Isolation and characterization of a γ-type phosphoinositide-specific phospholipase C (PLC-γ_2)

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A novel bovine spleen phosphoinositide-specific phospholipase C (PLC) has been identified with respect to immunoreactivity with four independent antibodies against each of the PLC isoenzymes, and purified to near homogeneity by sequential column chromatography. Spleen contains three of the isoenzymes: two different γ-types {γ_1 and γ_2, originally named as PLC-γ [Rhee, Suh, Ryu & Lee (1989) Science **244**, 546–550] and PLC-IV [Emori, Homma, Sorimachi, Kawasaki, Nakanishi, Suzuki & Takenawa (1989) J. Biol. Chem. **264**, 21885–21890] respectively} and δ-type of the enzyme, but PLC-γ_1 is separated from the PLC-γ_2 pool by the first DEAE-cellulose column chromatography. Subsequently, PLC-δ is dissociated on the third heparin–Sepharose column chromatography. The purified enzyme has a molecular mass of 145 kDa on SDS/polyacrylamide-gel electrophoresis and a specific activity of 12.8 μmol/min per mg with phosphatidylinositol 4,5-bisphosphate as substrate. This enzyme activity is dependent on Ca^{2+} for hydrolysis of all these phosphoinositides. None of the other phospholipids examined could be its substrate at any concentration of Ca^{2+}. The optimal pH of the enzyme is slightly acidic (pH 5.0–6.5).

INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) catalyses the hydrolysis of PtdIns(4,5)P_2 to generate intracellular second messengers such as diacylglycerol and Ins(1,4,5)P_3 (for reviews see refs. [1,2]). Several forms of PLC with different molecular masses, substrate specificities and Ca^{2+} requirement have been purified from various mammalian tissues [3,4]. These results indicate that multiple species of PLC molecules exist and constitute a PLC family. Recently, molecular cloning has been carried out for four types of PLC [5–9]. Among them, three PLC species (PLC-β, -γ and -δ) have two conserved X- and Y-regions [6–9], which are possibly involved in their basic functions such as catalytic activity. Additionally, PLC-γ contains a region (Z-region) similar in sequence to the *N*-terminal regulatory domain of non-receptor tyrosine kinase of the *src* family [8,9].

Recently, a new cDNA clone of PLC (originally named as PLC-IV [10,11]) has been isolated, and Northern-blotting analysis reveals that this form of PLC is expressed in various tissues. The primary structure deduced from this cDNA is similar to that of PLC-γ. Thus we named this new clone PLC-γ_2, in contrast with PLC-γ_1 for the PLC-γ previously reported [3]. However, PLC-γ_2 has not yet been purified and characterized. In this paper, we report the purification of this new type of PLC from bovine spleen, in which this enzyme has been shown to be most strongly expressed [12], and characterize its biochemical properties.

MATERIALS AND METHODS

Materials

[*Ins*-^{3}H]PtdIns, [*Ins*-^{3}H]PtdIns(4)*P* and [*Ins*-^{3}H]PtdIns(4,5)P_2 were purchased from New England Nuclear. PtdIns, all protease inhibitors used in this study and dithiothreitol were obtained from Sigma Chemical Co. PtdIns(4)*P* and PtdIns(4,5)P_2 were purified as described previously [13]. DEAE-cellulose and cellulose phosphate were obtained from Whatman. Toyopearl (HW 55 SF) was from Tosoh (Tokyo, Japan). The Mono Q column (5/5) was purchased from Pharmacia.

Assay of PLC activity

The assay conditions for PLC activity were as previously described [13]. For kinetic analysis, concentrations of substrates or effectors were varied as indicated in the text.

Purification procedures

Bovine spleen was freshly obtained from a local slaughterhouse. Spleen cells (200 g) were disconnected from fibrous connective tissue and homogenized with a Polytron homogenizer in 5 vol. of homogenization buffer, consisting of 50 mM-Tris/HCl (pH 7.6), 0.25 M-sucrose, 2 mM-EDTA, 0.5 mM-EGTA, 1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-di-isopropyl fluorophosphate, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, 10 μg of bestatin/ml and 10 μg of pepstatin A/ml. The homogenate was centrifuged at 105000 *g* for 1 h at 4 °C, and the supernatant was collected as the starting material.

The high-speed supernatant was diluted with an equal volume of column buffer A, consisting of 20 mM-Tris/HCl (pH 7.4), 1 mM-EDTA and 10 μg of leupeptin/ml, and applied to a DEAE-cellulose column (4.5 cm × 40 cm) previously equilibrated with buffer A. After washing, proteins were eluted with a 1500 ml linear gradient of 0–0.3 M-KCl in buffer A; 10 ml fractions were collected and assayed for activity, and their species was identified by reactivity with each of the antibodies (Fig. 1). Material reactive with anti-PLC-γ_2 antibody was observed at 0.20 M-KCl (Fig. 1*c*). To minimize the presence of PLC-γ_1 in this pool, the latter part of the peak should not be combined.

The pooled PLC-γ_2-containing fractions (fractions 83–95 in Fig. 1; 130 ml) were diluted with an equal volume of buffer A

Abbreviation used: PLC, phosphoinositide-specific phospholipase C.
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and applied to a cellulose phosphate column (2.5 cm × 20 cm) equilibrated with buffer B (0.1 M-NaCl added to buffer A). The column was washed with 5 vol. of the buffer, and then the sample was eluted with a 500 ml linear gradient of 0.1–0.6 M-NaCl in the same buffer; 7 ml fractions were collected and assayed for the activity. The pooled fractions (fractions 22–32 in Fig. 2) were diluted with 2 vol. of buffer A and subsequently applied to a heparin–Sepharose column (1.5 cm × 15 cm) equilibrated with buffer B. Proteins were eluted with a 200 ml linear gradient of 0.1–0.6 M-NaCl; 3 ml fractions were collected and PLC species were determined by immunoblotting. Two peaks of activity were separated, and it became clear that the former and the latter peaks contained PLC-γ_2 and PLC-δ respectively (Fig. 3).

The pooled fractions (fractions 17–25 in Fig. 3) were divided into three parts, and each part was subjected to gel filtration on Toyopearl HW 55 (3.5 cm × 90 cm), which was previously equilibrated with buffer B containing 0.1 % (w/v) sodium cholate. The sample was eluted in the same buffer, and 3 ml fractions were collected. The activity was detected as a single peak in fractions 120–127. These fractions were pooled and applied to a Mono Q column equilibrated with buffer B. The column was washed with 10 ml of buffer B and developed with a 25 ml linear gradient of 0.1–0.6 M-NaCl, at a flow rate of 1 ml/min; 1 ml fractions were collected and samples were assayed for the activity. PLC activity was detected at 0.20 M-NaCl as a single peak.

Antisera

Specific antibodies against four PLC isoenzymes were independently obtained as follows: four independent cDNA clones encoding four isoenzymes of rat PLC were isolated [11,12]. Expression of their fragments (amino acids 131–625 for PLC-β [7], 517–901 for PLC-γ_1 [9], 220–581 for PLC-δ [7] and 4–1265 for PLC-γ_2 [11]) in *Escherichia coli* was carried out as described previously [14]. PLC-β, -γ_1, -γ_2 and -δ proteins were separated on SDS/6 %-polyacrylamide-gel electrophoresis [15]. The stained band of PLC was excised as gel strips and ground into paste. PLC protein was eluted by sonication from the paste into 10 mM-Tris/HCl (pH 7.5) buffer containing 1 mM-EDTA, and freeze-dried. The freeze-dried PLC was independently dissolved and injected into New Zealand White rabbits with Freund's complete adjuvant at 3-week intervals. A sample of serum was taken from the rabbits and checked for its anti-PLC activity by Western blotting and immunostaining analysis.

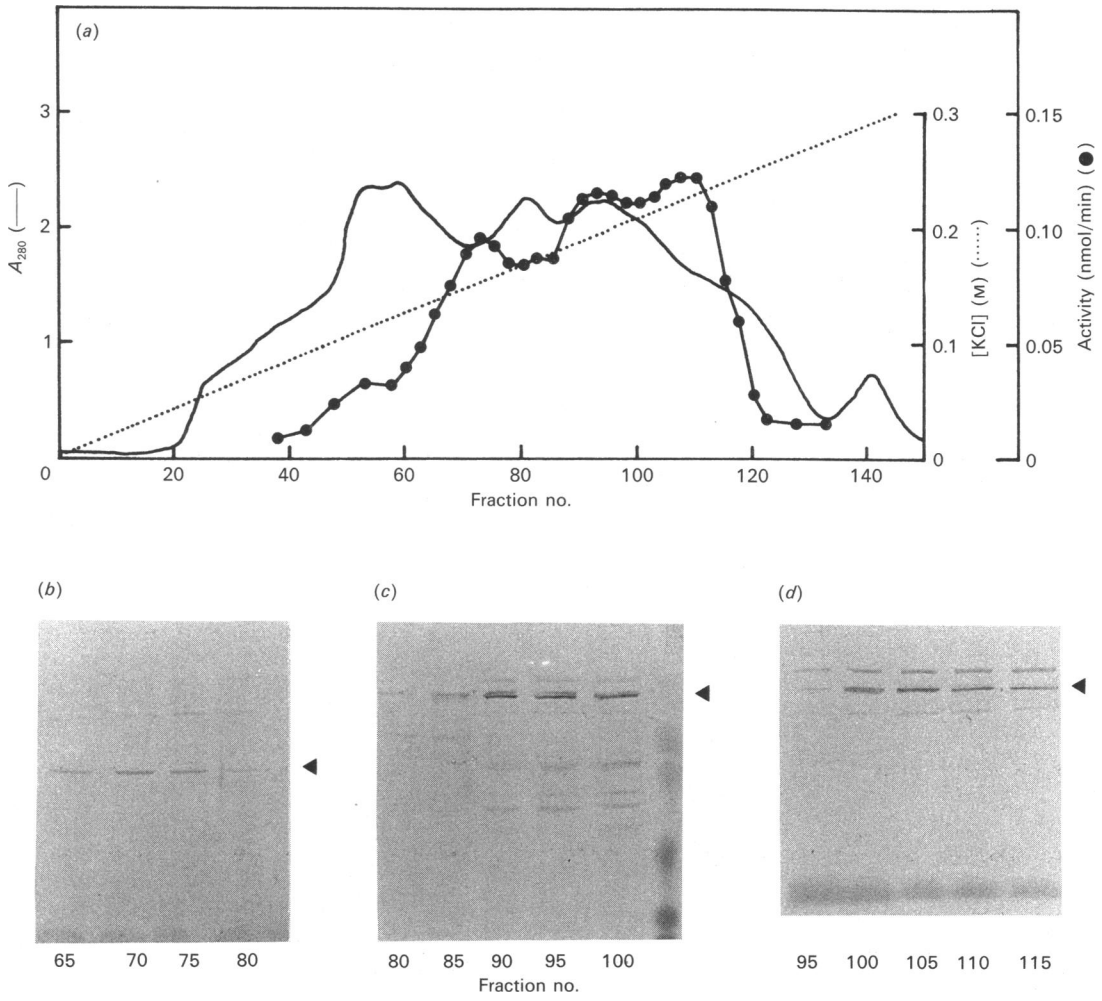

Fig. 1. DEAE-cellulose column chromatography

The crude extract was subjected to DEAE-cellulose column chromatography as described in the Materials and methods section (*a*). A sample (2 μl) of each fraction was assayed for PLC activity. A portion of each fraction was also investigated for PLC isoenzymes by immunoblotting with anti-PLC-δ (*b*), with anti-PLC-γ_2 (*c*) and with anti-PLC-γ_1 (*d*) antisera. Locations of isoenzymes are indicated by arrowheads.

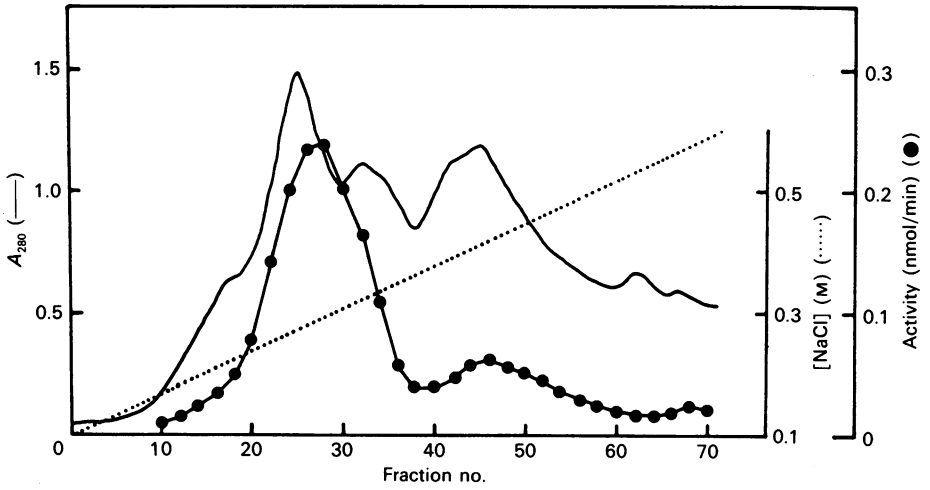

Fig. 2. Cellulose phosphate column chromatography

PLC-γ_2-containing fractions after DEAE-cellulose chromatography were applied to a cellulose phosphate column. A sample (2 μl) was assayed for the activity. Details are described in the Materials and methods section.

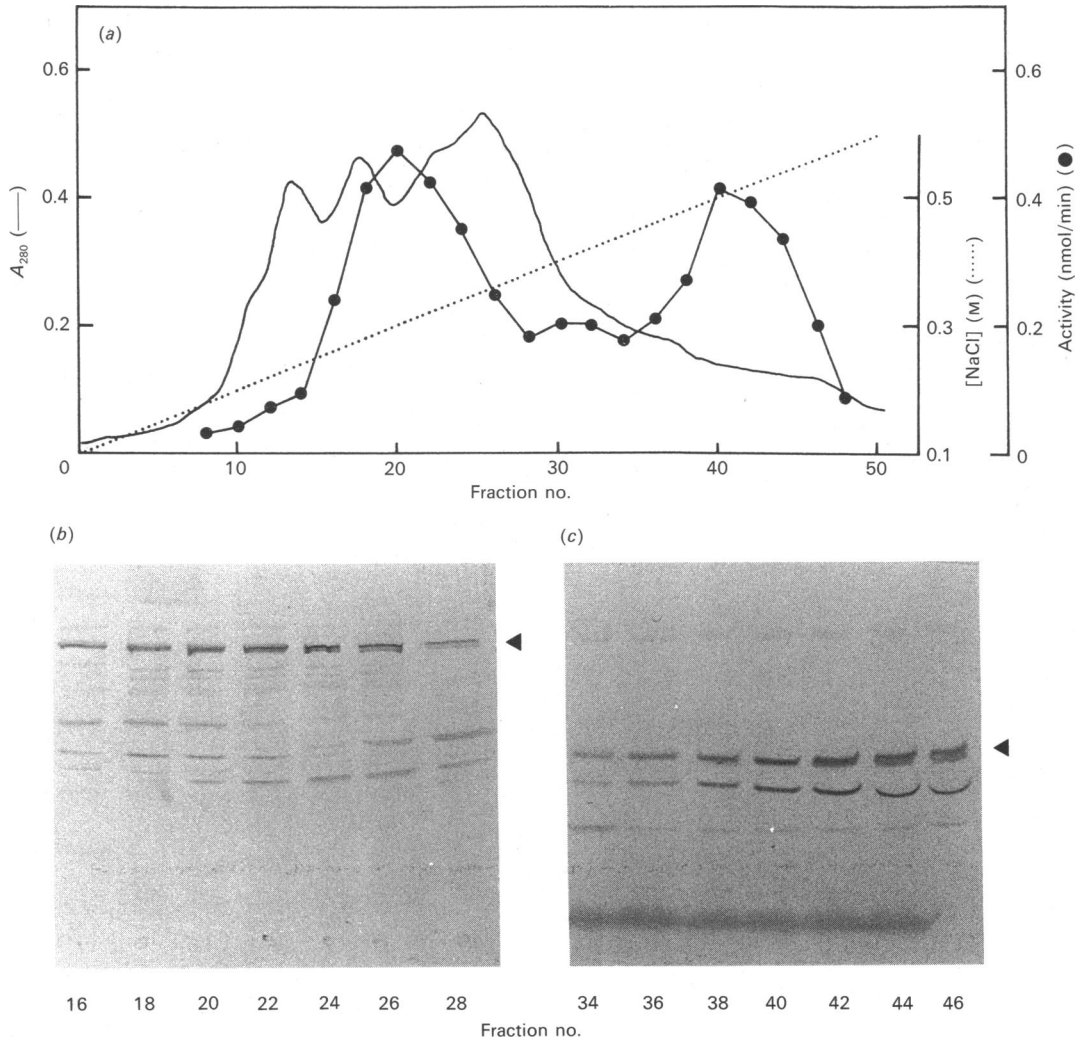

Fig. 3. Heparin–Sepharose column chromatography

The former peak of the activity from cellulose phosphate was pooled and applied to a heparin–Sepharose column (*a*). A sample (2 μl) of each fraction was assayed for the activity. A portion of each fraction was also investigated for isoenzymes with anti-PLC-γ_2 (*b*) and with anti-PLC-δ (*c*) antisera. Locations of isoenzymes are indicated by arrowheads.

Table 1. Purification of PLC-γ_2 from bovine spleen cells

Purification step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification (fold)	Yield (%)
Crude extract	16000	41.8*	0.00249	1	100
DEAE-cellulose	1300	17.8*	0.00913	3.67	42.6
Cellulose phosphate	125	6.88*	0.0550	22.1	16.5
Heparin–Sepharose	10.5	4.07*	0.388	156	9.74
Toyopearl HW 55	0.642	0.976	1.52	610	2.33
Mono Q	0.033	0.426	12.8	5140	1.02

* The relative contribution of each enzyme to the total activity was estimated from the area of each activity peak in Figs. 1, 2 and 3.

RESULTS

Purification of PLC-γ_2

To identify PLC isoforms, we developed specific antibodies against four types (β, γ_1, γ_2 and δ) of PLC. These antibodies were independently developed in rabbits which were immunized by the PLC proteins produced in *E. coli*. They reacted only with their antigenic proteins, but did not react with other PLCs (results not shown). They were also effective for determination of mammalian PLC isoforms. In each purification step, samples were employed for immunoblotting analysis with these antibodies as well as for measuring PtdIns(4,5)P_2-hydrolysing activity.

As shown in Fig. 1(*a*), several peaks of activity were eluted from the DEAE-cellulose column. To identify the PLC isoenzyme responsible for each peak, fractions were analysed by immunoblotting with four different antibodies. Although immunoreactive fractions were not observed with anti-PLC-β antibody in all fractions, the other three isoenzymes were detected by each of their specific antibodies (Figs. 1*b*–1*d*). PLC-δ was eluted from the column first, then PLC-γ_2 was found in fractions 80–105, and it was followed by PLC-γ_1. When active fractions were pooled for purification of PLC-γ_2 by the next chromatography, it was important to minimize contamination of PLC-γ_1 in the sample pool. On the contrary, the presence of PLC-δ in the same pool does not affect the purification, because PLC-δ can be separated from PLC-γ_2 on heparin–Sepharose chromatography.

On cellulose phosphate column chromatography (Fig. 2), both PLC-γ_2 and a relatively small amount of PLC-δ were co-eluted and formed the former peak (0.30 M-NaCl). Although another minor peak of the activity was observed at 0.42 M-NaCl, none of the four antibodies could detect any type of PLC by immunostaining, suggesting that the latter peak might contain PLC-α or an unidentified enzyme.

PLC-γ_2 and -δ could be completely separated on heparin–Sepharose (Fig. 3). The PLC-γ_2-containing pool was further purified by gel filtration. In this step, the activity migrated as a single peak, eluted coincidentally with a dimeric form of bovine serum albumin. The activity was finally purified and concentrated on a Mono Q column, yielding a single peak of activity. Our procedure achieved 5140-fold purification and 1 % recovery. The purification steps are summarized in Table 1. The purified enzyme has a specific activity of 12.8 μmol/min per mg with PtdIns(4,5)P_2 as substrate.

The final preparation was checked by SDS/polyacrylamide-

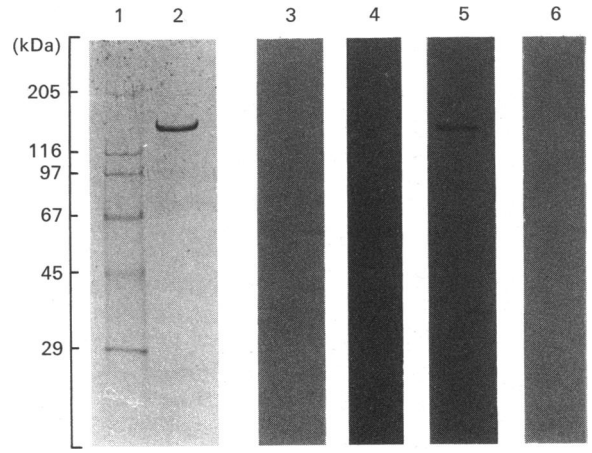

Fig. 4. Purity and immunoreactivity

The final preparation was subjected to SDS/polyacrylamide-gel (5–15 %) electrophoresis. The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular-mass standards: from the top, myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin and carbonic anhydrase; lane 2, the final preparation. Immunoreactivity was examined with antibodies against PLC-β (lane 3), PLC-γ_1 (lane 4), PLC-γ_2 (lane 5), or PLC-δ (lane 6).

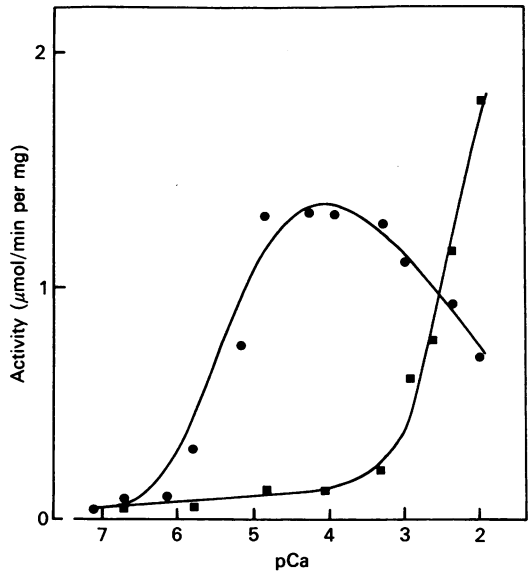

Fig. 5. Effect of Ca^{2+} on rate of hydrolysis of PtdIns and PtdIns(4,5)P_2

The activity was measured in buffer containing various concentrations of Ca^{2+} and 100 μM-PtdIns (■) or -PtdIns(4,5)P_2 (●). Various free Ca^{2+} concentrations below 10 μM were stabilized by using Ca–EGTA buffer [13]. The assay buffer was adjusted to pH 7.0.

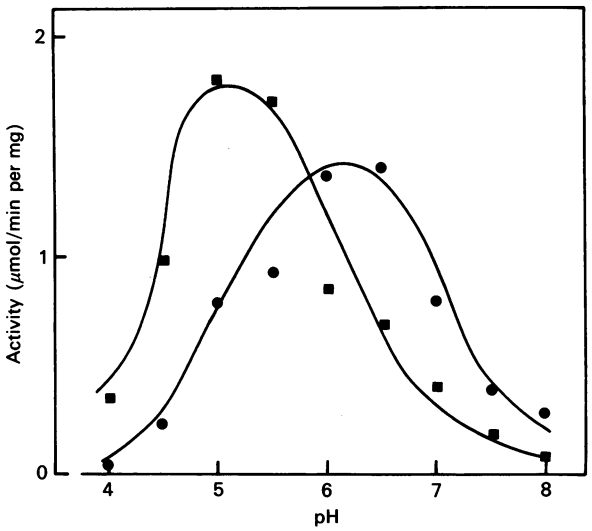

Fig. 6. Effect of pH on rate of hydrolysis of PtdIns and PtdIns(4,5)P_2

The activity was measured at various pH values. The reaction mixture contained 100 μM- or 10 mM-Ca^{2+} for the hydrolysis of PtdIns (■) or PtdIns(4,5)P_2 (●) respectively. The concentration of substrates was 100 μM.

gel electrophoresis (Fig. 4), and PLC-γ_2 was found to have an apparent molecular mass of 145 kDa. When the final preparation was analysed for its immunoreactivity with four independent antibodies, it reacted only with anti-PLC-γ_2 antibody, and no cross-reactivity was observed with other antibodies.

Characterization of PLC-γ_2

A sample of the enzyme preparation was assayed for requirement of Ca^{2+} for phosphoinositide-hydrolysing activity. As shown in Fig. 5, when PtdIns (4,5)P_2 was used as substrate, PLC-γ_2 had an optimal Ca^{2+} concentration of 100 μM. On the other hand, this enzyme was fully activated by 10 mM-Ca^{2+} with PtdIns as a substrate. Through the entire concentration ranges of Ca^{2+} examined, this enzyme could not hydrolyse phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine or phosphatidic acid. This PLC isoenzyme also hydrolysed three kinds of phosphoinositides in a pH-dependent manner (Fig. 6). The optimal pH was 5.0 and 6.5 for hydrolysis of PtdIns and PtdIns(4,5)P_2 respectively. At a neutral pH and 100 μM-Ca^{2+}, apparent K_m values for PtdIns and PtdIns(4,5)P_2 were 110 μM and 160 μM respectively. The activity was also measured in the two different conditions optimal for PtdIns hydrolysis (200 μM-PtdIns, pH 5.0 and 10 mM-Ca^{2+}) and for PtdIns(4,5)P_2 hydrolysis [200 μM-PtdIns(4,5)P_2, pH 6.5 and 100 μM-Ca^{2+}], and the apparent $V_{max.}$ values for PtdIns and PtdIns(4,5)P_2 were 18.1 and 12.8 μmol/min per mg respectively.

DISCUSSION

In the present study, we established a purification method for PLC-γ_2 from bovine spleen cytosol and examined its biochemical properties. In order to identify and distinguish it from other isoenzymes, we obtained specific antibodies against four PLC proteins independently produced in *E. coli* which expressed the corresponding rat PLC cDNA. These antibodies are revealed to be specific for their recognition and convenient for immunoblotting analyses. On the other hand, we chose spleen as the starting material for this purification, because PLC-γ_2 is mostly abundant in spleen, as shown by Northern-blotting analysis [12].

The above purification procedure yields an apparently homogeneous enzyme preparation, which catalyses the hydrolysis of PtdIns(4,5)P_2 with a specific activity of 12.8 μmol/min per mg. The recovery of this enzyme, however, is 1%, which is unexpectedly low, probably owing to the presence of digestive proteases derived from phagocytes. Recently, our preliminary results have demonstrated that γ_2-type enzyme can also be purified from thymus by the same procedure as described here and that the recovery of the enzyme is higher than from spleen. Therefore thymus, rather than spleen, might be recommended as a starting material for PLC-γ_2 purification.

In addition to PLC-γ_2 purification, we succeeded in purification of either PLC-γ_1 or PLC-δ from the peak of activity by the methods prevously reported by Ryu *et al.* [16,17]. These results and those obtained from Northern blotting [12] indicate that these three isoenzymes can contribute to PLC activity in the spleen cytosol. Additionally, since some minor peaks of the activity were separated during the whole purification process for three isoenzymes, it is still possible that spleen contains other types of the enzyme.

The physiological role of each PLC isoenzyme is not yet clear. However, the primary structures of PLC-γ_1 and PLC-γ_2 are quite similar, both of which contain a regulatory domain similar to the *N*-terminus of *src*-related tyrosine kinases. This fact suggests that the activation of these two isoenzymes should be regulated in a similar manner and that they exhibit similar functions. On the other hand, PLC-γ_2 is abundantly expressed in spleen, thymus and lung, whereas PLC-γ_1 is widely distributed in a variety of tissues [12]. These facts suggest both two enzymes may be involved in distinct pathways of cellular signal transduction. Recently, it has been reported that phosphorylation of PLC-γ_1 is induced with the stimulation of epidermal growth factor [18–21] or platelet-derived growth factor [20] by their receptor kinases. Structural similarity suggests that phosphorylation might also occur in PLC-γ_2 and exhibit a central role in cell growth.

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