Platelet Prothrombinase Activity and Intracellular Calcium Responses in Patients With Storage Pool Deficiency, Glycoprotein IIb-IIIa Deficiency, or Impaired Platelet Coagulant Activity—A Comparison With Scott Syndrome

By Harvey J. Weiss and Bruce Lages

The procoagulant activity of platelets induced by collagen, thrombin, and collagen plus thrombin, measured as their capacity to promote the conversion of prothrombin to thrombin in the presence of factors Va and Xa, was studied in patients with α, αδ, and δ storage pool deficiency (SPD), thrombasthenia, and in two new patients with isolated defects in platelet coagulant activity, and compared with that in Scott syndrome. The most significant abnormality in the new patients, whose defect may differ from that in Scott syndrome, is an impairment in collagen plus thrombin–induced prothrombinase activity in the absence of added factor Va. In one of these patients this may be caused by an abnormality in platelet α-granule factor V distinct from that described for factor V Quebec, αδ-SPD, or α-SPD (gray platelet syndrome). Prothrombinase activity in response to all agonists was impaired in α-SPD and was associated with an inability of these platelets to maintain elevated intracellular calcium levels. Both the rapid decline in agonist-induced [Ca\(^{2+}\)] levels and the impaired prothrombinase activation in α-SPD platelets were corrected by the addition of adenosine diphosphate (ADP) after stimulation. These findings suggest that secreted ADP may play an important role in the generation of prothrombinase activity by contributing to the maintenance of a critical [Ca\(^{2+}\)] level necessary to maintain aminophospholipids on the outer surface of the platelet membrane, and provide evidence that dense granules may be a major source of ADP which can contribute to calcium influx in stimulated platelets. Parallel alterations, including both increases and decreases, in the [Ca\(^{2+}\)] and prothrombinase responses were also observed in thrombasthenia, depending on the agonist and stirring conditions. Both responses were increased in collagen-stimulated, unstirred platelets, whereas an inability to maintain increased [Ca\(^{2+}\)] levels, associated with decreased prothrombinase activity in all but one atypical patient, was seen in stirred collagen plus thrombin-activated platelets. Although the parallel alterations in these responses in thrombasthenia, as in SPD, further show the close association between the generation of prothrombinase activity and the maintenance of increased intracellular Ca\(^{2+}\) levels, the specific role that GPIIb-IIIa may play in both these events remains unresolved.

Our findings of both enhancement and inhibition of these activation-related events in thrombasthenic platelets may be related to previous conflicting reports on the promotion or inhibition of fibrin formation by GPIIb-IIIa, and could be relevant to the use of specific inhibitors of GPIIb-IIIa as antithrombotic agents. In addition, the study provides further support for the concept that the development of agents that could induce a Scott syndrome defect in normal platelets may provide a new approach to antithrombotic therapy.

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An abnormality in the translocation of phosphatidyserine to the platelet surface, associated with impaired prothrombinase activity, has been described in a patient with an isolated platelet coagulant defect and a moderately severe bleeding disorder (Scott syndrome). Another patient who may have the same type of defect has also been described recently.

The relationship between the mechanisms involved in the transformation of GPIIb-IIIa to its high-affinity ligand-binding state and those responsible for the development of the platelet coagulant activity (PCA) is presently unclear. Early studies on patients with thrombasthenia, who are deficient in GPIIb-IIIa, reported abnormalities in PCA, designated as platelet factor 3 (PF3). These studies used a method in which PCA was induced by incubating platelet-rich plasma with platelet factor 3 (PF3). This method was used in a study on agonist-induced platelet prothrombinase activity, a conclusion that gained support from later studies on patients with thrombasthenia, who are deficient in GPIIb-IIIa.

To examine these questions further, we have performed studies on agonist-induced platelet prothrombinase activity in 5 patients with thrombasthenia and 20 patients with various types of SPD. Some of these studies have yielded conflicting results. The activated whole-blood clotting time was found to be prolonged in patients undergoing coronary angioplasty who received a monoclonal antibody (MoAb) to GPIIb-IIIa. However, Bevers et al reported normal prothrombinase activity in four patients with several types of thrombasthenia. Other studies reported that the absence or blockade of GPIIb-IIIa actually promoted fibrin formation on subendothelium and collagen, and in the early, but not the later, stages of hemostasis as measured in bleeding time wounds. A role for GPIIb-IIIa in promoting vesiculation of the platelet membrane, which has been linked in some studies to the development of prothrombinase activity, has also been proposed, but contrary evidence has been reported in other studies.

The role of platelet secretion and/or granule-bound substances in promoting PCA is also not clear. Early studies reported impaired PF3 availability (PF3a) in patients with secretion defects, some of whom had storage pool deficiency (SPD), a platelet disorder characterized by reduced contents of substances stored in dense granules (δ-SPD), dense granules and α-granules (αδ-SPD), or α-granules (α-SPD, grey platelet syndrome). The impaired PF3a in some of these patients was corrected by the addition of ADP. However, Bevers et al reported normal platelet prothrombinase activity in three patients with δ-SPD and three with α-SPD. These latter findings suggested that platelet granules, or their secretion, were not required for the development of prothrombinase activity, a conclusion that gained support from later studies on normal platelets.

To examine these questions further, we have performed studies on agonist-induced platelet prothrombinase activity in 5 patients with thrombasthenia and 20 patients with various types of SPD (α, αδ, and δ). The results obtained have been compared with our previously described patient with Scott syndrome in whom a defect in prothrombinase activity is not associated with any aggregation, secretion, or granule defect. In addition, we describe two new patients with this type of defect. In addition to measuring prothrombinase activity, parallel studies on the [Ca2+]c changes occurring during agonist-induced platelet activation were also performed.
Platelet Prothrombinase Activity

ACD PRP containing 1 μmol/L, prostaglandin E1 (PGE1) was gel-filtered into Ca-free Tyrode’s elution buffer, pH 7.35, containing 5 mmol/L HEPES, 1 mmol/L MgCl2, 0.1% glucose, and 0.2% bovine albumin as described previously. Prothrombinase activity in GFP was assayed by a modification of the method of Weidmer et al. Because the activities generated in aggregated (stirred), as well as nonaggregated (unstirred) platelets were being measured, platelet activation and the prothrombinase assay were performed in the same cuvette to avoid a potentially irreproducible transfer of an aliquot of aggregated platelets. Duplicate samples consisting of 280 μL GFP, 7.5 μL 100 mmol/mL CaCl2, 7.5 or 2.5 μL elution buffer, and 5 μL of agonist were activated with and without stirring in a Payton Dual Channel Aggregometer (Payton Associates, Buffalo, NY). The platelet (plt) concentrations of the GFP were adjusted for each agonist, as follows: unstirred (saline): 1.4 × 108 plt/mL; collagen: 1.1 × 107 plt/mL; thrombin, collagen plus thrombin: 7 × 107 plt/mL. Unstirred samples were mixed briefly by hand every 2 minutes during the activation period (see below). Activation was for 10 minutes at 37°C using 10 μg/mL “Horm” collagen, 0.1 μg/mL bovine thrombin, collagen plus thrombin, or saline. The extent of aggregation induced by thrombin in stirred normal GFP under these conditions was 53% ± 3% (n = 21), confirming the functional capacity of these platelets. After 9 minutes of activation, 50 μL of each of 27 μmol/L thrombin, 20 nmol/L factor Va, and 8 mmol/L CaCl2/6% albumin was added to each sample with brief mixing. The CaCl2/albumin was added to preserve the free Ca2+ concentration at 2.5 mmol/L and raise the albumin concentration to 1%. In some studies factor Va was not added, and was therefore provided entirely by the activated platelets. At 10 minutes, the stir bar was removed from the stirred sample and 50 μL of 20 mmol/L factor Xa was added to each sample. For exactly 1 minute (for thrombin and collagen plus thrombin) or 3 minutes (for collagen), 50-μL aliquots were transferred into 450 μL of ice-cold stopping buffer consisting of 10 mmol/L TES, 10 mmol/L EDTA, 0.15 mol/L NaCl, and 1% bovine albumin, pH 7.5. A 3-minute assay time for the collagen-stimulated samples was used because in preliminary studies, the amounts of thrombin generated during the first minute of the prothrombinase assay were found to vary substantially.

The amounts of thrombin generated were determined from the rate of increase of the absorbance at 405 nm in mixtures of 500 μL of 0.1 mmol/L Spectrozyme TH in 10 mmol/L TES, 0.15 mol/L NaCl, 1% bovine albumin, pH 7.5, and 25 μL of the 7.5-fold dilution of sample in stopping buffer, and were quantitated by comparison with the rates of increase produced by solutions of known activities of purified human α-thrombin or International Standard human α-thrombin. The platelet concentrations used were those found to maintain the amounts of thrombin generated by the activated and unactivated platelets within the linear region of the Spectrozyme TH-thrombin standard curves. Prothrombinase activity is expressed as picomoles of thrombin formed per 107 platelets in the 1-minute (thrombin, collagen plus thrombin) or 3-minute (collagen) assay periods. Agonist-specific activity was estimated by subtracting from the total activity the value obtained in a parallel saline-treated sample (constitutive activity). Except where specifically indicated, all values reported are for the total prothrombinase activity.

For most of the study, experiments were performed by a single operator. Experiments performed in a later part of the study (those investigating the effects of ADP on prothrombinase in SPD subjects) were performed by a second operator. The results obtained on normal subjects for stirred collagen plus thrombin-stimulated samples were comparable to those obtained by operator 1 (1,831 ± 125 vs 1,899 ± 113 pmol thrombin/107 platelets), while the values obtained in unstirred samples were higher (1,450 ± 183 vs 1,036 ± 90). Separate studies showed that the vigor with which the unstirred samples were hand-mixed strongly influenced the results obtained.

Intracellular Ca2+ Mobilization Using Fura-2-Loaded Platelets

Platelets were isolated from ACD PRP, loaded with fura-2/AM, and gel-filtered into Ca-free Tyrode’s elution buffer as described previously. The increases in [Ca2+]i, induced by collagen, thrombin, and collagen plus thrombin were measured at the same agonist concentrations, extracellular Ca2+ concentration (2.5 mmol/L), platelet concentrations, and activation times (10 minutes) as for the prothrombinase assay, in the presence and absence of stirring, according to the procedures described previously. The values of fura-2 fluorescence at 340 and 380 nm excitation were corrected for leakage of fura-2 using the changes in fluorescence at 340 nm produced by sequential additions of 100 μmol/L MnCl2 and 200 μmol/L DTPA-Ca2+ to estimate the amount of extracellular fura-2. It should be noted that, while most conditions in which [Ca2+]i, changes were measured were identical to those in which prothrombinase activity was measured, the stirring speed and efficiency could not be made identical because of the mechanical differences in the instruments used.

The average calcium concentration for the entire period of platelet activation, and for intervals during this period, was determined by numerical analysis of the curves generated by measuring [Ca2+]i at 2-second intervals. The method used was one in which the area under a curve is evaluated numerically by means of an integration formula obtained by interpolating parabolas through a number of pivotal points of the integrand, using the area under the parabolas as an approximation to the area under the integrand (Simpson’s rule), and dividing by the time interval over which the area was determined.

Patients

Twenty-eight patients with functional platelet defects were studied, including 20 with storage pool deficiency (18 δ-SPD, 1 αβ-SPD, 1 a-SPD), 5 with Glanzmann’s thrombasthenia, and 3 with an isolated defect in PF3a, including 2 previously unreported patients (MSn and MP). A patient with factor V deficiency, whose platelet defect is assumed to be a specific deficiency of α-granule factor V, was also studied.

δ-SPD

Eleven patients (5 males, 6 females) who have the albinism variant of δ-SPD (Hermansky-Pudlak syndrome, HPS) were studied. All have longstanding bleeding problems of varying severity. Seven of these patients have been the subjects of previous reports; four are new patients, two of whom are sisters with a longstanding history of colitis.

Seven non-HPS patients with δ-SPD (ES, NS, LG, EP, SKn, SKt, PD) are from a group of previously reported patients. The deficiencies of dense granule substances in the non-HPS patients are less pronounced than in those with the HPS variant. The average platelet values (±SE) for these substances, assayed as previously described, in the platelets of controls versus HPS versus non-HPS patients are as follows: adenosine triphosphate (ATP) (μmol/1011) = 4.86 ± 0.13 versus 3.61 ± 0.41 versus 4.28 ± 0.19; ADP (μmol/1011) = 2.56 ± 0.07 versus 0.41 ± 0.05 versus 0.83 ± 0.09; ATP/ADP = 1.91 ± 0.04 versus 10.88 ± 0.99 versus 5.46 ± 0.48; serotonin (nmol/1011) = 288 ± 8 versus 27 ± 5 versus 146 ± 18.

αβ-SPD

Patient JC has been described in previous reports as having severe combined deficiencies of both dense granule and α-granule constituents. Her platelets contain approximately 20% of the normal amount of α-granule number and substances.
alpha-SPD

One recently studied 29-year-old woman has a selective deficiency in platelet alpha-granules (gray platelet syndrome). Both vWF:Ag and fibrinogen are undetectable in her platelets. Platelet ATP, ADP, and serotonin values are normal. The platelet factor V value was 7 U/10^11 platelets (10% normal).

Thrombasthenia

Studies were performed on four previously described patients (MC, CG, LW, LM) whose platelet GPIIb-IIIa is markedly reduced^{11-14} and on one new patient (AE). The binding to their platelets of 7E3, an MoAb that reacts with both GPIIb-IIIa and alpha beta,^{15} and 10E5, which reacts only with GPIIb-IIIa^{16} (kindly performed by Dr Barry S. Coller, Mt Sinai School of Medicine, New York, NY), was (%) normal: LW 5.4, 2.6; MC 1.9, 11.4; CG 1.2, 3.4; AE 10.0, 9.0, and LM 5.8, 10.1. A GPIIb missense mutation (Leu 214 Pro) has recently been reported in patient LW.^{21} Patient AE is somewhat atypical in that its PRP aggregated to a small extent (5% to 13%) with 50 microl/L ADP on five occasions whereas only a shape change was observed in the other four patients. This small amount of platelet aggregation was eliminated completely by addition to his PRP of MoAbs against GPIIb-IIIa (7E3 from Dr Coller and JCIP8 from Dr Zaverio M. Ruggeri, La Jolla, CA). The PF3a value on patient AE was 51 seconds whereas it was 58 to 65 seconds in the other patients.

Isolated Defects in Platelet Coagulant Activity

Three patients with isolated PCA defects were studied, including the previously reported patient with Scott Syndrome^{28} and two more recently studied patients (MSn and MP).

Scott Syndrome

Patient MS is the originally reported patient who was found to have an isolated defect in PF3a.^{27} Subsequent studies showed that her activated platelets exhibited decreased numbers of newly exposed membrane binding sites for coagulation factors Va^{13,26} and VIIIa^{62} reflecting a diminished surface exposure of phosphatidylserine,^{29} and resulting in reduced membrane catalytic activity for both the tenase^{28} and prothrombinase complexes.^{25,26} In addition, the capacity of her platelets to generate microparticles^{33} and support fibrin formation on subendothelium^{39} is reduced. The various platelet studies, as well as those demonstrating decreased procoagulant activity in her RBCs^{63} and lymphocytes,^{44} that have been performed on this patient have been summarized recently.^{28}

Patient MP

MP is a 46-year-old woman who bled excessively after an appendectomy at age 16, a hemorrhoidectomy at age 22, cosmetic eye surgery at age 40, and after the birth of one of her two children. She does not bruise easily and did not bleed excessively at age 39 after extraction of a third molar tooth. There is no history of excessive bleeding in her parents, two children, or four siblings. Other medical conditions and diagnoses include fibromyalgia, herniated intervertebral disc, mitral valve prolapse, and a thyroid nodule, for Where studies on patients and controls were ... of patient Olive, NJ). Normal values were obtained for the bleeding time, and control groups were determined by Student’s t-test for either nonpaired or paired samples.

RESULTS

Platelet Prothrombinase and Other Coagulant Activities

Table 1 summarizes the results obtained for PF3a, platelet coagulant factor V, and thrombin or collagen plus thrombin-induced prothrombinase activity in stirred GFP.
Table 1. Platelet Coagulant Values

<table>
<thead>
<tr>
<th>Platelet Defect</th>
<th>Subject Disorder</th>
<th>PF3a (s)</th>
<th>Platelet Factor V (U/10^11)</th>
<th>Prothrombinase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Controls</td>
<td>x 43</td>
<td>68</td>
<td>1,057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD 4²</td>
<td>19</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SE 1</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n) (30)</td>
<td>(15)</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>PCA (PF3a) Scott</td>
<td>74†</td>
<td>51</td>
<td>543</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>57†</td>
<td>42</td>
<td>593</td>
</tr>
<tr>
<td></td>
<td>MSn</td>
<td>60†</td>
<td>62</td>
<td>759</td>
</tr>
<tr>
<td></td>
<td>Dense granules</td>
<td>HPS (n = 10)</td>
<td>48 ± 1†</td>
<td>63 ± 4</td>
</tr>
<tr>
<td></td>
<td>Non-HPS</td>
<td>ES 65†</td>
<td>60</td>
<td>574</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS 55†</td>
<td>60</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td>Other (n = 5)</td>
<td>42 ± 2</td>
<td>77 ± 7</td>
<td>671 ± 113</td>
</tr>
<tr>
<td></td>
<td>a- and dense</td>
<td>aβ-SPD</td>
<td>76†</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>granules</td>
<td>NS 55†</td>
<td>60</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td>a-Granules</td>
<td>a-SPD</td>
<td>45</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Factor V</td>
<td>65†</td>
<td>1,266</td>
</tr>
<tr>
<td></td>
<td>GPitbillia</td>
<td>TSA (n = 5)</td>
<td>58 ± 45</td>
<td>122 ± 18†</td>
</tr>
</tbody>
</table>

* Values are picomoles thrombin formed per 10^8 platelets during a 1-minute incubation period in the prothrombinase assay (see Materials and Methods). Platelet activation was for 10 minutes with stirring.

Normal Subjects

Prothrombinase activity was observed in platelets stimulated with collagen (Table 2) or thrombin alone, and in combination (Table 1, Fig 1). Collagen plus thrombin-induced prothrombinase activity in the absence of added factor Va was 72% of that in the presence of added factor Va, indicating that a substantial portion of total prothrombinase activity could be supported by platelet-derived factor Va. Prothrombinase activity was also measured in the absence of stirring, to minimize any effects of platelet aggregation on prothrombinase generation. For all agonists tested, the values obtained in unstirred GFP were less than those in stirred GFP (Fig 1A through C).

Isolated Defects in PCA

Patients in this group were found initially to have reduced PF3a, and all had reduced prothrombinase activity with one or more agonists.

In patient MS with Scott syndrome, the prothrombinase activities, ranging from 32% to 51% of normal, observed

Table 2. Collagen-Induced Platelet Prothrombinase in Thrombasthenia

<table>
<thead>
<tr>
<th>Stirring (– or +)</th>
<th>Total (pmol thrombin/10^8 platelets)</th>
<th>Agonist Specific*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol thrombin/10^8 platelets)†</td>
<td></td>
</tr>
<tr>
<td>Controls (x ± SE)</td>
<td>999 ± 99</td>
<td>183 ± 168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,373 ± 129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,065 ± 150</td>
</tr>
<tr>
<td>Thrombasthenics</td>
<td>LW</td>
<td>1,167</td>
</tr>
<tr>
<td></td>
<td></td>
<td>696</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,169</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,080</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>1,602</td>
</tr>
<tr>
<td></td>
<td></td>
<td>375</td>
</tr>
<tr>
<td></td>
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<td>3,315</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,455</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>2,049</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,095</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,510</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,787</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>2,847</td>
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<td></td>
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<td>1,539</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1,704</td>
</tr>
<tr>
<td></td>
<td>LM</td>
<td>1,257</td>
</tr>
<tr>
<td></td>
<td></td>
<td>933</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>879</td>
</tr>
<tr>
<td></td>
<td>x ± SE</td>
<td>1,784 ± 308†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>928 ± 195§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,979 ± 322</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,377 ± 173</td>
</tr>
</tbody>
</table>

* Agonist-specific values obtained by subtracting the value obtained for the saline-treated sample (constitutive) from the total activity.
† pmol thrombin formed per 10^8 platelets during a 3-minute incubation period in the prothrombinase assay (see Materials and Methods). P value v controls: † < .05, § < .01.
with all agonists were similar to the findings in previously reported studies.\textsuperscript{13,25,26} In patient MP, reduced prothrombinase activity was obtained with some agonists on each of three different occasions. The most significant reduction (average of 53% normal, >2 SD) was obtained with collagen plus thrombin in the absence of added factor Va. The coagulant factor V content of her platelets (Table 1) was lower than that in all but 1 of 15 normal subjects. In patient MSn, a low prothrombinase activity in the absence of factor Va (54% of normal), similar to that found in patient MP, was observed on two of four occasions. The coagulant factor V content of her platelets was normal.

**Dense Granule Defects**

Results are shown in Table 1 for patients with both the HPS and non-HPS variants of δ-SPD. The PF3a values in the HPS patients were, on average, only moderately abnormal. Among non-HPS patients, only patient ES and her daughter NS were abnormal.

Prothrombinase activities induced by all agonists in HPS patients were significantly decreased (45% to 71% normal) (P < .01 or .001) in both stirred and unstirred platelets (Table 1, Fig 1A through C). The prothrombinase activities in non-HPS patients ES and NS were also decreased, while less consistent decreases were obtained in the other patients.

**α-Granule Defects**

The common defect in these patients (α and αδ-SPD; factor V deficiency) was a variable reduction in platelet coagulant factor V and an impaired collagen plus thrombin-induced prothrombinase activity in the absence of added factor Va (Table 1). The addition of factor Va completely corrected the latter defect in the factor V−deficient patient, as reported previously,\textsuperscript{15} whereas only partial correction was achieved in the patient with α-SPD, and no correction was achieved in the patient with αδ-SPD.

**Thrombasthenia**

The average prothrombinase activities in stirred GFP in the five patients did not differ significantly from those in controls (Table 1 and Fig 1A through C). However, considerable variation in the individual values obtained with both thrombin and collagen plus thrombin was observed among these patients (data not shown). The values for patient AE were notably increased, and if this somewhat atypical patient (see Materials and Methods) was eliminated from the analysis, the mean collagen plus thrombin-induced activity would have been significantly decreased in both stirred samples (1,409 ± 118 v 1,899 ± 113, P < .05; patient AE = 2,452) and unstirred samples (718 ± 126 v 1,036 ± 90, P = .05; patient AE = 1,618).

The most consistent finding in thrombasthenia was an increased collagen-induced prothrombinase activity in the absence of stirring (1,784 ± 308 v 999 ± 99, P < .05) (Fig 1A). As shown in more detail in Table 2, this increase was even more striking when expressed as the agonist-specific prothrombinase activity, calculated by subtracting the values obtained for parallel saline-treated samples (constitutive activity) from the total activity (928 ± 195 v 183 ± 168, P < .01).

Figure 1A also shows that, even in thrombasthenic platelets, prothrombinase activities obtained in the presence of stirring were consistently greater than those obtained in the absence of stirring, indicating that the increased activities produced by stirring in all subjects (Fig 1) are probably not due solely to the occurrence of platelet aggregation per se.

Platelet coagulant factor V was also strikingly increased in patients with thrombasthenia (Table 1). This abnormality was not explored further. However, another α-granule constituent, vWF:Ag, was present in normal amounts (42 ± 3 v control values of 43 ± 2 U/10\(^1\)).

**Platelet \([\text{Ca}^{2+}]_{i}\) Responses**

**Normal subjects.** In both stirred and unstirred GFP, thrombin and collagen plus thrombin produced a rapid initial increase in \([\text{Ca}^{2+}]_{i}\), to generally similar values (Fig 2). This was followed by a gradual decrease in unstirred samples and in stirred samples stimulated with thrombin, whereas a secondary increase in \([\text{Ca}^{2+}]_{i}\), that was maintained throughout
Fig 2. Agonist-induced Ca\textsuperscript{2+} responses in a normal subject. GFP was activated with collagen (C), thrombin (T), or collagen plus thrombin (C + T), with (A) or without (B) stirring, as in Fig 1, and [Ca\textsuperscript{2+}], measured with fura-2. Typical responses obtained on a normal subject are shown. Initial peak values in controls with thrombin were 952 ± 85 nmol/L (+ stirring) and 807 ± 46 nmol/L (− stirring), and with collagen plus thrombin, the values were 953 ± 85 and 736 ± 58 nmol/L, respectively.

The activation period occurred in stirred GFP stimulated with collagen plus thrombin (Fig 2A). In contrast, collagen alone induced a delayed and slower increase in [Ca\textsuperscript{2+}], which reached peak values of 307 ± 35 nmol/L with stirring and 86 ± 7 nmol/L without stirring. Figure 1D through F shows the average [Ca\textsuperscript{2+}], values, calculated as described in Materials and Methods, for the entire period of activation.

δ-SPD. The Ca\textsuperscript{2+} responses in SPD-HPS patients were significantly less than those in controls under all conditions studied (Fig 1D through F). The basis for this defect is shown in more detail in Fig 3. As seen in Fig 3A, while the initial increase in [Ca\textsuperscript{2+}], to 821 ± 57 nmol/L in collagen plus thrombin-stimulated stirred HPS platelets was similar to that in normal platelets (953 ± 85 nmol/L), this was followed by a rapid decrease in the HPS platelets, in contrast to the secondary and sustained rise observed in normal platelets. A greater than normal decline was also seen in unstirred HPS platelets (Fig 3B) and the initial peak value was also somewhat decreased under these conditions.

Limited studies were obtained on four of the non-HPS patients with δ-SPD. Although the initial increase in [Ca\textsuperscript{2+}], was more variable than in the HPS patients, a greater than normal decrease from the initial peak value was also seen in these patients (data not shown).

As shown in Fig 1, the decreased [Ca\textsuperscript{2+}], response seen with all agonists in both stirred and unstirred HPS platelets was always associated with a corresponding decrease in prothrombinase activity.

Thrombasthenia. In the thrombasthenic patients, Ca\textsuperscript{2+} responses differing significantly from those in normals were observed in stirred, collagen plus thrombin–stimulated GFP (Fig 1F) and in unstirred, collagen-stimulated GFP (Fig 1D). The responses obtained under all other conditions of activation were similar to those in normal subjects (Fig 1D through F).

The initial peak [Ca\textsuperscript{2+}], value in stirred GFP stimulated with collagen plus thrombin was the same in thrombasthenics as in controls (1,026 ± 95 v 953 ± 85 nmol/L, Fig 3A). However, the secondary, sustained increase in [Ca\textsuperscript{2+}], seen in controls was not observed in thrombasthenics, resulting in an average [Ca\textsuperscript{2+}], significantly less than that in controls (642 ± 45 v 982 ± 71 nmol/L, P < .05). The decline in elevated [Ca\textsuperscript{2+}], levels was less pronounced in thrombasthenia, however, than in HPS (Fig 3A). This significantly decreased average [Ca\textsuperscript{2+}], value (Fig 1F) was associated with a corresponding decrease in prothrombinase activity (Fig 1C), which became statistically significant if the atypical patient AE was eliminated from the analysis.
gen plus thrombin was at the lower end of the 2-SD range of control values. Studies were not performed on patients with α-granule defects.

Correction of the impairment in prothrombinase and [Ca²⁺], responses in HPS by ADP. In previous studies we have shown that some activation-related abnormalities in δ-SPD, such as impaired acid hydrolase secretion, could be corrected by the addition of ADP. The effect of ADP on the impaired collagen plus thrombin-induced [Ca²⁺] responses and prothrombinase activity in SPD-HPS platelets was therefore studied. Addition of 5 μmol/L ADP to stirred platelets 30 seconds after stimulation resulted in a striking increase in the [Ca²⁺], response in both HPS and normal subjects (Fig 5). As seen in Fig 5B, ADP prevented the rapid decrease in [Ca²⁺], in the HPS platelets, and produced a secondary, sustained increase that was almost identical to that observed in the untreated controls (Fig 5A, −ADP). This increase in the [Ca²⁺], response (Fig 6C) was associated with a corresponding increase in prothrombinase activity (Fig 6A). Addition of ADP to unstirred HPS platelets also produced increases in both the average [Ca²⁺], values and prothrombinase activity (Fig 6D and B). In contrast, prothrombinase activity in normal platelets was not affected by addition of ADP, despite the increase in the [Ca²⁺], response (Fig 6). Enhancement of the [Ca²⁺], response by added ADP was not observed when HPS platelets were stimulated in a medium containing excess EGTA rather than 2.5 mmol/L Ca²⁺ (data not shown), thus suggesting that the effect of ADP on this response may be primarily via an increase in calcium influx.

DISCUSSION

Previous reports of a patient (Scott syndrome) with an isolated defect of PF3a whose platelets do not generate the prothrombinase and tenase activities associated with normal platelet procoagulant activity, and in whom surgically related bleeding has been controlled by platelet transfusion, have provided evidence that these platelet procoagulant properties are essential for normal hemostasis. A second patient with an isolated prothrombinase defect has also been reported.

In contrast to these decreased [Ca²⁺], responses, thrombasthenic platelets stimulated with collagen alone in the absence of stirring had an increased [Ca²⁺], response compared with that in normal platelets (Fig 4). The average [Ca²⁺], value in thrombasthenics was 97 ± 6 nmol/L, v 72 ± 5 nmol/L in controls (P < .01, Fig 1D), and this increased response was also associated with a corresponding increase in prothrombinase activity (Fig 1A). The correlation between the [Ca²⁺], and prothrombinase responses in the patients shown in Fig 4 was significant (r = .978, P < .05).

Other patients. No significant abnormalities in the Ca²⁺ responses were observed in patients MSn or MP (data not shown). The average [Ca²⁺], values were also normal in patient MS with Scott syndrome (data not shown), although the value obtained in stirred platelets stimulated with collagen plus thrombin was at the lower end of the 2-SD range of control values. Studies were not performed on patients with α-granule defects.

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DISCUSSION

Previous reports of a patient (Scott syndrome) with an isolated defect of PF3a whose platelets do not generate the prothrombinase and tenase activities associated with normal platelet procoagulant activity, and in whom surgically related bleeding has been controlled by platelet transfusion, have provided evidence that these platelet procoagulant properties are essential for normal hemostasis. A second patient with an isolated prothrombinase defect has also been reported.
Recently, we identified two new patients (MP and MSn) with relatively severe bleeding disorders in whom the only defects were impaired platelet PF3a and prothrombinase activities. Both patients had bled excessively after many surgical procedures, but with platelet coverage patient MP underwent an uncomplicated total abdominal hysterectomy and bilateral salpingo-oophorectomy, and patient MSn underwent an uncomplicated cholecystectomy. The clinical and laboratory findings in these patients therefore provide additional evidence that the expression of platelet prothrombinase activity is required for normal hemostasis.

The basis for the defect in factor Va binding that accounts for the impaired prothrombinase activity in the originally described patient with Scott syndrome remains under study, but appears to be due to an abnormality in the scramblase mechanism that promotes the translocation of phosphatidylinositol serine from the inner to the outer layer of the platelet, as well as the RBC membrane. The defect in patient MP may differ from that in Scott syndrome. The most significant prothrombinase defect in patient MP was observed with collagen plus thrombin in the absence of factor Va, and was partially corrected by the addition of factor Va. Her platelet coagulant factor V (64% normal) was also lower than all but 1 of 15 normal subjects. Thus, these findings suggest that a defect in α-granule factor V could be the basis for the impaired prothrombinase activity in patient MP. The importance of this protein in promoting prothrombinase activity is illustrated by the results obtained with and without added factor Va in collagen plus thrombin stimulated platelets from patients with αδ-SPD, α-SPD, and factor V deficiency (Table 1). The most selective and pronounced defect was observed in the patient with factor V deficiency, but this was completely corrected by addition of factor Va. A pronounced defect, which was only partially corrected by added factor Va, was also observed in α-SPD. The failure of factor Va to correct completely the prothrombinase defect in this latter patient was probably due to an almost significant (∼2 SD) decrease in the prothrombinase activity induced by thrombin alone, which is in contrast to the normal thrombin-induced prothrombinase activity reported in α-SPD by Bevers et al. However, the present findings are consistent with our recent observations and those of Srivastava et al that the platelets of some patients with α-SPD have a specific defect in thrombin-induced activation.

A prothrombinase defect has also been described in patients with factor V Quebec, a disorder characterized by deficiencies of platelet factor V and a factor V-binding protein (multimerin) found in α-granules and both quantitative and qualitative abnormalities in other α-granule proteins. In patient MP, both α and αδ-SPD are ruled out by the normal values obtained for dense granule substances and α-granule substances other than factor V, and her platelet multimerin content was also normal (studies kindly performed by Dr Catherine P.M. Hayward, McMaster University, Hamilton, Ontario, Canada). Thus, while the findings in patient MP suggest that some type of α-granule factor V defect may be the basis for her platelet prothrombinase abnormality, the nature of this defect remains to be determined. We have no explanation at present for the prothrombinase defect in patient MSn, whose platelet coagulant factor V is normal.

In addition to those patients with isolated defects in PCA, we also studied platelet prothrombinase activity in patients with δ-SPD and thrombasthenia. Although the clinical relevance of the prothrombinase findings in these latter patients is complicated by their other platelet abnormalities, these studies, as well as the parallel studies on intracellular Ca²⁺ responses, on patients with well-defined platelet defects may provide further information about the mechanisms of prothrombinase activation and Ca²⁺ responses in normal platelets.

Included in this study were 18 patients with δ-SPD, 11 of whom have the typical findings of SPD-HPs. Significantly impaired prothrombinase activity in the HPS patients, who are severely deficient in dense granule substances, was observed with collagen, thrombin, and collagen plus thrombin both in the presence and absence of stirring (Fig 1). However, these impairments were less pronounced than those in Scott syndrome (Table 1). More variable findings were obtained in the non-HPS δ-SPD patients, whose dense granule deficiencies are less severe than those in patients with the HPS. These results thus suggest that dense granules, or substances stored in them, may play a role in the full development of platelet prothrombinase activity. This con-
clusion is at variance with that of Bevers et al., who measured prothrombinase activity in a dilute PRP system and reported the pooled values on one HPS and two non-HPS patients. Of interest, even in their more limited study, the activities in the SPD patients were somewhat lower than normal, although the difference did not reach statistical significance. The possible mechanisms that could account for a prothrombinase defect in δ-SPD are discussed below.

The results obtained in five patients with thrombasthenia were greatly influenced by the agonist and stirring conditions. In addition, the molecular abnormalities of GPIIb-IIIa in thrombasthenia may be heterogeneous, and we observed considerable variation among these patients (Table 2). The average prothrombinase activity in collagen plus thrombin-stimulated, stirred GFP was not statistically different from that in controls, as also reported by Bevers et al. in patients with several types of thrombasthenia. However, patient AE, whose prothrombinase activity was greater than the mean control value, was somewhat atypical in that his platelets aggregated to a small extent with 50 μmol/L ADP. If this patient was eliminated from the analysis, the average prothrombinase value in the four remaining patients was significantly less than controls. In studies currently in progress, we have observed that the addition to normal platelets of MoAbs directed against GPIIIa also resulted in a statistically significant decrease in collagen plus thrombin-induced prothrombinase activity (unpublished results, June 1996). These results are similar to those reported recently by Revert et al. using a different method for measuring prothrombinase, and suggest that, under some conditions, GPIIIa may play a role in supporting prothrombinase activation. Whether this is solely a reflection of the absence of aggregation in thrombasthenic platelets is currently under investigation.

In contrast to these findings, prothrombinase activity induced by collagen alone in unstimred thrombasthenic platelets was consistently greater than that in controls. This increase was even more striking when prothrombinase was calculated as agonist-specific activity (Table 2). These observations of both increased and decreased prothrombinase activity in thrombasthenia are similar to the results obtained in previous studies, in which increased fibrinopeptide A formation versus that in controls was seen in early samples of blood emerging from bleeding time wounds in thrombasthenics, whereas decreased formation was seen in later samples. Although little is known about the comparative "strengths" of the collagen and thrombin stimuli that contribute to platelet activation in vivo, it is highly likely that the proportion and magnitude of these stimuli change considerably over time during normal hemostasis. Hence, the prothrombinase findings observed in thrombasthenia in this study could be relevant to those observed during hemostasis in these patients.

The reasons for the occurrence of increased and decreased prothrombinase activities, depending on the conditions of activation, in thrombasthenia remains to be determined. In addition to the possibility of a direct role for GPIIb-IIIa in supporting prothrombinase activity, the failure of thrombasthenic platelets to aggregate could also have modifying effects on the generation of this activity. For example, in the case of collagen stimulation, the absence of cell-cell contact (aggregation) might result in more factor Va binding sites exposed by single thrombasthenic platelets adherent to collagen than by the less activated platelets on the surface of a normal platelet aggregate, as suggested by others. In contrast, the stronger stimulus provided by collagen plus thrombin in stirred platelets may result in aggregation-dependent responses that enhance factor Va binding, and hence prothrombinase activity, in normal, but not in thrombasthenic platelets.

The results of parallel studies on the agonist-induced [Ca²⁺], changes in SPD and thrombasthenic platelets may provide some insights into the mechanisms that could contribute to the abnormal generation of platelet prothrombinase activity observed in these disorders. In addition, they may provide further insights into the mechanisms controlling activation-induced intracellular Ca²⁺ changes in normal platelets and the relationship of these changes to prothrombinase activation. The translocation of phosphatidylserine to the outer layer of the plasma membrane, which promotes the generation of procoagulant activity, is dependent on both an increase in [Ca²⁺] and the presence of extracellular Ca²⁺. Recent evidence suggests that the continuous presence of an elevated [Ca²⁺], level may be necessary to maintain exposure of PS on the outer membrane surface. The results obtained in SPD platelets suggest that their impaired prothrombinase response may be related to their inability to maintain a sufficiently elevated [Ca²⁺], level. The average [Ca²⁺], values for the period of activation were significantly decreased in the HPS patients in response to collagen, thrombin, and collagen plus thrombin (Fig 1), and with the latter agonist in particular, this was due primarily to a more rapid and extensive decline in elevated [Ca²⁺], following the initial increase (Fig 3). This enhanced decrease was also observed in non-HPS patients, in whom the initial [Ca²⁺], increases were more variable, perhaps reflecting the heterogeneous nature of SPD. For all six conditions of study, there was a significant impairment of both the [Ca²⁺], and prothrombinase responses in the HPS patients (Fig 1). Although these findings are not conclusive of a causal relationship, the consistent association of impaired prothrombinase activity and decreased average [Ca²⁺], levels with all agonists suggests that this may be the case.

It is unlikely that these impaired responses in δ-SPD are caused by the defective platelet aggregation that is characteristically observed in these patients, since both the prothrombinase and [Ca²⁺], defects were observed in stirred as well as unstirred GFP. In addition, both the average [Ca²⁺], values and prothrombinase activities in HPS patients were considerably less than those in the thrombasthenics (Fig 1), in whom aggregation does not occur, and impaired responses were found only in collagen plus thrombin-stimulated stirred samples. Both the enhanced decrease in [Ca²⁺], and the impaired prothrombinase activity in HPS platelets were corrected, however, by the addition of ADP after stimulation, providing further evidence suggesting that the failure to maintain an adequate [Ca²⁺], level may account for the prothrombinase defect in HPS platelets. ADP is believed to be unique among platelet agonists in opening a receptor-linked membrane Ca²⁺ channel, as opposed to second messenger-
linked Ca\(^{2+}\) channels activated by other agonists.\(^{13,16,74}\) Thus, the enhanced decay of [Ca\(^{2+}\)], in HPS platelets, which are virtually devoid of secretable dense granule ADP, may be due to the loss of an additional component of calcium influx initiated by the interaction of secreted ADP with membrane ADP receptors. Our observation that addition of ADP also increased the [Ca\(^{2+}\)], response, but not prothrombinase activity, in normal platelets stimulated with collagen plus thrombin (Fig 5) suggests that, while ADP receptors mediating Ca\(^{2+}\) influx may not be fully occupied by the amount of ADP secreted from normal dense granules under these conditions, the [Ca\(^{2+}\)], level achieved is sufficient for full development of prothrombinase activity. The results of these studies therefore strongly suggest that ADP secreted from normal platelets may play an important role in the generation of procoagulant activity by contributing to the maintenance of an elevated [Ca\(^{2+}\)], level sufficient to maintain aminophospholipids on the outer surface of the platelet membrane. They also provide evidence that the dense granules may be a major source of the ADP involved in opening receptor-operated calcium channels in stimulated platelets.

Parallel changes in [Ca\(^{2+}\)], and prothrombinase responses were also observed in thrombasthenia. The increased prothrombinase activity in collagen-stimulated unstirred platelets was associated with a corresponding greater increase in [Ca\(^{2+}\)], whereas the decreased mean prothrombinase activity in collagen plus thrombin–stimulated stirred platelets from four of the five patients was associated with a failure to maintain the elevated [Ca\(^{2+}\)], levels following an initially normal peak value. In all other conditions measured, both the [Ca\(^{2+}\)], and prothrombinase responses were normal. Although the parallel increases and decreases in these responses may be consistent with a causal relationship, the mechanisms(s) by which a deficiency of GPIIb-IIIa could produce such changes are not clear at present. Early studies indicated a role for GPIIb-IIIa in regulating [Ca\(^{2+}\)], levels in unstimulated platelets,\(^{75,76}\) but whether, and how, it may mediate [Ca\(^{2+}\)], increases in stimulated cells is controversial. Studies in liposomes\(^{77}\) have suggested that GPIIb-IIIa itself may act as a calcium channel, while other studies in stimulated platelets\(^{79}\) have implicated aggregation, or fibrinogen binding to GPIIb-IIIa, as promoting a component of calcium influx; however, these conclusions have been challenged.\(^{79}\) It is also difficult to reconcile our observations of an increased [Ca\(^{2+}\)], response in unstirred collagen-stimulated thrombasthenic platelets with these proposed roles for GPIIb-IIIa. Powling and Hardisty\(^{80}\) reported that calcium influx in response to weak agonists was decreased in normal platelets by an MoAb to GPIIb-IIIa, but normal in thrombasthenic platelets, thus suggesting that this influx may involve a channel in close proximity to GPIIb-IIIa. Similarly, Sinigaglia et al\(^{81}\) found that influx in response to ADP and thrombin was inhibited when GPIIb-IIIa was occupied by an inhibitory MoAb, an RGD-containing peptide, or fibrinogen. The possibility suggested by these studies, that occupancy of GPIIb-IIIa by inhibitory antibodies and peptides, or adhesive ligands, may negatively modulate calcium influx, is consistent with the increased [Ca\(^{2+}\)], response seen here in unstirred collagen-stimulated thrombasthenic platelets. As discussed above for the prothrombinase activity, the decreased [Ca\(^{2+}\)], response seen in collagen plus thrombin-stimulated thrombasthenic platelets could be related to the absence of aggregation, because it was seen only with stirring, i.e., under conditions in which aggregation occurs in normal platelets. Studies are currently underway to explore these questions further. Thus, the specific role(s) which GPIIb-IIIa may play in both prothrombinase activation and calcium influx remains unresolved at present. However, our findings of both enhancement and inhibition of these events in thrombasthenic platelets may be related to other conflicting reports describing the promotion\(^{94-91}\) or inhibition\(^{82}\) of fibrin formation by GPIIb-IIIa, and could be relevant to the use of specific inhibitors of GPIIb-IIIa as antithrombotic agents.\(^{83}\)

Finally, the observation that the most pronounced platelet prothrombinase abnormality was observed in Scott syndrome provides further support for the concept that pharmacologic induction of the Scott defect in normal platelets could provide a new approach to antithrombotic therapy.\(^{28,39}\)

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**ADDENDUM**

Mary Ann Scott died on November 12, 1996 at the age of 57. It was only her longstanding and selfless cooperation that made possible the many studies on the disorder that bears her name. This work is dedicated to her memory.

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Platelet Prothrombinase Activity and Intracellular Calcium Responses in Patients With Storage Pool Deficiency, Glycoprotein IIb-IIIa Deficiency, or Impaired Platelet Coagulant Activity — A Comparison With Scott Syndrome

Harvey J. Weiss and Bruce Lages