Platelet Activation with Combination of Ionophore A23187 and a Direct Protein Kinase C Activator Induces Normal Secretion in Patients with Impaired Receptor Mediated Secretion and Abnormal Signal Transduction

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Abstract

Defects in signal transduction mechanisms may underlie the impaired aggregation and secretion in patients with congenital platelet function defects (CPD). Both protein kinase C (PKC) induced pleckstrin phosphorylation and cytoplasmic Ca2+ mobilization play a major role in secretion. We postulated that combined platelet activation with a cell permeable direct PKC activator 1,2-dioctanoyl-sn-glycerol (DiC8) and ionophore A23187, which possibly bypass the steps involved in the intracellular synthesis of two major mediators (inositol trisphosphate, diacylglycerol), may induce normal dense granule secretion in patients with impaired receptor mediated secretion. We studied eight CPD patients with abnormal aggregation and secretion in response to several different surface receptor-mediated agonists despite the presence of normal dense granule contents. Receptor mediated Ca2+ mobilization and/or pleckstrin phosphorylation were abnormal in seven patients. Platelet activation with a combination of ADP (8 μM) with DiC8 (200 μM) or A23187 (10 μM) improved secretion in four patients. However, platelet activation with a combination of 200 μM DiC8 with 10 μM A23187, or 100 μM DiC8 with 5 μM A23187 induced normal secretion in platelet-rich plasma in all patients. These studies suggest that in such patients with CPD the ultimate process of exocytosis or secretion per se is intact and impaired secretion results from abnormalities in early signal transduction events, possibly upstream of diacylglycerol formation and calcium mobilization. Detailed studies are needed to delineate the specific abnormalities in these heterogeneous patients with signal transduction defects. © 1998 Elsevier Science Ltd.

Key Words: Platelet function disorders; Platelet activation; Signal transduction defects; Calcium mobilization; Inherited thrombocytopathy; Protein kinase C

Patients with congenital defects in platelets are characterized by mucocutaneous bleeding symptoms of variable intensity. The platelet dysfunction in such patients may arise by diverse mechanisms [1–3] including deficiencies of specific
membrane glycoproteins (thrombasthenia, Bernard Soulier Syndrome), deficiencies of dense and α granules (storage pool deficiency), and abnormalities in thromboxane A₂ production. Although these defects are well characterized, the majority of patients with congenital platelet function disorders studied by us have not had these abnormalities and have been generally characterized by impaired aggregation (or no second wave) in response to ADP, epinephrine, and platelet activating factor (PAF), and a uniformly decreased secretion of dense granule contents. Such patients have been encountered by others as well [4]. In these frequently encountered patients, who have been lumped under the rubric of platelet secretion defects or activation defects, the underlying mechanisms leading to the impaired responses remain unknown.

Interaction of platelet receptors with agonists leads to a series of events that ultimately results in the “end responses” of aggregation or secretion. These signal transduction events involve participation of the surface receptors, the G-proteins that modulate the transmission of the activation process, and the effector enzymes that are activated such as phospholipase C (PLC), phospholipase A₂, and adenylate cyclase. Activation of PLC through G-protein or tyrosine kinase mediated mechanisms results in the hydrolysis of membrane phosphatidylinositides and in the formation of two second messenger molecules-diacylglycerol (DG) which activates protein kinase C (PKC) to phosphorylate a 47 Kda protein pleckstrin, and inositol 1,4,5-trisphosphate (IP₃) which mediates Ca²⁺ mobilization [5,6]. Both PKC activation and Ca²⁺ mobilization have been considered to play a major role in platelet secretion [7–9]. We and others have postulated that defects in early signal transduction mechanisms, such as at the level of the receptors, the G-proteins or in activation of effectors, may underlie the impaired end-responses of aggregation and secretion in patients with platelet dysfunction. To test this hypothesis, we studied two early events on receptor activation, Ca²⁺ mobilization and pleckstrin phosphorylation, in eight patients with congenital platelet defects and impaired dense granule secretion despite presence of normal granule stores. We postulated also that if the primary abnormality leading to impaired secretion were to be in the early signal transduction processes, then bypassing the aberrant steps may lead to normal secretion. We tested the hypothesis that the ultimate process of exocytosis is normal in most of these patients but is compromised by abnormalities in the upstream signal transduction events. Thus, we studied the effect of platelet activation with a combination of a cell permeable direct PKC activator 1,2-dioctanoyl-sn-glycerol(DiCs) [8] and calcium ionophore A23187, which are considered to bypass the mechanisms leading to the synthesis of two major intracellular mediators (DG and IP₃). The results provide evidence that in the patients studied with congenital platelet dysfunction and normal dense granule stores, the ultimate process of exocytosis or secretion per se is intact and that the impaired secretion likely results from abnormalities in the early events of signal transduction, possibly upstream of DG formation and Ca²⁺ mobilization.

1. Patient Information

Eight patients (one male and seven females, ages 18 to 51 years) with longstanding mucocutaneous bleeding symptoms were studied. In all patients, platelet counts, prothrombin time (PT) and activated partial thrombin time (APTT) were normal. The bleeding times were prolonged on most occasions. Platelet function studies showed that aggregation in platelet-rich plasma (PRP) on exposure to several agonists, including ADP, epinephrine, PAF, U46619, and collagen, was diminished, generally characterized by absence of the second wave of aggregation, and ¹⁴C-serotonin secretion was markedly reduced or absent (Table 1). Several of the patients included in this report have been previously described in detail, including patients P1 [10–12], P2 and P3 [11,13–15], and P4 [16]. Patients P2 and P3 are related, as mother and son. All studies were performed after obtaining an informed consent from the subjects and have been approved by the Institutional Human Subjects Research Review Committee.

2. Materials and Methods

¹⁴C-Serotonin and ³²P-orthophosphate were purchased from Amersham Corporation, Arlington Heights, IL, and DuPont NEN Research Products,
Boston, Massachusetts, respectively. Thrombin was purchased from Armour Pharmaceutical Co., Kankakee, IL. PAF was from Avanti polar-lipids, Inc., Alabaster, AL. 1,2-dioctanoyl-glycerol (DioC8) was purchased from Bimol Research Laboratories, Plymouth Meeting, PA. Fura2/AM pentaacetoxy-methylester was purchased from CalBiochem Inc., San Diego. ADP, epinephrine, A23187, and all other chemicals used in this study were purchased from Sigma Chemical Co., St. Louis, MO.

2.1 Aggregation and Secretion

Blood was collected in 1/10 volume of 3.8% sodium citrate and centrifuged at 180×g for 15 minutes at room temperature to obtain PRP. The platelet counts in PRP were adjusted with autologous plasma to be 300,000/μl. Platelet aggregation responses were monitored using a Chrono-Log (Havertown, PA) dual channel aggregometer as described previously [10]. The extent of aggregation was expressed as a percentage taking the light transmission through PRP and platelet-poor plasma to be 0% and 100%, respectively. For assessing 14C-serotonin secretion, platelets were incubated for 30 minutes at room temperature with 0.25 μM of 5-hydroxy(side-chain-2-14C) tryptamine creatine sulfate (50 mCi/mmol). Secretion was expressed as a percent of total 14C-serotonin incorporated that is released on platelet stimulation [10]. Following exposure of platelets to the agonist, secretion was terminated by transferring the PRP to a mixture of EDTA-formaldehyde to prevent centrifugation-induced secretion. For these studies on aggregation and secretion in PRP, a relatively high concentration of A23187 (10 μM) was required to elicit the responses even in normal platelets because of the high avidity of A23187 for albumin.

Platelet contents of ATP and ADP were measured by the luciferase-luciferrin assay [17]. Thromboxane A2 production was measured using a radioimmunoassay for thromboxane B2 [18] in supernatants of PRP following incubation of PRP for 5 minutes at 37°C with stirring with thrombin (1 U/ml and 5 U/ml) or arachidonic acid (1 mM).

2.2 Measurement of Pleckstrin Phosphorylation

This was performed as described by us previously [14]. Briefly, blood was collected by venipuncture
into 1/7 volume of acid citrate dextrose buffer (85 mM trisodium citrate, 78 mM citric acid, 111 mM dextrose) and centrifuged at 180×g for 20 minutes to obtain PRP. PRP was centrifuged at 1000×g for 15 minutes in the presence of hirudin (0.05 U/ml) and apyrase (10 μg/ml) and the platelet pellet was resuspended in 1/3 volume of autologous plasma. Platelet suspension was incubated at 37°C for 60 minutes with 32P-orthophosphate (0.4 mCi/ml). The platelets were then filtered through a Sepharose 2B column equilibrated with Ca2+ free-Tyrode’s buffer, pH 7.4, containing 136 mM NaCl, 2.7 mM KCl, 0.47 mM NaH2PO4, 12 mM NaHCO3, 2 mM MgCl2, 5.5 mM dextrose, and 0.2% bovine serum albumin. The platelet counts were adjusted to 334×106/ml. Platelet suspensions labeled with 32P-P04 were activated with PAF (4 and 400 nM), thrombin (0.05 and 5.0 u/ml), DiC8 (200 μM) or A23187 (1 μM). The reactions were terminated at various intervals (0-300 seconds) by transferring 0.5 ml aliquots of the sample to tubes containing an equal volume of 0.6 N HClO. The samples were centrifuged in Eppendorf Centrifuge 5415 (Brinkmann Instrumental Inc. Westbury, NY) at 14,000×g for 5 minutes. The pellets were washed once with distilled water and subjected to 13.5% SDS-PAGE. The gels were dried and subjected to autoradiography using DuPont Cronex video imaging film (E.I. DuPont Company, Wilmington, DE). The bands on the gel corresponding to pleckstrin (47 kD) were cut, placed in liquid scintillation cocktail (Betafluor, National Diagnostic, Atlanta, GA), and counted for radioactivity. The results were expressed as fold of the basal radioactivity in pleckstrin.

2.3. Measurement of Cytoplasmic Ca2+ Concentration

Intracellular ionized calcium concentrations [Ca2+]i were measured by using fura-2 as the indicator as described previously [11,131]. Fluorescence was recorded (excitation 340 nm, emission 510 nm) using a spectrofluorimeter (model LS-5 Perkin Elmer, Oakbrook, IL). The [Ca2+]i concentrations were calculated as described [11].

2.4. Lactate Dehydrogenase Assay

To assess the extent of possible cell lysis following exposure to a combination of A23187 and DiC8, lactate dehydrogenase (LDH) activity was measured in parallel with platelet secretion under the same conditions. PRP was centrifuged (14000×g, 30 seconds) at 4 minutes after addition of the agonists and LDH activity in supernatant was measured with NADH and sodium pyruvate as substrates [19]. The change in optical density at 340 nm wave length was monitored using HP 8452A Diode Array Spectrophotometer. LDH activity was expressed as percentage of total LDH activity in PRP lysed with 0.2% Triton-X100.

2.5. Studies on Platelet GPIIb-IIIa by Flow Cytometry

Because the primary wave of aggregation appeared blunted in some of the patients, in five patients we assessed the platelet GPIIb-IIIa complexes by flow cytometry following the method described earlier [16]. Fluorescein isothiocyanate (FITC) labeled monoclonal antibody 10E5 (kindly provided by Dr. Barry Coller, Mount Sinai School of Medicine, New York, NY) was used to assess the GPIIb-IIIa complex on platelet surface. Samples (10000 platelets) were analyzed on EPICS Elite flow cytometer (Coulter Co., Miami, FL) for FITC fluorescence to quantitate the binding of the platelet antibody. In each patient study platelets from a normal subject were concurrently studied. The fluorescence histograms obtained in the patient and normal subjects, as well as the mean platelet fluorescence, were compared [16]. The mean platelet fluorescence represents an average of the antibody bound to platelets.

3. Results

In all patients, platelet aggregation and 14C-serotonin secretion in PRP in response to ADP, epinephrine, and PAF were abnormal; only a reversible primary wave or reduced aggregation and markedly decreased 14C-serotonin secretion were observed (Table 1). U46619 or collagen induced secretion was lower than the values in normal subjects in six and five patients, respectively. With divalent ionophore A23187 (10 μM), five patients showed diminished platelet secretion although substantial aggregation was noted in most patients. In
normal platelets, DiC₈, a direct PKC activator [8], induced a dose-dependent aggregation but negligible secretion.

In all patients, total platelet contents of ATP and ADP, and the ATP:ADP ratio were normal (not shown) thereby excluding dense granule storage pool deficiency. In patient P1 we have previously reported [10] that platelet thromboxane A₂ production was normal on exposure to arachidonic acid but decreased in response to thrombin and ADP, related to a defect in the release of arachidonic acid from phospholipids on activation. In all other patients, arachidonic acid and thrombin induced thromboxane A₂ production were normal.

### 3.1. Calcium Mobilization and Pleckstrin Phosphorylation

An early event on platelet activation is a rise in cytoplasmic Ca²⁺ concentration, which reflects release of Ca²⁺ from intracellular stores as well as influx of extracellular Ca²⁺ [7]. In three patients (P1, P2, and P3) the Ca²⁺ mobilization in the presence of 1 mM external Ca²⁺ was below the values observed in normal subjects in response to both thrombin (0.05 U/ml) and ADP (25 µM) (Figure 1). We have previously reported [11] that both internal release and influx of external Ca²⁺ are abnormal in these three patients. In two patients (P5, P6), Ca²⁺ release was impaired with ADP but not thrombin (Figure 1). Thus, Ca²⁺ mobilization was impaired at least with one agonist in five patients.

Another early event on platelet activation is PKC activation leading to phosphorylation of pleckstrin. In response to thrombin (0.05 U/ml), five of eight patients showed diminished pleckstrin phosphorylation (Figure 2A), and in three of these it was low even at a higher thrombin concentration (5 U/ml) (Figure 2B). Pleckstrin phosphorylation was impaired in four patients upon activation with 4 nM PAF, and in five patients with 400 nM PAF (Figures 2C and D). Overall, either Ca²⁺ mobilization or pleckstrin phosphorylation was abnormal in seven of eight patients following activation with receptor mediated agonists. Both processes were abnormal in two of eight patients (P2, P3). Pleckstrin phosphorylation was also studied in response to A23187 (1 µM) and DiC₈ (200 µM). Ionophore A23187-induced pleckstrin phosphorylation (Figure 3) was decreased in the same five patients as with PAF. Interestingly, on activation of PKC with DiC₈, pleckstrin phosphorylation appeared normal in all but one patient (PS) (not shown), indicating that PKC and its substrate pleckstrin per se are intact in their platelets.
Fig. 2. Agonist-induced platelet pleckstrin phosphorylation. Platelets in PRP were incubated with $^{32}$P-orthophosphate (0.4 mCi/ml) at 37°C for 60 minutes, gel-filtered and resuspended in Ca$^{2+}$-free-tyrode buffer (3–4×10⁸/ml). Pleckstrin phosphorylation was measured as described in Materials and Methods. Shown are the peak pleckstrin phosphorylation as a fold of basal upon activation with 0.05 U/ml thrombin (A), 5.0 U/ml thrombin (B), 4 nM PAF (C), and 400 nM PAF (D). Please see legend to Figure 1 for details. The dotted lines in each panel represent the highest and lowest values observed in studies in 7-16 normal subjects.

3.2. Platelet Aggregation and Secretion in Response to Combined Stimulation with A23187 and DiC₈

The above studies revealed that most of our patients had abnormalities in two signaling events linked to dense granule secretion [5–9]. To understand their impact on the decreased dense granule secretion in our patients, we studied the platelet responses (with specific emphasis on secretion) to combinations of ADP with either DiC₈ or A23187, two agonists that are considered to induce platelet responses by directly activating PKC and mobilizing Ca$^{2+}$, respectively, without interaction with cell surface receptors. These studies were performed in PRP because the patients were initially identified on the basis of impaired responses in PRP. It is relevant to note that negligible secretion was noted with DiC₈ alone even in normal platelets (Table 1). Secretion in response to the combination of ADP (8 μM) and DiC₈ (200 μM) was still lower than the values in normal subjects in six of eight patients (Figure 4), being strikingly abnormal in four of them. Normal secretion was noted in two patients (P1 and P5). Aggregation was lower than that in the normal subjects in five patients. Compared to the values with ADP alone (Table 1), secretion was improved in four subjects (P1, P4, P5, and P8, and Figure 4A). Aggregation was enhanced in seven subjects. Thus, combination of ADP and DiC₈ did not result in normal secretion in most patients. We examined the responses to a combination of ADP with A23187 which by itself is a more potent inducer of platelet responses than most agonists (see Table 1). In normal platelets both secretion and aggregation in response to this combination were only minimally enhanced over that with 10 μM A23187 alone. The combination of ADP and A23187 induced
higher secretion than with A23187 alone in several of the patients, but it was still below the values in normal subjects in 7 of 8 patients (Figure 5). Aggregation was enhanced in response to the combination in most patients (Figure 5).

The most striking findings were observed in response to the combination of 10 μM A23187 with 200 μM DiC8 (Figure 6); platelet secretion in all patients was clearly comparable to that in normal subjects. This increase was not due to cell lysis as evidenced by minimal release of total LDH (8%) in the samples. Studies were also performed using lower concentrations of A23187 and DiC8. Activation with a combination of DiC8 (100 μM) and A23187 (5 μM), neither of which causes secretion in normal PRP by itself, induced vigorous aggregation and secretion which were comparable to that in normal subjects. The secretion ranged from 33 to 72% in the seven patients studied compared to 32-69% in a normal subject. Moreover, in three patients, secretion was monitored in response to a combination of 50 μM ADP and 2 μM epinephrine (Figure 7), two receptor mediated agonists to which the responses were blunted in all patients when tested individually (Table 1). In six patients, the secretion responses remained abnormal (Figure 7). Interestingly, in 2 patients (P7 and P8) secretion was comparable to that in normal subjects, even though secretion on combination of ADP with DiC8 or A23187 was still low (Figures 4 and 5). These findings suggest that ADP and epinephrine have distinct pathways of activation that may be operating synergistically in these two patients to induce secretion.

3.3. Studies on Platelet GPIIb-IIIa

Because the primary aggregation appeared blunted in some patients, in five patients (P1, P2, P3, P4,
and P8), platelet GPIIb-IIIa was assessed by flow cytometry using antibody 10E5 (which recognizes both resting and activated forms of GPIIb-IIIa). The fluorescence histograms and mean platelet fluorescence obtained in each patient were comparable to that in the concurrently studied normal subject, indicating that they do not have a deficiency of platelet GPIIb-IIIa (not shown).

4. Discussion

Congenital platelet function defects represent a group of heterogeneous platelet disorders and are frequently encountered in clinical practice. Some of these patients have the well recognized abnormalities such as the membrane glycoprotein deficiency (e.g., thrombasthenia and Bernard-Soulier Syndrome) and intracellular granule deficiency (storage pool deficiency) [1–3]. However, in our experience such patients comprise a small fraction of patients with congenital platelet function defects, and in the vast majority, the underlying defects remain unknown. The bulk of the patients have impaired platelet aggregation (no second wave) and dense granule secretion on activation with several different agonists, despite presence of normal granule stores. It has been postulated that such patients may have defects in the early events following platelet activation that culminate in the...
end responses, such as secondary aggregation and secretion. Our present studies provide evidence of abnormalities in Ca^{2+} mobilization or PKC-induced pleckstrin phosphorylation, two key signaling events induced by mediators resulting from PLC activation, in seven of eight patients. Together with studies reported by others [20–23] and us [10–15], they establish the occurrence of aberrations in these early events in patients with congenital platelet dysfunction. In further studies in some of our patients we have provided additional corroboration and information regarding potential mechanisms. In patient P1, who was originally described in 1984, because of an abnormality in thrombin-induced release of arachidonic acid from phospholipids, recent evidence indicates an abnormality in G-protein α subunit function and a selective deficiency in membrane G_{αs}[12]. In this patient receptor-stimulated GTPase activity and GTP binding were impaired in platelets. We have demonstrated [14] that patients P2 and P3 have impaired production of IP_{3} and diacylglycerol on receptor activation consistent with a defect in PLC activation. In one of these patients we have delineated an unique deficiency in the expression of PLC-β2 isozyme, the predominant PLC-β isozyme in human platelets [15]. In patient P4 with impaired pleckstrin phosphorylation, we have demonstrated [16] an abnormality in receptor mediated activation of GPIIb-IIIa complex, despite the presence of adequate amounts of GPIIb-IIIa with intact ligand binding capacity on platelet surface. Signal transduction dependent activation of GPIIb-IIIa was impaired but not that induced by chymotrypsin which is independent of intracellular signaling mechanisms. Studies in patient P1 have also revealed an abnormality in the activation of GPIIb-IIIa[12]. Studies in these two patients provide evidence of the impact of intracellular signaling events on expression of GPIIb-IIIa on platelet surface and on platelet aggregation. Overall, the patients reported here provide a strong support for existence of discernible and specific abnormalities in platelet signaling mechanisms.

The second major finding in our studies is that the ultimate process of exocytosis per se is essentially intact in the patients described. This is evidenced by our finding that exposure of their platelets to a combination of two cell permeable agonists ionophore A23187 and DiC_{8}, which presumably bypass the early signaling events, induces entirely normal secretion even though responses to surface receptor mediated agonists are impaired. Thus, the impaired secretion observed in response to several different receptor mediated agonists is a reflection of the aberrations in the upstream signaling events that follow ligation of the surface receptor but precede exocytosis. The ability to possibly bypass the events of PLC activation and induce secretion may imply that the primary defect may occur in the areas consisting of the receptors, G-proteins, and effectors (e.g., PLC). The finding that combination of ADP with DiC_{8} or A23187 did not induce normal secretion in our patients, even though DiC_{8}+ A23187 combination was effective, is in line with
the paradigm that both Ca\(^{2+}\) mobilization and PKC activation are required for normal secretion [8–9].

If the key components of the platelet signal transduction mechanisms are the surface receptors, the G-proteins that modulate their link with intracellular effectors, and the effectors, evidence now exists for specific platelet abnormalities at each level in individual patients. Defects at the level of surface agonist receptors have been documented [24–31]. Hirata et al. [24] have described an Arg 60 to Leu mutation of the human thromboxane A\(_2\) receptor in a dominantly inherited bleeding disorder. Evidence has been presented by Cattaneo et al. [26] and Nurden et al. [27] for defects in the interaction of ADP and its receptors on platelets. Earlier studies have documented abnormalities at the level of the epinephrine receptors [1,28] and collagen receptors [29–32]. A defect at the level of G-protein function with platelet membrane deficiency in \(\text{Go}_q\) (but not \(\text{Go}_{12}, \text{Go}_{13}\)) and leading to impaired platelet responses has been recently demonstrated by us in patient PI[12]. A deficiency of PLC-\(\beta\)2 isozyme has been demonstrated by us in patient P2 [15] and constitutes a specific defect at an effector (PLC) level. Overall, these heterogeneous patients constitute a group appropriately labeled as signal transduction defects and as a group are likely to be encountered more frequently than the membrane glycoprotein deficiencies or the storage pool deficiency.

In summary, our studies provide further evidence for the existence of abnormalities in early signal transduction events in patients with impaired platelet responses. We conclude that in at least many of these patients, the ultimate process of exocytosis or secretion per se is intact but is compromised by aberrant upstream signaling events. There is a pressing need to carefully delineate the molecular defects in these heterogeneous patients with signal transduction defects, who constitute an untapped reservoir of new information into platelet physiology.

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