Purification and Characterization of Membrane-bound Phospholipase C Specific for Phosphoinositides from Human Platelets*

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Two peaks (mPLC-I and mPLC-II) of phosphatidylinositol 4,5-bisphosphate (PIP2)-hydrolyzing activity were resolved when 1% sodium cholate extract from particulate fractions of human platelet was chromatographed on a heparin-Sepharose column. The major peak of enzyme activity (mPLC-II) was purified to homogeneity by a combination of Fast Q-Sepharose, heparin-Sepharose, Ultrogel AcA-44, Mono Q, Superose 6-12 combination column, and Superose 12 column chromatographies. The specific activity increased 2,700-fold as compared with that of the starting particulate fraction. The purified mPLC-II had an estimated molecular weight of 61,000 on sodium dodecyl sulfate-polyacrylamide gels. The minor peak of enzyme activity (mPLC-I) was partially purified to 430-fold.

Both enzymes hydrolyzed PIP2 at low Ca2+ concentration (0.1–10 μM) and exhibited higher V_{max} for PIP2 than for phosphatidylinositol. PIP2-hydrolyzing activities of both enzymes were enhanced by various detergents and lipids, such as deoxycholate, cholate, phosphatidylethanolamine, and dimyristoylphosphatidylcholine. The mPLC-I and mPLC-II activities were increased by Ca2+, but not by Mg2+, while Mg2+, Fe3+, Cu2+, and La3+ were inhibitory. GTP-binding proteins (Gα, Gβ, and K-ras protein) had no significant effects on the mPLC-II activity.

A binding of specific agonist to cell surface receptor induces the rapid breakdown of phosphoinositide via a receptor-mediated process (1–4). The hydrolysis proceeds through activation of phosphoinositide-specific phospholipase C (PLC).1. The products of this reaction, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, act as second messengers involved in the activation of protein kinase C, respectively (5, 6). The activation of membrane-bound PLC is considered to be mediated by unspecified guanine nucleotide binding protein (7–14). Cytosolic PLCs have been purified to homogeneity from various sources (15–23) and well characterized, but the mechanism by which they are activated upon receptor stimulation remains to be explored.

The presence of particulate-bound PLC has been indicated by the observations that PIP2 hydrolysis occurs upon stimulation with agonists of isolated membranes (12, 13, 24–27). There are only a few reports on purification of membrane-bound PLC enzymes from murine thymocytes (28) and bovine brain (29). It was recently reported that the membrane-bound PLC seems to be a form of multiple cytosolic enzymes, based on biochemical characterization (30) of immunological analysis (29, 31).

In platelets, the majority of PLC activity was observed to be localized in the cytosol (32–34) and to exist in multiple molecular forms (35–37). Several investigators have recently presented evidence for the existence of membrane-bound PLC by demonstrating that endogenous or exogenous substrates ("H-phosphoinositides) are hydrolyzed in membrane preparations isolated from platelets (25, 38–40). Our previous paper has indicated the presence of a deoxycholate-extractable PIP2-PLC in human platelet membranes (41) and suggested the possible involvement of GTP-binding protein(s) in its activation (42).

In this study, we have extended the previous work and resolved two forms (mPLC-I and mPLC-II) of membrane-bound PLC from human platelets, and achieved purification of one (mPLC-II) of them to homogeneity, and the two enzymes were biochemically characterized.

EXPERIMENTAL PROCEDURES

Materials

Fast Q-Sepharose, heparin-Sepharose CL-6B, Ultrogel AcA-44, Mono Q HPLC (HR 10/10), and Superose 6 and 12 HPLC (HR 10/30) columns were purchased from Pharmacia LKB Biotechnology Inc. PI (soybean), PIP2 (bovine brain), phosphatidyethanolamine (PE, egg yolk), phosphatidylcholine (PC, egg yolk), dimyristoylphosphatidylcholine, phosphatidylserine (bovine brain), and arachidonic acid (porcine liver) were from Sigma. Dicylglycerol (1,2-diol) was from Funakoshi Chemical Company (Tokyo). Platelet membrane lipids were obtained by the method described by Deckmyn et al. (43). [3H]PI (specific activity; 16.3 Ci/mmol) was obtained from Amersham Corporation. [3H]PIP2 (specific activity; 3.5 Ci/mmol) was obtained from Du Pont-New England Nuclear. The purity of lipids was monitored by thin layer chromatography using pre-coated high performance thin layer chromatography plates of Silica Gel 60 (Merck).

Extraction of Phospholipase C from Particulate Fraction of Human Platelets

Outdated human platelet concentrate was centrifuged at 200 × g for 6 min to remove contaminating red blood cells. Platelets were then collected and washed twice with Tris bicarbonate buffer, pH 6.8, containing 5 mM EGTA by centrifugation at 2,000 × g for 15 min. Washed platelets were resuspended in 20 mM Tris maleate buffer, pH 7.0, 5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (buffer A) and were disrupted by sonication on ice for a total
of 2 min with 15-s bursts of a probe-type sonicator (Branson-Sonifier, B-12). After removing unbroken platelets by centrifugation at 3,500 × g for 10 min, the supernatant fraction was subjected to centrifugation at 105,000 × g for 60 min. The resulting pellet was resuspended in buffer A and stored frozen at −80 °C until enough was obtained for purification. The frozen pellets (about 15 g of protein) were thawed and resuspended in buffer A (500 ml) and spurn down the centrifugation at 105,000 × g for 60 min. The washed pellet was resuspended in 2 M KCl in buffer A and stirred for 2 h at 4 °C. Buffer A was then added to adjust KCl concentration to 1 M, and the suspension was centrifuged at 105,000 × g for 1 h. The residual pellet was suspended in buffer A (400 ml) and with an equal volume of buffer A containing 25% sodium cholate and 200 mM NaCl, pH 7.4. After stirring for 2 h at 4 °C, the suspension was sedimented by centrifugation at 105,000 × g for 60 min to obtain a cholae extract for the subsequent purification process.

Purification of Membrane-bound Phospholipase C

All procedures were carried out at 4 °C unless otherwise indicated. Fast Q-Sepharose Column Chromatography—The cholae extract was dialyzed overnight against buffer B containing 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 2 mM EDTA, 0.5 mM PMSF, 0.1 M NaCl, and 0.5% sodium cholate. The dialyzed solution (900 ml, 580 g of protein) was applied to a Fast Q-Sepharose column (4.0 × 25 cm) equilibrated with buffer B. After washing the column with 500 ml of the same buffer to remove unbound proteins, elution was performed with a linear concentration gradient of NaCl from 0.1 to 0.6 M in 1200 ml of buffer B. The majority of PLC activity for PI and PIP$_2$ was eluted between 0.2 and 0.3 M NaCl. The active fractions were pooled and dialyzed overnight against the buffer C: 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 M NaCl, 0.5% PMSF, 1 mM dithiothreitol, and 0.5% sodium cholate.

Heparin-Sepharose Column Chromatography—The dialysate from the Fast Q-Sepharose step was then applied to a heparin-Sepharose column (0.3 × 25 cm) equilibrated with buffer C, and the column was first washed with the same buffer and eluted with a 1000-ml NaCl linear gradient (0.1–0.7 M) in buffer C. The two activity peaks, mPLC-I and mPLC-I1, were eluted at 0.35–0.45 and 0.5–0.6 M NaCl, respectively. The fractions containing mPLC-I and mPLC-I1 were pooled separately and concentrated to 2–4 ml in an Amicon concentrator (YM-10 membrane).

Ultrogel AcA-44 Column Chromatography—The concentrated samples (mPLC-I and mPLC-I1) obtained as above were separately applied to Ultrogel AcA-44 columns (2.5 × 90 cm) equilibrated with buffer C containing 0.3 M NaCl. The active fractions were pooled and concentrated from about 2 ml. The concentrated mPLC-I and mPLC-I1 solutions were dialyzed against salt-free buffer D. The proteins were eluted at a flow rate of 1 ml/min by successive application of the increasing NaCl gradients from 0 to 0.3 M for 5 min, from 0.2 to 0.5 M for 30 min, and from 0.5 to 0.6 M for 5 min using a fast protein liquid chromatography system. The fractions coinciding with PLC activity were pooled and concentrated in a Centricon 10 (Amicon) to 100 μl.

Superox 6-12 Combination Column Chromatography—The mPLC-I and mPLC-I1 (100 μl) solutions pooled from Mono Q column step were separately applied to Superose 6-12 combination columns (HR 10–30) connected in series and equilibrated with buffer D containing 0.1 M NaCl. The columns were eluted with the same buffer at a flow rate of 0.4 ml/min, and 0.2-ml fractions were collected. The active fractions were pooled and concentrated to 50–100 μl in a Centricon 10.

Superox 12 Column Chromatography—The mPLC-II solution (100 μl) obtained from Superose 6-12 combination column was applied to a Superose 12 column equilibrated with buffer D containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml/min, and 0.1-ml fractions were collected. The active fractions were pooled and concentrated to 50 μl. Rechromatography on the same column gave rise to a nearly symmetric activity peak. This peak fraction was concentrated to a volume of less than 100 μl, separated into aliquots, and stored at −80 °C.

Assay for Phospholipase C Activity

Phospholipase C was assayed by measuring formation of radioactive inositol phosphates from [3H]P1 (16.8 Ci/mmol) or [3H]PIP$_2$ (3.5 Ci/mmol) as described previously (37). The standard assay for characterization of biochemical properties of PLCs was essentially performed as follows. The reaction mixture (68 μl) contained Tris maleate buffer (50 mM), pH 6.5, 80 mM KCl, sodium deoxycholate (1 mM/ml), [3H]PIP$_2$ (68,000 dpm; 0.25 mM) or [3H]PIP$_2$ (25,000 dpm; 0.2 mM), and enzyme protein. Final Ca$^{2+}$ concentrations of 1 mM and 10 μM were used for PI and PIP$_2$ hydrolyses, respectively. Where indicated, free Ca$^{2+}$ concentrations were adjusted to the desired level using Ca$^{2+}$/EGTA buffers containing 2 mM EGTA (5 mM concentration) to obtain the appropriate amount of CaCl$_2$ (44). After the incubation for 10 min at 37 °C, the reaction was terminated by addition of 0.24 ml of chloroform:methanol:concentrated HCl (109:100:0.6 by volume) and 0.1 ml of 5 mM EGTA, 1 nM HCl solution. The mixtures were vortex-mixed and then centrifuged for 10 min at 2,000 × g. A 0.2-ml portion of the upper aqueous phase was carefully transferred to a vial, mixed with 6 ml of scintillation fluid, and the radioactivity was determined in a liquid scintillation counter (Beckman LS-9000). The water-soluble reaction products from incubation of the mPLC-I and mPLC-II with PI and PIP$_2$ were analysed by chromatography on an AG 1-8 X10 column (45). More than 90% of the phospholipase C activity and inositol phosphates derived from PI and PIP$_2$ hydrolysis corresponded to the carrier inositol monophosphate and inositol 1,4,5-trisphosphate, respectively.

Other variations to this standard assay were indicated in the figure legends. For the purification steps on various columns described above, PLC activity was assayed using small unilamellar vesicles of PIP$_2$/PE as substrate. The PIP$_2$/PE vesicles were prepared as described by Irvine et al. (46). Briefly, PIP$_2$, PE (1:10, molar ratio), and [3H]PIP$_2$ were mixed in chloroform, evaporated under a nitrogen gas stream, and then dispersed into the assay buffer by vigorous vortexing and sonication for 2 min, to yield 2 nmol of [3H]PIP$_2$ (25,000 dpm) with 20 nmol of PE/assay. To examine the effects of various lipids on PLC activity, each lipid (2 nmol) was added to the assay mixture which contained 6 μM Ca$^{2+}$, 20 mM Tris maleate buffer, pH 6.0, 80 mM KCl, [3H]PIP$_2$ (25,000 dpm; 0.2 mM), and 0.001% sodium cholate.

Protein was measured using the Bio-Rad protein assay with γ-globulin as standard.

GTP-S Binding Activity

The GTP-S binding assay was essentially the same as described previously (42). Ten microliters of the samples was mixed with 90 μl of a solution such that the final concentrations of reagents were 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M dithiothreitol, 100 mM NaCl, 0.1% Lubrol PX, 25 mM MgCl$_2$, 10 μM App[NH]$_3$P, and 0.25 mM [3H]GTP-S (5,000–10,000 cpm/mol). The reaction mixture was incubated at 30 °C for 1 h. The reaction was terminated by rapid filtration on nitrocellulose filters (0.45 μm). The filters were then washed three times with ice-cold 20 mM Tris-HCl buffer, pH 8.0, containing 25 mM MgCl$_2$ and 100 mM NaCl, and re suspended in scintillation fluid for radioactivity measurement.

Other Methods

G, and G$_i$ were purified from rat brain by extraction with sodium cholate followed by successive chromatographies on DEAE-Sephalose, AcA-34 Ultragel, hydroxylapatite (1st), DEAE-Toyopearl 650(s), and hydroxylapatite (2nd) columns under the conditions as described by Katada et al. (47). Ki-ras protein was kindly supplied by Dr. Y. Nakano of Kyowa Hakko Company (Japan).

Analytical polyacrylamide gel electrophoresis was performed by the method of Laemmli (48) in 8–16% linear concentration of polyacrylamide slab gels containing sodium dodecyl sulfate. RESULTS

Purification of Phospholipase C—Our previous study of distribution of PIP$_2$-hydrolyzing activity in human platelet has shown that 20% of the total activity of the homogenate was associated with the particulate fraction (41). About one-fourth of this particulate-associated PIP$_2$-hydrolyzing activity was released by extraction with 2 M KC1. Furthermore, when the residual pellet obtained after the KCl extraction was treated with 1% sodium deoxycholate, more than 70% of the
PIP₂-hydrolyzing activity was solubilized.

In the present study, we have attempted to purify the membrane-bound PIP₂-PLC to homogeneity by modification of the procedure used previously (41). The residual pellet of the 2 M KCl treatment was treated with the buffer A solution containing 1% sodium cholate and 0.1 M NaCl. This treatment released nearly 80% of the PIP₂-PLC activity and about 40% of the total membrane protein. The cholate extract was directly applied to a Fast Q-Sepharose column. The elution profile showed a single major peak of PIP₂-PLC activity eluted between 0.2 and 0.3 M NaCl (Fig. 1A). Analysis of the column fractions for PI-hydrolyzing activity showed the same peak of PLC activity as detected with PIP₂ as substrate. Fractions containing this major PLC activity (70-100 in Fig. 1A) were combined and dialyzed to remove NaCl. The dialysate was then subjected to heparin-Sepharose column chromatography. The chromatography on a heparin-Sepharose column yielded two activity peaks (Fig. 1B): a small broad peak (mPLC-I) eluted between 0.35 and 0.45 M, and a major peak (mPLC-II) eluted between 0.5 and 0.6 M NaCl. A low PLC activity was detected in the unbound flow-through fractions, but this was not used for further purification. The heparin-Sepharose column step was very effective for purification of mPLC-II, because the PLC activity peak came out after elution of the bulk of proteins. On the other hand, the mPLC-I fractions coincided with overlapped proteins.

The mPLC-I and mPLC-II fractions separated on heparin-Sepharose column were subjected to Ultrogel AcA-44 gel filtration chromatography. The mPLC-II enzyme activity was eluted from the column as a single peak at position between NaCl concentration; *-*, absorbance at 280 nm.

FIG. 1. Fast Q-Sepharose column chromatography of the cholate extract from the human platelet particulate (A); heparin-Sepharose column chromatography of PLC obtained from Fast Q-Sepharose column chromatography (B). A, the cholate extract was applied to a Fast Q-Sepharose column and eluted as described under “Experimental Procedures.” Fractions of 7.0 ml were collected. PLC activities were measured at pH 7.0 in the presence of 0.1% sodium deoxycholate and 1 mM Ca²⁺ using [3H]PI as substrate (O--O) and at pH 6.5 in the presence of 0.1% sodium deoxycholate and 6 μM Ca²⁺ using [3H]PIP₂ as substrate (O--O). The active fractions, indicated by a bar, were pooled. ---, NaCl concentration; *-*, absorbance at 280 nm. B, the PLC solution eluted from Q-Sepharose column shown in A was applied to a heparin-Sepharose column and eluted as described under “Experimental Procedures.” Fractions of 5.0 ml were collected. The active fractions comprising peaks, mPLC-I and mPLC-II, were pooled separately. ---, NaCl concentration; *-*, absorbance at 280 nm; O--O, PLC activity for PI hydrolysis; O--O, PLC activity for PIP₂-hydrolysis.

At the subsequent step of HPLC ion exchange column (Mono Q) chromatography, both mPLC-I and mPLC-II were eluted with almost same NaCl concentration (between 0.38 and 0.45 M) as each single activity peak (Fig. 2, A and B). Then, mPLC-I and mPLC-II from Mono Q column were pooled separately and subjected to HPLC gel permeation.

FIG. 3. Superose 6-12 combination HPLC column chromatography of mPLC-I (A) and mPLC-II (B). mPLC-I (A) and mPLC-II (B) obtained from Mono Q column step shown in Fig. 2 were applied to Superose 6-12 combination columns and eluted as described under “Experimental Procedures.” Fractions of 0.2 ml were collected. Arrows indicate the positions of various molecular mass standard proteins (catalase, 240 kDa; aldolase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 25 kDa).

---, PLC activity for PIP₂ hydrolysis; ---, absorbance at 280 nm.

FIG. 2. Ion exchange chromatography on Mono Q HPLC column of mPLC-I (A) and mPLC-II (B). mPLC-I (A) and mPLC-II (B) obtained from Ultrogel AcA-44 columns were applied to Mono Q columns and eluted as described under “Experimental Procedures.” Fractions of 0.5 ml were collected. ---, NaCl concentration; O--O, PLC activity for PIP₂ hydrolysis; ---, absorbance at 280 nm.
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chromatography on Superose 6-12 combination column. The apparent molecular masses of mPLC-I and mPLC-II were estimated to be 61–63 kDa (Fig. 3, A and B). The mPLC-II obtained from the gel filtration column was then applied to a Superose 12 column, yielding a single symmetrical PLC activity peak centered at fraction 37 which had the highest PLC activity (Fig. 4A). Individual fractions from the Superose 12 column were analyzed by SDS-polyacrylamide gel electrophoresis. One major protein band (61 kDa) and other less stained bands (52, 70–71, and 83 kDa) as detected in Fig. 4B were appreciably decreased from the peak of enzyme activity in this rechromatography when analyzed on silver-stained SDS-gels. Thus, the 61-kDa band correlated best with PLC activity.

A summary of the purification steps is shown in Table I. The procedure achieved 2,740-fold purification of the mPLC-II present in the particulate fraction of human platelets, with a specific activity of 6,300 nmol of PIP2 cleaved per min/mg of protein (yield, 0.6%). On the other hand, the mPLC-I preparation obtained from the superose 6-12 combination column chromatography was enriched to 430-fold with a specific activity of 993.0 nmol of PIP2 cleaved per min/mg of protein (yield, 0.5%). Because the enzyme activity of mPLC-I seems to be less stable, further purification was not performed.

Biochemical Properties of the Purified mPLCs—The maximum activities of PIP2-hydrolysis of both mPLC enzymes (mPLC-I and mPLC-II) were obtained at pH 6.5 in the presence of 0.1% deoxycholate. The effects of various detergents and lipids were tested for their ability to support the hydrolysis of PIP2. As shown in Table II, sodium cholate and deoxycholate had pronounced activation effects on the PIP2-hydrolyzing activity of mPLC-I and mPLC-II. Deoxycholate

![Fig. 4. Chromatography on Superose 12 HPLC column of mPLC-II. A, mPLC-II obtained from Fig. 3 was applied to a Superose 12 and eluted as described under “Experimental Procedures.” Fractions of 0.1 ml were collected. -.-, PLC activity for PIP2 hydrolysis; —, absorbance at 280 nm. Molecular standards, aldolase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) were resolved in a parallel Superose 12 column. B, aliquots (20 μl) of fractions 32–39 from the Superose 12 column described above were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were stained with Coomassie Blue. Lanes 32–39 corresponded to fractions 32–39 from the Superose 12 column. Molecular mass standards from top to bottom; phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.5 kDa.

![Fig. 5. SDS-polyacrylamide gel electrophoresis of mPLC-II. Protein bands were visualized by means of the Coomassie Blue staining procedure. Lane 1, mPLC-II obtained by rechromatography on Superose 12 column; lane 2, molecular mass markers (94 kDa, phosphorylase b; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase). DF, dye front.]

| Purification step     | Total activity | Total protein | Specific activity | Yield Purification |
|-----------------------|----------------|---------------|-------------------|--------------------|
|                       | nmol/min       | mg            | nmol/min/mg       | % fold             |
| 105,000 x g pellet    | 33,603.6       | 14,610.3      | 2.3               | 1                  |
| Cholate extract       | 26,210.8       | 5,824.6       | 4.5               | 100                | 1.9               |
| Fast Q-Sepharose      | 17,037.0       | 946.5         | 18.0              | 64.9               | 7.8               |
| Heparin-Sepharose     | 3,525.8        | 131.1         | 26.9              | 13.5               | 11.7              |
| mPLC-I                | 4,591.1        | 27.8          | 165.0             | 17.5               | 71.7              |
| mPLC-II               | 1,310.5        | 17.8          | 73.5              | 5.0                | 32.2              |
| Ultragel AcA-44       | 1,560.1        | 4.1           | 380.0             | 6.0                | 167.4             |
| Mono Q HPLC           | 372.6          | 1.2           | 310.5             | 1.4                | 135.0             |
| mPLC-I                | 727.8          | 0.3           | 2,350.6           | 2.8                | 1,022.0           |
| mPLC-II               | 119.2          | 0.12          | 993.0             | 1.5                | 431.7             |
| Superose 6–12 HPLC    | 327.9          | 0.06          | 5,465.0           | 3.0                | 2,376.0           |
| Purification of mPLC from human platelet membranes

The PLC activity in each step was determined using [3H]PIP2 as a substrate as described under “Experimental Procedures.”

![Table I Purification of mPLC from human platelet membranes](image-url)

**Biochemical Properties of the Purified mPLCs**—The maximum activities of PIP2-hydrolysis of both mPLC enzymes (mPLC-I and mPLC-II) were obtained at pH 6.5 in the presence of 0.1% deoxycholate. The effects of various detergents and lipids were tested for their ability to support the hydrolysis of PIP2. As shown in Table II, sodium cholate and deoxycholate had pronounced activation effects on the PIP2-hydrolyzing activity of mPLC-I and mPLC-II. Deoxycholate
The activities of mPLC-I and mPLC-I1 were examined (Table II). The measurements were performed under "Experimental Procedures." The control values of 100% represent the specific activity of 0.02 nmol/min/mg of protein for mPLC-I and 1.21 nmol/min/mg of protein for mPLC-II. The values were the mean of triplicate experiments.

Table II

| Activator          | Concentration | mPLC-I | mPLC-II |
|--------------------|---------------|--------|---------|
| None               | 100           | 100    |         |
| Deoxycholate       | 1.6 mM        | 271.1  | 545.3   |
|                    | 8.0 mM        | 70.2   | 407.4   |
| Cholate            | 1.6 mM        | 180.6  | 264.5   |
|                    | 8.0 mM        | 542.7  | 308.2   |
| Lubrol PX          | 0.1%          | 136.6  | 342.8   |
| Triton X-100       | 0.1%          | 182.9  | 525.3   |
| Phosphatidylethanolamine (egg yolk) | 304.4 | 613.0 |
| Phosphatidylcholine (dimyristoyl) | 578.9 | 1,187.3 |
| Phosphatidylethanolamine (egg yolk) | 84.5 | 51.2 |
| Phosphatidylethanolamine (bovine brain) | 90.4 | 106.8 |
| Diacylglycerol (1,2-diolein) | 265.2 | 167.2 |
| Arachidonic acid   | 189.5         | 148.9  |         |
| Platelet membrane lipids | 85.3 | 75.3 |

Effects of detergents and lipids on mPLC-I and mPLC-II activities

The PI-hydrolyzing activities were measured as described under "Experimental Procedures" using the same preparations of mPLC-I and mPLC-II as in Table II. The reaction mixture consisted of 0.46 pmol/min/mg for mPLC-I and 3.2 pmol/min/mg for mPLC-II. The values were the mean of triplicate experiments.

Table III

| Activator          | Concentration | mPLC-I | mPLC-II |
|--------------------|---------------|--------|---------|
| None               | 100           | 100    |         |
| EGTA               | 2 mM          | 22.5   | 22.1    |
| EDTA               | 2 mM          | 15.8   | 20.1    |
| EGTA + Ca²⁺       | 2 mM + 1.6 mM | 193.7  | 166.4   |
| EDTA + Mg²⁺       | 2 mM + 1.6 mM | 29.7   | 38.7    |
| Hg²⁺              | 2 µM          | 9.1    | 8.9     |
| Hg²⁺ + dithiothreitol | 2 µM + 20 µM | 133.8  | 111.7   |
| Fe²⁺              | 50 µM         | 80.8   | 58.0    |
| Cu²⁺              | 50 µM         | 35.3   | 35.7    |
| La³⁺              | 50 µM         | 50.0   | 28.0    |
| NaF               | 5 mM          | 185.5  | 163.4   |

Effects of metal ions and chelators on mPLC-I and mPLC-II activities

PI₃-hydrolyzing activities were measured as described under "Experimental Procedures" using the same preparations of mPLC-I and mPLC-II as in Table II. The reaction mixture consisted of 0.46 pmol/min/mg for mPLC-I and 3.2 pmol/min/mg for mPLC-II. The values were the mean of triplicate experiments.

As shown in Fig. 6, PI₃, hydrolyses by mPLC-I and mPLC-II were Ca²⁺-dependent, with their maximal activities at about 10 µM Ca²⁺, and higher Ca²⁺ concentrations (over 0.5 mM) were rather inhibitory for PI₃-hydrolyzing activity. On the other hand, PI hydrolyses by mPLC-I and mPLC-II required millimolar concentrations of Ca²⁺ to obtain their maximum activities (data not shown). Thus, mPLC-I and mPLC-II were capable of hydrolyzing PI₃ at concentrations of free Ca²⁺ (0.1 mM) reported to be present in resting cells. At this Ca²⁺ concentration, mPLC-II showed about 30-fold higher activity to hydrolyze PI₃ than PI.

Under standard assay conditions, the apparent Kₘ values of mPLC-II obtained from a double-reciprocal plot (Fig. 7) were 0.75 (±0.08) mM for PI and 0.50 (±0.17) mM for PI₃ (mean ± S.D. of three experiments). The Vₘₐₓ values were 2.16 (±1.41) and 9.82 (±1.32) µmol of PI and PI₃ cleaved per min/mg of protein, respectively. The Kₘ of mPLC-II for PI was higher than that of platelet cytosolic PLC (60–80 µM).
The addition of PE decreased the $K_m$ for PIP$_2$ to 0.13 (±0.07) mM (data not shown).

**Effects of Nucleotides and GTP-binding Proteins on the mPLC-II Activity**—Neither GTPase activity nor GTP$_7$S-binding activity were detected in the mPLC-II obtained by rechromatography on Superose 12, suggesting that the purified mPLC-II enzyme was free from GTP-binding protein(s). The effects of GTP, GDP, GTP$_7$S, App(NH)$_2$P, and ATP on PIP$_2$ hydrolysis were examined for the mPLC-II in the presence of 2 mM EGTA or 6 μM Ca$^{2+}$. All nucleotides tested exerted small effects (10-20% stimulation) on the PLC activity in a nonspecific manner (data not shown).

The purified rat brain G$_s$, G$_o$, and K$_{i}$-ras protein were tested for effects on PIP$_2$-hydrolyzing activity of purified mPLC-II. The proteins were incubated with 10 μM GTP$_7$S and 5 mM MgCl$_2$ for activation. Each activated GTP-binding protein was added to the mPLC-II assay mixture, but PIP$_2$-hydrolyzing activity was unaffected. Albumin also had no effect on the PIP$_2$-hydrolyzing activity of the purified mPLC-II.

**DISCUSSION**

Many investigations have appeared on purification and characterization of cytosolic PLCs from various sources. Recent reports suggested the existence of several immunologically distinct isoenzymes in cytosol, for example, of sheep seminal vesicles (two forms) (16, 18), bovine brain (three forms) (19, 21, 22) and guinea pig uterus (two forms) (20). However, it has also been argued that multiple activity peaks of cytosolic PLC might be derived from proteolysis (49, 50) or oligomerization of a single enzyme species (19, 22, 56) or its association with other protein components (51, 52).

**Human Platelets**

Hakata et al. (17) reported a single PLC from bovine platelet cytosol with a molecular weight 143,000 as estimated on SDS-polyacrylamide gel. The PLC enzymes with similar molecular masses have been purified from bovine cytosol (PLC-I, 145,000; and PLC-II, 150,000) by Ryu et al. (19, 22). However, these PLCs were thought to be immunologically distinct from the platelet PLC (19). On the other hand, a purified PLC from sheep seminal vesicles (16) exhibited lower molecular mass (65 kDa). Other cytosolic enzymes with similar molecular size were also purified from rat liver (15), guinea pig uterus (20), and calf thymocytes (23); 70, 62, and 68 kDa, respectively. Furthermore, a PLC of 98 kDa was isolated from human platelet cytosol (53) and a purified bovine brain cytosolic PLC-III enzyme had a molecular mass of 85-88 kDa (21, 22). Thus, such variations in molecular mass may reflect tissue specificity or modifications during purification processes.

In contrast to the cytosolic PLC, scarce information has been available regarding purification of membrane-bound PLC. Recently, membrane-bound PLCs have been purified from murine thymocytes (28) and bovine brains (29, 31). The cytosolic PLCs, pig uterus PLC-I (28), and bovine brain PLC-I (31) were reported to be detected by immunoprecipitation in their membrane preparations. An identical protein was thought to be contained in the membrane-bound PLC-M1 and the cytosolic PLC-I of bovine brain (31). As for the molecular size, the mPLC-II of human platelet obtained here was similar to PLCs from murine thymocyte (70 kDa) and pig uterus (62 kDa), but it was distinct from the membrane-bound PLCs from bovine brain (145, 150, and 154 kDa). The molecular size of platelet mPLCs as estimated by gel filtration revealed difference between the crude preparation on Ultrogel AcA-44 and the purified mPLC on Superose 12 column; an apparent molecular mass of 240-110 kDa in the former and 63-61 kDa in the latter. However, when estimated by SDS-polyacrylamide gel electrophoresis, the molecular mass of purified mPLC-II was 61 kDa. Lenstra et al. (54) have observed that soluble PLC of human platelet showed an alteration of its molecular mass from 86 kDa in crude extract to 68 kDa when purified by electrofocusing in the presence of Triton X-100, suggesting that the PLC was associated with about molecular mass 20 kDa protein in crude extract. Furthermore, Wang et al. (51) has recently demonstrated that a GTP-binding protein was dissociated from soluble PLC of calf thymocytes by incubation with 1% sodium cholate. A similar effect might have occurred during purification of platelet mPLCs in the presence of 0.5% sodium cholate. These results suggest us to consider that mPLCs may be present in association with other protein component(s) in the crude preparation or that modifications or truncations may occur during the purification processes.

Activity of PLC has been shown to be modulated by addition of several types of reagents, e.g., detergents (15, 16, 19, 21, 26), phospholipids (15, 18, 28, 55, 56), and fatty acids (15, 55, 57). Similar results were obtained in our experiments with platelet mPLCs (mPLC-I and mPLC-II). PC is known to inhibit PLC activity, but we found that dimyristoyl-PC enhanced PIP$_2$-hydrolyzing activity (Table II). PE was a potent activator for the PIP$_2$-hydrolyzing activity of the platelet mPLCs. These observations suggest that the type of phospholipid molecules adjacent to substrate (phosphoinositides) in the vicinity of the membrane receptor might affect the membrane-bound PLC activity.

Rock and Jackowski (39) have demonstrated that chelators activated PIP$_2$-hydrolyzing activity of the crude human platelet membrane. However, our results showed that the purified mPLCs were inhibited by both EDTA and EGTA (Table III). Nevertheless, divalent cations, such as Hg$^{2+}$, Cu$^{2+}$, and La$^{3+}$, were inhibitory for platelet mPLCs and the inhibition by Hg$^{2+}$ may be due to −SH group of the enzyme, since addition of dithiothreitol completely overcame inhibition by Hg$^{2+}$. The mode of inhibitory action of Cu$^{2+}$ and La$^{3+}$ is unknown and either EDTA or dithiothreitol did not restore the activity (data not shown). These properties of platelet mPLCs resemble those of the bovine brain PLC-I and PLC-II as described by Ryu et al. (19). The human platelet mPLC-I was similar to mPLC-II in enzymatic properties except that the former was much more unstable than the latter. For this reason further purification was not made for mPLC-I.

The purified mPLC-II obtained from rechromatography on Superose 12 column, while the crude preparation was responsive to GTP and GTP$_7$S, was no longer stimulated by the guanine nucleotides. We also observed that this final preparation of mPLC-II had neither GTPase activity nor GTP$_7$S-binding activity (data not shown). Therefore, loss of GTP responsiveness of the purified mPLC-II suggests that an as yet unidentified GTP-binding protein might be associated with the PLC in the crude state. Our previous study showed that PIP$_2$-hydrolytic activity of a partially purified mPLC from human platelets was enhanced by addition of rat brain G$_s$ or G$_o$ (42). However, the mPLC-II purified to homogeneity in the present experiment elicited no significant augmentation in hydrolytic activity of PIP$_2$ by exogenously added G$_s$, G$_o$, or K$_{i}$-ras proteins. The reason for disappearance of responsiveness to GTP-binding proteins of mPLC-II is not known, yet it may be that certain unidentified membrane component(s) could be removed during purification process or that the interaction of the GTP-binding protein(s) with mPLC-II would not be adequate in the reconstitution system employed. To gain more insight into the interaction between GTP-binding protein(s) and membrane-bound PLCs (mPLCs),
GTP-binding proteins should be isolated from human platelets and its purification is under current progress.²

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