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

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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 Xa and 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 Xa required either PS:PC or platelets. The products of optimal factor VIII activation by the two enzymes, designated factor VIIIa and factor VIIIa, are kinetically different in activation by thrombin, and the kinetics of the factor IXa-VIIIa interaction.

MATERIALS AND METHODS

Tris base, bovine serum albumin (BSA; fatty acid free, fraction V), Lubrol-PX, Antifreeze, ethylenediamine tetraacetic 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). Tricinc [(N-tris(hydroxymethyl)-methylglycine, D-Phe-Pro-Arg-chloromethylketone (PPACK), dansyl-Glu-Gly-Arg-chloromethylketone, D-Phe-Phe-Arg-chloromethylketone, N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and morpholinopropanesulfonic 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, Spectrozyme Xa (methoxybenzyl-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 (Overland 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. 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. Human α-thrombin was prepared by a derivation of the method of Fenton et al, 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 filtration as follows. Two units (approximately 30 mL) of cryoprecipitate, obtained 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 µg/mL aprotinin. After removal of low-density lipid and particulate material by centrifugation at 10,000 g for 60 minutes at room temperature, the cryoprecipitate was loaded at 20 mL/h on a BioGel A15M column (3.4 x 90 cm) preequilibrated in buffer comprised of 0.05 mol/L NaCl, 0.5% PEG-6000 and 0.05 mol/L Tricine- HCl, 25 mmol/L MES-NaOH (pH 6.5), 0.5% PEG-6000 for two to three hours each dialyzed. The dialyzed protein was stored in small aliquots at -70°C. Under these storage conditions, >90% activity is retained for at least 4 months. The factor VIII activity of several preparations has been 3 to 4 U/mL by clotting assay relative to pooled normal human plasma. Overall recovery of factor VIII activity in the procedure was 70% to 80%. Clotting assays showed the fractions to be free of prothrombin, factor X, and antithrombin. SDS gel electrophoresis of the pooled fractions showed the product as being at least 80% von Willebrand factor (vWF), the remainder being almost entirely fibrinogen.

**Platelets**

These were isolated as follows. Thirty milliliters of whole blood were drawn from a volunteer into 4.5 mL of ACD. The blood was centrifuged at 650 x g for 4.5 minutes to obtain platelet-rich plasma (PRP). The PRP was carefully titrated with 1 mol/L acetic acid to a pH of 6.5, and then centrifuged at 1,800 x g for ten minutes to form a platelet pellet. The pellet was washed twice with 30 mL buffer containing 0.125 mol/L NaCl, 10 mmol/L HEPES-NaOH, 2.7 mmol/L KCl, 2 mmol/L MgCl, 25 mmol/L glucose, and 2 µg/mL aprotinin (pH 6.5). The final wash was vortexed in 1 mL of the same buffer, and a platelet count was obtained with a particle counter. Platelets isolated in this way were found to remain fully active for up to 12 hours in our assay system when kept with gentle agitation at room temperature, but were used within eight hours of their isolation. Platelet activations were performed at ten times the platelet count required in the factor X activation incubation by a two-minute exposure to 10 µmol/L A23187 at 37°C. Each activation was done immediately before the platelets' addition to the incubation as 1/2 the final volume.

**Assay of Factor X Activation**

This was performed with a p-nitroanilide substrate for factor X, (Spectrozyme X,) in a discontinuous amidolytic assay. Incubations were all performed at 37°C in a volume of 0.3 mL containing 0.1% BSA in a modified Tyrode's buffer pH 7.4, as previously described. Each incubation contained factor IX, (0.4 mmol/L unless otherwise specified), PS:PC or A23187-activated platelets, factor VIII/vWF, and 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 nmol/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 concentration in all experiments concerning phospholipid was 25 µmol/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 µL samples were removed at intervals into microplate wells (25°C, thermostatted) containing 100 µL 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, ΔA/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 (ΔA/min v [X,]) was linear to >0.1 µmol/L factor X, The reader should note that the "standard factor X," is purified human material, the concentration of which was determined from its absorbance and published extinction coefficient. Similarly, the concentration of the factor X was determined from its absorbance and published extinction coefficient. Given the margin of error in these four numbers, it should not be unexpected that a given amount of factor X might not generate an exactly equivalent amount of factor X, when maximally activated. We estimate the maximum error involved to be of the order of 20%.

A Dynatech MR600 microplate reader (Dynatech, Chantilly, VA), modified to include a thermostatted, Lucite plate holder, and controlled by an IBM PC, was used to measure absorbance changes. In some later experiments a similarly controlled Vmax reader (Molecular Devices, Palo Alto, CA) was used. The software used to control the readers and do the iterative regression will be described in detail elsewhere. All regression procedures, on both raw and derived data, were performed by the Marquardt method, as described by Bevington. The program used permits holding any parameter of a function constant, if required.

**Data Analysis**

To obtain values for Vmax (defined below) and Kd in studies of the factor IX-VIII interaction, factor X, generation rates at each concentration of factor VIII were measured as a function of factor IX, concentration and fitted to the quadratic form of an adsorption isotherm. In all these experiments the factor IX, concentrations were in the same range as the concentration of factor VIII, and the standard hyperbolic form of the adsorption isotherm, which requires one reactant always to be in large excess, could not be used for fitting the data. In this situation a quadratic form is necessary to account for the reduction in enzyme concentration (E) by the formation of enzyme-cofactor complex (EC), and the data were therefore fitted to the following equation, which was used by Morrison in a study of the factor X,-factor V interaction:

\[
EC = \frac{1}{2}(E_0 + C_0 + K_{d_{EC}}) \pm \sqrt{(E_0 + C_0 + K_{d_{EC}})^2 - 4E_0C_0} \]  

(1)
where $E_0$ and $C_0$ are initial factor IXa and VIIIi concentrations, $K_{d_{app}}$ is the apparent $K_d$ for the IXa-VIIIi interaction, and EC is the equilibrium concentration of the enzyme-cofactor complex. This model assumes that rate of substrate cleavage is proportional to EC, ie when (as in our experiments) $E_{max} > C_0$, $v = \frac{V_{max}}{EC/C_0}$. In the experiments described, factor VIIIi was held constant in each set of incubations, while factor IXa was varied. In fitting plots of $v$ vs $E_0$, $C_0$ was held constant at the respective factor VIIIi concentration used; this in turn was based on the assumption that 1 U factor VIII equals 1 pmol. The fitted parameters thus obtained were $V_{max}/EC_0$, 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 IXa; (2) activation of factor VIII by thrombin; and (3) the kinetics of the IXa-VIIIi equilibrium. In each section we compare the role of PS:PC with that of activated platelets.

Activation of Factor VIII by Factor IXa

Phospholipid and platelet dependence. The phospholipid and platelet dependence of factor VIII activation by factor IXa 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 x 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 IXa (1.3 nmol/L), ± 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 X 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 X-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 mmol/L factor Xa, and at 0.6 mmol/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
nmol/L. No difference was observed between the two, indicating a lack of thrombin interference (not shown). Omission of factor VIII from the incubation abolished any significant generation of factor Xa, as expected.

**Factor Xa dependence, platelets.** The experiments shown in Fig 2A were repeated, with activated platelets, \(2 \times 10^5\)/mL, replacing the PS:PC (Fig 2B). This low platelet count was chosen (1) to ensure that platelets were limiting in the mixture, and (2) to obtain maximal factor Xa activation rates approximately equivalent to those obtained with PS:PC. In the presence of platelets, factor VIII activation by exogenous factor Xa (0 to 0.6 nmol/L) shortened the lag time from ten minutes to about three minutes. However, this three-minute lag persisted even at the highest exogenous factor Xa level, 4.4 nmol/L. There are thus two salient differences between the factor Xa generation profiles with PS:PC and with platelets at \(2 \times 10^5\)/mL (Fig 2A and 2B). (1) A substantial lag persists in the presence of low numbers of platelets; this in contrast to the complete activation (zero lag) that can be attained at higher platelet counts (Fig 1B). (2) When factor VIII is eventually generated, even low numbers of activated platelets support a high rate of factor Xa activation.

**Thrombin Activation of Factor VIII**

**Phospholipid and platelet dependence.** The phospholipid and platelet dependence of factor VIII activation by thrombin were studied in a similar fashion. Factor VIII (0.05 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.

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 (circles). 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 (squares). Figure 3A also shows a control experiment confirming the phospholipid dependence of factor Xa 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 (circles).

Although these results showed thrombin able to activate...
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 x 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 (O); 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...
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 $K_{d_{app}}$ 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 X$_a$ generation rates were then determined as a function of varying factor IX$_a$ concentration (0 to 11 nmol/L). The factor X concentration was held constant at 90 nmol/L. Factor X$_a$ generation was monitored over four minutes, and initial rates were determined from the early linear phase of factor X$_a$ generation (as in Fig 5). Rates of factor X$_a$ generation were then plotted as a function of varying factor IX$_a$ concentration (Fig 7). Because factor IX$_a$ was varied over the same range as the factor VIII$_a$ concentration, fitting to a simple hyperbolic function was invalid. The data were therefore fitted to Equation 1 (see Materials and Methods), with $[C]_a$ (factor VIII$_a$) held constant in the fitting procedure in each case (see below). This analysis yielded the values for $V_{max(EC)}$ and $K_{d_{app}}$ (as defined in Material and Methods) given in Table 1.

In order to fix $[C]_a$ (factor VIII$_a$) in the fitting procedure, it was assumed that 1 U factor VIII equals 1 pmol. This was confirmed to be at least of the correct order of magnitude as follows. The data set obtained at 1 U/mL factor VIII (44 rate measurements in all) was also fitted to Equation 1 with all three parameters ($V_{max(EC)}$, $K_{d_{app}}$, and $[C]_a$) allowed to vary in the regression procedure. This gave a best estimate of $[C]_a = 1.0 \pm 0.5$ nmol/L. The large error estimate reflects the fact that even at this highest factor VIII concentration, the data are too noisy to justify general use of this approach.

**Platelets.** To determine whether activated platelets affect the factor IX$_a$-VIII$_a$ interaction, values of $V_{max(EC)}$ and $K_{d_{app}}$ were determined using fully activated platelets instead of PS:PC. However, in order to ensure that the platelet count was not limiting in these experiments, activation rates were first measured as a function of platelet count, up to $5 \times 10^8$/mL (Fig 8). A count of $2 \times 10^8$ per mL was chosen as producing near-maximum rate. With thrombin-activated factor VIII at 1 U/mL, activation rates were then measured as a function of factor IX$_a$ concentration. The remainder of each incubation was the same as used previously. A saturable dependency on factor IX$_a$ concentration was again observed (Fig 9), and nonlinear regression to Equation 1 yielded a $V_{max(EC)}$ of 73 nmol/L/min and a $K_{d_{app}}$ of 0.074 nmol/L (Fig 9 and Table 1). Fitting this data set to Equation 1, and allowing all three parameters to be fitted, gave an estimated factor VIII$_a$ concentration of 0.7 nmol/L—again in good agreement with the assumed value. Comparison of these kinetic parameters with those obtained with PS:PC showed no major difference in $V_{max(EC)}$ but did show a significant reduction in $K_{d_{app}}$. It should be noted that because the $K_{d_{app}}$ is very low, and less than $[C]_a$, the function (Equation 1, Materials and Methods) is almost linear at low enzyme concentration.
intrinsically activated factor X (IXa) generation was measured in each incubation as described for Fig 5. The final platelet counts were $2 \times 10^7$ (C), $5 \times 10^7$ (D), $2 \times 10^7$ (E), and $5 \times 10^7$ (F) per milliliter. Labels are $10^6$ platelets per mL.

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 IXa-VIIIa complex: (1) differences in the activation of factor VIII by factor IXa and thrombin, and (2) the kinetics of factor X activation by factor IXa-VIIIa. 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 VIIIa(T), and to factor IXa-activated factor VIII as factor VIIIa(IXa).

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 IXa. As can be seen from various experiments (e.g., Figs 2 and 5), the initial rates of factor IXa 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 VIIIa/vWF, and we wished to maintain this in studies in pure systems.

**Factor VIII Activation by Factor IXa, 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 IXa generation is a measure of the cofactor activity of the factor VIII 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 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 IXa 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 IXa 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 IXa 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 VIIIa 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 VIIIa 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 VIIIa 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 VIIIa species [VIIIa(T)/VIIIa(Xa)], 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., who showed the factor VIIIa(Xa) to be relatively independent of factor VIIIa(T), activity towards factor X.

Platelets

Although there are small differences between activated platelets and phospholipid in the relative rates of factor X activation with factors VIIIa(T) and VIIIa(Xa), 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 $2 \times 10^6$/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 mmol/L, and activated platelets, where studied, were used at a count of $2 \times 10^6$/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 model:

$$E + C \xrightarrow{K_c} EC \xrightarrow{K_s} ECS \xrightarrow{K_e} EC + P$$

where E, C, and S are factors IXa, VIIIa, and X, respectively, and in this case $K_{app} = K_{c} K_{s} / (K_{c} + S)$ and $V_{maxEC}$ 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 $V_{maxEC} (K_{eq}[EC])$ 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(T) 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_{app} with [VIIa] and [S].

Another point that deserves mention is the fact that while van Dieijen et al. 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_{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 IXα-VIIIα Interaction in the Presence of Platelets

This part of the study was done at a single concentration of factor VIII:C. Although a more comprehensive analysis of the effect of activated platelets (involving different concentrations of factor VIIIα and high factor X concentrations) is pending, the analysis at 1 U/ml of factor VIII:C is significant, showing the ability of A23187-activated platelets to change the activation kinetics of factor X by a reduction in K_{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,EC}, under these conditions between activation with 2 x 10^6 activated platelets per milliliter and activation with saturating PS:PC. These observations and conclusions regarding K_{app} are, of course, based on the premise that all the factor VIIIα, 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 IXα-VIIIα interaction. Nonetheless, the change in K_{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

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