Purification and characterization of phosphoinositide-specific phospholipase C from bovine iris sphincter smooth muscle

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Two forms (I and II) of phosphoinositide-specific phospholipase C (PLC) were purified from the cytosol of bovine iris sphincter by sequential chromatography on DEAE-Sepharose, EAH-Sepharose, heparin-Sepharose, Sephacryl S-200 gel filtration and Mono Q HR columns. The final step resulted in specific activities of PLC-I and PLC-II of 4.3 and 5.9 μmol of phosphatidylinositol (PI) cleaved/min per mg of protein, which represented up to 295-fold purification compared with that of the starting supernatant. The purified enzymes were further investigated for the presence of isoenzymes and characterized for molecular mass, substrate specificity, pH, Ca^{2+} requirements and kinetic parameters. Using monoclonal antibodies, PLC-I was identified as PLC- δ_1 . The apparent molecular mass of PLC-I as determined by SDS/PAGE and gel filtration was 85 kDa. PLC-II contained an apparently invisible protein band that reacted with the antibody against PLC- γ_1 , and a major 109 kDa protein band that was not recognized by any of the PLC monoclonal antibodies. Further purification of PLC-II by size-exclusion h.p.l.c. resulted in elution of the enzyme activity as a single peak which corresponded to 109 kDa position. Again, this PLC activity was not recognized by any of the PLC monoclonal antibodies. However, the 109 kDa protein activity was recognized by a polyclonal antibody raised against a rat PLC- γ_1 fragment (amino acids 1272–1287), thus suggesting that this protein is a

proteolytic product of PLC- γ_1 . PLC- δ_1 and PLC- γ_1 were identified in the supernatant fraction and PLC- β_1 in the membrane fraction of the iris sphincter. Although immunologically different, the catalytic properties of PLC-I and PLC-II were quite similar. The V_{max} and K_m values for phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis were three to five times greater than those for PI hydrolysis. Both forms preferred PIP and PIP_2 over PI and both were inactive against phosphatidylcholine. With PIP_2 as substrate, the optimal pH values for PLC-I and PLC-II were 6.5 and 7.5 respectively. Unlike PIP_2 , PI hydrolysis by both forms was dependent on the presence of free Ca^{2+} . The maximal hydrolysis of PI and PIP_2 by both forms occurred at 200 and 5 μM Ca^{2+} respectively. Incubation of the purified enzymes with the catalytic subunit of protein kinase A (PKA) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ resulted in increased phosphorylation of PLC-I and PLC-II, but it had no inhibitory effect on their enzyme activities. PLC in iris membranes did not act as a substrate for PKA. These studies indicate that the iris sphincter smooth muscle contains PLC- γ_1 , PLC- δ_1 , PLC- β_1 and a 109 kDa PLC which is a proteolytic product of PLC- γ_1 . The catalytic activities of these enzymes can be affected by pH, Ca^{2+} concentration and probably protein phosphorylation. The functional roles of the various PLC isoenzymes in signal transduction and cyclic AMP inhibition of smooth-muscle contraction has yet to be determined.

INTRODUCTION

In smooth muscle, as in several other tissues, interactions between a number of Ca^{2+} -mobilizing agonists and their appropriate receptors result in stimulation of phosphoinositide-specific phospholipase C (PLC), via a G-protein, and the production of the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), from phosphatidylinositol 4,5-bisphosphate (PIP_2), and contraction [1–4]. In the past few years a great deal of progress has been made in the purification and characterization of PLC from a wide variety of tissues [5–9]. Thus PLCs have been purified and characterized from liver [10–12], rat brain [11–14], bovine brain [15–19], rabbit brain [20], sheep seminal vesicular glands [21], platelets [22–26], porcine lymphocytes [27], *Drosophila* eye [28], rod outer segment [29], bovine heart [30,31], guinea-pig uterus [32], cultured vascular smooth muscle [33] and bovine aorta [34]. Evidence from direct protein isolation, immunological characterization and molecular cloning studies suggest the existence of four major types of PLC in mammalian tissues (α , β , γ and δ) all of which are single polypeptides [6]. The molecular masses of these isoenzymes determined by gel-filtration chromatography and SDS/PAGE are 62–68 kDa for PLC- α , 150–154 kDa for PLC- β , 145–148 kDa for

PLC- γ and 85–88 kDa for PLC- δ [6]. These isoenzymes have been shown to differ in subcellular distribution, Ca^{2+} requirement, pH-dependence and substrate specificity. There is accumulating evidence which indicates that PLC is coupled to its various receptors via GTP-binding proteins [35–38] and that it can be regulated through protein phosphorylation [6,39].

We have previously investigated the properties of PLC in cytosolic and membranous fractions from rabbit iris smooth muscle [40,41]. The PLC isoenzymes and their properties have not yet been investigated in this tissue. Recently, the occurrence of two distinct PLC forms in smooth-muscle preparations has been reported for guinea-pig uterus [32] and cultured vascular smooth-muscle cells [33], and three forms of the enzyme were separated from the cytosol of bovine aorta [34]. Cloning techniques have identified the low-molecular-mass peaks in the uterus [32] and the vascular smooth-muscle cells [33] as PLC- α . Over the past several years we have investigated in detail the role of PLC in signal transduction in the iris smooth muscle [2,39], and more recently we have demonstrated that agonist-stimulated IP_3 production and contraction in this tissue are inhibited by agents that raise the level of cyclic AMP (cAMP) [39]. Although the site of this inhibition is thought to be at the PLC level, its mechanism remains unknown. The purpose of the present study

Abbreviations used: PLC, phosphoinositide-specific phospholipase C; PI, phosphatidylinositol; PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_3 , inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; PKA, protein kinase A; DTT, dithiothreitol; GTP[S], guanosine 5'-[γ -thio]triphosphate; cAMP, cyclic AMP; PMSF, phenylmethanesulphonyl fluoride; TTBS, 1% gelatin/20 mM Tris/500 mM NaCl/0.05% Tween 20.

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was: (a) to purify and characterize PLC from the bovine iris sphincter, (b) to identify, through immunoblotting, the PLC isoenzymes in this tissue, and (c) to determine whether the purified PLC can serve as a substrate for cAMP-dependent protein kinase (PKA) and to show if PLC activity is attenuated as a result of the cAMP-dependent phosphorylation.

MATERIALS AND METHODS

Materials

Phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), PIP₂, catalytic subunit of PKA (bovine heart), leupeptin, aprotinin and phenylmethanesulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Monoclonal antibodies anti-(bovine PLC- β_1), anti-(bovine PLC- γ_1) and anti-(bovine PLC- δ_1) were from Upstate Biotechnology Inc., Lake Placid, NY, U.S.A. A polyclonal antibody raised against a PLC- γ_1 fragment was a gift from Dr. Alan Tarver. Mini-PROTEAN II Ready Gels (7.5%) for SDS/PAGE and goat anti-mouse IgG immunoblot assay kit were from Bio-Rad Laboratories, Richmond, CA, U.S.A. DEAE-Sepharose CL-6B, EAH-Sepharose 4B, heparin-Sepharose, Sephacryl S-200 and Mono Q HR 5/5 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. TSK gel G3000SW h.p.l.c. column was purchased from TOSOH AAS, Montgomeryville, PA, U.S.A. [³H]PI (specific radioactivity 5.2 Ci/mmol), [³H]PIP (specific radioactivity 4 Ci/mmol), [³H]PIP₂ (specific radioactivity 5.4 Ci/mmol) and [γ -³²P]ATP (specific radioactivity 3000 Ci/mmol) were obtained from DuPont New England Nuclear, Boston, MA, U.S.A. All other chemicals used were of reagent grade.

PLC assays

During purification, PLC activity was routinely assayed by measuring the formation of radioactive inositol phosphate from [³H]PI [2,32]. The reaction mixture, in a final volume of 100 μ l, contained 50 mM Tris/maleate (pH 7.0), 2 mM EGTA, 0.1 mM free Ca²⁺, 0.1% sodium deoxycholate, 100 μ M [³H]PI (30000 d.p.m.) and an appropriate amount of the enzyme. The substrate was prepared by mixing chloroform solutions of PI and [³H]PI in an appropriate ratio and evaporating the solvent under a stream of N₂. The phospholipid was suspended in the reaction buffer containing sodium deoxycholate by sonication. The reaction was initiated by addition of the enzyme preparation. The assays were conducted at 37 °C for 10 min and then terminated by the addition of 0.5 ml chloroform/methanol/conc. HCl (50:50:0.3, by vol). This was followed by the addition of 0.15 ml 1 M HCl containing 5 mM EGTA. The reaction mixture was thoroughly mixed and centrifuged. A portion (0.4 ml) of the upper aqueous phase was removed and counted for radioactivity. PLC activity against PIP or PIP₂ was assayed as described for PI except that the substrate concentration in the reaction mixture was 240 μ M.

Preparation of soluble and microsomal membrane fractions from bovine iris sphincter muscle

Bovine eyes, obtained from a local slaughterhouse, were transported to the laboratory packed in ice. The cornea was removed and the sphincter muscle of the iris was carefully cut and washed in ice-cold buffered saline. For each preparation of PLC, we employed approx. 200 g (wet weight) of the iris

sphincter. The methods of homogenization and subcellular fractionation were essentially the same as described previously [40]. Briefly, the tissues were first minced with scissors and suspended in 10 vol. of the buffer (pH 7.4) which contained 0.25 M sucrose, 10 mM Tris/HCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 10 μ g/ml leupeptin, 5 μ g/ml aprotinin and 0.1 mM dithiothreitol (DTT). The tissues were homogenized for 4 $\times$ 30 s with a Super Dispax tissue mixer model SDT-182 (Tekmar Co.) at two-thirds of the maximum speed. The homogenate was centrifuged at 600 g for 10 min. The pellet was discarded and the supernatant centrifuged at 10000 g for 30 min. The supernatant was removed and centrifuged again at 110000 g for 90 min. This yielded a soluble fraction (supernatant) and a microsomal fraction (pellet). The microsomal fraction was suspended in an appropriate volume of 50 mM Tris/maleate (pH 7.0)/50 mM KCl/2 mM EGTA/0.1 mM DTT, and the soluble fraction was dialysed overnight against 2 litres of 10 mM Tris/HCl/0.2 mM EDTA/20% glycerol/0.1 mM DTT (pH 7.6; buffer A). The dialysed soluble fraction was used as a source of PLC for purification.

Purification of PLC from the soluble fraction

The method used for purification of PLC was essentially the same as described by Bennett and Crooke [32] for guinea-pig uterus. Unless noted otherwise, all purification procedures were carried out at 4 °C.

Step 1: DEAE-Sepharose column chromatography

The dialysed supernatant was applied to a DEAE-Sepharose CL-4B column (5 cm $\times$ 20 cm) which had previously been equilibrated with buffer A. The column was washed with buffer A until the A_{280} returned to baseline. At this time, the column was eluted with 1200 ml of a 0–0.5 M linear concentration gradient of KCl in buffer A at a flow rate of 0.5 ml/min. Fractions (5 ml each) were collected and assayed for PLC activity. Fractions containing the enzyme activity were pooled separately for individual peaks, and then dialysed against 2 litres of 20 mM Hepes/20 mM NaCl/2 mM EGTA/20% glycerol/0.1 mM DTT (pH 6.8; buffer B).

Step 2: EAH-Sepharose chromatography

The dialysed samples were applied to an EAH-Sepharose 4B column (2.6 cm $\times$ 19 cm) previously equilibrated with buffer B. After washing with the same buffer, the elution was carried out with a 0.02–0.55 M linear gradient of NaCl in buffer B (300 ml) at a flow rate of 0.3 ml/min. The fractions (3 ml each) were collected and assayed for the enzyme activity. The fractions containing PLC activity were pooled and dialysed against 20 mM Hepes/50 mM KCl/0.5 mM EGTA/20% glycerol/0.1 mM DTT (pH 6.8; buffer C).

Step 3: Heparin-Sepharose chromatography

The dialysed samples from the previous step were applied to a heparin-Sepharose column (1.6 cm $\times$ 24 cm) equilibrated with buffer C. The column was washed with 250 ml of the same buffer and then eluted with 200 ml of 0.05–0.55 M linear gradient of KCl in buffer C at a flow rate of 0.3 ml/min. The fractions corresponding to enzyme activity were pooled and concentrated to about 3 ml by an Amicon ultrafiltration cell equipped with YM-30 filter.

Step 4: Sephacryl S-200 chromatography

The concentrated samples were applied to a Sephacryl S-200 column (1.6 cm × 90 cm) equilibrated with 20 mM Tris/HCl/75 mM KCl/0.2 mM EGTA/20% glycerol/0.1 mM DTT (pH 7.3; buffer D). The enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min and fractions (2.4 ml each) were collected and assayed for PLC activity.

Step 5: Mono Q column chromatography

The pooled samples from the previous step were diluted 1:1 with buffer E (20 mM Tris/HCl/1 mM EGTA/0.1 mM DTT, pH 7.6) and then concentrated to a volume of about 4 ml using an Amicon ultrafiltration cell fitted with a YM-30 filter. This procedure of diluting and concentrating the sample was repeated twice. Finally, the sample was injected into a Mono Q HR 5/5 f.p.l.c. column that had been equilibrated with buffer E. The proteins were eluted at a flow rate of 1 ml/min with a compound gradient from 0 to 1 M KCl in buffer E. This chromatographic step was carried out at room temperature. Fractions (1 ml each) were collected and immediately placed in ice. After PLC assay, the fractions containing enzyme activity were pooled, divided into suitable portions and stored frozen at -80 °C.

Immunoblots

Suitable samples from the purified PLCs and from the microsomal and soluble fraction were mixed with 2× sample buffer [125 mM Tris/HCl (pH 6.8)/20% glycerol/4% SDS/8 mM DTT] and heated in boiling water for 5 min. The proteins were separated on an SDS/polyacrylamide gel containing 7.5% polyacrylamide by the method of Laemmli [42]. The protein bands were visualized with Coomassie Blue R-250 or transferred to nitrocellulose membrane and probed with PLC antibodies. The nitrocellulose membranes were blocked with 3% gelatin in 20 mM Tris/500 mM NaCl (pH 7.5) for 1 h at room temperature. The blocked nitrocellulose membranes were incubated with monoclonal antibodies anti-(bovine PLC- β_1) (2 μ g/ml), anti-(bovine PLC- γ_1) (1 μ g/ml) or anti-(bovine PLC- δ_1) (1 μ g/ml) in 1% gelatin/20 mM Tris/500 mM NaCl/0.05% Tween 20 (pH 7.5; TTBS) overnight. Unbound antibodies were removed by washing the membrane twice in TTBS. The antibodies bound to nitrocellulose were detected by alkaline phosphatase-conjugated goat anti-mouse IgG and the chromogenic substrate 5-bromo-4-chloro-3-indoyl phosphate and Nitro Blue Tetrazolium.

Further purification of PLC-II by TSK G3000SW h.p.l.c. column chromatography

A sample of PLC-II was applied to a TSK gel G3000SW h.p.l.c. column (0.75 cm × 60 cm) pre-equilibrated with 50 mM Tris/HCl buffer (pH 7.5) containing 0.3 M NaCl. Protein was eluted at a flow rate of 0.3 ml/min and fractions of 0.15 ml were collected and assayed for PLC activity. Suitable samples of the fractions were subjected to SDS/PAGE and the proteins were either stained with silver stain or transferred to nitrocellulose membrane and probed with PLC antibodies.

Phosphorylation of PLC by cAMP-dependent protein kinase *in vitro*

Phosphorylation of purified PLC or microsomal fraction by the catalytic subunit of PKA was as follows: protein (400 ng) was

incubated, in a total volume of 50 μ l, with 50 mM Tris/maleate/50 mM KCl/2 mM EGTA/5 mM MgCl₂/0.1 mM free Ca²⁺/4 mM DTT (pH 7.0)/20 μ M [γ -³²P]ATP (specific radioactivity 100 Ci/mmol) in the presence or absence of a catalytic subunit of PKA at 37 °C for 5 min. The reactions were stopped by adding 2× sample buffer and proteins were processed for SDS/PAGE as described in the previous section. The radioactive protein bands corresponding to PLC were cut and counted for radioactivity.

In experiments where the effect of phosphorylation on activity of PLC was investigated, the enzyme protein was preincubated with unlabelled ATP in the presence or absence of PKA before assaying for PLC activity.

Other methods

In experiments where the effects of Ca²⁺ on PLC activity were to be determined, the low concentration of free Ca²⁺ in the assay mixture was maintained by using Ca²⁺/EGTA buffers. The buffers contained 2 mM EGTA, and the appropriate amount of CaCl₂ was added to give the desired concentration of free Ca²⁺. The free Ca²⁺ concentrations were calculated using the Bathe Constituents Program [43]. Protein concentration was determined by the method of Bradford [44] with BSA as standard.

RESULTS

Subcellular distribution of PLC in the bovine iris sphincter

To examine the subcellular distribution of PLC in the bovine iris sphincter, we assayed the enzyme activity against PI and PIP₂ in the various subcellular fractions. As shown in Table 1, the PIP₂-specific PLC activity was two to three times greater than the PI-specific PLC activity in all of the subcellular fractions investigated. The specific activity of PIP₂-PLC in the membranous fraction was about three times as high as that of the soluble fraction. In general, about 90% of the total PLC activity was recovered in the soluble fraction and the remainder was associated with the membrane fraction. We employed the soluble fraction for the purification of the enzyme.

Table 1 Subcellular distribution of PLC in bovine iris sphincter

Bovine iris sphincters were homogenized and centrifuged at 600 *g* for 15 min. The pelleted nuclear material and cell debris were discarded and the homogenate (nuclear supernatant) was centrifuged at 110 000 *g* for 60 min. The pelleted material was taken as the membrane fraction and the supernatant as soluble. The PLC activity, with PI and PIP₂ as substrates, was assayed as described in the Materials and methods section. Specific activity is given in nmol/min per mg of protein. The data are the means $\pm$ S.E.M. of three separate determinations.

Fraction	PI hydrolysis		PIP ₂ hydrolysis	
	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Total activity (nmol/min)
Homogenate (nuclear supernatant)	16 $\pm$ 1	3800 $\pm$ 27	33 $\pm$ 1	7300 $\pm$ 57
Membrane	55 $\pm$ 1	120 $\pm$ 2	179 $\pm$ 2	410 $\pm$ 11
Soluble (110 000 <i>g</i> supernatant)	39 $\pm$ 2	3800 $\pm$ 36	63 $\pm$ 1	6200 $\pm$ 54

Figure 1 DEAE-Sepharose chromatography of bovine iris sphincter soluble fraction

The dialysed 110000 *g* supernatant was loaded on to a DEAE-Sepharose column and eluted with an increasing KCl gradient (----) in buffer A. The fractions (5 ml each) were collected and assayed for PLC activity (●) as described in the Materials and methods section. —, A_{280} . The fractions corresponding to PLC-I and PLC-II were pooled separately and subjected consecutively to EAH-Sepharose, heparin-Sepharose, Sephacryl S-200 and Mono Q column chromatography.

Figure 2 Sephacryl S-200 chromatography

PLC-I (a) and PLC-II (b) fractions from the heparin-Sepharose column were pooled, concentrated to a volume of 3.0 ml and then loaded on to a Sephacryl S-200 column. The molecular markers used were: β -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa. The fractions were collected and assayed for PLC as described in the Materials and methods section. ●, PLC activity; —, A_{280} .

Figure 3 Mono Q column chromatography

The fractions corresponding to PLC-I (a) and PLC-II (b) were pooled, concentrated and applied to a Mono Q HR 5/5 f.p.i.c. column as described in the Materials and methods section. The proteins were eluted with a compound gradient from 0 to 1 M KCl (----). The fractions were pooled and stored frozen at -80°C . ●, PLC activity; —, A_{280} .

Purification of PLC

When the dialysed 110000 *g* supernatant was applied to the DEAE-Sepharose column and then washed with a 0–0.5 M linear gradient of KCl, the PLC activity was separated into two peaks (Figure 1). The minor peak (fractions 130–155) was eluted at 0.29 M KCl and designated PLC-I and the major peak (fractions 156–200) was eluted at 0.35 M KCl and designated PLC-II. The fractions corresponding to the two peaks were pooled separately and then subjected to further purification. The enzymes were loaded on to an EAH-Sepharose column and eluted with a linear gradient of NaCl. PLC-I and PLC-II were eluted individually as single peaks (results not shown). Fractions corresponding to PLC-I and PLC-II were pooled and then applied to a heparin-Sepharose column. PLC-I was bound to the heparin-Sepharose with a higher affinity and was eluted as a single peak with buffer containing 0.47 M KCl (results not shown). PLC-II, on the other hand, was eluted as a broad peak with 0.2–0.3 M KCl (results not shown). Fractions containing PLC_s activities were pooled and concentrated to a small volume and then chromatographed on a Sephacryl S-200 column. The column had previously been calibrated with standard proteins of known molecular mass. PLC-I (Figure 2a) and PLC-II (Figure 2b) were eluted from the column with apparent molecular masses of 92 kDa and 109 kDa respectively. The peak fractions were pooled, concentrated and then chromatographed on a Mono Q HR 5/5 ion-exchange f.p.i.c. column. On elution, several protein peaks emerged from the column, but the PLC-I (Figure

Table 2 Purification of PLC-I and PLC-II from bovine iris sphincter smooth muscle

The PLC activity after each purification step was determined using PI as substrate as described in the Materials and methods section.

Step	PLC	Protein (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Yield	Purification
110 000 g supernatant		1922	40.1	0.020	100	1
DEAE-Sepharose	I	215	5.3	0.025	13	1.2
	II	646	22.7	0.035	57	1.7
EAH-Sepharose	I	64	5.0	0.077	12	3.7
	II	129	9.4	0.073	23	3.5
Heparin-Sepharose	I	3.0	3.9	1.3	9.7	65.1
	II	15.0	9.9	0.66	25	33.0
Sephacryl S-200	I	1.83	3.0	1.64	7.4	80.9
	II	3.62	5.1	1.4	13	70.7
Mono Q HR 5/5	I	0.28	1.2	4.3	3.0	217.9
	II	0.29	1.7	5.9	4.1	295.0

3a) and PLC-II (Figure 3b) enzyme activities were eluted as single peaks with buffers containing 0.23 M and 0.38 M KCl respectively.

Typical data obtained from the above purification steps are summarized in Table 2. The final step (Mono Q HR) yielded approximately 300 μg of protein of each of PLC-I and PLC-II from 1.92 g protein in the starting 110 000 g supernatant. The final specific activities ($\mu\text{mol}/\text{min}$ per mg of protein) for PLC-I and PLC-II were 4.3 and 5.9 respectively. Although these specific activities represent a 218-fold (PLC-I) and 295-fold (PLC-II) increase in purification as compared with the starting cytosolic enzyme activity, the recovery of PLCs was only about 7%.

SDS/PAGE analysis and immunochemical properties of bovine iris sphincter PLCs

Although PLC-I and PLC-II were eluted at different positions in column chromatography indicating that we are dealing with two isoenzymes, the purity and identity of the PLCs were investigated by means of SDS/PAGE and immunoblotting. Figure 4(a) shows SDS/PAGE analysis of PLC-I and PLC-II. One major protein band corresponding to a molecular mass of 85 kDa and a minor band corresponding to a molecular mass of 54 kDa were observed in PLC-I. In PLC-II there was one major protein band corresponding to a molecular mass of 109 kDa and one minor band in the 76 kDa region. When samples from the 110 000 g supernatant and deoxycholate extracts from the membrane fraction were subjected to SDS/PAGE, several protein bands of varying molecular mass were observed (results not shown). The separated proteins from PLC-I, PLC-II, 110 000 g supernatant and microsomal membrane fractions were transferred to nitrocellulose paper and probed with monoclonal antibodies raised against PLC- β_1 , γ_1 and δ_1 from bovine brain. As can be seen from Figure 4b, PLC- δ_1 antibody reacted with the 85 kDa band in PLC-I; PLC- γ_1 antibody recognized an invisible protein band in the 145 kDa region of PLC-II, and none of the monoclonal antibodies recognized the major 109 kDa protein band in this enzyme preparation. However, a polyclonal antibody raised against a rat PLC- γ_1 fragment (amino acids 1272–1287) gave a positive reaction with the 109 kDa protein band (results not shown), suggesting that this protein is a proteolytic product of PLC- γ_1 . Monoclonal PLC- γ_1 antibody also reacted with a protein band corresponding to 145 kDa, and PLC- δ_1 antibody with the 85 kDa protein (results not shown) in the 110 000 g supernatant;

PLC- β_1 antibody reacted with a protein band corresponding to 150 kDa in the microsomal fraction. To investigate whether the enzyme activity in the PLC-II preparation was due to the 109 kDa protein, a sample of PLC-II was rechromatographed on a TSK gel G3000SW h.p.l.c. column. The protein was eluted as a single peak corresponding to 109 kDa with no peak in the 145 kDa position (results not shown). Estimation of PLC activity in different fractions revealed only one peak which coincided with the 109 kDa A_{280} absorption peak. Furthermore, SDS/PAGE of different fractions constituting the PLC peak revealed a major protein band at the 109 kDa position and no protein band corresponding to 145 kDa (Figure 5). Analysis of PLC in these fractions showed a positive correlation between the intensity of the 109 kDa band and the enzyme activity. For example, in fraction 78 (Figure 5a) where there was no 109 kDa protein we found very little PLC activity. On the other hand, in fraction 81 where the 109 kDa protein band was very prominent, the enzyme activity was also high. These data clearly indicate that the

Figure 4 (a) SDS/PAGE of purified PLC-I (lane 1) and PLC-II (lane 2) and (b) immunoblots of 110 000 g supernatant (lane 1), PLC-I (lane 2), PLC-II (lane 3) and deoxycholate-solubilized proteins from the membrane fraction (lane 4)

(a) The PLC-I and PLC-II obtained from the Mono Q column were applied to lanes 1 and 2 respectively of an SDS/polyacrylamide gel (7.5%). Molecular markers corresponding to myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa) and albumin (45 kDa) were applied to a separate lane. Protein bands were visualized by staining with Coomassie Brilliant Blue R₂₅₀. (b) The proteins were subjected to SDS/PAGE, transferred to nitrocellulose membranes and probed with antibodies against PLC- β_1 , PLC- γ_1 and PLC- δ_1 . Lane 1, 110 000 g supernatant probed with anti-(PLC- γ_1) antibodies; lane 2, PLC-I probed with anti-(PLC- δ_1) antibodies; lane 3, PLC-II probed with anti-(PLC- γ_1) antibodies (none of the antibodies recognized the 109 kDa protein band in lane 3); lane 4, deoxycholate (0.2%) solubilized proteins from the membrane fraction probed with anti-(PLC- β_1).

Figure 5 SDS/PAGE of PLC-II purified on a TSK G3000SW h.p.l.c. column and (b) PLC activity in fractions 70–90 obtained from the TSK G3000SW column

(a) Samples of fractions 78–83 were subjected to SDS/PAGE as described in the Materials and methods section. The protein bands were visualized with silver stain. The positions of the standard marker proteins is shown on the left. (b) The PLC enzyme activity was determined under standard assay conditions as described in the Materials and methods section.

enzyme activity in the PLC-II preparation is indeed due to the 109 kDa protein. Again, on Western blotting none of the monoclonal antibodies reacted with the 109 kDa protein. Therefore PLC-I contains PLC- δ_1 , and PLC-II contains a major 109 kDa protein band which is probably a proteolytic product of PLC- γ_1 . The 110000 g supernatant contains predominantly PLC- γ_1 , and the membrane fraction PLC- β_1 isoenzyme.

Substrate specificity and kinetic analysis of PLC-I and PLC-II

The substrate specificities of PLC-I and PLC-II were investigated using assay mixtures that contained 100 μ M PI, 240 μ M PIP or 240 μ M PIP₂ as substrates and 0.1 mM Ca²⁺ at pH 7.0. As shown in Table 3, both enzymes hydrolysed all three phosphoinositides; however, the activities against PIP and PIP₂ were substantially

Table 3 Substrate specificity of PLC-I and PLC-II

Purified PLCs (about 0.4 μ g) were incubated at 37 °C for 10 min in 50 mM Tris/maleate buffer that contained 0.1 mM Ca²⁺ and the ³H-labelled substrate as indicated in the Table. The reaction was stopped by addition of chloroform/methanol (2:1, v/v) and the radioactivity released into the aqueous phase was determined as described in the Materials and methods section. The data are the means $\pm$ S.E.M. of two experiments each conducted in triplicate.

Substrate	Specific activity (nmol/min per mg of protein)	
	PLC-I	PLC-II
PI (100 μ M)	115 $\pm$ 4	450 $\pm$ 24
PIP (240 μ M)	407 $\pm$ 22	1300 $\pm$ 27
PIP ₂ (240 μ M)	428 $\pm$ 8	1250 $\pm$ 32
PC (240 μ M)	0	0

Table 4 K_m and V_{max} values for PLC-I and PLC-II purified from bovine iris sphincter soluble fraction

PI and PIP₂ hydrolysis were measured by using purified PLC-I and PLC-II obtained from the final step in their purification procedure. PLC (0.15–0.3 μ g of protein) was incubated under the standard assay conditions (see the Materials and methods section) in a reaction mixture containing 50 mM Tris/maleate buffer (pH 7.0), 0.1 mM Ca²⁺ and ³H-labelled PI or PIP₂ (0.01–0.4 mM) in a final volume of 100 μ l for 5 min at 37 °C. The rate of phosphoinositide hydrolysis was measured at each substrate concentration and the K_m and V_{max} values obtained from a double-reciprocal plot.

	K_m (μ M)		V_{max} (μ mol/min per mg of protein)	
	PI	PIP ₂	PI	PIP ₂
PLC-I	30	150	3.4	13.7
PLC-II	60	160	3.7	16.1

greater than those against PI. Thus the relative rates of hydrolysis of PIP₂ by PLC-I and PLC-II were respectively about three to four times greater than that for PI. Neither of the enzymes hydrolysed phosphatidylcholine under the same assay conditions. The data demonstrate that PIP and PIP₂ are better substrates than PI for both PLC-I and PLC-II. The enzymes when stored at –80 °C were stable for several months. The apparent K_m and V_{max} values for PLC-I and PLC-II were estimated by the Lineweaver–Burk double-reciprocal plot using PI and PIP₂ as substrates. The substrate concentration ranged from 0.02 to 0.4 mM. The enzyme activity increased as a function of substrate concentration and reached a maximum at 0.2 and 0.4 mM PI and PIP₂ respectively. A summary of kinetic data obtained from these experiments is given in Table 4. When PI was the substrate, the apparent K_m was 30–60 μ M and V_{max} was approx. 3.5 μ mol/min per mg of protein for both enzymes. However, when PIP₂ was the substrate the K_m was about 150 μ M and V_{max} was approximately 15 μ mol/min per mg of protein for PLC-I and PLC-II.

Effects of pH on PLC-I and PLC-II activities

The effect of pH on PLC-I and PLC-II activities was investigated over a pH range of 5.0–8.5 with Tris/maleate buffer. The assay mixture contained either 100 μ M PI or 240 μ M PIP₂ with free Ca²⁺ concentration maintained at 100 μ M. As shown in Figure 6, when PI was the substrate, both PLC-I (Figure 6a) and PLC-II (Figure 6b) showed little enzyme activity below pH 6.5. As the pH was raised, the PLC activity increased rapidly to a maximum at pH 7.0–7.5. This was followed by a rapid decline in enzyme activity as the pH was raised above 7.5. When PIP₂ was used as a substrate, PLC-I hydrolysed the phospholipid at pH 5.0–6.0 at a rate which was about 60 % of the maximum hydrolysis observed at pH 6.5. The enzyme activity declined rapidly at higher pHs. In contrast with PLC-I, PLC-II showed little activity against PIP₂ at pH values below 5.5 but increased rapidly to a broad pH optimum with nearly constant enzyme activity between pH 6.5 and 8.0.

Ca²⁺ requirement for PLC activity

The requirement for Ca²⁺ in PLC-mediated hydrolysis of PI and PIP₂ was investigated by using EGTA/Ca²⁺ buffers. The ratios of Ca²⁺ to EGTA were varied to maintain low concentration of free Ca²⁺ in the standard assay mixture. As shown in Figure 7,

Figure 6 pH-dependence of PI (○) and PIP₂ (●) hydrolysis by PLC-I (a) and PLC-II (b)

The activities of PLC-I and PLC-II were determined by using either 100 μM PI or 240 μM PIP₂ as substrates. The free Ca²⁺ concentration in the assay mixture was 0.1 mM and Tris/maleate buffer was used to maintain the pH. The data were expressed as the means ± S.E.M. for two separate experiments each conducted in triplicate.

when PI was the substrate, both PLC-I and PLC-II showed an absolute requirement for Ca²⁺. Addition of 2 mM EGTA abolished PI hydrolysis and this was restored by addition of 1 μM free Ca²⁺. Increasing the free Ca²⁺ concentration resulted in a moderate increase in enzyme activity reaching a plateau at 1 mM Ca²⁺. The half-maximal activity was attained at about 2 μM free Ca²⁺ for both PLC-I and PLC-II. In contrast with PI, when PIP₂ was used as a substrate, significant hydrolysis of the phospholipid occurred in the absence of Ca²⁺ (Figure 7). In the presence of 2 mM EGTA, PIP₂ hydrolysis by PLC-I and PLC-II was 20 and 8% of their maximal rate respectively. Addition of 0.1 μM free Ca²⁺ resulted in a large increase in enzyme activity, maximal PIP₂ hydrolysis being elicited at 5 μM free Ca²⁺ concentration. Half-maximal activities of PLC-I and PLC-II were observed at 50 nM and 100 nM free Ca²⁺ respectively. Higher concentrations (> 10 μM) of Ca²⁺ exerted inhibitory effects on PIP₂ hydrolysis by both enzymes (Figure 7).

Figure 7 Ca²⁺-dependence of PI (○) and PIP₂ (●) hydrolysis by PLC-I (a) and PLC-II (b)

The activities of PLC-I (a) and PLC-II (b) were determined by using either 100 μM PI or 240 μM PIP₂ as substrates. Free Ca²⁺ concentrations shown on the abscissa were achieved by using Ca²⁺/EGTA buffers. The data are means ± S.E.M. for three experiments each conducted in triplicate.

Phosphorylation *in vitro* of PLC isoenzymes by the catalytic subunit of PKA and its effect on PIP₂ hydrolysis

To investigate whether or not PLC_s can serve as substrates for PKA, we incubated the purified PLC-I, PLC-II as well as PLC solubilized from the membrane fraction with the catalytic subunit

Table 5 Effect of catalytic subunit of PKA on phosphorylation of PLC-I, PLC-II and membrane PLC of the iris sphincter

Purified PLC (about 1.0 μg) and membrane PLC (20 μg) were incubated with 50 mM Tris/maleate buffer (pH 7.0) containing 20 μM [γ-³²P]ATP (specific radioactivity 100 Ci/nmol) and PKA catalytic subunit (5 units) as described in the Materials and methods section. After termination of the reaction, the proteins were analysed by SDS/PAGE. The radioactive protein bands corresponding to PLC were cut out and counted for radioactivity. The data are means ± S.E.M. of two experiments each conducted in triplicate.

Additions	³² P radioactivity (c.p.m.)		
	PLC-I	PLC-II	Membrane PLC
[γ- ³² P]ATP	512 ± 20	619 ± 37	524 ± 26
[γ- ³² P]ATP + PKA catalytic subunit	1626 ± 147	1597 ± 68	498 ± 19

Table 6 Effect of PKA-mediated phosphorylation on enzyme activities of purified PLC-I, PLC-II and membrane PLC

All experimental conditions were the same as in Table 4, except that the enzyme preparations were incubated with and without ATP and/or catalytic subunit for 2 min before determination of PLC activity against PIP₂. The results are the means $\pm$ S.E.M. of three separate experiments each conducted in triplicate. *Significantly different when compared with incubations in the presence of ATP or catalytic subunit alone, $P < 0.05$.

Additions	Specific activity (nmol/min per mg of protein)		
	PLC-I	PLC-II	Membrane PLC
None	338 $\pm$ 30	254 $\pm$ 8	34 $\pm$ 0.2
ATP (20 μ M)	323 $\pm$ 7	280 $\pm$ 14	35 $\pm$ 0.5
Catalytic subunit (5 units)	375 $\pm$ 29	267 $\pm$ 16	36 $\pm$ 0.6
ATP (20 μ M) + catalytic subunit (5 units)	449 $\pm$ 20*	268 $\pm$ 9	36 $\pm$ 0.1

of PKA. As shown in Table 5, in the presence of the catalytic subunit there was a large increase in phosphorylation of PLC-I and PLC-II; however, there was no change in the phosphorylation of PLC from the membrane fraction. The phosphorylation of PLC-I and PLC-II increased in a time-dependent manner with maximal phosphorylation achieved at 5 min (results not shown). Next, we investigated the effect of phosphorylation of PLC by PKA on the catalytic activities of the enzymes (Table 6). Addition of ATP alone or in the presence of the catalytic subunit (phosphorylation conditions) had no inhibitory effect on the activities of PLC-I, PLC-II or on membrane PLC. These data indicate that, although PLC-I and PLC-II are phosphorylated by PKA, this phosphorylation has no effect on the catalytic activities of the enzymes.

DISCUSSION

In sphincter smooth muscle of bovine iris, more than 90% of PLC activity was localized in the soluble fraction and the remainder in the membrane fraction. Two forms (I and II) of PLC were purified from the soluble fraction by means of sequential chromatography on DEAE-Sepharose, EAH-Sepharose, heparin-Sepharose, Sephacryl S-200 gel filtration and Mono Q HR columns. The specific activities of PLC-I and PLC-II were 4.3 and 5.9 μ mol of PI cleaved/min per mg of protein, and this represents about 200-fold purification. More work is required to obtain a homogeneous enzyme preparation from this tissue. Bennett and Crooke [32], working with guinea-pig uterus, purified PLC-I to homogeneity and PLC-II by 300-fold. PLC isoenzymes were partially purified from cultured vascular smooth-muscle cells [33] and from bovine aorta [34]. We have demonstrated that there are at least three immunologically distinct forms of PLC in the bovine iris sphincter, namely γ_1 and δ_1 (cytosolic) and β_1 (membranous). Thus, with the use of PLC monoclonal antibodies, PLC-I was identified as PLC- δ_1 , and PLC-II contained a major 109 kDa protein band that was not recognized by any of the PLC monoclonal antibodies. It is possible that the 109 kDa protein is simply a proteolytic segment of PLC- γ still retaining the catalytic activity. The latter possibility is supported by the fact that PLC- γ which is predominantly localized in the cytosolic fraction is barely detectable after being subjected to the purification procedures. Furthermore, the 109 kDa protein gave a positive reaction with a polyclonal antibody against a rat PLC- γ_1 fragment (amino acids 1272–1287). In both

guinea-pig uterus [32,45] and in cultured vascular smooth-muscle cells [33], PLC-I was identified by cDNA cloning as PLC- α . However, more recently it was found that cDNA for PLC- α actually encodes thiol:protein-disulphide oxidoreductase and that the published sequence for PLC- α may actually be the sequence for the latter enzyme [46]. In the iris sphincter, PLC- β_1 was found to be associated only with the membrane fraction. This could suggest that PLC- β_1 is the isoenzyme involved in mediating the action of Ca²⁺-mobilizing agonists in this tissue. Furthermore, this finding demonstrates that the membrane-bound and cytosolic PLCs are distinct enzymes. The significance of the presence of multiple PLC isoenzymes in different tissues is unknown.

PLC-I and PLC-II were further characterized for molecular mass, substrate specificity, pH, Ca²⁺ requirements and kinetic parameters. The molecular mass of PLC-I, as determined by gel filtration and SDS/PAGE, was 85 kDa, which corresponds to that of PLC- δ_1 in brain [6,9], and this is in accord with its identification by immunoblotting in the present work. Anti-(PLC- δ_1) antibodies detected an 85 kDa PLC in bovine heart homogenate [31]. Thus the 85 kDa PLC in heart and in iris sphincter could be the same as the PLC- δ_1 from brain [6]. The major band in PLC-II had a molecular mass of 109 kDa. In addition, there was a minor protein band in this preparation which corresponded to 76 kDa.

Although immunologically different, the catalytic properties of PLC-I and PLC-II were quite similar. Both isoenzymes preferred PIP and PIP₂ as substrate as compared with PI, whereas phosphatidylcholine was not hydrolysed by these enzymes. PLCs from guinea-pig uterus [32] and from bovine aorta [34] also preferentially hydrolysed PIP and PIP₂ as compared with PI, but the higher-molecular-mass (158 kDa) PLC from vascular smooth muscle showed much greater activity against PI than PIP₂ [33]. In general, the apparent K_m and V_{max} values for PIP₂ hydrolysis were three to five times higher than those for PI hydrolysis by both PLC-I and PLC-II. These data are consistent with kinetic data obtained from other PLC isoenzymes purified from various tissues [15,31,32]. With PIP₂ as substrate, the optimal pH values for PLC-I and PLC-II were 6.5 and 7.5 respectively. PLC- α from guinea-pig uterus exhibited a biphasic pH optimum using PI as a substrate, showing a major peak of activity between pH 6.0 and 7.0. Changing the free Ca²⁺ concentration from 4 μ M to 1 mM did not change the biphasic character of the pH profile. PLC from vascular smooth-muscle cells had a pH optimum for PIP₂ hydrolysis of 5.5–7.0, depending on the Ca²⁺ concentration [33]. In the bovine iris sphincter, hydrolysis of PI, but not of PIP₂, by PLC-I and PLC-II was dependent on the presence of free Ca²⁺ in the assay mixture. The maximal hydrolysis of PI and PIP₂ by these enzymes occurred at 200 and 5 μ M of the cation respectively. In general, in most tissues that have been investigated, PIP₂ hydrolysis occurred at a higher pH and at a lower Ca²⁺ concentration than did PI hydrolysis.

Our previous studies implicated PKA in the inhibitory effects of isoprenaline and forskolin on carbachol-induced IP₃ production and contraction in the iris sphincter [39,47]. This suggests a cross-talk between the two second-messenger systems. One possible target site for PKA action is PLC. Phosphorylation of PLC could inhibit the activity of the enzyme and reduce IP₃ production and consequently lead to muscle relaxation. Incubation of PLC preparations with the catalytic subunit of PKA and [γ -³²P]ATP resulted in increased phosphorylation of PLC-I and PLC-II, but it had no inhibitory effect on the catalytic activities of the enzymes. PLC in membrane fractions from the iris sphincter did not act as a substrate for PKA. Although direct

phosphorylation of PLC by PKA is a possibility, the mechanism by which cAMP blocks enhanced PIP_2 turnover and contraction in smooth muscle remains unknown.

In summary, we have purified two different forms (I and II) of PLC from bovine iris sphincter, investigated their properties, and for the first time identified multiple PLC isoenzymes including a 109 kDa PLC which did not react with any of the PLC monoclonal antibodies, but did react with a polyclonal antibody raised against a rat PLC- γ_1 fragment. The β_1 isoenzyme was found to be associated with the membrane fraction, whereas both δ_1 and γ_1 are localized in the cytosol. Whether in this tissue β_1 is the isoenzyme which is involved in signal transduction remains to be determined. Previously, we reported that PLC in the microsomal fraction of bovine sphincter could be stimulated by carbachol in the presence of low concentrations of GTP[S] and Ca^{2+} [48]. Phosphorylation of PLC isoenzymes by PKA may regulate the sphincter PLC activity and play a role in the inhibition of IP_3 production and consequently in muscle relaxation. However, further studies are needed to determine the target site of cAMP inhibition.

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