Decreased Expression of Phospholipase C-β2 Isozyme in Human Platelets With Impaired Function

By Sang Bong Lee, A. Koneti Rao, Kweon-Haeng Lee, Xu Yang, Yun Soo Bae, and Sue Goo Rhee

Platelets from a patient with a mild inherited bleeding disorder and abnormal platelet aggregation and secretion showed reduced generation of inositol 1,4,5-trisphosphate, mobilization of intracellular Ca2+, and phosphorylation of pleckstrin in response to several G protein mediated agonists, suggesting a possible defect at the level of phospholipase C (PLC) activation (see accompanying report). A procedure was developed that allows quantitation of platelet PLC isoforms. After fractionation of platelet extracts by high-performance liquid chromatography, 7 out of 10 known PLC isoforms were detected by immunoblot analysis. The amount of these isoforms in normal platelets decreased in the order PLC-γ2 > PLC-β2 > PLC-β3 > PLC-β1 > PLC-γ1 > PLC-δ1 > PLC-β4. Compared with normal platelets, platelets from the patient contained approximately one-third the amount of PLC-β2, whereas PLC-β4 was increased threefold. These results suggest that the impaired platelet function in the patient in response to multiple G protein mediated agonists is attributable to a deficiency of PLC-β2. They document for the first time a specific PLC isozyme deficiency in human platelets and provide an unique opportunity to understand the role of different PLC isoforms in normal platelet function.

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PLATELET ACTIVATION begins with the binding of an agonist to the cell surface and culminates in platelet aggregation and secretion and clot retraction. Binding of a platelet agonist, such as thrombin, collagen, platelet-activating factor (PAF), adenosine diphosphate (ADP), epinephrine, or thromboxane A2, to its cognate receptor results in the activation of phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to yield inositol 1,4,5-trisphosphate (IP3) and diacetyl-glycerol (DG). PI3 mobilizes Ca2+ from intracellular stores and DG activates protein kinase C, events that initiate bifurcating signaling pathways that function synergistically to produce a maximal biological effect. Prominent consequences of the activation of the two pathways in platelets include the phosphorylation of both the 20-kD myosin light chain by a Ca2+/calmodulin-dependent protein kinase and 47-kD pleckstrin by protein kinase C.

Like many other proteins important in signal transduction, PLC exists in multiple isoforms. The 10 mammalian PLC enzymes identified to date are all single polypeptides and can be divided into three types, PLC-β, PLC-γ, and PLC-δ, which include four, two, and four members, respectively. At least two distinct mechanisms link receptor occupancy to the activation of PLC isoforms: one mediated by heterotrimeric G proteins and the other by protein-tyrosine phosphorylation. Activation of PLC-β isoforms is dependent on either the α subunits of Gαi class G proteins (Gαi subunits) or the βγ subunits of G proteins (Gβγ subunits). Gαi subunits activate all four PLC-β isoforms, whereas Gβγ subunits activate PLC-β1, PLC-β2, and PLC-β3, but not PLC-β4. Activation by Gβγ is often inhibited by pertussis toxin, which modifies Gαi and Gαo and consequently prevents the generation of Gβγ subunits from heterotrimeric Gαi and Gαo; Gαi subunits do not possess the site for modification by pertussis toxin. Phosphorylation of PLC-γ1 and PLC-γ2 on several tyrosine residues results in their activation. The activation mechanism of PLC-δ isoforms is not known at the present time.

Defects in the early events of signal transduction have long been considered to be responsible for impaired platelet function in patients with bleeding disorders. However, such defects have not been clearly shown in these individuals. Several years ago, Rao et al. identified two patients (mother and son) with a mild bleeding disorder whose platelets showed defective aggregation and secretion responses to multiple agonists. The platelet dysfunction was postulated to be caused by defects at the level of PLC activation on the basis that the increase in cytosolic free Ca2+ concentration during platelet activation with several different agonists was significantly reduced in the two patients, but Ca2+ release induced by exogenous IP3, however, was normal. Defects in the granule storage pool or thromboxane production, which are known to occur in some individuals with inherited impairment of platelet activation, were not detected in the two patients. Furthermore, in an accompanying report, agonist-induced generation of IP3 and DG and the phosphorylation of pleckstrin are shown to be abnormal in the two patients.

In the present report, we have devised a method to fractionate and quantitate the amounts of PLC isoforms in human platelets. These studies provide new information on the relative amounts of various PLC isoforms in normal human platelets. With this approach, we show that the concentrations of PLC-β2 and PLC-β4 differ substantially between platelets from normal individuals and the patient investigated.

MATERIALS AND METHODS

Materials. Heparin-Sepharose CL-6B was purchased from Pharmacia (Piscataway, NJ). [3H]Phosphatidylinositol (PI) (specific ac-

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activity, 13.0 Ci/mmol) and [3H]PIP2 (8.8 Ci/mmol) were from Du Pont-NEN (Boston, MA). PI (soybean) was from Sigma (St Louis, MO). PIP2 (bovine brain) from Boehringer Mannheim (Indianapolis, IN), and phosphatidyethanolamine (bovine brain) from Avanti Polar Lipids (Alabaster, AL). [3H]-Labeled affinity-purified protein A (36 mCi/mg) was obtained from Amersham (Arlington Heights, IL) and alkaline phosphatase-conjugated secondary antibodies and phosphatase substrate system were from Kirkegaard & Perry (Gaithersburg, MD).

Preparation of platelets. Platelets were obtained from the patient, a 48-year-old white woman who has been previously described and six healthy volunteers. On each occasion blood (1 U) was collected from the patient and normal subjects by venipuncture into 1/7 volume of acid citrate dextrose buffer (85 mmol/L trisodium citrate, 78 mmol/L citric acid, 111 mmol/L dextrose) and centrifuged at 180g for 20 minutes. The platelet-rich plasma was then centrifuged at 1,000g for 15 minutes in the presence of hirudin (0.05 U/mL) and apyrase (10 nCi/mL) and the resulting platelet pellet was resuspended and washed three times with washing buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 µg/mL), and aprotinin (10 µg/mL)].

Isolation of PLC isozymes from platelets. Platelets were sonicated within 6 hours of preparation in 5 volumes of homogenization buffer (10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, leupeptin [10 µg/mL], aprotinin [10 µg/mL], and calpain inhibitors I and II [each at 4 µg/mL]), and the homogenate was centrifuged at 1,000g for 10 minutes. The supernatant was adjusted to 2 mol/L KCl by adding solid KCl, then centrifuged at 35,000g for 30 minutes. The resulting supernatant was dialyzed overnight against 4 L of homogenization buffer and recentrifuged. The supernatant (~80 mg of protein) was applied to a heparin-Sepharose CL-6B column (20 mL of gel packed in a 1.5- by 15-cm Econo column) that had been equilibrated with 20 mmol/L HEPES-NaOH (pH 7.0) containing 1 mmol/L EGTA and 0.1 mmol/L DTT. Bound proteins were eluted at a flow rate of 4 mL/min with the equilibration buffer containing 1.2 mol/L NaCl. Fractions (16 mL/fraction) were collected and assayed for PLC activity. Essentially all detectable PLC activity eluted in six fractions (~40 mg protein), which were pooled and concentrated in a stirred ultrafiltration cell fitted with a YM 30 membrane (Amicon, Danvers, MA). After the final salt concentration was then washed with equilibration buffer containing 1.2 mol/L NaCl, the concentrate was centrifuged at 35,000g for 30 minutes. The resulting supernatant was then dialyzed overnight against 4 L of homogenization buffer and recentrifuged. The supernatant (~80 mg of protein) was applied to a heparin-Sepharose CL-6B column (7.5 × 75 mm) that had been equilibrated with 20 mmol/L HEPES-NaOH (pH 7.0) containing 1 mmol/L EGTA, and 0.1 mmol/L DTT. Bound proteins were eluted at a flow rate of 1 mL/min by the application of equilibration buffer for 15 minutes followed by a stepwise linear NaCl gradient from 0 to 0.64 mol/L for 40 minutes and from 0.64 mol/L to 1 mol/L NaCl for 10 minutes. The column was then washed with equilibration buffer containing 1 mol/L NaCl. Fractions (0.5 mL) were collected and assayed for PLC activity (50 and 5 µL of each fraction were used for assay of PI- and PIP2-hydrolyzing activity, respectively).

PLC assay. PLC activity was assayed with either [3H]PI or [3H]PIP2 as substrate. PIP2-hydrolyzing activity was measured with mixed lipid vesicles of phosphatidyethanolamine:PIP2 in a molar ratio of 4:1. The lipids in chloroform were dried under a stream of nitrogen gas, suspended in 50 mmol/L HEPES-NaOH (pH 7.0), 120 mmol/L KCl, 10 mmol/L NaCl, and 1.6 mmol/L sodium deoxycholate, and subjected to sonication. Assays were performed for 10 minutes at 30°C in a 100-µL reaction mixture containing lipid micelles (12 µmol/L [3H]PIP2, 12,000 cpm), 50 mmol/L HEPES-NaOH (pH 7.0), 0.1% sodium deoxycholate, 120 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L MgCl2, 2 mmol/L EGTA, and 1.4 mmol/L CaCl2 (to give a final free Ca2+ concentration of 1 µmol/L). PI-hydrolyzing activity was measured in a 200-µL reaction mixture containing 150 µmol/L [3H]PI (20,000 cpm), 50 mmol/L HEPES-NaOH (pH 7.0), 3 mmol/L CaCl2, 2 mmol/L EGTA, and 0.1% sodium deoxycholate. The reaction mixture was incubated at 37°C for 10 minutes. Procedures for terminating the reactions with a mixture of chloroform, methanol, and HCl, and determination of water-soluble [3H]radioactivity were as described previously.

Immunoblot analysis. Monoclonal antibodies to PLC-y1 and PLC-y2 and rabbit antibera to PLC-b1, PLC-b2, PLC-b3, PLC-b4, PLC-y2, PLC-b5 (unpublished result), PLC-b3 (unpublished result), and PLC-b4 were prepared as described. These antibodies were highly specific for the corresponding antigen. Monospecific antibodies were purified from antisera with the use of purified PLC isozymes immobilized on nitrocellulose paper.

Proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (6% gel) and transferred to nitrocellulose membranes. After blocking with 3% (wt/vol) bovine serum albumin, the membranes were incubated with antibodies to PLC isozymes. The resulting immune complexes were either visualized with alkaline phosphatase-conjugated secondary antibodies or quantitated with [125I]-protein A autoradiography and a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

PLC isozymes in normal platelets. Our previous studies indicated that PLC isozymes associated with particulate fractions can be extracted with 2 mol/L KCl.11-13,17 Multiple forms of PLC have also been shown to be present in cytosolic and membrane fractions of human platelets but their relative amounts have hitherto not been characterized.18 Therefore, we extracted platelet homogenates with 2 mol/L KCl and then applied the soluble proteins, after dialysis, to a conventional heparin column. Bound proteins were eluted with a buffer containing 1.2 mol/L NaCl (Fig 1A). This procedure allowed us to recover PLC activity quantitatively in a small volume while eliminating one half of total protein and turbid material that interfered with the PLC assay, protein measurement, and subsequent HPLC analysis. Proteolytic degradation was minimized by including several protease inhibitors in the homogenization and dialysis buffers.

After concentration, the pooled fractions were subjected to HPLC analysis on a heparin column with a stepwise linear NaCl gradient. Each resulting fraction was assayed for PLC activity with either PI or PIP2 as substrate, and concentrated fractions were subjected to immunoblot analysis with isozyme-specific antibodies (Fig 1B). Overall, the activity profiles for both PI and PIP2 hydrolysis were similar. Two prominent activity peaks, centered at 32 and 45 minutes, were detected. Small peaks that were partially resolved but reproducible were also detected at 37, 51, and 54 minutes. Immunoblot indicated that the 32- and 45-minute peaks were mostly attributable to PLC-y2 (142 kD) and PLC-b2 (140 kD), respectively. PLC-y1 (145 kD) appeared to be associated with the shoulder peak at 37 minutes. PLC-b1 (150 kD) and PLC-b3 (152 kD) were eluted in the 51-minute peak, and PLC-b4 (130 to 135 kD) was detected as faint triplet bands that peaked at 54 minutes. PLC isozymes often appear as multiple bands on SDS-polyacrylamide gels as a result of alternative mRNA splicing, proteolysis, or phosphoryla-

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Fig 1. Separation and detection of PLC isozymes in normal platelets. A KCl extract of a homogenate of platelets from a normal individual was subjected to sequential chromatography on heparin-Sepharose CL-6B (A) and TSK heparin-5PW HPLC (B) columns as described under Experimental Procedures. Each fraction was assayed for PLC activity with either PI or PIP₂ as substrate. Proteins in the HPLC fractions were concentrated, separated on SDS-polyacrylamide gels, and subjected to immunoblot analysis with isozyme-specific antibodies (B, inset). The volumes of HPLC fractions applied to the gels varied depending on the PLC concentration and antibody sensitivity. Numbers above the immunoblots indicate HPLC retention time (minute).

A faint PLC-δ₁ (85 kD) band was apparent in fractions corresponding to the trough between the two major peaks. The other three PLC-δ isozymes (δ₂, δ₃, and δ₄) were not detected, suggesting that these isozymes are not present, or are present at extremely low concentrations in platelets.

Figure 2 shows comparison of PLC isozymes from platelets of two normal individuals. The two platelet protein samples were processed under identical conditions and as simultaneously as possible. The PLC-containing fractions pooled from the conventional heparin column chromatography of a single platelet preparation comprised slightly more than 40 mg of protein, which was sufficient for two independent HPLC analyses (20 mg of protein per analysis). The HPLC elution profiles were similar for two samples from the same individual (fig not shown). Furthermore, the areas under the PLC-γ₂ or PLC-β₂ peaks were reproducible within 20% when platelets from five normal individuals were analyzed as illustrated in Fig 2. To estimate the amount of each PLC isozyme in the HPLC fractions, portions of eight fractions were pooled for each PLC isozyme and the amount of isozyme in the pooled fraction was quantified by immunoblot analysis with [³⁵S]-protein A and purified PLC as a standard (data are not shown but were similar to those in Fig 4). The amount of each isozyme was expressed as nanograms of...
PLC defects in human platelets

The estimated values for abundant isoforms (PLC-γ2, PLC-β2, and PLC-β3) were reproducible within 15%; estimation of other isoforms was less accurate. The amounts of PLC isoforms decreased in the order PLC-γ2 > PLC-β2 > PLC-β3 > PLC-β1 > PLC-γ1 > PLC-β4. PLC-β1 could not be estimated because of its low concentration in platelets and its low reactivity with the corresponding antibody; nevertheless, the amount of PLC-β1 appeared to be greater than that of PLC-β4, which could be detected by immunoblot analysis with a high sensitivity (detection range of 0.5 ng). Whereas the concentration of PLC-β4 was much less than that of PLC-β3 or PLC-β1, its activity peak at 54 minutes was similar in magnitude to that of PLC-β3 and PLC-β1 at 51 minutes (Fig 1B), probably because PLC-β3 encroached into the PLC-β4 peak and because the catalytic activity of PLC-β4 is much greater than that of other PLC-β isoforms in the absence of ribonucleotides.

Comparison of platelet PLC isoforms between normal individuals and the proband. The KCl extracts of platelets from normal and normal individuals were chromatographed separately but in parallel on two identical conventional heparin columns. The pooled PLC-containing fractions from each column were divided into two, and each pair was fractionated on a TSK heparin-SPW HPLC column. PIP2-hydrolyzing activity was measured in each 0.5-minute fraction.

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amounts of these isozymes were not measured quantitatively. Moreover, we extend this information to show that human platelets express at least seven PLC isozymes and that their respective concentrations differ markedly. For more accurate determinations, the numbers in Table 1 should be corrected for the loss of PLC that might have occurred during the two chromatography steps. Comparison of the values in Tables 1 and 2 suggests that such loss ranges from 20% to 30%.

Detailed HPLC analyses of platelet proteins obtained from two separate occasions revealed that PLC-02 peak intensity was less than one-half that of the normal samples when measured using either PIP$_2$ or PI as the substrate (Fig 3). Immunoblot analysis of the HPLC fractions detected only one-third the amount of PLC-02 present in corresponding fractions from normal individuals (Fig 4 and Table 1). The PLC-02 deficiency was also apparent from the direct immunoblot analysis of both 2 mol/L KCl extracts and total homogenates (Figs 5 and 6 and Table 2). The amount of PLC-γ2 in patient platelets was about 70% to 80% of normal, by all three methods of estimation (immunoblot analysis of HPLC fractions, immunoblot analysis of crude extracts, and PLC activity measurement of HPLC fractions). Immunoblot analysis of HPLC fractions showed that the amount of PLC-γ1 was ∼75% of normal in patient platelets. However, the difference in PLC-γ1 is close to the experimental error for its measurement. The amounts of PLC-β1 and PLC-β3 in patient platelets were virtually identical to those in normal platelets. The increased concentration of PLC-β4 in patient platelets was shown by both immunoblot and activity assays.

Thus, the most striking differences between the patient and normal platelets are the decrease in PLC-β2 and increase in PLC-β4. PLC-γ2, the most abundant PLC isozyme, was present in our patient at 70% to 80% of normal. However, because this difference is close to the experimental error for its measurement and because we had only one patient in the study, whether this difference represents merely an individual variation or a decrease that has an impact on platelet responses is presently unknown. Little is known about the transcriptional and translational regulation of PLC isozymes. Although the possibility of multiple mutations resulting in aberrant expression of PLC-β2, PLC-β4 and, possibly, PLC-γ2 in patient platelets cannot be eliminated, a single mutation in an as yet unknown regulatory gene may also be responsible for this effect. Consistent with this latter suggestion, exposure of CCL39 Chinese hamster lung fibroblasts to conditions that generate single-site mutations produced a cell line 2A4b with deficient PLC-β1 but excessive PLC-δ1 expression. The increase in PLC-β4 in patient platelets may be compensatory, perhaps similar to the increase in PLC-δ1 noted in the CCL39 mutant 2A4b, which has defective thrombin-induced signaling.

Platelets from the patient described in the present study shows decreased Ca$^{2+}$ mobilization in response to activation with agonists such as ADP, PAF, the thromboxane A$_2$ analog.
U46619, collagen, and thrombin, despite a normal Ca\textsuperscript{2+} release in response to exogenous IP\textsubscript{3}, Early platelet responses to these agonists include PLC activation. As shown in the accompanying report,\textsuperscript{26} the generation of IP\textsubscript{3} and DG in response to thrombin and PAF was also significantly attenuated in platelets from the patient, further suggesting a possible defect in PLC activation. Moreover, Ca\textsuperscript{2+} release induced by GTP-\textgamma-\S was also blunted suggesting a defect in post-receptor events.

Receptor-mediated activation of PLC in platelets appears to be achieved through multiple mechanisms. The receptors for PAF, thrombin, and thromboxane A\textsubscript{2} belong to the super-family of G protein-coupled receptors. The receptors for thrombin and thromboxane A\textsubscript{2} appear to couple to PLC-\beta isoforms through G\textalpha\textgamma subunits, because antibodies to G\textalpha\textgamma inhibit PLC activation elicited by those two agonists.\textsuperscript{27,28} The

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**Table 2. Amounts of PLC Isozymes Measured Directly in KCl Extracts and Total Homogenates of Patient and Normal Platelets**

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>KCl Extract Patient</th>
<th>Normal</th>
<th>P/N</th>
<th>Total Homogenate Patient</th>
<th>Normal</th>
<th>P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC-\gamma2</td>
<td>190</td>
<td>204</td>
<td>79</td>
<td>110</td>
<td>150</td>
<td>77</td>
</tr>
<tr>
<td>PLC-\beta2</td>
<td>16</td>
<td>42</td>
<td>38</td>
<td>11</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>PLC-\beta3</td>
<td>16</td>
<td>17</td>
<td>94</td>
<td>12</td>
<td>12</td>
<td>92</td>
</tr>
</tbody>
</table>

Data shown in Fig 5 (KCl extract) and Fig 6 (Total homogenate) were expressed as nanograms of PLC per milligram of KCl extract and per milligram of total homogenate, respectively. Immunoblot analysis was repeated four times for the KCl extract and three times for the total homogenate. Reproducibility was within 15%.
PLC response to PAF and thrombin is affected by pertussis toxin, suggesting a role for $G_{o}$ or $G_{i}$ proteins. Indeed, the Ca$^{2+}$ response to thrombin in CCL39 cells was partially inhibited by microinjection of antibodies to $G_{o}$.28 In addition, treatment of platelets with thrombin or collagen results in tyrosine phosphorylation of PLC-γ2.23,24 The direct participation of a protein-tyrosine kinase (Src) in PAF-induced PLC activation in platelets has also been shown.29 These observations suggest that stimulation of PLC in platelets by Ca$^{2+}$-mobilizing agonists may be mediated by all three PLC activation mechanisms: PLC-β isoforms by $G_{o}$ subunits, PLC-β (except PLC-β4) isoforms by $G_{βγ}$ subunits, and PLC-γ isoforms by protein-tyrosine kinases. Because PLC-γ2 and PLC-β2 are the major PLC isoforms in platelets the reduced expression of either of them may cause the attenuated Ca$^{2+}$ response to various agonists and other defects observed in the patient platelets. Based on the marked deficiency of PLC-β2 and the blunted Ca$^{2+}$ release in response to GTP-γS, the impaired platelet function in response to several G protein mediated agonists in the patient is attributable to a deficiency of PLC-β2. A contribution from the possible minimal decrease in PLC-γ2 needs to be defined. Platelets from this patient document for the first time a deficiency of a specific PLC isozyme in human platelets and provide an unique opportunity to understand the role of the different PLC isoforms in normal platelet function. Our studies provide a hitherto unavailable detailed analyses of the various PLC isozymes in normal human platelets.

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Decreased expression of phospholipase C-beta 2 isozyme in human platelets with impaired function

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