Isolation and Characterization of One Soluble and Two Membrane-Associated Forms of Phosphoinositide-Specific Phospholipase C from Human Platelets†

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ABSTRACT: Two forms (mPLC-I, mPLC-II) of phosphoinositide-specific phospholipase C have been purified, 1494- and 1635-fold, respectively, from plasma membranes of human platelets. Purified mPLC-I and mPLC-II had estimated molecular weights by gel filtration and sodium dodecyl sulfate–polyacrylamide gels of 69 000 and 63 000, respectively. Two cytosolic forms (PLC-I and PLC-II) of phosphoinositide-specific phospholipase C were also resolved on a phenyl-Sepharose column. The major cytosolic form present in outdated platelets, PLC-II, was purified to homogeneity by chromatography on Fast Q–Sepharose, cellulose phosphate, heparin–agarose, phenyl-Sepharose, Superose 12, DEAE-5PW, and hydroxylapatite. Purified PLC-II had a molecular weight of 57 000 on sodium dodecyl sulfate–polyacrylamide gels. mPLC-I, mPLC-II, and PLC-II hydrolyzed both PI and PIP_2. The V_{max} for PIP_2 hydrolysis was similar for all three forms of PLC and was approximately 5-fold greater than for PI hydrolysis. The K_m for PIP_2 hydrolysis was also similar for the three enzymes. In contrast, the K_m for PI hydrolysis by PLC-II was 10-fold lower than by mPLC-I and mPLC-II. In addition, antibody prepared against PLC-II did not cross-react with either mPLC-I or mPLC-II. These data indicate that platelets contain membrane-associated phosphoinositide-specific phospholipases C that are distinct from at least one cytosolic form (PLC-II) of the enzyme.

Agonist stimulated phosphoinositide hydrolysis has been demonstrated in a number of cells and tissues (Cockcroft & Gomperts, 1985; Litosch & Fain, 1986; Majerus et al., 1986). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)[1] by phospholipase C (PLC) results in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglyceride, which serve as second messengers for biochemical reactions associated with early events of cellular activation (Streb et al., 1984; Nishizuka, 1984). The phosphoinositide-specific phospholipases C that catalyze the hydrolysis of inositide phospholipids therefore have an important role in signal transduction.

Several studies have indicated the presence of both soluble and membrane-associated PLC (Lee et al., 1987; Bennett & Crooke, 1987; Wang et al., 1987). Cytosolic phosphoinositide-specific PLCs from numerous sources have been isolated and characterized (Hofmann & Majerus, 1982; Takenawa & Nagai, 1981; Ryu et al., 1987; Bennett & Crooke, 1987; Homma et al., 1987). At present the role of the cytosolic enzyme in agonist-coupled PIP_2 breakdown is unclear. The importance of the membrane-associated PLC in signal transduction, however, is supported by the observations that addition of specific agonists to isolated membranes induces the hydrolysis of PIP_2 at physiological Ca^{2+} concentrations (Litosch et al., 1985; Melin et al., 1986; Smith et al., 1987; Baldassare & Fisher, 1986). Membrane-associated PLCs have been purified from murine thymocytes (Wang et al., 1986) and bovine brain (Lee et al., 1987; Katan & Parker, 1987). Recently, two membrane-associated forms of PLC have been identified in human platelets (mPLC-I and mPLC-II), and mPLC-II has been purified to homogeneity (Banno et al., 1988). The physical and functional relationships among the soluble and membrane-associated PLCs in platelets are currently unknown.

In the present study, we have purified two membrane-associated forms (mPLC-I and mPLC-II) and two soluble PLC forms (PLC-I and PLC-II) of PLC from human platelets. We demonstrate that by biochemical and immunological criteria the membrane-associated forms are distinct from the soluble enzymes.

Experimental Procedures

Materials. L-α-Phosphatidyl[2-^{3}H]inositol and phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate were purchased from New England Nuclear (Boston, MA). Phosphatidylethanolamine and phosphatidylinositol were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), and checked for purity on silica gel 60 plates (Merck) (Jolles et al., 1981). PIP_2 was purchased from Sigma Chemical Co. (St. Louis) and purified by chromatography on Neomycin-linked glass beads as described by Schacht (1978). Fast Q–Sepharose and Superose 12 were purchased from Phamacia LKB Inc. (Piscataway, NJ). All other chemicals were obtained from Sigma.

Preparation of Cytosol and Plasma Membranes from Human Platelets. Outdated human platelets were lysed by sonication (Sonic 300 Dismembrator, Artek Systems Corp., Farmingdale, NY) at 90 W on ice for 3 × 30 s in 20 mM Tris-HCl buffer (pH 7.5) with 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM PMSF, and leupeptin (100 ng/mL) (buffer A). The platelet sonicate was centrifuged at 100000*g* for 2 h at 4 °C. Protein content of the supernatant fraction was

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[1] Abbreviations: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PE, phosphatidylethanolamine; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Table I: Purification of Soluble PLC-I and PLC-II from Human Platelets[a]

step	protein (mg)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	purifn (x-fold)	yield (%)
cytosol	9400	24400	2.6	1	100
Fast Q–Sepharose	1530	20700	13.5	5	85
cellulose phosphate	208	15100	72.8	28	61
heparin–agarose	77	12800	165.8	64	52
phenyl-Sepharose					
PLC-I	4.5	1600	351	135	6
PLC-II	10.1	6000	592	228	24
Superose 12 (PLC-II)	2.1	3400	1658	638	14
DEAE-5PW (PLC-II)	0.5	1600	3151	1212	6.6
hydroxylapatite (PLC-II)	0.23	1300	5493	2113	5.3

[a] Phosphoinositide-specific PLC was purified from the 100000*g* supernatant of sonicated human platelets. PLC activity was assayed with [^{3}H]-PI/PE (1:5 mol %) vesicles as described under Experimental Procedures.

adjusted to 10 mg/mL and applied to a Fast Q–Sepharose column as described below. The 100000*g* pellet was resuspended in buffer A containing 0.25 M sucrose, layered onto 25% sucrose, and spun at 100000*g* at 4 °C for 18 h. Membranes were collected from the 25% sucrose/buffer interface, diluted 3-fold with buffer A, and centrifuged at 100000*g* at 4 °C for 1 h. The membrane pellet was then resuspended in buffer A with 2 M KCl and incubated for 2 h at 4 °C. The KCl concentration was adjusted to 1 M with buffer A and the suspension centrifuged for 1 h at 100000*g* at 4 °C. The platelet membrane pellet was resuspended at 10–15 mg of protein/mL in buffer A containing 1% deoxycholate and incubated at 4 °C for 2 h with constant stirring. The suspension was centrifuged at 100000*g* for 1 h and the supernatant dialyzed overnight against 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EGTA, 1 mM PMSF, 100 mM NaCl, and 0.5% sodium cholate for subsequent purification of mPLC-I and -II.

Phospholipase C Assay. Unless stated otherwise, phospholipid vesicles (25–50 μg of phospholipid) containing either [^{3}H]PI/PE (1:5 mol %) or [^{3}H]PIP_2/PE (1:5 mol %) were prepared as described previously (Baldassare & Fisher, 1986). The final assay mixture (150 μL) contained 100 mM Tris–maleate, pH 6.8, 80 mM KCl, and sodium deoxycholate (1 mg/mL). Final Ca^{2+} concentrations were adjusted by the addition of Ca^{2+}/EGTA buffers containing 2.5 mM EGTA (Raaflaub, 1960). Reactions were initiated by the addition of enzyme and incubated for 5 min at 37 °C. The reactions were terminated by the addition of 2 mL of chloroform/methanol/HCl (200:100:0.75 v/v), and vortexed, and 500 μL of the upper phase containing [^{3}H]inositol phosphate was removed for liquid scintillation counting.

Preparation of Antibodies to PLC-II. Antiserum against PLC-II was prepared by immunizing an adult New Zealand white rabbit with 50 μg of purified PLC-II. The rabbit was boosted at 4 and 5 weeks after the primary immunization with 30 μg of PLC-II in Freund's incomplete adjuvant. Serum was obtained 9 weeks after the initial injection and screened by solid-phase radioimmunoassay analyses with purified PLC-II as described by Newman et al. (1985). IgG was then purified by chromatography on protein A–Sepharose (Hjelm et al., 1972).

Immunoprecipitation of PLC Activity. Purified PLCs were incubated with increasing amounts of anti-PLC-II IgG in 50 mM Tris-HCl (pH 7.0), 100 mM KCl, 0.5 mM EGTA, and 1 mg/mL BSA for 30 min at 37 °C. Cross-reactive products were removed by addition of *Staphylococcus aureus* cells followed by centrifugation at 10000*g* for 5 min. Residual PLC activity was then measured with PIP_2/PE vesicles as described above.

Purification of Soluble Phospholipase C Activities (PLC-I, PLC-II). Platelet high-speed supernatant (total protein 10 g) was applied to a Q–Sepharose column (2.5 cm × 30 cm). The column was washed with 1.5 L of buffer A containing 100 mM NaCl. PLC activity was eluted with a 3-L increasing salt gradient of 100–500 mM NaCl. Active fractions were pooled, concentrated to 300 mL with an Amicon PM-10 membrane, dialyzed against 20 mM Hepes (pH 6.8), 1 mM EGTA, 0.1 mM dithiothreitol, and 100 ng/mL leupeptin (buffer B), and further purified by cellulose phosphate chromatography. PLC activity was applied to a cellulose phosphate column (5 cm × 20 cm) equilibrated with buffer B plus 100 mM NaCl and eluted with a linear NaCl gradient (100–500 mM). Active fractions (0.25–0.35 M NaCl) were pooled and dialyzed against buffer B and then applied to a heparin–agarose column (2.5 cm × 15 cm) and eluted with a step gradient of 300 mM NaCl. Heparin–agarose fractions containing PLC activity were pooled, $(NH_4)_2SO_4$ was added to a final concentration of 1 M, the pH was adjusted to 7.6 with 1 M NaOH, and the fractions were applied to a phenyl-Sepharose CL-4B column equilibrated with buffer B plus 1 M $(NH_4)_2SO_4$. The column (2 cm × 30 cm) was washed with buffer B and activity eluted with a decreasing $(NH_4)_2SO_4$ gradient (1 M to 0). Chromatography on phenyl-Sepharose resolved PLC activity into two fractions, PLC-I and PLC-II. PLC-I eluted near the end of the gradient (0.25 M), and PLC-II eluted after completion of the $(NH_4)_2SO_4$ gradient. PLC-I and PLC-II were each concentrated and dialyzed against buffer B plus 100 mM NaCl. Either PLC-I or -II was applied to a Superose 12 column (1.5 cm × 90 cm) and eluted with buffer B plus 100 mM NaCl. PLC-II from the Superose 12 column was pooled and concentrated to 1 mL with an Amicon Centriprep 30 concentrator. The activity was applied to a Beckman DEAE-5PW column and eluted with a linear gradient of NaCl from 200 mM to 1 M. Pooled DEAE-5PW PLC-II fractions (0.25 M NaCl) were dialyzed against 50 mM sodium phosphate buffer (pH 6.8) and 100 mM NaCl and applied to a hydroxylapatite column (1 cm × 6 cm) equilibrated with 50 mM phosphate and 100 mM NaCl, pH 6.8. PLC activity was eluted with an increasing linear phosphate gradient (50–300 mM).

Other Methods. Protein was determined as described by Bradford et al. (1985). Analytic polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Electrophoretic transfer to nitrocellulose sheets and immunoblotting using ^{125}I-labeled protein A was carried out as described by Towbin et al. (1979).

Results

Purification of Soluble PLC Activites (PLC-I, PLC-II) from Platelet Cytosol. Two forms of soluble phospholipase C were identified in human platelets, PLC-I and PLC-II, and PLC-II was purified to homogeneity (Table I). The final preparation

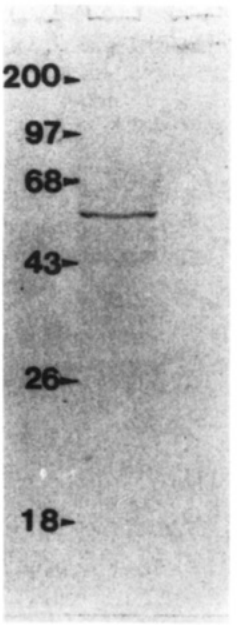

FIGURE 1: SDS–PAGE gel electrophoresis of purified PLC-II. PLC-II (5 μg) was electrophoresed on a 9% SDS–PAGE gel in the presence of mercaptoethanol as described under Experimental Procedures. A similar pattern was seen in the absence of mercaptoethanol (data not shown).

of PLC-II yielded about 200 μg of protein and had a specific activity of 5.493 μmol min^{-1} (mg of protein)$^{-1}$ (Table I). The molecular weight of PLC-II was 58 000 as determined by SDS–PAGE (Figure 1). The other form of the soluble PLC, PLC-I, was separated from PLC-II by hydrophobic chromatography on phenyl-Sepharose CL-4B. The molecular weight of PLC-I as estimated by gel filtration chromatography on Superose 12 was 200 000. Due to its low amount and lack of stability, it was not possible to purify PLC-I to homogeneity.

Physical Properties of PLC-II. Purified PLC-II was stable at protein concentrations above 500 μg/mL when stored at −80 °C. Chromatography of PLC-II on Superose 12 following storage for several weeks at −80 °C at concentrations below 500 μg/mL revealed several higher molecular weight forms. SDS–PAGE analysis, however, continued to yield a single band with molecular weight of 58 000 (data not shown).

Purification of Membrane-Associated Phospholipase C Activities (mPLC-I, mPLC-II) from Human Platelet Membranes. Approximately 15% of the total platelet PLC activity (assayed with PIP_2 as substrate) was found to be associated with purified KCl-washed platelet plasma membranes. This activity was solubilized by incubation of the membranes with 1% deoxycholate (DOC) for 2 h at 4 °C. This treatment released over 85% of membrane-associated PLC activity and approximately 50% of the membrane protein. The DOC extract was precipitated with 25–55%-saturated $(NH_4)_2SO_4$ and redissolved (5 mg/mL protein) in buffer A containing 0.5% sodium cholate (buffer C). The DOC extract was dialyzed overnight against buffer C containing 100 mM NaCl and applied to a Fast Q–Sepharose column (4.0 × 20 cm) equilibrated with buffer C. After the column was washed with 500 mL of buffer C, the PLC activity was eluted with a 1-L linear gradient from 0.1 to 0.5 M NaCl. PLC activity eluted at approximately 0.26 M NaCl (Figure 2). Active fractions were pooled and diluted to 0.15 M NaCl with buffer C and applied to a heparin–agarose column (2.5 × 20 cm). After the column was washed with buffer C containing 0.3 M NaCl, enzyme activity was eluted with 0.5 M NaCl (data not shown). Active fractions were pooled and concentrated to 1 mL with an

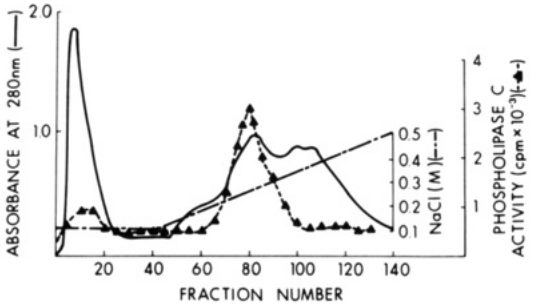

FIGURE 2: Fast Q–Sepharose column chromatography of the deoxycholate extract from human platelet plasma membranes. The deoxycholate extract was applied to a Q–Sepharose column (4.0 cm × 20 cm) and eluted with a linear NaCl gradient (100–500 mM NaCl) as described under Results. The flow rate was 2 mL/min. Fractions were collected, and PIP_2 hydrolysis was measured at pH 7.0 in the presence of 0.1% sodium deoxycholate and 20 μM Ca^{2+}. (-·-) NaCl; (—) absorbance at 280 nm; (▲) PIP_2 hydrolysis.

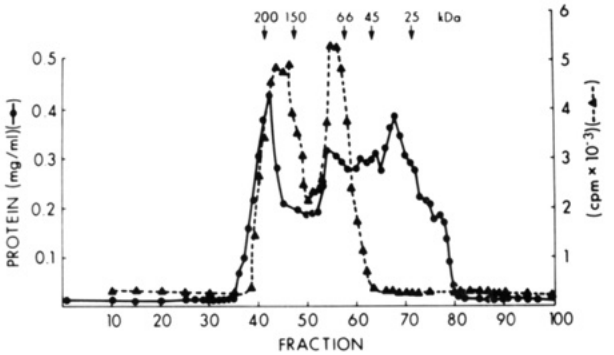

FIGURE 3: Superose 12 gel filtration chromatography of PLC. Fractions eluted from heparin–agarose containing PLC activity were pooled and concentrated to 1 mL with Amicon Centreprep 10 concentrator and applied to a Superose 12 column (1.5 cm × 90 cm). The flow rate was 250 μL/min. Fractions (2 mL) were assayed for PLC activity with PIP_2 as substrate as described under Experimental Procedures. The proteins used to calibrate the column were carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase 150 kDa; and amylase 200 kDa. The exclusion limit was determined with blue dextran, 2 × 10^3 kDa. (●) Protein; (▲) PIP_2 hydrolysis.

Amicon Centriprep 10 concentrator and subjected to gel filtration chromatography on a Superose 12 column (1.5 × 90 cm) equilibrated with buffer C plus 100 mM NaCl. Superose 12 chromatography yielded two peaks of activity, mPLC-I and mPLC-II (Figure 3). mPLC-I eluted between myosin (200 kDa) and alcohol dehydrogenase (150 kDa), and mPLC-II eluted between alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa).

The mPLC-I and mPLC-II activities were pooled separately, each concentrated to 1 mL with an Amicon Centriprep 10 concentrator, and further purified on a Beckman TSK phenyl-5PW column (Figure 4). Both mPLC-I and -II were eluted with a linearly decreasing 20-mL gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in buffer C. mPLC-I and mPLC-II activities eluted as broad peaks toward the end of the gradients (Figure 3). The mPLC-I and -II activities were dialyzed against buffer C, and each was applied to a Beckman DEAE-5PW column eluted with a linear 20-mL NaCl gradient (0.25–0.5M). Both PLC activities eluted at 0.32 M NaCl (data not shown). mPLC-I and mPLC-II were pooled separately, each concentrated to 100 μL, and subjected to gel filtration chromatography on a Spherogel TSK 2000SW column (Toyo Soda). Fractions from this column were analyzed for activity with PIP_2 as substrate and by SDS–polyacrylamide gel electrophoresis (Figures 5 and 6). A single symmetrical protein peak

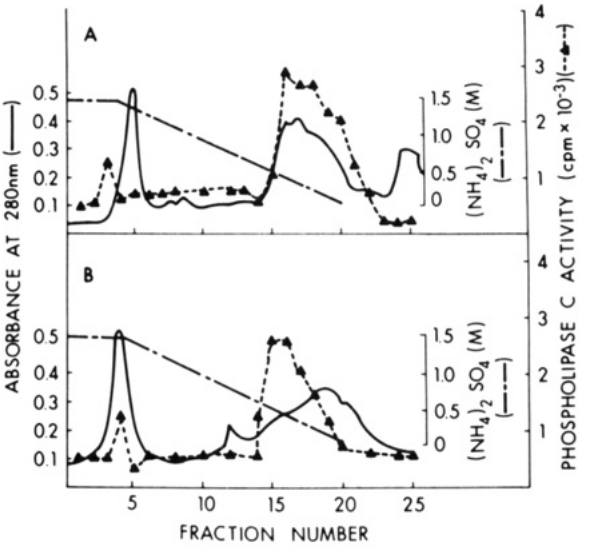

FIGURE 4: Chromatography of mPLC-I (A) and mPLC-II (B) on TSK phenyl-5PW. mPLC-I(A) and mPLC-II(B) eluted from Superose 12 were dialyzed against 1.0 M $(NH_4)_2SO_4$ applied separately to a phenyl-5PW column and eluted with a reverse $(NH_4)_2SO_4$ gradient; the flow rate was 1 mL/min. Fractions of 1 mL were collected. (-·-) $(NH_4)_2SO_4$ concentration; (—) absorbance at 280 nm; (▲) PIP_2 hydrolysis.

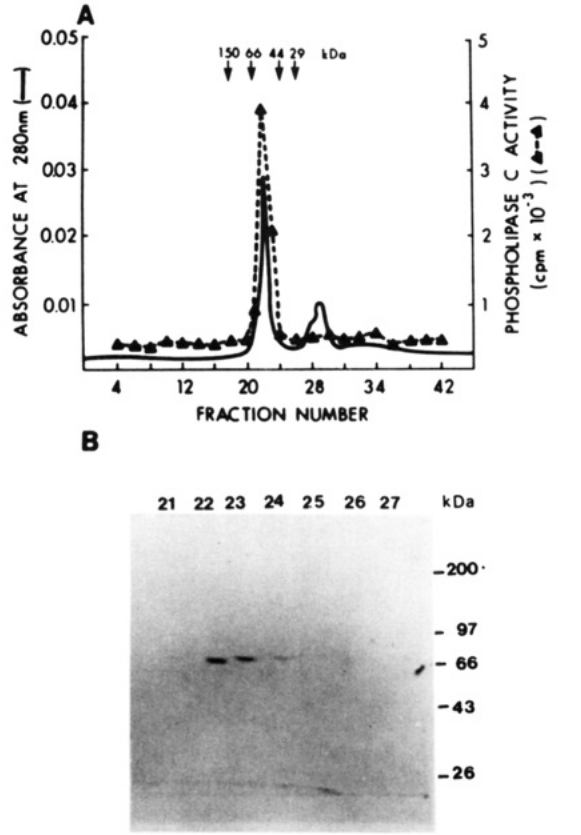

FIGURE 5: Gel filtration chromatography of mPLC-II on TSK 2000SW. (A) mPLC-II from DEAE-5PW column was concentrated with Amicon Centreprep 10 concentrator to 0.1 mL and applied to a TSK 2000SW column. The flow rate was 0.5 mL/min. Fractions of 350 μL were collected, assayed for PLC activity with PIP_2 as substrate, and subjected to SDS–PAGE analysis. Standards used were aldolase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa. (—) Absorbance at 280 nm; (▲) PIP_2 hydrolysis. (B) Aliquots (20 μL) of fractions 19–22 from the TSK 2000 column were subjected to SDS–PAGE as described under Experimental Procedures.

(molecular weight between 66 000 and 44 000) that contained PLC activity was observed with mPLC-II (Figure 5A).

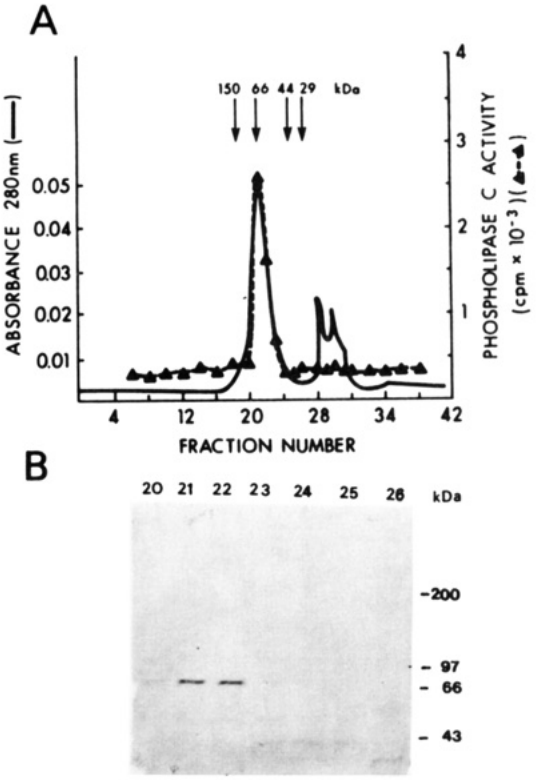

FIGURE 6: Gel filtration chromatography of mPLC-I on TSK 2000SW column. (A) mPLC-I from DEAE-5PW column was concentrated with Amicon Centreprep 10 concentrator to 0.1 mL and applied to a TSK 2000SW column. All other details were the same as described in the legend to Figure 5. (B) Aliquots (20 μL) of fractions 19–22 from the TSK 2000 column were subjected to SDS–PAGE as described under Experimental Procedures.

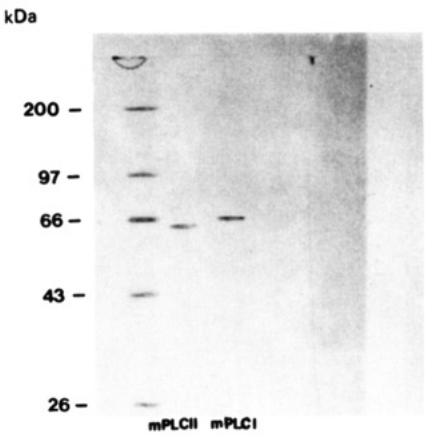

FIGURE 7: SDS–PAGE gel electrophoresis of purified mPLC-I and mPLC-II. Five micrograms of mPLC-I and mPLC-II were electrophoresed on a 9% SDS–PAGE gel in the presence of mercaptoethanol as described under Experimental Procedures. Gels were stained with Coomassie blue. A similar pattern was seen in the absence of mercaptoethanol (data not shown).

Protein peaks at molecular weight less than 29,000 were also seen, but did not contain PLC activity (Figure 5A). A major protein band of 63 000 was detected by Coomassie blue staining of SDS gels in the protein peak containing mPLC-II activity (Figure 5B). A major protein peak was observed with mPLC-I at an apparent molecular weight of 69 000 (Figure 6A). SDS–PAGE analysis showed a single protein band of 69 000 in those fractions containing PLC-I activity (Figure 6B).

Table II summarizes the purification of mPLC-I and mPLC-II. This purification protocol achieved 1494- and

Table II: Purification of mPLC-I and mPLC-II from Human Platelet Plasma Membranes[a]

step	protein (mg)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	purifn (x-fold)	yield (%)
plasma membrane	3058	11620	3.2	–	–
DOC extract	1836	11387	5.1	1.6	100
$(NH_4)_2SO_4$ (30–55)	1005	10757	9.5	2.9	94
Fast Q–Sepharose	218	7679	35.1	10.9	66
heparin–agarose	15	3268	213	66.8	281
Superose 12					
mPLC I	2.2	1033	469	146	9.0
mPLC II	1.8	789	438	137	7.0
phenyl-5PW					
mPLC-I	0.39	418	1080	387	3.6
mPLC-II	0.12	373	3113	972	2.7
DEAE-5PW					
mPLC-I	0.09	309	3457	1080	2.5
mPLC-II	0.03	149	4981	1556	1.2
TSK 2000SW					
mPLC-I	0.03	143	4783	1494	1.2
mPLC-II	0.02	104	5233	1635	0.9

[a] Phosphoinositide-specific PLC was purified from KCl-washed plasma membranes from human platelets. PLC activity was assayed with [^{3}H]-PIP_2/PE (1:5 mol %) vesicles as described under Experimental Procedures.

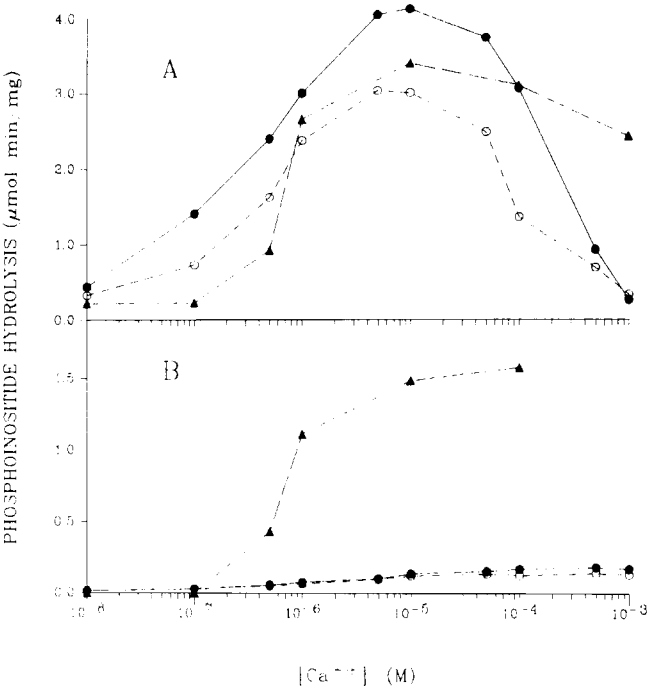

FIGURE 8: Ca^{2+} dependence of PI- and PIP_2 hydrolysis by mPLC-I, mPLC-II, and PLC-II. mPLC-I (70 ng), mPLC-II (80 ng), and PLC-II (90 mg) were incubated with [^{3}H]PIP_2/PE vesicles or [^{3}H]PI/PE vesicles for 10 min at 35 °C. The reaction was stopped, and [^{3}H]inositol phosphates were isolated as described under Experimental Procedures. Calcium concentrations were maintained with Ca^{2+}/EGTA buffers as described under Experimental Procedures. (○) mPLC-I; (●) mPLC-II; (▲), PLC-II. (A) [^{3}H]PIP_2/PE (1:5 mol %). (B) [^{3}H]PI/PE (1:5 mol %). Results are expressed as means for three separate experiments.

1635-fold purifications and 0.8% and 0.6% recoveries of mPLC-I and mPLC-II, respectively. The final preparations were nearly homogeneous as judged by Coomassie blue staining of SDS gels (Figure 7). A minor band at 80 000 was detected on silver-stained gels for PLC-I (data not shown). Silver-stained gels of mPLC-II showed a minor protein band at an apparent molecular weight of 52 000 (data not shown).

Characterization of Soluble and Membrane-Associated PLCs from Human Platelets. The Ca^{2+} requirements for mPLC-I, mPLC-II, and PLC-II were analyzed with PI and PIP_2 as substrates (Figure 8). PIP_2 hydrolysis by mPLC-I, mPLC-II, and PLC-II were Ca^{2+} dependent with maximal rates of hydrolysis between 5 and 10 μM Ca^{2+}. While higher

Table III: K_m and V_{max} Values for mPLC-I, mPLC-II, and PLC-II from Human Platelets[a]

	K_m (mM)		V_{max} (μmol min^{-1} mg^{-1})	
	PI	PIP_2	PI	PIP_2
mPLC-I	0.74 ± 0.15	0.52 ± 0.13	1.34 ± 1.08	8.62 ± 1.09
mPLC-II	0.67 ± 0.12	0.48 ± 0.15	1.29 ± 1.24	9.29 ± 1.17
PLC-II	0.072 ± 0.09	0.41 ± 0.11	2.06 ± 1.12	7.84 ± 1.26

[a] PI and PIP_2 hydrolysis were measured by using purified mPLC-I and mPLC-II obtained from the final step in their purification protocol. The reaction mixture contained 1 mM and 10 mM free Ca^{2+} for PI and PIP_2, respectively. Free Ca^{2+} concentrations were maintained with Ca^{2+}/EGTA buffer as described under Experimental Procedures. PI and PIP_2 hydrolysis was determined as described under Experimental Procedures. Data are mean values ± SE for at least three separate experiments.

Ca^{2+} concentrations inhibited all three enzymes, mPLC-I and mPLC-II were more markedly affected by Ca^{2+} concentrations above 10 μM. Addition of 1 mM Mg^{2+} decreased the activity by approximately 50% throughout the Ca^{2+} concentration curves for all three PLCs (data not shown). PI hydrolysis by mPLC-I, mPLC-II, and PLC-II was also Ca^{2+} dependent with maximal activity at 1 mM Ca^{2+}. In contrast to PIP_2 hydrolysis, addition of Mg^{2+} had little effect on PI hydrolysis (data not shown).

The substrate kinetics for PIP_2 and PI hydrolysis by PLC-II, mPLC-I, and mPLC-II were determined at 10 μM and 1 mM Ca^{2+}, respectively, since these conditions gave maximal activation. The apparent K_m values of mPLC-I were 0.74 mM for PI and 0.52 mM for PIP_2. The K_m values of mPLC-II for PI and PIP_2 were 0.67 and 0.48 mM, respectively. The V_{max} values of mPLC-I were 1.34 μmol of PI and 8.62 μmol of PIP_2 hydrolyzed min^{-1} (mg of protein)$^{-1}$. The V_{max} values of mPLC-II were 1.29 μmol min^{-1} mg^{-1} for PI and 9.29 μmol min^{-1} mg^{-1} for PIP_2. Within error the substrate kinetics of mPLC-I and mPLC-II were virtually identical. The V_{max} values of PLC-II for PI (2.05 μmol min^{-1} mg^{-1}) and PIP_2 (7.84 μmol min^{-1} mg^{-1}) were similar to those reported here for mPLC-I and mPLC-II. The K_m of PLC-II for PIP_2 (0.41 mM) was also similar to those for mPLC-I and mPLC-II. The K_m of PLC-II for PI (0.07 mM), however, was approximately 10-fold less than that found for mPLC-I and mPLC-II.

To determine immunological relatedness among the membrane and cytosolic PLCs, rabbit polyclonal antibodies directed against PLC-II were prepared. Western blot analysis of purified PLC-II with anti-PLC-II antibodies shows a protein band with molecular weight of 57 000 (Figure 9). However,

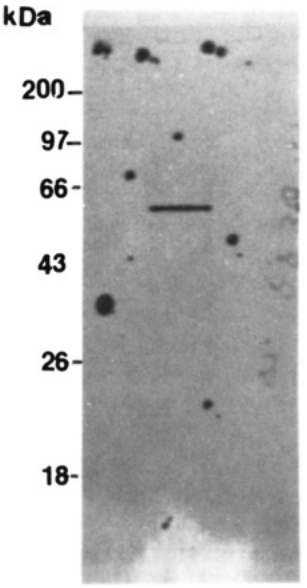

FIGURE 9: Immunoblot of PLC-II. PLC-II was subjected to SDS–PAGE electrophoresis and was immunoblotted with anti-PLC-II antibodies as described under Experimental Procedures.

Table IV: Failure of Antibodies against PLC-II To Inhibit mPLC-I or mPLC-II[a]

	PLC act. (nmol/min)	
	control (normal rabbit serum)	anti-PLC-II IgG
mPLC-I (0.35 μg)	1.08	0.93
mPLC-II (0.2 μg)	1.1	1.12
PLC-I (0.6 μg)	0.66	0.08
PLC-II (0.2 μg)	1.07	0.13

[a] Purified phospholipases C from human platelet cytosol and membranes were incubated for 20 min at 37 °C with 35 μg of rabbit anti-PLC-II IgG or 20 μL of control rabbit antiserum in 100 μL of 50 mM Hepes (pH 7.0) and 100 mM NaCl plus 0.1% DOC; 80 μL of 20% *Staphylococcus aureus* in 50 mM Hepes, pH 7.0, and 100 mM NaCl plus 0.1% DOC was then added. After an additional 30 min, the mixture was centrifuged, and the supernatant fractions were assayed for PLC activity with PIP_2 as substrate as described in Experimental Procedures. Data are average values from two measurements.

Western blot analysis of a crude preparation of PLC-II shows several protein bands, which indicates that our antibody is not monospecific for PLC-II (data not shown). As shown in Table IV, incubation of PLC-I or PLC-II with antibodies directed against PLC-II significantly inhibited PIP_2 hydrolysis by either enzyme preparation. This demonstrates cross-reactivity between the two soluble forms of the enzyme. Incubation of mPLC-I or mPLC-II with antibodies directed against PLC-II, however, had no effect on PIP_2 hydrolysis by either mPLC-I or mPLC-II, indicating that the antibodies do not cross-react with purified mPLC-I or mPLC-II. These data suggest that platelet membranes contain PLC activities that are immunologically distinct from those of the soluble PLCs.

Discussion

Two forms of soluble phosphoinositide-specific phospholipase C have been identified in human platelets. One of these, PLC-II, was purified to homogeneity with a final specific activity of 5.493 μmol min^{-1} (mg of protein)$^{-1}$. The molecular weight of the purified PLC-II by SDS–PAGE was found to be 57 000. PLC-I was resolved from PLC-II by several chromatographic procedures. However, due to its instability and low abundance, this activity was not purified to homogeneity. The molecular weight of PLC-I on Superose 12 was 200 000. The two supernatant enzymes appeared to have similar biochemical properties (Ca^{2+} requirements, pH optima, substrate specificity; data not shown), and both were inhibited by antibody to PLC-II.

Two forms of phosphoinositide-specific phospholipase C, mPLC-I and mPLC-II, were identified and purified from platelet plasma membranes. The molecular weights of mPLC-I and mPLC-II by gel filtration chromatography on a TSK 2000SW column and by SDS–PAGE analysis were 69 000 and 63 000, respectively. The molecular weight for mPLC-II of 63 000 is in agreement with the molecular weight of mPLC-II reported by Banno et al. (1988). Banno et al. (1988) did not isolate mPLC-I to homogeneity but reported an approximate molecular mass of 61 kDa, which is similar to the value reported here for mPLC-I.

Significant differences in the apparent molecular weight of mPLC-I, as determined by gel filtration, were found during the early and late stages of purification. Molecular masses of 170 and 70 kDa were estimated by gel permeation chromatography for crude and purified preparations of mPLCI, respectively. This discrepancy between the molecular masses of the crude and purified mPCL-I was also observed by Banno et al. (1988).

Data from several laboratories have indicated that PLCs can associate with other proteins or be modified during purification. Low et al. (1986) have found that a high M_r form of cytosolic PLC from human platelets is converted to a lower form by a Ca^{2+}-dependent protease. Lenstra et al. (1984) showed that the molecular weight of the soluble PLC from platelets changed from 86 000 in crude extracts to 68 000 when purified in the presence of Triton X-100. In addition, a GTP-binding protein has been shown to dissociate from PLC of calf thymocytes upon addition of detergent (Wang et al., 1987, 1988). We (Baldassare et al., 1988) have also found that a specific GTP-binding protein cochromatographed on heparin–agarose with the soluble PLC from platelets in the absence of detergent. However, the G-protein separated from PLC upon rechromatography on heparin–agarose after addition of 1% sodium cholate (Baldassare et al., 1988). These data suggest that mPLC-I may be associated with other proteins in crude preparations or proteolytically modified during purification.

Little information concerning the relationship between the cytosolic and membrane-associated phosphoinositide PLCs is currently available. Bennett and Crooke (1987) have demonstrated that antibodies specific for PLC-I from guinea pig uterus cytosol (Bennett & Crooke, 1987) cross-reacted with uterus membrane-associated PLC. PLC activities from bovine brain particulate fraction were recognized by antibodies directed against one of the soluble PLC isoenzymes, PLC-I (Lee et al., 1987). The membrane and cytosolic PLC enzymes from murine thymocytes, however, have been distinguished from one another on the basis of pH optima and Ca^{2+} requirements. Our data clearly demonstrate that the membrane-associated PLCs from human platelets are distinct from one of the soluble forms of the enzyme (PLC-II), since the K_m of the soluble enzyme (PLC-II) for PI was 10-fold lower than for the membrane-associated PLCs and also anti-PLC-II antibodies, which inhibited PLC-I and PLC-II activity, did not inhibit either mPLC-I or mPLC-II. We and Banno et al. (1988) have found only two membrane-associated PLCs in human platelets. However, we cannot exclude the possibilities of other membrane-associated forms and that these forms would not be distinct from PLC-II.

The functional roles of the different species of phosphoinositide-specific PLC are currently unknown. Abundant

evidence exists suggesting that agonist activation of PLC is mediated by one or more GTP-binding proteins. Whether all forms of phosphoinositide-specific PLC participate in signal transduction and are regulated by G-proteins is not known. The identification of multiple forms of GTP-binding proteins and PLCs has added to the complexity of this issue. The resolution and purification of these multiple forms is therefore a necessary first step in studying the regulation of PLC by GTP-binding proteins in reconstituted systems.

Registry No. PLC, 63551-76-8.

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