The Role of Activated Human Platelets in Prothrombin and Factor X Activation

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The effect of activated human platelets in intrinsic factor X activation was compared with their effect in prothrombin activation. Compared with unstimulated platelets, platelets triggered by the combined action of collagen plus thrombin showed a tenfold activity increase in prothrombin activation, and a 20-fold rate enhancement in factor X activation. Treatment of collagen plus thrombin-stimulated platelets with N.naja phospholipase A₂ almost completely abolished their activity in prothrombin and factor X activation. Since no significant cell lysis occurs during phospholipase treatment, this indicates that platelet phospholipids, exposed at the membrane exterior, play an essential role in the interaction of platelets with the proteins of the prothrombin and factor X-activating complexes. The time course of generation of the procoagulant platelet surface was different when the amount of coagulation factors present in the assay systems was varied. At suboptimal concentrations of coagulation factors, maximum platelet activity was reached after a shorter time period than at saturating concentrations. When measured at suboptimal amounts of coagulation factors, the platelet activity in prothrombin and factor X activation is also more sensitive to phospholipase treatment. Experiments with synthetic phospholipid mixtures show that prothrombin and factor X activation are optimal at low mol% phosphatidylserine when high concentrations of factor Va and factor VIIIa are employed. The optimal mol% phosphatidylserine increases when the concentrations of nonenzymatic protein cofactors are lowered. These findings are discussed in relation to a model in which phosphatidylserine, exposed at the outer surface of activated platelets, plays an essential role in prothrombin and factor X activation. It is proposed that this phosphatidylserine is not homogeneously distributed in the platelet outer membrane, but that areas with different phosphatidylserine density participate in coagulation factor activation.

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The interaction between blood platelets and coagulation factors is essential for hemostatic plug formation. Activated platelets promote the catalytic two sequential reactions of the blood coagulation cascade: the activation of factor X into factor Xa by a complex of factor IXa, factor VIIIa, and Ca²⁺ and the conversion of prothrombin into thrombin by a complex of factor Xa, factor Va, and Ca²⁺.

Detailed information is available on the interactions of proteins of the prothrombinase complex with the platelet membrane. Unstimulated bovine and human platelets reversibly bind factor Va with high affinity. Platelet-bound factor Va, either derived from added factor Va or released from platelets upon activation with thrombin, is required for high-affinity factor Xa binding. Kinetic experiments suggest that the platelet-bound factor Xa–factor Va complex participates in prothrombin activation.

The nature of the components that comprise the binding site for factor Va and factor Xa on the platelet membrane is not yet defined. Similarities between the effects of platelets and negatively charged phospholipid vesicles in prothrombin activation led to the assumption that negatively charged phospholipids might also be involved in the assembly of the prothrombinase complex at the platelet membrane. Negatively charged (procoagulant) phospholipids are, however, virtually absent from the outer surface of unstimulated platelets. They are almost exclusively located at the inner surface of the platelet plasma membrane and in intracellular membranes. This explains why intact unstimulated platelets show little procoagulant activity compared with lysed platelets. However, when platelets are stimulated by the combined action of collagen and thrombin, about 25% of the platelet phosphatidylserine molecules become exposed at the platelet outer surface, presumably by a mechanism involving transbilayer movement of phosphatidylserine. This process is accompanied by an increase of activity of platelets in prothrombin activation.

Since negatively charged phospholipids have similar effects in factor X and prothrombin activation, it could be anticipated that the appearance of phosphatidylserine at the platelet outer surface would stimulate both activities. In the present study, we compared the effect of platelets, stimulated with various activators, in prothrombin and factor X activation. We report that those platelet activators that increase the activity of platelets in prothrombin activation also increase the capacity of platelets to enhance intrinsic factor X activation. The data support the concept that the exposure of phosphatidylserine at the outer surface of...
platelets is essential to obtain an optimal activity of platelets in prothrombin and factor X activation.

MATERIALS AND METHODS

Materials

S2238, p-h-D-Phenylalanyl-t-pipeolyl-l-arginine-p-nitroanilide hydrochloride, and S2337, N-benzoyl-1-isoleucyl-1-glutamyl-(-p-piperidyl)-glycyl-l-arginine-p-nitroanilide hydrochloride, were purchased from AB Biokastica, Stockholm. p-Nitrophenyl-p'-guanidinobenzoate hydrochloride (p-NPGB) was purchased from ICN Nutritional Biochemicals (Cleveland). DEAE-Sephadex A-50, QAE-Sephadex A-50, and Sephadex G-100 and G-200 were products of Pharmacia, Uppsal, Sweden. Factor VIII-deficient plasma was from King King Biological, Overland Park, Kan. *Echis carinatus*, Russell’s viper venom, soy bean trypsin inhibitor (STI), and fat-free human serum albumin were from Sigma, St Louis. Naja naja venom was from Koch Light. Cohnbrook Bucks, England). Reaction tubes were 2-mL plastic flat-bottom tubes obtained from Sterilin Ltd, Teddington, Middlesex, England. Teflon-coated magnetic spinning bars, 7 x 2 mm were purchased from Bel-Art Products, Pequannock, N.J. Phosphatidylinositol and sphingomyelin were purchased from Koch Light. Phosphatidylicholine (18:1ω/18:1ω-phosphatidylicholine) was from Sigma. [3H]Serotonin creatine sulfate (58 Ci/mol) was obtained from Amersham Corp. Phospholipase A2 was purified from *Naja naja* venom according to Kane et al.23 Single bilayer vesicle solutions were prepared according to de Kruyff,18 as described earlier.27 Phospholipid concentrations were determined by phoshate analysis according to Böttcher et al.19 Extraction of total phospholipid from platelets was done according to Reed et al.26 Lipid analysis was carried out as described previously.14

Proteins

Phospholipase A2 was purified from Naja naja venom according to Zwaal et al.20 One international unit is defined as that amount of enzyme that degrades 1 μmol of egg phosphatidylicholine/min at 37°C. In the purified phospholipase A2, which had a specific activity of 700 U/mg, no proteolytic activity could be detected.27 The vitamin K-dependent factors, prothrombin, factor IX, and factors X, and X, were purified from bovine blood according to established procedures20 as described previously.14,27 The factor X activator from Russell’s viper venom was purified as described by Schifman et al.19 The purifications of thrombin, factor Xa, factor IXa, and factor XIa (contact product) were described earlier.14,20 Bovine Factor V was purified according to a method similar to that described by Kane et al., which is published in detail.24 Factor VIII was purified from bovine blood according to the method of Vehar and Davie,23 with slight modifications.22 Factor V and factor VIII were activated with thrombin as described earlier.20 The specific clotting activities of the coagulation factors were: 6 U/mg (prothrombin), 30 U/mg (factor V), 100 U/mg (factor X), 145 U/mg (factor IX), and 324 U/mg (factor VIII). One clotting unit is defined as the amount of coagulation factor present in 1 mL normal bovine plasma. Thrombin had a specific activity of 2360 NIH U/mg (1 mL thrombin is 0.005 NIH units).

Factor V was stored at -70°C in 50 mmol/L Tris, 175 mmol/L NaCl, and 10 mmol/L CaCl2 (pH 7.9) and factor VIII in 10 mmol/L morpholino ethanesulfonic acid (MES), 175 mmol/L NaCl, and 10 mmol/L CaCl2, adjusted to pH 6.5 with 1 mol/L Tris. The other protein preparations were stored at -70°C after dialysis against 50 mmol/L Tris-HCl, 175 mmol/L NaCl at pH 7.9. Before storage at -70°C, prothrombin and the factor X-, X, and IX preparations were passed through a column of STI-agare (0.5 x 10 cm) to reduce the small amounts of factor Xa that might be present in these preparations. No thrombin or factor Xa could be detected in our protein preparations as determined with the chromogenic substrates S2238 and S2337.

Protein Concentrations

Thrombin, factor Xa, and factor IXa concentrations were determined by active site titration with p-NPGB.27 Prothrombin concentrations were determined after complete activation with *E. carinatus* venom, followed by active site titration with p-NPGB. Similarly, factor X concentrations were obtained by active site titration after complete activation of factors X, or X, by RVV-X. Factor Va concentrations were determined by kinetic analysis.22 The molar concentration of factor VIIIa was determined by kinetic analysis similar to that employed for factor Va.14

Isolation of Platelets

Human platelets were isolated by differential centrifugation as described earlier.24 Platelets were kept at room temperature in a calcium-free buffer, pH 7.5, containing 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 10 mmol/L HEPES, 5 mmol/L glucose, and 0.06% fatty acid-free human serum albumin, conditions at which no loss of platelet activity was found for at least 15 hours. Platelet concentrations were determined with a Coulter counter (Coulter Electronics, Hialeah, Fla). Bovine platelets were isolated according to the same procedure.

Phospholipid and Phospholipid Vesicle Preparations

Phosphatidylethanolamine and phosphatidylserine (both 18:1ω/18:1ω) were prepared from phosphatidylcholine by enzymatic synthesis by the method of Comfurius and Zwaal.17 Single bilayer vesicle solutions were prepared according to de Kruyff,18 as described earlier.27 Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al.19 Extraction of total phospholipid from platelets was done according to Reed et al.26 Lipid analysis was carried out as described previously.14

Platelet Stimulation and Determination of Prothrombin and Factor X Converting Activity

Platelet activity in prothrombin and factor X activation was measured in 2-mL flat-bottom plastic tubes, in which the reaction mixtures were stirred at 350 rpm with Teflon stirring bars. To a reaction tube containing 292.5 g/L of a platelet suspension, 1.3 g/L of human serum albumin, 0.5 g/L of CaCl2, and 3 g/L of NaCl, 2.9 g/L of glucose, 1.2 g/L of MgCl2, 6 g/L of NaCl, 1.6 g/L of KCl, 2.9 g/L of glucose, 1.2 g/L of MgCl2, 6 g/L of NaCl, and 0.5 g/mL human serum albumin at pH 7.9. The final reaction mixture (500 μL) contained: 20 mol/L Tris, 6 mol/L HEPES, 150 mmol/L NaCl, 1.6 mmol/L KCl, 2.9 mmol/L glucose, 1.2 mmol/L MgCl2, 6 mmol/L CaCl2, 0.5 g/mL human serum albumin at pH 7.9 and amounts of platelets, factor Va, factor Xa, and thrombin indicated in the legends of the tables and figures. Fifteen and 30 seconds after initiating prothrombin activation, aliquots (usually 5 or 10 μL) were taken from the reaction mixture and transferred to cuvettes to

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determine the amount of thrombin formed. The cuvettes (thermo-

stated at 37 °C) contained a buffer of 50 mmol/L Tris-HCl, 175 

mmol/L NaCl, 0.5 mg/mL ovalbumin, and 20 mmol/L EDTA, pH 

7.9, in such amounts that the final volume was 2 mL; 335 μL of 

the thrombin-specific chromogenic substrate S2328 was also pre-

sent. Further activation of prothrombin is prevented by dilution 

and the presence of EDTA. The absorbance change recorded at 405 

minus 500 nm on an Aminco DW2 spectrophotometer (set in the 

dual wavelength mode) is a measure of the amount of thrombin 

present in the aliquots. From a calibration curve made with known 

amounts of active site-titrated thrombin, determined under the 

same conditions as described above, the amount of thrombin present 

in the reaction mixture is calculated. Under the conditions used, 

the formation of thrombin in the reaction mixtures was linear in time, 

and the rate of thrombin formation was calculated from the amount 

of thrombin present after 15 and 30 seconds.

**Assay of Platelet Activity in Factor X Activation**

Forty-five seconds before measuring the effect of platelets in 

factor X activation, 25 μL factor IXa and 33.4 μL CaCl₂ (60.6 

mmol/L) were added to the platelet suspension. Factor X activation 

was started with 16.6 μL factor Xa and 100 μL activated factor VIII.

The final reaction mixture (500 μL) had the same composition as 

described above for prothrombin activation and contained amounts 

of platelets, factor IXa, factor VIIIa, and factor X indicated in the 

legends of the tables and figures. Forty-five and 90 seconds after 

initiating factor X activation, aliquots from the reaction mixture 

were transferred to cuvettes to determine the amount of factor Xa 

formed. The experimental set-up to measure factor Xa is essentially 

the same as described for the measurement of thrombin formation, 

with the exception that the absorbance change at 405 minus 50 nm 

was measured using the factor Xa-specific chromogenic substrate 

S2337 (final concentration 192 μmol/L). Factor Xa formation is 

linear in time after a lag period of about 15 seconds after starting the 

reaction. Therefore, the interval between 45 and 90 seconds after 

initiating factor X activation was used to calculate the rate of factor 

Xa formation. Control experiments were carried out to check whether 

or not the concentration of factor Xa is adequately mea-

sured in the presence of activated platelets or artificial phospholipid 

vesicles. A known amount of factor Xa was added to artificial 

phospholipid vesicles plus Ca²⁺ or to platelets that were subsequently 

activated with thrombin plus collagen. All added factor Xa was 

recovered in the chromogenic assay described above, which indicates 

that the binding of factor Xa to platelets and phospholipids is either 

reversible (EDTA is present in the assay system) or does not 

interfere with the chromogenic assay.

**Stimulation of Platelets by Collagen**

The collagen used in most of the experiments presented in this 

article was horse tendon collagen obtained from Hormon Chemie, 

München. Also, collagen prepared from bovine achilles tendon 

obtained from Sigma and Merck, Germany, as well as highly 

purified (type I) collagen from calf skin (a gift from Professor J. 

Caen) were employed in these studies. Collagens were added in the 

nonfibrillar form, dissolved in acidic dilution buffer obtained from 

Hormon Chemie.

**Platelet Aggregation**

Platelet aggregation studies were carried out in a lumiaggregom-

er from Chronolog Corp, Havertown, Pa. Platelets suspended in a 

HEPES buffer at pH 7.5 (see above) were brought in a cylindrical 

siliconized glass cuvette in a volume of 614 μL and stirred with a 

Teflon-coated spinning bar. This cuvette was placed in the aggre-

gometer, thermostated at 37 °C, and 26 μL of a 75-mmol/L CaCl₂ 

solution was added. The platelets were prewarmed for five minutes 

and stirred. Then, 10 μL platelet stimulator was added, and the 

aggregation pattern was recorded.

**Measurement of Serotonin Release and Leakage of Lactate Dehydrogenase**

The conditions for platelet stimulation are the same as described 

by the determination of platelet prothrombin and factor X converting 

activity. Release of [³⁵S]serotonin was determined in the platelet 

supernatant after centrifugation at 7,000 g for two minutes in an 

Eppendorf microfuge. Leakage of lactate dehydrogenase was mea-

sured as described earlier.

**RESULTS**

**Activity of Stimulated Platelets in Prothrombin and Factor X Activation**

In a previous article, we have shown that activation of platelets with thrombin plus collagen resulted in the formation of a procoagulant surface that is highly active in prothrombin activation. It was proposed that the exposure of the negatively charged phospholipid phosphatidylserine at the outer surface of the platelets, which accompanied the generation of the procoagulant surface, is essential for the observed increase of platelet activity in prothrombin activation. In order to compare the activity of platelets in prothrombin and intrinsic factor X activation, assay systems were developed in which rates of thrombin and factor Xa formation were proportional with the amount of procoagulant phospholipid present. At high concentrations of coagulation factors, this condition was satisfied. Figure 1A shows that with 15 nmol/L factor Xa, 30 nmol/L factor Va, and 4 μmol/L prothrombin present in a reaction mixture, rates of prothrombin activation are proportional to the amount of procoagulant phospholipid when a platelet lysate, a platelet lipid extract, or synthetic vesicles, containing 2.5 or 40 mol% phosphatidylserine, are used as a phospholipid source. At phospholipid concentrations below 2.5 μmol/L, rates of prothrombin activation are linear with the amount of phospholipid present. A further increase of the concentration of coagulation factors had no effect on the rate of thrombin formation, which indicates that all available sites on the phospholipid surface participate in prothrombin activation. For intrinsic factor X activation, saturation with coagulation factors and a linear relation between rates of factor Xa formation and the phospholipid concentration was observed at 150 nmol/L factor IXa, 15 nmol/L factor VIIIa, and 0.5 μmol/L factor X. Due to a limited supply of factor VIII, it was not possible to regularly use the saturating factor VIIIa concentrations. We succeeded, however, in selecting coagulation factor concentrations at which rates of factor X activation, though not saturated with respect
Materials and Methods.

Lipid extraction from platelets, lipid analysis, and the assay systems for prothrombin and factor X activation are described in detail. Vesicles composed of 40% phosphatidylserine, 4% phosphatidylethanolamine, 18% sphingomyelin, and cholesterol in a 1:1 molar ratio to total phospholipid are used as a source of phospholipid in the assay systems described above. In this experiment, factor X activation is measured at saturating factor VIIIa concentrations (15 nmol/L). Release of serotonin and platelet aggregation was determined in a separate experiment. Platelets stimulated by the combined action of 10 μg/mL collagen and 1.3 nmol/L thrombin exhibited a tenfold activity increase in prothrombin activation and were 20 times more active in factor X activation than unstimulated platelets. Platelets stimulated only with thrombin show little rise in activity, but platelet stimulation with collagen alone resulted in activities that were approximately 30% of that observed with platelets stimulated by collagen plus thrombin. In a recent article, Bevers et al have shown that there is a limited exposure of phosphatidylserine when platelets are stimulated with collagen alone. Although the standard deviation for the determination of the amount of phosphatidylserine exposed was relatively high, it is possible that it accounts for the procoagulant activity observed for collagen-stimulated platelets. However, it can also not be ruled out that activities found with collagen-stimulated platelets were in fact the result of a combined action of collagen and small amounts of thrombin present in the assay system. In the prothrombinase assay, 2 nmol/L thrombin is formed in less than five seconds, while thrombin is also present in the assay system in which factor X activation is measured, because the factor VIIIa preparation still contained the small amounts of thrombin that were used for the activation of factor VIII. With other platelet activators (adenosine diphosphate, serotonin, epinephrine, either alone or in combination), activities similar to unstimulated platelets were observed (data not shown). It is essential that platelets are stirred during the activation process. Nonstirred platelets incubated with thrombin plus collagen show no activity increase in prothrombin and factor X activation. It should be emphasized that the prothrombin and factor X converting activities mainly reside with the stimulated platelets, since less than 10% of the activity remained in the supernatant after sedimenting the platelets by centrifugation at 7,000 g.

A possible explanation for the effect of collagen plus thrombin could be that platelets stimulated with this trigger are subject to increased cell lysis. This could explain the increased prothrombin and factor X converting activities, since lysed platelets are highly active in prothrombin and factor X activation (Fig 1A and B). We used leakage of lactate dehydrogenase, a cytoplasmic platelet enzyme, as a marker for cell lysis during platelet activation. Approximately 1% to 2% of the total lactate dehydrogenase present leaks out of platelets that were stimulated with thrombin or col-
The activity of prothrombin and factor X activation in platelets is not due to cell lysis. To test the proposal that the procoagulant surface of platelets is not due to cell lysis, experiments were carried out with bovine platelets. The effects of thrombin, collagen, and collagen plus thrombin on the prothrombin and factor X converting activities of bovine platelets were the same as on human platelets.

Dependence of Platelet Activation on the Concentration of Thrombin, Collagen, and the Source of Collagen

The activity of platelets in prothrombin and factor X activation was measured after stimulation with variable amounts of collagen, either in the absence of thrombin or at a constant thrombin concentration (5 nmol/L). As shown in Fig 2, both activities rise parallel when the amount of collagen is increased and reach a maximum at a collagen concentration of 8 µg/mL. The collagen used in this experiment was in the nonfibrillar form. When collagen was first allowed to form fibrils at neutral pH and subsequently used for platelet stimulation, the same activity in prothrombin and factor X activation was found. The collagen was obtained from horse tendon (Hormon Chemie). Collagens prepared from bovine achilles tendon (Sigma

Table 1. Effect of Platelets in Prothrombin and Intrinsic Factor X Activation: A Comparison With Platelet Aggregation and Serotonin Release

<table>
<thead>
<tr>
<th>Platelet Stimulator</th>
<th>Prothrombin Activation (nmol/L, Xa/min)</th>
<th>Factor X Activation (nmol/L, Xa/min)</th>
<th>Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xa†</td>
<td>Xa-Va†</td>
<td>IXa§</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>34.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1.9</td>
<td>40.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Collagen</td>
<td>7.9</td>
<td>98.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Thrombin + collagen</td>
<td>24.5</td>
<td>351.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Thrombin + collagen + phospholipase A₂</td>
<td>0.4</td>
<td>17.4</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

ND, not determined.

*0.5 nmol/L factor Xa, no factor Va, 4 µmol/L prothrombin.
† 15 nmol/L factor Xa, 30 nmol/L factor Va, 4 µmol/L prothrombin.
‡ 150 nmol/L factor IXa, no factor Villia, 0.5 µmol/L factor X.
§ 150 nmol/L factor IXa, 15 nmol/L factor Villia, 0.5 µmol/L factor X.

Human platelets at a concentration of 5 x 10⁶/mL were stimulated for 15 minutes with 1.3 nmol/L thrombin and/or 10 µg/mL collagen. In the experiment in which phospholipase was used, platelet stimulation was followed by a 15-minute incubation with 3 IU/mL phospholipase A₂ (Naja naja). Platelet aggregation was measured in a separate experiment at 5 x 10⁶ platelets/mL. Platelet aggregation, serotonin release, and rates of prothrombin and factor X activation were determined as described in Materials and Methods.
The concentrations of thrombin and collagen required to expose the procoagulant surface are similar to those required to induce platelet aggregation and release. Table 2 summarizes the concentrations of thrombin or collagen that produce threshold, half-maximal, and maximal effects in platelet aggregation and release. The combined effect of thrombin plus collagen on these platelet functions is synergistic. Combination of concentrations of collagen and thrombin below the threshold concentrations for the individual components already produce half-maximal platelet aggregation and release.

**Time Course of Appearance of Platelet Activity in Prothrombin and Factor X Activation**

The generation of a procoagulant surface was followed in time for platelets stimulated with thrombin or collagen plus thrombin. Since the effect of collagen alone could not be singled out from the combined collagen and thrombin effect (see above), collagen-stimulated platelets were not further considered. The activity of platelets in prothrombin activation was measured with different concentrations of factor Xa and factor Va present in the prothrombinase assay. The prothrombin activating mixtures contained platelets, 4 μmol/L prothrombin, and 0.5 nmol/L factor Xa; no added factor Va (Fig 4A), 0.5 nmol/L factor Xa, 1 nmol/L factor Va (Fig 4B); or 15 nmol/L factor Xa, 30 nmol/L factor Va (Fig 4C). In all assay systems, platelets became active upon stimulation with collagen plus thrombin. As expected, the highest activities were found at saturating factor Xa and factor Va concentrations (Fig 4C). Unstimulated platelets exhibited the lowest prothrombin converting activity.

<table>
<thead>
<tr>
<th>Platelet Aggregation and Release</th>
<th>Aggregation Effect</th>
<th>Half-Maximal Effect</th>
<th>Maximal Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Activator</td>
<td>Threshold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>0.5 nmol/L</td>
<td>0.75 nmol/L</td>
<td>1.2 nmol/L</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0 μg/mL</td>
<td>3.3 μg/mL</td>
<td>5.0 μg/mL</td>
</tr>
<tr>
<td>Thrombin plus collagen</td>
<td>0.4 nmol/L plus</td>
<td>0.5 μg/mL</td>
<td></td>
</tr>
</tbody>
</table>

Platelets were activated by thrombin and/or collagen at a concentration of 5 × 10⁷ platelets/mL, and the platelet aggregation and release reaction was measured as described in Materials and Methods.
Stimulation of platelets with thrombin alone resulted in a slow rise of prothrombin converting activity when added factor Va is present in the prothrombinase assay mixture (Fig 4B and C). When prothrombin activation is measured in the absence of added factor Va, the situation is somewhat different (Fig 4A). Unstimulated platelets are completely inactive in prothrombin activation, presumably due to the virtual absence of both a procoagulant surface and factor Va. Thrombin stimulation of platelets gives rise to an appreciable activity of platelets in prothrombin activation when this is measured in the absence of added factor Va and compared with the activity of unstimulated platelets. However, also in the absence of added factor Va, the highest activity was observed with platelets stimulated by thrombin plus collagen. We propose that in platelet experiments carried out in the presence of a saturating concentration factor Va, the rate of prothrombin activation is determined by the procoagulant surface already present in the case of unstimulated platelets or the procoagulant surface generated in case of platelets stimulated with thrombin plus collagen. In the absence of added factor Va, both the presence or appearance of the procoagulant surface and the release of platelet factor Va is probed. We have no explanation for the origin of the procoagulant surface of unstimulated platelets. It is either present on platelets circulating in the blood or exposed upon platelet lysis or platelet activation during venipuncture of the blood or the isolation of the platelets.

The platelet procoagulant activity increases rapidly, without a lag period, directly upon activation. We have extended our measurements over a period of 15 to 30 minutes to determine the maximal prothrombin and factor X converting activities that can be reached in order to calculate and compare the maximal number of procoagulant sites that can be exposed (see Discussion and Table 4, below). It should be emphasized, however, that two minutes after platelet activation by thrombin plus collagen the prothrombin converting activity has already risen to 100 nmol/L thrombin formed/min/6.6 x 10^6 platelets/mL (Fig 4B and C). Taking into account that the amount of platelets in whole blood is 2 x 10^9/mL and that the plasma prothrombin concentration is approximately 2,000 nmol/L, it is obvious that the platelet prothrombin converting activity reached after two minutes would be more than sufficient to account for in vivo thrombin formation.

One other feature of the time generation curves has to be mentioned here. The appearance of platelet prothrombin converting activity is apparently more rapid when prothrombinase activity is assayed in a system containing low amounts of factor Xa and factor
Va. Half-maximal activities were obtained after 2½ minutes (Fig 4A), four minutes (Fig 4B), and nine minutes (Fig 4C). Compared with prothrombin converting activity, factor X converting activity appeared even more rapidly when platelets were stimulated by thrombin plus collagen (Fig 4D). Within two minutes after platelet activation, half-maximal factor X converting activity was observed in an assay system, in which suboptimal factor VIII concentrations were used. Also, in factor X activation, unstimulated platelets exhibit a constant and low activity, and stimulation with thrombin alone results in a slow rise of platelet activity.

The effect of platelet concentration in prothrombin and factor X activation is shown in Fig 5A and B. Collagen plus thrombin stimulated platelets show a linear increase of both activities with the platelet concentration, until rates of thrombin and factor Xa formation were reached at which clotting factor activation in these assay systems is no longer linearly proportional to the amount of phospholipid present (cf, 

Effect of Phospholipase A$_2$ on the Activity of Stimulated Platelets in Prothrombin and Factor X Activation

The results presented in Table 1 show that the activity of platelets in prothrombin and factor X activation could be completely abolished by treating the collagen plus thrombin stimulated platelets with phospholipase A$_2$. The course of disappearance of platelet prothrombin and factor X converting activities was followed when platelets stimulated for 30 minutes with thrombin plus collagen were subsequently treated with a small amount of phospholipase A$_2$ (0.04 IU/mL). After different time intervals of phospholipase treatment, coagulation factors were added to measure the remaining activity of platelets in prothrombin and factor X activation. Figure 6 shows that the prothrombinase activity of stimulated platelets is more sensitive to phospholipase treatment when measured in the absence of factor Va than at suboptimal and saturating factor Xa and factor Va concentrations. It seems that...
part of the prothrombinase activity measured at saturating factor Xa and factor Va is less sensitive to phospholipase treatment. The prothrombinase activity measured at saturating factor Xa and factor Va could, however, be completely abolished when the stimulated platelets were treated with higher amounts of phospholipase A₂ (2 IU/mL). Figure 6 also shows that the factor X converting activity of stimulated platelets measured at suboptimal factor VIIIa is highly sensitive to treatment with low amounts of phospholipase A₂.

The results shown in Fig 6 cannot be due to a detrimental effect of phospholipase A₂ on the coagulation factors, since their activity was not affected by prolonged incubation with phospholipase. This can actually also be concluded from the experiment itself. For each data point in the figure, there is an equal time of incubation of coagulation factors with phospholipase A₂. This enzyme is added at time 0 (after activation of platelets for 30 minutes with thrombin plus collagen), and the coagulation factors are added at 2, 4, 6, 8, 10, or 15 minutes to probe the remaining procoagulant surface. Also, the fact that after proper dilution phospholipase-treated control platelets still become active in prothrombin and factor X activation and show a normal response in aggregation and release upon stimulation with thrombin and/or collagen demonstrates that the action of phospholipase A₂ is restricted to phospholipids exposed at the membrane exterior (Table 3). Platelet aggregation and release are even not affected when control platelets are treated at a ten-fold higher phospholipase A₂ concentration.

In our laboratory, we confirmed the finding of Verhey et al.² that the degradation products of phospholipases (lysophospholipids) do not interfere with coagulation factor activation and are therefore not anticoagulant. Membrane bilayers composed of lysophosphatidylserine and lysophosphatidylcholine plus the appropriate fatty acids neither stimulate nor inhibit prothrombin activation. The abolishment of the prothrombin and factor X converting activities of thrombin plus collagen stimulated platelets upon treatment with phospholipases is therefore indicative for a “phospholipid-like” nature of the procoagulant surface.

**Effect of Different Synthetic Phospholipid Mixtures in Prothrombin and Factor X Activation**

It was investigated whether differences in time course of generation of a procoagulant platelet surface probed at varying coagulation factor concentrations and the accompanying different sensitivities to phospholipase A₂ treatment could be related to differences in phospholipid requirement. Therefore, prothrombin and factor X activation were measured in the presence of phospholipid vesicles with varying lipid composition. These vesicles were composed of lipids present in platelet membranes. The mole fraction of each phospholipid was fixed at the same value as found in a total lipid extract of platelets, with the exception of phosphatidylserine and phosphatidylincholine. Prothrombin activation was measured in the absence of factor Va and at the suboptimal and saturating factor Xa and factor Va concentrations also employed in Fig 4. Factor X activation was measured in the absence of factor VIIIa and at the same coagulation factor concentrations as used in Fig 4D.

Large differences between prothrombin and factor X activation at different coagulation factor concentrations were observed when the mole fraction phosphatidylserine in a vesicle was varied at the expense of phosphatidylcholine (Fig 7A and B). The mole fraction phosphatidylserine required for maximal stimulation of prothrombin and factor X activation varied at different coagulation factor concentrations. In prothrombin activation, the optimal phosphatidylserine mole fraction varied from 2.5 mol% at saturating

<table>
<thead>
<tr>
<th>Platelet Stimulator</th>
<th>Prothrombin Activation (nmol/L·min⁻¹)</th>
<th>Factor X Activation (nmol/L·min⁻¹)</th>
<th>Aggregation</th>
<th>Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated platelets</td>
<td>46.0</td>
<td>0.42</td>
<td>50.2</td>
<td>0.60</td>
</tr>
<tr>
<td>Thrombin</td>
<td>50.2</td>
<td>0.60</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin plus collagen</td>
<td>385.4</td>
<td>9.45</td>
<td>+</td>
<td>72</td>
</tr>
<tr>
<td>Phospholipase A₂-treated platelets</td>
<td>16.2</td>
<td>&lt;0.1</td>
<td>23.4</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

*Human platelets at a concentration of 5 x 10⁹/mL were incubated at 37 °C for 70 minutes with 3 mmol/L CaCl₂, in either the absence or presence of 0.1 IU/mL phospholipase A₂ (Naja naja). After treatment, the platelets were diluted to 5 x 10⁹/mL (for measurement of platelet aggregation and release) or 5 x 10⁹/mL (for measurement of their procoagulant properties). The diluted platelet suspensions were stimulated for 15 minutes with 1.3 nmol/L thrombin and/or 10 μg/mL collagen. The prothrombin and factor X converting activities were measured as described in Materials and Methods at 15 nmol/L factor Xa, 30 nmol/L factor Va, 4 μmol/L prothrombin, and 50 nmol/L factor IXa, 0.1 nmol/L factor VIIIa, and 0.5 μmol/L factor X, respectively.*
factor Xa and factor Va concentrations to more than 40 mol% when prothrombin activation was measured in the absence of factor Va (Fig 7A). Phospholipid vesicles with more than 40 mol% phosphatidylserine were not used in this experiment because they aggregate in the presence of 10 mmol/L CaCl2. For factor X activation, the same phenomenon was observed. The phosphatidylserine optimum was dependent on the amounts of factor IXa and factor VIIIa present in the assay system for factor X activation (Fig 7B). At high concentrations of factor IXa and factor VIIIa, the optimum is observed at 10 mol% phosphatidylserine, while similar to prothrombinase, the optimum in the absence of the nonenzymatic cofactor factor VIIIa is at 40 mol% phosphatidylserine or higher. These differences in phosphatidylserine requirement at different coagulation factor concentrations can explain the observed differences in time course of generation of a procoagulant platelet surface and sensitivity to phospholipase treatment measured under the same conditions (see Discussion).

**DISCUSSION**

The experiments presented in this article show that platelets stimulated with thrombin plus collagen expose a procoagulant surface that highly promotes prothrombin and intrinsic factor X activation. With unstimulated and thrombin-stimulated platelets, markedly lower activities were observed. In previous articles, we have shown that the negatively charged phospholipid phosphatidylserine, which is localized in unstimulated platelets in the inner membrane monolayer, becomes exposed in the outer membrane monolayer when platelets are activated with collagen plus thrombin. Negatively charged phospholipids are required in synthetic phospholipid vesicles that are used as a procoagulant surface in prothrombin and factor X activation. We therefore propose that the phosphatidylserine exposed at the outer surface of activated platelets plays an important role in the interactions of the platelet membrane with the proteins of the prothrombin and factor X activating complexes and that these interactions are essential for the observed rate enhancements of prothrombin and factor X activation. This concept is supported by the finding that the activity of stimulated platelets in prothrombin and factor X activation is almost completely abolished when the collagen plus thrombin stimulated platelets are treated with phospholipase A2 (Table 1). In this experiment, phospholipase action was limited to phospholipids of the outer membrane monolayer, and the membrane integrity was left intact.

Our experiments do not rule out an involvement of platelet membrane proteins in the interactions with the coagulation factors. Majerus and coworkers described a patient with a bleeding disorder whose platelets lack a surface component required for the binding of the factor Xa–Va complex. In a recent paper, they proposed that this component presumably is a protein. The group of Mann reported that unstimulated bovine platelets bind factor Va three orders of magnitude tighter than synthetic phospholipid vesicles. Although “a highly specialized phospholipid structure” at the platelet surface can cause this affinity difference, it can also be visualized that a platelet membrane protein is responsible for this phenomenon. Tuszyński et al demonstrated that platelet-bound factor Va is asso-
associated with components from the platelet cytoskeleton. The ability of the cytoskeleton to promote factor Xa-catalyzed prothrombin activation is, however, still dependent on the presence of lipid. Our experiments were designed in such a way that rates of prothrombin and factor X activation are limited by the availability of a negatively charged procoagulant phospholipid surface. Saturating coagulation factor concentrations were employed so that the binding affinities of the membrane for the coagulation factors became less important. Therefore, we cannot rule out the possibility that besides phosphatidylserine, other membrane components play a role in the assembly of the prothrombin and factor X activating complexes at the surface of platelets stimulated with collagen plus thrombin.

Kinetic experiments have shown that the activation of prothrombin and factor X in the absence of phospholipids is a very inefficient process. Phospholipids dramatically stimulate these reactions because they promote the assembly of the prothrombin and factor X activating complexes in such a way that phospholipid-bound substrates (prothrombin or factor X) are activated by phospholipid-bound enzymes (factor Xa–Va or factor IXa–VIIIa). In experiments with thrombin plus collagen stimulated platelets, such high rates of prothrombin and factor X activation were observed that it seems justified to assume that the activation of coagulation factors takes place at the platelet membrane surface. This would mean that in analogy with the model system, platelet-bound prothrombin and factor X are activated by a platelet-bound factor Xa–Va and factor IXa–VIIIa complex, respectively. Binding of prothrombin to the platelet membrane, however, has not yet been demonstrated. If the binding affinity of platelets for prothrombin is similar to that of synthetic phospholipids, it is possible that it escaped detection because the affinity would then be three to four orders of magnitude lower than observed for factor Xa and Va.

Both bovine and human platelets possess high-affinity binding sites for factor Va. Some 1,000 to 2,000 binding sites with an approximate $K_d$ of $10^{-10}$ mol/L are detected per platelet, independent of whether or not the platelets are stimulated with thrombin. Platelet-bound factor Va is the receptor for factor Xa. Two hundred to 300 binding sites for factor Xa are present per platelet either from human or bovine sources. In these studies, there was no investigation of platelets stimulated with collagen plus thrombin. The platelet-bound factor Xa participates in prothrombin activation. From reports of other laboratories, it can be calculated that thrombin-stimulated human and bovine platelets, in which factor V released from platelets is the source of factor Va, activate prothrombin at a rate of 4 to 8 nmol/L thrombin formed per minute with 10$^7$ platelets/mL. This correlates very well with the 1.9 nmol/L thrombin formed per minute that we observed with $0.3 \times 10^7$ thrombin-stimulated platelets/mL when we measured prothrombin activation in the absence of added factor Va (Table I). Platelets have, however, a much higher capacity to promote prothrombin activation. Table I also shows that platelets stimulated with collagen plus thrombin exhibit a 170-fold higher activity. In this experiment, saturating amounts of factor Xa and factor Va were present. It is essential that a limited amount of platelets is used in these experiments. At high platelet concentrations ($5 \times 10^7$/mL), such a large number of procoagulant sites is exposed that even if enough factor Xa and factor Va could be added to saturate these sites, the prothrombin available in the assay systems would be completely converted within a few seconds. It should also be emphasized that platelets have to be stirred during stimulation in order to obtain procoagulant activity. Stirring of platelets is also essential for the expression of other platelet functions. Nonstirred platelets activated with the proper stimuli do not aggregate. Also, the release of serotonin is drastically diminished or even absent if nonstirred platelets are activated with collagen, adenosine diphosphate (ADP), or the ionophore A23187. We have ruled out possible artifacts like platelet lysis and platelet fragmentation. Activation of stirred platelets with thrombin plus collagen does not induce liberation of cytoplasmic lactate dehydrogenase, and fragmentation of platelets is unlikely because more than 90% of the procoagulant activity sediments with platelets upon centrifugation. We do not know why stirring of platelets is required for the expression of procoagulant activity. It is possible that an efficient interaction of platelets with collagen fibers requires stirring or that a minimal shear stress is essential for the generation of a procoagulant surface. The latter could be an important biologic phenomenon, since circulating platelets in vivo are also subject to shear stress. Since the flow regimen of a solution stirred with a stirring bar consists of very complex time-varying laminar flow streams, it is, however, not possible to express procoagulant activities as a function of the hydrodynamic force on the platelets and to relate the shear force induced by stirring with that acting on platelets in the circulation.

The data presented in Table I can be used to quantitate the number of functional sites that participate in prothrombin and factor X activation at the platelet surface. Since the coagulation factors were present in saturating amounts, both activities were measured at conditions approaching $V_{\text{max}}$. For this...
calculation, the $V_{\text{max}}$ of prothrombin activation is taken as 2,700 mol thrombin/min/mol factor Xa, and the $V_{\text{max}}$ of factor X activation as 500 mol factor Xa/min/mol factor IXa, and it is assumed that the platelet-bound factor Xa–Va and factor IXa–VIIa complexes have the same activity as found in the model systems. The latter assumption seems to be justified because, so far, we have never found a procoagulant surface (either from synthetic or platelet origin) that gave $V_{\text{max}}$ values higher than those published earlier. The $V_{\text{max}}$ of prothrombin activation by factor Xa in the presence of synthetic phospholipids and a saturating concentration of factor Va is constant when the amount of phosphatidylserine present in the phospholipid vesicles is varied between 2 and 30 mol%. The results of these calculations are presented in Table 4. Unstimulated and thrombin-stimulated platelets have a low number of functional binding sites for the prothrombin and factor X activating complexes (1,000 to 3,000 per platelet). This number correlates very well with the number of factor Va binding sites (2,000), determined in a binding study under these conditions. We do not know, however, whether this correlation is purely accidental or if it means that the factor Va binding sites detected on unstimulated platelets are functional sites that participate in prothrombin activation. For collagen plus thrombin stimulated platelets, approximately 26,000 functional binding sites for the prothrombinase complex and approximately 20,000 for the factor X activating complex are calculated. An identical number of functional sites for prothrombin and factor X activation is to be expected when the phosphatidylserine exposed is an essential component of the procoagulant site. (Also, the fact that the collagen and thrombin requirements for generation of platelet activity in prothrombin and factor X activation are the same supports this.)

It cannot be excluded that phosphatidylserine is also part of the procoagulant site (factor Xa–Va receptor) at the surface of unstimulated platelets. This possibility is supported by the following observations: (a) A small amount of phosphatidylserine (probably sufficient to explain the low rate of prothrombin activation) is determined in the outer monolayer of unstimulated human platelets. This phosphatidylserine can even be present in domains with high density (see below). (b) Prothrombin converting activity of unstimulated platelets is abolished upon treatment with phospholipases. (c) Factor Xa, a factor Xa derivative that lacks γ-carboxyglutamyl residues, does not bind to the platelet factor Xa receptor. We do not feel that the experiments in which a monoclonal antibody against negatively charged phospholipids does not block platelet prothrombin activation and factor Xa binding can be used as an argument against phospholipid involvement. This antibody only blocks prothrombin activation on synthetic phospholipid vesicles and does not interact with platelet membrane preparations, for which it can hardly be argued that negatively charged phospholipids are absent. The authors suggest that the phosphatidylserine in platelet membranes may be relatively inaccessible to the antibody. Moreover, Dahlbäck et al recently reported a case with a polyclonal lupus anticoagulant with specificity for negatively charged phospholipids that inhibited platelet prothrombinase activity.

Two other phenomena observed during our studies on the properties of the procoagulant sites of platelets need further discussion.

1. The time course of generation of the procoagulant surface on collagen plus thrombin stimulated platelets is different when prothrombin and factor X activation are measured at different coagulation factor concentrations (Fig 4).

2. Phospholipase treatment of thrombin plus collagen stimulated platelets more rapidly destroys the procoagulant surface when this is measured at low coagulation factor concentrations than when it is measured at high concentrations. The results obtained with synthetic phospholipid vesicles show that there are remarkable differences in phosphatidylserine requirement in the assay systems employed in the above-mentioned experiments. At low concentrations of factor Xa–factor Va or factor IXa–

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**Table 4. Procoagulant Sites on Human Platelet Membranes**

<table>
<thead>
<tr>
<th>Platelet Stimulator</th>
<th>Prothrombin Activation</th>
<th>Factor X Activation</th>
<th>Factor Xa</th>
<th>Factor Va</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2,550</td>
<td>920</td>
<td>&lt;10*</td>
<td>2,000–3,000†</td>
</tr>
<tr>
<td>Thrombin</td>
<td>2,990</td>
<td>1,240</td>
<td>200–300*</td>
<td>ND</td>
</tr>
<tr>
<td>Thrombin + collagen</td>
<td>26,120</td>
<td>19,000</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The number of procoagulant sites per platelet is calculated with the formula: number of sites/platelet = $v/V_{\text{max}} \times 10^{-8}$ x Avogadro's number/platelets times $1^{-1}$, in which $v$ is the rate of prothrombin or factor X activation determined in the experiment presented in Fig 1, and $V_{\text{max}}$ is the maximal rate of prothrombin activation or factor X activation. Data shown represent the functional sites per platelet (this paper).

ND, not determined.

*Binding sites from reference 5.
†Binding sites from reference 4.
factor VIIIa, procoagulant surfaces containing low amounts of phosphatidylserine hardly stimulate prothrombin and factor X activation. Optimal rates are observed at 40 mol% phosphatidylserine or higher. At high concentrations of coagulation factors, the optimal mol% phosphatidylserine required is much lower (as low as 2.5% for prothrombin activation at saturating factor Xa and factor Va concentrations). Under these conditions, procoagulant surfaces with low phosphatidylserine still have an appreciable activity. Based on these observations, it can be hypothesized that the phosphatidylserine exposed at the surface of collagen plus thrombin stimulated platelets is not homogeneously distributed in the outer lipid leaflet of the membrane bilayer. Domains with high phosphatidylserine density, which rapidly appear at the surface of stimulated platelets are measured at low coagulation factor concentrations. Domains with low phosphatidylserine density that appear slower can only be probed at high coagulation factor concentrations. This would also explain why the activity of the procoagulant surface measured at low coagulation factor concentrations is more sensitive to phospholipase treatment. It has been shown that phosphatidylserine monolayers, at surface pressures found in the platelet membrane (34 dyne/cm\(^{2}\)), are more rapidly degraded by phospholipase A\(_2\) than monolayers of phosphatidylcholine.\(^{32,41}\) It is therefore likely that platelet membrane surface areas containing high local phosphatidylserine concentrations are more susceptible to phospholipase A\(_2\) than surface areas containing low phosphatidylserine concentrations.

If such lateral phase separations occur in the outer leaflet of the platelet plasma membrane after or during platelet activation, it presumably requires other components to induce or maintain them. An involvement of extracellular calcium in this process cannot be excluded, but it is also possible that membrane proteins participate in the properties of activated platelets to produce at least two different lipid domains with different phospholipid compositions.

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REFERENCES

18. de Kruijf B, Cullis PR, Radda GK: Differential scanning calorimetry and \(\text{^3}P\)NMR studies on sonicated and unsonicated phosphatidylcholine liposomes. Biochim Biophys Acta 406:16, 1975
The role of activated human platelets in prothrombin and factor X activation

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