Comparison of Anticoagulant and Procoagulant Activities of Stimulated Platelets and Platelet-Derived Microparticles

Guido Tans, Jan Rosing, M. Christella L.G.D. Thomassen, Mary Jo Heeb, Robert F.A. Zwaal, and John H. Griffin

Activation of human platelets considerably enhanced their ability to accelerate factor Va inactivation by activated protein C (APC). The anticoagulant activity of platelet suspensions was markedly dependent on the kind of agonist used to activate platelets. APC-catalyzed factor Va inactivation in free solution was characterized by an apparent second-order rate constant of 2 x 10^9 (mol/L)^2 (seconds)^{-1}. Nonstimulated platelets (2.4 x 10^9/mL) and platelets stimulated with aden sine diphosphate or adrenalin accelerated factor Va inactivation fourfold. Rates of factor Va inactivation were increased 11-fold by thrombin-stimulated platelets, 29-fold after platelet stimulation with collagen, and about 60-fold by platelets stimulated with the Ca^2+-ionophore A23187. At low platelet concentrations (3 x 10^7/mL) only background levels of anticoagulant activity of platelets were observed in platelet suspensions that were nonstimulated or stimulated with thrombin or collagen. However, when such reaction mixtures were stirred during the activation procedure, platelet anticoagulant activity was increased more than 10-fold. Independent of platelet stimulation and stirring conditions, exogenously added purified plasma protein S increased platelet-dependent factor Va inactivation approximately twofold. Addition of a neutralizing antiprotein S antibody had little effect on the anticoagulant activity of platelets. This indicates that, under the reaction conditions tested, protein S did not contribute to factor Va inactivation. Approximately 25% of the anticoagulant activity of stimulated platelet suspensions appeared to be associated with microparticles that were released on platelet activation. Such microparticles may provide an important source of anticoagulant activity. A similar distribution of procoagulant, ie, prothrombinase, activity between platelets and microparticles was observed for the same platelet suspensions. Because platelet stimulation and stirring also had the same overall effects on the ability of platelets and platelet microparticles to promote prothrombin activation and factor Va inactivation, it appears likely that the generation of potential platelet anticoagulant and procoagulant activities is coupled to the same platelet stimulation reactions.

ONE OF THE MAJOR functions of blood platelets in hemostasis is to provide a membrane surface that accelerates blood coagulation and that promotes the formation of the fibrin network in the hemostatic plug. It has been shown that platelets considerably enhance both prothrombin and intrinsic factor X activation. The procoagulant activity of platelets is increased after stimulation of platelets with thrombin, collagen, or the Ca^2+-ionophore A23187, and optimal expression of procoagulant activity is observed when platelets are subject to limited shear stress. However, the procoagulant activity of platelet suspensions is not exclusively associated with intact platelet membranes. Recently, Sims et al have shown that a significant part of the prothrombin-converting activity resides on small membrane vesicles (microparticles) that are shed from the plasma membrane on platelet activation.

It has been reported that platelets also stimulate proteolytic inactivation of the prothrombinase cofactor factor Va by activated protein C (APC). This activity of platelets may be of great physiologic importance because APC appears to be an important hemostatic regulatory protein. In model systems APC-catalyzed factor Va inactivation is considerably accelerated by the presence of negatively charged phospholipids and by the nonenzymatic protein cofactor, protein S. With respect to the requirement for protein S in the presence of platelets there appears to be a difference between the bovine and the human system. Harris and Esmo reported that APC-catalyzed inactivation of bovine factor Va on bovine platelets is only observed in the presence of protein S, while Solyms et al, in a study with human proteins and human platelets, observed that platelets considerably stimulate factor Va inactivation by APC in the absence of exogenously added protein S. For the human system it was also reported that there were no significant differences between the effects of nonstimulated and thrombin-stimulated platelets on APC-catalyzed factor Va inactivation under the conditions studied.

It is as yet unknown whether the ability of platelets to accelerate factor Va inactivation by APC can be further increased when platelets are activated with agonists other than thrombin. Therefore, in the present report we have compared the anticoagulant activities of human platelets stimulated with different platelet agonists in stirred and nonstirred reaction mixtures. Here we describe that a considerable part of the anticoagulant activity generated in platelet suspensions is associated with microparticles that are released on platelet activation. Furthermore, we have quantitated the anticoagulant and procoagulant activities of both the intact platelet membranes and the shed microparticles, and we report that platelet concentration and stirring conditions are important determinants of the activities observed.

MATERIALS AND METHODS

Materials. D-Phenylalanyl-L-pipeocyI-L-arginine-p-nitroanilide (S-2238), N-benzoyl-isoleucyl-L-glutamyl (piperidide) glycyI-L-
arginie-p-nitroanilide (S-2336), and N-dansyl-p-guanidino)-phenylalanine-phenylalanine-phenylhydrazine hydrochloride (I-2581) were obtained from AB Kabi Diagnostica (Stockholm, Sweden). Ovalbumin (grade V), fatty acid-free bovine serum albumin (BSA), soybean trypsin inhibitor (type IS), 1,2-dioleoyl-sn-glycero phosphocholine, phosphatidylserine (Folch fraction III) from bovine brain, egg-yolk phosphatidylethanolamine, prostateserin A (PGE), and the calcium ionophore, A23187, were purchased from Sigma (St Louis, MO). Adrenalin was from Nogepha Centrachemie (Cuyyk, The Netherlands) and adenosine diphosphate (ADP) was obtained from Boehringer Mannheim (Mannheim, Germany). Phenylalanine-prolyl-arginine chloromethylketone (PPACK) was obtained from Calbiochem (La Jolla, CA). Horse tendon collagen (type I) was from Hormon Chemie (Munich, Germany).

Platelet preparations. Washed human platelets were obtained from healthy consenting donors by venapuncture. Six volumes of blood were collected into 1 vol anticoagulant (42 mmol/L EDTA, 42 mmol/L trisodiumcitrate, pH 6.6). The blood was centrifuged for 15 minutes at 250g at room temperature after which the platelet-rich plasma (PRP) was collected and centrifuged an additional 5 minutes at 160g to remove any remaining erythrocytes and leukocytes. The PRP was subsequently centrifuged for 15 minutes at 900g and the platelet pellet was resuspended into a buffer consisting of 9 vol 10 mmol/L HEPES (pH 6.6), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, 0.5% BSA, and 1 vol anticoagulant. Platelets were washed twice using this procedure and were then resuspended at a final concentration of 3 × 108 platelets/ml in 10 mmol/L HEPES (pH 7.5 at room temperature), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, and 0.5% BSA. Platelet concentrations were determined with a Coulter counter (Coulter Electronics Munich, Germany).

Platelet preparations. Washed human platelets were obtained from healthy consenting donors by venapuncture. Six volumes of blood were collected into 1 vol anticoagulant (42 mmol/L EDTA, 42 mmol/L trisodiumcitrate, pH 6.6). The blood was centrifuged for 15 minutes at 250g at room temperature after which the platelet-rich plasma (PRP) was collected and centrifuged an additional 5 minutes at 160g to remove any remaining erythrocytes and leukocytes. The PRP was subsequently centrifuged for 15 minutes at 900g and the platelet pellet was resuspended into a buffer consisting of 9 vol 10 mmol/L HEPES (pH 6.6), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, 0.5% BSA, and 1 vol anticoagulant. Platelets were washed twice using this procedure and were then resuspended at a final concentration of 3 × 108 platelets/ml in 10 mmol/L HEPES (pH 7.5 at room temperature), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, and 0.5% BSA. Platelet concentrations were determined with a Coulter counter (Coulter Electronics Munich, Germany).

Phospholipid preparations. Single bilayer phospholipid vesicles (20% 1,2-dioleoylphosphatidylserine/80% 1,2-dioleoylphosphatidylcholine; molar/molar) were prepared by sonication according to de Kruijf et al.16 Phospholipid vesicles used in the factor Va assay (10% bovine brain phosphatidylserine/90% egg-yolk phosphatidylcholine; molar/molar) were prepared in the same manner.

Proteins. Proteins used in this study were purified from fresh frozen human plasma. Prothrombin and factor X were purified according to DiScipio et al.19 α-Thrombin was isolated from prothrombin activation mixtures in which purified prothrombin (20 mg) was activated at 37°C in 10 mL of a buffer solution containing 50 mmol/L Tris (pH 7.5 at 37°C), 100 mmol/L NaCl, 2 mmlol/L CaCl2, 50 μmol/L phosphatidylserine (20% brain phosphatidylserine/80% egg-yolk phosphatidylcholine; molar/molar), 2.5 mmol/L factor Xa, 40 mmol/L factor Va, and 20 μmol/L 1,2581. The reversible thrombin inhibitor 1,2581 was present to prevent autocalytic degradation of α-thrombin. Prothrombin activation was complete within 10 minutes, after which the α-thrombin was purified from the reaction mixture as described by Fletcher and Nelsestuen.21 Human protein C was prepared, activated, and assayed as previously described.22 APC anticoagulant activity (250 U/mg) was equivalent to that of normal plasma protein C activated with Protac (Pentapharm, Basel, Switzerland).22 Human protein S was obtained from Enzyme Research Laboratories (South Bend, IN). A reference protein S was purified by barium adsorption of normal plasma.14 Goat antiserum against protein S was prepared as previously described.23 An IgG-rich fraction was prepared from the antiserum by precipitation with 40% saturated ammonium sulfate, followed by re suspension in water and dialysis against Tris-buffered saline, pH 7.4. APC and protein S preparations were greater than 95% pure, and APC was greater than 90% activated, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Factor Xa was obtained from purified factor X after activation with the purified factor X activator from Russel’s viper venom (RVV-X) and was isolated from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose (Sigma) as described by Bock et al.23 Bovine factor Xa, which was used in the factor Va assay (see below), was purified as described by Fujikawa et al.24 RVV-X was purified from the crude venom of Russel’s viper as described by Schifman et al.25 Factor V was purified essentially as described by Dahlbäck et al26 and Suzuki et al,27 with minor modifications. The irreversible thrombin inhibitor PPACK was added to fresh frozen plasma to a final concentration of 2 μmol/L. All buffer solutions used in the isolation procedure contained 250 mmol/L PPACK. The final purification step (chromatography on AcA22) was performed with a buffer solution that did not contain PPACK. The presence of PPACK was necessary to obtain reproducible preparations of homogeneous and single-chain factor V (> 95%). Factor Va was prepared by activation of factor V with thrombin. Factor V (0.3 mg/mL) was incubated for 30 minutes with 30 mmol/L thrombin in 50 mmol/L Tris (pH 7.9 at 20°C), 175 mmol/L NaCl, 20% (vol/vol) glycerol, and 5 mmol/L CaCl2. After activation, 30 mmol/L PPACK was added to inhibit thrombin. The small amount of remaining PPACK did not interfere with the reactions conducted with the factor Va preparations. Factor Va was subsequently diluted 1/10 in 10 mmol/L HEPES (pH 7.5 at room temperature), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, and 5 mg/mL BSA and stored at –80°C. Factor Va was stable for several months at –80°C and lost about 10% of its activity when kept on ice for 6 hours.

Protein preparations were homogeneous and greater than 95% pure as judged by AGE in the presence of SDS according to Laemmli.28 Protein concentrations were routinely determined according to Lowry et al.29 using BSA as a standard. Molar concentrations of prothrombin and factor Xa were determined by active-site titration with p-nitrophenylglycinesuebenzoate.30 The APC concentration was determined with S-2366 using kinetic parameters reported by Sala et al.31 Concentrations of factor Va were determined kinetically as described for bovine factor Xa Va by Lindhout et al.32

Inactivation of factor Va by APC. The rate of factor Va inactivation was determined in reaction mixtures of 350 μL containing 10 mmol/L HEPES (pH 7.5 at room temperature), 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L MgCl2, 25 mmol/L glucose, 0.5% BSA, and platelets, platelet agonists, and factor Va at the desired concentration. Incubations were conducted in flat-bottom polystyrene tubes with a diameter of 0.9 cm. When indicated the reaction mixtures were stirred with a Teflon-coated magnetic stirring bar (length 0.7 cm, diameter 0.2 cm; Tamson, Zoetermeer, The Netherlands) at 350 rpm. Because platelets contain about 1 nmol factor V/1010 platelets36 that might be released and activated on platelet stimulation, the amount of factor Va present in the inactivation mixture was kept constant by the addition of purified plasma factor Va. When present, platelet agonists (10 mmol/L thrombin, 10 μg/mL collagen, or 3 μmol/L of the Ca2+-ionophore A23187) were added and incubated for 10 minutes at 37°C with the platelets to allow complete platelet stimulation. Before the addition of APC, two 10-μL aliquots were withdrawn to determine factor Va (see below). Inactivation of factor Va was started by adding APC to the reaction mixture. The decrease in factor Va activity was monitored in 10-μL aliquots taken from the reaction mixture each 30 seconds over a period of 3.5 minutes. From the progressive decrease in factor Va activity, the rate of factor Va inactivation, expressed as percentage factor Va inactivated (%V) per minute, was calculated. During our studies it became apparent that the rate of factor Va inactivation varied greatly with the different platelet agonists used. These differences were such that it was impossible to quantitate the rate...
of the reaction at a single set of experimental conditions (ie, during a fixed time period at fixed platelet and APC concentrations). When large variations in reaction rates are to be accurately measured with as little experimental variation as possible, three options exist: ie, variation of the time period of measurement at fixed platelet and APC concentrations or measurement at a fixed time interval, and either varying the platelet or the APC concentration. Variation of the time interval was undesirable because, with a minimum reaction time of approximately 2 to 3 minutes for ionophore-stimulated platelets, it would require reaction times of more than 60 minutes in the case of nonstimulated platelets (see Table 1). Such long reaction times would introduce problems with the stability of both the proteins and the platelet anticoagulant activity. We also considered activity measurements at widely different platelet concentrations not desirable, because in that case variations of platelet-platelet interactions and of the release of different amounts of platelet components might have different effects on APC-catalyzed factor Va inactivation. To accurately determine and compare the various rates of factor Va inactivation we have, therefore, varied the amount of APC present in the reaction mixtures and used concentrations of APC at which the rate of factor Va inactivation was linear in time (<20% factor Va became inactivated) and proportional to the amount of APC present. The rate of factor Va inactivation was subsequently expressed as percentage factor Va inactivated (%Vi) per minute per nmol/L of APC. Factor Va inactivation rates were determined in triplicate and the experimental data presented in this report represent average values determined with five different donors.

Factor Va assay. Factor Va was determined via its cofactor activity in the activation of prothrombin by factor Xa. For convenience, bovine factor Xa was used in the assay. Amounts of factor Xa, phospholipid vesicles, and prothrombin present in the assay were such that the rate of prothrombin activation was linearly dependent on factor Va, was constant in time, and was not influenced by small variations of the factor Xa, phospholipid, and prothrombin concentrations. In a typical experiment factor Va was quantitated as follows: to 230 μL of a buffer solution (prewarmed at 37°C) containing prothrombin and phospholipid 10 μL bovine factor Xa was added, and after 15 seconds prothrombin activation was started by the addition of a 10-μL aliquot containing factor Va. Final concentrations reached in the reaction mixture were: 50 mmol/L Tris (pH 7.9 at room temperature), 175 mmol/L NaCl, 2 mmol/L CaCl₂, 5 mmol/L factor Xa, 100 μmol/L phospholipid vesicles (10% brain phosphatidylserine/90% egg-yolk phosphatidylcholine; molar/molar), 0.5 μmol/L prothrombin, 0.5 μmol/L aprotinin, 20 μmol/L I-2581, 5 μmol/L PGE₃, and the factor Va from the aliquot. PGE₃ and the reversible thrombin inhibitor I-2581 were present to prevent release and activation of platelet factor V by the thrombin that is formed in the assay. Prothrombin activation in the assay mixture was determined by measuring the amount of thrombin formed after 1 minute using the chromogenic substrate S-2238. The amount of thrombin formed in this assay was linearly dependent on factor Va (up to 60 pmol/L factor Va present in the assay mixture), and from a calibration curve made with known amounts of factor Va the amount of factor Va present in the reaction mixture could be calculated. The I-2581 present in the aliquots taken from the factor Va assay mixtures did not interfere with the thrombin determination because I-2581 is a reversible inhibitor whose inhibitory action in the thrombin assay was greatly reduced by dilution in the cuvette and by the presence of a large excess of the thrombin-specific chromogenic substrate S-2238.

RESULTS

The effect of platelets on factor Va inactivation by APC. In the experiment shown in Fig 1 we have compared time courses of APC-dependent inactivation of factor Va in free solution, of factor Va that was added to a platelet suspension, and of factor Va that was released by platelets. The platelets present in the latter two incubations were activated with the Ca²⁺-ionophore A23187. A small amount of thrombin was present to ensure complete release and activation of platelet factor Va. To distinguish between the inactivation of platelet-derived and plasma factor Va, the platelet experiments were performed in the absence of added factor Va or in the presence of a 10-fold excess of added purified factor Va. The inactivation reaction was started by the addition of APC and the remaining amount of factor Va (expressed as percentage of the amount present at time zero) was determined as a function of time. APC-catalyzed inactivation of factor Va in free solution was a rather slow reaction that was greatly enhanced by the presence of A23187-stimulated platelets (Fig 1). Identical time courses of inactivation were obtained with platelet-derived factor Va and plasma factor Va. These identical time courses indicate that platelet-derived and plasma-derived factor Va were equally susceptible to inactivation by APC and were inactivated with the same pseudofirst-order rate constant. However, the rates of factor Va inactivation in the presence of platelets were too high to allow accurate calculation of the rate constant of APC-catalyzed inactivation of factor Va. To quantitate the effect of platelets on factor Va inactivation the subsequent experiments were performed at APC concentrations that were chosen such that rates of factor Va inactivation by APC were linear in time (<20% inactivation of factor Va) and proportional to the amount of APC present (see also Materials and Methods).

Factor Va inactivation by APC in the presence of platelets stimulated with different platelet agonists. To assess the
effect of stimulated platelets on the inactivation of factor Va by APC, the rate of factor Va inactivation was determined in the absence or presence of 2.4 \times 10^7/mL platelets that were activated with a variety of platelet agonists (Table 1). In the absence of platelets the rate of inactivation of factor Va was rather low (1.3\% factor Va inactivated/min/nmol/L APC). The presence of nonstimulated platelets or platelets stimulated with ADP or adrenalin caused a threfold to fourfold increase of the rate of factor Va inactivation. Other platelet agonists were more effective in generating platelet anticoagulant activity. Thrombin stimulation of platelets resulted in an 11-fold acceleration of APC-catalyzed factor Va inactivation and the rate of inactivation was increased 29-fold by collagen-stimulated platelets. Optimal rate enhancements (61-fold) were observed with platelets that were stimulated with the Ca^{2+} ionophore A23187. For comparison the rate of APC-catalyzed factor Va inactivation was also determined in the presence of negatively charged phospholipid vesicles (10\% phosphatidylserine/90\% phosphatidylcholine, molar/molar). On these vesicles the rate of factor Va inactivation was 3.6 times higher than obtained with A23187-stimulated platelets.

**Effect of protein S on platelet-dependent inactivation of factor Va by APC.** It is well known that APC-catalyzed inactivation of factor Va is accelerated by the protein cofactor protein S^{10,18} and that platelet \(\alpha\)-granules contain protein S that can be released on platelet activation.\cite{39,40} Therefore, we have investigated whether the rate of platelet-dependent inactivation of factor Va by APC is affected by protein S that is released from platelets and whether the reaction rate can be further enhanced by exogenously added protein S. To this end we have determined the effect of stimulated and unstimulated platelets on the rate of APC-catalyzed factor Va inactivation both with and without added protein S and in the presence of an amount of a polyclonal antibody against protein S (antiprotein S) that completely inhibits the action of protein S in a model system (ie, factor Va inactivation on phospholipid vesicles composed of 20 mol\% phosphatidylserine and 80 mol\% phosphatidylcholine).

In agreement with earlier reports,\cite{17,18} we found that protein S does not promote APC-catalyzed inactivation of factor Va in free solution and that the stimulatory effect of protein S is confined to reaction mixtures that contain a so-called anticoagulant surface, ie, platelets or phospholipid vesicles (Table 2). Platelet-dependent inactivation of factor Va by APC was accelerated twofold to threefold by protein S, irrespective of whether the platelets were unstimulated or stimulated with thrombin, collagen, or A23187. Antiprotein S antibodies did not affect APC-catalyzed inactivation of factor Va in the presence of platelets. This finding indicates that it is unlikely that under our experimental conditions platelet protein S significantly contributes to the rate of factor Va inactivation.

**Platelet-dependent inactivation of factor Va by APC as a function of the platelet concentration.** The experiments presented thus far were performed at a single platelet concentration (2.4 \times 10^7/mL). Figure 2 shows the effect of varying amounts of nonstimulated and stimulated platelets on APC-catalyzed factor Va inactivation. With A23187-activated platelets optimal rates of factor Va inactivation were observed at 3 \times 10^7 platelets/mL. For the other platelet activators much higher platelet concentrations were required to promote factor Va inactivation. With collagen-stimulated platelets, half-maximal rates of factor Va inactivation were obtained at 6 \times 10^7 platelets/mL. Nonstimulated and thrombin-stimulated platelets did not accelerate APC-catalyzed factor Va inactivation at low platelet concentrations. With these platelet preparations significant rate enhancements were observed only at platelet concentrations.

### Table 1. Effect of Platelets on Factor Va Inactivation by APC

<table>
<thead>
<tr>
<th>Anticoagulant Surface</th>
<th>Rate of Va Inactivation (%Vi/min/nmol/L APC)</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without platelets*</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Nonstimulated platelet(\uparrow)</td>
<td>4.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Platelets stimulated with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (\mu)mol/L ADP(\uparrow)</td>
<td>5.5</td>
<td>4.2</td>
</tr>
<tr>
<td>10 (\mu)g/mL adrenaline(\uparrow)</td>
<td>5.6</td>
<td>4.3</td>
</tr>
<tr>
<td>10 nmol/L thrombin(\uparrow)</td>
<td>14.5</td>
<td>11.0</td>
</tr>
<tr>
<td>10 (\mu)g/mL collagen(\uparrow)</td>
<td>38.0</td>
<td>29.0</td>
</tr>
<tr>
<td>3 (\mu)mol/L A23187(\uparrow)</td>
<td>79</td>
<td>61</td>
</tr>
<tr>
<td>Phospholipid vesicles</td>
<td>283</td>
<td>218</td>
</tr>
</tbody>
</table>

APC-catalyzed inactivation of 10 nmol/L of factor Va was determined in the absence of platelets or in the presence of 2.4 \times 10^7/mL platelets that were not stimulated or that were stimulated for 10 minutes in unstimred reaction mixtures with the platelet activators that are indicated in the table. For comparison, the rate of factor Va inactivation was also determined in the presence of 50 \(\mu\)mol/L phospholipid vesicles (20 mol\% phosphatidylserine/80 mol\% phosphatidylcholine). Factor Va inactivation was started by the addition of: (*) 5 nmol/L APC; (\(\uparrow\)) 1 nmol/L APC; (4) 0.5 nmol/L APC; (6) 0.1 nmol/L APC; or (l) 0.02 nmol/L APC. Further experimental details are described in the Materials and Methods section.

### Table 2. Effect of Protein S and Antiprotein S on Platelet-Dependent Factor Va Inactivation by APC

<table>
<thead>
<tr>
<th>Anticoagulant Surface</th>
<th>Rate of Factor Va Inactivation (%Vi/min/nmol/L APC)</th>
<th>- Protein S</th>
<th>+ Protein S</th>
<th>+ (\alpha)-Protein S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without platelets*</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Nonstimulated platelet(\uparrow)</td>
<td>6.3</td>
<td>11.0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Platelets stimulated with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nmol/L thrombin(\uparrow)</td>
<td>16.4</td>
<td>27.9</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>10 (\mu)g/mL collagen(\uparrow)</td>
<td>49.3</td>
<td>74</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>3 (\mu)mol/L A23187(\uparrow)</td>
<td>92</td>
<td>229</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Phospholipid vesicles</td>
<td>336</td>
<td>664</td>
<td>305</td>
<td></td>
</tr>
</tbody>
</table>

APC-catalyzed factor Va inactivation was determined in the absence of platelets or in the presence of 2.4 \times 10^7/mL platelets that were either unstimulated or stimulated in unstimred reaction mixtures for 10 minutes with the platelet agonists indicated in the table. Rates of factor Va inactivation were also determined in the presence of 50 \(\mu\)mol/L phospholipid vesicles (20 mol\% phosphatidylserine/80 mol\% phosphatidylcholine). The inactivation of 1 nmol/L of factor Va was measured with: (*) 5 nmol/L; (\(\uparrow\)) 1 nmol/L; (6) 0.5 nmol/L; (l) 0.1 nmol/L; or (l) 0.02 nmol/L APC with or without 100 nmol/L protein S or 150 \(\mu\)g/mL antiprotein S (\(\alpha\)-Protein S). Further experimental details are described in the Materials and Methods section.
PLATELET ANTICOAGULANT ACTIVITY

started with APC. To which the various platelet suspensions stimulated factor Va was determined at varying concentrations of platelets in unstirred reaction mixtures. Platelets were either unstimulated or stimulated for 10 minutes with different platelet agonists. The amount of factor Va present in the platelet suspension was kept constant by the addition of purified plasma Va and the inactivation reaction was started with APC. Unstimulated platelets, 5 nmol/L APC; (△,△), platelets stimulated with 10 nmol/L thrombin, 1 nmol/L APC; (○,○), platelets stimulated with 10 μg/mL collagen, 0.3 nmol/L APC; (●,●), platelets stimulated with 3 μmol/L A23187, 0.1 nmol/L APC. Further experimental conditions are given in the Materials and Methods section.

The effect of different platelet concentrations on APC-catalyzed factor Va inactivation was determined at varying concentrations of platelets in unstirred reaction mixtures. Platelets were either unstimulated or stimulated for 10 minutes with different platelet agonists. The amount of factor Va present in the platelet suspension was kept constant by the addition of purified plasma Va and the inactivation reaction was started with APC. Unstimulated platelets, 5 nmol/L APC; (△,△), platelets stimulated with 10 nmol/L thrombin, 1 nmol/L APC; (○,○), platelets stimulated with 10 μg/mL collagen, 0.3 nmol/L APC; (●,●), platelets stimulated with 3 μmol/L A23187, 0.1 nmol/L APC. Further experimental conditions are given in the Materials and Methods section.

Effect of stirring on platelet-dependent inactivation of factor Va by APC. It has been shown that introduction of shear stress (ie, stirring of platelets) may greatly influence the procoagulant activity of activated platelets. Therefore, we have determined the effect of stirring on the ability of platelets to accelerate APC-catalyzed factor Va inactivation (Table 3). These experiments were performed at a platelet concentration (3 × 10⁷/mL) at which the weaker agonists, thrombin and collagen, had a minor stimulatory effect under nonstirring conditions. The data presented in Table 3 show that stirring of platelets during activation with thrombin or collagen resulted in an 8- to 15-fold increase of the rate of platelet-dependent factor Va inactivation. With A23187, stirring had a minor effect on the platelet anticoagulant activity. The ability of stirred platelets to accelerate APC-catalyzed factor Va activation was increased by a further twofold to threefold by 100 nmol/L of protein S, whereas the presence of antiprotein S did not inhibit the anticoagulant activity of the platelets (data not shown). This finding indicates that platelet protein S did not contribute to platelet anticoagulant activity under either stirring or nonstirring conditions.

Localization of the anticoagulant and procoagulant activity of platelets. When platelets are incubated with platelet agonists they shed microparticles from the plasma membrane that contain a significant part of the procoagulant activity generated in platelet suspensions. It has been shown that platelets and platelet-derived microparticles have vastly different centrifugal properties, and although the centrifugation conditions vary somewhat between the different studies a good separation between platelets and microparticles can apparently be achieved. Centrifugation at 3,000g for 10 minutes (30,000g · min), 800g for 10 minutes (8,000g · min), 11,500g for 1.5 minutes (16,000g · min), and 15,600g · min was used to quantitatively spin down the platelets and obtain a supernatant containing the platelet-derived microparticles. In a control experiment we centrifuged our platelet suspensions at 9,000g for various time intervals and determined both the anticoagulant and procoagulant activity (see also below) remaining in the supernatant. The activity of the platelet suspension decreased on centrifugation due to the removal of platelet-associated activity. For all the different triggers used, more than 98% of the platelets were removed from the supernatant within 1 minute (as determined with a Coulter counter) and the activities remaining in the supernatant reached a value that was constant up to 5 minutes of centrifugation (data not shown). This result is in agreement with the earlier data on the separation of platelets and microparticles. In the subsequent experiments we routinely used centrifugation at 9,000g during 2 minutes (18,000g · min) to spin down the platelets and obtain the microparticle-containing supernatant. Table 4 summarizes studies showing the ability of noncentrifuged and centrifuged platelet suspensions to promote APC-catalyzed factor Va inactivation. Independent of the agonist used to activate the platelets, approximately 75% of the anticoagulant activity was sedimentable and appeared to be associated with intact platelets, while some 25% of the activity was associated with microparticles. In the same platelet suspensions we have also measured the procoagulant activity of the platelets and of the shed microparticles assayed as their ability to promote prothrombin activation by the factor Xa-Va complex (Table 4). The overall effects of the different platelet agonists on platelet anticoagulant and procoagulant activity are shown in Table 3.

Table 3. Effect of Stirring on Platelet Anticoagulant Activity

<table>
<thead>
<tr>
<th>Anticoagulant Surface</th>
<th>Rate of Factor Va Inactivation (% / min / nmol/L APC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without platelets*</td>
<td>1.4</td>
</tr>
<tr>
<td>Nonstimulated platelets†</td>
<td>1.9</td>
</tr>
<tr>
<td>Platelets stimulated with:</td>
<td></td>
</tr>
<tr>
<td>10 nmol/L thrombin§</td>
<td>4.3</td>
</tr>
<tr>
<td>10 μg/mL collagen§</td>
<td>4.8</td>
</tr>
<tr>
<td>3 μmol/L A23187§</td>
<td>61.3</td>
</tr>
</tbody>
</table>

APC-catalyzed factor Va inactivation was determined in the absence or presence of unstimulated platelets or platelets that were stimulated with the platelet agonists indicated in the table. The platelet mixtures (3 × 10⁷/mL platelets) were either unstimulated or stirred during the activation procedure. The inactivation of 1 nmol/L of factor Va was initiated with: (⁎) 5 nmol/L; (†) 5 nmol/L; (‡) 2 nmol/L; (§) 2 nmol/L; or (‖) 0.1 nmol/L. APC in unstirred reaction mixtures or (⁎⁎) 5 nmol/L; (††) 0.2 nmol/L; (‡‡) 0.2 nmol/L; or (‖‖) 0.1 nmol/L APC in stirred reaction mixtures. Further experimental details are given in the Materials and Methods section.

Fig 2. The effect of different platelet concentrations on APC-catalyzed factor Va inactivation. The inactivation of 1 nmol/L of factor Va was determined at varying concentrations of platelets in unstirred reaction mixtures. Platelets were either unstimulated or stimulated for 10 minutes with different platelet agonists. The amount of factor Va present in the platelet suspension was kept constant by the addition of purified plasma Va and the inactivation reaction was started with APC. Unstimulated platelets, 5 nmol/L APC; (△,△), platelets stimulated with 10 nmol/L thrombin, 1 nmol/L APC; (○,○), platelets stimulated with 10 μg/mL collagen, 0.3 nmol/L APC; (●,●), platelets stimulated with 3 μmol/L A23187, 0.1 nmol/L APC. Further experimental conditions are given in the Materials and Methods section.

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with the different activated platelet preparations were of activities were determined at 1 nmol/L factor Va and A23187. Furthermore, the rate enhancements obtained procoagulant activity appeared to be similar. The effective-

Methods section.

distributed in a similar manner between platelets and microparticles.

and procoagulant activities were determined in the total platelet suspension (platelets plus microparticles) and in the supernatant (microparticles). The anticoagulant activity was measured at 1 nmol/L factor Va and (+) 5 nmol/L; (f) 0.7 nmol/L; (g) 0.2 nmol/L; or (h) 0.1 nmol/L APC. The procoagulant activities were determined at 1 μmol/L prothrombin, 2 nmol/L factor Va, and 5 nmol/L factor Xa. Microparticle- and platelet-associated activities were expressed as percent of the activity present in the total platelet suspension. The activities of intact platelets were calculated from the difference between the activities measured in the total platelet suspension and in the supernatant. Further experimental details are given in the Materials and Methods section.

procoagulant activity appeared to be similar. The effectiveness with which the various platelet agonists generated platelet anticoagulant and procoagulant activity increased in the order of no activator < thrombin < collagen < A23187. Furthermore, the rate enhancements obtained with the different activated platelet preparations were of the same order of magnitude and the two activities were distributed in a similar manner between platelets and microparticles.

DISCUSSION

The data presented in this report show that blood platelets considerably enhance APC-catalyzed factor Va inactivation and that the anticoagulant activity of platelet suspensions is greatly dependent on platelet activation with platelet agonists. The nonphysiologic platelet activator A23187 induces the highest platelet anticoagulant activity. In comparison with factor Va inactivation in free solution, a 61-fold rate enhancement is observed with A23187-stimulated platelets. Nonstimulated platelets or platelets that are stimulated with ADP or adrenaline accelerated factor Va inactivation approximately fourfold. Intermediate activities are observed with thrombin- or collagen-stimulated platelets, which increase the inactivation rates 11-fold and 29-fold, respectively. We do not know whether these activities, which are obtained at high concentrations of activated platelets, represent true maximal values, because under these reaction conditions visible aggregation of platelets occurs, a phenomenon that may have masked anticoagulant sites present at the platelet surface.

Exogenously added protein S has little effect on APC-catalyzed factor Va inactivation in the presence of platelets. Saturating concentrations of protein S increase the rate of factor Va inactivation approximately twofold. The fact that the presence of antiprotein S antibodies had a negligible effect on platelet-dependent factor Va inactivation indicates that this reaction does not require participation of protein S that is released from platelets. This result is in agreement with observations of Solymoss et al.2 on human platelets. However, it has been reported that APC-catalyzed inactivation of factor Va on bovine platelets11 and bovine aortic endothelial cells45 depends on the presence of protein S, which indicates that protein S is an essential cofactor for factor Va inactivation by APC in the bovine system, but not in the human system. In the latter system protein S appears to be an important cofactor when factor Xa is present in the inactivation mixture. Factor Xa protects factor Va from inactivation by APC,15,16,45 an effect reported to be nullified by the presence of protein S.12 In our study we did not investigate APC-catalyzed factor Va inactivation in the presence of factor Xa and, therefore, we have no information on the possible cofactor function of protein S in our reaction systems.

Stirring of platelets greatly influences their anticoagulant activity. This is especially true for thrombin- or collagen-stimulated platelets at low platelet concentrations. Compared with unstirred reaction mixtures, stirring causes an additional 8- to 15-fold increase of the anticoagulant activity of platelets that are stimulated with thrombin or collagen at low platelet concentrations (3 × 107 platelets/mL). With A23187 as platelet activator there is little difference between the anticoagulant activities of stirred and unstirred platelet suspensions. Similar stirring effects are observed when the prothrombinase or the intrinsic factor X-converting activity of activated platelets is measured.2 Stirring of platelets greatly increases the procoagulant activity of platelet suspensions that are stimulated with thrombin or with collagen. It has been suggested that the combination of shear stress (stirring) and platelet activation promotes transmembrane bilayer movement of phosphatidyserine molecules from the inner platelet membrane monolayer to the outer membrane monolayer and that the exposed phosphatidyserine molecules participate in the formation of functional procoagulant sites.4,44 Such a phenomenon may also be important for the generation of platelet anticoagulant activity.

Platelet-dependent factor Va inactivation by APC is a rather efficient reaction. When 3 × 107 platelets/mL were
activated with collagen in a stirred reaction medium in the absence of protein S, APC-catalyzed factor Va inactivation proceeded at a rate of 73% Vi/min/nmol/L APC (Table 3). Because these reaction rates were determined at a factor Va concentration (1 nmol/L) that was far below the reported Km for factor Va (12 nmol/L),\(^{12}\) this result corresponds to an apparent second-order rate constant of about 1.3 \(\times 10^7\) (mol/L\(^{-1}\)) \(^{-1}\) (seconds\(^{-1}\)). This is only four times lower than the rate constant obtained at an optimal concentration of phosphatidylserine-containing phospholipid vesicles (Table 1).

In the experiments reported here we have also compared the anticoagulant and procoagulant activities of platelets stimulated with different platelet agonists. It appears that platelet-dependent factor Va inactivation by APC and prothrombin activation by the factor Xa-Va complex share many properties. Both activities increase when platelets are stimulated with platelet activators. The effectiveness of the various platelet agonists to generate platelet procoagulant and anticoagulant activities increases in the order of no activator = ADP = adenalin < thrombin < collagen < A23187. When platelets were subject to limited shear stress during activation with thrombin or collagen their ability to promote prothrombin activation and factor Va inactivation was significantly increased.

In a recent study, Sims et al\(^7\) reported that activated platelets release small membrane vesicles that are highly enriched in factor Va binding sites and that exhibit prothrombinase activity. Here it is demonstrated that platelet microparticles also possess potent anticoagulant activity. This is concluded from experiments in which we have determined the extent to which procoagulant and anticoagulant activities of platelet suspensions stimulated with different platelet agonists are associated with platelets and with platelet microparticles. Although the ability of platelet suspensions to promote prothrombin activation and factor Va inactivation greatly increased on platelet stimulation, the relative distribution of anticoagulant and procoagulant activity between platelets and microparticles remained approximately the same. For all reaction conditions studied (ie, stirred and nonstirred platelet suspensions stimulated with different platelet agonists), approximately 20% to 30% of both activities appeared to be associated with the microparticles. Thus, we did not find activation conditions that resulted in a selective shedding of microparticles with different activities in the anticoagulant and procoagulant pathways. However, it should be emphasized that the current methods would not identify subpopulations of microparticles that are selectively enriched in either procoagulant or anticoagulant activities because the techniques that we have used measure the average activity of all particles or cells present in the reaction mixture. It is not possible to infer whether the microparticles are in effect anticoagulant or procoagulant in vivo because influences of blood flow (shear stress), adhesive reactions, or soluble factors including coagulation factors, cofactors, and inhibitors ultimately would determine the activity of these vesicles. Therefore, further experimentation will be necessary to obtain information about the physiologic roles of microparticles for either procoagulant or anticoagulant processes.

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REFERENCES


19. de Kruijf F, Cullis PR, Radda GK: Differential scanning


Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles

G Tans, J Rosing, MC Thomassen, MJ Heeb, RF Zwaal and JH Griffin