Impaired Platelet Response to Thromboxane-A_2 and Defective Calcium Mobilization in a Patient With a Bleeding Disorder

By Bruce Lages, Curt Malmsten, Harvey J. Weiss, and Bengt Samuelsson

Platelet aggregation, secretion, and thromboxane formation induced by various agonists, including arachidonate, prostaglandin-G_2 (PGG_2), and thromboxane-A_2 (TxA_2), were examined in a patient with a bleeding disorder who was previously reported to have a TxA_2-related defect. Aggregation and ^14C-SHT secretion were decreased, and no TxB_2 formation occurred in response to adenosine diphosphate (ADP), epinephrine, or collagen. Arachidonate-induced aggregation and TxB_2 formation, and PGG_2-induced aggregation (but not TxB_2 formation) were impaired at low agonist concentrations. The patient's platelets did not aggregate in response to TxA_2 generated from arachidonate in normal platelets, but were capable of synthesizing TxA_2 from both arachidonate and PGG_2. In addition, aggregation and secretion induced by low concentrations of the ionophore A23187 were impaired in platelet-rich plasma (PRP) and in gel-filtered platelets in the absence of extracellular calcium; these responses became normal at higher A23187 concentrations or, in GFP, at low A23187 concentrations in the presence of exogenous calcium. These findings indicate that the TxA_2 defect in this patient does not result from a thromboxane synthetase deficiency, but may be due to impaired mobilization of platelet calcium, and thus are consistent with the possibility that TxA_2 may act as a calcium ionophore.

THE ACTIVATION of the prostaglandin synthetic pathway in human platelets by aggregation and secretion inducing agents results in the enzymatic liberation of arachidonate from platelet phospholipids and its subsequent conversion via cyclooxygenase and thromboxane synthetase into the endoperoxides PGG_2 and PGH_2 and to thromboxane-A_2 (TxA_2). Since these latter compounds are themselves potent inducers of aggregation and secretion, their formation is believed to be an important process regulating platelet reactivity. However, the mechanisms for this regulation remain largely unknown. Based on the premise that platelet contraction and secretion are initiated by the mobilization of intracellular calcium, Gerrard et al. have presented evidence that TxA_2 may function as a calcium ionophore, transporting stored calcium from the dense tubular system to the cytoplasmic locations of the contractile proteins.

In addition to this possible association with TxA_2, calcium has also been shown to stimulate the liberation of arachidonate from platelet phospholipids, particularly phosphatidylinositol, through activation of either phospholipase A_2 or, as recently described, a calcium-dependent diglyceride lipase.

The essential role of PGG_2/PGH_2 and TxA_2 in platelet function has also been demonstrated by studies such as those of Malmsten et al., in which a hemostatic defect was found to be associated with a deficiency of platelet cyclooxygenase. Similar patients have subsequently been described by others. In addition, other studies have also described defects in the formation of prostaglandins, prostaglandin endoperoxides, and TxA_2 associated with characterized platelet disorders such as storage pool deficiency, thrombasthenia and the Bernard-Soulier syndrome.

We (H.J.W., B.L.) have previously reported a patient with a mild bleeding disorder whose platelets showed impaired aggregation responses to arachidonate and PGG_2. These aggregation defects showed mutual correction with platelets from a patient with an apparent cyclooxygenase deficiency or from a normal subject who had ingested aspirin. We postulated that the defect in this patient was either in the production of, or response to, TxA_2. The studies reported here have examined the aggregation and secretion responses of this patient's platelets by various agents, including arachidonate, PGG_2, and TxA_2, as well as the formation of thromboxanes in response to these various agonists. In addition, we also studied the aggregation and secretion responses of his platelets to the ionophore A23187. Our findings suggest that the TxA_2 abnormality in this patient is due to impaired mobilization of intracellular calcium.

MATERIALS AND METHODS

Collection of Blood and Preparation of Platelet-Rich Plasma (PRP)

After the subjects gave informed consent, blood was collected by venipuncture into 1/10 vol of 3.2% sodium citrate dihydrate. PRP was prepared by centrifugation of whole blood at 1500 g for 3 min at room temperature. For measurements of platelet cyclic AMP, 9-ml aliquots of blood were collected into 1 ml of 25 U/ml heparin (Heparin Sodium, Upjohn, Kalamazoo, Mich.) in 0.15 M saline.

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and PRP prepared by centrifugation at 120 g for 15 min at 37°C. This PRP was kept at 37°C prior to assay for cyclic AMP.

**Aggregation, 14C-5-Hydroxytryptamine Secretion, and Thromboxane Formation in PRP**

Aggregation was monitored at 37°C in a Payton Dual Channel Aggregation Module (Payton Associates, Buffalo, N.Y.). For determination of 14C-5-hydroxytryptamine (14C-SHT) secretion and thromboxane-B2 (TxB2) formation in response to ADP (10 μM), collagen (20 μg/ml), epinephrine (1 μM), and thrombin (2 IU/ml), a portion of PRP was labeled with 2-14C-SHT (58 mCi/mmol, Amersham, Arlington Heights, Ill.) as described previously. Duplicate aliquots (1.0 ml) of labeled and unlabeled PRP were aggregated with the above agonists for 7 min, after which the unlabeled sample was diluted with 5 vol of ice-cold ethanol containing 70 μM indomethacin and frozen for subsequent radioimmunoassay of TxB2. The 14C-SHT-labeled aliquot was transferred to an ice-cold centrifuge tube containing 1/20 vol of 0.1 M EDTA, pH 7.4, and centrifuged at 12,000 g for 2 min; the supernate was assayed for 14C as described previously, and secretion was expressed as a percent of the radioactivity taken up by the platelets.

Aggregation and TxB2 formation in PRP were also determined in response to varying concentrations of PGG2 and the ammonium salt of arachidonic acid (20:4-NH4). Indomethacin (24 μM) was added to PRP prior to addition of PGG2 to prevent secondary effects of endogenous arachidonate oxidation. In addition, the dose dependency of aggregation and 14C-SHT secretion responses to the divalent cation ionophore A23187 was studied, which had been preincubated with 24 μM indomethacin for 2 mm. Unlabeled arachidonic acid was obtained from Nu Chek Prep., Inc., Elysian, Minn., and the labeled acid from from Nu Chek Prep. Inc., Elysian, Minn., and the labeled acid from

**Formation of 14C-TxB2, From 14C-Arachidonic Acid and 14C-PGG2**

One milliliter aliquots of washed platelets in Krebs-Henseleit medium without calcium (0.118 M NaCl, 4.7 mM KCl, 1.1 mM KH2PO4, 1.1 mM MgSO4, 24.8 mM NaHCO3, equilibrated with 5% CO2) were incubated at 37°C for 30 min in the presence or absence of 0.05 U/ml of phosphodiesterase (from bovine heart, Sigma Chemical Co., St. Louis, Mo.), following which 1 ml of 40% trichloroacetic acid was added, the samples centrifuged at 17,500 g for 10 min at 4°C, and the supernates extracted with 6 × 40 ml of acidified water-saturated ether. The aqueous layer was evaporated to dryness in vacuo and redisolved in 1.0 ml of 0.05 M Tris/4 mM EDTA, pH 7.5. Cyclic-AMP was assayed in these extracts using a protein-binding assay kit (Amersham, Arlington Heights, Ill.). The percent yield of cyclic-AMP was determined in aliquots of PRP containing 50 μM EGTA to which varying concentrations of CaCl2 in CFT were added 1 mm prior to A23187.

**Platelet Cyclic-AMP and Calcium**

Duplicate aliquots of heparin-PRP (4.0 ml) were incubated at 37°C for 30 min in the presence or absence of 0.05 U/ml of phosphodiesterase (from bovine heart, Sigma Chemical Co., St. Louis, Mo.), following which 1 ml of 40% trichloroacetic acid was added, the samples centrifuged at 17,500 g for 10 min at 4°C, and the supernates extracted with 6 × 40 ml of acidified water-saturated ether. The aqueous layer was evaporated to dryness in vacuo and redisolved in 1.0 ml of 0.05 M Tris/4 mM EDTA, pH 7.5. Cyclic-AMP was assayed in these extracts using a protein-binding assay kit (Amersham, Arlington Heights, Ill.). The percent yield of cyclic-AMP was determined in aliquots of PRP to which known amounts of 3H-cyclic-AMP were added. The levels of platelet cyclic-AMP were taken as those obtained in phosphodiesterase-treated PRP.

**RESULTS**

**Aggregation, 14C-SHT Secretion, and Thromboxane Formation in PRP**

ADP-, epinephrine-, and collagen-induced aggregation in normal PRP were associated with both 14C-SHT secretion and TxB2 formation (Table 1). In PRP from patient K.G., both the initial rate and maximum extent of aggregation and 14C-SHT secretion in response to these agonists were decreased, and no TxB2 formation occurred. On 2 of 8 occasions,
Table 1. Platelet Aggregation, **14**C-5HT Secretion, and TxB₂ Formation in PRP in Patient K.G. (P) and in a Control Subject (C)

<table>
<thead>
<tr>
<th>Aggregant</th>
<th><strong>14</strong>C-5HT Aggregation*</th>
<th>TxB₂ Formation (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Rate</td>
<td>Max. Extent</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>62</td>
<td>85</td>
</tr>
<tr>
<td>Epinephrine (1 μM)</td>
<td>11</td>
<td>83</td>
</tr>
<tr>
<td>Collagen (20 μg/ml)</td>
<td>--</td>
<td>75</td>
</tr>
<tr>
<td>Thrombin (2 IU/ml)</td>
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</tbody>
</table>

*Initial rate and maximum extent are expressed in chart U/min. and percent (PRP -- PPP = 100), respectively.

Fig. 1. (A) Aggregation and TxB₂ formation in PRP from patient K.G. (P) and from control subjects (C) in response to arachidonic acid at the indicated concentrations. TxB₂ formation in ng/ml is shown for each of two controls and for patient K.G. in the boxes beside each tracing. Similar aggregation responses were obtained in both control subjects. (B) Same as (A), showing aggregation and TxB₂ formation in response to PGG₂ at the indicated concentrations.
biphasic aggregation responses to epinephrine have been observed in this patient's platelets. The formation of TxB₂, but not ¹⁴C-5HT secretion, was also decreased in patient PRP in response to thrombin. The aggregation responses to this inducer could not be quantitated, however, because of clot formation.

Arachidonate- and PGG₂-Induced Aggregation and TxB₂ Formation in PRP

Arachidonate, in concentrations ranging from 50 to 125 μg/ml, induced maximum extents of aggregation and dose-dependent formation of TxB₂ in normal PRP (Fig. 1A). In contrast, neither aggregation nor TxB₂ formation was induced in patient PRP in response to 50 μg/ml 20:4-NH₄. However, the defects in both responses were progressively overcome by higher concentrations of 20:4-NH₄, so that aggregation and TxB₂ formation in the patient's PRP were completely normal in response to 125 μg/ml 20:4-NH₄.

A similar impairment of aggregation, which was corrected by higher concentrations of aggregant, was found in PRP of K.G. in response to PGG₂ (Fig. 1B). Very small amounts of TxB₂ were formed in response to these concentrations of PGG₂; however, except for the value of 25.0 ng/ml obtained in one control in response to 0.7 μM PGG₂, no clear differences in TxB₂ formation between PRP from the patient and from controls could be detected.

Formation of and Response to TxA₂

Suspensions of washed platelets aggregated with 20:4-NH₄ generate TxA₂ activity, which is maximal after 30 sec of aggregation.² When 0.1 ml of the filtrate from a suspension of washed normal platelets stirred for 30 sec with 20:4-NH₄ was added to indomethacin-treated normal PRP, a rapid and complete aggregation response was induced (Fig. 2), whereas only a very weak response was observed when this same filtrate was added to indomethacin-treated PRP from K.G. However, when washed patient platelets were used to generate TxA₂ activity, the aggregation response induced in normal PRP was identical to that obtained using washed normal platelets to generate TxA₂. Thus, despite their inability to respond to TxA₂, this patient’s platelets were capable of synthesizing significant, and perhaps normal, amounts of TxA₂.

Further evidence for the ability of K.G.'s platelets to synthesize TxA₂ was obtained from analysis of the labeled metabolites produced in suspensions of washed platelets following incubation with ¹⁴C-arachidonic acid or ¹⁴C-PGG₂. In both cases, platelets from the patient synthesized ¹⁴C-TxB₂ in amounts that did not significantly differ from those produced in control platelets (Table 2). However, it should be stressed that the radioimmunoassay of TxB₂ indicates that TxB₂ formation in the patient’s PRP in response to low concentrations of arachidonic acid is decreased when compared with two normal subjects (Fig. 1A). This difference in the relative amounts of TxB₂ formed in the patient’s platelets is probably accounted for by the greater potency of a given concentration of arachidonic acid in platelet suspensions than in PRP (due to the decreased amounts of plasma proteins that bind arachidonate).

A23187-Induced Aggregation and ¹⁴C-5HT Secretion in PRP

Aggregation and ¹⁴C-5HT secretion in PRP from K.G. were also impaired in response to ionophore A23187 (Fig. 3). The pattern of this impairment was similar to that found for 20:4-NH₄ and PGG₂-induced responses (Fig. 1). Thus, little or no aggregation or secretion was induced in PRP from K.G. by concentrations of A23187 (4–6 μM), which produced maximum aggregation and 40%–50% secretion in normal PRP, whereas both aggregation and ¹⁴C-5HT secretion in

<table>
<thead>
<tr>
<th>¹⁴C-TxB₂ from Normal PRP (30 μM)</th>
<th>¹⁴C-5HT from Normal PRP (30 μM)</th>
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<tbody>
<tr>
<td>K.G. Incubated with ¹⁴C-Arachidonic Acid (30 μM) and ¹⁴C-PGG₂ (1.5 μM)</td>
<td>K.G. Normal 100 100 100</td>
</tr>
<tr>
<td>Normal 104.0 104.0 104.0</td>
<td>Normal 97.3 97.3 97.3</td>
</tr>
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</table>

Platelet suspensions were incubated with ¹⁴C-arachidonic acid or ¹⁴C-PGG₂ for 5 min and then extracted as described in the text. Suspensions tested with ¹⁴C-PGG₂ were preincubated with 24 μM indomethacin for 2 min.

Values given for patient K.G. are from three different incubations from the same preparation of platelets. Normal values were obtained as the means of five incubations from two normal subjects.
the patient's platelets were normalized in the presence of higher (10–12 μM) concentrations of A23187.

A23187-Induced Aggregation and ³¹C-5HT Secretion in GFP

In order to investigate the possible relationship of this impairment of A23187-induced responses to defects in mobilization of platelet calcium, the effects of A23187 were also examined in GFP, in the presence and absence of extracellular calcium. To exclude contributions from extracellular Ca, GFP was prepared in Ca- and Mg-free Tyrode's buffer (residual Ca level, 32.4 ± 4.8 μM), and 50 μM EGTA was added prior to addition of A23187. Under these conditions, A23187-induced ³¹C-5HT secretion was accompanied by increases in light transmission that were not associated with the formation of platelet aggregates, as confirmed by visual inspection of the cuvette contents. This phenomenon has also been observed in other studies.20,21 While these changes in light transmission were similar in GFP from K.G. and from controls (data not shown), ³¹C-5HT secretion in the patient's GFP (Fig. 4), as in his PRP, was decreased in response to low, but not high, A23187 concentrations. Whether secretion induced by 0.5 μM A23187 is also decreased in K.G., however, cannot be determined due to the large variations in the response of control GFP to this ionophore concentration.

Addition of 0.5 or 1 mM exogenous calcium to normal GFP markedly increased the magnitude of the light transmission changes induced by 1 μM A23187 (Fig. 5) and resulted in a true aggregation response, as evidenced both by the oscillations of the aggregometer tracing and by the visible formation of platelet aggregates. Addition of calcium produced identical changes in light transmission in GFP from patient K.G. (Fig. 5). Addition of calcium did not significantly alter the extent of ³¹C-5HT secretion in normal GFP. However, in the patient's GFP, the extent of secretion in the presence of calcium, in clear distinction to that in its absence, was normal (Fig. 5). These findings are thus consistent with the possibility that the impaired responses to A23187 in patient K.G. result from a defect in the mobilization of intracellular platelet calcium.

Contents of Platelet Cyclic-AMP and Calcium

No evidence was found for increased levels of cyclic-AMP in the patient's platelets. Platelet cyclic-
AMP values, determined in heparin-PRP in the presence of phosphodiesterase, were 22.0 pmole/10^9 platelets for K.G. versus 27.1 ± 6.2 (SD) in 10 normal subjects. In addition, the calcium content of GFP from K.G. (22.5 μmole/10^11 platelets) was also similar to that of normal GFP (19.2 ± 2.5, n = 6).

**DISCUSSION**

Based on preliminary studies in which the aggregation responses to both arachidonic acid and PGG_2 were found to be impaired, it was concluded that the platelet defect in patient K.G. did not result from a cyclooxygenase deficiency as described by Malmsten et al., but rather from an inability to either synthesize TxA_2 from PGG_2, or respond to TxA_2. The present studies demonstrate that, under certain conditions, this patient’s platelets are capable of synthesizing normal amounts of TxA_2. Thus, while TXB_2 formation was decreased in response to ADP, epinephrine, low concentrations of collagen and 20:4-NH_4, and thrombin, normal amounts of TXB_2, as measured by both radioimmunoassay and thin-layer radiochromatography, were produced in response to PGG_2 and higher concentrations of 20:4-NH_4 (Tables 1 and 2, Fig I). In addition, incubation of washed platelets from K.G. with 20:4-NH_4 produced an aggregating activity, previously shown to be TxA_2, identical to that generated in washed normal platelets. However, PRP from patient K.G. failed to respond to the TxA_2 activity generated in normal platelets (Fig 2). These results indicate that the defect in this patient’s platelets cannot result solely from an inability to convert PGG_2 to TxA_2, i.e., a thromboxane synthetase deficiency, but is clearly related to an inability to respond to TxA_2. Thus, this patient is not the same as one recently described by Mestel et al., in whom no formation of thromboxane was found even in response to high concentrations of arachidonic acid or PGH_2.

In addition to their inability to respond to TxA_2, platelets from patient K.G. were also found to have impaired responses to the divalent cation ionophore A23187. His platelets showed decreased aggregation and/or 14C-5HT secretion responses in PRP and GFP with low concentrations of A23187, but normal responses with higher levels of ionophore (Figs. 3 and 4). This pattern was similar to that seen for aggregation and/or TXB_2 formation in response to 20:4-NH_4 and PGG_2 (Fig 1). In GFP, the impairment of A23187-induced responses was observed under conditions that presumably eliminated the contributions of extracellular calcium. With the addition of calcium, however, aggregation and 14C-5HT secretion in response to low concentrations of A23187 were the same as those of normal platelets (Fig. 5). Since A23187 is generally held to activate platelets by either the direct transport of extracellular Ca or the mobilization of intracellular Ca into the cytoplasm, these findings suggest that the abnormal A23187 responses in this patient’s platelets result from impaired mobilization of intracellular calcium. The normal content of calcium in K.G.’s platelets further suggests that the impairment is not due to a reduced amount of intracellular calcium, and hence may be in the mobilization process itself. It is also possible, however, that the impairment may be due to a defect in a calcium effector, such as calcium-dependent protease or calmodulin, which is overcome at the greater cytoplasmic calcium levels produced by higher concentrations of A23187 or in the presence of extracellular calcium.

The association of an impaired intracellular calcium mobilization with the defective responses to TxA_2 in K.G.’s platelets would be consistent with the hypothesis of Gerrard et al. that TxA_2 acts as an ionophore, transporting intracellular calcium from its dense tubular storage site to the cytoplasmic locations of contractile proteins.

Although these studies provide evidence for both defective TxA_2 responses and impaired mobilization (or utilization) of intracellular calcium in K.G’s platelets, it is possible only to speculate on the relationship of these defects to the abnormalities of aggregation and secretion. Although Ca mobilization in platelets is believed to be closely interrelated with cyclic-AMP levels, these abnormalities were not associated with increased basal levels of platelet cyclic-AMP. We propose that impairment of calcium mobilization or a subsequent Ca-dependent process is the primary defect in this patient’s platelets. The lack of response to TxA_2, therefore, in accord with the hypothesis of Gerrard et al., might result from an impaired ability of dense tubular calcium to complex with TxA_2. Since calcium is also required for the phospholipase and/or diglyceride lipase activities that liberate arachidonate from platelet phospholipids, an impairment of calcium mobilization might also prevent the stimulation or activation of these enzymes. Such an effect would account for the decreased aggregation and secretion responses and the absence or decrease of TXB_2 formation in K.G.’s platelets in response to ADP, epinephrine, collagen, and thrombin (Table 1).

In contrast to these aggregating agents, arachidonic acid and PGG_2 can bypass the phospholipase-mediated liberation of arachidonate, and thus the abnormal responses to these stimuli could be directly related to TxA_2-calcium interactions. The induction of normal aggregation and secretion responses by higher concentrations of arachidonate and PGG_2 (and also A23187) in K.G.’s platelets implies that the calcium
defect is not irreversible; hence, the decreased responses observed in the presence of low concentrations of these stimuli (and A23187) may reflect an insufficient amount of ionophore (either TxA2 or A23187) to overcome the calcium impairment. Although not tested because of technical reasons, it is entirely possible that the impaired TxA2-induced aggregation of the patient’s platelets would also become normal in response to higher concentrations of TxA2.

This hypothesis, however, does not account for the decreased TxB2 formation observed in the patient’s platelets (in PRP) at low concentrations of 20:4-NH4 (Fig. 1A). While such decreases could be accounted for by a calcium dependency of thromboxane synthetase, similar to that for phospholipase, this may not be the case, since thromboxane synthetase activity is not inhibited by EDTA.26 It is possible, however, that phospholipase can be activated by exogenous 20:4-NH4 via a feedback type of mechanism, resulting in the formation of TxB2 from both exogenous and endogenous arachidonate. This feedback mechanism may not operate in platelets from K.G. due to impaired calcium stimulation of phospholipase, and thus TxB2 formation, occurring only from exogenous arachidonate, would be decreased relative to that in normal platelets. Such feedback effects would not contribute to TxB2 formation from PGG2 because of the presence of indomethacin, and indeed, TxB2 formation from PGG2 was not decreased in K.G.’s platelets.

The defective TxA2 responses of our patient’s platelets appear to closely parallel those of arachidonate-insensitive dog platelets, which do not aggregate in response to arachidonate and its metabolites, despite normal synthesis of TxA2.29 This arachidonate-insensitivity also is not associated with alterations in basal levels of platelet cyclic AMP,30 however, since agents such as ADP and epinephrine, which lower elevated cyclic AMP, abolish the arachidonate-insensitivity, it has been postulated that these unresponsive dog platelets somehow retain the effects of a prior in vivo elevation of platelet cyclic AMP due to exposure to prostacyclin.30 Whether such retention also might occur in our patient’s platelets is not known, but the ability of calcium to enhance the impaired responses of his platelets is not inconsistent with a cyclic-AMP-dependent mechanism.27

Impaired calcium mobilization has also been suggested as a cause of platelet dysfunction in a brief report of three patients studied by Deykin et al.,31 based on their observations that A23187 plus exogenous calcium increased the diminished thrombin-induced phospholipase activity in these patients. Incubation with labeled arachidonate showed that, although their platelets produced decreased amounts of 20:4 metabolites in response to thrombin (cf Table 1), the distribution of the label among these metabolites was normal. Thus, while it is not known whether these patients, like K.G., also fail to respond to TxA2, their platelets appear to be capable of forming thromboxanes. Hence, it is likely that the platelet defect in these three patients is similar to that in K.G.

Our studies confirm that impaired mobilization or utilization of intracellular calcium may be a cause of platelet dysfunction and also provide further evidence for the existence of an intimate association of calcium mobilization with endoperoxide/thromboxane mediation of normal platelet functional responses.

ACKNOWLEDGMENT

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