ies to specific PLC isozymes. Furthermore, amino acid sequences of all 12 tryptic peptides derived from the PLC-β4 preparations exhibiting multiple bands could be found in the amino acid sequence deduced from the rat cDNA. These results suggest that PLC-β4 might exist in multiple forms derived from alternatively spliced mRNAs or from proteolysis; PLC-β1 exists in 150, 140, and 100-kDa forms.

[19] Purification of 110 kDa Phosphoinositide Phospholipase C Activated by G-Protein βγ Subunits

By J. L. BLANK and J. H. EXTON

Introduction

The β isoforms of phosphoinositide-specific phospholipase C (PLC) are activated by heterotrimeric G-protein α subunits of the G_q family in response to a variety of Ca^{2+}-mobilizing agonists.[1-5] Subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) yields two intracellular second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively. This signaling pathway is unaffected by pertussis toxin (PTX) because α subunits of the G_q class lack the site for PTX-catalyzed ADP-ribosylation.[2]

The PTX-sensitive G proteins are also implicated in transducing the signal from certain Ca^{2+}-mobilizing agonists to PLC.[5] Experiments suggest that βγ subunits derived from PTX-sensitive G proteins (e.g., G_i and G_o) mediate this activation.[6-12] Using a reconstitution assay that measures

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[11] A. Carozzi, M. Camps, P. Gierschik, and P. J. Parker, *FEBS Lett.* **315,** 340 (1993).
[12] D. Park, D.-Y. Jhon, C.-W. Lee, K.-H. Lee, and S. G. Rhee, *J. Biol. Chem.* **268,** 4573 (1993).

G-protein-stimulated PIP_2 hydrolysis, we describe the purification of a 110-kDa PLC from bovine brain cytosol that is markedly activated by $\beta\gamma$ subunits.[13]

Assay of Phospholipase C Activation

The PLC assay is based on that originally described by Taylor and Exton.[14] Phosphatidyl[^{3}H]inositol 4,5-bisphosphate (100 μM [^{3}H]PIP_2) (900 cpm/nmol) from New England Nuclear (Boston, MA) is used as substrate and is incorporated into lipid vesicles containing phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the molar ratio 1 : 4 : 1. The unlabeled PIP_2 is commercially available (Sigma, St. Louis, MO; Boehringer-Mannheim, Indianapolis, IN), or may be prepared from mixed phosphoinositides (Sigma) on neomycin-linked glass beads (glyceryl-CPG-240 Å, 200–400 mesh, Fluka, Ronkonkoma, NY) as described by Schacht.[15] The lipids PE (bovine liver) and PS (bovine brain) are supplied by Avanti Polar Lipids (Birmingham, AL).

The assay is performed at 37° for 15 min or less in a final volume of 200 μl. The phospholipids are dried under a stream of N_2 and vesicles prepared at twice the final desired concentration by sonication into assay buffer containing 75 mM HEPES, pH 7.0, 150 mM NaCl, 4 mM EGTA, and 1 mg/ml bovine serum albumin (BSA). The $CaCl_2$/EGTA buffer system maintains the free Ca^{2+} concentration at 220 nM, as calculated from the COMICS program.[16] To monitor the enzyme during purification, fractions from each column step are assayed for PLC activity in the absence and presence of purified bovine brain $\beta\gamma$ subunits. The purification of bovine brain $\beta\gamma$ subunits is described elsewhere.[7] Routinely, $\beta\gamma$ subunits are added to the assay mixture in 10 μl or less to give a final concentration of 60 nM. Reactions are initiated by addition of 15 μl or less of column fraction such that the total volume of $\beta\gamma$ plus PLC is 20 μl. The reaction is terminated by adding 200 μl of 10% (w/v) trichloroacetic acid, followed by 100 μl of 1% (w/v) BSA. After 5 min on ice, the mixture is centrifuged (900 g for 4 min), and 400 μl of supernatant is counted.

Purification of $\beta\gamma$-Stimulated Phospholipase C

Preparation of Bovine Brain Cytosol. All steps in the preparation of the phospholipase C are performed at 4°. Cerebra (~1 kg) from four bovine

[13] J. L. Blank, K. Shaw, A. H. Ross, and J. H. Exton, *J. Biol. Chem.* **268,** 25184 (1993).
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[15] J. Schacht, *J. Lipid Res.* **19,** 1063 (1978).
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brains are homogenized using a Waring blender in 2 liters of 20 m*M* PIPES (1,4-piperazinediethanesulfonic acid), pH 6.8, containing 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* dithiothreitol (DTT), 2.7 m*M* KCl, 10 μg/ml leupeptin, 10 μg/ml antipain, 5 μg/ml aprotinin, and 5 μg/ml APMSF (4-amidinophenylmethanesulfonyl fluoride). The homogenate is centrifuged at 13,700 *g* for 30 min and the supernatant recentrifuged at 100,000 *g* for 60 min.

Heparin-Sepharose Chromatography. The 100,000 *g* supernatant (~1.5 liters; 10 g total protein) is loaded at 5 ml/min onto a 300-ml heparin-Sepharose CL-6B (Pharmacia-LKB, Piscataway, NJ) column (2.6 × 57 cm) equilibrated with 20 m*M* PIPES, pH 6.8, 1 m*M* EDTA, 1 m*M* DTT (buffer A), containing 2.7 m*M* KCl, 10 μg/ml leupeptin, 10 μg/ml antipain, 5 μg/ml aprotinin, and 5 μg/ml APMSF. After washing overnight with 3 liters of buffer A, the column is developed with a linear gradient from 0 to 300 m*M* NaCl in 1140 ml of this buffer, and fractions of 12 ml are collected at a flow rate of 3 ml/min. Fractions eluting from heparin-Sepharose column are assayed for PLC activity in the absence and presence of purified bovine brain βγ subunits. Fractions are pooled on the basis of βγ-stimulated PLC activity, which elutes as a single peak at approximately 200–250 m*M* NaCl. Heparin-Sepharose removes PLC-β1, PLC-γ1, and PLC-δ1 from the principal βγ-stimulated PLC activity, as assessed by Western blotting with corresponding monoclonal antisera (Upstate Biotechnology Inc., Lake Placid, NY). The procedure for immunoblot analysis of PLC isozymes has been described elsewhere.[7] The enzymes remain bound to heparin-Sepharose under the conditions described, and they elute in order PLC-γ1, PLC-β1, then PLC-δ1 when the column is developed with a second gradient of NaCl, between 400 and 700 m*M*.

Phenyl-Sepharose Chromatography. The pool from the heparin-Sepharose step (~168 ml) is brought to 35% saturation with $(NH_4)_2SO_4$ (20.9 g/100 ml) and stirred for 30 min at 4°C; insoluble proteins are removed by centrifugation at 10,800 *g* for 20 min. The supernatant is loaded at 3 ml/min onto a 150-ml phenyl-Sepharose 6 Fast Flow-Low Sub (Pharmacia-LKB) column (2.6 × 28 cm) equilibrated in buffer A containing 1.2 *M* $(NH_4)_2SO_4$. The column is washed with 300 ml of column equilibration buffer at the same flow rate and eluted at 2 ml/min with a reverse gradient of $(NH_4)_2SO_4$ from 1.2 to 0 *M* in 800 ml of buffer A. Elution is completed with a further 150 ml of buffer A, and fractions of 10 ml are collected. The βγ-stimulated PLC activity elutes as a single peak at approximately 0.4 *M* $(NH_4)_2SO_4$. Peak fractions (~90 ml) are pooled and dialyzed overnight against two changes of 2 liters of buffer A containing 2.7 m*M* KCl.

Q-Sepharose Chromatography. The dialyzed proteins are applied at 4 ml/min to a 100-ml Q-Sepharose Fast Flow (Pharmacia-LKB) column

(2.6 × 19 cm), equilibrated with buffer A containing 2.7 m*M* KCl. After washing with 200 ml of equilibration buffer containing 50 m*M* NaCl, the column is eluted at 2 ml/min with an 800-ml gradient from 50 to 300 m*M* NaCl, followed by a 150-ml gradient to 1 *M* NaCl in this buffer. The fraction size is 10 ml. A peak of basal ($\beta\gamma$-independent) PLC activity is detected in fractions containing the trailing edge of the $\beta\gamma$-stimulated activity which, on some occasions, appears as a partially resolved double peak. Therefore, fractions containing the peak and leading edge of the $\beta\gamma$-stimulated PLC activity, which display little or no basal PLC activity, are pooled (~150 ml) to partly avoid this second activity.

Hydroxylapatite Chromatography. The Q-Sepharose pool is concentrated to approximately 20 ml using an Amicon (Danvers, MA) filtration cell fitted with a YM30 membrane and loaded onto a 24-ml column of high-performance liquid chromatography (HPLC)-grade hydroxylapatite (Calbiochem, La Jolla, CA) (1.0 × 30 cm) equilibrated in 20 m*M* PIPES, pH 6.8, containing 0.1 m*M* EDTA, 1 m*M* DTT, 2.7 m*M* KCl, and 100 m*M* NaCl (buffer B). The column is washed with 50 ml of this buffer and eluted at 2 ml/min with a linear gradient of K_2HPO_4 from 0 to 250 m*M* in 320 ml of buffer B, collecting 4-ml fractions. Hydroxylapatite chromatography resolves the $\beta\gamma$-dependent PLC activity into three distinct peaks. The second and third minor peaks elute later in the gradient and are associated with a significantly higher basal activity than that detected in the first, major $\beta\gamma$-stimulated peak. Fractions containing the principal $\beta\gamma$-stimulated PLC activity eluting at approximately 110 m*M* K_2HPO_4 are pooled (~28 ml) and dialyzed overnight against 2 liters of buffer A.

Blue-Sepharose Chromatography. The dialyzed protein solution is loaded at 1 ml/min onto an 8-ml column of blue-Sepharose Fast Flow (Pharmacia-LKB) (1.0 × 10 cm) that has been equilibrated in buffer A. After washing, the column is eluted at 2 ml/min with a linear gradient from 0 to 1.2 *M* NaCl in 240 ml of buffer A, and 4-ml fractions are collected. The $\beta\gamma$-stimulated activity elutes as a broad peak between approximately 340 and 600 m*M* NaCl; active fractions are pooled (~50 ml) and concentrated to approximately 2–3 ml using a Centriprep 30 concentrator (Amicon).

Sephacryl S-300 Gel Filtration. The concentrate is applied to a 320-ml Sephacryl S-300 HR HiLoad 26/60 column (Pharmacia-LKB) that has been equilibrated in 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* DTT (buffer C) containing 150 m*M* NaCl. The column is eluted at 0.5 ml/min, and 3-ml fractions are collected. The $\beta\gamma$-stimulated PLC activity elutes as a symmetrical peak centered at fractions 60–61 (~180 ml elution volume).

Mono Q Chromatography. Active fractions are pooled (12 ml), diluted with 2 volumes of buffer C, and applied to a 1-ml Mono Q HR5/5 column

TABLE I
PURIFICATION OF PHOSPHOLIPASE C

Column	Total activity[a] (μmol/min)	Total protein[b] (mg)	Specific activity (μmol/min/mg)	Purification (-fold)	Yield (%)
Heparin-Sepharose	2.82	663	0.0043	1	100
Phenyl-Sepharose	2.41	71.8	0.0336	8	86
Q-Sepharose	1.33	1.11	1.20	279	47
Hydroxylapatite	0.62	0.095	6.53	1520	22
Blue-Sepharose	0.51	N.D.	—	—	18
Sephacryl S-300	0.19	N.D.	—	—	6.7
Mono Q	0.12	0.010[c]	12.0	2790	4.3

[a] Determined in the presence of 63 n*M* βγ.
[b] Determined by AMIDO black staining. N.D. indicates that protein concentrations were not detectable above blank values.
[c] Estimated by silver staining using PLC-β1 of known concentration to construct a standard curve.[13]

(Pharmacia-LKB) equilibrated with this buffer. The column is washed with 10 ml of buffer C containing 100 m*M* NaCl, then eluted with a linear gradient from 100 to 350 m*M* NaCl in 25 ml of buffer C, followed by a 5-ml gradient to 1 *M* NaCl. Fractions of 1 ml are collected at a flow rate of 1 ml/min. Fractions containing the peak of PLC activity, which elutes at approximately 240–280 m*M* NaCl, are supplemented with 20% (v/v) glycerol, divided into aliquots, frozen in liquid N_2, and stored at −70°. Omission of glycerol or repeated freezing and thawing results in a complete loss of enzyme activity after purification.

As summarized in Table I, this procedure results in an approximately 2000-fold purification of the enzyme from the pool obtained by heparin-Sepharose chromatography of bovine brain cytosol, with a final yield of approximately 4%. Approximately 10 μg of the purified enzyme is obtained from four bovine brains. Because the enzyme has a negligible basal activity in the absence of βγ, purification is assessed using a fixed concentration of βγ in the reconstitution assay, routinely 60 n*M*. Values are calculated based on the pool of activity obtained by heparin-Sepharose chromatography of bovine brain cytosol, from which the basal (βγ-independent) PLCs have been largely removed.

Properties of βγ-Stimulated Phospholipase C

The enzyme migrates on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels with an apparent molecular mass of 110 kDa and is essentially pure as judged by silver stain analysis (Fig. 1). The enzyme, designated

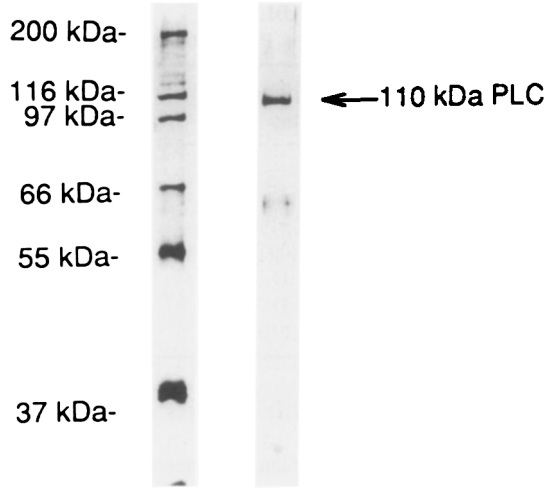

FIG. 1. Silver stain analysis of purified $\beta\gamma$-stimulated PLC-110. Purified $\beta\gamma$-stimulated PLC from Mono Q chromatography (36 ng PLC-110, right-hand lane) was subjected to 10% polyacrylamide–SDS gel electrophoresis and stained with silver according to previously published procedures.[7] The left-hand lane shows the marker proteins myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (55 kDa), and lactate dehydrogenase (37 kDa).

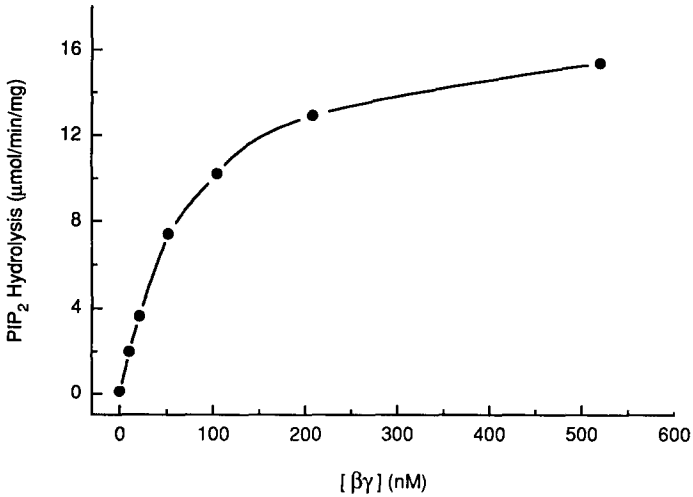

FIG. 2. Stimulation of purified PLC-110 by bovine brain $\beta\gamma$ subunits. The hydrolysis of PIP_2 by 12 ng PLC-110 was determined in the presence of varying amounts of purified brain $\beta\gamma$ subunits as described in the text. The basal ($\beta\gamma$-independent) specific activity of PLC-110 in the experiment shown was 0.1 μmol/min/mg, and maximal activation by $\beta\gamma$ was approximately 150-fold.

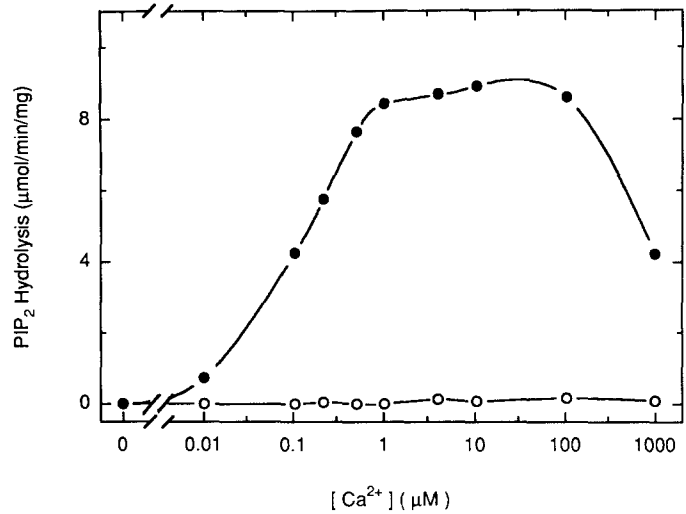

FIG. 3. Calcium dependence of βγ-stimulated PLC-110. The hydrolysis of PIP_2 by 19 ng PLC-110 was measured in the absence (○) and presence (●) of 63 n*M* βγ as described in the text, except that Ca^{2+}/EGTA buffers were used to vary the free calcium concentration, as indicated. Basal PLC-110 activity was in the range of 0–0.16 μmol/min/mg.

PLC-110, is not recognized by monoclonal antisera raised to bovine brain PLC-β1, PLC-γ1, and PLC-δ1, which have apparent molecular masses of 150, 145, and 85 kDa, respectively.[3] An antiserum to a synthetic peptide corresponding to a region of the Y domain conserved among mammalian PLC isozymes[17] cross-reacts with PLC-110, confirming its relatedness to these enzymes. PLC-110 is also recognized by two antisera selective for N-terminal amino acid residues 58–67 (GRYARLPKDP) and for residues 550–561 (TDPKKPTTDEGT) in PLC-β3,[18] indicating that PLC-110 is related to PLC-β3. However, PLC-110 is not recognized by an antiserum raised to a sequence corresponding to amino acid residues 1206–1217 (ADSESQEENTQL) at the C-terminus of PLC-β3,[18] indicating that PLC-110 is a C-terminal truncated form of PLC-β3. Further proof that PLC-110 is derived from PLC-β3 is provided by the sequences of 15 tryptic peptides obtained from the 110-kDa protein (J. L. Blank, S. Afendis, C. Moomaw, C. A. Slaughter, and J. H. Exton, unpublished findings). These show the presence of the PLC-β3 sequence up to residue 856,

[17] D. Park, D.-Y. Jhon, R. Kriz, J. Knopf, and S. G. Rhee, *J. Biol. Chem.* **267,** 16048 (1992).
[18] D.-Y. Jhon, H.-H. Lee, D. Park, C.-W. Lee, K.-H. Lee, O. J. Yoo, and S. G. Rhee, *J. Biol. Chem.* **268,** 6654 (1993).

consistent with PLC-110 being a C-terminally truncated form of the enzyme.

Activation of PLC-110 by $\beta\gamma$ is direct and produces a greater than 100-fold stimulation of PIP_2 hydrolysis over basal. Half-maximal activation requires approximately 60 n*M* $\beta\gamma$, and full activation is observed at about 500 n*M* $\beta\gamma$ (Fig. 2). Purified liver and brain $\beta\gamma$ are equipotent. PLC-110 has no activity with phosphatidylinositol as substrate, and it hydrolyzes phosphatidylinositol 4-phosphate and PIP_2 maximally at 1–100 μM Ca^{2+}. The calcium dependence of PLC-110 with PIP_2 as substrate is shown in Fig. 3. Whereas $\beta\gamma$ subunits produce a dramatic activation of PLC-110, GTPγS-liganded α_q, which is a potent activator of PLC-β1[3–5] and PLC-β3,[18] has no effect. This observation indicates that the site at which $\beta\gamma$ interacts with PLC-110 is distinct from that at which α_q regulates the activity of PLC-β isozymes. As experiments have demonstrated that the α_q interaction site is located in the C terminus of PLC-β1,[19,20] our findings support the conclusion that PLC-110 is a PLC-β isozyme that has lost this domain but has retained that involved in $\beta\gamma$ interaction.

[19] D. Park, D.-Y. Jhon, C.-W. Lee, S. H. Ryu, and S. G. Rhee, *J. Biol. Chem.* **268,** 3710 (1993).

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[20] Amplification of Phosphatidylinositol-Specific Phospholipase C-β Isoforms Using Degenerate Primers

By HAI-WEN MA, RAVI IYENGAR, and RICHARD T. PREMONT

Introduction

Hydrolysis of phosphatidylinositol (PI) on the inner leaflet of the cell membrane to liberate phosphorylated inositols and diacylglycerol is catalyzed by a family of calcium-dependent, phosphatidylinositol-specific phospholipase C (PLC) enzymes. Three distinct classes of PI-specific phospholipase C enzymes, called β, γ, and δ, have been characterized by both protein purification and cDNA cloning and expression.[1] Multiple members of each class have been identified. Hormonal stimulation of PI

[1] S. G. Rhee, *Trends Biochem. Sci.* **16,** 297 (1991).