A Comparison of Phospholipid and Platelets in the Activation of Human Factor VIII by Thrombin and Factor Xa, and in the Activation of Factor X

By Pierre Neuenschwander and Jolyon Jesty

Two aspects of the activation of factor X by the intrinsic clotting pathway have been studied in purified human systems, in the presence of either purified phosphatidylserine:phosphatidylcholine vesicles (PS:PC) or platelets activated with ionophore A23187: (1) the activation of factor VIII by factor X and by thrombin, and (2) the activation of factor X by the factor IXa/VIIIa complex. Factor VIII activation by thrombin was unaffected in either rate or extent by the presence of PS:PC or activated platelets. In contrast, factor VIII activation by factor X required either PS:PC or platelets. The products of optimal factor VIII activation by the two enzymes, designated factor VIIIa and factor IXa, are kinetically different in the activation of factor X by factor IXa, factor VIIIa being approximately twice as active (in factor X activation) as factor VIIIa in the presence of PS:PC or platelets. Factor VIIIa can be converted to the more active VIIIa by thrombin treatment, but the activity of factor VIIIa is unchanged by factor X treatment. Factor X activation was also studied with optimally activated factor VIIIa, in the presence of PS:PC or activated platelets, as a function of factor IXa concentration in order to determine the apparent dissociation constant for the factor IXa-VIIIa interaction in the two cases. Activated platelets increased the apparent affinity more than fivefold.

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Materials and Methods

Tris base, bovine serum albumin (BSA; fatty acid free, fraction V), Lubrol-PX, Antifreeze C emulsion, ethylenediamine tetracetic acid (EDTA; tetrasodium salt), aprotinin, and A23187 were obtained from Sigma Chemical Co, St Louis. Anticoagulant-citrate-dextrose (ACD) solution was obtained from Fenwal Inc (Ashland, MA) and polyethylene glycol 6000 (PEG) was purchased from J.T. Baker Chemical Co (Phillipsburg, NJ). Tricine [N-tris(hydroxymethyl)-methylglycine], D-Phe-Pro-Arg-chloromethylketone (PPACK), dansyl-Glu-Gly-Arg-chloromethylketone, D-Phe-Phe-Arg-chloromethylketone, N-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and morpholinoethanesulfonic acid (MES) were obtained from Calbiochem-Behring Corp, San Diego. All chloromethylketone inhibitors were made up as 1 mmol/L solutions in 10 mmol/L HCl and stored at −20°C. Standard CaCl2 solution (1 mol/L) was from BDH Ltd (Gallard-Schlesinger, Carle Place, NY), and synthetic phosphatidylserine and phosphatidylcholine were purchased from Supelco, Inc (Bellefonte, PA) and prepared as a sonicated equimolar mixture (PS:PC) of 2 mg/mL in 0.1 mol/L NaCl, 0.05 mol/L tris-HCl pH 7.5 (tris-buffered saline; TBS) containing 1% PEG. All other chemicals were of reagent grade. The chromogenic substrate for factor Xa was Spectrozyme Xa (methoxy carbonyl-D-cyclohexylglycyl-Gly-Arg-p-nitroanilide acetate), obtained from American Diagnostica, Greenwich, CT. BioGel A-15M was obtained from BioRad Laboratories.

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Richmond, CA. The factor-VIII–deficient plasma for clotting assays (GK812-37D) was purchased from George King Biomedical (O\-verland Park, KS), and the activator for clotting assays was Platelet Plus from General Diagnostics, Morris Plains, NJ.

Human factors IX, and X were prepared by the method of Morrison and Jesty.23 The concentration of factor IX, was determined by reference to a standard factor IX, that had been assayed by titration with bovine antithrombin III. Preparation of factor X, was performed as described by Jesty.24 Human \-\alpha-thrombin was prepared by a derivation of the method of Fenton et al,25 using chromatography on CM-Sephadex C50. Its specific activity was 3,100 National Institutes of Health (NIH) U/mg.

Factor VIII/von Willebrand Factor

Partial purification was performed by gel filtration26’27 as follows. Two units (approximately 30 mL) of cryoprecipitate, obtained frozen from the Long Island Blood Center, were allowed to thaw at 37°C in the presence of 200 mmol/L PPACK, 200 mmol/L Phe-\-Phe-Arg-chloromethylketone, and 0.1 mmol/L sodium citrate, 0.05 mol/L NaCl, 0.5 mmol/L CaCl\(_2\). Thrombin (1.3 mmol/L, unless otherwise specified) was used to activate factor VIII and was added to the incubation 30 seconds before the addition of factor X (90 mmol/L) to start the reaction. Factor X, (0 to 4.4 mmol/L) was used as the factor VIII activator in place of thrombin for experiments designed to study factor X, feedback. The PS:PC:Ca\(_2\) system in all experiments concerning phospholipid was 25 mmol/L, which has been shown to be optimal for factor X activation in porcine, bovine, and human systems. After activation was started by the addition of factor X, 20-\muL samples were removed at intervals into microplate wells (25°C, thermostatted) containing 100 \muL 0.5 mmol/L factor X, substrate in “well buffer” for assay of factor X, activity. The well buffer consisted of 50 mmol/L tricine-HCl, 25 mmol/L NaCl, 25 mmol/L PPACK, 14 mmol/L EDTA, and 2% Lubrol-PX, pH 8.3.

Absorbance at 410 nm was measured every 0.7 to 1.5 minutes for 20 to 45 minutes, and the data were then fitted by iterative polynomial regression to obtain an initial slope, \(\text{DA}_{max}/\text{min}\), in each well. Factor X, generation curves were obtained by making a secondary plot of this data as factor X, activity \(v\) the time of the sampling from the incubation. Rates of factor X, generation were determined from these secondary plots by measuring the rate, after any observed lag (usually <30 seconds), by linear regression. The units for factor X, generation (mmol/L) were obtained by normalizing all the data to a factor X, standard assayed daily. The standard line \(\left(\text{DA}_{max}/\text{min}\cdot v\right)\) was linear to >0.1 mmol/L factor X,.
where \( E_0 \) and \( C_0 \) are initial factor IXa and VIIIa concentrations, \( K_{d_{app}} \) is the apparent Kd for the IXa-VIIIa interaction, and \( EC \) is the equilibrium concentration of the enzyme cofactor complex. This model assumes that rate of substrate cleavage is proportional to EC, i.e., when (as in our experiments) \( E_{max} \gg C_0 \), \( V \approx V_{max}EC/EC/C_0 \). In the experiments described, factor VIII was held constant in each set of incubations, while factor IXa was varied. In fitting plots of \( V \) vs. \( E_p \), \( C_0 \) was held constant at the respective factor VIII concentration used; this in turn was based on the assumption that 1 U factor VIII equals 1 pmol. The fitted parameters thus obtained were \( V_{max} \), which refers to the velocity at saturation of cofactor with enzyme, as opposed to the \( V_{max} \) for saturation with substrate, and \( K_{d_{app}} \). The meaning of \( K_{d_{app}} \) is considered further in the Discussion section.

RESULTS

The results are divided into three parts: (1) activation of factor VIII by factor Xa; (2) activation of factor VIII by thrombin; and (3) the kinetics of the IXa-VIIIa equilibrium. In each section we compare the role of PS:PC with that of activated platelets.

Activation of Factor VIII by Factor Xa

Phospholipid and platelet dependence. The phospholipid and platelet dependence of factor VIII activation by factor Xa was studied in the presence or absence of saturating amounts of PS:PC (25 \( \mu \)mol/L) or a near-plasma level of A23187-activated platelets (2 \( \times \) 10^9/mL). In order to ensure that factor VIII was limiting in these experiments, so that observed differences reflected differences in the activation of factor VIII, its concentration was held at 5% of its normal plasma level. Factor VIII (0.05 U/mL) was treated for two minutes with factor Xa (1.3 nmol/L), plus phospholipid or activated platelets, in the presence of factor IXa and Ca^{2+}. Factor Xa was then added to start factor Xa generation. When factor VIII was activated in the absence of phospholipid or platelets the appropriate component (PS:PC or activated platelets) was added with the factor Xa and was thus present during factor Xa generation. In each experiment factor Xa generation was monitored over a 12-minute time course.

Figure 1 shows the resulting factor Xa generation curves with PS:PC (A) or activated platelets (B). In the incubations containing exogenous factor Xa, the exogenous enzyme level was subtracted from the data to show only factor Xa generated. The top line in each panel shows factor Xa generation after factor VIII activation in the presence of PS:PC or platelets. In neither case do we see any measurable lag phase, which indicates that factor VIII was activated under these conditions. The middle lines in contrast show factor Xa generation after factor VIII activation performed in the absence of PS:PC or platelets, and clearly show a very low initial rate of factor Xa activation and a significant lag phase. The low initial rate strongly suggests that in the absence of PS:PC or platelets, factor Xa did not activate significant amounts of factor VIII during the two-minute preincubation. The fact that the lags observed in these incubations (B) are shorter than in the control experiments (C) can be explained by the fact that the latter contained no exogenous factor Xa; PS:PC or platelets were present in all incubations during the factor Xa activation phase.

Factor Xa dependence, PS:PC. The dependence of factor VIII activation on factor Xa concentration was studied in the presence of PS:PC in a way similar to that in Fig 1, but with shorter (30-second) preincubation times. Figure 2A shows the resulting factor Xa generation curves, the initial exogenous factor Xa concentration having been subtracted in each case. (This subtraction accounts for the fact that at high exogenous enzyme levels the activation curves may not exactly extrapolate to zero enzyme generated at zero time; the error, however, is clearly small.) The six- to eight-minute lag observed in the absence of exogenous enzyme was shortened to about four minutes by the addition of a mere 0.06 nmol/L factor Xa and at 0.6 nmol/L it was no longer apparent. At factor Xa concentrations of >2 nmol/L, no significant changes in factor Xa generation rate were observed, suggesting complete activation of factor VIII to have occurred. It should be noted that in contrast with its effect on the lag time, the factor Xa concentration had little effect on the maximal (postlag) rate of factor Xa generation. This probably reflects the fact that factor Xa is eventually generated in all mixtures at concentrations in excess over the initial addition of exogenous enzyme.

Because trace contamination of the proteins, particularly the factor Xa, with very small quantities of prothrombin could theoretically produce enough thrombin to feedback-activate factor VIII and interfere with the effect of factor Xa on factor VIII, experiments were also done at high factor IXa and factor VIII concentrations (11 nmol/L and 1 U/mL, respectively), and factor Xa generation was measured in the presence and absence of the thrombin inhibitor PPACK, 100
Phospholipid and platelet dependence. The phospholipid and platelet dependence of factor VIII activation by thrombin were studied in a similar fashion. Factor VIII was activated for 30 seconds with thrombin ± phospholipid or activated platelets, in the presence of factor IXa and Ca\(^{2+}\). Factor X containing 50 nmol/L PPACK was then added to inactivate the thrombin and start factor Xa generation. At this concentration PPACK has no significant effect on factor Xa. In the case of factor VIII activation in the absence of phospholipid or platelets, the missing component (PS:PC or activated platelets) was added with the factor X, as described for Fig 1.

Factor Xa generation was monitored over a 12-minute time course, and the resulting data are shown in Fig 3A and B for PS:PC and platelets, respectively. As before, when factor VIII was not initially activated (ie, no exogenous thrombin) a substantial lag was observed: six minutes for PS:PC and four minutes for platelets (data not shown; see Fig 1A and B, circles). Activation of factor VIII by thrombin reduced this lag to zero in both cases, showing maximal factor VIII activation by thrombin (■). In marked contrast to factor VIII activation by factor Xa, however, this was also observed when phospholipid and platelets were absent from the factor VIII activation step (□). Figure 3A also shows a control experiment confirming the phospholipid dependence of factor X activation. Factor VIII was maximally activated with 5.3 nmol/L thrombin in the absence of phospholipid and was added (with factor IXa and Ca\(^{2+}\)) to factor X (containing 50 nmol/L PPACK) in the absence of phospholipid. This resulted in no significant factor Xa generation over a time course of 12 minutes (○).

Although these results showed thrombin able to activate U/mL) was activated for 30 seconds with thrombin ± phospholipid or activated platelets, in the presence of factor IXa and Ca\(^{2+}\). Factor X containing 50 nmol/L PPACK was then added to inactivate the thrombin and start factor Xa generation. At this concentration PPACK has no significant effect on factor Xa. In the case of factor VIII activation in the absence of phospholipid or platelets, the missing component (PS:PC or activated platelets) was added with the factor X, as described for Fig 1.

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factor VIII maximally in the absence of phospholipid or activated platelets, any small effect of either phospholipid or activated platelets would not be detectable. To test the possibility that phospholipid or activated platelets might affect factor VIII activation, these experiments were repeated at a lower thrombin concentration (0.2 nmol/L), all other reaction components remaining as before. This concentration was chosen to ensure that the lag time was in the region of its greatest sensitivity to thrombin. The results of these experiments are shown in Fig 4A and 4B. Although a small difference in the extents of factor X activation can be seen, the lag phases are almost identical for each pair with both PS:PC and activated platelets.

Thrombin dependence. PS:PC. The dependence of factor VIII activation on thrombin concentration was studied in the same way as that with factor Xa, but consideration of the results is more difficult because of the factor Xa generated in the system. Varying concentrations of thrombin, 0 to 5.3 nmol/L, were used to preactivate factor VIII, with the final concentrations of other incubation components identical to those used in the factor Xa experiments (Fig 2). The shortening of the lag time with rising thrombin concentration (Fig 5A) is very similar to that seen with factor Xa (Fig 2A), but the final rate of factor X activation at saturating levels of thrombin is significantly greater, suggesting that in the presence of PS:PC, factor VIII maximally activated with thrombin is more active than factor VIII generated solely by factor Xa (see the Discussion section).

Thrombin dependence. Platelets. The effect of fully activated platelets, $2 \times 10^7$/mL, on factor VIII activation by thrombin was studied similarly; the results are shown in Fig 5B. The lag time observed with no activator was progressively shortened, as with PS:PC, until it was no longer detectable at 5.3 nmol/L thrombin; and once again, the maximum rate attainable was significantly higher than that attained by factor Xa activation of factor VIII (Fig 2B). It should also be noted that even at a platelet count of <10% the normal plasma count, the maximum rate of factor X activation is somewhat higher than with saturating levels of PS:PC (cf Fig 5A).

These observations together strongly suggest that factor VIII optimally activated by thrombin is of higher activity than that activated by factor Xa. This led us to compare directly the activity of the two through sequential activation experiments (Fig 6). Factor VIII (0.05 U/mL) was (1) maximally activated by factor Xa and then, 3.5 minutes after addition of factor Xa, exposed to thrombin (○); or (2) maximally activated with thrombin and then immediately exposed to factor Xa added along with the factor X (□). PS:PC was used as the phospholipid source, with all other reaction components remaining the same. Thrombin-activated factor VIII showed no apparent change in activity on exposure to exogenous factor Xa. On the other hand, factor Xa-activated factor VIII showed a significant increase in activity on subsequent exposure to thrombin. This new activity corresponded closely with the activity of thrombin-activated factor VIII alone and could not be duplicated by the addition of further factor Xa in place of thrombin (not shown).

Kinetics of the Factor IXa-VIIIa Interaction

In this section we describe studies concerning the factor IXa-factor VIIIa interaction and determination of the apparent dissociation constant. As described below, a real Kd value

![Fig 4](https://www.bloodjournal.org/bloodjournal/article-pdf/75/4/1765/405586/1765.pdf)

![Fig 5](https://www.bloodjournal.org/bloodjournal/article-pdf/75/4/1765/405586/1765.pdf)
cannot be determined kinetically at a fixed substrate (factor X) concentration. The results do, however, give us a good indication of the kinetics of the IXa-VIIIa interaction at the normal plasma concentration of factor X. In that sense, it is Kdapp that is meaningful.

PS:PC. In this series of experiments factor VIII (0.05 to 1 U/mL, ~0.05 to 1 nmol/L) was optimally activated with thrombin, and factor Xa generation rates were then determined as a function of varying factor IXa concentration (0 to 11 nmol/L). The factor X concentration was held constant at 90 nmol/L. Factor Xa generation was monitored over four minutes, and initial rates were determined from the early linear phase of factor Xa generation (as in Fig 5). Rates of factor Xa generation were then plotted vs factor IXa concentration, showing a saturable dependency at each factor VIII concentration (Fig 7). Because factor IXa was varied over the same range as the factor VIII concentration, fitting to a simple hyperbolic function was invalid. The data were therefore fitted to Equation 1 (see Materials and Methods), with [C]0 (factor VIII) held constant in the fitting procedure at its respective assumed value. Comparison of these kinetic parameters with those obtained with PS:PC showed no major difference in Vmax(EC) but did show a significant reduction in Kdapp. It should be noted that because the Kdapp is very low, and less than [C]0, the function (Equation 1, Materials and Methods) is almost linear at low enzyme

Table 1. Parameters of the Factor IXa-VIIIa Interaction in the Presence of PS:PC and Activated Platelets

<table>
<thead>
<tr>
<th>Component</th>
<th>[VIII] (U/mL)</th>
<th>Vmax ± SD (nmol/L/min)</th>
<th>Kdapp ± SD (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS:PC</td>
<td>0.05</td>
<td>8.9 ± 1.0</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>PS:PC</td>
<td>0.2</td>
<td>26.5 ± 4.3</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>PS:PC</td>
<td>0.5</td>
<td>59.3 ± 6.4</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>PS:PC</td>
<td>1.0</td>
<td>65.1 ± 6.7</td>
<td>0.55 ± 0.30</td>
</tr>
<tr>
<td>Platelets</td>
<td>1.0</td>
<td>73.7 ± 4.7</td>
<td>0.074 ± 0.069</td>
</tr>
</tbody>
</table>

The factor X activation data shown in Fig 7 (with PS:PC, 25 µmol/L) and Fig 9 (with A23187-activated platelets, 2 × 10⁷/mL) were fitted to Equation 1 to obtain Vmax and Kdapp for the equilibrium between factors IXa and VIIIa. For fitting purposes, it was assumed that 1 U factor VIII = 1 pmol (see Methods). Estimates of SD were derived from the nonlinear regression procedure. 29
concentrations. Thus, under these conditions, the half-maximal velocity does not correspond with $K_{d,app}$.

**DISCUSSION**

This study concerned two aspects of the activation of human factor $X$ by the human factor $IX_a$-VIII$_a$ complex: (1) differences in the activation of factor VIII by factor $X_a$ and thrombin, and (2) the kinetics of factor $X$ activation by factor $IX_a$-VIII$_a$. In both parts the role of PS:PC was compared with that of activated platelets. For the purposes of the discussion, we will refer to thrombin-activated factor VIII as factor VIII$_{a(T)}$ and to factor $X_a$-activated factor VIII as factor VIII$_{a(Xa)}$.

The reader will have noted that the factor VIII used in this study was a relatively crude preparation of factor VIII/vWF. This was chosen for three reasons.

1. We required that the preparation could be activated to a maximal extent by thrombin and factor $X_a$. As can be seen from various experiments (eg, Figs 2 and 5), the initial rates of factor $X_a$ generation in the absence of exogenous activator are much less than 1% of the maximum rate attained with optimal activator levels. Purer preparations of factor VIII tried initially were always less satisfactory in this regard and showed shorter lag times in factor $X$ activation.

2. We also required that the recovery of factor VIII activity during the preparation be as high as possible, so that we could be sure that the factor VIII used was not a selected preparation.

3. Finally, we noted that the natural plasma form of factor VIII is VIII/vWF, and we wished to maintain this in studies in pure systems.

**Factor VIII Activation by Factor $X_a$,Thrombin**

This section of the study was rather more qualitative than the subsequent investigation of factor $X$ activation, but nevertheless permitted significant conclusions. Two assumptions are crucial to the discussion that follows:

1. The initial rate of factor $X$ activation, and the closely related lag time, reflect the extent of factor VIII activation. Although we cannot yet quantitatively relate lag times to kinetic parameters of factor VIII activation, this assumption is intuitively reasonable.

2. The second assumption is that, other components being equal, the maximum (postlag) rate of factor $X_a$ generation is a measure of the cofactor activity of the factor VIII$_{a(Xa)}$ generated. This is perhaps not such a safe assumption; it is possible, for instance, that differences in factor $X$ activation rate might reflect differences in binding of factor VIII$_a$ to PS:PC and activated platelets, or to enzyme or substrate.

**Phospholipid, Platelet Dependence**

The results shown in Fig 1 demonstrate conclusively that the activation of factor VIII by factor $X_a$ requires a source of phospholipid. Although this has not to our knowledge been previously described, it is perhaps unsurprising when we consider the phospholipid requirement in the action of factor $X_a$ on factors VII, IX, X, and prothrombin. In contrast, thrombin activation of factor VIII is not measurably affected by PS:PC or activated platelets (Figs 3 and 4).

The observation that activated platelets have no effect on factor VIII activation by thrombin is at odds with the conclusions of Hultin, who studied factor VIII activation by thrombin in the presence and absence of platelets under several conditions. Although the data presented by Hultin support the general conclusion of an effect of platelets on factor VIII activity, we would like to mention some hazards in drawing specific conclusions from the shape of “generation curves,” such as are typical of the rise and decay of factor VIII activity in the presence of thrombin. These curves can be modeled at their simplest with a three-parameter two-exponential function such as we have used to study factor $X_a$ generation curves (Jesty, Equation 2). Lollar et al used a similar function to analyze the activation and inactivation of porcine factor VIII. However, these functions have the problem that as long as the generation and decay rate constants are unequal, the equations always have two solutions, with reversed rate constants: without knowledge of the amplitude parameter, the rate constants cannot be unequivocally assigned. For generation curves showing
different maximal factor VIII activities, it is thus impossible to know whether it is the rate of activation or inactivation that has changed. In the case of Hultin's observation that platelets increased the extent of factor VIII activation by thrombin, it is feasible that the cause was not an increase in the rate of factor VIII activation, but a reduction-in the rate of its inactivation in the presence of platelets.

**Enzyme Dependence, PS:PC, and Platelets**

From the dependence of the lag times on preactivation of factor VIII with varying factor Xa or thrombin concentrations, it appears that the kinetics of factor VIII activation by the two enzymes, as opposed to the activities of the different products, are very similar when PS:PC is used as the phospholipid source. The effect of platelets on factor VIII activation by thrombin was relatively small: the lag times at varying thrombin concentration were almost unchanged, even though the subsequent maximal rate of factor X activation was somewhat higher, as mentioned. Although with factor Xa as the factor VIII activator a very large difference was seen between low numbers of platelets and PS:PC, a tenfold increase in platelet count allowed rapid factor VIII activation by factor Xa, and abolished any observed lag time in factor Xa generation (Fig 1B).

**Factor VIIIa(Xa) vs Factor VIIIa(T) in Factor X Activation**

Before comparing the two factor VIII species, we should mention the difficulties in distinguishing between them in some of our experiments. In these studies, it is feasible that at the lower thrombin concentrations the factor Xa generation curves represent factor VIIIa(Xa) more than factor VIIIa(T) because of the generation of sufficient factor Xa to overshadow the effects of thrombin within the first few minutes. However, the most significant conclusions were obtained at high thrombin and factor Xa levels.

**PS:PC**

From the difference in maximum factor Xa generation rates in the presence of PS:PC (Figs 2A and 5A) and platelets (Figs 2B and 5B), we conclude that the factor VIII species produced by optimal activation with thrombin and factor Xa are kinetically different, factor VIIIa(Xa) being significantly less active in factor X activation than factor VIIIa(T). This is confirmed by the sequential-activation experiments (Fig 6), which clearly show the ability of thrombin to activate factor VIIIa(Xa) further, producing a factor VIII species that is indistinguishable in activity from factor VIIIa(T). Factor Xa does not have the ability to change the activity of factor VIIIa(T) to any significant degree. The activity ratio for the different factor VIII species [VIIIa(Xa)/VIIIa(T)], produced in the presence of PS:PC (Figs 2A and 5A), approaches 2. This observation confirms in an all-human system the results of Lollar et al. with purified porcine factor VIII:C. Although the obvious conclusion from these observations is that the species have different true cofactor activity, there are other equally plausible possibilities. For instance, the two species could bind differently to phospholipid, or indeed to factor IXa of factor X. As long as $K_d > [VIIIa]$, such differences in affinity would appear as differences in activity.

**Platelets**

Although there are small differences between activated platelets and phospholipid in the relative rates of factor X activation with factors VIIIa(Xa) and VIIIa(T), it is difficult to assign significance to them. However, one thing is abundantly clear: the cofactor effect of optimal (saturating) levels of PS:PC can be equaled or exceeded at a platelet count as low as $2 \times 10^7$/mL.

**Factor IXa-VIIIa Interaction**

The major focus of this section was a quantitative description of the interaction of factors IXa, and VIIIa(T). The study was largely limited to conditions reasonably close to the conditions in plasma—factor X was held constant at 90 nmol/L, $Ca^{2+}$ at $5\text{ mmol/L}$, and activated platelets, where studied, were used at a count of $2 \times 10^7$/mL. In terms of the general approach, the study was straightforward, but certain problems require discussion.

Previous examples of the study of enzyme-cofactor interactions in coagulation have been done mainly in the field of prothrombin activation, where the levels of the substrate (prothrombin) are much higher, and, with fluorimetric assay, continuous generation curves can be obtained. In contrast, the substrate concentration in this study was 90 nmol/L throughout. Moreover, because of the need to maximize sensitivity in the determination of initial rates, the assay method is discontinuous. With these restrictions, it has been impossible so far to determine activation rates with sufficiently small errors to test definitively the model we have used. Equation 1 of the methods can be derived from this mechanism:

$E + C \rightarrow EC \rightarrow ECS \rightarrow EC + P$

where E, C, and S are factors IXa, VIIIa, and X, respectively, and in this case $K_{app} = K_E/([E] + S)$ and $V_{max(E)}$ refers to the velocity of factor X activation when cofactor is saturated with enzyme; it does not refer to saturation with factor X. This model predicts that $K_{app}$ should remain constant and $V_{max(E)}$ ($K_E([E]S)$) vary, as E and C are varied at any given substrate concentration. Although our results tend to support this prediction at 0.5 to 1 U/mL factor VIII, they do not at 0.05 to 0.2 U/mL. It should be pointed out, however, that because of uncertainties, particularly in the assumption of factor VIII concentration, the estimation of parameters is not as reliable as one might wish for.

The results differ from similar studies by van Dieijen et al., who showed the $K_{app}$ for bovine factors IXa and VIIIa, to be relatively independent of factor VIIIa concentration. Aside from the difference in species, the cause of this discrepancy might be the different factor X concentrations used: our studies were done at approximately plasma concentrations (90 nmol/L), while van Dieijen et al. used 0.5 $\mu$mol/L factor X. Under the conditions most similar in the two studies, the difference in estimated $K_{app}$ is reasonable—about 50%. However, to reconcile these differences, it is clearly necessary to perform exhaustive studies of the type described here at varying substrate concentrations. In order
to analyze such data, a more complex model will be required to account for any variation of $K_{d_{app}}$ with [VIII] and [S].

Another point that deserves mention is the fact that while van Dieijen et al.13 used a simple hyperbolic function, we used the more complex quadratic function to fit our data. The reason for the difference is simple: in their experiments, factor VIII was held at about 10 pmol/L, which is below the range of factor IX concentrations studied; while in ours, the factor VIII was as high as 1 nmol/L, well within the range of factor IX concentrations used. Under the latter condition, use of a hyperbolic function is invalid, since it fails to account for changes in enzyme concentration resulting from formation of the enzyme-cofactor complex. It should be emphasized that Equation 1 is not a hyperbola and so $K_{d_{app}}$ cannot be determined simply by estimation of $K_{1/2}$; nor can it be estimated from a double-reciprocal or other basic transform of a hyperbola.

Factor IX-VIII Interaction in the Presence of Platelets

This part of the study was done at a single concentration of factor VIII. Although a more comprehensive analysis of the effect of activated platelets (involving different concentrations of factor VIII and high factor X concentrations) is pending, the analysis at 1 U/mL of factor VIII is significant, showing the ability of A23187-activated platelets to change the activation kinetics of factor X by a reduction in $K_{d_{app}}$. While the error on the parameter estimate is fairly large, visual inspection of the curve confirms a marked leftward shift and clearly demonstrates increased affinity. There was no significant difference in $V_{max}$ or in the conditions between activation with $2 \times 10^6$ activated platelets per milliliter and activation with saturating PS:PC. These observations and conclusions regarding $K_{d_{app}}$ are, of course, based on the premise that all the factor VIIIs or at least the same fraction, is bound to phospholipid surface, whether it be PS:PC vesicles or activated platelets. However, it is quite feasible that the differences we have observed are caused by differences in binding, rather than to differences in the factor IX-VIII interaction. Nonetheless, the change in $K_{d_{app}}$ lends kinetic support to the suspicion of many that the platelets' role in intrinsic factor X activation extends beyond that of merely supplying phospholipid.

REFERENCES


A comparison of phospholipid and platelets in the activation of human factor VIII by thrombin and factor Xa, and in the activation of factor X

P Neuenschwander and J Jesty