Platelet Receptor Occupancy with Factor IXa Promotes Factor X Activation*

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To investigate the activated platelet surface as a locus for factor X activation, the functional consequences of factor IXa binding to platelets were studied. The concentration of factor IXa required for half-maximal rates of factor X activation in the presence of factor VIIIa and thrombin-activated platelets was 0.53 nM, which is close to the $K_d$ (0.56 nM) for factor IXa binding to platelets under identical conditions, determined from equilibrium binding studies. In direct comparative experiments, there was a close correspondence between equilibrium binding of factor IXa to thrombin-activated platelets in the presence of factor VIIIa and kinetic determinations of factor X activation rates. Analysis by polyacrylamide gel electrophoresis revealed that $^{125}$I-labeled factor IXa bound to platelets was structurally intact and did not form covalent complexes with platelet proteins. Factor IXa active site-inhibited by 5-dimethylaminonaphthalene-1-sulfonil glutamyl-glycylarginyl chloromethyl ketone was shown to be a competitive inhibitor of factor IXa binding in the absence ($K_i$ = 2.3 nM) and presence ($K_i$ = 0.43 nM) of factor VIIIa and factor X and of factor X activation ($K_i$ = 0.4 nM) by factor IXa in the presence of factor VIIIa, indicating that the generation of factor Xa is not required for factor IXa binding and that factor IXa bound tightly to activated platelets in the presence of factor VIIIa is closely coupled with rates of factor X activation. We conclude that factor IXa bound tightly to a platelet receptor in the presence of factor VIIIa is the enzyme active in factor X activation.

The interaction between blood platelets and coagulation factors is essential for normal coagulation and hemostasis. Activated platelets promote the catalysis of two sequential reactions in the blood coagulation cascade: the activation of factor X to factor Xa by a complex of factor IXa, factor VIIIa, and calcium ions (1–6), and the conversion of prothrombin to thrombin by a complex of factor Va, factor V, and calcium ions (7–12). Platelets possess specific, high affinity, saturable receptors for factor Xa (8, 9, 12), factor V (Va) (8, 11, 12), factor VIII (13), factor IX, and factor IXa (14). Although there is ample evidence that factor VIII and platelets (1–6) or phospholipids (15, 16) are essential cofactors that promote the proteolytic activation of factor X by factor Xa, the molecular mechanisms involved in the assembly of the factor X activating enzyme-cofactor complex on the platelet membrane are poorly understood.

Previously we have demonstrated rapid, reversible binding of both factor IX and factor IXa to thrombin-activated, gel-filtered platelets that requires the presence of physiologic concentrations of calcium ions (14). Factor IX was shown to bind to ~300 sites/platelet with a $K_d$ ~ 2.5 nM, either in the absence or in the presence of saturating concentrations of thrombin-activated factor VIII and factor X. In contrast, factor IXa was shown to bind to ~550 sites/platelet (including ~250 sites/platelet not shared by factor IX) with a $K_d$ ~ 2.5 nM in the absence of factor VIIIa and factor X (or in the presence of either protein alone), whereas in the presence of saturating concentrations of factor VIIIa (0.5–5.0 units/ml) and factor X (0.15–15 μM) together, factor IXa was bound to the same number of sites (~550/platelet) but with 5-fold higher affinity ($K_d$ ~ 0.5 nM). These results suggest the hypothesis that the enzymatic species active in factor X activation consists of factor IXa tightly bound ($K_d$ ~ 0.5 nM) to activated platelets in the presence of both factor VIIIa and factor X. To test this hypothesis we have now characterized the bound factor IXa both functionally and structurally.

EXPERIMENTAL PROCEDURES

Materials—d-Phenylalanyl-prolyl-arginy1-chloromethyl ketone (PPACK)1 and dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone-2 HCl (DEGR-CK) were purchased from Behring Diagnostics. The chromogenic substrate S2357 (Bz-Ile-Glu-(γ-piperidyl)-Gly-Arg-p-nitroanilide) was purchased from AB Kabi Diagnostica. Other materials were the same as reported previously (14).

Proteins—Details of the purification, assay, and characterization of human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin were previously published (14). All proteins were >98% pure as judged by polyacrylamide slab gel electrophoresis in NaDodSO4. Factor IXa (22.2 μM) was inactivated by incubation with DEGR-CK (600 μM) for 3 h at 25 °C (dansyl-Glu-Gly-Ang-factor IXa or DEGR-factor IXa) as described by Lollarr and Fass (17). For binding studies, $^{125}$I-labeled factor IXa (2.5 × 10$^{-6}$ cpn/μg) was prepared as previously described (14). Protein concentrations were determined by the BioRad dye binding assay according to the instructions provided by the manufacturer. Polyclamydlide slabs gel electrophoresis in NaDodSO4 was carried out according to the procedure of Laemml (18).

Binding Experiments—In a typical binding experiment, gel-filtered platelets (5–4 × 10$^{10}$/ml) in calcium-free HEPES Tyrode's buffer, pH 7.4, were incubated at 37 °C in a 1.5-ml Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa, divalent

1 The abbreviations used are: PPACK, d-phenylalanyl-prolyl-arginy1 chloromethyl ketone; dansyl, 5-dimethylaminonaphthalene-1-sulfonlyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEGR-CK, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone-2 HCl; DEGR-factor IXa, factor IXa active site inhibited with DEGR-CK; NaDodSO4, sodium dodecyl sulfate

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lations, platelet stimuli, and other proteins and platelets were separated from unbound proteins as previously described (14). The data were analyzed and the number of binding sites and dissociation constants (Kd) were calculated as previously described (14) using a Mac Plus Computer.

Measurements of Rates of Factor Xa Formation.—The activation of factor X by platelet-bound factor IXa was determined in the presence of CaCl2 and thrombin-activated factor VIII at concentrations of reactants indicated in the Figure legends. After stopping the reaction by the addition of 6 mM EDTA, the rate of factor X activation was measured from unbound proteins as previously described and the number of binding sites and dissociation constants were analyzed and the number of binding sites and dissociation constants were calculated as previously described (14) using a Mac Plus Computer.

The results of our kinetic studies were analyzed using two different sets of assumptions. Assuming relatively weak binding (or the situation in which the concentration of binding sites is smaller than the Kd), the apparent Kd can be expressed as:

\[ K_d = \frac{(E_0 - EC) - Co}{EC} \]  

where E0, total IXa concentration; Co, concentration of total IXa receptors; EC, concentration of IXa-receptor complex. In this case EC is small compared with Co and EC can be determined as:

\[ EC = E_0 - Co - K_d + Co \]  

where EC is proportional to initial velocity. However, when binding is tight or E0 is large compared with Kd, EC is not insignificant compared with Co and the expression becomes:

\[ K_d = \frac{(E_0 - EC) - (Co - EC)}{EC} \]  

This can be solved for EC (or velocity) to give:

\[ EC = \frac{(E_0 + Co + K_d) \pm \sqrt{(E_0 + Co + K_d)^2 - 4E_0Co}}{2} \]  

We solved this expression using a nonlinear regression program based on the method of Marquardt (19) and a TRS-80 computer to give a value of Kd based on the kinetic data, as previously described (6, 20, 22). Alternatively, the Kd was determined from graphical analysis using double-reciprocal plots of rates of factor Xa formation versus added factor IXa or from nonlinear regression analysis (19) of the hyperbola described in Equation 2. Values of Kd obtained from graphical analysis and using the two equations were in good agreement (i.e. within 15% of one another). Inhibition of factor X activation by DEGR-factor IXa was analyzed by Dixon plots as previously described by Stern et al. (22).

RESULTS AND DISCUSSION

Functional Characterization of Bound Factor IXa.—To characterize bound factor IXa functionally, platelets were incubated with factor IXa (0.18-3.3 nM), thrombin (0.1 unit/mL), and CaCl2 (5 mM) and after neutralization of thrombin with PPACK (50 nM), examined for their capacity to support factor X activation in the presence of thrombin-activated factor VIII as described under “Experimental Procedures.” The purpose of the present studies was to determine whether the apparent Kd of factor IXa binding from our equilibrium binding experiments corresponds with the concentration of factor IXa required for half-maximal rates of factor X activation in the presence of thrombin-activated platelets. The results (Fig. 1A), presented as a double-reciprocal plot of the rate of factor Xa formed versus the concentration of factor IXa added, indicate that the concentration of factor IXa required for half-maximal rates of factor Xa formation is 0.53 nM. This value is close to the Kd previously reported from equilibrium binding studies of factor IXa in the presence of factor VIIIa.
and factor X (14). Analysis of similar experiments carried out at factor X concentrations of 0.15, 0.5, and 1.5 μM, and factor VIIa concentrations of 0.5–5.0 units/ml gave similar results. Thus, the observed rates of factor X activation in this experiment should not reflect a significant contribution from free factor IXa. Consistent with this possibility are the results of a detailed kinetic analysis of the contributions of activated platelets to factor X activation (25). We have shown that the bound and the rates of factor Xa formation, both showing a "∼1,000-fold higher than the physiological concentration of factor X) is decreased to below the plasma factor X concentration (1.5 μM), as described in the legend to Fig. 1. Binding was determined as described under "Experimental Procedures." Maximal binding (100%) was determined by subtracting the nonspecific binding, i.e. the binding determined in the presence of excess unlabeled factor IXa or DEGR-factor IXa (0.4 μM) from total binding. The results shown represent residual factor IXa binding in the presence of unlabeled factor IXa in the absence (open circles) or presence (closed circles) of factor VIIIa and factor X and in the presence of unlabeled DEGR-factor IXa in the absence (open triangles) and presence (closed triangles) of factor VIIIa and factor X. The results presented are the mean ± S.E. of three separate experiments, each done in triplicate.

To study further the relationship between factor IXa binding and factor X activation, we carried out experiments in which both factor IXa binding and factor X activation were examined in the same incubation mixture, consisting of thrombin-activated platelets, 125I-labeled factor IXa, thrombin-activated factor VIII, factor X, PPACK (added to neutralize thrombin after platelet activation and before addition of factor VIIIa), and CaCl2. The results (Fig. 1B) demonstrate a close correspondence between the amount of factor IXa bound and the rates of factor Xa formation, both showing a hyperbolic relationship to the concentration of factor IXa added. By dividing rates of factor Xa formation by the amount of factor IXa bound at each concentration of added factor IXa, we could calculate turn over numbers (in moles of factor Xa per min/mol of factor IXa bound), which proved to be independent of enzyme concentration (Fig. 1B, inset) as should be the case for saturating substrate concentration. The mean (±S.E.) of the six Ks values shown in Fig. 1B (inset) is 2391 (±90) min⁻¹. In kinetic studies of factor X activation reported by Neuschwander and Jesty (6), the Ks for factor IXa binding of factor VIIIa in the presence of thrombin-activated platelets was found to be ∼7-fold tighter (Ks = 74 pm) than that found in the present study. In any case, the

[Fig. 2. Autoradiogram of 125I-factor IX, 125I-factor IXa, and platelet-bound 125I-factor IXa. Autoradiograms of 15% polyacrylamide gel electrophoresograms in NaDodSO₄ of 125I-labeled factor IX (nonreduced, Lane 1), 125I-labeled factor IXa (nonreduced, Lane 2), bound 125I-labeled factor IXa (reduced, Lane 3), and bound 125I-labeled factor IXa (reduced, Lane 4). Gel filtered platelets (3.8 × 10⁹/ml) were incubated for 10 min at 37 °C with 125I-labeled factor IXa (17 nm), thrombin (0.1 unit/ml), factor X (0.15 μM), factor VIIIa (1.0 unit/ml), and CaCl₂ (5 mM). After centrifugation through 20% sucrose, the platelet pellet was solubilized in 2% NaDodSO₄ containing 1 mM dithiothreitol, 1 mM benzamidine, and 1 mM EDTA, and applied to the gel. Mn, apparent molecular weights of protein standards.

Fig. 3. A, competition by unlabeled factor IXa and DEGR-factor IXa for 125I-labeled factor IXa binding sites on thrombin-activated platelets in the absence and presence of factor VIIIa and factor X. Gel filtered platelets (3.8 × 10⁹/ml) were incubated for 20 min at 37 °C with human α-thrombin (0.1 unit/ml), CaCl₂ (5 mM), 125I-labeled factor IXa (3.3 nM), various concentrations of unlabeled factor IXa or DEGR-factor IXa, and PPACK (50 nM) in the presence or absence of thrombin-activated factor VIII (2 units/ml) and factor X (0.15 μM), as described in the legend to Fig. 1. Binding was determined as described under "Experimental Procedures." Maximal binding (100%) was determined by subtracting the nonspecific binding, i.e. the binding determined in the presence of excess unlabeled factor IXa or DEGR-factor IXa (0.4 μM) from total binding. The results shown represent residual factor IXa binding in the presence of unlabeled factor IXa in the absence (open circles) or presence (closed circles) of factor VIIIa and factor X and in the presence of unlabeled DEGR-factor IXa in the absence (open triangles) and presence (closed triangles) of factor VIIIa and factor X. The results presented are the mean ± S.E. of three separate experiments, each done in triplicate. B, inhibition of platelet-bound factor IXa (FIXa)-factor VIIIa-mediated factor X activation by DEGR-factor IXa. The rate of factor Xa formation was determined in the presence of various concentrations (0.5–50 nM) of DEGR-factor IXa in the presence of 5 × 10⁻¹⁰/ml of thrombin-activated (0.1 unit/ml), gel filtered platelets at 37 °C in a reaction volume of 100 μl containing 50 mM Tris, pH 7.9, 175 mM NaCl, 5 mM CaCl₂, 1.5 μM factor X, 5 units/ml of factor VIII (activated with thrombin as described in the legend to Fig. 1), and 0.5 mg/ml human serum albumin. Platelets were stimulated with 0.1 unit/ml thrombin in the presence of CaCl₂ (5 mM). Factor IXa, 0.5 nM (open circles), 1.5 nM (closed circles), and DEGR-factor IXa (0.5–50 nM) were preincubated with platelets for 10 min at 37 °C. Excess thrombin was neutralized with 50 nM PPACK prior to addition of factor VIIIa and performance of the assay.

Structural Characterization of Bound Factor IXa—To char-
characterize the bound factor IXa structurally, platelets were incubated with thrombin, CaCl$_2$, factor VIIIa, and factor X for 10 min at 37°C and centrifuged through 20% sucrose to separate bound from free ligand. Platelet pellets were solubilized in NaDdSO$_4$ and were analyzed by polyacrylamide gel electrophoresis and autoradiography. More than 96% of $^{125}$I was solubilized and applied to the gel. The bound radioligand migrated as an M$_r$ 44,000 protein on nonreduced gels (Fig. 2, lane 2) and was indistinguishable from free factor IXa (Fig. 2, lane 2). After reduction, the bound factor IXa migrated as two polypeptides of M$_r$ 27,000 and 17,000 (Fig. 2, lane 4). $^{125}$I-Labeled factor IXa, which was a single band at M$_r$ 57,000 is shown for comparison (Fig. 2, lane 1). When factor X and factor VIIIa were excluded from the incubation mixture, identical results were obtained. This experiment provides no evidence for the formation of high molecular weight covalent complexes or for proteolytic degradation of factor IXa by platelets, and confirms that the bound radioactivity consists entirely of factor IXa and not a radiolabeled contaminant. The fact that the bound factor IXa is structurally intact and indistinguishable from free factor IXa provides strong evidence that the functional factor X activating complex consists of factor IXa bound to platelets in the presence of factor VIIIa.

Competition Studies with Factor IXa and DEGR-Factor IXa—We carried out competition studies with unlabeled factor IXa and DEGR-factor IXa by incubating thrombin-stimulated platelets in the presence of CaCl$_2$ for 20 min at 37°C with $^{125}$I-labeled factor IXa and various concentrations of unlabeled proteins in the presence or absence of factor X and thrombin-activated factor VIII. When residual binding of $^{125}$I-labeled factor IXa was determined (Fig. 3A), it was apparent that excess factor IXa and DEGR-factor IXa prevented $>$95% of $^{125}$I-labeled factor IXa binding. The presence of factor VIIIa and factor X significantly decreased the concentration of unlabeled factor IXa required to displace bound $^{125}$I-labeled factor IXa from thrombin-activated platelets. Addition of excess unlabeled DEGR-factor IXa in the presence of factor VIIIa and factor X showed displacement curves identical to those observed with excess unlabeled factor IXa, suggesting that active site-blocked factor IXa competes equally well with unlabeled factor IXa and that the active site is not required for binding of factor IXa to thrombin-stimulated platelets. From the results presented in Fig. 3A, it is estimated that the concentration of factor IXa and DEGR-factor IXa required for half-maximal inhibition of factor IXa binding in the absence of factor VIIIa and factor X were 2.5 and 2.6 nM, respectively, and the concentration of factor IXa and DEGR-factor IXa required for a similar effect in the presence of factor VIIIa and factor X were 0.5 and 0.6 nM, respectively. Furthermore, we have determined the inhibition constant (K$_i$) for both factor IXa and DEGR-factor IXa both in the presence and absence of factor VIIIa and factor X using a computer fit displacement curve as described by Rodbard (23) and modified to calculate the K$_i$ using the formula of Cheng and Prusoff (24). These values indicate that the K$_i$ for factor IXa and the K$_i$ for DEGR-factor IXa in the absence of factor VIIIa and factor X were 2.2 and 2.3 nM, respectively, and the K$_i$ for factor IXa and for DEGR-factor IXa in the presence of factor VIIIa and factor X were 0.38 and 0.43 nM, respectively.

Finally, we examined the effect of DEGR-factor IXa on the activation of factor X by factor IXa in the presence of thrombin-activated platelets and factor VIIIa (Fig. 3B). The resulting Dixon plot suggests that DEGR-factor IXa is a competitive inhibitor of factor IXa with a K$_i$ of 0.4 nM. Thus, both in competition binding studies (Fig. 3A) and in factor X activation (Fig. 3B), DEGR-factor IXa behaved as a competitive inhibitor of factor IXa binding. These results also indicate that factor IXa binding to platelets is essential for and is closely correlated with factor X activation. Equally important is the conclusion that the conversion of factor X to factor Xa is not required for the high affinity binding of factor IXa to its platelet receptor.

The studies reported herein and previously by our laboratory (1, 2, 14, 25) and by other investigators (3–6, 13) give rise to the hypothesis depicted schematically in Fig. 4. This presents a two-receptor model in which factor IXa and factor VIII can bind independently to two separate and distinct sites on the surface of activated platelets. As shown by Nesheim et al. (13), factor VIII can bind saturably, reversibly, and specifically to ~450 sites/platelet with a K$_D$ of ~5.0 nM. The effects of factor IXa and factor X on factor VIII binding have not been reported, nor have studies of factor VIIIa binding been published. The requirement of factors VIIIa and X that we have reported (14) for the induction of high affinity binding sites for factor IXa on platelets is similar to the requirement for factors VIII and X reported by Stern et al. (22) for the induction of high affinity factor IXa binding sites on endothelial cells. Direct comparative experiments have not yet been reported to examine the relationship between factor VIII (or VIIIa) receptor occupancy and rates of factor X activation. However, we have shown that, whereas by itself, factor VIIIa has no effect on the kinetics of factor X activation by factor IXa, the addition of saturating concentrations of factor VIIIa (13, 14) to incubation mixtures containing thrombin-activated platelets decreases the K$_m$ of factor IXa-catalyzed factor X activation.

Fig. 4. Hypothesis depicting platelet receptor-mediated factor X activation by factor IXa. The symbols R1 and R2 refer to platelet membrane binding sites for factor IXa and factor VIII, respectively. Each reversible equilibrium is designated by arrows with dissociation constants (Kd) or Michaelis constants (Km) indicated. Roman numerals refer to coagulation proteins. Unidirectional arrows are enzymatic reactions characterized by catalytic constants (kcat). Details are discussed in the text.
Factor X Activation by Platelet-bound Factor IXa

Our present experiments (Fig. 1B) suggest that the true $k_{cat}$ (i.e., the maximal rate of factor Xa formed in moles/min/mol of factor IXa bound or $\sim 2400$ min$^{-1}$) may be considerably higher than that previously estimated (i.e., $\sim 500$ moles of factor Xa formed per min/mol of total factor IXa added; Ref. 25). Although further details of this hypothesis (Fig. 4) remain to be worked out, we believe its central features are supported by our present and previous studies (1, 2, 14, 25) and those reported by others (3-6, 13), i.e., that factor X activation by factor IXa is a platelet-receptor-mediated process tightly coupled to receptor occupancy by factor IXa and factor VIIIa.

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