The authors have investigated the ability of platelets to enhance factor Xa-catalyzed activation of factor VII. Unstimulated platelets were without effect, whereas freeze/thawed platelets substantially enhanced activation. Anti-factor V antibodies did not diminish the enhancement. Platelets activated by thrombin, collagen, or calcium ionophore A23187 also enhanced factor Xa-catalyzed activation of factor VII. In contrast to their lack of effect upon freeze/thawed platelets, anti-factor V antibodies abolished augmented factor VII activation induced by activated platelets. Adding exogenous factor V to unstimulated platelets failed to enhance factor Xa-catalyzed activation of factor VII; nor did adding exogenous factor V to activated platelets augment activation beyond that observed with activated platelets alone. These observations can be interpreted as follows: (1) factor V does not function as a cofactor for factor Xa-catalyzed activation of factor VII; (2) anionic phospholipids are a known cofactor for factor Xa-catalyzed activation of factor VII, and freeze/thawed platelets probably enhance activation by making anionic phospholipids on disrupted platelet membranes available to function as a cofactor; (3) the presumed binding of factor Xa to exogenous factor V on unstimulated platelets is insufficient in itself to augment factor Xa-catalyzed activation of factor VII; (4) activated platelets augment factor Xa-catalyzed factor VII activation because activation allows both factor Xa to bind to released platelet factor Va and makes available a surface membrane component, probably anionic phospholipids, with which the bound factor Xa interacts.

The Effect of Platelets Upon Factor Xa-Catalyzed Activation of Factor VII in vitro

By L. Vijaya Mohan Rao and Samuel I. Rapaport

MATERIALS AND METHODS

Reagents. Carrier-free Na\(^{251}\)I was obtained from Amersham Corporation (Arlington Heights, IL). Collagen (calf skin), adeno-

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Submitted November 19, 1988; accepted February 19, 1988.

Supported by grant HL 27324 from the National Heart, Lung and Blood Institute.


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0006-4971/88/7202-0127$3.00/0

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platelet. Five to seven milliliters of plasma were applied directly onto a 1.75 x 30-cm Sepharose-2B column that had been previously equilibrated with HEPES balanced salt buffer. This buffer contained NaCl 8 g/L, KCl 0.2 g/L, MgCl₂ - 0.2 g/L, dextrose 1.0 g/L, albumin 3.5 g/L, Na₂HPO₄ 0.45 g/L, HEPES 0.9 g/L. The platelets were eluted with the same buffer. Platelets were counted either electronically or by phase microscopy.

Endothelial cells. Human umbilical vein endothelial cells (HUVEC) were cultured as monolayers essentially according to the method of Jaffe et al.²

Activation of factor VII. In most experiments platelets, at a final concentration of 2 x 10⁸/mL, were stimulated by an agonist for ten minutes in the presence of calcium ions (5 mmol/L) at 37°C with continuous stirring. Then factor Xa, 5 nmol/L (equivalent to activation of 3% of plasma factor X concentration), was added, followed by [¹²⁵I]-factor VII, 10 nmol/L (plasma concentration). The final volume of reaction mixtures was 0.5 mL. Stirring was continued, and at regular intervals 30-μL aliquots were removed and added to an equal volume of SDS-sample buffer containing 5% 2-mercaptoethanol and 6 mmol/L EDTA. Control mixtures contained either unstimulated platelets or HEPES balanced salt buffer in place of activated platelets. Modifications of the above in specific experiments are described in the "Results" section.

Activation of factor VII was quantitated from radioactivity gel profiles obtained by subjecting test samples to SDS-PAGE. Gels were cut in 5-mm slices, which were counted in a Tracor (Elk Grove, IL) analytic 1197 gamma counter. Percent factor VII activation was calculated as:

\[
cpm \text{ in heavy plus light chain} \times 100
\]

Different preparations of [¹²⁵I]-factor VII contained small amounts of contaminating material, primarily activated factor VII, migrating in the position of the heavy and light chains. If counts from such material were less than 2% to 3% of total counts in a zero time sample, then they were disregarded. If they amounted to 3% to 5% of total counts, then they were subtracted from the counts obtained at later incubation times in the calculation of percent factor VII activation.

Approximately 90% factor VII cleavage measured by this technique has been shown to correspond to an approximately 25-fold increase in factor VII coagulant activity, as determined from a VIIC/VIIC ratio.¹⁰

SDS-PAGE. PAGE in the presence of SDS was performed according to the method of Laemmli¹³ with the use of 12% acrylamide gels.

RESULTS

Activation of factor VII by factor Xa in the presence of platelets. Gel-filtered platelets not treated further (unstimulated platelets) at a concentration of 2 x 10⁸/mL had no effect upon the activation of factor VII by factor Xa. The time course of activation was similar to that obtained in control reaction mixtures containing buffer instead of platelets (Fig 1). In contrast, platelets lysed by freezing and thawing substantially enhanced the rate of factor VII activation (Fig 1). The enhancement was factor Xa dependent; factor VII was not activated in a reaction mixture containing freeze/thawed platelets, calcium, but no factor Xa. The enhanced rate of factor Xa-catalyzed activation of factor VII obtained with freeze/thawed platelets was about one half of the enhanced rate obtained with a 0.2 mmol/L concentration of phosphatidyserine, 40% phosphatidylcholine, 60% vesicles (Table 1).

Platelets activated by exposure to thrombin, collagen, or both materials also acquired the ability to enhance factor Xa-induced activation of factor VII (Fig 1). Stirring was required for maximum effect. Enhanced activation was not

Table 1. The Effect of Platelets Activated by Different Agonists on Factor Xa-Catalyzed Activation of Factor VII

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Mean Factor VII Activation (nmol/L/min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No platelets</td>
<td>0.075 ± 0.028</td>
<td>6</td>
</tr>
<tr>
<td>PS/PC</td>
<td>2.055 ± 0.195</td>
<td>4</td>
</tr>
<tr>
<td>Platelets, no agonists</td>
<td>0.095 ± 0.034</td>
<td>20</td>
</tr>
<tr>
<td>Platelets, freeze/thawed</td>
<td>0.970 ± 0.210</td>
<td>5</td>
</tr>
<tr>
<td>Platelets, with agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>0.281 ± 0.067</td>
<td>4</td>
</tr>
<tr>
<td>Thrombin</td>
<td>0.269 ± 0.064</td>
<td>19</td>
</tr>
<tr>
<td>A23187</td>
<td>0.311 ± 0.075</td>
<td>11</td>
</tr>
<tr>
<td>Thrombin + collagen</td>
<td>0.280*</td>
<td></td>
</tr>
<tr>
<td>Thrombin + A23187</td>
<td>0.400 + 0.019</td>
<td>4</td>
</tr>
<tr>
<td>ADP</td>
<td>0.091 + 0.013</td>
<td>3</td>
</tr>
<tr>
<td>ADP + fibrinogen</td>
<td>0.095*</td>
<td></td>
</tr>
</tbody>
</table>

Platelets, 2 x 10⁸/mL, in HEPES buffer were incubated with 5 mmol/L calcium and the agonist shown for ten minutes at 37°C. Concentrations were PS/PC, phosphatidyserine, 40%/phosphatidylcholine, 60%, vesicles at a concentration of 0.2 mmol/L, collagen, 100 μg/mL; thrombin, 1 U/mL; calcium ionophore A23187, 10 μmol/L; ADP, 10 μM; human fibrinogen, 1.8 mg/mL. Then factor Xa, 5 nmol/L, and [¹²⁵I]-factor VII, 10 nmol/L, were added. Subsamples were removed over time for determination of factor VII activation, as described in "Materials and Methods." *Mean of two experiments.
observed in stirred control reaction mixtures: (1) a mixture containing factor Xa, calcium, a platelet agonist, but no platelets; and (2) a mixture containing calcium, a platelet agonist, platelets, but no factor Xa. Adding a 100-fold excess of active site-inhibited factor Xa (500 nmol/L DEGR-CK-treated factor Xa) blocked the ability of activated platelets to enhance factor Xa-catalyzed activation of factor VII; under this condition activation was comparable to activation in a reaction mixture containing 5 nmol/L factor Xa and unstimulated platelets.

The effect of treating platelets with different agonists is summarized in Table 1. Thrombin, collagen, calcium ionophore A23187, and combinations thereof all induced a threefold to fourfold increase in rate of factor VII activation. In contrast, adenosine diphosphate (ADP), with or without fibrinogen, had no effect upon the rate of factor VII activation. Although substantial, the increase in rate induced by activating platelets with thrombin, collagen, or A23187 did not achieve that observed with freeze/thawed platelets.

Enhanced factor VII activation induced by thrombin-activated platelets varied with platelet concentration (Table 2). When the platelet concentration in reaction mixtures was reduced to 0.25 × 10⁸/mL, the rate of factor VII activation with 5 nmol/L factor Xa did not differ from a buffer control.

Effect of antithrombin III. Adding a plasma concentration of antithrombin III, 2.5 μmol/L (150 μg/mL), to a reaction mixture containing factor VII, 10 nmol/L; factor Xa, 5 nmol/L; and Ca²⁺, 5 mmol/L, prevented the activation of factor VII. When thrombin-activated or A23187-activated platelets, 2 × 10⁶/mL, were added with the antithrombin III, activation of factor VII was still blocked. Similar results were obtained when 50 nmol/L antithrombin III (which was still ten times the factor Xa concentration) and 1 U/mL of heparin were added to reaction mixtures containing activated platelets. Incubating the factor Xa with thrombin-activated platelets and Ca²⁺ for 30 minutes before adding the antithrombin III and heparin did not prevent the inactivation of the factor Xa. In a parallel experiment with thrombin-activated platelets in which prothrombin, 75 μg/mL, instead of factor VII, was the substrate, antithrombin III, 50 nmol/L, plus heparin, 1 U/mL, prevented factor Xa-catalyzed activation of prothrombin.

Role of factor Va. Because factor Va on platelets serves as a high-affinity binding site for factor Xa, the investigators examined whether antifactor V antibodies influenced the ability of platelets to enhance factor Xa-induced activation of factor VII. Antifactor V antibodies did not impair the ability of freeze/thawed platelets to enhance activation of factor VII by factor Xa (Fig 2). In contrast, antifactor V antibodies markedly impaired the ability of activated platelets to enhance factor Xa-induced activation of factor VII (Fig 3A). Data from four experiments are summarized in Table 3.

As the investigators have reported elsewhere, binding of factor Xa to cultured human umbilical vein endothelium also enhances the ability of factor Xa to activate factor VII. In contrast to the observations with activated platelets, incubating endothelium with antifactor V antibodies did not impair the ability of endothelium to enhance factor Xa-induced activation of factor VII (Fig 3B).

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**Table 2. The Effect of Different Concentrations of Activated Platelets Upon Factor Xa-Catalyzed Activation of Factor VII**

<table>
<thead>
<tr>
<th>Concentration of Platelets</th>
<th>Factor VII Activation (nmol/L/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 × 10⁶</td>
<td>0.22</td>
</tr>
<tr>
<td>0.5 × 10⁶</td>
<td>0.14</td>
</tr>
<tr>
<td>0.25 × 10⁶</td>
<td>0.11</td>
</tr>
<tr>
<td>None</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Platelets were activated with 1 U/mL thrombin. Other conditions were as described in Fig 1.

*Mean of two experiments.

---

**Fig 2. Failure of antifactor V antibodies to affect the ability of freeze/thawed platelets to enhance factor Xa-catalyzed activation of factor VII.**

Human antifactor V antibodies (Δ) or control human IgG (○) at a concentration of 100 μg/mL was added to 2 × 10⁶/mL freeze/thawed platelets. After 20 minutes Ca²⁺, 5 mmol/L; factor Xa, 5 nmol/L; and 10⁻¹-factor VII, 10 nmol/L were added, and activation of factor VII was monitored.

**Fig 3. Effect of antifactor V antibodies upon factor Xa-catalyzed activation of factor VII in the presence of (A) activated platelets or (B) a monolayer of unperturbed cultured HUVEC.**

Platelets, 2 × 10⁶/mL, were activated with 1 U/mL thrombin. Human antifactor V antibodies (Δ) or control IgG (○) were added to activated platelets or to HUVEC at a final concentration of 100 μg/mL. After 20 minutes factor Xa, 5 nmol/L, and 10⁻¹-factor VII, 10 nmol/L were added, and activation of factor VII was monitored. As a further control for the experiment with activated platelets, activation of factor VII by factor Xa in the presence of unstimulated platelets was also measured (A, ○). As a further control for the experiment with HUVEC, activation of factor VII by factor Xa in the absence of HUVEC (albumin-coated Petri dish) was also measured (B, ○).
PLATELETS AND ACTIVATION OF FACTOR VII

Table 3. Effect of Antifactor V Antibodies Upon Factor Xa-Catalyzed Activation of Factor VII in the Presence of Thrombin-Activated Platelets or Freeze/Thawed Platelets

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Mean Factor VII Activation (nmol/L/min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ control IgG</td>
<td>0.297 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>+ antifactor V IgG</td>
<td>0.164 ± 0.03*</td>
<td>4</td>
</tr>
<tr>
<td>Freeze/thawed platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ control IgG</td>
<td>0.504 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>+ antifactor V IgG</td>
<td>0.470 ± 0.03†</td>
<td>4</td>
</tr>
</tbody>
</table>

*P < .025.
†P > 0.2.

Antifactor V IgG or control IgG, 100 μg/mL, was added to 2 x 10^9/mL freeze/thawed or thrombin-activated platelets. After 20 minutes factor Xa, 5 nmol/L; [125I]-factor VII, 10 nmol/L; and calcium, 5 mmol/L were added, and activation of factor VII was monitored.

Factor Va, not factor V, is the high-affinity binding site on platelets for factor Xa.14 Yet platelets treated with A23187 were equally or more effective in enhancing factor Xa-induced activation of factor VII than were platelets treated with thrombin, an agonist that not only activates platelets but also activates factor V. To investigate this further the investigators compared the coagulant activity, as measured in the investigators' one-stage factor V assay, of the factor V released from platelets by each agonist.

On two occasions stirred normal platelets were treated for 15 minutes with thrombin, 0.25 U/mL, or with A23187, 10 μmol/L, and with Ca2+, 5 mmol/L, and were then assayed for factor V activity. The results, normalized to 2 x 10^9 platelets, were: for thrombin, 1.08 and 1.00 U; for A23187, 0.38 and 0.40 U; and for a thrombin/A23187 activity ratio, 2.8 and 2.5. In one experiment a subsample of platelets incubated with A23187 was also assayed for factor V activity after further incubation for 2.5 minutes with 5 nmol/L factor Xa. The incubation with factor Xa yielded a further sixfold increase in factor V activity. (Adding factor Xa directly to the substrate plasma in the same concentration as that carried over in the diluted test sample did not affect the clotting time of the assay).

Role of platelet activation. When stirred platelets were exposed to agonists in our experiments, the platelets not only released factor V but presumably underwent surface-membrane changes associated with platelet activation. The investigators knew from the above experiments on the role of factor Va that factor V release was required for platelet enhancement of factor Xa-induced factor VII activation, presumably to make available the high-affinity factor Xa–surface-membrane binding site. An additional experiment was carried out to determine whether surface membrane changes associated with platelet activation were also needed.

Since exogenous factor Va allows high-affinity factor Xa binding to unstimulated platelets,16,17 the investigators determined whether adding exogenous factor Va would enhance factor Xa-catalyzed activation of factor VII in reaction mixtures containing stirred but otherwise unstimulated platelets. As shown in Fig 4, adding 3 μg/mL of factor Va to unstimulated platelets failed to increase the rate of factor VII activation over that observed with unstimulated platelets in the absence of added factor Va. One also notes in Fig 4 that exogenous factor Va failed to increase the degree of enhancement obtained with activated platelets.

DISCUSSION

The known cofactor role of anionic phospholipids in factor Xa-catalyzed activation of factor VII14 and the known location of anionic phospholipids on the cytoplasmic face of the surface membrane of unstimulated platelets20 can explain why unstimulated platelets failed to augment factor Xa-catalyzed activation of factor VII, whereas freeze/thawed platelets markedly augmented the activation. However, freezing and thawing platelets also liberate platelet factor V(a).21,22 Therefore before concluding that freeze/thawed platelets increase factor Xa-catalyzed activation of factor VII solely by making available anionic phospholipids, the investigators had to show that antifactor V antibodies were without effect upon the reaction. As is evident from Fig 2, unlike its role in factor Xa-catalyzed activation of prothrombin, factor Va is not a cofactor for factor Xa-catalyzed activation of factor VII.

Platelets stimulated with thrombin, collagen, or the calcium ionophore A23187 also enhanced factor Xa-catalyzed activation of factor VII. In striking contrast to freeze/thawed platelets, antifactor V antibodies abolished the enhancement (Fig 3A, Table 3). The function of factor Va as a high-affinity binding site for factor Xa on the platelet-
surface membrane can account for this difference if such high-affinity binding is required for factor Xa to interact with a surface membrane component of platelets serving as a cofactor for factor VII activation.

Exogenous factor Va can bind to unstimulated platelets and can function on unstimulated platelets as a high-affinity binding site for factor Xa. Yet the investigators failed to observe augmented factor VII activation in reaction mixtures containing unstimulated platelets, purified plasma factor Va, and factor Xa (Fig 4). This presumably reflects the absence of unstimulated platelets of the surface membrane component with cofactor activity present on activated platelets.

Considerable evidence exists that platelet activation reorients phospholipids in the surface membrane, making anionic phospholipids available to interact with clotting factors on the outer surface of the membrane. Therefore, since phospholipid and Ca²⁺ are the only known cofactors for factor Xa-induced activation of factor VII, the investigators suggested that the platelet-surface component of activated platelets enhancing factor Xa-catalyzed activation of factor VII is anionic phospholipid. If so, then one must also conclude that whereas factor Xa can interact directly with anionic phospholipids on disrupted platelet membranes, it cannot interact with anionic phospholipids on the surface membrane of activated platelets without first binding to factor Va. It is of interest that adding exogenous factor Va to a reaction mixture containing a saturating concentration of factor Xa and activated platelets did not augment the rate of factor VII activation beyond that observed in a reaction mixture in which the activated platelets were the only source of factor Va (Fig 4).

Factor Va, but not factor V, can function as the high-affinity platelet binding site for factor Xa. When thrombin was used as the platelet agonist in our experiments, platelet factor V was both released and activated. When 10 μmol/L A23187 was the agonist, platelet factor V was released but presumably only partially activated, since it was about one half to one third as active in a one-stage factor V assay as thrombin-released factor V. Yet A23187-activated platelets augmented factor Xa-catalyzed factor VII activation at least as well, if not better, than thrombin-treated platelets. This may reflect a rapid further activation of platelet factor V by factor Xa in the reaction mixture. The failure of ADP-treated platelets to augment factor Xa-catalyzed activation of factor VII probably reflects the inability of ADP to induce secretion of factor V from platelets suspended in a calcium-containing medium.

Incubation of factor Xa with thrombin-treated platelets has been reported to preserve factor Xa’s ability to activate prothrombin in the presence of antithrombin III. In the reported experiments the antithrombin III concentration only slightly exceeded that needed to neutralize stoichiometrically the initial concentrations of factor Xa and thrombin in the reaction mixtures. However, when investigators added a 3-μg/mL concentration of antithrombin III to reaction mixtures (2% of plasma concentration yet in approximately fivefold excess over the combined concentrations of factor Xa and thrombin in reaction mixtures), they could not demonstrate activation of factor VII, nor could they demonstrate activation of prothrombin (data not shown). Therefore if factor Xa-catalyzed activation of prothrombin on the platelet surface is believed to be important for physiologic hemostasis, then one should not conclude from the antithrombin III data that the ability of activated platelets to enhance factor Xa-induced activation of factor VII lacks potential physiologic significance.

Miller et al have presented data compatible with the hypothesis that the circulating blood of normal persons, but not of hemophilic patients, contains traces of factor VIIa. This would imply that minute amounts of factor VIIa are being formed continuously in normal persons from an activation of factor VII by traces of factor Xa generated in the intrinsic pathway on the surface of platelets or other cells. If so, then enhanced factor Xa-catalyzed activation of factor VII arising from an interaction of factor Xa with the surface of activated platelets could play a physiologically meaningful role in hemostasis.

ACKNOWLEDGMENT

We thank An Hoang for his technical assistance and Angela Wakeham for the preparation of the manuscript.

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The effect of platelets upon factor Xa-catalyzed activation of factor VII in vitro

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