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Intrinsic versus extrinsic coagulation

Kinetic considerations

Bonnie J. WARN-CRAMER* and S. Paul BAJAJ†‡

*Department of Medicine (H-811K), University of California at San Diego, La Jolla, CA 92093, and †Section of Medicine, Biochemistry and Pathology, St. Louis University School of Medicine, St. Louis, MO 63104, U.S.A.

A study to compare the kinetics of activation of factor IX by Factor XIa/Ca^{2+} and by Factor VIIa/tissue factor/Ca^{2+} has been undertaken. When purified human proteins, detergent-extracted brain tissue factor and tritiated-activation-peptide-release assays were utilized, the kinetic constants obtained were: K_m = 310 nM, $k_{cat.}$ = 25 min^{-1} for Factor XIa and K_m = 210 nM, $k_{cat.}$ = 15 min^{-1} for Factor VIIa. The kinetic constants for the activation of Factor X by Factor VIIa/brain tissue factor were: K_m = 205 nM, $k_{cat.}$ = 70 min^{-1}. Predicted rates for the generation of Factor IXa and Factor Xa were obtained when human monocytic tumour U937 cells (source of tissue factor) and Factor VIIa were used to form the activator. In other experiments, inclusion of high-M_r kininogen did not increase the activation rates of Factor IX by Factor XIa in the presence or absence of platelets and/or denuded rabbit aorta. These kinetic data strongly indicate that both Factor XIa and Factor VIIa play physiologically significant roles in the activation of Factor IX.

INTRODUCTION

During normal human blood clotting, Factor IX may be activated by either Factor XIa, requiring Ca^{2+} [1,2] or Factor VIIa, requiring Ca^{2+} and tissue factor (Scheme 1; [3]). The activation results from two peptide-bond cleavages (Arg^{145}–Ala^{146} and Arg^{180}–Val^{181}) in the single-chain Factor IX glycoprotein (415 residues) to yield a two-chain disulphide-linked Factor IXa and a 35-residue activation glycopeptide [4]. Both enzymes initially cleave the Arg–Ala bond, and the subsequent cleavage of the Arg–Val bond represents the rate-limiting step [1,5]. However, the relative importance of the two enzymes in activating Factor IX during haemostasis *in vivo* is not clear. In the present study we examined activation of Factor IX by Factor VIIa and by Factor XIa under a variety of experimental conditons to assess the potential contributions of each enzyme to human haemostasis at this step.

During normal clotting, Factor X, as shown in Scheme 1, may also be activated by either Factor IXa, requiring Ca^{2+}, PL and VIIICa, or Factor VIIa, requiring Ca^{2+} and TF. The activation by both enzymes results from cleavage of the Arg^{52}–Ile^{53} bond in the heavy chain (303 residues) of human Factor X and release of a 52-residue activation glycopeptide; the light chain (139 residues) remains unaltered during this process [6–8]. We have determined the kinetic constants for the activation of human Factor X by Factor VIIa/TF and have contrasted these values with those obtained for the activation of Factor IX. In addition, we have compared the reported kinetic constants for the activation of Factor X by the factor IXa–Ca^{2+}–PL–VIIICa enzymic complex [9] with the kinetic constants obtained by the Factor VIIa–TF enzymic complex. The analyses indicate that the generation of both Factor XIa and Factor VIIa represent important initiation events in human haemostasis.

EXPERIMENTAL PROCEDURES

Materials

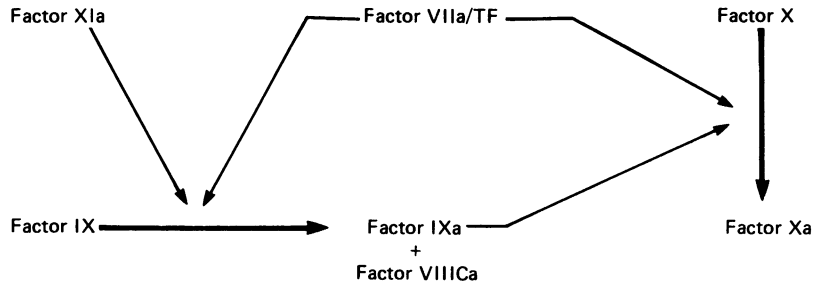
$Na^{125}I$ and NaB^3H_4 were purchased from Amersham Corp. Hereditary-clotting-factor-deficient plasmas were obtained from George King Biomedical, Overland Park, KS, U.S.A. Cephalin was obtained from Sigma and the PL concentration determined by the analysis of P_i [10]. All other reagents were of the highest grade commercially available.

Proteins

The protocol used to purify human factor XII was that of Fujikawa & Davie [11], with the exception that the proteinase inhibitors were not included wherever called for. The preparation contained ~70% of Factor XIIa, which was separated from Factor XII by benzamidine–agarose chromatography [11]. Upon 'reduced' SDS polyacrylamide-gel-electrophoretic analysis [12], the final Factor XIIa preparation revealed two bands corresponding to M_r ~ 50000 and ~ 30000. Human Factor XI was purified as outlined by Kurachi & Davie [13], except that the last step, benzamidine–agarose column chromatography, was replaced by high M_r-kininogen (HM_rK) chromatography [14]. Factor XI was activated by Factor XIIa as outlined [13], kept at 4 °C and used within 24 h. On SDS/polyacrylamide-gel electrophoretic analysis Factor XIa showed two bands corresponding to M_r ~ 47000 and ~ 37000. When Factor XIIa (1 μg/ml, final concn.) was incubated with [^{3}H]Factor IX

Abbreviations used: PL, phospholipid; TF, tissue factor; HM_rK, high-M_r kininogen; Tris/NaCl, 0.05 M-Tris/0.15 M-NaCl, pH 7.5; Tris/NaCl/Alb, Tris/NaCl containing 1 mg of bovine serum albumin/ml; Hepes/NaCl, 0.05 M-Hepes/0.15 M-NaCl, pH 7.5; Hepes/NaCl/Alb, Hepes/NaCl containing 1 mg of bovine serum albumin/ml; VIIICa; activated Factor VIIIC.

‡ To whom correspondence and reprint requests should be sent.



Scheme 1. A condensed outline depicting the generation of Factor Xa through the intrinsic (Factor XIa) and the extrinsic (Factor VIIa) pathways of blood clotting

Factor Xa formed together with Ca^{2+}, PL and Factor Va converts prothrombin into thrombin, which, in turn, converts fibrinogen into fibrin (for details, see the text and reference [6]).

(5 μg/ml, final concn) and Ca^{2+} (5 mM) for 1 h, less than 1% of Factor IX was found to be activated, as determined by the tritiated-peptide-release assay [2] and by the SDS/polyacrylamide-gel radioactivity profiles [5]. Since Factor XIIa did not activate Factor IX, it was not removed from Factor XIa. The molarity of Factor XIa was determined as described [2]. HM_rK was purified by the procedure of Kerbiriou & Griffin [15]. As determined by SDS/polyacrylamide-gel-electrophoretic analysis [12] the preparation contained ~ 50% unactivated protein (M_r ~ 110000) and ~ 50% activated protein (~ 65000-M_r heavy chain and ~ 56000-M_r light chain).

Human Factors VII, IX and X were purified as described [16–18]. Factor Xa [16], thrombin [16] and Factor VIIa [17] were prepared as described in the references cited. ^{125}I-Factor VII (2.1×10^9 c.p.m./mg) was prepared by using Pierce Iodogen reagent. The method of Fraker & Speck [19] was followed as outlined by the manufacturer. [^{3}H]Sialyl-Factor IX (3×10^8 c.p.m./mg) and [^{3}H]sialyl-Factor X (2×10^8 c.p.m./mg) were prepared by the general technique of Van Lenten & Ashwell [20] as described [21]. All of the three labelled proteins retained more than 80% of the coagulation activity of the non-labelled controls.

TF

Human brain TF was prepared as follows. First, acetone- and water-soluble contaminants were removed as described by Bach *et al.* [22]. Next the centrifuged pellet was extracted with 0.045% Triton in Tris/NaCl, washed with acetone and dried to a powder. The powder was homogenized in Tris/NaCl and the suspension was heated for 20 min at 56 °C and centrifuged at 900 *g* for 10 min. The supernatant was centrifuged at 27000 *g* for 20 min and the pellet was suspended in Tris/NaCl; it contained 11 mM (as P_i)-PL and clotted recalcified plasma in 25 s. The preparation was stored in small portions at −20 °C. Human monocytic tumour U937 cells for use as a source of membranous TF were cultured by Ms. Lory Minning in Dr. D. Hudig's laboratory as described [23].

Platelets and vessel-wall preparation

Human platelets were prepared and washed by centrifugation as described [24]. The washed platelets were suspended in cold Hepes/NaCl buffer (~ 5×10^8 platelets/ml) and used within 4 h. Platelets were incubated with thrombin (0.2 μg/ml) for 2 min before use. Vessel-wall preparation was denuded rabbit aorta. The aorta [about 2.5 cm (1 in) long] was obtained at the time the rabbits were killed, cleaned of attached tissue, rinsed with Hepes/NaCl, slit lengthwise and the inner wall scraped with a scalpel. The denuded vessel was minced to particles of ~ 1 mm^2 in size and washed with Hepes/NaCl until the wash contained no detectable Factor-VII, -IX, -X or -XI clotting activities. It was kept at 4 °C in Hepes/NaCl and used immediately.

[^{3}H]Activation-peptide-release assays

Rates of activation of factors IX and X were monitored by the release of ^{3}H-labelled activation peptide from [^{3}H-]Factor IX [25] and [^{3}H-]Factor X [26]. Minor modifications of the assays were the same as those described previously [2].

Table 1. Steady-state kinetic constants for activation of Factor IX and Factor X

For details, see the text.

Enzyme	Factor IX			Factor X		
	K_m (nM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ (min^{-1}·nM^{-1})	K_m (nM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ (min^{-1}·nM^{-1})
Factor XIa	310	25	0.08	–	–	–
Factor VIIa–TF	210	15	0.07	205	70	0.34
Factor IXa–PL–VIIICa*	–	–	–	140	25	0.18

* From ref. [9].

Table 2. Initial rates of activation of [^{3}H]Factor IX by Factor XIa/Ca^{2+} in the presence or absence of HM_r K, PL, platelets and vessel wall

The concentration of the enzyme was 0.12 nM (active site) and that of Ca^{2+} was 5 mM. Plus or minus signs indicate the presence or absence of additional components. When added their concentrations were: HM_rK, 0.36 nM; PL, 50 μM; platelets, 1.4×10^8/ml; vessel-wall preparation, 10 pieces/ml. Platelets were activated with thrombin (0.2 μg/ml) before use. The concentration of [^{3}H]factor IX was 88 nM (A) or 175 nM (B). Abbreviation used: N.D., not determined.

HM_rK	PL	Platelets	Vessel wall	Initial rate (nmol·l^{-1}·min^{-1}) A	B
−	−	−	−	0.69	1.19
+	−	−	−	0.64	1.25
−	+	−	−	0.32	N.D.
+	+	−	−	0.39	N.D.
−	−	+	−	0.43	0.82
+	−	+	−	0.37	0.85
−	−	−	+	N.D.	1.05
+	−	−	+	N.D.	0.99
−	−	+	+	N.D.	0.85
+	−	+	+	N.D.	0.80

RESULTS

Activation of Factor IX by Factor XIa

Reaction mixtures were made containing 20 different concentrations of Factor IX (mixtures of [^{3}H]Factor IX and non-labelled Factor IX) ranging from 35 nM to 5 μM, 0.60 nM-Factor XIa (active site) and 5 mM-Ca^{2+}. Initial rates of activation of Factor IX were measured and the Michaelis–Menten kinetic constants were calculated from a Lineweaver–Burk plot prepared by using linear-regression analysis [2]. Values (Table 1) were: K_m = 310 nM, $k_{cat.}$ = 25 min^{-1} (correlation coefficient, r, 0.983).

In additional experiments, the initial rates of activation of Factor IX by Factor XIa/Ca^{2+} were determined in the presence or absence of HM_rK, PL or platelets and/or vessel-wall preparation. In order to prevent activation of Factor IX by a possible trace contamination of Factor VIIa/TF, each reaction mixture was preincubated with antibodies to Factor VII [17] before adding Factor XIa. The concentrations of platelets and Factor XIa were such that more than 90% of Factor XIa was potentially bound to the platelets [27]. The initial-rate data are presented in Table 2. Under the conditions of our experiments, addition of HM_rK alone or HM_rK ± (PL or platelets) ± vessel wall did not result in an increase in the activation of Factor IX by Factor XIa. Hower, in reaction mixtures containing platelets or PL, 30–50% decrease was observed in the initial rate of activation (Table 2).

Activation of Factor IX and of Factor X by Factor VIIa/TF

The initial experiments were aimed at determining the concentration of active Factor VIIa-binding sites in our TF preparation. Two sets of experiments were performed for this purpose. As demonstrated by Silverberg *et al.* [26], the assumption was made that only Factor VIIa complexed to TF is the active enzymic species. In one set of experiments, TF concentration was varied and Factor VIIa concentration was kept constant at 1 nM. The rates of activation of [^{3}H]Factor IX (5 μg/ml) or [^{3}H]Factor X (5 μg/ml) were measured. Double-reciprocal analyses of the data are shown in Fig. 1(*a*) for Factor IX activation and in Fig. 1(*c*) for Factor X activation. The maximum velocity for each substrate obtainable under these conditions at 1 nM-Factor VIIa (saturation with TF) was then determined from the inverse of the y-intercepts of these plots. The values were: 2.8 nM·min^{-1} for Factor IX activation and 15 nM·min^{-1} for Factor X activation. Furthermore, the calculated TF concentration required for $\frac{1}{2} V_{max.}$ for Factor IX and Factor X activation was essentially the same (~ 3% TF).

In a second set of experiments, Factor VIIa concentration was varied and TF concentration was kept constant at 3% of the reaction volume. The rates of activation of Factor IX (5 μg/ml) and of Factor X (5 μg/ml) were again measured. Double-reciprocal plots of the data are shown in Fig. 1(*b*) for Factor IX activation and Fig. 1(*d*) for Factor X activation. The maximal velocity for each substrate at 3% TF saturated with Factor VIIa was determined from the inverse of the y-intercepts of each of these plots. The values were: 5 nM·min^{-1} for Factor IX activation and 24 nM·min^{-1} for Factor X activation. From these maximal-velocity data, our 3% solution of TF was calculated to contain 1.8 nM (5/2.8) or 1.6 nM (24/15) equivalent binding sites for Factor VIIa.

From the amount of Factor VIIa added and from the observed velocity (Figs. 1*b* and 1*d*), we could now calculate the amount of Factor VIIa present in the bound (i.e., complexed with TF) and in the free form at any given concentration of Factor VIIa and analyse the data by the Scatchard method. An average apparent K_d for the interaction of TF and Factor VIIa using the data of Fig. 1(*b*) was 1.1×10^{-10} M and using the data of Fig. 1(*d*) was 4.6×10^{-10} M.

Michaelis–Menten kinetic constants were also determined for the activation of Factor IX and Factor X by the Factor VIIa–TF complex. Since high PL concentrations in the incubation mixtures are known to increase the K_m for the reaction [25], we used low concentrations of TF in these experiments. For Factor IX (seven different concentrations ranging from 17 to 880 nM) studies it was 1% (115 μM-PL) and for factor X (six different concentrations ranging from 35 to 530 nM) studies it was 0.5% (58 μM-PL). The concentration of Factor VIIa used was 1 nM for both Factor IX and Factor X activations. The concentration of Factor VIIa complexed with TF was calculated by using the data of Fig. 1 and was 0.86 nM for Factor IX and 0.55 nM for Factor X activation mixtures. The apparent steady-state kinetic constants for activation of Factor IX were: K_m = 210 nM, $k_{cat.}$ 15 min^{-1} (r = 0.933) and for activation of factor X were: K_m = 205 nM, $k_{cat.}$ = 70 min^{-1} (r = 0.966). These values are listed in Table 1.

In further experiments, undisrupted U937 cells of the human monocytic tumour cell line were used as a source of TF. The cells (10^8/ml) were incubated with ^{125}I-Factor VIIa (10 nM) for 30 min at room temperature. The cells were then spun down at 5000 *g* for 10 min, washed three times with 50 ml of buffer (Hepes/NaCl/Alb containing

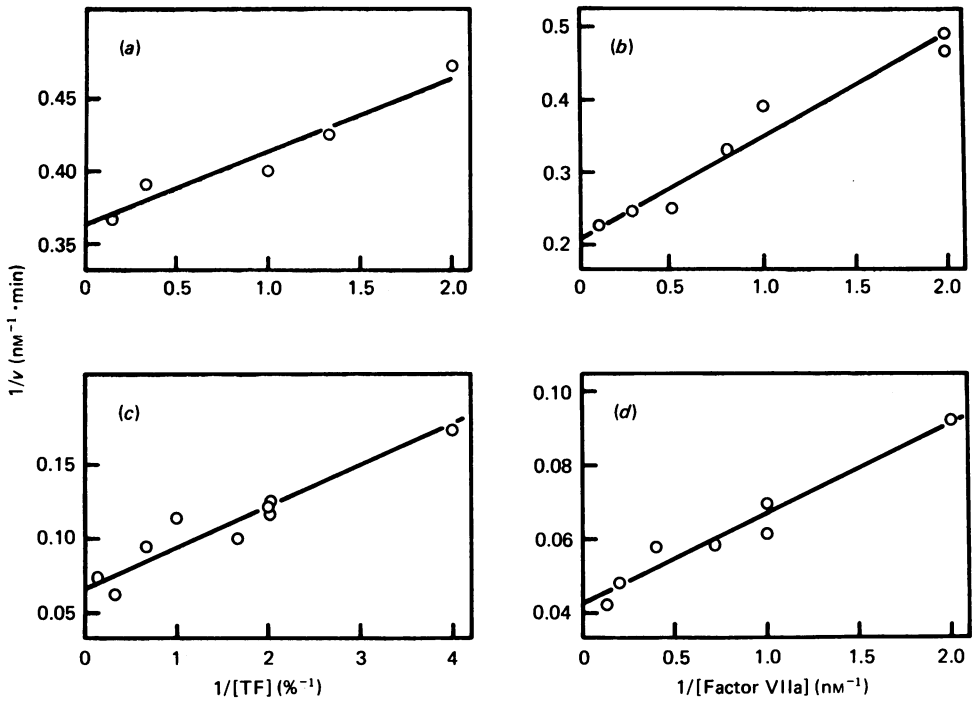


Fig. 1. Double-reciprocal graphic analyses of the rates of activation of [^{3}H]Factor IX and [^{3}H]Factor X

(*a*) Activation of Factor IX (5 μg/ml) at 1 nM-factor VIIa and various concentrations of TF (0.5–9%, v/v); (*b*) activation of Factor IX (5 μg/ml) at 3% (v/v) TF and various concentrations of Factor VIIa (0.5–10 nM); (*c*) activation of factor X (5 μg/ml) at 1 nM-Factor VIIa and various concentrations of TF (0.25–12%, v/v); (*d*) activation of Factor X (5 μg/ml) at 3% (v/v) TF and various concentrations of Factor VIIa (0.5–10 nM). The buffer used was Tris/NaCl/Alb containing 5 mM-Ca^{2+}. For details see the text.

5 mM-glucose and 5 mM-Ca^{2+}) and resuspended (2.8×10^8/ml) in Tris/NaCl/Alb containing 5 mM-Ca^{2+}. These cells were then used as a source of Factor VIIa–TF to study the activation of Factor IX and Factor X. The reaction mixture contained either [^{3}H]Factor IX (88 nM) or non-labelled Factor X (85 nM), U937 cells (1.3×10^8/ml) and Ca^{2+} (5 mM). (A 200 μl portion of the reaction mixture gave 3700 c.p.m., corresponding to a concentration of 176 pM-^{125}I-Factor VIIa.) The initial rate of Factor IX activation measured by the tritiated-peptide-release assay [2] was 0.71 nmol of Factor IXa formed/min and the initial rate of Factor X activation measured by the clotting assay [28] was 3 nmol of Factor Xa formed/min. Utilizing these initial-velocity data and the kinetic constants (Table 1), we calculate the effective concentration of Factor VIIa to be 160 pM in Factor IX and 146 pM in Factor X activation mixture, values which are in reasonable agreement with the value obtained from the radioactivity measurement.

DISCUSSION

Haemostasis involves coagulation proteins, Ca^{2+}, platelets, exposed subendothelial surface and injured endothelial cells [6]. Cultured bovine aortic endothelial cells have been reported to propagate a coagulant pathway starting with Factor XIa [29]. The effect of PL on the activation of Factor IX by Factor XIa has also been examined. In one study [30] neither PL nor HM_rK had any effect on the activity of Factor XIa towards Factor IX. In another study [31] it was noted that PL accelerates Factor IX activation by surface-bound Factor XIa about 2-fold. We have extended these studies herein and have examined the effect of platelets and/or vessel-wall preparations (in the absence and presence of HM_rK) on the activation of Factor IX by Factor XIa (Table 2). The rates of activation of Factor IX in all of our reaction mixtures (> 10) that contained PL or platelets were decreased. The lower rates of activation in our experiments may be related to the binding of Factor IX to PL, which in turn could decrease the effective concentration of the substrate; this is consistent with the previous observations of activation of Factor IX by Factor VIIa–TF containing different concentrations of PL [25]. The reasons why a decrease in the Factor IX activation rate was not observed in previous studies are not evident, but may be due to lower (unspecified) concentrations of PL [30,31]. Consistent with this thought is our own observation that when we decreased the number of platelets from 1.4×10^8/ml (Table 2) to 1.2×10^7/ml in the reaction mixtures, only $\sim 15\%$ decrease in the rate of Factor IX activation was observed. However, HM_rK, platelets and vessel wall separately, or in any combination thereof, did not increase the rate of Factor IX activation by Factor XIa (Table 2). Since each active subunit of Factor XIa binds one molecule of HM_rK in a fluid phase [32] and since this interaction is required for Factor XIa binding to thrombin-activated platelets [27] one could infer from these data that HM_rK may simply serve to protect Factor XIa from plasma proteinase inhibitors [33] and to localize Factor XIa on platelets for activation of Factor IX.

In the present study, the kinetic constants observed for the activation of Factor IX by Factor XIIa-activated Factor XIa are $K_m = 310$ nM, and $k_{cat.} = 25$ min^{-1}. These values are different from the values of 11 min^{-1} ($k_{cat.}$) and 2 μM (K_m) obtained with trypsin-activated Factor XIa [2]. The aim of the previous investigations was to examine the role of Ca^{2+} in Factor IX activation. In those studies, in a series of kinetic experiments, the range of Factor IX concentration (4–26 μM) was kept the same at several constant levels of Ca^{2+}. We now have determined that this substrate concentration range is adequate to determine the kinetic constants at zero and at subsaturating concentrations of Ca^{2+} only. At higher concentrations of Ca^{2+} (> 1 mM) the substrate concentratons of 4–26 μM are too high (relative to the K_m) to yield an accurate K_m value [34]. This explains the reason for obtaining a high K_m value in the previous study [2]. An approximate 2-fold difference in the $k_{cat.}$ value could stem from the use of trypsin-activated Factor XIa in the earlier study [2]. Alternatively, since this 2-fold difference is not large, it could simply stem from variations in the two enzyme preparations.

Walsh *et al.* [35] have recently reported the kinetic constants of the activation of Factor IX by Factor XIIa-activated Factor XIa to be: $K_m = 490$ nM and $k_{cat.} = 230$ min^{-1}. Their K_m value is in reasonable agreement with ours (310 nM), but their $k_{cat.}$ value is 9-fold higher than our value of 25 min^{-1}. The reason for this difference is unknown. However, we wish to point out that the rates (4.1–6.1 mol of Factor IXa/min per mol of enzyme) of Factor IX activation by Factor XIIa-activated Factor XIa observed by van der Graff *et al.* [30] are in good agreement with the rate (4.3 mol of Factor IXa/min per mol of enzyme) predicted from our kinetic constants, but not with the rate (26.7 mol of factor IXa/min per mol of enzyme) predicted from kinetic constants given by Walsh *et al.* [34].

The average K_d (2.8×10^{-10} M) for the interaction of Factor VIIa and TF obtained kinetically (Fig. 1) is in excellent agreement with the K_d (1×10^{-10} M) of binding of Factor VII/VIIa to the TF on human monocytes [36]. Kinetic analysis of the activation of Factor IX and Factor X by Factor VIIa/TF gave essentially the same values for K_m, but different values for $k_{cat.}$ (Table 1). The same values for K_m suggest that Factor VIIa–TF will be partitioned according to plasma concentrations of Factors IX and X. Since the plasma concentration of human Factor IX (70 nM) is approximately half that of Factor X (130 nM), only about one-third of Factor VIIa–TF will be available to activate Factor IX, Our kinetic data for the activation of Factor IX by Factor XIa and Factor VIIa–TF suggest that the apparent catalytic efficiencies of these enzymes ($k_{cat.}/K_m$; Table 1) are quite similar. However, the plasma concentration of Factor VII (10 nM) is 6-fold less than the plasma concentration of catalytic subunit of Factor XI (60 nM). The above considerations would give ~ 18-fold potential edge to the intrinsic pathway for the activation of Factor IX. However, how much Factor XIa as against Factor VIIa–TF is produced during coagulation *in vivo* is not known. Nonetheless, it would appear that Factor IX activation by Factor XIa is an important step in haemostasis. Furthermore, the known activator of Factor XI *in vitro* is Factor XIIa [6]. Since deficiency of Factor XII (the zymogen form of Factor XIIa) is not associated with a bleeding disorder [6], a hitherto unidentified activator of Factor XI may exist in the vascular system.

Factor XI deficiency is a mild bleeding disorder and primarily becomes manifest after major surgery [37]. This means that coagulation initiated by the native Factor VII–TF [6] is almost adequate to control bleeding under normal circumstances. It has been postulated [6] that native Factor VII–TF is capable of activating Factor X, albeit slowly; the Factor Xa generated then converts Factor VII to Factor VIIa, which, when complexed with TF, efficiently activates both Factors IX and X. Thus, in the absence of Factor XI, one-third of Factor VIIa–TF engaged in an obligatory fashion in the activation of Factor IX could generate enough Factor IXa molecules that are known to catalyse efficiently the conversion of Factor X into Factor Xa (Table 1; ref. [9]). However, generation of Factor IXa only by the Factor VIIa–TF pathway is apparently not adequate during major bleeding episodes [37]. Interestingly, patients who have either abnormal Factor IX variants such as those described previously [21] or have normal Factor IX but lack Factor VIIIC in addition to consuming Factor XIa in generating non-productive Factor IXa molecules will also consume in a non-productive obligatory fashion one-third of available Factor VIIa/TF in activating Factor IX. Thus, under such conditions, both the intrinsic and the extrinsic pathways are affected, which leads to severe bleeding disorders [37].

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