The contribution of Ca^{2+} and phospholipids to the activation of human blood-coagulation Factor X by activated Factor IX

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The role of the cofactors Ca^{2+} and phospholipid in the activation of human Factor X by Factor IXa was investigated. By use of a sensitive spectrophotometric Factor Xa assay, it was demonstrated that human Factor IXa can activate Factor X in the absence of cofactors. The presence of Ca^{2+} as the only cofactor resulted in a 7-fold stimulation of the Factor Xa formation. Kinetic analysis of the Ca^{2+} -stimulated reaction showed that the apparent K_m of Factor X was $4.6\ \mu\text{M}$, whereas the apparent V_{max} for Factor Xa formation was $0.0088\ \text{mol of Xa/min per mol of IXa}$. The presence of phospholipid as the only cofactor had no effect on the rate of Factor Xa formation. However, a several-hundred-fold stimulation was observed when Ca^{2+} and phospholipid were present in combination. The activation of Factor X in the presence of Ca^{2+} and phospholipid was found to be kinetically heterogeneous, involving both phospholipid-bound and free reactants. Quantitative data concerning the phospholipid binding of Factors IXa and X were used to study the relation between the rate of Factor Xa formation and the binding of enzyme and substrate to the phospholipid membrane. The results support the hypothesis that phospholipid-bound Factor X is the substrate in the phospholipid-stimulated reaction; however, phospholipid-bound and free Factor IXa seem to be equally efficient in catalysing the activation of phospholipid-bound Factor X.

Factor X is a plasma glycoprotein that is involved in both the intrinsic and the extrinsic pathway of blood coagulation. It is the zymogen of Factor Xa, the serine proteinase that, as part of a complex with Factor V, Ca^{2+} and phospholipid (the 'prothrombinase' complex), catalyses the conversion of prothrombin into thrombin (Davie *et al.*, 1979; Jackson & Nemerson, 1980).

In the intrinsic coagulation pathway, Factor X is activated by a complex consisting of Factor IXa, Factor VIII, Ca^{2+} and phospholipid. By analogy with the prothrombinase complex, the 'intrinsic Factor X activator' is generally supposed to be a

complex in which Factor IXa is the Factor X-activating enzyme, whereas Factor VIII, Ca^{2+} and phospholipid function as cofactors (Jackson & Nemerson, 1980). Evidence for this concept has been based on studies of Factor Xa formation by the complete Factor X-activating complex (Hemker & Kahn, 1967; Østerud & Rapaport, 1970; Chuang *et al.*, 1972). However, the coagulation assays used in those studies for the detection of Factor Xa did not permit investigation of the role of the individual components of the complex. This limitation has been overcome since the introduction of specific spectrophotometric (Aurell *et al.*, 1977) and radiochemical (Silverberg *et al.*, 1977) methods to monitor Factor X activation. Both for the human (Suomela *et al.*, 1977; Griffith *et al.*, 1982; Mertens & Bertina, 1982) and for the bovine (Hultin & Nemerson, 1978; Neal & Chavin, 1979; van Dieijen *et al.*, 1981; Link & Castellino, 1983) system it has been reported that Factor IXa can activate Factor X in the presence of Ca^{2+} and phospholipid, and that Factor VIII stimulates this

Abbreviations used: PS, L- α -phosphatidyl-L-serine; PC, L- α -phosphatidylcholine; S-2337, *N*-benzoyl-L-isoleucyl-L-glutamyl(piperidyl)-L-glycyl-L-arginine *p*-nitroanilide hydrochloride; SDS, sodium dodecyl sulphate.

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reaction considerably. For the bovine system, the effect of Ca^{2+} and phospholipid on the kinetics of Factor X activation by Factor IXa has been studied in detail (van Dieijen *et al.*, 1981; Link & Castellino, 1983). However, the mechanism by which phospholipid membranes contribute to Factor Xa formation has not yet been established.

The aim of the present study was to describe the contribution of Ca^{2+} and phospholipid to the activation of Factor X by Factor IXa in the human system, and thereby to obtain a well-defined reference system for studies concerning the role of the third cofactor, Factor VIII. In the preceding paper (Mertens *et al.*, 1984), we studied the phospholipid-binding properties of human Factor IXa and Factor X. In the present paper, these binding data were used for the interpretation of kinetic experiments concerning the effect of Ca^{2+} and phospholipid on the activation of Factor X by Factor IXa. By this approach it was possible to study the relation between the rate of Factor X activation and the binding of enzyme and substrate to the phospholipid membrane.

Experimental

Materials

Tris (TRIZMA base), L- α -phosphatidyl-L-serine (PS) and L- α -phosphatidylcholine, type I-EH (PC), were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ovalbumin (five-times-recrystallized) was purchased from Koch-Light Laboratories, Colnbrook, Slough, Berks., U.K. The chromogenic substrate S-2337 was a product of Kabi Diagnostica, Stockholm, Sweden. All other chemicals were analytical-grade products from Merck, Darmstadt, Germany.

Phospholipid vesicles. PS/PC vesicles were prepared from the purified components as described in the preceding paper (Mertens *et al.*, 1984). Unless stated otherwise, the vesicles contained equimolar amounts of PS and PC.

Purified coagulation factors. Human Factor X and Factor IXa were prepared as described in the preceding paper (Mertens *et al.*, 1984).

Methods

Determination of the rate of Factor Xa formation. The rate of activation of human Factor X by Factor IXa in the presence and absence of Ca^{2+} and/or PS/PC vesicles was measured by incubating these components in 100 mM-NaCl/50 mM-Tris/HCl, pH 7.5, at 37°C in silicone-treated glass tubes. The phospholipid and Ca^{2+} were pre-incubated for 10 min in 60% of the final volume; subsequently, buffer and Factor IXa were added, and 15 s later the reaction was started by the addition of Factor X. The final volume was at least

250 μl . After different incubation times, 50 μl samples were drawn and assayed for Factor Xa (see below). From the increase of the Factor Xa concentration with time, the initial rate of Factor Xa formation was calculated. During the measuring period, less than 1% of the Factor X was converted into Factor Xa, whereas no autocatalytic cleavage products of Factor X (Mertens & Bertina, 1980; Mertens *et al.*, 1982) were detected by SDS/polyacrylamide-gel electrophoresis. The preincubation of Ca^{2+} and phospholipid was essential to avoid non-linearity during the initial phase of the Factor Xa formation, due to Ca^{2+} -induced changes of the PS/PC surface (cf. Mertens *et al.*, 1984). By using pre-incubated vesicles, Factor Xa formation was linear for at least 60 min. Longer incubation times were avoided, since Factor IXa was not stable on prolonged incubation.

Spectrophotometric Factor Xa assay. Factor Xa activity was measured by adding 50 μl samples to cuvettes containing 5 mM-EDTA, 100 μg of ovalbumin/ml and 0.22 mM-S-2337 in 0.2 M-NaCl/0.05 M-Tris/HCl, pH 8.0 (final volume 1 ml) and by recording A_{405} at 37°C with a type-PM6 spectrophotometer (Zeiss, Oberkochen, Germany) equipped with a Servogor RE 511 recorder (Goerz, Vienna, Austria). Factor Xa was stable in the cuvette mixture for at least 30 min. The relation between Factor Xa activity and Factor Xa concentration was determined by using a freshly activated active-site-titrated Factor X reference preparation (Mertens *et al.*, 1984). The lower detection limit of this assay was 2.3 fmol of Factor Xa per 50 μl of sample.

Spectrophotometric Factor IXa assay. Factor IXa was assayed by measuring its Factor X-activating activity in the presence of 10 mM- CaCl_2 , 180 μM -PS/PC vesicles and 0.4 μM -Factor X. After determination of the rate of Factor Xa formation (see above), the concentration of Factor IXa was determined by intrapolation on a calibration curve relating nm-Factor Xa/min to Factor IXa concentration, as obtained by using an active-site-titrated reference preparation. The detection limit of this assay was 0.36 pmol of Factor IXa per ml of sample.

Calculation of the concentrations of phospholipid-bound and free Factors IXa and X. As described in the preceding paper (Mertens *et al.*, 1984), Factors IXa and Factor X can be regarded as two ligands that compete for binding to a single class of independent sites on PS/PC membranes. In this model (see Mertens *et al.*, 1984), the expression for conservation of binding sites is:

$$[\text{P}]_0 = [\text{P}] + [\text{X-P}] + [\text{IXa-P}] \quad (1)$$

where $[\text{P}]_0$ and $[\text{P}]$ are the concentrations of total

and free binding sites respectively, and $[X-P]$ and $[IXa-P]$ represent the concentrations of phospholipid-bound Factors X and IXa; these are given by:

$$[X-P] = \frac{[P] \cdot [X]_0}{K_1 + [P]} \quad (2a)$$

and

$$[IXa-P] = \frac{[P] \cdot [IXa]_0}{K_2 + [P]} \quad (2b)$$

where $[X]_0$ and $[IXa]_0$ are the total concentrations of Factors X and IXa, and K_1 and K_2 represent the dissociation constants of the complexes of phospholipid with Factor X and Factor IXa respectively. After substitution of eqns. (2a) and (2b) in eqn. (1), the concentration of free binding sites, $[P]$, can be rearranged into the equation:

$$[P]^3 + a[P]^2 + b[P] + c = 0 \quad (3)$$

where

$$\begin{aligned} a &= K_1 + K_2 + [X]_0 + [IXa]_0 - [P]_0 \\ b &= K_1 K_2 + [IXa]_0 K_1 + [X]_0 K_2 - [P]_0 (K_1 + K_2) \\ c &= -[P]_0 K_1 K_2 \end{aligned}$$

The binding parameters K_1 , K_2 and $[P]_0$, the number of available binding sites at a given PS/PC concentration, have been obtained experimentally for the conditions of the kinetic experiments (Mertens *et al.*, 1984). Thus a , b and c could be calculated for any set of experimental conditions. After solving eqn. (3), the calculated value of $[P]$ was used in eqns. (2a) and (2b) to obtain the concentrations of phospholipid-bound Factor X and Factor IXa; the concentrations of free Factors IXa and X then were calculated as the difference between total and phospholipid-bound concentrations.

Results

Effect of Ca^{2+} on the rate of Factor Xa formation

When various amounts of Ca^{2+} were added to reaction mixtures containing Factor X and Factor IXa, a sigmoid dose-response curve was obtained that reached a plateau at 2.5 mM- Ca^{2+} (Fig. 1a). In the absence of Ca^{2+} , the rate of Factor Xa formation was 0.00018 mol of Xa/min per mol of IXa; this Factor X activation in the absence of cofactors was found to be linear with respect to time and proportional to the Factor IXa concentration (results not shown). On the plateau of Fig. 1(a), the rate of Factor Xa formation was 0.0012 mol of Xa/min per mol of IXa, which is 7-fold the rate observed in the absence of Ca^{2+} . When Ca^{2+} was added to reaction mixtures that, in addition to Factors IXa and X, also contained

Fig. 1. Effect of Ca^{2+} on the rate of Factor Xa formation. Factors IXa and X were incubated at 37°C in 100 mM-NaCl/50 mM-Tris/HCl, pH 7.5, containing various amounts of $CaCl_2$; at 0 mM- Ca^{2+} , EDTA (5 mM) was present in the reaction mixture. (a) Factor X (0.4 μM) and Factor IXa (25 nM) were incubated in the absence of phospholipids. (b) Factor X (0.4 μM) and Factor IXa (3.9 nM) were incubated in the presence of PS/PC vesicles (140 μM). The rates of Factor Xa formation were calculated from the Factor Xa concentrations after 0, 10, 30 and 60 (a) or 0, 10 and 20 (b) min of incubation.

PS/PC vesicles, a 320-fold increase in the rate of Factor Xa formation was observed (Fig. 1b). The sigmoid dose-response curve had a plateau between 5 and 10 mM- Ca^{2+} . At higher Ca^{2+} concentrations (25 mM), flocculation of the PS/PC vesicles was observed. This was accompanied by a decrease in the reaction rate; such a decrease did not occur in the absence of phospholipid (Fig. 1a). These results demonstrate that Ca^{2+} functions as a cofactor in the Factor Xa formation; however, its cofactor effect is markedly enhanced by the presence of phospholipid. For further experiments, the Ca^{2+} concentration was fixed at 10 mM.

Effect of phospholipid on the rate of Factor Xa formation

Fig. 2 shows the phospholipid-dependence of the rate of Factor Xa formation (v) by Factor IXa in the presence of Ca^{2+} ; the experiment was

Fig. 2. Effect of the phospholipid concentration on the rate of Factor Xa formation

Factor IXa (2.7 nM) and Factor X (●, 0.1 μ M; ○, 0.4 μ M; ■, 1.0 μ M) were incubated with various amounts of PS/PC vesicles at 37°C in 10 mM- CaCl_2 /100 mM- NaCl /50 mM- Tris/HCl , pH 7.5. The rates of Factor Xa formation were calculated from the Factor Xa concentrations after 0, 10 and 20 min of incubation.

performed at three different Factor X concentrations. In all three experiments, an increase of the PS/PC concentration resulted in an increase of the rate of Factor Xa formation. Both the amount of phospholipid needed to saturate the system, and the level of the plateau, were dependent on the Factor X concentration. At phospholipid concentrations exceeding 0.3 mM, flocculation of the PS/PC vesicles was observed on the addition of Ca^{2+} ; above 0.7 mM-PS/PC, the formation of large phospholipid precipitates was accompanied by a decrease in the rate of Factor X activation (cf. Fig. 1b). For further experiments, the phospholipid concentration was chosen below 0.2 mM, thus avoiding conditions of flocculating PS/PC membranes. In the absence of Ca^{2+} , PS/PC vesicles had no stimulating effect on the Factor Xa formation (see also Fig. 1b); this indicates that Ca^{2+} is required for the expression of the cofactor effect of phospholipid. When comparing vesicles composed of 25% (mol/100 ml) PS/75% PC, 50% PS/50% PC and a phospholipid extract from human brain, the activity of the 25% PS preparation was only 15% of that of the other two preparations (conditions of Fig. 1b; results not shown). Vesicles containing 50% PS ('PS/PC') were used in all further experiments.

Fig. 3. Effect of the Factor X concentration on the rate of Factor Xa formation by Factor IXa in the presence of Ca^{2+} . Factor X (0.4–24 μ M) was incubated with Factor IXa (15.8 nM) at 37°C in 10 mM- CaCl_2 /100 mM- NaCl /50 mM- Tris/HCl , pH 7.5. The rates of Factor Xa formation were calculated from the Factor Xa concentrations after 10 and 20 min of incubation. The inset shows a double-reciprocal plot of the same data.

Kinetics of the activation of Factor X in the absence of phospholipid

When the Factor X concentration was varied at constant Factor IXa and saturating Ca^{2+} concentrations, a hyperbolic plot of initial rate of Factor Xa formation versus substrate concentration was obtained (Fig. 3). The rate of Factor X activation was found to be constant for at least 30 min, and proportional to the Factor IXa concentration as tested at various enzyme (0.1–15.8 nM) and substrate (0.4–15 μM) concentrations. The K_m of Factor X was 4.6 μM (see Fig. 3, inset), which equals 46 times the plasma concentration. The V_{max} of 0.0088 mol of Xa/min per mol of IXa indicates that the reaction is extremely inefficient: at saturating Factor X concentrations, one turnover of the enzyme would require incubation for about 2 h, whereas even longer incubations would be needed at lower Factor X concentrations. However, such long incubation times cannot be applied because of the limited stability of enzyme and product. Since the initial rates of Fig. 3 were obtained from experiments where the enzyme made less than one turnover, the calculated kinetic constants strictly should be regarded as apparent Michaelis–Menten parameters.

Kinetics of the activation of Factor X by Factor IXa in the presence of Ca^{2+} and phospholipid

Fig. 4 shows the effect of the Factor X concentration on the rate of Factor Xa formation at several phospholipid concentrations. In the absence of phospholipid, when the reaction proceeds in solution, v was linear with the Factor X concentration up to 1.2 μM (cf. Fig. 3). When the same experiment was performed in the presence of 100 μM -PS/PC vesicles, v increased more rapidly on increasing the Factor X concentration until a plateau was reached at 0.4 μM -Factor X. The half-maximal rate was obtained at 0.2 μM -Factor X, which would mean that the apparent K_m of Factor X had been decreased considerably, owing to the presence of phospholipid. However, the relation between v and Factor X concentration was not hyperbolic; accordingly, the Lineweaver–Burk plot of these data was non-linear. Similar results were obtained at 10 and 25 μM -phospholipid. At these low phospholipid concentrations, heterogeneity of the Factor X-activating system is suggested by the shape of the dose-response curves: when the Factor X concentration was gradually increased, v initially increased in a PS/PC concentration-dependent manner; however, at higher Factor X concentrations, v did not reach a plateau, as in the presence of 100 μM -PS/PC, but increased further, just as in the absence of phospholipid.

In further experiments, it was checked whether

Fig. 4. Effect of the Factor X concentration on the rate of Factor Xa formation in the presence of Ca^{2+} and phospholipid

Various amounts of Factor X were incubated with Factor IXa (15.8 nM) in the presence of 0 (■), 10 (□), 25 (●) and 100 (○) μM -PS/PC vesicles in 10 mM- CaCl_2 /100 mM- NaCl /50 mM- Tris/HCl , pH 7.5, at 37°C. The rates of Factor Xa formation were calculated from the Factor Xa concentrations after 10 and 20 min of incubation.

the rate of Factor Xa formation was linear with the Factor IXa concentration under the conditions of Fig. 4. In the presence of 0.4 μM -Factor X, linearity was observed between 0.5 and 15 nM-Factor IXa, at PS/PC concentrations between 20 and 180 μM . However, when the Factor X concentration was reduced 10-fold, the rate of Factor Xa formation was non-linear with the Factor IXa concentration (0.5–15 nM-Factor IXa, 180 μM -PS/PC), resulting in a decreasing turnover of Factor IXa at the higher enzyme concentrations.

It was concluded that, in the presence of Ca^{2+} and phospholipid, the activation of Factor X by Factor IXa cannot be easily described in terms of added concentrations of reactants. For a more adequate interpretation of the results of Fig. 4, it is necessary to know the distribution of enzyme and substrate between solution and the phospholipid membrane.

Relation between the rate of Factor Xa formation and the binding of Factor X and Factor IXa to the phospholipid membrane

In the preceding paper (Mertens *et al.*, 1984), it was demonstrated that Factor IXa and Factor X

compete for binding to a single class of sites on PS/PC membranes. The interaction of two ligands with one category of binding sites is given by the mathematical model described in the Experimental section. Since the required binding parameters of Factor IXa and Factor X are known for the experimental conditions of Fig. 4, the model can be used to calculate the concentrations of free and phospholipid-bound enzyme and substrate in the kinetic experiments. Fig. 5 shows the results of these calculations for two curves of Fig. 4: the data concern the phospholipid binding of Factor X (top panels) and Factor IXa (middle panels) at PS/PC concentrations of 100 and 25 μM (Figs. 5a and 5b respectively). The contribution of the reaction between free Factor IXa and free Factor X to the overall rate of Factor Xa formation can be calculated by using the kinetic parameters of the reaction in the absence of phospholipid (see Fig. 3). The difference between the experimentally observed reaction rate and the calculated rate of Factor Xa formation in solution (see Fig. 5, bottom panels), must be attributed to the participation of

phospholipid-bound factors in the reaction. The curves in Fig. 5 show that the phospholipid-dependent rate of Factor Xa formation, which will be referred to as Δv , and the concentration of phospholipid-bound Factor X reach a plateau at the same added Factor X concentration. On the plateau, the concentrations of free and bound Factor IXa are calculated to be variable, depending on the Factor X concentration. Therefore, the plateau for Δv cannot be explained by assuming that either free or bound Factor IXa converts bound Factor X, nor by considering free Factor X as the substrate for bound Factor IXa. Taking these considerations into account, we propose that, in the phospholipid-stimulated reaction, bound Factor X is the substrate for both free and bound Factor IXa.

By reploting data from Fig. 5, a linear relation was found between Δv and the concentration of phospholipid-bound Factor X. As can be seen in Fig. 6, this relation is independent of the phospholipid concentration; this indicates that Δv is dependent on the total concentration of phospho-

Fig. 5. Calculated phospholipid binding of Factor X and Factor IXa under the experimental conditions of Fig. 4 at (a) 100 and (b) 25 μM -phospholipid

Top panels: free (○) and bound (●) Factor X; middle panels: free (△) and bound (▲) Factor IXa; bottom panels: experimentally observed rate of Factor Xa formation (■, see Fig. 4) and calculated rate of the reaction between free Factor IXa and free Factor X (□).

Fig. 6. Relation between the concentration of phospholipid-bound Factor X and the rate of phospholipid-dependent Factor Xa formation (Δv)

The data concern the experiments of Fig. 4, at 25 (○) and 100 (●) μM -PS/PC vesicles. The concentration of bound Factor X was obtained from Fig. 5 (top panels). Δv was obtained by subtracting the calculated rate of the reaction between free Factors IXa and X from the experimentally observed rate of Factor Xa formation (see Fig. 5, bottom panels).

lipid-bound Factor X, but independent of the amount of phospholipid surface to which the Factor X is bound (see also the Discussion section). Fig. 6 further shows that the phospholipid-dependent reaction was not saturable with bound Factor X under the conditions of Fig. 4. This makes it difficult to estimate the kinetic parameters of the phospholipid-dependent reaction from these data. In Fig. 6, the Δv at low bound Factor X concentrations already exceeds the $V_{\max}$ of the reaction in solution. Obviously, the $V_{\max}$ of Factor Xa formation has been increased considerably due to the binding of Factor X to phospholipid. However, no conclusion can be drawn with regard to the K_m of Factor X in the phospholipid-bound system. This would require much higher concentrations of phospholipid-bound Factor X, and thus higher phospholipid concentrations, than in Fig. 6. However, this could not be achieved, because of the tendency of PS/PC vesicles to flocculate at high phospholipid concentrations (cf. Fig. 2).

Discussion

By using a sensitive spectrophotometric Factor Xa assay, we have demonstrated that human Factor IXa activates human Factor X in the absence of cofactors, and that Ca^{2+} stimulates this reaction (Fig. 1a). This observation is in agreement with the results of van Diejen *et al.* (1981), who

described similar experiments using bovine Factors IXa and X. However, in contrast with the bovine system, the human system has a sigmoid Ca^{2+} -dependence: at concentrations below 0.5 mM, Ca^{2+} does not stimulate the reaction (Fig. 1a). Similar sigmoid Ca^{2+} -dependences have been described for the activation of human Factor X by the Factor X-activating proteinase from Russell's viper (*Vipera russelli*) venom (Morris *et al.*, 1978) and for the activation of human Factor IX by the same venom proteinase or by Factor XIa (Amphlett *et al.*, 1981; Bajaj, 1982), and are probably associated with conformational changes that occur on the filling of a number of co-operative Ca^{2+} -binding sites on human Factors IX (Bajaj, 1982) and X (Morris *et al.*, 1978). It can be concluded from Fig. 3 that the Ca^{2+} -stimulated reaction is an inefficient process, with a high apparent K_m of Factor X (4.6 μM), and a low $V_{\max}$ for Factor Xa formation (0.0088 mol of Xa/min per mol of IXa). For the bovine system, the K_m of Factor X has been reported to be 181 μM (van Diejen *et al.*, 1981) and 16 μM (Link & Castellino, 1983), which is in both cases higher than for the human system; however, the reported values for $V_{\max}$ of the bovine system are similar to that found in our studies.

In addition to its function in the absence of phospholipid (Fig. 1a), Ca^{2+} is also involved in the expression of the cofactor effect of phospholipid (Figs. 1b and 2). It has been generally recognized that Ca^{2+} mediates the interaction of vitamin K-dependent proteins with membranes that contain negatively charged phospholipids (Stenflo & Suttie, 1977; Nelsestuen *et al.*, 1978; Dombrose *et al.*, 1979). In the preceding paper (Mertens *et al.*, 1984), we have determined the binding parameters for the Ca^{2+} -mediated interaction of human Factor IXa and Factor X with PS/PC membranes. The experimental conditions (pH, temperature, ionic strength, Ca^{2+} concentration) of the binding studies were identical with those of the kinetic experiments described in the present paper. Therefore the binding parameters could be used to interpret the effects of phospholipid on the Factor Xa formation in terms of binding of enzyme and substrate to the phospholipid membrane. By this analysis, the phospholipid-stimulated Factor Xa formation was found to be related to the phospholipid binding of Factor X, but not to that of Factor IXa (Figs. 5 and 6). When free and bound Factor IXa are equally efficient enzymes, the cofactor function of phospholipid must be associated exclusively with its Factor X-binding properties. In our experiments, the same relation between the phospholipid-dependent reaction rate and the concentration of phospholipid-bound Factor X has been observed at 25 and 100 μM -phospholipid (Fig. 6); apparently, a 4-fold change in the density of

Factor X on the PS/PC membrane has no effect on the Factor IXa-catalysed reaction, whereas a 4-fold increase in the concentration of bound Factor X has. It has further been observed that the addition of binding sites in excess over Factor X (thus decreasing the Factor X density on the membrane) is not associated with inhibition of the Factor Xa formation (see Fig. 2: PS/PC between 0.2 and 0.7 mM, at 0.1 μ M-Factor X). These results indicate that the rate of the phospholipid-dependent reaction is determined by the total concentration of bound Factor X rather than by the Factor X density on the phospholipid membrane. Figs. 2 and 6 further show that the rate of the phospholipid-dependent reaction can largely exceed the $V_{\max}$ of the reaction in solution (cf. Fig. 3). We therefore propose that, on binding to phospholipid, the substrate properties of Factor X change in such a way that the catalytic efficiency of the reaction increases; free and phospholipid-bound Factor IXa then are equally efficient in catalysing the activation of the phospholipid-bound Factor X (see Fig. 5).

The proposed model for the role of phospholipid in the activation of human Factor X by Factor IXa in the presence of Ca^{2+} differs from current concepts of phospholipid-dependent reactions in blood coagulation, as based on studies concerning the activation of Factor II (prothrombin) by the 'prothrombinase' complex. This complex, which consists of Factor Xa, Ca^{2+} , phospholipid and activated Factor V, is believed to be highly analogous to the intrinsic Factor X-activating complex (see Jackson & Nemerson, 1980). The importance of phospholipid for the assembly of the prothrombinase complex has been well-established, and it co-concentrates the enzyme (Factor Xa) and cofactor (Factor Va) on the membrane surface, thus forming the catalytically efficient Factor Xa-Factor Va complex (Jobin & Esnouf, 1967; Nesheim *et al.*, 1981; Lindhout *et al.*, 1982). It is not completely clear whether the substrate of this complex is free Factor II (Pusey & Nelsestuen, 1983) or phospholipid-bound Factor II (Tucker *et al.*, 1983). Recently, Nesheim *et al.* (1984) presented a mathematical model describing the prothrombinase reaction to occur in a perivesicular shell in which local substrate and enzyme concentrations determine the reaction rate. Such a 'concentration model' does not explain our observation that the concentration of bound Factor X, but not the Factor X density on the membrane, is related to the phospholipid-dependent reaction rate (see above). In the 'concentration model' for the prothrombinase complex, only the phospholipid-bound fraction of Factor Xa, in complex with Factor Va, contributes significantly to the activation of Factor II. If a similar model would apply to

the activation of Factor X by Factor IXa, increasing the Factor X concentration, which is associated with a decrease of the fraction of Factor IXa that is phospholipid-bound, would result in a decreasing reaction rate (substrate inhibition; see also Nesheim *et al.*, 1984). This clearly is in contrast with the results of Fig. 5, where free and bound Factor IXa appeared to be equally efficient enzymes. However, it should be realized that the studies concerning the prothrombinase complex involved the complete complex, thus including Factor Va, whereas in our studies the corresponding cofactor, Factor VIIIa, was not present. An explanation for the discrepancy between the prothrombinase model and our results might therefore be that only the phospholipid-bound fraction of the enzyme is stimulated by the presence of the cofactor, thus making the free enzyme relatively less efficient. With regard to this hypothesis, it will be of interest to investigate how the addition of activated Factor VIII to the system consisting of Factor IXa, Factor X, Ca^{2+} and phospholipid will affect the enzyme specificity of Factor IXa and/or the substrate properties of Factor X.

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