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Research Article

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Platelet Receptor-mediated Factor X Activation by Factor IXa

High-Affinity Factor IXa Receptors Induced by Factor VIII Are Deficient on Platelets in Scott Syndrome

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Abstract

We have studied factor IXa binding and factor X activation with normal platelets and with platelets obtained from a patient with a bleeding disorder and an isolated deficiency of platelet procoagulant activity termed Scott syndrome. In the absence of factor VIIIa and factor X, normal, thrombin-treated platelets exposed 560±35 sites for factor IXa with a K_d of 2.75±0.27 mM, compared with 461±60 sites per patient platelet with K_d of 3.2±0.33 nM. The addition of factor VIIIa and factor X resulted in a decrease in the K_d for normal platelets to 0.68 nM but had no effect on the K_d for patient platelets. The concentrations of factor IXa required for half-maximal rates of factor X activation for normal (0.52 nM) and patient platelets (2.5 nM) were similar to those determined from equilibrium binding studies. Kinetic parameters for factor X activation by factor IXa showed that the K_m and k_{cat} were identical for normal and patient platelets in the absence of factor VIIIa. In the presence of factor VIIIa, and k_{cat} for patient platelets (163 min^{-1}) was only 33% of that for normal platelets (491 min^{-1}). This result can be explained by the difference in affinity for factor IXa between normal and patient platelets in the presence of factor VIIIa, suggesting impaired factor VIIIa binding to Scott syndrome platelets.

Introduction

Platelets promote the catalysis of two sequential, calcium-dependent reactions in blood coagulation: the activation of factor X by a complex of factor IXa and factor VIIIa (1–6) and the conversion of prothrombin to thrombin by a complex of factor Xa and factor Va (7–12). The contribution of platelets to prothrombin activation is receptor mediated, since platelets possess specific, high-affinity, saturable binding sites for factor Xa (7, 10) and factors V and Va (11, 12) and receptor occupancy is closely correlated with rates of prothrombin activation (7–12). Recently we have demonstrated that activated human platelets also have specific, saturable receptors for factor IXa involved in factor X activation (13). The presence of factor VIII, which also binds to specific, high-affinity sites on activated platelets (14), together with factor X, increases fivefold the affinity of factor IXa binding to activated platelets (13). In order to investigate in more detail the mechanism of factor X activation by platelet-bound factor IXa, we have carried out studies of factor IXa binding and factor X activation with normal platelets and with platelets obtained from a patient with a bleeding disorder termed Scott syndrome (15, 16). These platelets, which have previously been shown to have an isolated deficiency of platelet procoagulant activity (15), lack factor Xa binding sites (17) and are deficient both in their capacity to promote prothrombin activation (17, 18) and factor X activation (18) and to expose phosphatidylserine at the outer surface of stimulated platelets (18).

Methods

Platelets. Gel-filtered platelets were isolated as described previously (13). The patient, MS, who has been reported in detail by Weiss et al. (15), is a 45-yr-old woman with a moderate bleeding disorder now referred to as Scott syndrome (16). She visited Philadelphia and her platelets were studied on three separate occasions in direct comparison with control platelets adjusted to the same platelet count.

Purification of proteins. Factor IX (333 U/mg) was purified to apparent homogeneity from human plasma by immunoaffinity chromatography and was converted to factor IXa by incubation with purified factor XIa as previously described (13). Factor IX was radiolabeled with ^{125}I by the Iodogen method (19), as previously described (13). The majority of the free ^{125}I was then separated from the protein by passage through a 1-ml G-25 column (20), and the labeled protein was further dialyzed in the presence of ovalbumin (1 mg/ml). Purified human factor X had a specific activity of 225 U/mg and appeared as a single band with an apparent molecular weight of 72,000 on nonreduced SDS gels as reported previously (13). Purified human factor VIII, kindly provided by Dr. J. E. Brown (Cutter Biological, Miles, Inc., Berkeley, CA), had a specific activity of 1,850 U/mg, was devoid of von Willebrand factor antigen, and consisted of protein bands with molecular masses of 230, 92, 80, and 40 kD on SDS-PAGE as previously described (13). The factor VIII activity was increased 38-fold in 1 min by incubation of the protein with 0.05 U/ml of human α-thrombin, was stable for ~ 3 min and remained within 50% of its maximal activity for at least 10 min, during which time all binding and factor X activation experiments were completed.

Binding experiments. These were carried out as reported in detail earlier (13) in triplicate on three separate occasions using platelets obtained from MS and from three separate normal donors. Saturation binding data were analyzed by the method of Scatchard (21) as previously reported using linear regression analysis and assuming a single class of binding sites.

Measurement of rates of factor Xa formation. Activation of factor X by factor IXa in the presence of stimulated platelets either in the presence or absence of factor VIIIa (for further experimental condi-

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tions, see figure legends) was monitored in incubation mixtures containing platelets, factor IXa, $CaCl_2$, and thrombin at 37°C for 10 min in a buffer containing 50 mM Tris-HCl, 175 mM NaCl, and 0.5 mg/ml human serum albumin (pH 7.4). Excess thrombin was neutralized with 50 nM D-Phe-Pro-Arg-chloromethylketone (PPACK,[1] Calbiochem-Behring Corp., San Diego, CA) prior to the addition of factor VIIIa and factor X to the reaction mixture. After 3 min (presence of factor VIIIa) or 10 min (absence of factor VIIIa) at 37°C 10 mM EDTA was added to prevent further activation of factor X by factor IXa. During this time period rates of factor Xa formation were linear. The release of *p*-nitroanilide from 0.5 mM S2337 (factor Xa–specific chromogenic substrate from AB Kabi Diagnostica, Stockholm, Sweden) by factor Xa was followed for 10 min, during which period the rates of cleavage of S2337 were linear. The reaction was stopped by the addition of glacial acetic acid (5.2% final concentration) and the amount of pNA released was measured at 405 nm in a 96-well microtiter plate (No. 3075 plates, Falcon Labware, Oxnard, CA) using a Vmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA). Factor IXa alone had no amidolytic activity against S2337. From a calibration curve made with known amounts of factor Xa under the same conditions as described above, the amount of factor Xa present in the aliquot was calculated. The rates of factor Xa formation in the reaction mixture were calculated from the amounts of factor Xa present in the samples. The amount of factor IXa present in the incubation mixture was chosen such that < 10% of added factor X was converted to factor Xa during the time course of the experiment.

Calculation of kinetic constants. The derivation of kinetic constants for factor X activation by factor IXa was based on a one-enzyme, one-substrate model. Values for the Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated from the mean±standard error of the mean of three independent determinations by the Lineweaver-Burk method (22) and were calculated by using least-squares fit and the FORTRAN program of Cleland (23) with a TRS 80 computer. Values of kinetic constants obtained by the Cleland program and by graphical analysis were in close agreement (i.e., within 5%). The apparent dissociation constant for factor IXa binding to platelets was also determined from kinetic measurements of factor Xa formation by treating factor IXa binding to its receptor as a simple adsorption isotherm in which the concentration of the variable ligand (factor IXa) is well in excess of the other reactant (the factor IXa binding site). Under these conditions the binding constant could be determined from a linear transformation of a simple hyperbola expressing factor X conversion versus factor IXa concentration. Justification for this approach is as follows. The concentration of factor X is sufficiently high that < 10% is transformed to factor Xa during the time of assay at any concentration of factor IXa. Consequently, the concentration of factor X is essentially constant, always saturating, and its effect on factor IXa binding does not change. The rate of factor Xa formation is linear and is proportional to the fraction of factor IXa bound to the receptor since binding to the receptor is required to give a significant rate of factor X conversion (13). The concentration of receptor can be calculated as follows, assuming about 500 receptor sites per platelet. The platelet count is 5×10^7/ml or 5×10^{10}/liter, so that the total number of receptors per liter is 2.5×10^{13}. Dividing by Avogadro's number gives about 0.04 nM receptor. This is well below the dissociation constant (13) for factor IXa binding (0.5 to 2.5 nM) so that the concentration of free factor IXa can be considered equal to the total concentration of factor IXa. As a result it is not necessary to analyze the curves by quadratic expressions (24, 25) that take into account a reduction in factor IXa concentration through its binding to receptor.

Results and Discussion

We have previously demonstrated that ^{125}I-labeled factor IXa binds in a saturable and reversible manner to thrombin-activated platelets and that equilibrium is attained in 5–10 min. Scatchard analysis indicated the presence of 515 sites per platelet with a K_d of 2.68 nM in the absence of factor VIIIa and factor X, and 551 sites per platelet with a K_d of 0.56 nM in the presence of factor VIIIa and factor X. Both factor VIIIa and factor X were required to give the observed fivefold increase in affinity for factor IXa, indicating that perhaps a complex of these two proteins modified an existing binding site for factor IXa (13). In the present work, we have compared binding of ^{125}I-labeled factor IXa to normal platelets with binding to Scott syndrome platelets (Fig. 1 *A*). With normal platelets we obtained 560 sites per platelet with a K_d of 2.75 nM in the absence of factor VIIIa and X; and 538 sites per platelet with a K_d of 0.68 nM in the presence of factor VIIIa and factor X, in excellent agreement with our previous findings (13). With Scott syndrome platelets we obtained 461 sites per platelet with a K_d of 3.2 nM in the absence and 412 sites per platelet with a K_d of 2.5 nM in the presence of factor VIIIa and factor X. The most significant difference observed between normal and Scott syndrome platelets was the absence of enhanced affinity of binding of factor IXa induced by factor VIIIa and factor X in the patient platelets (Fig. 1 *A* and Table I).

In this study we also determined an apparent K_d for factor IXa binding by kinetic studies of factor Xa formation in the presence of saturating factor X and factor VIIIa (Fig. 1 *B*). The kinetic approach gave similar results to the binding studies (13) and its use is justified under Methods. Hence, kinetic studies were performed in the presence of saturating levels of factor X and factor VIIIa. The apparent K_d was determined as 0.52 nM for normal platelets and 2.5 nM for Scott syndrome platelets, indicating again that the affinity of the patient platelets for factor IXa was not enhanced by factor X and factor VIIIa (Fig. 1 *B* and Table I).

We also determined the kinetic parameters for factor X activation by factor IXa in the presence of normal or patient platelets either in the absence (Fig. 2 *A*) or presence (Fig. 2 *B*) of thrombin-activated factor VIII. Studies were performed at a factor IXa concentration of 0.01 nM, which is well below the apparent dissociation constant for binding of factor IXa to normal or patient platelets. In the absence of factor VIIIa, the K_m and k_{cat} were almost identical for normal and patient platelets (Table I). In contrast, in the presence of saturating concentrations of factor VIIIa, while the K_m for patient platelets decreased from 0.5 to 0.11 μM in a manner comparable to normal platelets, the k_{cat} for the patient's platelets (163 min^{-1}) was only 33% of that for normal platelets (491 min^{-1}). This finding can be explained by the difference in affinity for factor IXa between normal and patient platelets in the presence of factor VIIIa. Hence, assuming hyperbolic binding of factor IXa that is directly related to the rate of factor Xa formation, the velocity (v) at saturating factor X can be determined from the expression: $v = V_{max} \cdot [IXa]/(K_d + [IXa])$, where V_{max} is the velocity obtained at saturating factor IXa concentration (and saturating factor X) and K_d is the dissociation constant for binding of factor IXa to platelets.

In normal platelets, in the presence of factor VIIIa, K_d is 0.52 nM and at 0.01 nM factor IXa, v can be calculated as 1.9% of V_{max}. For patient platelets under the same conditions, v is 0.4% of V_{max}. Hence, at 0.01 nM factor IXa, the velocity determined at saturating factor X for patient platelets would be expected to be $100 \times 0.4/1.9$ or 21% of that determined for normal platelets, which is close to the value of 33% that is

1. *Abbreviation used in this paper:* PPACK, D-phenylalanylprolylarginyl chloromethyl ketone.

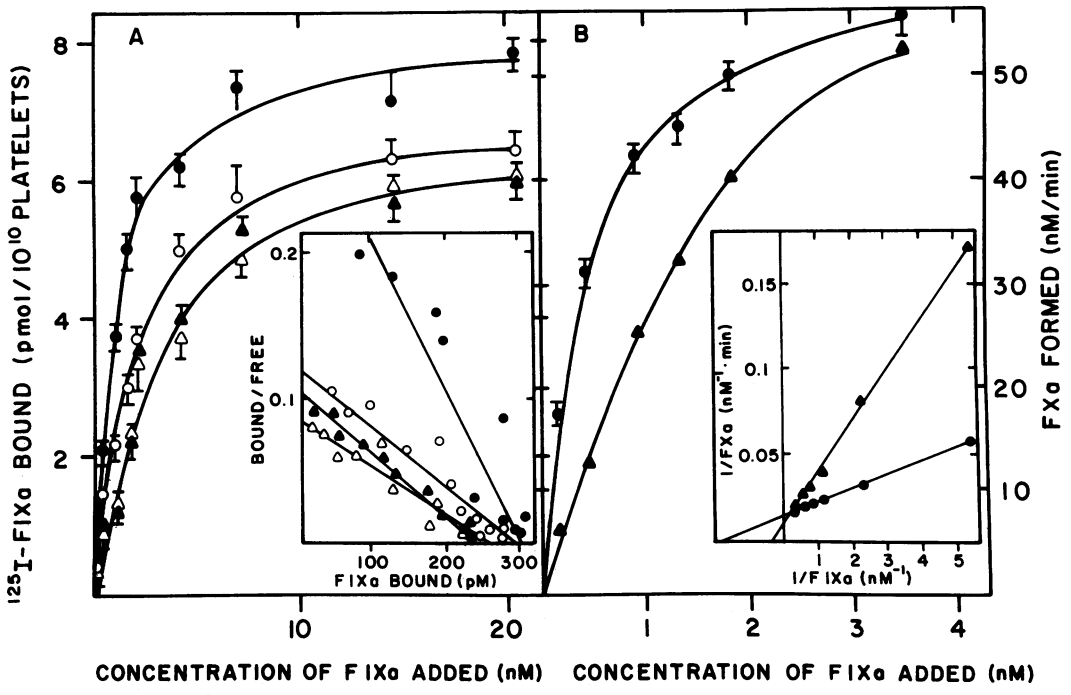

Figure 1. (*A*) Specific binding of ^{125}I-factor IXa to thrombin-activated normal human platelets and in Scott syndrome in the absence and presence of factor VIIIa and factor X. Gel-filtered platelets (3.5×10^8/ml) were incubated at 37°C with human α-thrombin (0.1 U/ml), $CaCl_2$ (5 mM), and ^{125}I-factor IXa in the presence or absence of thrombin-activated factor VIII (5 U/ml) and factor X (1.5 μM). Binding was determined as detailed under Methods. Nonspecific binding was determined in the presence of excess unlabeled factor IXa (0.44 μM; 25 μg/ml) and was subtracted from total binding to obtain specific binding. The results shown represent specific binding of factor IXa in the absence (○) or presence (●) of factor VIIIa and factor X to normal platelets and in Scott syndrome in the absence (△) and presence (▲) of factor VIIIa and factor X. Results represent means±SEM for three separate experiments done on three separate days with three separate normal donors. (*Inset*) Scatchard plot of the same data including data obtained at higher ligand concentration. (*B*) Rates of factor Xa formation by factor IXa in the presence of thrombin-activated platelets and factor VIIIa. The rate of activation of human factor X by varying concentrations (0.17–3.5 nM) of factor IXa was determined in the presence of 5×10^7/ml stimulated platelets in 37°C in a reaction volume of 100 μl containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 1.5 μM factor X, 5 U/ml of factor VIII, and 0.5 mg/ml human serum albumin. Platelets were stimulated with 0.1 U/ml thrombin in the presence of $CaCl_2$ (5 mM), and factor IXa was preincubated with platelets for 10 min at 37°C. Excess thrombin was neutralized with 50 nM PPACK before addition of factor VIIIa and performance of the assay. For experimental details, see Methods. The results presented are the means±SEM of duplicate observations with platelets from three separate normal donors (●) and means of duplicate observations with MS platelets (▲). (*Inset*) Double reciprocal plot of the same data.

actually observed. By this reasoning, the rates of factor Xa formation by both normal and patient platelets should be identical when the factor IXa site is saturated. This is borne out by the data in Fig. 1 *B* that shows the same rate of factor Xa formation by normal and patient platelets as factor IXa approaches saturation. Moreover, we have actually calculated the turnover numbers for factor X activation by factor IXa bound to normal or patient platelets from the data presented in Fig. 1 *A* and *B*. Thus, at five different concentrations of added factor IXa (0.44–3.5 nM) the rates of factor Xa formed (in picomoles per 10^9 platelets per minute, Fig. 1 *B*) were divided by the amounts of factor IXa bound (in picomoles per 10^9 platelets, Fig. 1 *A*). The resulting turnover numbers (in moles of factor Xa formed per mole of factor IXa bound per minute) were independent of enzyme concentration, as they should be at saturating substrate concentration. Furthermore, the mean (±SEM, $n = 5$) value for normal platelets was 2,280 (±138), compared with 2,361 (±88) for Scott syndrome platelets.

Our studies were carried out at a single concentration of factor VIIIa (5 U/ml), which is near the plasma concentration of factor VIII. This concentration of factor VIII has been

Table I. Kinetic Parameters and Binding Constants for Normal and Scott Syndrome Platelets

Platelets	Factor VIII	Number of sites per platelet	K_d		K_m	k_{cat}*	k_{cat}‡	k_{cat}*/K_m	k_{cat}‡/K_m
			Equilibrium	Kinetic					
			nM		*μM*		*min*$^{-1}$	*μM*$^{-1}$·*min*$^{-1}$	
Normal	Absent	560 (±35)	2.75 (±0.27)		0.5	0.25		0.5	
	Present	538 (±50)	0.68 (±0.07)	0.52	0.104	491	2,280 (±138)	4,721	21,923
Scott syndrome	Absent	461 (±60)	3.2 (±0.33)		0.59	0.29		0.49	
	Present	412 (±44)	2.5 (±0.19)	2.5	0.11	163	2,361 (±88)	1,482	21,463

Results presented represent means (±SEM) of three separate determinations. k_{cat}* is expressed as moles of factor Xa formed per mole of total factor IXa per minute. k_{cat}‡ is expressed as moles of factor Xa formed per mole of platelet-bound factor IXa per minute. See text for explanation.

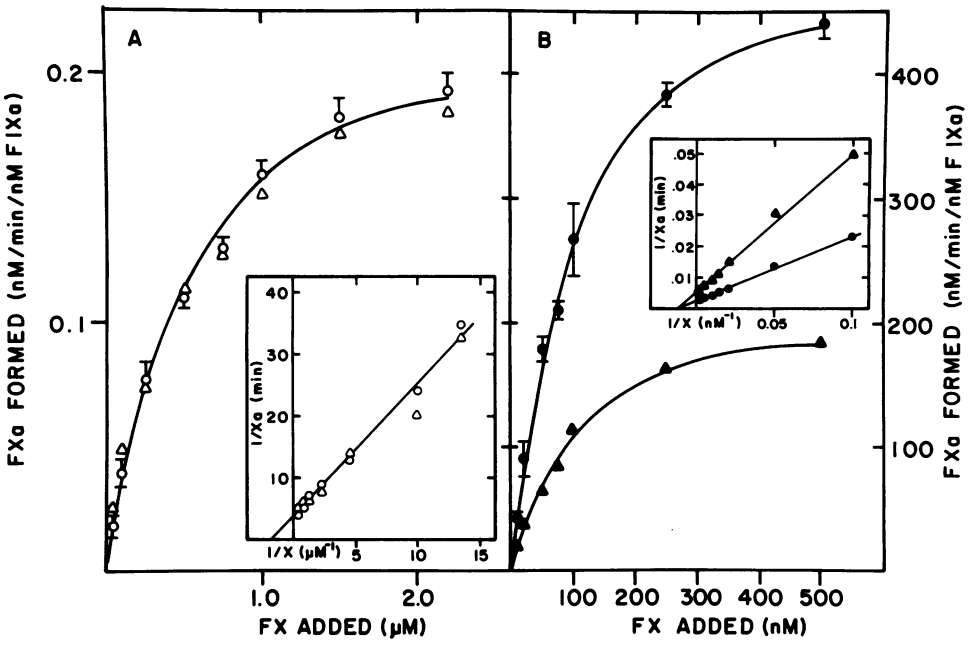

Figure 2. (*A*) Factor Xa formation by factor IXa in the presence of thrombin-activated platelets and absence of factor VIIIa. Thrombin-stimulated (0.1 U/ml), gel-filtered platelets (5×10^7/ml) were preincubated with factor IXa (0.01 μM) and 5 mM $CaCl_2$ at 37°C for 10 min, and with 50 nM PPACK for 2 min at 37°C prior to addition to a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml human serum albumin (pH 7.9) and various concentrations of factor X (0.005–0.5 μM). The details are described in Methods Procedures. The results presented are the means±SEM of duplicate observations with platelets from three separate normal donors (○) and means of duplicate observations with MS platelets (△). (*B*) Factor Xa formation by factor IXa in the presence of thrombin-activated platelets and factor VIIIa. Thrombin-stimulated (0.1 U/ml), gel-filtered platelets (5×10^7/ml) were preincubated with factor IXa (0.01 nM) and 5 mM $CaCl_2$ at 37°C for 10 min and with 50 mM PPACK for 2 min at 37°C prior to addition to a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml human serum albumin (pH 7.9), factor VIIIa (5 U/ml final concentration separately incubated at 37°C for 1 min at a concentration of 210 U/ml with 0.01 U/ml thrombin before addition to a reaction mixture) and various concentration of factor X (0.075–2 μM). The details are described in Methods. The results presented are the means±SEM of duplicate observations with platelets from three separate normal donors (●) and means of duplicate observations with MS platelets (▲).

shown to be saturating for normal platelets (14) and saturating in our own experiments of factor IXa binding (13) and factor X activation (this paper). It remains to be seen what the effect of higher or lower concentrations of factor VIIIa will have on the differences observed between normal and Scott syndrome platelets.

We conclude that the major difference between normal and Scott syndrome platelets lies in the ability of factor VIIIa, alone or in combination with factor X, to enhance factor IXa binding to normal but not patient platelets to give a more active form of factor IXa. This result suggests the possibility of an impairment of factor VIIIa binding or a diminished affinity of the factor VIIIa–IXa complex for Scott syndrome platelets in the presence of factor X. These possibilities will be examined by direct factor VIIIa binding studies to Scott syndrome platelets.

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