Thrombin and Ionophore A23187-Induced Dense Granule Secretion in Storage Pool Deficient Platelets: Evidence for Impaired Nucleotide Storage as the Primary Dense Granule Defect

By Bruce Lagos, Holm Holmsen, Harvey J. Weiss, and Carol Dangelmaier

The secretion of the dense granule constituents ATP, ADP, calcium, pyrophosphate (PP), and orthophosphate (P), and the release of magnesium induced by thrombin and the divalent cation ionophore A23187 have been quantitated directly in gel-filtered platelets from patients with storage pool deficiency (SPD). Both the contents and the maximal amounts of the dense granule constituents securable by thrombin were decreased in all the patients studied, while the nonsecurable, retained amounts of these substances were identical in SPD and normal platelets. In response to both thrombin and A23187, the amounts of securable ATP and ADP were strongly correlated in the platelets of individual patients; in contrast, securable calcium showed no correlation with the nucleotides, and significant amounts of calcium were secreted in the total absence of nucleotide secretion in the platelets of several patients.

Storage pool deficiency (SPD) in human platelets has been well characterized by decreased numbers of platelet dense granules and decreased contents of securable, granule-bound substances. In addition to adenosine triphosphate (ATP), adenosine diphosphate (ADP), and serotonin, these substances include calcium and pyrophosphate. Recent studies have shown that platelets from several SPD patients, in addition to their dense granule deficiencies, are also deficient to varying extents in the number and contents of α-granules.

While the decreased number of dense granules in SPD platelets has been clearly established, the nature of this granule defect is not as well characterized. Whether the observed deficiencies result from an absence of, or reduction in, the granular structures themselves, or from defects in the storage of granule-bound substances due to abnormal granular structures remains to be determined. Evidence for the latter view has been presented by Lorez et al. and Rendu et al., both of whom observed atypical dense granule structures in SPD platelets using electron microscopic and mepacrine fluorescence techniques. In addition, Lorez et al., based on cytochemical studies utilizing the fluoride-specific reagent uranaffin, have suggested that these atypical organelles represent nucleotide and amine-deficient granular structures. In contrast, Rendu et al. have postulated that the abnormal granules might reflect an inability of calcium to penetrate or be stored in the dense granules.

The present studies were undertaken to characterize further the deficiencies of securable substances in SPD platelets. Unlike previous studies in which these deficiencies were calculated from the measured contents in SPD versus normal platelets, we have determined directly the amounts of ATP, ADP, calcium, pyrophosphate, and orthophosphate secreted from SPD platelets in response to thrombin and ionophore A23187 and have thus quantitated the securable and nonsecurable pools of each substance. Our results provide further evidence for the existence of abnormal dense granule structures in SPD platelets and suggest that this granule abnormality may result from a nucleotide transport defect.

Materials and Methods
Preparation of Platelet-Rich Plasma (PRP) and Gel-Filtered Platelets (GFP)

These studies were approved by the Research Committees of St. Luke’s-Roosevelt Hospital Center and Temple University Health Sciences Center. After obtaining informed consent from SPD patients and control subjects, blood was collected into 1/10 volume of 3.2% sodium citrate dihydrate. PRP was prepared and labeled with U-14C-adenine (281 mCi/m mole, Amersham, Arlington Heights, Ill.) as described previously. GFP was also prepared.
according to previously described methods, with the following modifications: 30 ml of 14C-adenine-labeled PRP was applied to a Sepharose 2B column of approximately 2.6 x 20 cm, and 28 ml of GFP collected into Tyrode's buffer, pH 7.4, Sepharose 2B column of approximately 2.6 x 20 cm, and 28 ml of DENSE GRANULE SECRETION IN SPD PLATELETS 155

Thrombin-Induced Secretion in GFP

Aliquots (2.4 ml) of GFP were incubated at 37°C for 3 min, during which 1.4 mM (f.c.) EDTA was added to prevent subsequent degradation of secreted adenine nucleotides. Then 0.25 ml of either saline or bovine thrombin (Parke-Davis, Detroit, Mich.), 0.04, 0.3 or 5.0 U/ml (f.c.) was added for an additional 3 min, after which the samples were transferred to an ice bath and, except for one saline treated aliquot, centrifuged at 17,500 g for 10 min at 4°C. In some experiments with both thrombin and ionophore A23187 (see below), 0.1 µM U-3H-ATP (268 mCi/mmole, Amersham) was added prior to the inducer.

The supernates and uncentrifuged sample were extracted and assayed as follows: 0.6 ml was mixed with 0.06 ml of 6.6% perchloric acid at 0°C, centrifuged at 12,000 g for 2 min in an Eppendorf microcentrifuge, and 550 µl of the supematant neutralized with 2 M K2CO3 for determination of pyrophosphate and orthophosphate by a Malachite green-phosphomolybdate assay. 0.2 ml was mixed with 0.2 ml of 9:1 ethanol:0.1 M EDTA for determination of ATP and ADP by a fire-fly-luciferase assay and for determination of 14C-adenine metabolites; 1.0 ml was mixed with 1.0 ml of 0.5% Triton X-100, 5 M EDTA in 0.15 M saline for determination of calcium and magnesium by atomic absorption spectroscopy. These extracts were kept at −20°C for at least 1 day before analysis and were stable for at least 3 mo at −20°C. Thrombin was stored as a 100 U/ml stock in 0.15 M saline at −20°C and diluted just prior to use. Thawed stock solutions were discarded after use.

With some subjects, the time course of thrombin-induced calcium secretion was obtained using a calcium electrode (Radiometer, Copenhagen, Denmark) in the apparatus described by Akkerman et al.

A23187-Induced Aggregation in PRP

Aliquots (1.0 ml) of PRP were incubated at 37°C in a Payton Dual Channel Aggregometer (Payton Associates, Buffalo, N.Y.) with stirring for 1 min. Aggregation was initiated by addition of 1 µl of the divalent cation ionophore A23187, as the free acid (a generous gift of Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, Ind.) in dimethylsulfoxide (DMSO) to give final concentrations of 5 and 10 µM.

A23187-Induced Secretion in GFP

Aliquots (2.0 ml) of GFP were incubated at 37°C without stirring for 1 min, following which 2 µl of DMSO or A23187 (4 or 12 µM, f.c.) were added. After 3 min, 0.1 ml of 0.1 M EDTA was added, the samples transferred to an ice bath, and all except one DMSO-treated aliquot centrifuged at 17,500 g for 10 min at 4°C. The supernates and the uncentrifuged sample were extracted and assayed as described above. A23187 was stored as a 20 mM stock in DMSO at −20°C and diluted just prior to use.

Patients

Seventeen patients with SPD are included in this study. Most have been described elsewhere as follows: M.V., L.V. (sisters); D.C., R.C., S.C. (mother, daughter, son); J.C., J.D., R.Z., and L.G.; H.P., E.P., C.P. (father, two sons) in Ingerman,11 where they are identified as III-4, IV-5, and IV-6, respectively; F.S., L.S. (mother, daughter), and C.F. in Pareti et al.12 and H.S. in Day et al.13 F.M., a patient of Dr. M. Johnson, Wilmington Medical Center, Wilmington, Del., is a 33-yr-old Puerto Rican female with oculocutaneous albinism (Hermansky-Pudlak syndrome) who has had a life-long history of epistaxis and easy bruising. Her Ivy bleeding time was 20 min and her platelet count was normal. Aggregation in response to collagen was typically decreased, whereas a biphasic aggregation response was induced by 4 µM epinephrine. A similar pattern of biphasic epinephrine aggregation and decreased collagen aggregation has also been observed in patient J.D.10 In addition to F.M., patients M.V., L.V., and R.Z. also represent the Hermansky-Pudlak variant of SPD. D.C., R.C., S.C., and J.C. have been found to have variable deficiencies of α-granules and α-granule constituents in addition to dense granule deficiencies. Control subjects were selected from laboratory and hospital personnel who denied taking any medication for 7 days prior to study.

Table 1. Platelet Content, Maximal Thrombin Secretable and Retained Amounts of Dense Granule Substances and Mg in SPD and Control GFP

<table>
<thead>
<tr>
<th></th>
<th>ATP (µM)</th>
<th>ADP (µM)</th>
<th>Ca (µM)</th>
<th>PP (µM)</th>
<th>P, (µM)</th>
<th>Mg (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet content</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPD (no.)</td>
<td>3.2 ± 0.22*</td>
<td>0.46 ± 0.09</td>
<td>9.9 ± 1.0</td>
<td>0.58 ± 0.13</td>
<td>3.33 ± 0.16</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Control (no.)</td>
<td>4.1 ± 0.22</td>
<td>2.5 ± 0.20</td>
<td>20.3 ± 1.1</td>
<td>1.49 ± 0.18</td>
<td>4.12 ± 0.20</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td><strong>Maximal secretable</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPD (no.)</td>
<td>0.18 ± 0.05</td>
<td>0.12 ± 0.04</td>
<td>2.1 ± 0.4</td>
<td>0.34 ± 0.10</td>
<td>0.14 ± 0.06</td>
<td>0.91 ± 0.26</td>
</tr>
<tr>
<td>Control (no.)</td>
<td>1.4 ± 0.11</td>
<td>2.0 ± 0.17</td>
<td>13.7 ± 1.1</td>
<td>1.33 ± 0.22</td>
<td>0.89 ± 0.18</td>
<td>0.72 ± 0.18</td>
</tr>
<tr>
<td><strong>Retained</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPD (no.)</td>
<td>3.0 ± 0.27</td>
<td>0.34 ± 0.13</td>
<td>7.8 ± 1.4</td>
<td>0.24 ± 0.23</td>
<td>3.19 ± 0.22</td>
<td>5.8 ± 0.58</td>
</tr>
<tr>
<td>Control (no.)</td>
<td>2.7 ± 0.33</td>
<td>0.50 ± 0.37</td>
<td>6.6 ± 2.2</td>
<td>0.16 ± 0.40</td>
<td>3.23 ± 0.38</td>
<td>5.8 ± 0.58</td>
</tr>
</tbody>
</table>

Maximal secretable amounts were determined as the quantities present in the extracellular medium after incubation of GFP containing 1.5 mM EDTA with 5.0 U/ml thrombin for 3 min at 37°C and are corrected for the extracellular amounts in saline-treated suspensions. Retained amounts are calculated as the difference between platelet content and maximal secretable amounts. Values are given as means ± SE.

*µmole/1011 platelets.

Units apply to all values.
Definitions

The term secretion is used specifically to denote the extrusion of granule-bound substances in response to an activating agent in the absence of losses of nongranular and cytoplasmic substances (i.e., cell lysis). The appearance of either granule-bound or nongranular material in the extracellular medium by any other process is termed liberation or release.

RESULTS

Thrombin-Induced Secretion

Secreted and Retained Quantities of Dense Granule Constituents in SPD Platelets

Maximal secretable amounts of dense granule constituents were measured directly in GFP from SPD patients and normal subjects as the amounts secreted in response to 5 U/ml thrombin. As shown in Table 1, the maximal secretable amounts, as well as the total platelet contents of ATP, ADP, calcium, and PP, were reduced in SPD platelets. For each of these substances, however, the nonsecretable, retained amounts, calculated as the difference between the content and maximal secretable amounts, were not significantly different in SPD and control platelets. The contents and maximal secretable amounts of orthophosphate (P1) in SPD platelets were also decreased (81% and 15%, respectively, of control values), while the retained amounts were the same in patients and controls. Thus, a portion of platelet P1 (approximately 22%) also is probably contained in, and secreted from, the dense granules.

In contrast to these constituents, both the contents and releasable amounts of Mg were similar in SPD and normal platelets, and the amounts of Mg liberated by thrombin (11% in controls and 14% in SPD patients) were much lower than those of other secretable substances.

Cell Lysis During the Thrombin–Platelet Interaction

A small degree of cell lysis is known to occur during platelet secretion, as demonstrated by the appearance extracellularly of lactate dehydrogenase (LDH), 51Cr from 51Cr-labeled platelets, and radiolabeled nucleotides from platelets prelabeled with 3H- or 14C-adenine. Since the secretable dense granule nucleotides are not labeled by 14C-adenine during the short time course of the experiments reported here, percent lysis was calculated as the radioactivity in extracellular ATP, ADP, AMP, and IMP relative to the total platelet-bound radioactivity of these nucleotides. For all thrombin concentrations studied, the mean percent lysis was less than 1% in both SPD and normal platelets. However, lysis in SPD platelets was significantly less than that in control platelets in response to both 0.3 (0.04% versus 0.46%, p < 0.025) and 5 (0.16% versus 0.73%, p < 0.025) U/ml thrombin.

Specific Radioactivities of Secreted ATP and ADP

Platelets from several SPD patients released significant amounts of ATP and ADP in response to thrombin (Fig. 1), as also observed in previous studies. However, the source of these released nucleotides was not investigated in the earlier studies. We therefore determined the specific radioactivities of both total platelet ATP and ADP and the released nucleotides (Fig. 2). The specific radioactivities of ATP and ADP in unstimulated SPD platelets were typically increased, due to the decreased amounts of granule-bound nucleotides, which are not labeled under these conditions. In those patients whose platelets released measurable quantities of ATP and ADP, the specific radioactivities of these nucleotides were, with two exceptions, very low or zero, similar to the values obtained in controls. The two patients in whom the specific radioactivities were elevated were those with
the greatest extents of cell lysis. Thus, ATP and ADP released from SPD platelets were predominantly unla-
beled, indicating that they originated from a nonmeta-
bolic source.

Comparative Secretion of Dense Granule Substances in SPD Platelets

According to the above results, thrombin induced the specific secretion of dense granule ATP and ADP from some SPD platelets. The relation between the maximal secretable amounts of ADP, Ca, and ATP in individual patients is shown in Fig. 1. Secretable ADP showed a strong linear correlation with secretable ATP in 14 of 17 patients \((r = 0.81, p < 0.001)\). Nine patients, including all four Hermansky-Pudlak syndrome patients, were found to have no measurable ATP and ADP secretion. The ATP/ADP ratio of the secretable nucleotides in SPD patients \((1.21 \pm 0.13 \text{ SE}, n = 7)\) was significantly higher \((p < 0.05)\) than that in controls \((0.81 \pm 0.06 \text{ SE}, n = 14)\). This decreased or absent nucleotide secretion in SPD platelets did not result from an enzymatic degradation of the secreted nucleotides, as shown by comparable recoveries of ATP radioactivity in the supernates of SPD and control GFP treated with \(^{14}\text{C}-\text{ATP} \text{ prior to induction of secretion (data not shown).}

In marked contrast, there was no correlation between the amounts of secretable Ca and ATP in platelets from 13 SPD patients \((r = 0.08)\). Furthermore, Ca was secreted in significant amounts even from SPD platelets, which showed no measurable ATP and ADP secretion. The time course of this calcium secretion, measured with a calcium electrode \(^{14}\text{Ca}\text{ in the platelets of one SPD patient, was identical to that in normal platelets (Fig. 3).}

Comparison of the maximal secretable amounts of PP, with those of both ATP and Ca in SPD platelets showed that secreted PP, was strongly correlated with both secreted ATP \((r = 0.71, p < 0.01)\) and secreted Ca \((r = 0.78, p < 0.05)\), despite the lack of correlation between these latter two substances. Secretable P, however, was not as clearly correlated with either ATP \((r = 0.63, p < 0.1)\) or Ca \((r = 0.53, \text{NS})\).

A23187-Induced Aggregation and Secretion

A23187-Induced Aggregation in PRP

Since, to our knowledge, no detailed study of the responses of SPD platelets to ionophore A23187 has been made, we examined the aggregation as well as secretion responses of SPD platelets to this agonist. The maximum extent of aggregation in PRP induced by 5 \(\mu\text{M} \text{ A23187 was decreased in all but one (J.D.) of}}

Fig. 3. The time course of calcium secretion, recorded as calcium ion electrode output (mV), in GFP from an SPD patient and a normal subject in response to 5 U/ml thrombin. The downward deflection of the tracing denotes increasing extracellular calcium concentration. Thrombin was added at the time point indicated by the arrows.
the SPD patients (Fig. 4). In response to 10 μM A23187, the extent of aggregation was normal in 5 patients but remained decreased in 11 others. The initial rates of the aggregation response, while also decreased in some patients, were not as markedly abnormal at either concentration of A23187. Platelets from patient J.D., in addition to their normal aggregation response to A23187, have also consistently shown biphasic aggregation response to ADP and epinephrine.

**A23187-Induced Dense Granule Secretion**

The secretion of ATP, ADP, and calcium was measured in response to A23187 (Table 2). The amounts of these substances released by 12 μM A23187 in both SPD and normal GFP were generally similar to the maximal amounts secreted by thrombin (Table 1), although A23187 induced a greater release of Ca from SPD GFP than did thrombin.

**Table 2. A23187-Induced Release of Dense Granule Substances in SPD and Normal GFP**

<table>
<thead>
<tr>
<th>A23187 (μM)</th>
<th>ATP (μ mole)</th>
<th>ADP (μ mole)</th>
<th>ATP/ADP</th>
<th>Ca (μ mole)</th>
<th>Percent Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.22</td>
<td>0.19</td>
<td>1.67</td>
<td>2.1</td>
<td>3.5</td>
</tr>
<tr>
<td>12</td>
<td>(0.07)</td>
<td>(0.06)</td>
<td>(0.58)</td>
<td>(0.6)</td>
<td>(2.0)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.20</td>
<td>1.69</td>
<td>0.72</td>
<td>11.8</td>
<td>6.4</td>
</tr>
<tr>
<td>12</td>
<td>(0.15)</td>
<td>(0.17)</td>
<td>(0.07)</td>
<td>(0.9)</td>
<td>(1.4)</td>
</tr>
</tbody>
</table>

Mean (± SE) amounts of each substance, in μmole/10^11 platelets, found extracellularly in GFP incubated for 3 min with A23187 at 37°C without stirring, corrected for the extracellular amounts in DMSO-treated suspensions.

Percent lysis calculated as the percent of platelet-bound 14C-labeled ATP, ADP, IMP, and AMP found extracellularly.

As with thrombin, the ATP/ADP ratio of the nucleotides released by A23187 was increased in SPD GFP (Table 2) relative to that in normal GFP. However, the specific radioactivities of ATP and ADP liberated by A23187 (Fig. 5) were markedly greater than those of the thrombin-secretable nucleotides for both patients and controls. Also, while the specific radioactivities of thrombin-secretable ATP and ADP decreased with increasing thrombin concentration (Fig. 2), those of ATP and ADP liberated by A23187 increased with increasing A23187 concentration. These increased values thus reflect a greater degree of cell lysis, as indicated by the amounts of extracellular cytoplasmic nucleotides (Table 2) induced by A23187 in comparison to thrombin. Hence, the values for ATP, ADP, and Ca in Table 2 also include material liberated via cell lysis, and these releasable values are thus probably greater than the amounts of each substance secreted in response to A23187.

Despite this increased extent of lysis, the correlation of the amounts of A23187-liberated ATP with those of ADP and Ca in individual patients (Fig. 6) was similar to that observed with thrombin (Fig. 1). Thus, in response to both 4 and 12 μM A23187, liberation of ATP was strongly correlated with that of ADP, but was not significantly correlated with Ca liberation. In addition, as with thrombin-induced secretion, Ca liberation by A23187 was observed in several patients (R.Z., F.M., L.S.) in the absence of ATP and ADP liberation.

**A23187-Induced Liberation of Mg**

Previous studies have shown that A23187 liberates much greater amounts of Mg from human platelets.
Significantly less Mg liberated from SPD than from control platelets than does thrombin. We observed a dose-dependent liberation of Mg from both SPD and control platelets in response to A23 187 (Table 3), but found significantly less Mg liberated from SPD than from control platelets. In both cases, however, the extent of Mg liberation was clearly greater than the loss of cytoplasmic nucleotides (Table 2), suggesting that Mg liberation was due to an A23 187-specific process (such as cation transport) different from cell lysis.

**DISCUSSION**

To further characterize the dense granule defect in storage pool deficient platelets we have measured directly the amounts of dense granule constituents secreted from SPD platelets in response to thrombin and the divalent cation ionophore A23187. Previous studies have characterized the deficits in the granule-bound pool of these constituents in SPD platelets obtained indirectly from the differences in total platelet contents in SPD versus normal platelets. The present studies, in contrast, have examined specifically the granule-bound pool that is present in SPD platelets, measured directly as the amounts secretable by thrombin and A23187.

In accord with their decreased total platelet contents, the maximal amounts of dense granule substances (ATP, ADP, Ca, and PP) secreted by thrombin were decreased or zero in all patients studied (Table 1). Both the small extent of cell lysis and the very low specific radioactivities of extracellular ATP and ADP observed in SPD GFP after thrombin treatment confirmed that these substances were specifically secreted and did not originate from nongranular sources. In addition, the reduced amounts of secretable ATP and ADP could not be attributed to enzymatic degradation subsequent to secretion. While the maximal secretable amounts of each constituent varied among the patients (Fig. 1), the nonsecretable, retained amounts, calculated as the difference between the total content and maximal secretable amounts, were identical to those in normal platelets (Table 1). Hence, the deficiencies in all these constituents in SPD platelets, as shown previously for ATP and ADP, are clearly restricted to the granule-bound secretable pools. The similar pattern of decreased contents and maximal secretable amounts and normal retained amounts of orthophosphate (P) in SPD platelets provides further evidence for a dense granule localization of a portion of platelet P, as proposed by Fukami et al.

In contrast to the released nucleotides, the low specific radioactivity of which identifies their granular origin, there is no direct means to deduce the origin of released calcium. The identical time courses of thrombin-induced calcium release in SPD and normal platelets (Fig. 3) provide strong evidence for a granular origin of this released calcium in SPD platelets. However, other possibilities must also be considered. Since platelet activation is very likely mediated by an increase in the cytoplasmic calcium concentration, and the platelet membrane undergoes a transient increase in permeability following thrombin stimulation, releasable calcium in SPD platelets could also result from diffusion of cytoplasmic calcium. The maximal secretable amount of calcium in SPD platelets (2.1 μmole/10¹¹ cells, Table 1), however, represents an average increment of 6.3 μM calcium in the extracellular medium, a greater concentration than the

**Table 3. A23187-Induced Liberation of Magnesium in SPD and Normal GFP**

<table>
<thead>
<tr>
<th>Group</th>
<th>Extracellular Mg</th>
<th>Significance of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPD patients</td>
<td>1.7 ± 0.3</td>
<td>p &lt; 0.025</td>
</tr>
<tr>
<td>Controls</td>
<td>2.8 ± 0.3</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Percent of content</td>
<td>43.1</td>
<td>69.2</td>
</tr>
<tr>
<td>A23187 (μM)</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

Concentrations (means ± SE) and percents of platelet content of extracellular Mg in GFP incubated for 3 min at 37°C without stirring with A23187, corrected for the amounts in DMSO-treated supernates. The p values refer to the differences between patient and control values.
1.4 µM and 0.2–0.3 µM levels of cytoplasmic calcium measured in thrombin-stimulated pig⁴⁶ and human⁴¹ platelets, respectively. Assuming that no major shifts in the equilibrium between intracellular bound and free calcium are associated with this transient increase in membrane permeability, the diffusion and subsequent dilution of cytoplasmic calcium into the extracellular medium thus cannot account for the amount of calcium released from SPD platelets. Alternatively, platelets may have an energy-driven calcium pump at the level of the plasma membrane, analogous to that found in other cells.³² If so, calcium released into the cytoplasm from intracellular, nongranular stores in response to thrombin could be actively transported into the extracellular medium and accumulate to concentrations considerably higher than those in the cytoplasm. However, we know of no clear evidence for such a pump in platelets, and it is not likely that the time course of such calcium pumping would show saturation kinetics as in Fig. 3.

Another possibility is that, although secretable calcium in normal platelets is clearly localized in the dense granules,³³ calcium released from SPD platelets may originate from the α-granules and/or the acid hydrolase-containing vesicles. However, calcium release was not more noticeably decreased in those patients previously found to be deficient in α-granules as well as dense granules,² and the kinetics of calcium secretion are clearly different from those of acid hydrolase secretion.³⁴ Finally, calcium mobilized from the dense tubular system in response to thrombin stimulation could also be a possible source of this released calcium. Such mobilization is generally held to be an intracellular process, however, and has been observed in the absence of any release of calcium to the extracellular medium.³⁵ Our observations thus confirm most closely with a thrombin-induced exocytosis of dense granule calcium in SPD platelets.

Comparison of the maximal thrombin-secretable amounts of adenine nucleotides and calcium in platelets of individual SPD patients (Fig. 1) revealed marked differences between these substances. The amounts of secretable ATP and ADP were strongly correlated in 14 of 17 patients, whereas secretable calcium showed no correlation with the nucleotides. Moreover, significant amounts of calcium were secreted in the total absence of nucleotide secretion in the platelets of 8 patients, including those with Hermansky-Pudlak syndrome. An absence of calcium secretion was found in only 2 patients. Similar relationships among secretable ATP, ADP, and Ca were also seen in the ionophore studies (Fig. 6). These studies, however, were complicated by higher extents of cell lysis and also could include an additional mechanism for calcium liberation, i.e., ionophore-mediated transport (see below). We have therefore based our conclusions primarily on the correlations among the secretable constituents obtained in the thrombin studies.

Thus, for a majority of the patients studied, these findings suggest the presence in SPD platelets of abnormal dense granule structures that sequester calcium and, according to our results, PP, also, but which are wholly or partially devoid of ATP and ADP. Hence, the principal dense granule defect in these patients could be the transport of substances into the developing granule, as proposed by Rendu et al.,¹⁰ although our results suggest that this defect is in the nucleotide rather than the calcium transport mechanism. Since most of the granule-bound calcium and nucleotides in human platelets appear to exist in an insoluble form,³⁶ precipitating as Ca-ATP and Ca-ADP, this nucleotide storage defect could also be directly responsible for the decreased amounts of calcium in the granules of SPD platelets. Thus, calcium transport per se may be normal in these platelets.

Although the strong correlation between secretable ATP and ADP suggests that this granule transport defect may be common to both nucleotides, the ratio of secreted ATP to secreted ADP was markedly increased in SPD platelets relative to that in normal platelets. Thus, the transport or storage of one nucleotide, most likely ADP, could be more severely impaired than that of the other. However, as an alternative to such separate transport mechanisms, it is also possible that ATP only is taken up into the granule and subsequently converted to ADP via the reaction ATP + P → ADP + PP. This reaction readily occurs in precipitates of calcium phosphate,³⁷ an environment that strongly resembles human platelet dense granules. Thus, the higher ATP/ADP ratio of the secretable nucleotides in SPD platelets could also be explained by an impaired transport of ATP alone, resulting in reduced intragranular concentrations that would not give maximum rates of conversion to ADP. Such a mechanism would also account for the increased ratio of P/PP, content in SPD platelets (Table 1).

The presence of abnormal dense granule structures rather than a reduced number of normal granules in SPD platelets, which is suggested by our results as well as by the studies of Lorez et al.,⁹ and Rendu et al.,¹⁰ is further supported by recent studies of platelets from cattle with the SPD-associated Chediak-Higashi syndrome (CHS).³⁸ These CHS platelets, as shown previously for human SPD platelets,³⁹ were found to take up serotonin into a secretable compartment with an initial velocity indistinguishable from that of normal cattle platelets.⁴⁰ Subcellular fractionation of CHS
platelets incubated with $^{14}$C-serotonin prior to homogenization produced a labeled vesicular fraction that had a distinctly lower density than the radiolabeled fraction from normal cattle platelets. Furthermore, the presence of secretable cations and the absence of secretable nucleotides in the CHS platelets suggests that in SPD this species, impaired nucleotide storage may also be the primary dense granule defect.

The present studies have also shown that about 12% of total platelet magnesium was released by thrombin in both SPD and normal platelets (Table 1), an amount that cannot be explained by cell lysis. This observation, together with the comparable contents of Mg in SPD and normal platelets and our previous finding that the thrombin-induced release of Mg does not follow the same pattern as that of secretable constituents, provides further evidence that released Mg does not originate from storage granules. The increase in permeability to calcium that occurs after thrombin-induced secretion has also been observed for La$^{3+}$ as well as for Na after exposure to ADP. Thus, this release of Mg may result from a thrombin-induced increase in the permeability of cations in general. The magnitude of the Mg release in our experiments may be exaggerated, however, since no extracellular Mg was present.

In contrast to thrombin, A23187 induced a massive release of Mg, up to 70% of the platelet content in normal platelets and 46% in SPD platelets. Since A23187 is a divalent cation ionophore that transports Mg almost as well as Ca, this release is most likely due to the specific transport of the ion by A23187. A loss of cytoplasmic Mg of this extent, however, would undoubtedly have severe adverse metabolic effects and may constitute a serious side effect of A23187 in intact cells.

Finally, our results clearly show that certain responses to A23187 are impaired or diminished in SPD platelets. These include the aggregation response in PRP (Fig. 4), the ionophore-specific release of Mg (Table 3), and the extent of cell lysis induced by low concentrations of A23187 (Table 2). In addition, we have found that A23187-induced acid hydrolase secretion is also impaired in SPD platelets and that this impairment is more severe than that of thrombin-induced acid hydrolase secretion.

Whether these impairments are directly related to the granule defects in SPD is unknown. The mechanism(s) by which A23187 induces secretion in platelets is presently obscure. It has been demonstrated that A23187 can sensitize platelets so that they undergo secretion during centrifugation, and that this centrifugation-induced secretion is abolished by prior fixation with formaldehyde. Recent studies indicate that secretion also occurs when A23187-sensitized platelets are allowed to aggregate. Thus, close cell contact, resulting from either packing during centrifugation or aggregation, is one mechanism by which A23187 induces secretion. In the present studies, secretion was induced by A23187 in the absence of aggregation, but the platelets were not fixed prior to centrifugation in order to compare directly the A23187 and thrombin-induced responses. Hence, the impaired responses to A23187 in SPD platelets may be related to abnormalities in mechanisms initiated by close cell contact. Studies are currently underway to characterize further these aspects of A23187-induced responses in both normal and SPD platelets.

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Thrombin and ionophore A23187-induced dense granule secretion in storage pool deficient platelets: evidence for impaired nucleotide storage as the primary dense granule defect

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