Phospholipase Abolishes the Effect of Stimulated Platelets on the Thrombin Activation of Factor VIII

By Margaret E. Rick and Dennis M. Krizek

Factor VIII functions as a cofactor in the intrinsic coagulation pathway and must first be activated to function optimally in this capacity. Low concentrations of thrombin activate factor VIII, and the presence of stimulated platelets is known to enhance the activation of factor VIII complexed to von Willebrand factor. The current studies show that platelets stimulated by thrombin, collagen, or calcium ionophore will increase the activation of isolated factor VIII by thrombin. Ongoing platelet release is not necessary for the enhanced factor VIII activation, nor is platelet von Willebrand factor or platelet membrane glycoproteins Ib or IIb/IIIa. Platelet membrane phospholipids, on the other hand, are important for the enhanced activation of factor VIII by thrombin because the effect of stimulated platelets is abolished by incubation of the stimulated platelets with phospholipases. These results suggest that the enhanced activation of factor VIII by thrombin in the presence of stimulated platelets may be mediated by factor VIII binding to platelet phospholipid or to a receptor whose functional integrity is dependent on surrounding membrane phospholipid.

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MATERIALS AND METHODS

Diisopropylfluorophosphate (DFP), rabbit brain cephalin, prostaglandin E1 (PGE1), trasyloc, and heparin were obtained from Sigma Chemical Co. (St Louis). β-Thromboglobulin radioimmunoassay kit was obtained from Amersham Corp. (Arlington Heights, IL). Phospholipase A2 (bee venom, 2,400 U/mg) and phospholipase C (Bacillus cereus, 2,000 U/mg) were obtained from Boehringer Mannheim (Indianapolis). Lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). Collagen (calf skin) was obtained from Cooper Biomedical (Malvern, PA). Factor VIII deficient substrate and factor VIII inhibitor plasma (for inhibition studies) were obtained from George King Biomedical (Overland Park, KS). Human α-thrombin was the kind gift of Dr John Fenton II (Albany, NY) and had a specific activity of 2,100 U/mg. The monoclonal antibody to platelet GPIb, 6D1, was kindly supplied by Dr Barry Coller (Stoney Brook, NY). All other reagents were of reagent grade or higher.

Buffers included VBS 0.125 mol/L NaCl, 0.015 mol/L barbital, and 0.01 mol/L sodium barbital, pH 7.5 (VBS), borate saline (0.036 mol/L boric acid, 0.005 mol/L NaOH, and 0.159 mol/L NaCl, pH 7.8), and HEPES buffer (3.8 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KC1, 2.5 mmol/L MgCl2, 2.0 mol/L CaCl2, 1.0 g/L dextrose, and 3.5 g/L albumin, pH 7.5).

Factor VIII preparation. Factor VIII was prepared from blood collected directly into heparin (5 U/mL); after separation of plasma at 4°C, DFP (2 mmol/L) and trasylol (100 U/mL) were added, and factor VIII was separated from other plasma proteins on a solid-phase anti-vWF column as previously reported. The bovine serum albumin (BSA) used as a carrier protein was fatty acid free. Factor VIII preparations contained no measurable vWF antigen by radioimmunoassay (RIA; <0.002 U/mL, 20 ng/mL) and had a ratio of factor VIII activity to antigen (VIII:C:Ag) of 1.0 to 2.1. The factor VIII activity ranged from 0.5 to 1.1 U/mL.

Preparation of platelets. Informed consent was obtained from all blood donors. In several experiments the normal donor was given 650 mg aspirin orally at 24, 12, and 2 hours before phlebotomy. Nine parts whole blood were collected into one part MgCl2 (final concentration, 25 mmol/L) containing 10 μmol/L PGE1. Platelet-rich plasma (PRP) was prepared by centrifugation at 600 g for three minutes at room temperature; the PRP was removed and HEPES buffer containing the same final concentrations of MgCl2 and PGE1 was added to the remaining cells, which were gently mixed and centrifuged at 600 g for three minutes. This wash was combined with...
the original PRP, and the mixture was layered on a 10% to 20% discontinuous arabinogalactan gradient containing the anticoagu-
ulant-inhibitor mixture and centrifuged for 30 minutes at 2,000 g at room temperature. After centrifugation the platelet layer was cut from the tubes, 10 mL of HEPES buffer was added, and the platelets were pelleted by centrifugation at 2,000 g for ten minutes. They were resuspended in HEPES buffer at a concentration of 1 to 2 x 10^9/µL and were further treated as follows: (a) one portion was stimulated for five minutes at 37°C; agonists included thrombin (0.1 U/10^6 platelets), collagen (10 µg/10^6 platelets), or A23187 (10 µmol/L). The concentrations of thrombin and collagen were chosen after preliminary experiments indicated that the same effect on the thrombin activation of factor VIII was achieved when using platelets stimulated with tenfold greater concentrations of either agonist and that lesser responses were observed with lower concentrations. Similarly, the stimulation of platelets by the combination of thrombin (0.1 U/10^6 platelets) and collagen (10 µg/10^6 platelets) showed the same degree of enhancement on the activation of factor VIII by thrombin as either agonist alone. When thrombin was used to stimulate the platelets, they were subsequently washed with HEPES buffer containing hirudin (five- to tenfold molar excess) and then twice with HEPES buffer alone; when other agonists were used, they were washed with HEPES buffer. (b) A second aliquot was stimulated and washed as before and followed by incubation with phospholipase A2 (0.008 to 0.01 U/mL) or phospholipase C (0.1 U/mL) for 20 minutes at 37°C, and (c) a third aliquot had no further additions. The latter constituted the “unstimulated” platelets. The concentrations of phospholipases were chosen as the minimal concentrations that would abolish the effects of activated platelets in the activation of factor VIII by thrombin. In several control experiments, the phospholipases were incubated at the same concentrations with the isolated platelets before stimulation by thrombin; the platelets were washed once before the addition of thrombin.

In the experiments using formalin-fixed platelets, the platelets were fixed either after the arabinogalactan step or after stimulation and washing. Platelets were suspended in 15 mL of 1% paraformaldehyde in HEPES buffer (without BSA) and incubated at 37°C for 15 minutes and subsequently at 4°C until use. They were spun at 2,000 g for ten minutes and washed twice with HEPES buffer just before use.

As an indication of platelet activation, β-thromboglobulin was measured in platelet pellets and supernatants after thrombin stimulation (0.1 U/10^6 platelets). The platelets were prepared through the arabinogalactan step and then divided into two aliquots: one was resuspended in HEPES buffer, and the second was fixed with formalin before the addition of thrombin. Timed samples were removed after thrombin addition, spun, and separated; the pellets were frozen and thawed three times before assay.

**Factor VIII activation.** The factor VIII (0.4 to 0.6 U/mL) and isolated platelets (treated as indicated in each figure; 150,000 to 250,000/µL) were added to a polypropylene tube in a total volume of 400 µL and gently mixed at 37°C; a subsample was removed for factor VIII activity and antigen assays. Thrombin, 0.01 vol (0.1 U/mL final concentration), was added, and subsamples were removed at the indicated times for immediate factor VIII activity assay and for the VIII:C Ag assay. Samples for the VIII:C Ag assay were immediately diluted in borate saline containing 5 U/mL hirudin and were snap-frozen at -70°C for up to seven days before assay. In some experiments lysophospholipids (0.01 mg/mL) were added to factor VIII and platelets before the addition of thrombin. With each experiment, control incubations of factor VIII plus thrombin plus buffer, factor VIII plus buffer plus platelets, and buffer plus platelets plus thrombin were included.

**Assays.** Factor VIII activity was measured by using a one-stage assay based on the activated partial thromboplastin time, and VIII:C Ag was measured by RIA. The VIII:C Ag assay uses an antibody purified from a hemophiliac with a high-titer inhibitor.

In the experiments to demonstrate the specificity of the apparent factor VIII activity during thrombin activation, timed subsamples from an incubation mixture (see previous methods) were diluted into borate saline containing a concentrated IgG fraction from a factor VIII inhibitor plasma. The IgG (38 mg/mL) was prepared from citrated plasma by caprylic acid precipitation. The subsamples were allowed to incubate at 37°C for two minutes, a final dilution was made into VBS, and the factor VIII activity assay was performed immediately.

In a parallel experiment the subsamples were diluted into borate saline containing nonimmune IgG, incubated, and then further diluted in VBS before the VIII:C activity assay.

**RESULTS**

Preliminary studies evaluating the effect of thrombin on the platelet release reaction in our system showed that thrombin caused a rapid release of β-thromboglobulin from fresh platelets in HEPES buffer (43.7% at 30 seconds) that progressed to approximately 70% by five minutes (Table 1). Release of β-thromboglobulin by formalin-fixed platelets remained low (<10%) during the first five minutes of incubation.

**Effect of platelets on thrombin activation of factor VIII.** Activation of factor VIII by thrombin (0.1 U/mL final concentration) in the presence of unstimulated platelets is slightly but not significantly increased when compared with buffer (Fig 1A). On the other hand, when platelets are initially stimulated with thrombin (0.1 U/10^6 platelets), washed, and then incubated with factor VIII and a second aliquot of thrombin, a marked increase in the activity of factor VIII is observed (Fig 1A). The enhanced activation is seen at the initial time point (30 seconds) and during the first three minutes after thrombin addition. The same effect could be achieved by stimulation of the platelets with either collagen (10 µg/10^6 platelets) or A23187 (10 µmol/L), which indicated that different agonists could be used to stimulate the platelets to enhance factor VIII activation (Fig 1B). In addition, platelets that were obtained from an aspirin-treated donor and subsequently activated by thrombin, collagen, or A23187 still fully supported the enhanced activation of factor VIII (data not shown). Factor VIII:C Ag levels did not increase or decrease significantly during the time course of thrombin activation whether platelets were present or absent (Fig 2).

| Table 1. Release of β-Thromboglobulin From Platelets After Thrombin Addition |
|-----------------------------|-----------------------------|-----------------------------|
| Time (min) | Fresh Platelets | Fixed Platelets |
| | Platelet | SN (%) | Platelet | SN (%) |
| Prethrombin | 96.0* | 2.4 | 57.8 | 5.7 |
| 0.5 | 78.7 | 43.7 | 61.7 | 5.9 |
| 3 | 53.5 | 6.8 | 63.1 | 8.5 |
| 5 | 38.9 | 72.4 | 62.1 | 10.0 |
| 20 | 33.5 | 67.7 | 74.7 | 13.6 |

*Platelet pellets and supernatants (SN%) were assayed for β-thromboglobulin before and at varying times after the addition of thrombin (0.1 U/mL). Values are expressed as ng/2.5 × 10^6 platelets and as the percentage in the SN from 2.5 × 10^6 platelets.
FACTOR VIII, PLATELETS, AND PHOSPHOLIPASES

Fig 1. Thrombin activation of factor VIII in the presence of buffer and unstimulated or stimulated platelets. (A) Thrombin (0.1 U/mL) was added to factor VIII (0.4 to 0.6 U/mL) and either HEPES buffer (○), unstimulated platelets (●), or thrombin-stimulated, washed platelets (△) (150,000 to 250,000/μL). Subsamples were removed and assayed for factor VIII activity. Curves represent means and SD of 22 experiments each. (B) Identical conditions except that platelets were initially activated with collagen (●) (10 μg/10^6 platelets) or A23187 (●) (10 μM) before addition to factor VIII and thrombin. Curves represent means of three experiments each.

Specificity of the enhanced clotting activity after thrombin stimulation was verified by showing that the activity was inhibited by an antibody to factor VIII (Table 2). Control incubations (see Materials and Methods) indicated no generation of clotting activity unless factor VIII was present and no increase in factor VIII activity unless thrombin was present; the factor VIII activity did not vary by greater than 25% over the time course when incubated with platelets or buffer alone.

Influence of platelet release and membrane glycoproteins. The possible contribution of ongoing platelet activation or release to the enhanced activation of factor VIII by thrombin was examined with the use of stimulated platelets that had been fixed in formalin. When these platelets were incubated with factor VIII and thrombin, the enhanced activation of factor VIII occurred as previously noted with fresh stimulated platelets (Fig 3). Platelets prepared from a patient with Glanzmann’s thrombasthenia and from a patient with severe von Willebrand’s disease (vWD) (plasma and platelet vWF, <0.002 U/mL) that were stimulated with thrombin each supported the enhanced activation of factor VIII in a similar manner to that seen with normal stimulated platelets (Fig 4). The increased activation was also seen when stimulated normal platelets were preincubated with monoclonal antibody to GPlb (Fig 4).

Effect of phospholipase. In contrast to the aforementioned findings, incubation of stimulated platelets with phospholipases before their incubation with factor VIII and thrombin abolished the enhancement of factor VIII activation. Both phospholipase A2 and phospholipase C eliminated the effects of stimulated platelets on the thrombin activation of factor VIII (Fig 5A). Control experiments showed that factor VIII was stable when incubated with either phospholipase and that neither phospholipase had any effect on the thrombin activation of factor VIII in the presence of unstimulated platelets or HEPES buffer. Interestingly, the phospholipases also abolished the enhancement seen when formalin-fixed platelets were used in the incubation mixture (Fig 5B). Phospholipase C appeared to show a dose response in that 0.01 U/mL showed no effect on stimulated platelets, 0.1 U/mL reduced the activation of factor VIII to approximately the level seen with unstimulated platelets, and 1.0 U/mL reduced the stimulation to less than 20% to 30% of that seen with unstimulated platelets. Phospholipase A2 (0.008 to 0.01 U/mL) reduced the stimulation to the level seen with unstimulated platelets, and higher concentrations had no further inhibitory effect on the activation of factor VIII by thrombin. Lower concentrations of phospholipase A2 had less or no effect. No significant change was observed in the VIII:C Ag levels when platelets had been incubated with phospholipase before their addition to factor VIII and thrombin (Fig 2).

Table 2. Specificity of Factor VIII Activity After Addition of Thrombin to Factor VIII and Stimulated Platelets With or Without Phospholipase A2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Anti-VIII</th>
<th>Control</th>
<th>Anti-VIII</th>
<th>Control</th>
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<td>0.59</td>
<td>0.59</td>
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<td>5.9</td>
<td>&lt;0.02</td>
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<td>&lt;0.02</td>
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<td>&lt;0.02</td>
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Timed samples from an incubation mixture of factor VIII, platelets, and thrombin were diluted in buffer containing normal (nonimmune) IgG or anti–factor VIII IgG (see Materials and Methods). After a further two-minute incubation at 37°C, a factor VIII activity assay was performed.

*Prethrombin samples were diluted into nonimmune control IgG to obtain baseline factor VIII values.
Fig 4. Thrombin stimulation of factor VIII in the presence of platelets from normal (△), Glanzmann’s (ο), and vWd (□) donors; △, stimulated normal platelets incubated with anti-GPlb. All platelet preparations were initially stimulated with thrombin and washed; other conditions are identical to those in Fig 1A. Curves represent means of four (Glanzmann’s), three (vWd), and two (anti-GPlb) experiments.

Further experiments showed that preincubation of unstimulated platelets with phospholipase A2 or phospholipase C had no inhibitory effect on the ability to stimulate the platelets so that they would still enhance factor VIII activation. Additionally, the inclusion of lysophospholipids (0.01 mg/mL) in the incubation mixture with the stimulated platelets and factor VIII also had no inhibitory effect on the enhancement of factor VIII activation.

DISCUSSION

A number of studies have documented that factor VIII binds to phospholipids and platelet membranes. The functional consequences of the binding of factor VIII to phospholipids and platelet membranes have not been fully defined, although it appears that this interaction protects factor VIII activity when antibodies to factor VIII are present and that the activation of factor VIII that is complexed with vWF is enhanced by the presence of stimulated platelets.

Fig 5. Effect of phospholipases on the thrombin activation of factor VIII in the presence of stimulated platelets. (A) Thrombin-activated, washed, normal platelets were incubated with HEPES buffer (△), phospholipase A2 (□) (0.008 U/mL), or phospholipase C (○) (0.1 U/mL) for 20 minutes at 37°C before addition to factor VIII. Other conditions are as described in Fig 1A. Curves represent means and SD of 22 (HEPES), 14 (phospholipase A2), and five (phospholipase C) experiments. (B) Thrombin-stimulated, washed platelets were fixed in 1% formalin and then incubated with buffer (△), phospholipase A2 (□), or phospholipase C (○) before addition to factor VIII.

Our current studies demonstrate that factor VIII (not bound to vWF) is activated to a greater degree by thrombin when stimulated platelets are present and indicate that the integrity of platelet membrane phospholipid is important in mediating this effect. Previous studies had shown that the factor VIII/vWF complex was activated to a greater extent by the addition of platelets that were stimulated by washing and centrifugation, thrombin, or calcium ionophore. Our studies show that factor VIII does not need to be bound to vWF to demonstrate this effect and that collagen-stimulated platelets can also support this enhanced activation. The alteration in platelets that occurs upon stimulation with thrombin is also observed with platelets prepared from a donor who had taken pharmacological doses of aspirin. Additionally, because stimulated, washed, formalin-fixed platelets also enhance the thrombin activation of factor VIII, ongoing platelet release or other metabolic processes do not account for the observed increase in factor VIII activation.

Platelets membrane proteins including vWF, GPlb, and GPIIb/IIIa do not appear to be important in the functional enhancement of the thrombin–factor VIII reaction. This is shown in our experiments with platelets lacking vWF or GPIIb/IIIa and by inhibition of the glycoconjugate portion of GPlb with monoclonal antibody; all of these platelets still increased the thrombin activation of factor VIII. These functional studies are in agreement with binding studies that also showed no inhibition by monoclonal antibodies directed against GPlb or GPIIb/IIIa.

A recent study examining the binding of radiolabeled factor VIII to platelet membranes has indicated that increased binding occurs after thrombin proteolysis of factor VIII and that platelet aggregation induced by A23187 does not further increase binding; on the other hand, Muntean et al. have shown that activating platelets with thrombin and collagen increases factor VIII binding as assessed by factor VIII:C Ag measurements. Whether the binding observed in these studies precedes or follows the functional effect of enhanced thrombin activation of factor VIII reported herein is not clear because activity assays were not followed over a time course in these studies.

Platelet membrane phospholipids, on the other hand, are important in the platelet effect on the factor VIII activation reaction. Relatively low concentrations of both phospholipase A2 and phospholipase C abolish the effect of stimulated platelets on the reaction. Whether this is a direct effect of altering the potential interaction of factor VIII with the platelet membrane phospholipid or whether it might alter the conformation of a putative membrane glycoprotein receptor for factor VIII is not clear. The current experiments have ruled out any direct inhibitory effect of phospholipases (at these concentrations) on the stimulation of platelets, on factor VIII, or on the activation of factor VIII by thrombin. Also, potential negative feedback on the reaction by lysophospholipids is not a factor because no inhibition is observed when they are added to stimulated platelets and factor VIII during thrombin activation.

Studies with factor V, a coagulation factor that has structural and functional homologies with factor VIII, have indicated that the light chain of factor Va binds to phosphati-
dylcholine/phosphatidylserine (3:1) vesicles but not to vesicles composed of only phosphatidylcholine.19,20 During the activation of platelets, phosphatidylserine is exposed at the outer surface of the platelet membrane21 and might be expected to bind factor Va light chain or homologous structures in factor VIII. This potential of binding between factor VIII and activated platelets may account for our reported effect on factor VIII activation and for the loss of this effect when phospholipases are present. Evidence for this hypothesis requires direct binding studies of factor VIII and platelets.

ACKNOWLEDGMENT

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REFERENCES

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ME Rick and DM Krizek