

Recombinant human factor VIIa (rFVIIa) can activate factor FIX on activated platelets

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Summary. The studies reported here show that factor (F)VIIa can activate factor (F)IX on activated platelets in the absence of tissue factor. Both FIX and FIXa bind to the activated platelet surface with a K_d of 8 nM and 2 nM, respectively. With factor (F)VIIIa, FIXa binds more tightly to platelets (K_d 0.6 nM). At rFVIIa concentrations < 100 nM, no direct binding to the activated platelet surface can be detected with electrophoretic light scattering. However, in the presence of FIX, rFVIIa binding to platelets at concentrations as low as 10 nM rFVIIa can be detected. This is reflected by a decrease in the FIX K_d from 8 to 1.6 nM. When rFVIIa is added to activated platelets in the presence of both FIX and FVIIIa, the K_d for FIX decreases to 0.6, suggesting that rFVIIa activates FIX on the surface of activated platelets in the absence of tissue factor. The activation of FIX by FVIIa on activated platelets can also be demonstrated by a functional assay for FIXa. These data show that pharmacological doses of rFVIIa result in the direct activation of FIX by rFVIIa to form additional tenase complexes ultimately resulting in improved thrombin generation. These results may explain, at least in part, the mechanism of action of rFVIIa in hemorrhagic conditions seen in otherwise normal patients who develop an acquired coagulopathy due to trauma, surgery or a variety of other events in which rFVIIa has been found to be effective.

Keywords: factor FVIIa, factor IX, light scattering, platelet.

Introduction

The modern view of the initiation and assembly of coagulation activation complexes such as the tenase complex suggests that cellular surfaces are of fundamental importance for the formation and localization of thrombin generation on the

platelet surface resulting in a hemostatic plug [1]. Coagulation initiation involves cell-bound tissue factor (TF) and activated coagulation factor VII (FVIIa) [2]. The occupancy of the TF receptor by FVIIa is important because the TF–FVIIa complex can activate both zymogen factor (F)X for participation in the prothrombinase complex and zymogen factor (F)IX for participation in the tenase complex. In the case of either congenital or acquired deficiencies or inhibition of components of the tenase or prothrombinase complex, either replacement of the missing factor or the use of agents thought to ‘by-pass’ certain deficiencies may restore normal hemostasis. There is now increasing evidence that the administration of pharmacological doses of recombinant human FVIIa (rFVIIa) may act as a by-passing agent. Evidence to date suggests that the ‘by-passing’ action of rFVIIa is due to both the enhancement of the TF pathway as well as the direct activation FX by rFVIIa on the surface of activated platelets [3].

FIX activation also proceeds through interaction with the TF–FVIIa complex as well as by factor (F)XIa, the latter occurring on the surface of activated platelets [4–12]. FIXa binds to the activated platelet surface with a K_d of 2.5 nM, but in the presence of recombinant factor (rF)VIIIa, FIXa binding is tighter with a K_d of 0.6 nM [6,13–17]. The number of binding sites on the activated platelet surface for FIX and FIXa depends on how the platelet is activated [13,18, Gabriel, unpublished data].

It is widely accepted that complex formation between FVIIa and TF is an obligatory step for the expression of FVIIa activity [5,19,20]. However, several studies have demonstrated the TF independent activation of FX by high doses of FVIIa in the presence of calcium ion alone or calcium and phospholipid vesicles [21–24].

Experiments shown in this report were designed to explore if platelet-bound zymogen IX could be activated by FVIIa in the absence of TF and if the mechanism of activation involved FIX as a receptor. By monitoring changes in the binding constant of FIX to activated platelets during its activation by rFVIIa, we show that factor FIX can be converted to FIXa. We demonstrate that unactivated FIX bound to activated platelets provides an interaction site for rFVIIa and that the rFVIIa–FIX interaction leads to activation of FIX. Once FVIIa binds

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to and activates FIX, the newly formed FIXa in the presence of FVIIIa can be assembled into additional tenase complexes, thus enhancing thrombin production.

Experimental procedures

Platelet isolation

Fresh gel-filtered platelets were used in all experiments. Blood from healthy donors was collected in acid-citrate-dextrose and prostacyclin (PGI₂) (5 µg mL⁻¹; Sigma Chemical Co., St Louis, MO, USA). Platelet-rich-plasma (PRP) was obtained by centrifugation of the anticoagulated blood at 150 × *g* for 15 min. The platelets were isolated from PRP by centrifugation at 650 × *g* for 20 min, and resuspended in citrated saline (13 mM citrate, 123 mM NaCl, 33 mM dextrose) buffer containing 5 µg mL⁻¹ PGI₂, washed twice and then resuspended in a small amount of calcium-free albumin-free Tyrode's buffer (137 mM NaCl, 12 mM NaHCO₃, 5.5 mM glucose, 2 mM KCl, 1 mM MgCl₂, 0.3 mM Na₂HPO₄, pH 7.4) containing 5 µg mL⁻¹ PGI₂. Gel-filtered platelets were obtained from the application of washed platelets to a Sepharose CL-2B column (Pharmacia Inc., Uppsala, Sweden) equilibrated with calcium-free, albumin-free Tyrode's buffer. Platelets for light scattering experiments were suspended in buffer containing 20 mM NaCl, 265 mM sucrose, 2 mM HEPES, pH 7.4 and activated with human α-thrombin (0.2 NIH U mL⁻¹) [25]. Fresh gel-filtered platelets were shown to activate and aggregate normally with 1 U mL⁻¹ of human thrombin and 10 µM ADP. Electrophoretic quasi elastic light scattering (EQELS) spectra of the gel-filtered platelets gave a single homogeneous mobility indicating the lack of microparticles. We found no change in the platelet mobility spectrum on the addition of rFVIIa over the concentration range where rFVIIa would be expected to bind to TF if it was present, indicating an absence of TF in our preparation.

Proteins

Blood coagulation FIX and FIXa were purified as described [26]. rFVIIa was the generous gift of U. Hedner of Novo Nordisk (Copenhagen, Denmark). rFVIII was a gift from Bayer Laboratories Inc. (Clayton, NC, USA).

Electrophoretic quasi elastic light scattering (EQELS)

Electrophoretic quasi ELS offers the ability to monitor changes in the surface of blood cells resulting from cell activation and ligand binding [27,28]. The electrophoretic mobility of activated platelets changes when exposed to a known ligand and the mobility change is the result of ligand binding. It is the change in the platelet surface charge density and hence its electrophoretic mobility caused by ligand binding and not the ligand itself that is monitored. Under our experimental conditions, resting gel-filtered platelets have an electrophoretic mobility of $-0.9 (\mu\text{-cm})/(\text{V-s})$ and activated platelets have a mobility of $-0.65 (\mu\text{-cm})/(\text{V-s})$. Loading the platelet surface with a ligand,

such as a FIX or FVIII, changes the platelet electrophoretic mobility. While the absolute mobility varies slightly from donor to donor, there is minimal variation in a given donor. The dependence of the change in the platelet electrophoretic mobility on the addition of FIX permits calculation of the binding constant for FIX to the activated platelet. It is not the magnitude of the change in the platelet electrophoretic mobility, μ , that determines the binding constant, but how the change in μ depends on the concentration of the ligand FIX. The effect of ligand binding on the platelet mobility varies with the ligand, and is dependent on the extent of ligand binding, the extent of surface modification caused by ligand binding, and on the net charge of the ligand itself.

EQELS measurements were made on a multiangle quasi elastic light scattering spectrometer (DELSA 440; Coulter Electronics, Inc., Hialeah, FL, USA) as described in previous reports [25,28].

Debye limit

Charged particles in solution orient oppositely charged counter ions about their surface so that the electrical potential of the particle's surface decreases with the distance from the particle surface. At a distance defined by the Debye-Huckel theory, the particle no longer has an influence on the solution counter ions. The distance from this point to the particle surface is called the Debye-Huckel length, also called the electrical double layer, and for platelets is estimated to be 8D [29,30]. The electrophoretic mobility for particles the size of blood cells, where the ratio of the particle diameter to the Debye screening length is > 30 , assuming a platelet diameter of at least 10 000D, is governed by the magnitude of the surface charge density and not by frictional factors [31-33].

Binding of proteins to platelets

Activated platelets and activated FVIII were prepared by activation with 0.2 NIH U mL⁻¹ human α-thrombin for 5 min. After activation, residual thrombin was inactivated by addition of 2.5 µM phenylalanyl prolinyl arginine chloromethyl ketone (PPACK; Calbiochem, La Jolla, CA, USA). FIX, FIXa, FVIIIa, and rFVIIa were incubated with platelets for 10 min before ELS measurements.

The binding coefficient (K_d) of the proteins to platelets was determined by fitting the data from the binding experiment to: $\mu = \mu_0 + \Delta\mu [\text{Ligand}/(K_d + \text{Ligand})]$, where μ is the electrophoretic mobility, μ_0 is the mobility in the absence of added protein, and $\Delta\mu$ is the calculated maximal change in mobility at the saturating concentration of protein. This model assumes one class of binding sites for the specific protein ligand on the platelets. Data were fitted using the non-linear module (NLIN) of the analysis program SAS (SAS Institute Inc., Cary, NC, USA).

Zymogen FIX at varying concentrations was added to the thrombin-activated platelets that in some experiments included FVIIIa followed by a 10-min incubation period. After this

incubation period, rFVIIa was added to activated platelets coated with zymogen FIX. The ELS spectrum was then obtained.

Functional assays for activation of FIX and FX

All assays were run in Tyrode's buffer [15 mM HEPES (pH 7.4), 3.3 mM Na_2HPO_4 , 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , and 5.5 mM glucose] with 2 mM CaCl_2 . Platelets were activated with 0.2 NIH U mL^{-1} of human α -thrombin for 10 min at 37 °C. FVIII at 5 U mL^{-1} was included with the platelets and thrombin. Thrombin was neutralized with hirudin from a solution titrated against an α -thrombin standard. FVIIa at varying concentrations was added to the platelets for 10 min. Plasma concentrations of FIX at 80 nM were added and incubated for varied times. FIXa was assayed in a two-stage assay. Platelets with activated FIX were incubated with plasma concentrations of FX for 5 min, then the amount of FXa was measured by adding plasma concentrations of prothrombin along with a prothrombin substrate (0.5 mM Perfachrome Th). Pilot studies showed that: FXa generation was linear over the 5-min assay period, FXa generation was dependent on FIX, thrombin generation was dependent on the addition of FX, and there was no background cleavage of substrate in the absence of prothrombin. Thrombin generation was measured as the change in the absorbance as the p-nitroanilide substrate was cleaved as a function of time. As expected, substrate cleavage fitted a second order pattern and the rate of thrombin generation was determined from the first derivative of the absorbance vs. time data.

Results

In Fig. 1 the binding profile is shown for zymogen FIX interaction with activated platelets in the presence of 4 mM calcium and in the presence and absence of rFVIIIa. In the absence of rFVIIIa, unactivated FIX bound with a K_d of 7.9 nM [confidence interval (CI) 6.948, 8.851]. When 3 nM FVIIIa was present, the binding of FIX to activated platelets was slightly tighter, as seen by a decrease in the K_d to 5.8 nM (CI 5.183, 6.419). These K_d s are different based on comparison of non-overlapping 95% CIs.

Figure 2 depicts the binding of activated FIX to activated platelets. In contrast to activated platelets, resting platelets did not bind either FIX or FIXa (data not shown). In the presence of 4 mM calcium ion, but no rFVIIIa, FIXa bound to activated platelets with a K_d of 1.9 nM (SE = 0.05, CI 1.741, 2.059) [3–7,10,13,14,17,20,21]. However, as shown in Fig. 2, in the presence of 4 mM calcium and 3 nM rFVIIIa, the K_d for FIXa binding to activated platelets decreased from 1.9 nM to 0.569 nM (SE = 0.185, CI 0.188, 0.950) showing tighter binding. The CIs for the K_d s for FIXa binding with and without rFVIIIa are statistically different at $\alpha = 0.05$. Because of its significant charge, rFVIIIa conferred a higher surface charge to the activated platelets, observed by the downward shift of the binding curve when rFVIIIa was present [25].

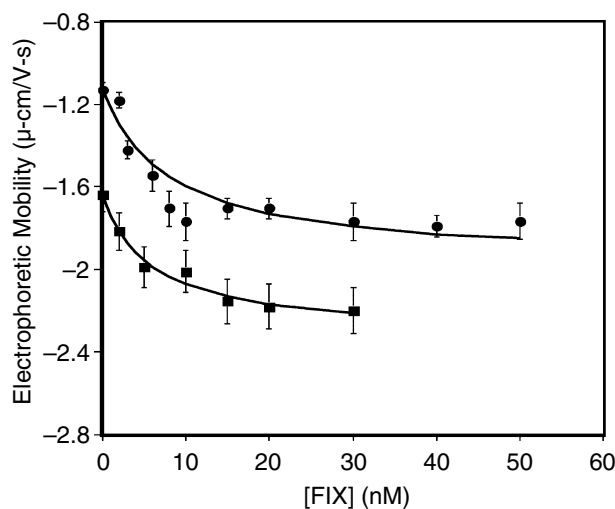


Fig. 1. The effect of recombinant factor (r)FVIIIa on the binding of zymogen factor (F)IX to activated platelets in the presence of 4 mM calcium chloride. Platelets were activated with 0.2 NIH U mL^{-1} thrombin followed by complete inhibition of thrombin with phenylalanyl prolinyl arginine chloromethyl ketone (PPACK). In the absence of rFVIIIa, the binding constant for zymogen FIX was 7.9 nM (●) compared with a K_d of 5.8 nM when rFVIIIa was present (■).

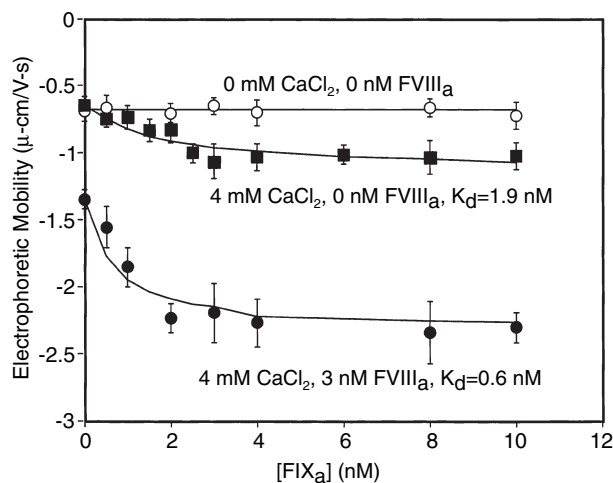


Fig. 2. The effect of calcium and recombinant factor (r)FVIIIa on the binding constant for activated factor IX (FIXa). In the absence of calcium, no change in the platelet electrophoretic mobility occurred, indicating no binding of FIXa. In the presence of 4 mM calcium, but no rFVIIIa, the K_d for a single-state binding model was 1.9 nM. In the presence of 3 nM (5 U mL^{-1}) rFVIIIa and at 0 nM of FIXa, the platelet mobility was increased resulting from rFVIIIa binding. As the concentration of FIXa was increased, the mobility was further increased, reflecting FIXa binding. In the presence of rFVIIIa, a K_d of 0.569 nM was calculated.

Because of the difference in the binding constant between FIX and FIXa and since rFVIIa does not bind to activated platelets at 10 nM, the following experiments were performed to investigate the role of rFVIIa in the activation of platelet-bound zymogen FIX. A decrease in the K_d of FIX was used to detect activation of zymogen FIX by rFVIIa.

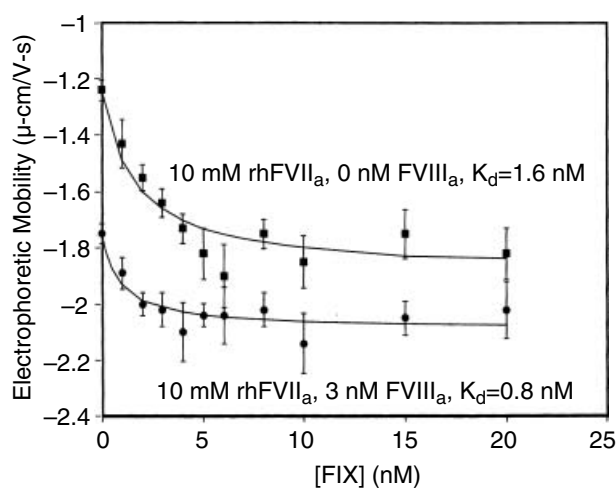


Fig. 3. Recombinant factor (rF)VIIa activation of platelet-bound factor (F)IX. rFVIIa at 10 nM, calcium at 4 mM, and 10 nM FIX were added to activated platelets. No activation of FIX resulted in a binding curve and binding coefficient would be similar to that shown in Fig. 1. In the absence of recombinant factor (rF)VIIIa, a binding coefficient of 1.62 nM was observed similar to that shown in Fig. 2 for the binding of FIXa to activated platelets. When 3 nM rFVIIIa was present, the FIX K_d was 0.84 nM, similar to that for FIXa plus rFVIIIa shown in Fig. 2. These experiments indicate activation of FIX by rFVIIa.

Figure 3 shows the effect of a high concentration of rFVIIa on the activation of zymogen FIX. In these experiments 10 nM rFVIIa and a variable amount of zymogen FIX (0–20 nM) were added to thrombin-activated platelets and incubated for 10 min followed by the determination of the electrophoretic mobility. When rFVIIa was present at a concentration of 10 nM, FIX bound to activated platelets with a K_d observed for FIXa in the absence of rFVIIIa ($K_d = 1.62$, CI 1.491, 1.748) compared with a K_d of 1.9 for FIXa shown in Fig. 2. When 3 nM rFVIIIa was first added to the activated platelets, incubated for 10 min, followed by addition of zymogen FIX and rFVIIa and reincubated for 10 min, the binding was tighter ($K_d = 0.84$, CI 0.287, 1.393). The difference between these binding curves is also statistically significantly different. The tighter binding was reflected in the decrease in the K_d and was similar to that observed for FIXa binding to platelets in the presence of rFVIIIa (compare with Fig. 2 and Table 1). These experiments suggest that at pharmacological doses of rFVIIa, zymogen FIX is activated by rFVIIa on activated platelets in the absence of TF.

In Fig. 4a the time dependence for the activation of zymogen FIX (10 nM) at different concentrations of rFVIIa is shown. The rate constants for these reactions are shown in Fig. 4b. At 0.075 nM rFVIIa minimal activation of zymogen FIX was observed at approximately 30 min (Fig. 4a and the first data point in Fig. 4b). When the concentration of rFVIIa was increased to 1 nM, further activation of zymogen FIX was detected. When the concentration of rFVIIa was further increased to 10 nM, the conversion of zymogen FIX to activated FIX occurred at a still more rapid rate.

TF was not present on the surface of activated platelets in the experiments presented. The evidence for this is that addition of rFVIIa over a concentration range of 0–40 nM did not change the electrophoretic mobility of platelets. If TF was present, rFVIIa would bind to TF and induce an alteration in the platelet surface charge that would be observed as a change in platelet mobility.

Figure 5 provides additional functional evidence for the activation of platelet-bound zymogen FIX by rFVIIa. In the absence of rFVIIa zymogen FIX is not activated (filled circles, Fig. 5). As rFVIIa is added at 10 nM (squares), 20 nM (diamonds), 30 nM (inverted triangles), and 40 nM (triangles), the activation of zymogen FIX is shown to be linear in time and directly proportional to the concentration of added rFVIIa. The rate constant for FIX activation as a function of the concentration of added rFVIIa is shown in the inset of Fig. 5. Although not shown in Fig. 5, we have also shown that FXa and thrombin are produced in this system and also dependent on the concentration of added rFVIIa [39]. Data shown in Fig. 5 confirm the ELS result that platelet-bound FIX is activated by rFVIIa.

Discussion

A complete understanding of hemostasis, including contributions from platelets, soluble phase coagulation factors, surface effects and fluid dynamics, has been hindered by the complexity of the system. A recent theory for coagulation proposed by Nemerson and others [2,34–37] supports TF as the initiating event in hemostasis. Physical separation of the amplifying tenase and prothrombinase complexes from the TF–FVIIa trigger complex provides a spatial feature for regulation of hemostasis. It seems likely that soluble zymogen FIX is first activated by the TF–FVIIa complex at a site distant to the platelet surface and then translocation of activated FIX to the platelet surface occurs. The activated platelet participates in

Table 1 Summary of binding coefficients for factor (F)IX and FIXa interaction with activated platelets

Conditions	FIX	FIXa	FIX + FVIIa*
No Ca^{2+} , no FVIIIa	–	–	–
4 mM Ca^{2+} , no FVIIIa	7.9 (CI 6.95, 8.85)	1.9 (CI 1.74, 2.06)	1.6 (CI 1.49, 1.750)
4 mM Ca^{2+} , 3 nM FVIIIa	5.8 (CI 5.18, 6.42)	0.57 (CI 0.19, 0.95)	0.84 (CI 0.29, 1.39)

*The K_d for FIX in the presence of factor (F)VIIa is very similar to that for direct activation of FIX and indicates that FIX is activated by FVIIa on the activated platelet surface.

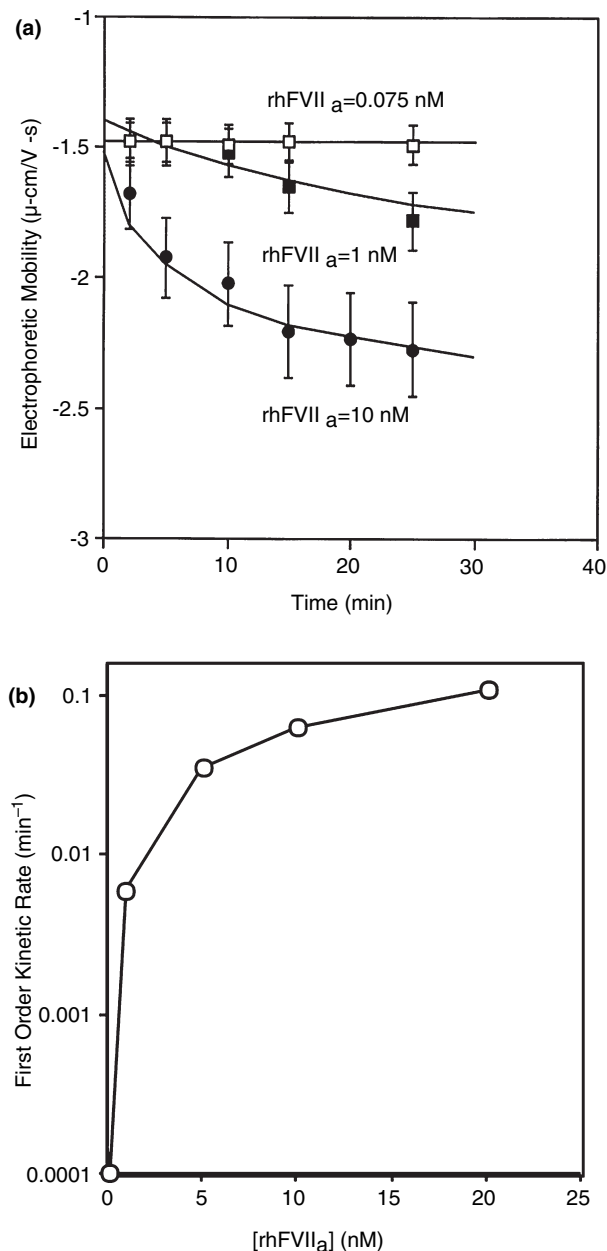


Fig. 4. (a) The time and concentration dependence of recombinant factor (rF)VIIa on the activation of zymogen factor (F)IX. Platelets were activated with thrombin followed by inhibition of thrombin with phenylalanyl prolinyl arginine chloromethyl ketone (PPACK) as before. The activated platelets were then loaded with $10 \mu\text{g mL}^{-1}$ of zymogen FIX followed by the addition of rFVIIa [0.075 nM (\square), 1 nM (\blacksquare), or 10 nM (\bullet)] and monitored as a function of time. The mobility at zero time is the mobility for platelets loaded with zymogen FIX. rFVIIa did not bind to activated platelets in the rFVIIa range 0.075–10 nM. Changes in the platelet mobility resulted from FIX activation. (b) First-order rate constants were calculated for the conversion of zymogen FIX to activated FIXa by rFVIIa. Four different concentrations of rFVIIa are shown, 0.075 nM, 1 nM, 10 nM, and 20 nM.

this sequence through tight binding of coagulation factors to specific binding sites, so that amplification complexes can be sequestered, spatially oriented, and protected from plasma inhibitors.

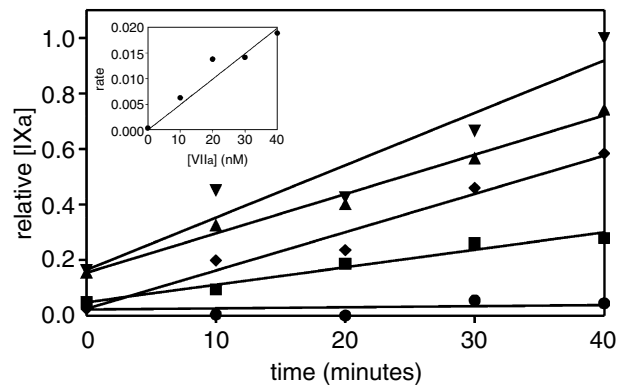


Fig. 5. Chromogenic assay to show activation of factor (F)IX by recombinant factor (rF)VIIa in the absence of tissue factor. The amount of FIX activated is shown as a function of time and at different concentrations of rFVIIa that are varied from 0 nM to 40 nM. The inset shows the rate constant for FIX activation that corresponds to a specific concentration of rFVIIa.

In the special case where high concentrations of rFVIIa are infused, as in the treatment of hemophilic patients or for bleeding episodes in non-hemophiliacs, the circumstances of FIX activation may be different. In this case rFVIIa may be bound to other sites in addition to TF. In this scheme, zymogen FIX bound to the activated platelet surface could provide a binding site for rFVIIa, since rFVIIa at physiological concentrations binds only weakly to the platelet surface. We and others have shown that at very high concentrations, rFVIIa will bind to platelets in the presence of calcium with a K_d of 123 nM [38] (unpublished data). We estimate that the concentration of rFVIIa after therapeutic infusion for a bleeding diathesis is approximately 30 nM, borderline for binding directly to the activated platelet surface. Under these conditions rFVIIa may bind directly to zymogen FIX bound to the platelet surface. Monroe and colleagues have previously reported evidence that rFVIIa bound to activated platelets can directly activate FX [39].

Platelets are intimately involved in several steps of the coagulation pathway. It was found that activated platelets promote the activation of FX to form FXa by a complex of FIXa, FVIIIa, and calcium [40–43]. Activated platelets not only provide the phospholipid surface, but also presumably possess specific, high-affinity, saturable binding sites for FXa [44,45], factor (F)Va [45,46], FVIIIa [25,47], and FIXa [40]. TF-bearing microparticles possibly derived from leukocytes appear to be significant in thrombus propagation, but not in the initiation of the thrombus [2]. Microparticles containing TF that localize to activated platelets that normally do not contain TF have been shown to be mediated by P-selectin on the platelet surface and CD15 on the microparticle [2,48–51].

The changes observed in the platelet surface charge as the concentration of FIXa is increased (Fig. 2) are due to binding of FIXa to the platelet surface since: (1) the binding is saturable, (2) the change requires the presence of calcium, (3) platelet activation is required, (4) saturation with other proteins does not inhibit the effect, and (5) a protein highly homologous

to FIXa, i.e. FVIIa does not change the platelet surface charge under identical conditions. The possible role of TF-containing microparticles as a source for this effect under our conditions is unlikely since we do not see a mobility corresponding to microparticles in the mobility spectrum. Additionally, no change in platelet mobility occurs when we add rFVIIa at concentrations that should bind TF. Further support for specific FIXa binding is seen from the increased affinity of activated platelets for FIX in the presence of rFVIIIa (1.9 nM to 0.569 nM) [40].

Platelet activation results in a reduction in the surface charge, probably due to the exposure of newly activated molecules [25,27]. The exact mechanism for the platelet surface charge modification during platelet activation is not known. It is known that many surface events occur with platelet activation, e.g. exposure of CD62, FVa, various receptors, appearance of phosphatidylserine, etc., all of which could contribute to changes in the platelet surface charge. After platelet activation is complete, the surface charge on the platelet surface stabilizes until a ligand is bound to the activated surface.

One important result of these experiments is that high concentrations of rFVIIa can activate FIX even in the absence of TF, which may explain its effect in the correction of bleeding in non-hemophiliacs who have a severe hemorrhagic diathesis due to trauma, surgery or many other acquired hemorrhagic conditions in which rFVIIa has a demonstrated benefit.

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