platelets and 2−10-fold on lipid vesicles. Treatment of two factor IX preparations with active-site inhibitors did not affect these observations. Autoradiographs of PAGE-separated reactions containing either ^{125}I-labeled factor IX or ^{125}I-labeled factor X showed that the increased factor X activation was not due to factor Xa-mediated feedback activation of factor IX and that there was increased cleavage of factor X heavy chain in the presence of factor IX in comparison with control reactions but only in the presence of both the enzyme and the surface. Since plasma concentrations of prothrombin, factor VII, protein C, or protein S did not by themselves potentiate factor Xa generation and did not interfere with the potentiation of the reaction of factor IX, the effect is specific for factor IX and is not attributable to the Gla domain of all vitamin K-dependent proteins. These observations indicate that under physiologic conditions, plasma levels of the zymogen factor IX specifically increase the affinity of factor IXa for the intrinsic factor X activation complex.

Enzymatic reactions, including those involved in hemostasis, are usually investigated using simplified in vitro systems to justify basic Michaelis−Menten analysis of the data. However, the complexities of the hemostatic enzyme cascade include not only cellular and cell matrix surface elements but also hemodynamics supplying elements that positively and negatively help to balance pro- and anticoagulant forces in the process of ensuring a closed but continually fluid transport and delivery system. Surface-supported catalysis has been studied by adapting Michaelis−Menten principles, although surface as a fourth element in a complex containing enzyme, substrate, and cofactor introduces two-dimensionality in which simple Michaelis−Menten diffusion-dependent complex formation is complicated by many possible surface binding events. Indeed, a single surface in vivo can support many simultaneous enzymatic reactions producing products used in series on neighboring binding sites. Resident molecules produced on or bound by the surface can provide modulatory elements that positively or negatively affect neighboring bound complexes. This paper identifies one such modulating element.

While studying the effect of surface on the activation of factor X by factor IXa, it had been determined that factor IX binds to only half the number of sites on activated platelets as factor IXa and that factor IX competes for only half the sites occupied by factor IXa; whereas, factor IXa competes for all the sites occupied by factor IX (*1*). Also, an N-terminal peptide of the Gla-domain[1] of factor IX competes for only one-half the factor IXa binding sites, at a K_i of 3.5 nM (*2*) but competes for all the platelet binding sites of factor IX. These observations led to the hypothesis that although factor IX and factor IXa both bind to a site on activated platelets determined by residues 5−7 and 9−11 within the Gla-domain of both proteins (*3*), there is an additional binding site on activated platelets that is specific for factor IXa (*2*). Moreover, in functional assays, it was determined that an excess of factor IX does not inhibit the activation of factor X by the intrinsic complex assembled on the activated platelet surface, leading to the conclusion that these additional factor IXa sites on activated platelets are specific for the functional activity of the enzyme factor IXa (*2*).

To pursue this observation in more detail, factor IX was titrated into a factor IXa-catalyzed factor X activation assay and was found not to inhibit the enzymatic cleavage of factor X but to potentiate it at physiologic concentrations of

† This study was supported by research grants from the National Institutes of Health: HL56914, HL56153, and HL46213 (to PNW) and from the American Heart Association: #9951297U (to F.S.L.).

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S2765, chromogenic substrate; *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide-dihydrochloride; PMSF, phenylmethyl sulfonyl fluoride; EGR-CK, Glu−Gly−Arg-chloromethyl ketone; PPACK, Phe−Pro−Arg-chloromethyl ketone; ATIII, antithrombin III.

10.1021/bi000245o CCC: $19.00
Published on Web 07/20/2000

zymogen. This potentiation was observed in either the presence or the absence of the cofactor, factor VIII, and was absolutely dependent on the presence of surface, either activated platelets or negatively charged phospholipid vesicles, implying that it does not require surface protein. These studies were extended kinetically, and the potentiation of the enzyme by the zymogen was attributed to its ability to lower the $K_{d,appFIXa}$ for the surface-dependent reaction. This result suggests that in the vasculature where only minute fractions of zymogen are converted to active enzyme, the remaining zymogen contributes to a more efficient utilization of low enzyme concentrations. This conclusion was supported by specificity studies that determined that plasma concentrations of other vitamin K-dependent coagulation proteins neither mimicked the effect of factor IX nor interfered with it.

EXPERIMENTAL PROCEDURES

Proteins. Factors VII, IX, X, XIa, prothrombin, protein S, and protein C were obtained either from Enzyme Research Laboratories (South Bend, IN) or from Hematologic Technologies, Inc. (VT) dissolved in 20 mM Tris, 150 mM NaCl, with or without 1mM benzamidine. Proteins were dialyzed free of benzamidine. Purity was assessed by SDS−PAGE, and protein concentration was confirmed by bicinchoninic acid assay from Pierce Chemical Co. (Rockford, IL). Factor IX was activated to factor IXa by incubation with factor XIa at a 50−100:1 molar ratio in 20 mM Tris and 150 mM NaCl, pH 7.8, containing 5 mM $CaCl_2$ at 37 °C for 0.5 h, as described previously (*1*), quick-frozen in 5−10 μL aliquots, and stored at −80 °C for no more than 30 days. Completion of activation was assessed by SDS−PAGE. Factor X was adsorbed with soybean trypsin inhibitor coupled to Affigel-15 beads (Biorad Laboratories Inc., Hercules, CA) to remove any contaminating factor Xa. Recombinant factor VIII (FVIII) was kindly provided by Baxter Healthcare Corp. (Duarte, CA), stored in 10 mM HEPES, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 6.5, and activity was determined by a one-stage clotting assay (*4*). Human α-thrombin (Sigma Chemical Co., St. Louis, MO) was stored at 200 U/mL at −80 °C. The thrombin receptor hexapeptide SFLLRN-amide was synthesized using [(9-fluorenyl)methoxy]-carbonyl (FMOC) chemistry on an Applied Biosystems 430A synthesizer, and by reverse phase HPLC was purified to >99.9% homogeneity. All proteins were stored in small aliquots at −80 °C and thawed only once before use.

Recombinant Factor IX. Human wt-factor IX was expressed in human embryo kidney cells as described previously (*5*) and purified from cell supernatants by calcium gradient elution (*6*). Protein was characterized by Gla analysis (*7*) and compared with plasma-derived factor IX for rate of activation by factor XIa and resulting enzymatic activity. Coomasie Blue staining of SDS−PAGE-separated wt-factor IX showed a single band at 58 kDa.

Platelet Preparation. Washed, gel-filtered platelets were prepared as described previously (*8*) and resuspended in a 15 mM HEPES-buffered Tyrodes solution (0.13 M NaCl, 3 mM KCl, 1 mM MgCl, and 0.4 mM monosodium phosphate) (buffer A), pH 7.2, with bovine serum albumin (BSA) (1 mg/mL).

1:3 PS/PC Phospholipid Vesicles. Large unilamellar vesicles of porcine brain phosphatidylserine (PS) and L-α-dioleoyl-phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, AL), mixed in a molar ratio of 1:3 in buffer A were prepared by extrusion as described previously (*8*). Each lipid preparation was titrated into the factor Xa generation assay for selection of a concentration of lipid that supported factor Xa generation in the lower linear portion of the titration curve. For lipid-supported assays performed in the presence of factor VIIIa, a concentration of lipid was chosen that produced approximately equal amounts of factor Xa as when activated platelets were used at 5×10^7/mL.

Factor Xa Generation Assay. Factor Xa generation was assayed as described previously (*8*). All proteins were diluted in 50 mM HEPPS and 175 mM NaCl, pH 8.1 (buffer B). For assay in the absence of factor VIIIa, platelets or lipid vesicles in buffer A containing 1 mg/mL BSA were combined with factor IX or buffer B, factor IXa at various concentrations, and $CaCl_2$ (5 mM) in microtiter wells. The plate was prewarmed at 37 °C before a further 5 min incubation with thrombin receptor peptide (5 μM), and the reaction was begun by addition of factor X. After incubation for 20 min, the reaction was stopped by addition of EDTA (10 mM) and removal onto ice. For assay in the presence of factor VIIIa, the factor VIII was added to the wells containing the surface, factor IXa and factor IX (and/or other Gla-containing protein) or buffer. Platelets were activated by thrombin receptor peptide at 5 μM for 4 min, before addition of thrombin at 0.1 U/mL for 1 min to activate the factor VIII. The reaction was begun by addition of factor X and stopped after 1 or 2 min by addition of EDTA and removal of the plate as above. Fluid phase reactions were performed in the absence of factor VIIIa, substituting buffer for a surface. Reactions were proven to be linear with respect to product formation under these conditions.

The factor Xa generated in all reaction mixtures was assayed for its rate of hydrolysis of chromogenic substrate *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide-dihydrochloride (S2765) (AB Kabi Diagnostica, Stockholm, Sweden). Aliquots diluted into buffer B were prewarmed before addition of substrate to 300 μM, and the hydrolysis was followed kinetically on a ThermoMax microplate reader (Molecular Devices Corp., Menlo Park, CA) using the manufacturer's Softmax software. Reactions were assayed in duplicate at dilutions that assured initial rates of chromogenic substrate hydrolysis, and the rates of hydrolysis were converted to factor Xa concentration by comparison to a standard curve resulting from hydrolysis of S2765 by known concentrations of factor X fully activated by Russell's Viper Venom (Sigma Chemical Co., St. Louis, MO). Data were collected into an Excel spreadsheet (Microsoft Corp., Redmond, WA) where velocity results were converted to nanomolar factor Xa generated per minute. Fluid phase factor X hydrolysis was subtracted from total platelet- or phospholipid-potentiated factor X activation performed in the absence of factor VIIIa and constituted no more than 10% of the total. Data from three to seven experiments were averaged for each analysis.

With the exception of Figures 1 and 6, where curves were interpolated through data points, titration curves were generated by KaleidaGraph (Synergy Software, PCS Inc., Reading, PA) run on a Macintosh Quadra 900 (Apple Corp., Cupertino, CA), using a nonlinear least-squares fit of data points to the following equation: $y = ax/(b + x)$.

Specificity Studies. Factor IXa titrations were performed (i) in the presence and absence of either 100 nM factor IX or 1 μM prothrombin, 72 nM protein C, 145 nM protein S, or 10 nM factor VII, or (ii) in the presence of 100 nM factor IX in combination with one of the other Gla-containing proteins at the above-mentioned concentrations. Reaction mixtures were set up as described above, and results of S2765 cleavage assays were analyzed by KaleidaGraph titration curves. Reactions containing prothrombin also contained hirudin 5−8 U/mL to neutralize any thrombin formed by factor Xa. Kinetic parameters generated were compared for the effect of additions.

Treatment of Zymogen Preparations with Protease Inhibitors. Different preparations of plasma-derived factor IX were treated with protease inhibitors to inactivate any factor IXa or other contaminating serine proteases present. Twenty nanomolar factor IX was incubated with 1 mM phenylmethyl sulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) or with Glu−Gly−Arg-chloromethyl ketone (EGR-CK) (Calbiochem-Novabiochem, La Jolla, CA) 4 h at 37 °C, then overnight at 4 °C, or with Phe−Pro−Arg-chloromethyl ketone (PPACK) (Calbiochem-Novabiochem, La Jolla, CA) for 1 h prior to use in kinetic assays as described above. A factor IXa preparation, identically treated with EGR-CK and retaining no residual enzymatic activity, served as a control in factor IX and factor IXa titrations.

Radiolabeling Factor IX and Factor X. Proteins were iodinated by the Iodogen (Pierce Chemical Co., Rockford, IL) method as reported previously (*9*). Specific activity was determined from CPM/vol and from protein concentration and was found to be in the range of $2-2.5 \times 10^6$ cpm/μg. SDS−PAGE of iodinated factor X showed a single heavy band at 58 kDa when electrophoresed under nonreducing conditions and a heavy chain and a light chain at 28 and 18 kDa, respectively, when electrophoresed under reducing conditions. SDS−PAGE of iodinated factor IX under reducing conditions showed minor bands (constituting <0.5% of total cpm) as well as the predominant band at 58 kDa. A polyclonal antibody against factor IX used in a Western blot of iodinated protein recognized all minor bands, as well as the major band.

Following the Enzyme Reaction Radiometrically. Radioiodinated factor X or factor IX were included in reaction mixtures to visualize factor X heavy chain cleavage in the presence and absence of factor IX or to examine the possibility of feedback activation of factor IX. Enzyme reactions were initiated and sampled at 1, 2, 3, and 5 min into reducing electrophoresis sample buffer kept on ice. These samples were boiled, electrophoresed on 13% polyacrylamide SDS gels that were then dried, and visualized by autoradiography on X-ray film (Dupont-NEN, Wilmington, DE).

Active-Site Titration using Antithrombin III (ATIII). Factor IX preparations were analyzed for contaminating factor IXa by ATIII active-site titration as described in (*10*). Briefly, various concentrations of standardized ATIII were incubated for 20 min at 37 °C with 1 μM factor IX. The residual factor X activating capability of these reactions was determined by mixing them with factor VIIIa, phospholipid vesicles, and factor X, followed by assay of the S2765 cleavage activity generated. The linear response of S2765 cleavage rates plotted against ATIII concentration intersected with the abscissa at the factor IXa concentrations contained in the original factor IX preparation.

RESULTS

Factor IX Titrations. When factor IX was titrated into factor X activation mixtures containing a constant amount of factor IXa, roughly equal to the $K_{d,appFIXa}$ for the respective condition, factor Xa activity was gradually increased until a peak of 2−3-fold potentiation was achieved at 100−200 nM zymogen on activated platelets and at 25−125 nM zymogen on PS/PC vesicles (Figure 1). At greater than 200 nM factor IX, there was a gradual loss of potentiation with factor Xa generation returning to baseline levels at between 500 and 1000 nM factor IX addition. This general pattern was seen in the absence (Figure 1, panels c and d) or presence (Figure 1, panels a and b) of the cofactor factor VIIIa and was seen using either SFLLRN-activated platelets (Figure 1, panels a and c) or phosphatidylserine/phosphatidylcholine (PS/PC) vesicles (Figure 1, panels b and d) at a concentration that in the presence of factor VIIIa represented a similar concentration of binding sites as in platelet reactions (determined from lipid and platelet titration curves). Hence, it was concluded that the effect of factor IX must be either on the enzyme, on the substrate, or on the surface phospholipid of platelets or vesicles.

Higher concentrations of factor IX (400 nM) retained more potentiating ability on platelets in the presence of FVIIIa (Figure 1a) than under all other conditions. With PS/PC vesicles in the absence of factor VIIIa (Figure 1d), where two titrations were carried out past 1 μM, there was eventual inhibition of factor X activation. However, at 100 nM, an approximately physiologic concentration, factor IX potentiated the reaction 2−3-fold under all conditions.

Effect of 100 nM Factor IX on Factor IXa Titrations. Factor IX (100 nM) was used to test its effect on kinetic parameters of factor IXa activation of factor X. When factor IXa titrations were performed in the presence and absence of 100 nM factor IX (Figure 2), the presence of factor IX lowered the $K_{d,appFIXa}$ for the complex 4−5-fold on activated platelets, in the presence or absence of factor VIIIa (Figure 2, panels a and c, respectively) and decreased it on PS/PC vesicles 8−10-fold in the presence of factor VIIIa (Figure 2b) and 20-fold in its absence (Figure 2d) (See Table 1).

Effect of 100 nM Factor IX on Factor X Titrations. If the presence of factor IX resulted in the formation of more factor IXa complexes for a given concentration of factor IXa added, then factor X titrations performed in the presence of factor IX should show an increase in both $K_{m,mapp}$ and in V_{max} (*11*). Factor X was titrated into factor VIIIa-containing reactions in the presence or absence of factor IX and reactions lacking the enzyme but containing factor IX were subtracted from those containing zymogen and enzyme together (Figure 3). The addition of factor IX resulted in a 70% higher V_{max} and $K_{m,mapp}$ on both activated platelets (Figure 3a) and on PS/PC vesicles (Figure 3b). The kinetic parameters calculated from fitted curves (see Table 1) suggested that zymogen led either to (i) greater catalytic efficiency for the same number of bound enzymatic complexes or (ii) that for a given added factor IXa concentration a larger number of factor IXa complexes assembled on both surfaces in the presence of factor IX than assembled in its absence.

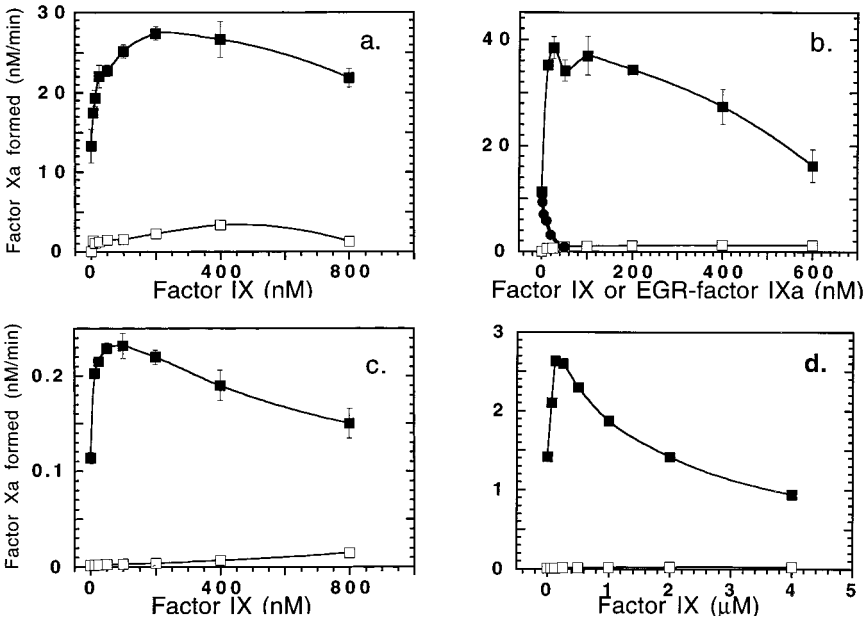

FIGURE 1: Titrating factor IX into factor-X activations. Factor IX at the indicated concentrations was added to incubations containing components of the factor X activating complex: panels a and b, in the presence of factor VIIIa (6 U/mL), panels c and d, in its absence. Factor Xa generated was assayed by cleavage of chromogenic substrate S2765, and activities were calculated by comparison to cleavage of substrate by reference factor Xa. (□) incubations without factor IXa; (■) the difference between incubations lacking factor IXa and those containing both factor IXa and factor IX; (●) titration of EGR-factor IXa into reactions containing factor IXa, from which reactions containing EGR-factor IXa alone have been subtracted. (a) Platelets 5×10^7/mL, factor IXa 1 nM or 0, factor X 200 nM; (b) phospholipid vesicles 1 μM, factor IXa 1 nM or 0, factor X 150 nM; (c) platelets 3e8/mL, factor IXa 3 nM or 0, factor X 200 nM; (d) phospholipid vesicles 25 μM, factor IXa 163 nM, factor X 200 nM. Curves were generated by nonlinear least-squares analysis of means ± SE derived from 3−5 experiments (panels a−c) or a representative experiment of two performed (panel d).

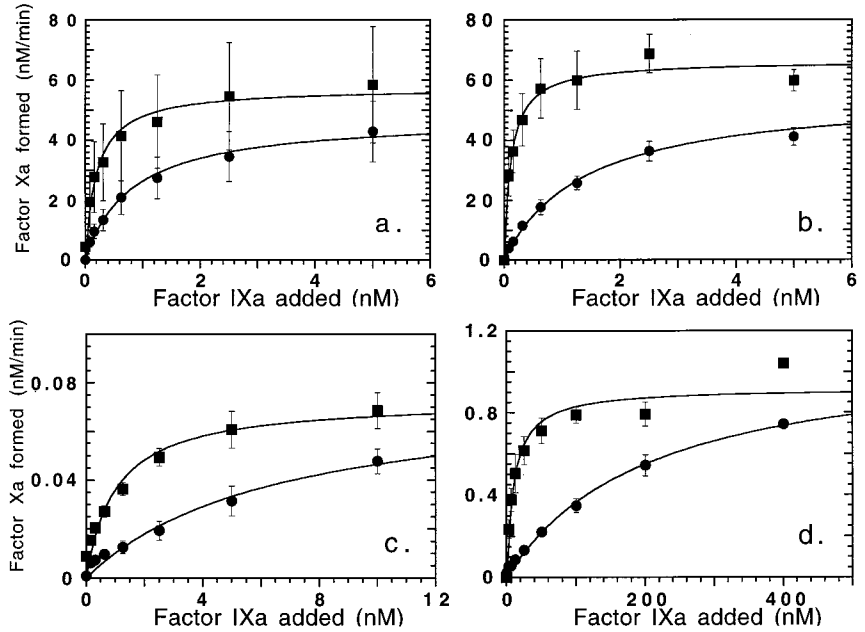

FIGURE 2: Effect of plasma levels of factor IX (100 nM) on factor IXa titrations. Factor IXa was titrated into factor X activations in the absence (●) or presence (■) of 100 nM factor IX, in the presence of thrombin-activated factor VIII (6 U/mL) (panels a and b) or in its absence (panels c and d). Reaction mixtures contained SFLLRN-activated platelets (panels a and c) or PS/PC vesicles (panels b and d). (a) 5×10^7 platelets/mL, 150 nM factor X; (b) 1 μM PS/PC, 150 nM factor X; (c) 3e8 platelets/mL, 200 nM factor X; (d) 10 μM PS/PC, 150 nM factor X. Reaction mixtures were assayed for factor Xa generation by chromogenic substrate cleavage. Curves were generated by nonlinear least-squares analysis of means ± SE derived from 4−7 experiments, from which kinetic parameters could be derived. Fluid phase factor X activation by factor IXa was subtracted from factor IXa titration data obtained in the absence of factor VIII.

Investigating the Nature of the Potentiation by the Factor IX Preparations. Since factor X titrations in the presence of 100 nM zymogen but in the absence of enzyme appeared to produce some factor X activation (Figure 3, open squares), a series of experiments was designed to determine whether (i) the S2765 cleavage assayed was attributable to increased factor Xa produced; (ii) the factor IX preparations contained sufficient activated species to account for the increase in factor X activation; and (iii) the factor Xa produced was capable of feedback activation of the excess factor IX present.

Radioiodinated factor X was added as substrate to factor X activating complexes assembled on PS/PC vesicles, and

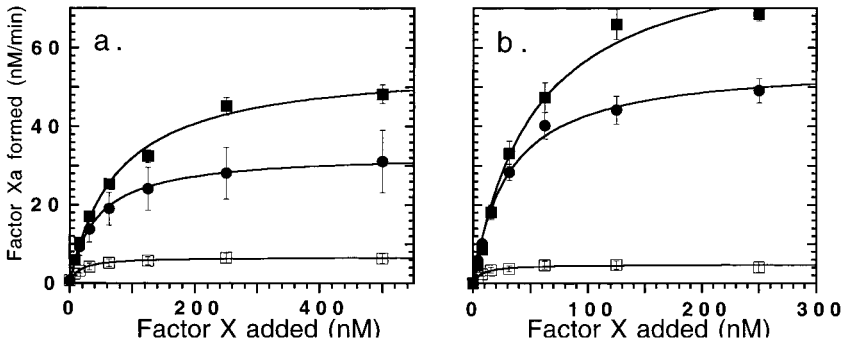

FIGURE 3: Effect of plasma levels of factor IX (100 nM) on factor X titrations. Factor X was titrated into factor X activations containing thrombin-activated factor VIII and either SFLLRN-activated platelets at 5×10^7/mL (a) or 1 μM PS/PC (b) and either factor IXa alone (●) (2 nM in panel a, 2.5 nM in panel b), 100 nM factor IX in place of factor IXa (□), or both (■). Reactions were assayed for factor Xa by chromogenic substrate cleavage compared to that of a standard factor Xa preparation. Data from 4−5 experiments were averaged and analyzed for standard error. Data from factor IX alone were subtracted from data obtained with both enzyme and zymogen, and nonlinear least-squares analysis was used to construct curves from which kinetic parameters could be derived.

Table 1: Effect of 100 nM Factor IX on Kinetic Parameters of Factor X Activation

	Kd,appFIXa (nM)[a]		V_{max} (nM/min)[a]	
factor IX (100 nM)	(−)	(+)	(−)	(+)
platelets + factor VIIIa	0.8 ± 0.1	0.20 ± 0.04	47.8 ± 2.1	57.6 ± 2.5
platelets − factor VIIIa	6.5 ± 2.2	1.0 ± 0.25	0.08 ± 0.01	0.11 ± 0.01
PS/PC + factor VIIIa	1.35 ± 0.1	0.12 ± 0.02	55.6 ± 1.7	66.3 ± 2.1
PS/PC − factor VIIIa	204 ± 30	10.7 ± 2.4	1.1 ± 0.09	0.92 ± 0.05
	$K_{m,app}$ (nM)[a]		V_{max} (nM/min)[a]	
factor IX (100 nM)	(−)	(+)	(−)	(+)
platelets + factor VIIIa	43.2 ± 2.9	75.1 ± 7.6	33.0 ± 0.6	55.9 ± 1.9
PS/PC + factor VIIIa	30.7 ± 2.8	52.9 ± 7.5	55.9 ± 1.6	87.1 ± 4.6

[a] Kinetic parameters were derived from fitted factor IXa titration data (Figure 2) and from fitted factor X titration data (Figure 3) using KaleidaGraph software.

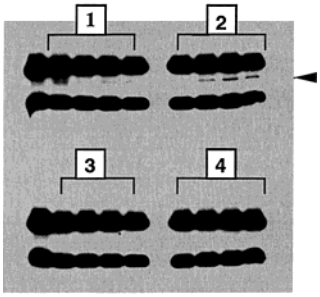

FIGURE 4: Visualizing the fate of substrate added to factor X activations. ^{125}I-labeled factor X was added as 150 nM substrate to reaction mixtures: (1) the complete mixture contained 1 μM PS/PC, 2 nM factor IXa, thrombin-activated factor VIII (6 U/mL); (2) contained 100 nM factor IX in the complete mixture; (3) duplicated reaction 2 without factor IXa; (4) duplicated reaction 2 without PS/PC vesicles. Incubations were sampled at 1, 2, 3, and 5 min into reducing buffer for electrophoresis on 13% polyacrylamide gels that were visualized by autoradiography. Lane 1 of each gel contained labeled factor X only. Arrow indicates cleaved heavy chain.

reactions were sampled at 1, 2, 3, and 5 min for electrophoresis and autoradiography to visualize the effect of incubation on substrate added. Results shown in Figure 4 revealed small amounts of cleaved heavy chain representing initial rates of factor X activation in complete mixtures incubated in the absence of factor IX (Figure 4, reaction 1) but more cleavage of factor X heavy chain in mixtures incubated in its presence (Figure 4, reaction 2). Either the absence of enzyme or the absence of surface resulted in no

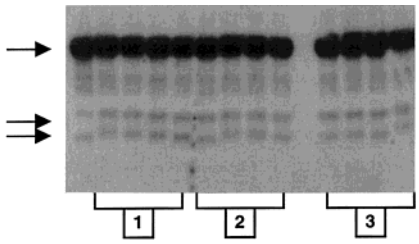

FIGURE 5: Visualizing the fate of factor IX added to factor X activations. ^{125}I-labeled factor IX was included in incubation mixtures that were sampled at 1 min (lanes 2, 6, and 10), 2 min (lanes 3, 7, and 11), 3 min (lanes 4, 8, and 12) and 5 min (lanes 5, 9, and 13), mixed with reducing buffer, and electrophoresed through 13% polyacrylamide gels that were visualized by autoradiography. Lanes 2−5 (reaction 1) represent the complete enzyme complex of 1 μM PS/PC, 2 nM factor IXa, thrombin-activated factor VIII (6 U/mL), and 200 nM factor X; lanes 6−9 (reaction 2) represent incubations lacking the enzyme; lanes 10−13 represent incubations lacking substrate factor X. Lane 1 contained radiolabeled factor IX. Arrows top to bottom represent single chain factor IX, factor IXa heavy chain, and light chain.

accumulation of cleaved heavy chain under these standard conditions used to calculate initial rates of activation (Figure 4, reactions 3 and 4, respectively). Thus, the increased S2765 cleavage appeared to result from increased factor X activation.

It was determined by ATIII active-site titrations performed on two plasma-derived factor IX preparations that there was 1.2 and 1.5% contamination with factor IXa active sites. If correct, this would mean that in 100 nM factor IX there was about 1.5 nM factor IXa present. Overexposed autoradiography of radioiodinated factor IX electrophoresed under reducing conditions revealed four minor bands in addition to the major overexposed band representing factor IX (Figure 5, lane 1). A Western blot performed on electrophoresed factor IX probed with a goat antihuman factor IX identified all bands as related to factor IX, and two bands comigrated with authentic heavy and light chains of factor IXa (data not shown). However, activity generated by the factor IX preparations in the absence of factor IXa (Figure 3, panels a and b) is not equivalent to 1−1.5 nM factor IXa, and this activity when subtracted from the factor X titration curves does not account for the potentiation seen (Figure 3, closed squares compared to Figure 3, closed circles). Moreover, if the results of factor IXa titrations potentiated by 100 nM zymogen are plotted combining 1.5 nM active sites with the

Table 2: Treatment of Factor IX with Protease Inhibitors Does Not Remove Its Potentiating Effect on Factor X Activation[a]

conditions	$K_{d,appFIXa}$ (nM)	V_{max} (% of control)[b]
factor IXa only	0.92	100
(+) factor IX	0.29	128
(+) PMS-factor IX	0.32	178
(+) PPA-factor IX	0.18	110
(+) EGR-factor IX	0.44	101
(+) rwt-factor IX	0.32	86
(+) EGR−factor IXa	2.83	91[c]

[a] Preparations of plasma-derived factor IX both untreated and treated with protease inhibitors (see Experimental Procedures) were compared at 100 nM with recombinant wild-type factor IX at 100 nM or with 10 nM EGR-factor IXa, as additions to factor IXa titrations containing PS/PC 1 μM, thrombin-activated factor VIII (6 U/mL), and 150 nM factor X. Chromogenic substrate cleavage data were plotted and analyzed by KaleidaGraph software for kinetic parameters presented here. [b] 100% represents 44.4 nM/min. [c] 100% represents 30 nM/min.

factor IXa added, the two curves still do not superimpose (data not shown).

Radioiodinated factor IX was included in factor X activating reactions assembled on PS/PC vesicles, and reactions were sampled at 1, 2, 3, and 5 min for electrophoresis and autoradiography to determine whether the factor Xa formed by factor IXa activity could be activating some of the zymogen preparation added. Results shown in Figure 5 show the minor bands present in the preparation before incubation with the factor X activation mixture (lane 1) but show no additional accumulation of factor IXa heavy and light chains over the 5 min incubation in the complete mixture (Figure 5, lane 2−5) or in mixtures lacking the enzyme (Figure 5, lanes 6−9) or the substrate (Figure 5, lanes 10−13).

Treatment of the Factor IX Preparation with Protease Inhibitors. To rule out contamination of the plasma-derived factor IX with proteases that could be responsible for the additional factor Xa produced in reactions containing zymogen and to inactivate the trace amounts of factor IXa detected, the factor IX was treated with various active-site serine protease inhibitors. Either EGR-CK or PMSF was incubated with factor IX for 4 h at 37 °C, then overnight at 4 °C, or PPACK was incubated with factor IX for 1 h at 37 °C, before inclusion in factor IXa titrations for comparison with the effect of untreated factor IX and with rwt-factor IX on the $K_{d,appFIXa}$ in factor X activations. Results presented in Table 2 show that rwt-factor IX, prepared entirely differently than plasma-derived factor IX and appearing on reducing gels as a single band (data not shown), lowers the $K_{d,appFIXa}$ to the same extent as untreated plasma-derived factor IX. Additionally, treatment of factor IX with protease inhibitors does not change its ability to reduce the $K_{d,appFIXa}$. EGR-factor IXa, prepared by treatment of factor IXa with EGR-CK exactly as described for factor IX under Experimental Procedures, increased the $K_{d,appFIXa}$ when included in a factor IXa titration, consistent with competition for binding sites on the surface (Table 2). Titrating EGR-factor IXa into factor Xa generation reactions resulted in exponential inhibition of product formation in contrast to the potentiation seen with EGR-factor IX (Figure 1b).

Binding Site Concentration. Since the potentiating effect of factor IX appeared to be surface-dependent, we investigated the role of binding site availability on factor IX potentiation of factor IXa-catalyzed factor X activation. PS/PC vesicles were titrated into factor Xa generation reactions using three different concentrations of factor IXa and in the presence and absence of 100 nM factor IX (Figure 6). With 100 pM factor IXa (Figure 6a), factor Xa generation increased with increasing phospholipid binding sites up to a peak at 5 μM lipid, then decreased at higher phospholipid in both the absence and presence of factor IX. At higher concentrations of factor IXa, 250 and 625 pM (Figures 6, panels b and c), in the absence of factor IX, the reaction remained saturated above 5 μM phospholipid until 100 μM at which concentration there was evidence of inhibition. The presence of factor IX not only increased the reaction at all phospholipid concentrations but also protected against inhibition at high phospholipid, especially with 625 pM enzyme. Furthermore, the potentiating effect of factor IX was most evident at 100 pM enzyme where there was a 10-fold increase in product formed at 5 μM phospholipid. As the enzyme concentration was increased, the potentiating effect of 100 nM factor IX was less at all phospholipid concentrations, as predicted by factor IXa titration curves. Factor IXa titration curves performed with three different phospholipid concentrations: 1, 20, and 100 μM confirmed the ability of factor IX to lower the $K_{d,appFIXa}$ even at excess phospholipid binding sites (data not shown).

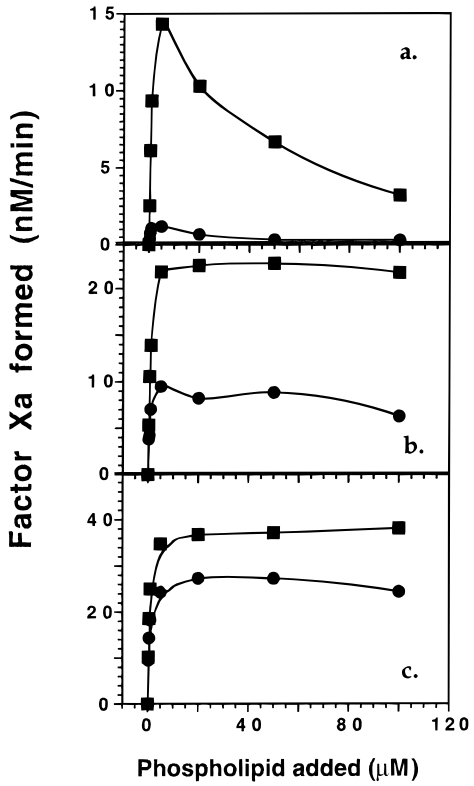

FIGURE 6: Effect of binding site concentration on factor IX potentiation of factor IXa-catalyzed factor X activation. PS/PC (25:75) vesicles were titrated into factor Xa generation mixtures containing factor VIIIa (5.5 U/mL), factor X (150 nM), and either 0 (●) or 100 nM (■) factor IX, in the presence of factor IXa at either 100 (a), 250 (b), or 625 pM (c). Factor Xa formed, assayed by cleavage of chromogenic substrate S2765, was plotted against phospholipid concentration with curves generated by interpolation through data points by KaleidaGraph. Data are representative of two sets of similar data collected.

Specificity of the Zymogen Effect. Since the observed effect of the factor IX preparations appears to be attributable to the zymogen factor IX, factor IX was compared with other

Table 3: Specificity of the Factor IX Effect on Factor IXa-Catalyzed Factor Xa Generation[a]

platelets		$K_{d,app}$ FIXa (nM)		V_{max} (nM/min)	
protein added	nM	FIX (−)	FIX (+)	FIX (−)	FIX (+)
buffer		1.34 ± 0.11		20.4 ±0.54	
factor IX	100		0.35 ± 0.03		20.3 ± 0.48
protein C	72	1.27 ± 0.11	0.39 ± 0.06	24.4 ± 0.86	23.2 ± 1.01
protein S	145	1.29 ± 0.10	0.44 ± 0.03	17.2 ± 0.54	18.1 ± 0.39
factor VII	10	0.99 ± 0.10	0.70 ± 0.17	21.2 ± 0.78	23.4 ± 1.87
prothrombin	1000	0.99 ± 0.11	0.18 ± 0.03	14.8 ± 0.62	10.5 ± 0.18
PS/PC (25:75)		$K_{d,app}$ FIXa (nM)		V_{max} (nM/min)	
protein added	nM	FIX (−)	FIX (+)	FIX (−)	FIX (+)
buffer		0.76 ± 0.06		59.2 ± 1.57	
factor IX	100		0.17 ± 0.01		56.7 ± 0.67
protein C	72	0.72 ± 0.05	0.33 ± 0.03	61.6 ± 1.50	65.4 ± 1.44
protein S	145	1.05 ± 0.05	0.38 ± 0.02	49.7 ± 0.92	58.8 ± 0.89
factor VII	10	0.57 ± 0.02	0.24 ± 0.02	51.0 ± 0.44	65.1 ± 1.28
prothrombin	1000	2.18 ± 0.09	0.49 ± 0.04	47.3 ± 0.91	56.0 ± 1.42

[a] Factor IXa titrations containing thrombin-activated factor VIII (6 U/mL) and either SFLLRN-activated platelets (5×10^7/mL) and 200 nM factor X or 0.5 μM PS/PC and 150 nM factor X were performed in the presence and absence of each plasma-derived Gla-containing coagulation factor or in the presence of both factor IX and each Gla-containing coagulation factor. Kinetic parameters derived from KaleidaGraph fitting of factor Xa generation data represent the means of 3−7 experiments.

vitamin K-dependent coagulation proteins to determine whether the potentiation seen is attributable to a nonspecific effect of the Gla-domain on the surface. Factor IX (100 nM) was compared with protein C (72 nM), protein S (145 nM), factor VII (10 nM), and prothrombin (1 μM) for an effect on the $K_{d,appFIXa}$ calculated from factor IXa titrations performed in the presence of excess factor VIIIa on either SFLLRN-activated platelets or on PS/PC vesicles. Hirudin (5−8 U/mL) was added to reactions containing prothrombin to avoid cleavage of chromogenic substrate by any thrombin generated. As shown in Table 3, whereas factor IX lowered the $K_{d,appFIXa}$ ~4-fold on both surfaces, neither protein C, protein S, nor factor VII had a similar effect, and prothrombin raised the $K_{d,appFIXa}$ approximately 2-fold on phospholipid vesicles. When protein C (72 nM) or protein S (145 nM) or factor VII (10 nM) or prothrombin (1 μM) was added to factor IXa titrations containing factor IX (100 nM), they were found not to interfere with the ability of zymogen to potentiate the reaction by lowering the $K_{d,appFIXa}$. On lipid vesicles, factor IX obviated the increase in the $K_{d,appFIXa}$ observed in the presence of prothrombin alone. These results suggest that factor IX specifically lowers the $K_{d,appFIXa}$ by a mechanism independent of general Gla-domain induced conformations.

DISCUSSION

These results indicate that factor IX potentiates activation of factor X by small quantities of factor IXa. The consequence of the ability of zymogen factor IX to lower the $K_{d,appFIXa}$ is to increase the amount of factor Xa formed by initial rates of factor IX activation (<2% of available zymogen factor IX converted to factor IXa). When the factor IXa present approaches saturation levels for available sites, the potentiation by zymogen factor IX is less apparent. Results indicate the possibility that the presence of factor IX slightly increases the V_{max} of the reaction at surface saturation with enzyme. This may be a consequence of stabilizing the equilibrium binding and favoring occupation of sites by increasing the on rate or decreasing the off rate. These possibilities will be the focus of future studies.

These experiments were undertaken to probe the functional consequences of having two types of equilibrium binding sites for the Gla-domain containing proteins coagulation zymogen factor IX and its active enzyme form factor IXa. One site requires residues 5−7 and 9−11 of the Gla-domain (*2*, *3*) and can be occupied both by factor IX and by factor IXa (n = ~250 sites/platelet; K_d ~2.5 nM). The other site is specific for factor IXa (n = ~250 sites/platelet; K_d ~2.5 nM in the absence of factor VIIIa, 0.5 nM in its presence) (*3*). The results presented here provide further proof that factor IX does not interfere with assembly of the functional factor X activation complex on the specific factor IXa site (*2*). EGR-factor IXa, the active-site inhibited enzyme that would be expected to compete for enzyme sites, does inhibit the functional factor X activation complex competitively by raising the $K_{d,appFIXa}$.

The inability of factor IX to compete with factor IXa on both activated platelets and on PS/PC vesicles suggests that the two types of binding sites can be minimally determined solely by phospholipid and do not necessarily involve platelet surface protein in direct binding. The lipid configuration patterns utilized by enzyme for factor X activation complex formation must be different than those responsible for zymogen Gla-domain binding on both phospholipid vesicles and on activated platelets.

It is also deducible from these results that activating cleavage of factor IX not only results in exposure of substrate binding and catalytic sites in the heavy chain but also results in more N-terminal conformational changes in the light chain that result in different surface recognition patterns on both phospholipid vesicles and on activated platelets. Evidence exists for interdomain interactions in factor IX that support this concept. Medved et al. (*12*, *13*) using Gla- and EGF-domain fragments showed by fluorescently monitored protein unfolding, circular dichroism, and differential scanning calorimetry that the Gla-domain folding is stabilized by interaction with the EGF-1 domain and that the two EGF domains fold independently but interact with one another. Point mutations in one EGF domain lead to impairment of functions associated with the other EGF domain, i.e., a salt bridge between Glu 78 in EGF-1 and Arg 94 in EGF-2 appears necessary for proper interactions with cofactor light chain (*14*). There is evidence that cleavage of factor IX at Arg 145 converts factor IX into a factor VIII binding enzyme, since factor IXα, cleaved only at Arg145, was capable of normal interaction with factor VIII light chain, whereas, factor IXaα, cleaved only at Arg 180, showed reduced affinity for it (*15*). Thus, the cleavage of factor IX at Arg 145 frees its light chain to assume an optimal conformation for binding factor VIII light chain. It is possible that this conformational change involves areas more N-terminal that interact with surface, despite the report that upon activation of factor IX, Fab fragments to heavy and light chain domains reveal conformational changes only in the heavy chain (*16*).

There is evidence that factor Xa in the presence of lipid can cleave the Arg 145−Ala 146 bond in zymogen factor IX, thus allowing interaction of the light chain of product factor IXα with the light chain of factor VIIIa (*15*). However,

autoradiography of radioiodinated factor IX included in the reaction showed no increase in any of the minor bands present in the preparation, especially at a molecular weight associated with factor IXα. Also, one would presume that such cleavage would result in inhibition of cofactor-supported reactions due to competition for factor VIIIa. Indeed, the ability of zymogen factor IX to improve the $K_{d,appFIXa}$ in the absence of factor VIIIa precludes an explanation that involves the cofactor.

In this work, painstaking efforts were made to ensure that the potentiation of factor X activation seen was attributable to the zymogen. (i) Three different preparations of plasma-derived factor IX showed the same potentiating activity. Extended treatment with protease inhibitors EGR-CK, PMSF, and PPACK did not reduce their ability to potentiate the reaction. On the contrary, factor IXa treated identically with EGR-CK, and retaining no enzymatic activity, inhibited the reaction by raising the $K_{d,appFIXa}$, confirming an earlier report that dansyl-EGRCK-treated factor IXa serves as a competitive inhibitor in both binding of factor IXa to activated platelets and in factor Xa generation assays ($K_i = 0.4$ nM) (*17*). (ii) Recombinant wild-type factor IX, prepared and purified differently from plasma-derived factor IX, showed the same ability as plasma-derived factor IX. (iii) Finally, the trace factor IXa contamination determined by active site titration with ATIII, visualized by autoradiography, and assayed in factor X titration reaction mixtures lacking factor IXa cannot explain the level of factor X activation achieved by the combination of enzyme and zymogen from which activity attributable to zymogen alone was subtracted. Peak activity achieved by optimal concentration of zymogen added to mixtures containing $K_{d,app}$ levels of enzyme (Figure 1) did not reach the V_{max} attainable by saturation of all binding sites (Figure 2). This argues against increased complex formation formed from contaminating factor IXa. The low affinity of factor IXa for the phospholipid vesicles in the absence of factor VIIIa necessitates the use of high concentrations of enzyme that would be only minutely increased by trace levels of factor IXa introduced by zymogen addition. Thus, the potentiation of the reaction observed in the presence of zymogen cannot be attributed to enzyme contamination of the zymogen preparations.

The activated platelet surface binds several coagulation enzymes and either passively supports their activities or actively potentiates them by increasing local concentrations, by orienting reactants optimally in a two-dimensional system, by stabilizing more effective conformations, or perhaps by providing a combination of these effects. Thus, one could envision in a physiologic environment a series of complexes on activated platelets: (i) factor XI being activated by thrombin (*18*), (ii) factor XIa activating factor IX (*19*), (iii) factor IXa assembled with factor VIIIa for the activation of factor X (*17*), and (iv) factor Xa assembled with factor Va for activation of prothrombin (*20*, *21*). The shared factor IX zymogen and enzyme sites identified by equilibrium and competition binding may possibly be the factor IX activation sites that could then feed enzyme-specific sites more efficiently than could be accomplished by three-dimensional diffusion through the buffer or plasma compartment.

In these experiments, excess zymogen would be expected to saturate shared zymogen/enzyme binding sites. It is possible that competition with enzyme for these sites allows undetoured access to enzyme-specific sites, in effect increasing the concentration of enzyme available to these specific sites, much as would be accomplished in vivo if shared sites were factor IX activation sites. If occupation of activation sites represented the mechanism, addition of 100 nM factor IX should result in no more potentiation than from 25−30 nM (10-fold above $K_{d,app}$), which should be sufficient to saturate available sites. However, increasing activity was noted with up to 100−200 nM factor IX (Figure 1), suggesting that saturation of shared sites does not explain the potentiation. Addition of excess binding sites should make saturation of shared sites more difficult. In the absence of factor IX, confirming previous observations, excess lipid is inhibiting (Figure 6a), presumably because the dilution effect of excess sites causes separation of substrate factor X from enzyme factor IXa. Although there is a continued dilution effect in the presence of factor IX at low enzyme concentration (Figure 6a), evidence that there is excess lipid present, there is continual potentiation by factor IX at all lipid concentrations. Thus, the presence of excess binding sites does not compromise the potentiating effect of factor IX, suggesting that competition for shared factor IX/factor IXa sites does not explain the mechanism. In preliminary studies, a cyclized synthetic peptide containing residues 3−12 of the amino terminus of the Gla domain, which was previously shown to compete with factor IX for shared sites ($K_i = 3$ nM) but required 165 μM to half-maximally inhibit factor X activation (*2*), had no potentiating or inhibiting effect, at up to 1 μM, on factor IXa-catalyzed factor X activation. Therefore, the three observations support the conclusion that mere occupation of shared sites is insufficient to account for potentiation by zymogen. Additional experiments are underway to investigate this possibility further.

Whatever the mechanism, the effect of zymogen on factor X activation appears to be specific to factor IX. Neither protein C, protein S, nor factor VII at plasma concentrations had a potentiating effect. Prothrombin at 1 μM inhibited factor X activation on phospholipid by raising the $K_{d,appFIXa}$, presumably by competing with factor IXa for binding sites (*22*) and inhibited the reaction on activated platelets by lowering the V_{max}, presumably by competing with factor X for shared sites (*22*). The fact that each of these Gla-containing proteins when present with factor IX could not compete with the observed effect of factor IX argues against a mechanism of potentiation involving a general Gla domain conformation.

It thus appears that, in the context of the vasculature, where only a small proportion of available zymogen is activated and localized to a surface that is fed continuously with circulation-borne supplies of fresh zymogen, zymogen factor IX may play a role in increasing the efficiency of intrinsic pathway coagulation kinetics. We have shown that zymogen is more effective at low enzyme concentrations and at limiting surface binding sites, suggesting its usefulness in initial responses to vessel injury. Tissue factor/factor VIIa can activate a small quantity of factor X to factor Xa as well as factor IX to factor IXa before it is inhibited by tissue factor pathway inhibitor. As the primary platelet plug accumulates, it presents a small but growing surface of activated platelet membrane that can support coagulation enzymatic reactions leading to both an initial burst of thrombin production, through tissue factor/factor VIIa derived

factor Xa, and subsequent thrombin production through intrinsic derived factor Xa. Under those conditions, the presence of plasma levels of zymogen factor IX (90 nM) would ensure more rapid conversion of factor X to factor Xa than in the absence of zymogen factor IX. Although factor IX potentiation was accompanied by increases in the $K_{m,app}$, it is clear in Figure 3 that, even at relatively high levels of factor IXa, ~2.0 nM, factor IX allows a 150% increase in factor Xa formed at plasma levels (130 nM) of factor X added. Since $K_{m,app}$ is lower for fewer surface complexes (*11*), plasma factor X availability is at least 6-fold above the $K_{m,app}$ for initial levels of factor IXa produced (considerably less than 1 nM). Studies have been designed to test the validity of this hypothesis using reconstituted plasma systems. Mann et al. have shown using a reconstituted plasma system that the presence of excess factor IX delays the onset of thrombin production and decreases the total amount of thrombin produced by a tissue-factor driven system (*23*). The results described here suggest that the importance of intrinsic factor X activation may be weighted not only by the activity of tissue factor pathway inhibitor that shuts down the extrinsic system for factor Xa production (*24*) but also by the role of factor IX zymogen, which, by competing with factor X in the extrinsic system, results in availability of small amounts of factor IXa and, by potentiating the intrinsic system, makes the factor IXa produced more efficient at activating factor X.

ACKNOWLEDGMENT

We are grateful to Frank Wilkinson for expression, purification, and characterization of rwt-factor IX; to Patricia Pileggi and Virginia Sheaffer for assistance in manuscript preparation; and to Baxter Healthcare Corporation for the generous gift of recombinant factor VIII.

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BI000245O