Human Platelet Signaling Defect Characterized by Impaired Production of Inositol-1,4,5-Triphosphate and Phosphatic Acid and Diminished Pleckstrin Phosphorylation: Evidence for Defective Phospholipase C Activation

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Signal transduction on platelet activation involves phosphoinositide-specific phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositides and formation of inositol 1,4,5-triphosphate ([I(1,4,5)P₃]) and diacylglycerol (DG), which activates protein kinase C (PKC) to phosphorylate a 47-kD protein (Pleckstrin). We studied these events in two related patients previously reported (Blood 74:664, 1989) to have abnormal aggregation and ¹⁴C-serotonin secretion, and impaired intracellular Ca²⁺ mobilization in response to several agonists. Thrombin-induced [I(1,4,5)P₃] and phosphatidic acid formation were diminished. Pleckstrin phosphorylation was impaired on activation with thrombin, platelet-activating factor, and ionophore A23187, but was normal with PKC activator 1,2-dioctanoyl-sn-glycero-(Dio₃). Ca²⁺ mobilization induced by guanosine triphosphate (GTP) analog guanosine 5'-O-(3-thiotriphosphate) (GTP₃S) did not correct the impaired adenine diphosphate-induced secretion; however, upon stimulation with A23187 plus Dio₃, pleckstrin phosphorylation and secretion were normal, indicating that both PKC activation and Ca²⁺ mobilization are essential for normal secretion. We conclude that these patients have a unique inherited platelet defect in formation of two key intracellular mediators [I(1,4,5)P₃] and DG) and in the responses mediated by them due to a defect in postreceptor mechanisms of PLC activation.

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Interaction of platelets with an agonist initiates the production of several intracellular messenger molecules, including Ca²⁺ ions, products of phosphoinositide hydrolysis (1,2 diacylglycerol [DG] and inositol 1,4,5-triphosphate [I(1,4,5)P₃]) and cyclic nucleotides (cAMP). Phosphoinositides constitute a small fraction of the platelet membrane lipids and are hydrolyzed on platelet activation by a phosphoinositide-specific phospholipase C (PLC) to DG and various inositol phosphates including I(1,4,5)P₃. DG is phosphorylated by DG kinase to form phosphatidic acid (PA), which then recycles to phosphatidylinositol (PI) or is hydrolyzed by lipases to free arachidonic acid and glycerol. In thrombin-stimulated platelets, the bulk of PA formed is from PLC catalyzed hydrolysis of phosphoinositides, with only a minimal (~13%) contribution from hydrolysis of phosphatidylcholine (PC) by phospholipase D. Platelet activation results in an increase in the cytoplasmic calcium concentration [Ca²⁺], which encompasses two processes, ie, the release of Ca²⁺ from intracellular stores and the influx of external Ca²⁺, resulting in the release of calcium from intracellular stores, the dense tubular system. DG activates protein kinase C (PKC), which results in the phosphorylation of a protein of molecular weight approximately 47 kD (pleckstrin). PKC activation is considered to play a major role in platelet secretion and in the expression of platelet surface fibrinogen binding sites (consisting of glycoproteins [GPs] IIb-IIIa), which is a prerequisite for platelet aggregation.

In platelets, free arachidonic acid is liberated by the hydrolysis of phospholipids (predominantly PC) by phospholipase A₂, which is a Ca²⁺-dependent enzyme; this is the rate-limiting step in thromboxane (TXA₂) synthesis. These mechanisms are set into motion on platelet activation and modulate the end responses such as aggregation and secretion.

Inherited defects in platelet function may arise by several different mechanisms. Specific deficiencies in membrane GPs such as GPIb (Bernard-Soulier syndrome) and GPIb-IIIa (thrombasthenia) result in impairment of platelet adhesion and aggregation, respectively, but they are rare. A large proportion of patients with inherited platelet function defects are characterized by mild to moderate bleeding symptoms and variably prolonged bleeding times associated with the absence of the secondary wave or impaired extent of aggregation on platelet activation with agonists such as adenine diphosphate (ADP), epinephrine, platelet-activating factor (PAF), a TXA₂ analog U46619, and collagen. In parallel, the affected individuals have diminished secretion of dense granule contents. A small proportion of these patients have abnormalities of dense granule contents (storage pool deficiency) or defects in TXA₂ production due to deficiencies of cyclooxygenase or thromboxane synthetase. In the vast majority of the rest of the patients, the mechanisms leading to the dysfunction remain unknown. These patients have...
been generally lumped under the rubric of platelet secretion defects or platelet activation defects.\(^{16-20}\) Defects in early events of platelet activation, such as in the formation of messenger molecules I(1,4,5)P\(_3\) and DG, Ca\(^{2+}\) mobilization, and protein phosphorylation, have been postulated but hitherto not well documented in such patients.

We have previously described\(^{21}\) an impaired agonist-stimulated increase in cytoplasmic [Ca\(^{2+}\)] in two related patients with impaired aggregation (no second wave) and dense granule secretion in platelet-rich plasma (PRP) in response to several different agonists. The dense granule contents of adenosine triphosphate (ATP) and ADP were normal, and TxA\(_2\) production was at the lower limits of the normal range. Subsequent studies indicated that the blunted increase in [Ca\(^{2+}\)] on activation was due to the defects both in Ca\(^{2+}\) release from intracellular stores and influx of external Ca\(^{2+}\).\(^{22}\) However, in response to exogenous I(1,4,5)P\(_3\), Ca\(^{2+}\) mobilization was normal, indicating that the platelet Ca\(^{2+}\) storage organelles had intact ability to respond to I(1,4,5)P\(_3\). We postulated\(^{22}\) that defects in the mechanisms leading to and including PLC activation and I(1,4,5)P\(_3\) production may underlie the impaired Ca\(^{2+}\) mobilization in these patients. In the present study, we document that their platelets indeed have a hitherto undescribed defect in agonist-stimulated I(1,4,5)P\(_3\) formation and, in addition, in phosphatidic acid production and pleckstrin phosphorylation. We conclude that their platelets have a defect in postreceptor mechanisms of PLC activation.

**MATERIALS AND METHODS**

**Patient information and previous studies.** Both patients have been described previously.\(^{22}\) The propositus, patient A, is a 47-year-old white woman with a life-long history of easy bruising, bleeding from the gums on brushing her teeth, and menorrhagia. Her brother, paternal aunt, and grandmother had excessive bleeding after surgical procedures. Laboratory studies showed the platelet counts, prothrombin time (PT), and activated partial thromboplastin time (APTT) to be within normal limits. The bleeding times (Simplate; General Diagnostics, Morris Plains, NJ) have ranged from 6 to 14 minutes (normal range, 2 to 7 minutes). Patient B, 28-year-old son of the propositus, denied a history of easy bruising. There is no known history for patient B of excessive bleeding after surgery for Meckel's diverticulum, appendectomy, and extraction of wisdom teeth. His platelet counts, PT, and APTT were within normal limits. The bleeding times ranged from 5 to greater than 15 minutes.

Both patients have abnormal platelet aggregation (no second wave) and diminished \(^{14}\)C-serotonin secretion in PRP in response to ADP, epinephrine, PAF, arachidonic acid, U46619, divalent ionophore A23187, and collagen.\(^{21}\) The platelet dense granule contents of ATP and ADP as well as the ratio of ATP to ADP were normal, thereby excluding dense granule storage pool deficiency. TxA\(_2\) synthesis, measured using a radioimmunoassay for TxB\(_2\), was at the lower limits of the normal range on thrombin activation.\(^{21}\) In studies using quin2- and fura-2–loaded platelets, the basal or resting [Ca\(^{2+}\)] was normal in both subjects; however, the increase in [Ca\(^{2+}\)] in response to several agonists, including ADP, PAF, thrombin, U46619, and collagen, was diminished.\(^{21}\) This defect was subsequently shown\(^{22}\) to be related to abnormalities in both the release of Ca\(^{2+}\) from intracellular stores (internal release) and in the influx of extracellular Ca\(^{2+}\).

The control subjects were healthy laboratory personnel and medical students who denied ingesting any medication for at least 10 days. All subjects were studied after an informed consent was obtained. All studies were performed after approval was given by the institutional Human Research Review Committee.

**Materials.** Carrier-free \(^{32}\)P-orthophosphate (−285 Ci/mg P) was obtained from ICN Biomedicals Inc (Irvine, CA). \(^{3}H\)-arachidonic acid (180 to 240 Ci/mmol) and I(1,4,5)P\(_3\) \(^{3}H\) radioreceptor assay kit were from New England Nuclear (Boston, MA). 1,2-dioctanoyl-sn-glycerol (DiC\(_4\)) was purchased from Biomol Research Laboratories, Inc (Plymouth Meeting, PA). PAF and PA were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Fura 2/AM pentaacetylatedoxymethylster was purchased from Calbiochem, Inc (San Diego, CA). Thrombin (bovine) was obtained from Armour Pharmaceutical Co (Kankakee, IL). All reagents for electrophoresis were from Bio-Rad (Melville, NY). All other reagents were purchased from Sigma Chemical Co (St Louis, MO).

**Preparation and labeling of human platelets with \(^{32}\)P-orthophosphate and \(^{3}H\)-arachidonic acid.** Blood was collected by venipuncture into 1/7 vol of acid citrate dextrose buffer (85 mmol/L trisodium citrate, 78 mmol/L citrate acid, and 111 mmol/L dextrose) and centrifuged at 180g for 20 minutes to obtain PRP. PRP was centrifuged at 1,000g for 15 minutes in the presence of hirudin (0.05 U/mL) and apyrase (10 \(\mu\)g/mL), and the platelet pellet was resuspended in 1/3 vol of autologous plasma. Platelet suspension was incubated at 37°C for 60 minutes with either \(^{32}\)P-orthophosphate (0.4 mCi/mL) alone or both \(^{32}\)P-orthophosphate (0.25 mCi/mL) and \(^{3}H\) arachidonic acid (1 mCi/mL),\(^{23}\) depending on the studies performed. The platelets were then filtered through a Sepharose 2B column equilibrated with Tyrode’s buffer, pH 7.4, containing 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.47 mmol/L NaH\(_2\)PO\(_4\), 12 mmol/L NaHCO\(_3\), 2 mmol/L MgCl\(_2\), 5.5 mmol/L dextrose, and 0.2% bovine serum albumin (BSA) but no added Ca\(^{2+}\). The platelet counts were adjusted to 3 to 4 \(\times\) 10\(^9\)/mL.

**Studies of phosphatidic acid formation.** Platelet suspensions labeled with \(^{32}\)P-orthophosphate and \(^{3}H\)-arachidonic acid were equilibrated at 37°C for 5 minutes before adding thrombin (0.1 or 5.0 U/mL). One-milliliter aliquots of platelet suspensions were removed at intervals (0 to 300 seconds), extracted for phospholipids according to the procedure of Bligh and Dyer,\(^{24}\) and dried with nitrogen. The extracts were dissolved in 50 \(\mu\)L of CHCl\(_3\) and subjected to thin-layer chromatography (TLC) on Silica Gel 60 plates (Thomas Scientific, Swedesboro, NJ) using the solvent system of Hauser and Eichberg,\(^{25}\) with some modification: CHCl\(_3\):CH\(_3\)OH:CH\(_3\)COOH (80:20:30). The phosphatidic acid on the TLC plate was visualized with phosphomolybdic acid and the band corresponding to \(^{32}\)P radioactivity was scraped into liquid scintillation fluid and counted for radioactivity. The results were expressed as the fold increase relative to the basal level.

**Measurement of inositol trisphosphate production.** Platelet I(1,4,5)P\(_3\) production in response to thrombin (0.5 or 5.0 U/mL) was measured by radioreceptor assay (NEN Research Products, Boston, MA). PRP prepared from blood collected in 1/7 vol of ACID as described above was centrifuged and resuspended in HEPES-Tyrode’s buffer (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\(_2\), 0.5 mmol/L NaHPO\(_4\), 5.5 mmol/L glucose, and 0.2% BSA, pH 7.4). Aliquots (0.5 mL) of platelet suspension (10\(^9\) platelets/mL) were equilibrated to 37°C with stirring (600 rotations per minute) and stimulated with thrombin in the presence of 1 mmol/L CaCl\(_2\). One hundred seventy-five microliters of ice-cold trichloroacetic acid (20%) was added at different time intervals to terminate the reaction. To remove trichloroacetic acid, the supernatants were mixed with 2 vol of a mixture of trichloro-tribufluoro-triethanotriacetylene (3:1); the samples were centrifuged (2 minutes at 1,000g) and the upper phase was removed. I(1,4,5)P\(_3\) levels were measured in the extracts according to the instructions provided in the kit.

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Measurement of pleckstrin and myosin light chain phosphorylation. Platelet suspensions (4 x 10^6 platelets/mL) labeled with ^32P-PO_4 (0.4 mCi/mL) as described above were activated with thrombin (0.05 or 5.0 U/mL) or PAF (4 or 400 nmol/L). The reactions were terminated at various intervals (0 to 300 seconds) by transferring 0.5-mL aliquots of the sample to tubes containing an equal volume of 0.6 N HClO_4. The sample was centrifuged in Eppendorf Centrifuge 5415 (Brinkmann Instrumental Inc, Westbury, NY) at 14,000g for 5 minutes. The pellet was washed once with distilled water and subjected to autoradiography using DuPont Cronex video imaging film (E.I. DuPont Co, Wilmington, DE). The bands on the gel corresponding to 47 kD (pleckstrin) and 20 kD (myosin light chain [MLC]) were cut, placed in liquid scintillation fluid, and counted for radioactivity. The results were expressed as the fold of the basal radioactivity in pleckstrin and MLC.

GTPγS-induced Ca^{2+} mobilization. GTPγS-induced Ca^{2+} mobilization was measured as previously described. Platelet suspensions were permeabilized with saponin (10 to 15 μg/mL) in the presence of 1 mmol/L ATP, 1 μmol/L CaCl_2, and 6 μmol/L Quin2 acid. For measurement of Ca^{2+} concentrations, fluorescence was recorded (excitation, 339 nm; emission, 492 nm) using a Perkin-Elmer spectrofluorometer (Oak Brook, IL).

Other methods. Platelet aggregation was studied in PRP using an aggregometer (ChronoLog Corp, Haverford, PA) and secretion was monitored in platelets labeled with ^3H-serotonin, as described previously. Levels of cytoplasmic ionized Ca^{2+} concentration were monitored in platelets labeled with fura-2, as described previously.

**RESULTS**

PA formation. Agonist-stimulated production of PA has been reported to occur by PLC-catalyzed hydrolysis of phosphoinositides in conjunction with DG kinase and by the hydrolysis of PC by phospholipase D. However, in human platelets stimulated with thrombin, PLD contributes only a minor portion (~13%) of the PA formed. Thus, in platelets, PA formation is largely an indicator of DG production. The[^32P] content of PA has been shown to be directly proportional to the mass of PA. Thrombin (0.1 U/mL)-induced[^32P]-PA formation was diminished in both patients (Fig 1). Even at a relatively high thrombin concentration (5 U/mL), PA levels in patient B were lower than those in normal controls at 300 seconds. At 300 seconds,[^32P]-PA levels in normal platelets ranged from 29- to 59-fold of basal levels, and remained elevated for more than 300 seconds (mean, 41.3-fold; n = 15). In response to another agonist, PAF (400 nmol/L), pleckstrin phosphorylation in patients' platelets was consistently lower than in the normal subjects (Fig 3A). It remained low compared with normal subjects even on activation with a 100-fold higher concentration of thrombin (5 U/mL); at 60 seconds in the normal platelets, there was a 3.5- to 5.8-fold increase over the basal levels (mean, 5.4-fold; n = 10), whereas the increase in both patients was 2.9-fold. In response to another agonist, PAF (400 nmol/L), pleckstrin phosphorylation in normal platelets peaked at 30 seconds and returned to the basal level by 300 seconds (Fig 3B). In both patients, pleckstrin phosphorylation in response to PAF at concentrations of 400 nmol/L (Fig 3B) and 4 nmol/L (data not shown) was impaired.

Platelet activation with agonists such as thrombin and PAF is mediated by the platelet surface receptors and the resulting responses of pleckstrin phosphorylation and Ca^{2+} mobilization are induced by formation of secondary intracellular messengers DG and I(1,4,5)P_3, respectively. We therefore assessed the effects of a cell-permeable PKC activator (DiCa) and that of Ca ionophore A23187, which induce their effects directly without interaction with platelet surface receptors. In normal platelets, 200 μmol/L DiCa induced substantial pleckstrin phosphorylation at 60 seconds (2.4- to 6.2-fold of basal levels; mean, 4.0-fold; n = 7; Fig 4). A23187 (1 μmol/L)
Fig 2. Thrombin-induced \([1,4,5]P_3\) formation in platelets. Aliquots (0.5 mL) of platelet suspensions (10^9 platelets/mL) in HEPES-Tyrode's buffer were stimulated with 5.0 (A) and 0.5 U/mL (B) of thrombin for 0 to 15 seconds. \([1,4,5]P_3\) was extracted with trichloroacetic acid and measured in duplicate by radioreceptor assay (NEN Research Products). The symbols are the same as in Fig 1. The values in normal controls are expressed as the mean ± SD (n = 7 to 10).

L) also induced substantial pleckstrin phosphorylation (2.1- to 3.6-fold increase over basal levels; mean, 2.7-fold; n = 7; Fig 4). Interestingly, pleckstrin phosphorylation induced by A23187 but not that induced by DiC₈ was inhibited by pretreatment of platelets with aspirin, indicating its dependence on TxA₂ production. Stimulation of normal platelets with a combination of DiC₈ and A23187 induced no further increase in pleckstrin phosphorylation over that by DiC₈ alone; this finding was not altered by aspirin. In both patients, pleckstrin phosphorylation was normal on stimulation with DiC₈ but was lower than in the control subjects on exposure to A23187 (Fig 4). The combination of DiC₈ plus A23187 induced little further phosphorylation than that with DiC₈ alone (Fig 4).

In normal platelets, MLC phosphorylation peaked around 30 to 60 seconds on activation with 0.05 and 5.0 U/mL thrombin. In patient A, MLC phosphorylation appeared normal at both thrombin concentrations. In patient B, the peak MLC phosphorylation on exposure to thrombin (5.0 U/mL) was lower than that in normal subjects (patient, 1.86-fold of

Fig 3. Thrombin- and PAF-induced pleckstrin phosphorylation in platelets. \(^{32}P\)-orthophosphate-labeled platelet suspensions (4 x 10^9/mL) were stimulated with 0.05 U/mL of thrombin (A) and 400 nmol/L of PAF (B). The reactions were terminated at different intervals by transferring 0.5 mL aliquots of the sample to an equal volume of 0.6 N HClO₄. The precipitate was washed and subjected to 13.5% SDS-PAGE. The gels were dried and subjected to autoradiography. The band corresponding to pleckstrin was cut and counted for radioactivity. The results are expressed as the fold of the basal (unstimulated) radioactivity in pleckstrin. The symbols are the same as in Fig 1. The values in normal controls are expressed as the mean ± SD (n = 8 to 11). The mean basal radioactivities (in cpm) were as follows: normal subjects, 318 (range, 94 to 647, n = 11); patient A, 462 and 370; and patient B, 488 and 443.
basal level; normal subjects, 2.30- to 4.70-fold; mean, 3.60-fold; n = 18). On activation with 0.05 U/mL thrombin, the peak MLC phosphorylation was slightly lower (1.88-fold of basal) than the values noted in 13 normal subjects (mean, 2.70-fold; range, 2.00- to 3.00-fold).

**GTPyS-induced Ca\(^{2+}\) mobilization.** The GTPyS-induced increase in ionized Ca\(^{2+}\) levels studied in patient A was diminished on platelet exposure to 1 μmol/L GTPyS (patient, 9 nmol/L; normal subjects, mean of 40 nmol/L and range of 14 to 81 nmol/L; n = 5), 2.5 μmol/L GTPyS (patient, 35 nmol/L; normal subjects, mean of 140 nmol/L and range of 66 to 299 nmol/L; n = 13), and 5 μmol/L GTPyS (patient, 46 nmol/L; normal subjects, mean of 161 nmol/L and range of 75 to 300 nmol/L; n = 15).

**Relationship between secretion, PKC activation, and Ca\(^{2+}\) mobilization.** To study the relationship of platelet secretion to PKC activation and Ca\(^{2+}\) mobilization, we studied dense granule secretion induced in normal and patient PRP by ADP, DiC\(_8\), and A23187 alone and in combinations. Our goal was to determine if the combination of a receptor-dependent agonist (ADP) with DiC\(_8\) (which directly activates PKC) or A23187 (which induces an increase in Ca\(^{2+}\) levels) would normalize the defective secretion in our patients whose platelets have both impaired pleckstrin phosphorylation and Ca\(^{2+}\) mobilization. It is important to take into account that, in studies performed in patient B using fura-2, the peak [Ca\(^{2+}\)]\(_i\) on activation with the Ca ionophore ionomycin (24 nmol/L) was normal (patient B, 421 nmol/L; normal subjects, 257 to 604 nmol/L; n = 10).

In normal PRP, DiC\(_8\) alone induced a concentration-dependent (25 to 200 μmol/L) aggregation but minimal secretion (Fig 5A). A23187 at 5 μmol/L induced reversible aggregation (extent of aggregation, 10% to 78%; n = 5) with no secretion, whereas 10 μmol/L A23187 caused substantial aggregation (88% to 98%; n = 5) and secretion (47% to 82%; n = 5; Fig 5A). These relatively high concentrations of A23187 are needed to induce responses because the studies were performed in PRP and A23187 is avidly bound to albumin. As described previously, platelets from both patients consistently showed abnormal aggregation (no second wave) and little or no secretion in responses to 8 μmol/L ADP and A23187 (Fig 5A). In normal platelets, the combination of DiC\(_8\) and ADP did not induce any increase or decrease in secretion over that with ADP alone; the combinations of 5 μmol/L A23187 + ADP and 10 μmol/L A23187 + ADP induced, as expected, more secretion than did either ADP or A23187 alone (Fig 5B). We then studied the effect of combining ADP with DiC\(_8\) or A23187 in patient platelets to determine if this would reverse the abnormal secretion in response to ADP. In both patients, neither DiC\(_8\) nor A23187 corrected the impaired secretion noted with ADP (Fig 5B). Patient A showed no secretion with these combinations. Although some secretion (30%) occurred in patient B when ADP was combined with the higher A23187 concentration (10 μmol/L), this was still substantially lower than that observed in normal platelets with A23187 alone (75%) or with the combination (Fig 5A). Moreover, the combination of two receptor-mediated agonists, 4 μmol/L ADP and 2 μmol/L epinephrine, also did not result in any secretion in patient platelets (data not shown). However, exposure of platelets to DiC\(_8\) + A23187 induced a completely normal secretion in both patients (Fig 5B). These findings indicate that both intact PKC activation and Ca\(^{2+}\) mobilization are essential requirements for normal secretion.

**DISCUSSION**

The two patients described here have abnormal platelet aggregation and \(^{14}\)C-serotonin secretion in response to several agonists that are not due to a deficiency of dense granule contents (storage pool deficiency) or a major deficiency in TxA\(_2\) synthesis.\(^{21,22}\) Two mechanisms generally recognized to lead to abnormal platelet responses. We have previously shown\(^{21}\) that the Ca\(^{2+}\) mobilization in the patients' platelets was abnormal in response to stimulation with thrombin, ADP, PAF, and U46619 as a result of defects in both the internal release and the influx of Ca\(^{2+}\), whereas the response to exogenous I(1,4,5)P\(_3\) was normal. In the present study, we provide the crucial information that I(1,4,5)P\(_3\) production, the major mediator of the Ca\(^{2+}\) increase, is impaired on platelet activation (Fig 2). This provides a cogent explanation for the impaired increase in [Ca\(^{2+}\)]\(_i\) on platelet activation. I(1,4,5)P\(_3\) is formed by the hydrolysis of phosphoinositides by PLC with simultaneous formation of DG, which is phosphorylated to PA. We show that thrombin-induced PA formation is also diminished (Fig 1) at both low and a relatively high thrombin concentrations when measured by either \(^{3}H\) or \(^{32}P\) radioactivity of PA. In human platelets, the bulk of PA formed on activation arises from PLC-induced DG formation and its subsequent conversion to PA by DG kinase.\(^{5}\) Therefore, taken together, the diminished production
of \(I(1,4,5)P_3\) and PA lead us to suggest that the patient platelets have a defect in early signal transduction involving one or more events that constitute receptor-effector coupling and PLC activation.

The abnormalities observed in our patients\(^{21,22}\) are in response to several G-protein–linked receptors, including those for ADP, thrombin, PAF, and U46619. Thrombin and PAF both activate PLC in platelets in part via a pertussis toxin-sensitive G protein, presumably Gi.\(^{21}\) The TXA\(_2\)/U46619 and thrombin activate PLC by toxin-resistant G-proteins, which belong to the Gq family.\(^{31-33}\) These findings indicate that signaling occurring through at least four different receptors and two different G-protein families is abnormal. Because of this multitude of surface receptors and the different mediator G-proteins involved, it is likely that the abnormality lies in the postreceptor events encompassing the G-proteins and the effector enzyme PLC. The abnormal Ca\(^{2+}\) mobilization noted with GTP\(\gamma\)S provides a further support for this conclusion. We therefore postulate that the specific abnormality lies at the level of the G-proteins or PLC or their coupling.

PI-specific PLC activities in cells belong to a family of isozymes that are distinct gene products and divided into at least three structural types (\(\beta, \gamma, \) and \(\delta\)) and multiple subtypes.\(^{34,35}\) The PLC types and subtypes differ in their relative substrate specificity [PI to \(I(1,4,5)P_2\)], tissue distribution, and activation. The two PLC-\(\gamma\) isozymes are phosphorylated by tyrosine kinases and are not involved in G-protein–mediated signal transduction, and activation. The role of G-proteins in PLC-\(\delta\) activation remains unknown. Seven of ten known PLC isoforms can be shown in human platelets with amounts decreasing in the order PLC-\(\gamma_2 > PLC-\beta_2 > PLC-\beta_3 > PLC-\beta_1 > PLC-\gamma_1 > PLC-\delta_1 > PLC-\beta_4\).\(^{36,37}\) The relative importance and roles of these PLC isozymes on platelet activation remains unknown. Recently, a Chinese hamster fibroblast mutant with defective thrombin-induced signaling has been described\(^{39}\) that has several features in common with our patients, including blunted production of inositol phosphates and impaired Ca\(^{2+}\) mobilization. These mutant cells were found to be deficient in PLC-\(\beta_1\). A quantitative or qualitative abnormality in platelet PLC-\(\beta\) isozymes may lead to the observed diminished synthesis of \(I(1,4,5)P_3\) and PA in our patients.\(^{38}\)

A functional consequence of DG formation is PKC activa-
tion and pleckstrin phosphorylation. The impaired PA formation in parallel with decreased pleckstrin phosphorylation on activation with thrombin and PAF (Fig 3) indicate that the abnormality in the patient platelets is not agonist-specific. In contrast, pleckstrin phosphorylation was entirely normal on exposure of platelets to PKC activator DiC8, suggesting that the enzyme and the substrate involved in pleckstrin phosphorylation are intact and that the impaired phosphorylation is secondary to blunted DG production. Another important implication of these studies is that the reductions in I(1,4,5)P3 and DG (PA) formation, even at the magnitude noted, do manifest themselves by discernible impairments in the specific functional responses mediated by them.

Numerous studies have linked PKC activation to platelet secretion including in granule membrane fusion. The evidence for this has come largely from studies of secretion after activation of platelets with synthetic diacylglycerols alone or in combination with low concentrations of A23187 and from studies using PKC inhibitors. The latter studies are somewhat affected by the nonspecific effects of some of the inhibitors on other kinases as well. Our patients provided us with a unique opportunity to directly explore the role of Ca2+ mobilization and PKC activation in platelet secretion. Platelet secretion and Ca2+ mobilization in both patients were markedly abnormal on activation with ADP. Exposure of platelets to a combination of ADP (8 μmol/L) and a concentration of DiC8 (200 μmol/L) known to induce maximal effects failed to correct the impairment in secretion (Fig 5). We attribute the inability of the combination to induce secretion to the uncorrected abnormality in Ca2+ mobilization. In our studies in normal platelets loaded with fura-2, DiC8 by itself caused little increase in [Ca2+]i (Rao et al, unpublished observations). Thus, PKC activation by itself is inadequate to induce secretion in the absence of an adequate increase in [Ca2+]i. Similarly, the combination of ADP and the Ca2+ ionophore A23187 failed to induce normal secretion (Fig 5), most likely due to uncorrected deficient PKC-mediated protein phosphorylation, which is abnormal in our patients with A23187 as well (Fig 4). In contrast, the combination of DiC8 and A23187 induced completely normal secretion (Fig 5); in parallel studies, the diminished A23187-induced pleckstrin phosphorylation was normalized by its combination with DiC8 (Fig 4). These reconstitution studies strongly support our suggestion that the primary platelet abnormality in the patients is an inability to synthesize the two key intracellular mediators and that the exocytotic (secretion) process per se is intact. Moreover, they provide a strong direct evidence that both PKC activation and Ca2+ mobilization are prerequisites for and function together to induce normal granule secretion. Studies in this biologic model constitute an important and unique validation of the physiologic role of I(1,4,5)P3 and PKC activation in normal platelet responses, particularly dense granule secretion.

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X Yang, L Sun, S Ghosh and AK Rao