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

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ABSTRACT

Platelets from a patient with a mild inherited bleeding disorder and abnormal platelet aggregation and secretion show reduced generation of inositol 1,4,5-trisphosphate (IP_3), mobilization of intracellular Ca^{2+}, and phosphorylation of pleckstrin in response to several G protein mediated agonists, suggesting a possible defect at the level of phospholipase C (PLC) activation. A procedure was developed that allows quantitation of platelet PLC isozymes. After fractionation of platelet extracts by high-performance liquid chromatography, seven, out of ten 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 isozymes in normal platelet function.

INTRODUCTION

Platelet activation begins with the binding of an agonist to the cell surface and culminates in platelet aggregation and secretion and clot retraction.[1,2] Binding of a platelet agonist, such as thrombin, collagen, platelet-activating factor (PAF), ADP, epinephrine, or thromboxane A_2, to its cognate receptor results in the activation of PLC, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield IP_3 and diacylglycerol (DG).[1-3] IP_3 mobilizes Ca^{2+} 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-kDa myosin light chain by a Ca^{2+}/calmodulin-dependent protein kinase and 47-kDa pleckstrin by protein kinase C.[1,2]

Like many other proteins important in signal transduction, PLC exists in multiple isoforms.[4,5] 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 isozymes: one mediated by heterotrimeric G proteins and the other by protein-tyrosine phosphorylation.[4,5] Activation of PLC-β isozymes is dependent on either the α subunits of G_q class G proteins ($G\alpha_q$) or the βγ subunits of G proteins (Gβγ). $G\alpha_q$ subunits activate all four PLC-β isozymes, 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 $G\alpha_o$ and $G\alpha_i$ and consequently prevents the generation of Gβγ from heterotrimeric G_o and G_i; $G\alpha_q$ 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 isozymes 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.[6,7] However, such defects have not been clearly demonstrated in these individuals. Several years ago, Rao and colleagues[8,9] 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 Ca^{2+} concentration during platelet activation with several different agonists was significantly reduced in the two patients, but Ca^{2+} release induced by exogenous IP_3, 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,[6] were not detected in the two patients.[8] Furthermore, agonist-induced generation of IP_3 and DG and the phosphorylation of pleckstrin are shown to be abnormal in the two patients.

In the present study, we have devised a method to fractionate and quantitate the amounts of PLC isozymes in human platelets. These studies provide new information on the relative amounts of various PLC isozymes 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.

METHODS

Preparation of platelets

Platelets were obtained from the patient, a 48-year-old white woman who has been previously described[8,9] and six healthy volunteers. On each occasion blood (1 unit) was collected from the patient and normal subjects 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 min. The platelet-rich plasma was then centrifuged at 1000 × *g* for 15 min in the presence of hirudin (0.05 U/ml) and apyrase (10 μg /ml) and the resulting platelet pellet was resuspended and washed three times with washing buffer [50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin (10 μg /ml), and aprotinin (10 μg /ml)].

Isolation of PLC isozymes from platelets

Platelets were sonicated within 6 h of preparation in 5 volumes of homogenization buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM 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 1000 × *g* for 10 min. The supernatant was adjusted to 2 M KCl by adding solid KCl, stirred for 2 h at 4°C, and then centrifuged at 35,000 x g for 30 min. The resulting supernatant was dialyzed overnight against 4 liters 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 mM Hepes-NaOH (pH 7.0) containing 1 mM EGTA and 0.1 mM DTT. Bound proteins were eluted at a flow rate of 4 ml/min with the equilibration buffer containing 1.2 M 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). After the final salt concentration was adjusted to 50 mM NaCl, the concentrate was centrifuged at 100,000 x g for 10 min. Proteins (20 mg, unless otherwise mentioned) from the supernatant were injected into a TSKgel heparin-5PW HPLC column (7.5 × 75 mm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.0), 1 mM EGTA, and 0.1 mM DTT. Proteins were eluted, at a flow rate of 1 ml/min, by the application of equilibration buffer for 15 min followed by a stepwise linear NaCl gradient from 0 to 0.64 M for 40 min and from 0.64 M to 1 M NaCl for 10 min. The column was then washed with equilibration buffer containing 1 M 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 PIP_2-hydrolyzing activity, respectively).

PLC assay

PLC activity was assayed with either [^{3}H]PI or [^{3}H]PIP_2 as substrate. PIP_2-hydrolyzing activity was measured with mixed lipid vesicles of phosphatidylethanolamine: PIP_2 in a molar ratio of 4:1. The lipids in chloroform were dried under a stream of nitrogen gas, suspended in 50 mM Hepes-NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, and 1.6 mM sodium deoxycholate, and subjected to sonication. Assays were performed for 10 min at 30°C in a 100-μl reaction mixture containing lipid micelles (12 μM [^{3}H]PIP_2, 12,000 cpm), 50 mM Hepes-NaOH (pH 7.0), 0.1% sodium deoxycholate, 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, and 1.4 mM $CaCl_2$ (to give a final free Ca^{2+} concentration of 1 μM). PI-hydrolyzing activity was measured in a 200-μl reaction mixture containing 150 μM [^{3}H]PI (20,000 cpm), 50 mM Hepes-NaOH (pH 7.0), 3 mM $CaCl_2$, 2 mM EGTA, and 0.1% sodium deoxycholate.[11] The

reaction mixture was incubated at 37°C for 10 min. Procedures for terminating the reactions with a mixture of chloroform, methanol, and HCl, and determination of water-soluble ^{3}H radioactivity were as described previously.[11]

Immunoblot analysis

Monoclonal antibodies to PLC-γ1 and PLC-δ1[11] and rabbit antisera to PLC-β1,[12] PLC-β2,[13] PLC-β3,[14] PLC-β4,[15] PLC-γ2,[16] PLC-δ2 (unpublished result), PLC-δ3 (unpublished result), and PLC-δ4[17] 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 SDS-polyacrylamide gels (6% gel) and transferred to nitrocellulose membranes. After blocking with 3% (w/v) 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 ^{125}I-protein A autoradiography and a PhosphorImager (Molecular Dynamics).

RESULTS

PLC isozymes in normal platelets

Our previous studies indicated that PLC isozymes associated with particulate fractions can be extracted with 2 M KCl.[12-14,18] 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.[19,20] We, therefore, extracted platelet homogenates with 2 M KCl and then applied the soluble proteins, after dialysis, to a conventional heparin column. Bound proteins were eluted with a buffer containing 1.2 M NaCl (Fig 1A). This procedure allowed us to recover PLC activity quantitatively in a small volume while eliminating 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 PIP_2 as substrate, and concentrated fractions were subjected to immunoblot analysis with isozyme-specific antibodies (Fig 1B). Overall, the activity profiles for both PI and PIP_2 hydrolysis were similar. Two prominent activity peaks, centered at 32 and 45 min, were detected. Small peaks that were partially resolved but reproducible were also detected at 37, 51, and 54 min. Immunoblot indicated that the 32- and 45-min peaks were mostly attributable to PLC-γ2 (142 kDa) and PLC-β2 (140 kDa), respectively. PLC-γ1 (145 kDa) appeared to be associated with the shoulder peak at 37 min. PLC-β1 (150 kDa) and PLC-β3 (152 kDa) were eluted in the 51-min peak, and PLC-β4 (130 to 135 kDa) was detected as faint triplet bands that peaked at 54 min. PLC isozymes often appear as multiple bands on SDS-polyacrylamide gels as a result of alternative mRNA splicing, proteolysis, or phosphorylation.[12-14] A faint PLC-δ1 (85 kDa) 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 be detected, suggesting that these isozymes are not present, or are present at extremely low concentrations, in platelets.

Fig 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 (figure 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 ^{125}I-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 per milligram of protein in the KCl extract (Table 1). 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 isozymes decreased in the order PLC-γ2 > PLC-β2 > PLC-β3 > PLC-β1 > PLC-γ1 > PLC-β4. PLC-δ1 could not be estimated because of its low concentration in platelets and its low reactivity with the corresponding antibody; nevertheless, the amount of PLC-δ1 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 min was similar in magnitude to that of PLC-β3 and PLC-β1 at 51 min (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 isozymes in the absence of ribonucleotides.[21]

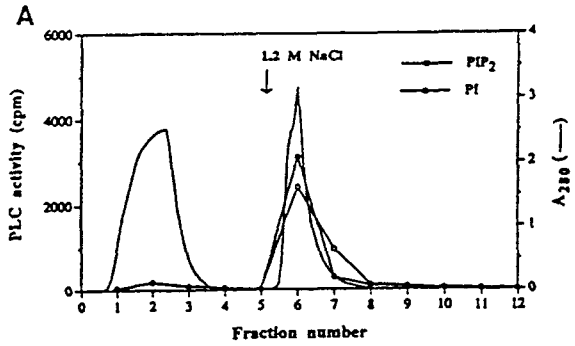
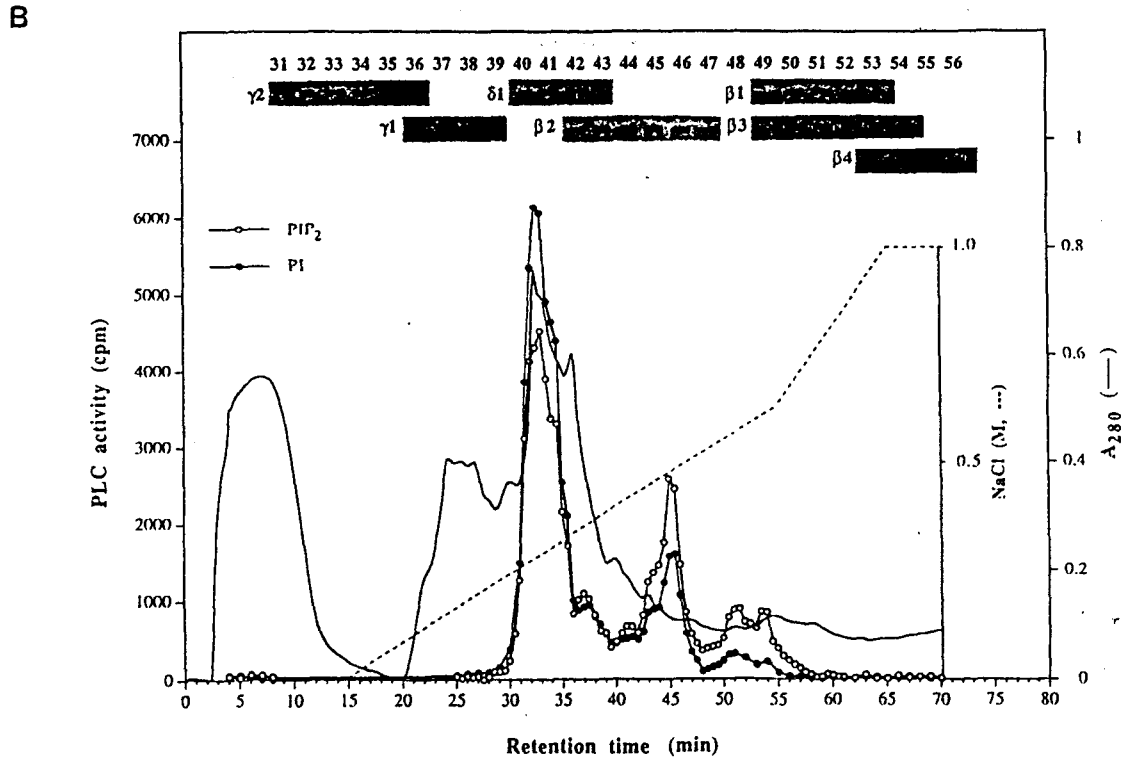


Fig 1. Separation and detection of PLC isozymes in normal platelets. A KCl extract of a homogenate of platelets from a normal individual was subjected to sequential chromatography on heparin-Sepharose CL-6B (A) and TSK heparin-5PW HPLC (B) columns as described under Experimental Procedures. Each fraction was assayed for PLC activity with either PI or PIP_2 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).



Comparison of platelet PLC isozymes between normal individuals and the proband

The KCl extracts of platelets from patient 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 an HPLC heparin column. The profiles of PIP_2-hydrolyzing activities eluted from the HPLC column are shown in Fig 3.

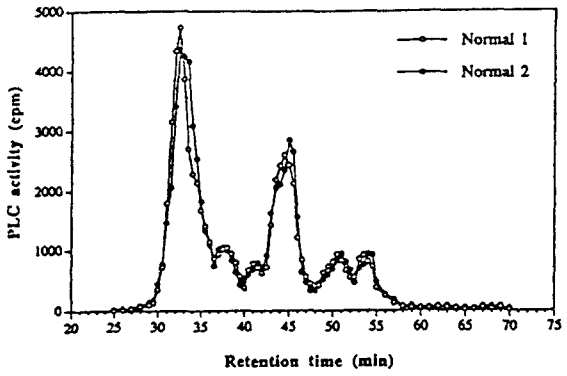


Fig 2. Comparison of PIP_2-hydrolyzing activity profiles after heparin HPLC chromatography of platelet proteins from two normal individuals. Platelet proteins (20 mg each) from two normal individuals, after chromatography on heparin-Sepharose CL-6B, were separated on a TSK heparin-5PW HPLC column. PIP_2-hydrolyzing activity was measured in each 0.5-minute fraction.

Table 1. Amounts of PLC Isozymes Measured in the HPLC Fractions of Patient and Normal Platelets

	Patient	Normal 1	Normal 2	Average of Normals	P/N
		ng/mg of Extract			%
PLC-γ2	150	210	190	200	75
PLC-β2	11	35	31	33	33
PLC-β3	15	14	15	14.5	103
PLC-β1	0.80	0.77	0.73	0.75	107
PLC-γ1	0.47	0.63	0.60	0.62	76
PLC-β4	0.13	0.03	0.05	0.04	325

Data (Patient and Normal 1) shown in Fig 4 were expressed as nanograms of PLC per milligram of KCl extract and are means of triplicate measurements. Platelets from another normal subject (Normal 2) were analyzed by the procedure described in Fig 4. Reproducibility of the immunoblot analysis was within 15% for major isoforms (PLC-γ2, -β2, and -β3) and within 30% for others.

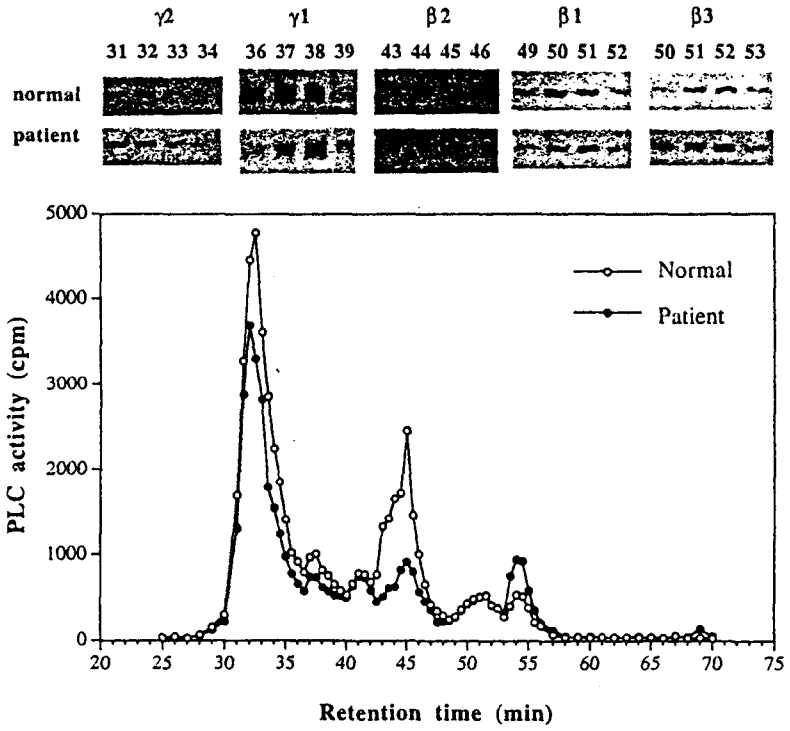


Fig 3. Comparison of PIP_2-hydrolyzing activity profiles after heparin HPLC chromatography of platelet proteins from a normal individual and the patient. Proteins (20 mg) pooled after chromatography of extracts of normal and patient platelets on heparin-Sepharose CL-6B were separated on a TSK heparin-5PW HPLC column and PIP_2-hydrolyzing activity was measured in each 0.5-minute fraction. Proteins in the HPLC fractions were also concentrated, separated on SDS-polyacrylamide gels, and subjected to immunoblot analysis with isozyme-specific antibodies. Numbers above the immunoblots indicate HPLC retention time (minute). The volumes of HPLC fractions applied to the gels varied depending on the PLC concentration and antibody sensitivity.

The most marked difference between the normal and patient activities was observed in the 44-min peak (PLC-β2); the peak height of the patient sample was less than one-half that of the normal sample. In addition, the patient platelets exhibited slightly lower activities than normal platelets in the 32-min (PLC-γ2) and 37-min (PLC-γ1) peaks, whereas activity in the 54-min (PLC-β4) peak was greater in patient platelets. The 51-min peak (PLC-β1 and PLC-β3) was similar in size between normal and patient platelets. The HPLC column fractions were also assayed for PI-hydrolyzing activity (data not shown). The 32- and 44-min peaks were again smaller for patient platelets; the activities of the 51- and 54-min peaks were too low for meaningful comparison. These studies were performed in the patient on two separate occasions with similar findings with respect to PLC isozymes.

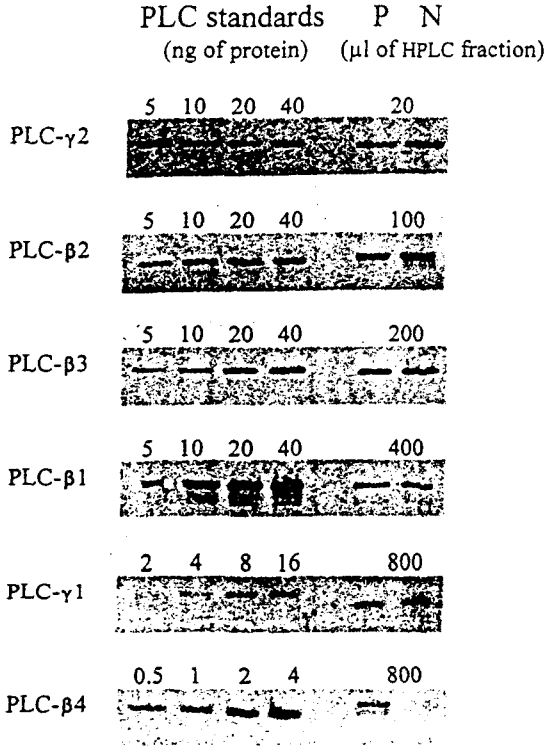


Fig 4. Quantitation of PLC isozymes in pooled HPLC peak fractions from normal and patient platelets. Portions of each HPLC fraction from Fig 3 were first subjected to immunoblot analysis to detect each isozyme. Portions of eight fractions corresponding to each isozyme were then pooled and concentrated. Proteins in the concentrated fractions and four different amounts of each purified PLC isozyme were separated on SDS-polyacrylamide gels and transferred to nitrocellulose filters, which were probed with isozyme-specific antibodies and ^{125}I-protein A. The filters were subjected to autoradiography and the PLC bands are shown. The numbers above each autoradiogram indicate nanograms of PLC standard or microliters of pooled HPLC fractions for patient (P) and normal (N) platelets that were loaded on the gel. The relative radioactivity in the PLC bands was determined with a Phosphoimager. From comparison with the relative radioactivity values of purified PLC, the amount of PLC loaded on the gel was calculated: PLC-γ2, 22 ng (P), and 31 ng (N); PLC-β2, 8.5 ng (P), and 26 ng (N); PLC-β3, 22 ng (P), and 21 ng (N); PLC-β1, 2.4 ng (P), and 2.3 ng (N); PLC-γ1, 2.8 ng (P), and 3.8 ng (N); and PLC-β4, 0.8 ng (P), and 0.2 ng (N).

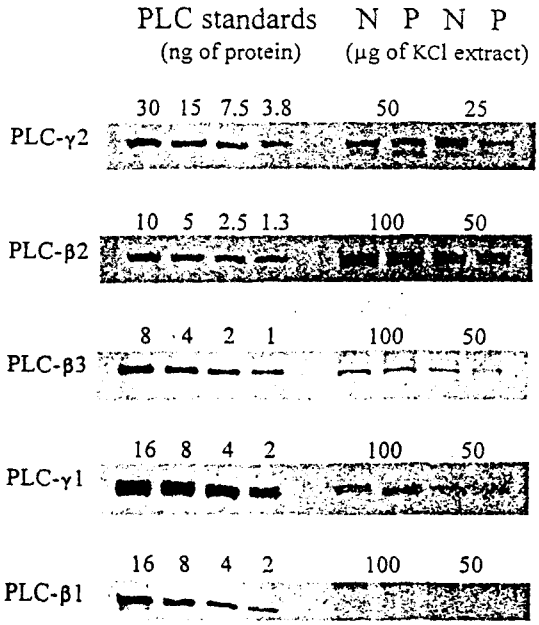


Fig 5. Quantitation of PLC isozymes in KCl extracts of normal and patient platelets. The amounts of PLC isozymes in 2 mol/L KCl platelet extracts were measured directly by quantitative immunoblot analysis. The numbers above each autoradiogram indicate nanograms of PLC standard or micrograms of 2 mol/L KCl extract protein for normal (N) and patient (P) platelets that were loaded on the gel. From comparison with the relative radioactivity values of purified PLC, the amount of PLC loaded on the gel was calculated: PLC-γ2, 13.1 ng (N), and 9.4 ng (P) in 50 μg, and 5.4 ng (N) and 4.8 ng (P) in 25 μg; PLC-β2, 3.7 ng (N), and 1.4 ng (P) in 100 μg, and 2.3 ng (N) and 0.9 ng (P) in 50 μg; and PLC-β3, 1.7 ng (N), and 1.7 ng (P), in 100 gmg, and 0.8 ng (N) and 0.7 ng (P) in 50 μg.

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

	KCl Extract			Total Homogenate		
	Patient	Normal	P/N	Patient	Normal	P/N
	ng/mg of Extract		%	ng/mg of Total Homogenate		%
PLC-γ2	190	204	79	110	150	77
PLC-β2	16	42	38	11	24	41
PLC-β3	16	17	94	12	12	92

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%.

Immunoblot analyses were also performed on samples from two occasions. In the first study, the blots showed PLC-β2 to be decreased. On the second blot, we measured the amount of each PLC isozyme present in pooled HPLC fractions by quantitative immunoblot analysis (Fig 4). The average values from triplicate analyses of two normal individuals and the proband are shown in Table 1. Relative to normal platelets, patient platelets contained approximately one-third the amount of PLC-β2, three times the amount of PLC-β4, three-fourths the amounts of PLC-γ1 and PLC-γ2, and similar amounts of both PLC-β1 and PLC-β3.

To confirm that the differences in the amounts of PLC isozymes observed with the HPLC fractions were not attributable to artifacts of the two column chromatography steps, the amounts of PLCs in the KCl extracts were assessed directly by immunoblot analysis (Fig 5). By comparison with the immunoblot intensities of purified enzymes, the concentrations of PLC-γ2, PLC-β2, and PLC-β3 were estimated and the results (Table 2) were consistent with those obtained by analysis of HPLC fractions. PLC-γ1 was detectable only as a faint band and PLC-β1, PLC-β4, and PLC-δ1 were not detectable at all in the KCl extracts. We also measured the amounts of the three major isoforms in the total homogenates. The reduced expression of PLC-β2 in patient platelets compared to normal platelets could be seen clearly in the total homogenates (Fig 6). The homogenates were extracted with 2M KCl as described in Fig 5, and the resulting extract and pellet were also subjected to immunoblot analysis. The results shown in Fig 6 clearly indicate that the three enzymes were quantitatively extracted by KCl from both normal and patient platelets, suggesting that the reduction of PLC-β2 detected in the KCl extracts of patient platelets was not due to inefficient extraction.

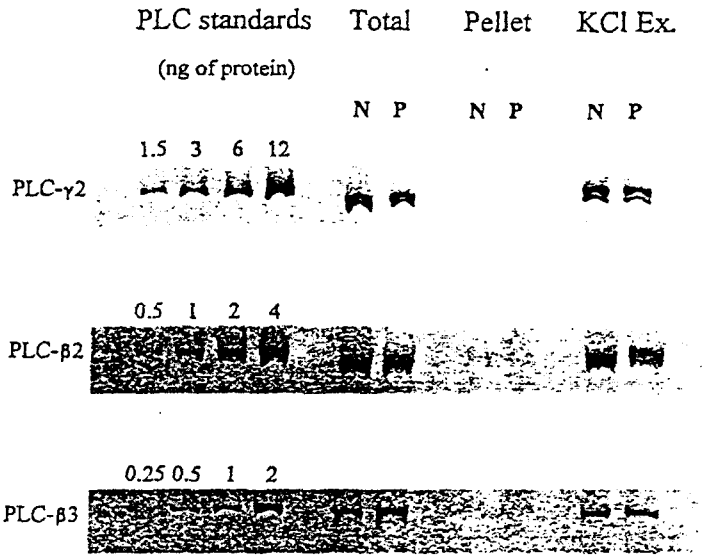


Fig 6. Quantitation of PLC isozymes in total homogenates of normal and patients platelets. Platelets from normal (N) and patient (P) were sonicated in 10 volumes of homogenization buffer (20 mmol/L Hepes [pH 7.2], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, leupeptin (10 μg/mL), aprotinin (10 μg/mL), and calpain inhibitors I and II (each at 20 μg/mL)). Portions of the homogenates (Total) were saved for immunoblot analysis. The remaining portions were adjusted to 2 mol/L KCl by adding solid KCl, stirred for 1 hour at 4°C, and then centrifuged at 100,000*g* for 10 minutes. The resulting supernatant (KCl Ex.) and the pellet (Pellet) were also subjected to immunoblot analysis. The amount of total homogenate loaded on the SDS gel was 50 μg for PLCg2 and 100 μg for PLC-β2 and PLC-β3. The protein amounts represented in the Pellet and the KCl Ex. lanes were derived from 50 μg of total homogenate in the case of PLC-γ2 and 100 μg of total homogenate in the cases of PLC-β2 and PLC-3. Proteins measurements indicate that the KCl extracts carry approximately twice as much proteins as do the pellets. The numbers above each autoradiogram indicate nanograms of PLC standards that were loaded on the gel. The fig shown is representative of three different experiments. From comparison with the relative radioactivity values of purified PLC, the amount of PLC in the total homogenate that was loaded on the gel was calculated: PLC-γ2, 5.9 ng (P), and 7.9 ng (N); PLC-β2, 1.0 ng (P), and 2.2 ng (N); and PLC-β3, 1.1 ng (P), and 1.1 ng (N). The amount of the three isozymes in the KCl extract was also estimated: PLC-γ2, 6.2 ng (P), and 8.1 ng (N); PLC-β2, 0.9 ng (P), and 2.4 ng (N); and PLC-β3, 1.2 ng (P), and 1.4 ng (N). The amount of PLC isozymes in the pellet was too low to be quantitated.

DISCUSSION

The presence of PLC-γ1, PLC-γ2, and PLC-β3 in human platelets has been demonstrated previously,[19, 20, 22-25] although the 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-β2 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-β2 present in corresponding fractions from normal individuals (Fig 4 and Table 1). The PLC-β2 deficiency was also apparent from the direct immunoblot analysis of both 2 M 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 70-80% 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.[26] 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.[26]

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^{2+} release in response to exogenous IP_3.[8, 9] Early platelet responses to these agonists include PLC activation. As shown in the accompanying paper,[10] the generation of IP_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^{2+} release induced by GTPγS 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_2 belong to the superfamily of G protein-coupled receptors. The receptors for thrombin and thromboxane A_2 appear to couple to PLC-β isoforms through $G\alpha_q$ subunits, because antibodies to $G\alpha_q$ inhibit PLC activation elicited by those two agonists.[27,28] The 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 $G\alpha_o$.[28] In addition, treatment of platelets with thrombin or collagen results in tyrosine phosphorylation of PLC-γ2.[22-25] The direct participation of a protein-tyrosine kinase (*Src*) in PAF-induced PLC activation in platelets has also been demonstrated.[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 isozymes by $G\alpha_q$ subunits, PLC-β (except PLC-β4) isozymes by $G\beta\gamma$ subunits, and PLC-γ isozymes by protein-tyrosine kinases. Because PLC-γ2 and PLC-β2 are the major PLC isozymes 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 GTPγS, 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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