Phosphoinositide Binding Specificity among Phospholipase C Isozymes as Determined by Photo-Cross-Linking to Novel Substrate and Product Analogs†

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ABSTRACT: We tested for the presence of high-affinity phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and PI(3,4,5)P_3 binding sites in four phospholipase C (PLC) isozymes (δ_1, β_1, β_2, and β_3), by probing these proteins with analogs of inositol phosphates, D-Ins(1,4,5)P_3, D-Ins(1,3,4,5)P_4, and InsP_6, and polyphosphoinositides PI(4,5)P_2 and PI(3,4,5)P_3, which contain a photoactivatable benzoyldihydrocinnamide moiety. Only PLC-δ_1 was specifically radiolabeled. More than 90% of the label was found in tryptic and chymotryptic fragments which reacted with antisera against the pleckstrin homology (PH) domain, whereas less than 5% was recovered in fragments that encompassed the catalytic core. In separate experiments, the isolated δ_1-PH domain was also specifically labeled. Equilibrium binding of D-Ins(1,4,5)P_3 to PLC-δ_1 indicated the presence of a single, high-affinity binding site; binding of D-Ins(1,4,5)P_3 to PLC-β_1, -β_2, or -β_3 was not detected. The catalytic activity of PLC-δ_1 was inhibited by the product D-Ins(1,4,5)P_3, whereas no inhibition of PLC-β_1, -β_2, or -β_3 activity was observed. These results demonstrate that the PH domain is the sole high-affinity PI(4,5)P_2 binding site of PLC-δ_1 and that a similar site is not present in PLC-β_1, -β_2, or -β_3. The data are consistent with the idea that the PH domain of PLC-δ_1, but not the β isozymes, directs the catalytic core to membranes enriched in PI(4,5)P_2 and is subject to product inhibition.

The eukaryotic phosphoinositide-specific phospholipase C (PI-PLC)[1] isozymes cleave the phosphodiester bond of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], releasing two intracellular second messenger molecules, D-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol. These isozymes can be grouped by sequence into three types: β, γ, and δ (Lee & Rhee, 1995; Exton, 1996). Each group appears to be subject to a unique mode of cellular regulation. The β isozymes are controlled by the G_α or $G_{\beta\gamma}$ subunits of heterotrimeric GTP-binding proteins (G-proteins), which are coupled to receptors that possess seven transmembrane-spanning segments. The γ isozymes are substrates for receptor tyrosine protein kinases, exemplified by the receptor for platelet-derived growth factor. Calcium and/or G-proteins may be involved in controlling the δ isoforms. The recent identification of a G-protein (G_h) that activates PLC-δ_1 suggests a pathway linking this enzyme to cell surface receptors (Feng et al., 1996).

The PLC isozymes are soluble proteins that bind to the membrane/solution interface where they operate in two dimensions, hydrolyzing numerous substrate molecules before returning to the bulk solution. This highly efficient catalytic mode has been termed "scooting" behavior (Gelb et al., 1995). For some PLC isoforms, the polyphosphoinositides themselves may serve as both substrates and membrane anchoring sites. PLC-δ_1 (Rebecchi et al., 1992; Pawelczyk & Lowenstein, 1993), and PLC-β isolated from turkey erythrocytes (James et al., 1995), bind strongly to PI(4,5)P_2 through a site that does not appear to be catalytic. Their PI(4,5)P_2 hydrolytic activities, measured in membrane and detergent/phospholipid mixed micelle assays, are consistent with a bisubstrate model in which at least one high-affinity PI(4,5)P_2 binding site tethers the low-affinity catalytic site to the membrane surface (Cifuentes et al., 1993; James et al., 1995). Studies of the δ_1 isozyme support this idea. Deletion of the amino-terminal 60 amino acids abolishes high-affinity PI(4,5)P_2 binding and reduces scooting behavior, although the catalytic site remains functional (Cifuentes et al., 1993, 1994; Yagisawa et al., 1994). The 60-amino

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[1] Abbreviations: PH domain, pleckstrin homology domain; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; Ins(1,4,5)P_3, D-myoinositol 1,4,5-trisphosphate; [3H]BZDC-Ins(1,4,5)P_3, [3H]-1-*O*-[3-(4-benzoyldihydrocinnamidyl)propyl]-Ins(1,4,5)P_3; PC, phosphatidylcholine; PI(4)P, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; TEAB, triethylammonium bicarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BCA, bicinchronic acid; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; DTT, dithiothreitol; LUVs, large unilamellar vesicles; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; β-ARK, β-adrenergic receptor kinase; G-proteins, GTP-binding proteins.

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acid region deleted in these experiments is now recognized as part of a domain with pleckstrin homology.

The amino-terminal regions of all mammalian PLC isozymes contain a single pleckstrin homology (PH) domain of 130−140 residues (Lee & Rhee, 1995). PH domains, first described as duplicated regions within the platelet protein pleckstrin (Haslam et al., 1993), are found in many polypeptides that function in cell signaling and cytoskeletal structure (Musacchio et al., 1993; Gibson et al., 1994; Shaw, 1996). Like SH2 and SH3 domains, they are modular units that assume a common structure which is independent of the surrounding protein (Gibson et al., 1994; Ferguson et al., 1995a; Cohen et al., 1995). Despite their low sequence conservation, all PH domains form a pseudo-β-sandwich containing a carboxy-terminal α-helix that has an invariant tryptophan residue.

A few well-studied examples support the idea that PH domains bind polyphosphoinositides and thereby anchor their host proteins to membrane surfaces. The PH domains of pleckstrin (N-terminal and C-terminal), T-cell-specific tyrosine kinase, ras GTPase activating protein, β-adrenergic receptor kinase (β-ARK) (Harlan et al., 1994), β-spectrin (Hyvönen et al., 1995), and PLC-δ_1 (Garcia et al., 1995; Lemmon et al., 1995) bind to phospholipid vesicles containing either PI(4,5)P_2 or PI(4)P. The relative order of affinities is PLC-δ_1 > β-spectrin > pleckstrin, β-ARK, or other PH domains. A molecular explanation of this specificity and affinity is provided by crystallographic studies. The three-dimensional structure of the PLC-δ_1 PH domain, complexed with Ins(1,4,5)P_3, reveals a deep binding pocket formed by β-strands 1 and 2 and the loop connecting them (Ferguson et al., 1995b). Six residues that line this pocket contribute 12 hydrogen bonds to the complex with Ins(1,4,5)P_3, seven of which are involved in recognizing the 5-position phosphate. The PH domain of β-spectrin also contains a binding site that accommodates the PI(4,5)P_2 polar head group (Hyvönen et al., 1995). Formed by β-strands 1 and 2 and the loop connecting them, this shallow groove contains four residues that contribute six hydrogen bonds to the complex with Ins(1,4,5)P_3. Similar residues in the N-terminal PH domain of pleckstrin are perturbed upon binding of Ins(1,4,5)P_3 (Harlan et al., 1994).

The amino acid residues that line the PI(4,5)P_2 binding pocket of the PLC-δ_1 PH domain are not well conserved in other PLC isozymes. This is surprising since a PLC-β isoform, isolated from turkey erythrocyte membranes, binds to PI(4,5)P_2 with an affinity similar to that reported for PLC-δ_1 (James et al., 1995); the kinetics of PI(4,5)P_2 hydrolysis are also consistent with a bisubstrate model of catalysis. Similar kinetic results were reported for PLC-γ_1 (Wahl et al., 1992). These observations suggest that the PH domains of PLC-β, -γ, and -δ_1 are more similar than can be inferred from sequence comparisons or that other regions of the β and γ isozymes, besides the catalytic site, bind polyphosphoinositides. Direct measurements of the affinities of the mammalian PLC-β or -γ isozymes for PI(4,5)P_2 or other phosphoinositides have not been reported.

The active site residues that bind the PI(4,5)P_2 polar head group were recently identified in the three-dimensional structure of the PLC-δ_1 catalytic core (Essen et al., 1996). Within this site, a network of hydrogen bonds and specific charge−charge interactions ligate inositol ring substituents. Lys 438, Lys 440, Ser 522, and Arg 549, which are conserved in PLC-β and -γ isozymes, form hydrogen bonds with the 4- and 5-position phosphates. In our kinetic model of PLC-δ_1 catalytic activity, the affinity of the active site for substrate or product is predicted to be lower than that for other regions involved in membrane binding (Cifuentes et al., 1993). Although weaker active site binding can be inferred from the crystallographic and deletion studies of PLC-δ_1, the affinity of this site has not been directly measured in the native protein.

In this study, we use analogs of PI(4,5)P_2 and PI(3,4,5)P_3, and their polar head groups, containing the photoactivatable moiety, [^{3}H]benzoyldihydrocinnamide ([^{3}H]BZDC), to probe the PLC isozymes for evidence of multiple high-affinity binding sites (Prestwich, 1996; Prestwich et al., 1996). Peptide mapping of the δ_1 isozyme, labeled with these probes, demonstrates that the PH domain is the only accessible high-affinity site. No specific labeling of PLC-β_1, -β_2, or -β_3 is detected under these same conditions. From these and equilibrium binding studies, we conclude that a high-affinity site, which recognizes the PI(4,5)P_2 or PI(3,4,5)P_3 polar head group, is not detectable in the PLC-β isozymes or in the catalytic core of PLC-δ_1. These data strongly suggest that, among the PLC isozymes, PLC-δ_1, through its PH domain, possesses a uniquely high affinity for membranes enriched in PI(4,5)P_2.

EXPERIMENTAL PROCEDURES

Synthesis of [^{3}H]BZDC-Inositol Polyphosphates and Phosphoinositides

Syntheses of [^{3}H]BZDC-Ins(1,4,5)P_3, P-1-tethered Ins(1,3,4,5)P_4, and P-2-tethered Ins(1,2,3,4,5,6)P_6 were carried out as previously described (Mourey et al., 1993; Estevez & Prestwich, 1994; Dormán et al., 1995; Hammonds-Odie et al., 1996; Prestwich et al., 1996; Prestwich, 1996). The products were purified on a short column of DEAE-cellulose (HCO_3^- form) using increasing concentrations (0.1 to 0.6 M) of aqueous triethylammonium bicarbonate (TEAB) (Olszewski et al., 1995). The concentrations of fractions containing the [^{3}H]BZDC-labeled compounds (eluting at 0.4−0.5 M TEAB) were determined by liquid scintillation counting (ca. 0.03−0.05 mCi/mL) and verified by analytical HPLC on a C8 reverse phase column interfaced with a diode array detector and a BetaRAM radiochemical detector. The specific activities of the photoactivatable ligands were 35−44 Ci/mmol on the basis of the specific activity of the reagent [^{3}H]BZDC-NHS ester. Fractions were stored as triethylammonium salts at 5 °C in the eluting buffer. The concentration of the ligand was adjusted to the required value (0.5−1.0 μM) immediately prior to use by dilution with water or concentration under a stream of nitrogen. [^{3}H]BZDC-PI(4,5)P_2 and [^{3}H]BZDC-PI(3,4,5)P_3 triesters and acyl-[^{3}H]-BZDC-PI(4,5)P_2 were synthesized by methods described elsewhere (Chen et al., 1996; Gu & Prestwich, 1996).

Expression of the Recombinant PLC Isozymes and the PLC-δ_1 PH Domain

Expression and purification of recombinant human PLC-δ_1 are described below. Recombinant human PLC-β_1, -β_2, and -β_3 were expressed in Sf9 insect cells using baculovirus vectors (Runnels et al., 1996). Baculovirus, containing the human PLC-β_3 sequence, was generously provided by S. G.

Rhee (NIH). The PLC-δ_1 PH domain was expressed in *Escherichia coli* strain BL-21(DE3) and purified as previously described (Garcia et al., 1995).

Expression of Human PLC-δ_1

Human PLC-δ_1 was expressed in the BL-21(DE3) strain of *E. coli* using the pET3a vector (Studier, 1990). Because this vector contained no affinity tag, the native sequence was completely retained in the expressed protein. A single, transformed colony, selected for growth inhibition by isopropyl β-D-thiogalactopyranoside (IPTG) (Studier, 1990), was used to inoculate 4 L of superbroth (32 g of bactotryptone/L, 20 g of bactoyeast/L, 5 g of NaCl/L, and 5 mL of 1 N NaOH/L) containing 150 μg/mL ampicillin. The growing culture was cooled to 20 °C and induced with IPTG (0.4 mM). An additional dose of ampicillin (150 μg/mL) was added at this time. Expression continued for 24 h at 20 °C (expression of the native soluble enzyme was optimal by 24 h at 20 °C; the bulk of the enzyme was insoluble when expression was attempted at 37 °C). The cells were harvested by centrifugation and resuspended in 180 mL of homogenization buffer [200 mM NaCl, 20 mM HEPES (pH 7.6), 1 mM EDTA, 2 mM PMSF, 5 mM EGTA, 0.5 mM DTT, 5 mM benzamidine, 5 μg/mL leupeptin, 2.5 μg/mL pepstatin, 2 μg/mL aprotinin, and 500 μg/mL lysozyme]. Cells were then flash frozen in liquid nitrogen and then thawed in cold water.

(1) Homogenization. Cells were disrupted by a single pass through a French press or by sonication. The lysate was then subjected to centrifugation at 35000*g* (17 000 rpm) in a Beckman JA-20 rotor for 30 min at 4 °C.

(2) Heparin−Sepharose. Supernatant fluid obtained at step 1 was diluted 2-fold in ice-cold water and pumped at 3 mL/min over a 30 mL bed of Heparin−Sepharose (25 mm × 30 mm) equilibrated in buffer A [20 mM HEPES (pH 7.2), 1 mM EGTA, 1 mM EDTA, 5 mM DTT, 5 μg/mL leupeptin, and 2.5 μg/mL pepstatin] which contained 100 mM NaCl. The column was washed sequentially with 60 mL portions of buffer A that contained 200 and 400 mM NaCl. The protein was eluted with 120 mL of buffer A containing 700 mM NaCl at 3 mL/min. Eluted material was collected in 10 mL fractions. Nearly all the PLC activity appeared in fractions 4−9.

The fractions containing PLC activity were pooled, transferred to dialysis tubing (50 kDa molecular mass cutoff), and dialyzed for approximately 3 h against 5 L of buffer B [20 mM TRIS-HCl (pH 7.6), 1 mM EGTA, and 1 mM DTT]. It was important that the conductivity of the sample did not fall below 3 mS at 4 °C; otherwise, the PLC formed an insoluble precipitate. Following dialysis, the sample was subjected to centrifugation at 17 000 rpm in a JA-20 rotor for 15 min to remove any precipitated protein. Glycerol was added to the supernatant fluid to a final concentration of 10% by volume. Leupeptin, pepstatin, and DTT were added to final concentrations of 5 μg/mL, 2.5 μg/mL, and 4 mM, respectively.

(3) FPLC HQ-Poros. A 4.6 mm × 100 mm HQ-Poros column (PerSeptive Biosystems), connected to a Waters 650 protein purification system, was equilibrated with buffer A containing 50 mM NaCl, 4 mM DTT, and 10% glycerol at 4 °C. The sample from step 2 was pumped over the column at 3 mL/min. The protein was eluted with a linear gradient of 50 to 300 mM NaCl over 32 min at a rate of 1 mL/min. PLC-δ_1 eluted between 14 and 20 min of the gradient, constituting at least 80% of the protein at this step.

(4) FPLC Mono-S. A 4.6 mm × 100 mm Mono-S column (Pharmacia) was equilibrated with buffer C [10% glycerol, 20 mM MES (pH 6.1), 1 mM EGTA, 5 mM DTT, 5 μg/mL leupeptin, and 2.5 μg/mL pepstatin] which contained 0.1 M NaCl. Fractions containing PLC activity from step 3 were pooled and diluted 5-fold with buffer C. The sample was applied at 0.7 mL/min and eluted with a linear gradient from 0.1 to 0.5 M NaCl at a rate of 0.7 mL/min for 20 min. PLC-δ_1 eluted between 15 and 17 min. Samples of the peak fractions were subjected to SDS−PAGE and stained with Coomassie Brilliant Blue dye. A single protein band with an apparent molecular mass of 85 kDa was detected.

Approximately 2 mg of pure PLC was recovered from each liter of *E. coli* culture. The molar extinction coefficient, 116 262 M^{-1} cm^{-1} at 280 nm, was determined using homogeneous preparations of PLC-δ_1 dissolved in 8 M guanidine hydrochloride (Gill & von Hippel, 1989). This constant was used to calculate the PLC concentration. The PLC activity was measured with a detergent/PI(4,5)P_2 mixed micelle assay as described previously (Cifuentes et al., 1993). The final protein preparation was subjected to SDS−PAGE, electroblotted onto nitrocellulose, and probed with sequence-specific antisera as previously described (Cifuentes et al., 1993). An 85 kDa immunoreactive band corresponding to the purified protein was detected with all specific sera. *E. coli*-expressed human PLC-δ_1 showed characteristics similar to those of PLC-δ_1 isolated from bovine brain cytosol. These characteristics included calcium activation and PI(4,5)P_2 or Ins(1,4,5)P_3 binding (Rebecchi et al., 1992; Cifuentes et al., 1993; Garcia et al., 1995). The specific activity of this enzyme, however, was at least 10-fold greater than those reported for the purified bovine brain and liver enzymes (Rebecchi & Rosen, 1987; Fukui et al., 1988). Similar results were reported by Ellis et al. (1993) for the GST fusion protein expressed in *E. coli*. They attributed the higher activity to a lack of post-translational modification.

Photo-Cross-Linking of [^{3}H]BZDC-Ins(1,4,5)P_3, -Ins(1,3,4,5)P_4, or -Ins(1,2,3,4,5,6)P_6 to Recombinant PLC Isozymes

Comparable amounts (0.5−3.0 μg) of purified PLC-δ_1, -β_1, -β_2, or -β_3 were incubated in total reaction volumes of 24 μL with 0.12−0.25 μM [^{3}H]BZDC-Ins(1,4,5)P_3, [^{3}H]-BZDC-Ins(1,3,4,5)P_4, or [^{3}H]BZDC-Ins(1,2,3,4,5,6)P_6 in the presence or absence of (10−60 μM) unlabeled D-Ins(1,4,5)-P_3, D-Ins(1,4)P_2, or Ins(1,2,3,4,5,6)P_6 for 10 min at room temperature in buffer containing 100 mM NaCl and 125 mM HEPES at pH 7.2. The samples, in silanized borosilicate glass test tubes, were irradiated with 350 nm light (36 W) for 20 min in a Rayonet photoreaction cell at 4 °C. After irradiation, the samples were boiled in SDS sample buffer and subjected to electrophoresis in a 7.5% polyacrylamide gel. The gel was fixed in methanol/acetic acid/water (3:1:6), stained with Coomassie Brilliant Blue dye, impregnated with Entensify, an aqueous fluor solution (NEN), dried, and subjected to autoradiography. Each photo-cross-linking experiment was repeated at least twice with similar results.

Photo-Cross-Linking of [^{3}H]BZDC-Ins(1,4,5)P_3, -Ins(1,3,4,5)P_4, or -Ins(1,2,3,4,5,6)P_6 to the PLC-δ_1 PH Domain

Purified PLC-δ_1 PH domain (2.5 μg) was incubated in a total reaction volume of 20 μL with 0.12−0.25 μM [^{3}H]-BZDC-Ins(1,4,5)P_3, [^{3}H]BZDC-Ins(1,3,4,5)P_4, or [^{3}H]BZDC-Ins(1,2,3,4,5,6)P_6 in the presence or absence of (10−60 μM) unlabeled D-Ins(1,4,5)P_3, D-Ins(1,4)P_2, or Ins(1,2,3,4,5,6)P_6 for 10 min at room temperature in buffer containing 100 mM NaCl and 125 mM HEPES at pH 7.2. The samples were irradiated and processed as described above.

Mapping the Ins(1,4,5)P_3 Binding Site

PLC-δ_1 (5 μg) was incubated with 0.5 μM [^{3}H]BZDC-Ins(1,4,5)P_3 for 10 min at room temperature in buffer containing 100 mM NaCl, 1 mM EGTA, and 70 mM HEPES at pH 7.2. Samples were irradiated as described above. Each microgram of PLC was then digested for 2−4 h with 0.025 μg of either chymotrypsin or trypsin. Digestions were carried out in a total volume of 30 μL in digest buffer containing 5 mM $CaCl_2$, 5 mM DTT, 0.8% octyl glucoside, 100 mM NaCl, and 20 mM HEPES at pH 7.2. The digestions were terminated by addition of PMSF and SDS sample buffer. One-half of each sample was subjected to SDS−PAGE on a 12% acrylamide gel, transferred to nitrocellulose, and immunoblotted using the specific antisera described previously (Cifuentes et al., 1993). The immunoreactive protein bands were excised, transferred to scintillation vials, dissolved in methanol, and counted. The other half of each sample was subjected to SDS−PAGE on a 12% acrylamide gel and stained with Coomassie Brilliant Blue dye. Individual lanes were cut into 2 mm sections and transferred to counting vials. The slices were digested in H_2O_2 for 2.5 h at 55 °C and then counted in a liquid scintillation spectrometer.

Photo-Cross-Linking of [^{3}H]BZDC-PI(4,5)P_2 Acyl or Triester Derivatives or [^{3}H]BZDC-PI(3,4,5)P_3 Triester to Recombinant PLC Isozymes

Comparable amounts (1−2 μg) of purified PLC-δ_1, -β_1, -β_2, or -β_3 or δ_1-PH domain were incubated in reaction volumes of 24−26 μL with 0.12−0.25 μM [^{3}H]BZDC-PI-(4,5)P_2 triester, [^{3}H]BZDC-PI(3,4,5)P_3 triester, or acyl [^{3}H]-BZDC-PI(4,5)P_2 in the presence or absence of unlabeled 30 μM PIP_2 or 30 μM PI for 2.5 min at room temperature in buffer containing 220 μM dodecyl maltoside, 100 mM NaCl, 1 mM EGTA, and 25 mM HEPES at pH 7.2. The samples, in silanized borosilicate glass test tubes, were irradiated with 350 nm light for 20 min at 4 °C and processed as described above for the [^{3}H]BZDC-inositol phosphates.

Mapping of the Binding Site Cross-Linked to [^{3}H]-BZDC-Lipid

After irradiation with UV light, an equivalent volume of (0.01 μg/mL) trypsin in digest buffer was added directly to the samples. Digestions were carried out at 30 °C for 2−3 h, terminated, and processed as described above for mapping of the Ins(1,4,5)P_3 binding site.

Binding of [^{3}H]Ins(1,4,5)P_3 to PLC-δ_1, -β_1, -β_2, and -β_3

Purified PLC isozymes (6.5 μg of β_1, 10.5 μg of β_2, 9.4 μg of β_3, and 8.4 or 12.9 μg of δ_1) were equilibrated for 10 min at 30 °C in 100 μL of binding buffer containing 100 mM NaCl, 1 mM DTT, 20 mM HEPES (pH 7.5), and [^{3}H]-Ins(1,4,5)P_3 ranging in concentration from 0.18 to 30 μM. Conditions of the binding reaction were similar to those described by Yagisawa et al. (1994). Each reaction mixture was placed on ice; 100 μL of ice-cold 36% (w/v) PEG-8000 and 10 μL of 15 μg/μL human γ-globulin (Sigma) were subsequently added. Samples were incubated on ice for 15 min and then subjected to centrifugation in a microfuge (12000*g*) for 5 min at 4 °C. Under these conditions, ~75% of each PLC was precipitated. The supernatants were aspirated, and the pellets were washed twice with 250 μL of 18% (w/v) PEG-8000. Each pellet was solubilized in 0.1 N NaOH, transferred to a scintillation vial, and counted. The data were analyzed using eq 1:

$$\nu = \frac{[\mathrm{InsP_3}]_{\mathrm{free}}}{[\mathrm{InsP_3}]_{\mathrm{free}} + K_{\mathrm{d}}}$$

where ν = the number of molecules of $InsP_3$ bound per molecule of PLC.

Assay of PLC Catalytic Activity

LUVs composed of PE/PC/[^{3}H]PIP_2 (15:4:1) were prepared by extrusion through polycarbonate filters as previously described (Cifuentes et al., 1993) to yield a final PI(4,5)P_2 concentration of 60 μM. The reactions, which were initiated by addition of substrate vesicles, were carried out in buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.5), 2.5 mM DTT, 0.1% gelatin, 120 μM $CaCl_2$, and 4 μg/mL leupeptin and pepstatin, in the presence or absence of 30 μM Ins(1,4,5)-P_3.

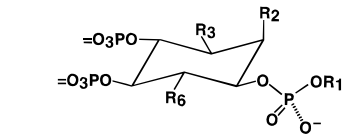
Protein Concentration Measurements

The concentration of PLC-δ_1 was calculated using the experimentally determined PLC-δ_1 extinction coefficient at 280 nm (ϵ = 116 262 M^{-1} cm^{-1}). The concentrations of PLC-β_1, -β_2, and -β_3 were estimated by BCA assay (Pierce) using BSA as a standard. Concentrations of the PLC-δ_1 PH domain were measured by the Bradford assay (Bradford, 1976).

RESULTS

[^{3}H]BZDC-Ins(1,4,5)P_3, which contains the PI(4,5)P_2 polar head group linked by an aminopropyl tether to the photoreactive BZDC moiety (Figure 1a), has been used to label specifically Ins(1,4,5)P_3 receptors from rat brain and map the ligand binding sites (Mourey et al., 1993). We took advantage of this compound and probed the PLC isozymes for high-affinity PI(4,5)P_2 binding sites. We (Rebecchi et al., 1992) and others (Pawelczyk & Lowenstein, 1993) had previously shown that PLC-δ_1 binds to PI(4,5)P_2 with high affinity and that binding is inhibited competitively by Ins-(1,4,5)P_3 (Cifuentes et al., 1994; Kanematsu et al., 1992). In this study, we compared the ability of [^{3}H]BZDC-Ins-(1,4,5)P_3 to label covalently three PLC isozymes: δ_1, β_1, and β_2 (Figure 2). Only the δ_1 isozyme was specifically radiolabeled (lane 5, upper portion). Labeling of the δ_1 isozyme was observed in reaction mixtures containing both PLC-β_1 and -δ_1 (lane 7, upper portion) or in reaction mixtures that contained substrate vesicles (7:3 PE/PI) and/or calcium (50−200 μM) (data not shown). No labeling of PLC-β_1 or

a



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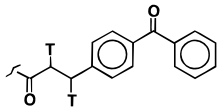
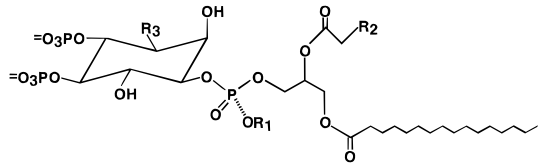
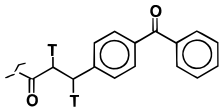


Inositol polyphosphate	R_1	R_2	R_3	R_6
[^{3}H]BZDC-Ins(1,4,5)P_3	aminopropyl-BZDC	OH	OH	OH
[^{3}H]BZDC-Ins(1,3,4,5)P_4	aminopropyl-BZDC	OH	OPO_3=	OH
[^{3}H]BZDC-Ins(1,2,3,4,5,6)P_6	H	phosphoamino-hexylBZDC	OPO_3=	OPO_3=

b



[^{3}H]BZDC =



Polyphosphoinositide	R_1	R_2	R_3
[^{3}H]BZDC-PI(4,5)P_2-triester	aminopropylBZDC	$(CH_2)_{13}CH_3$	OH
[^{3}H]BZDC-PI(3,4,5)P_3-triester	aminopropylBZDC	$(CH_2)_{13}CH_3$	OPO_3=
[^{3}H]BZDC-PI(4,5)P_2-acyl	H	$(CH_2)_4NHBZDC$	OH

FIGURE 1: (a) Chemical structures of synthetic analogs of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,2,3,4,5,6)P_6 containing the photoactivatable [^{3}H]BZDC moiety (T = tritium). (b) Chemical structures of synthetic analogs of PI(4,5)P_2 and PI(3,4,5)P_3 containing [^{3}H]-BZDC (T = tritium).

-β_2 was observed under any of these conditions. Ins(1,4,5)P_3 competitively inhibited labeling of PLC-δ_1 by [^{3}H]BZDC-Ins(1,4,5)P_3 (Figure 2) and was more effective than Ins(1,4)P_2 (Figure 3). At 60 μM Ins(1,4)P_2, the labeling was decreased ~50% when the bands were excised and counted directly in a liquid scintillation spectrometer (data not shown). By contrast, labeling was reduced >80% by addition of 10 μM Ins(1,4,5)P_3. This is consistent with the higher affinity of native PLC-δ_1 and its PH domain for inositol phosphates containing a phosphomonoester group at the position 5- of the inositol ring (Rebecchi et al., 1992; Cifuentes et al., 1994; Garcia et al., 1995; Lemmon et al., 1995).

A panel of sequence-specific antibodies (described in Figure 4) was used to map the sites in PLC-δ_1 labeled in the photo-cross-linking reaction with [^{3}H]BZDC-Ins(1,4,5)P_3. The labeled enzyme was digested with either chymotrypsin or trypsin to generate a series of fragments analyzed for their content of radiolabel, relative molecular mass, and immunoreactivity (Table 1a,b).

One major product of chymotrypsin digestion (Table 1a) was a 70 kDa fragment that contained ~1% of the total radiolabel and reacted with antisera against the X and Y boxes but not with antiserum against the PH domain. Several highly radioactive protein bands migrated between 80 and 85 kDa and reacted with antisera against the PH domain and the X and Y box regions. A lightly stained protein band,

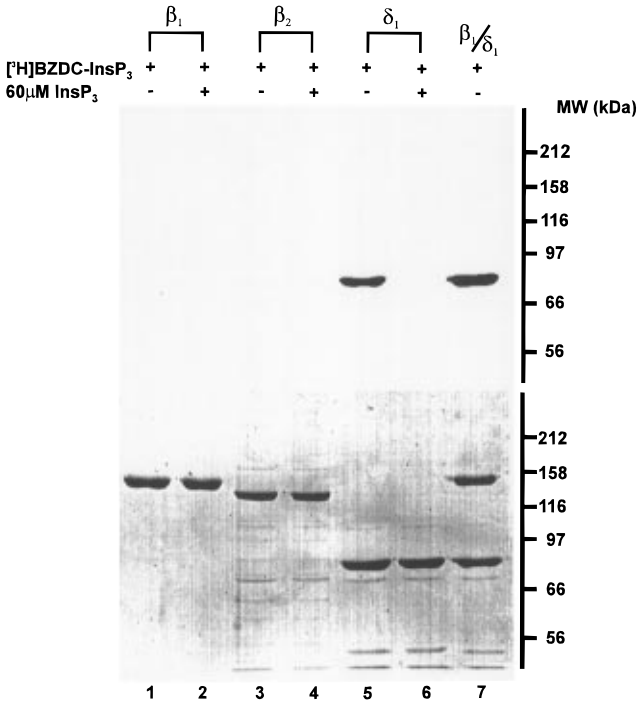


FIGURE 2: Reaction of PLC-δ_1, -β_1, or -β_2 with [^{3}H]BZDC-Ins(1,4,5)P_3. The PLC isozymes were incubated with [^{3}H]BZDC-Ins(1,4,5)P_3 (0.5 μM) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, and 6) of 60 μM unlabeled Ins(1,4,5)P_3 and exposed to 350 nm light. Samples were subjected to SDS−PAGE and autoradiography. The acrylamide gel, stained with Coomassie Brilliant Blue dye (bottom), contained PLC-β_1 (lanes 1 and 2), PLC-β_2 (lanes 3 and 4), PLC-δ_1 (lanes 5 and 6), or a mixture of PLC-β_1 and PLC-δ_1 (lane 7). Autoradiograph of the same gel (top). Only the δ_1 isozyme contained detectable radioactivity. Mobilities of molecular mass standards are shown in kilodaltons.

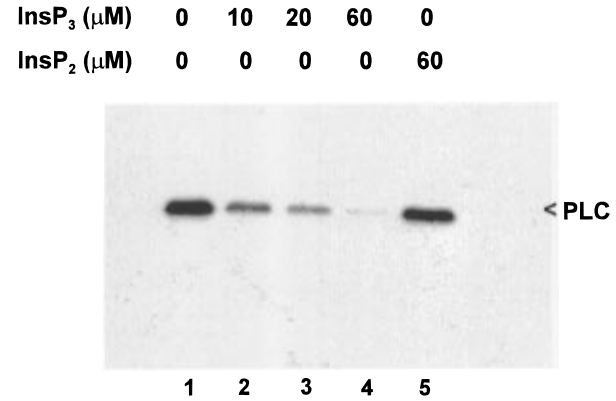


FIGURE 3: Reaction of PLC-δ_1 with [^{3}H]BZDC-Ins(1,4,5)P_3. PLC-δ_1 was incubated with [^{3}H]BZDC-Ins(1,4,5)P_3 (0.5 μM) alone (lane 1) or in the presence of unlabeled Ins(1,4,5)P_3 (lanes 2−4) or Ins(1,4)P_2 (lane 5) at the indicated concentrations and irradiated with 350 nm light. Samples were subjected to SDS−PAGE and autoradiography. PLC-δ_1 labeling was specifically competed for by Ins(1,4,5)P_3.

migrating with an apparent molecular mass of ~60 kDa, contained 20% of the incorporated label and reacted with antisera against the amino-terminal sequence of the PH domain, and a portion of the X box, but not the Y box region. Minor protein bands of 40, 30, and 28 kDa were also detected by Coomassie staining but contained low amounts of radioactivity.

Digestion with trypsin yielded major fragments of 60 and 30 kDa (Table 1b). The 60 kDa fragment reacted with amino-terminal and X box antisera, while the 30 kDa

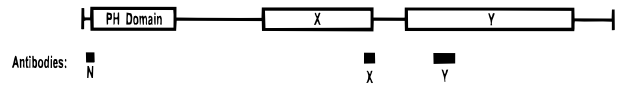


FIGURE 4: Regions of PLC-δ_1 recognized by sequence-specific antibodies. Open boxes indicate portions of the enzyme that comprise the PH domain, the X box, and the Y box. Dark filled boxes indicate the positions of the sequences of PLC-δ_1 used to design the synthetic peptides to which the antibodies N (residues 3−16), X (residues 430−442), and Y (residues 511−530) were produced, as described in Cifuentes et al. (1993).

Table 1: Analysis of the Proteolytic Fragments Generated by Digestion of Radiolabeled PLC-δ_1 with (a) Chymotrypsin and (b) Trypsin[a,b]

apparent molecular mass (kDa)	cpm of ^{3}H	Coomassie stain	N ab	X ab	Y ab
(a) 80−85	43876	++++	+	+	+
70	722	+++	−	+	+
60	12805	+	+	+	−
40	1647	+	−	−	−
30	342	+	−	−	−
28	601	+	−	−	−
20	3240	−	−	−	−
(b) 85	2678	+	+	−	−
60	27505	++++	+	+	−
55	381	++	−	+	−
39	677	++	−	+	−
38	1784	++	−	+	−
30	131	++++	−	−	+
28	111	++	−	−	−
20	2864	−	−	−	−

[a] + indicates relative intensity of Coomassie staining or antibody (ab) reactivity. [b] The radioactivity was determined by cutting the gel into 2 mm slices and counting in a liquid scintillation spectrometer. The amounts of radiolabel incorporated into immunoreactive bands detected were also determined and gave similar results (data not shown).

fragment only reacted with Y box antisera. The 60 kDa fragment contained approximately 50% of the incorporated radiolabel, whereas the 30 kDa fragment contained less than 0.5%. This result proved that the Y box was not labeled significantly by [^{3}H]BZDC-Ins(1,4,5)P_3. We deduce from these results that the site radiolabeled by [^{3}H]BZDC-Ins-(1,4,5)P_3 lies within the amino-terminal PH domain. This contention is most strongly supported by the observation that the 70 kDa product of chymotrypsin digestion, encompassing the X and Y boxes and missing the first ~100 amino acid residues, did not contain significant levels of radioactivity. By exclusion, the region of PLC-δ_1 covalently cross-linked to [^{3}H]BZDC-Ins(1,4,5)P_3 or [^{3}H]BZDC-Ins PI(4,5)P_2 lies within the PH domain (Table 1a,b). This interpretation is further supported by the demonstration that the isolated domain binds PI(4,5)P_2 and Ins(1,4,5)P_3 with an affinity and specificity that are comparable to those of the native enzyme (Garcia et al., 1995; Lemmon et al., 1995).

Minor products of the trypsin digestion, such as the 55, 39, and 38 kDa fragments, contained low levels of radioactivity, reacted with the X box antibody, and were not detected with antibodies against either the PH domain or the Y box region. It is unlikely that the low levels of radioactivity, present in these minor bands, represent labeling of the X box, since even less radioactivity was recovered in a major 70 kDa chymotrypsin-generated fragment that reacted strongly with the X box antiserum. It is likely that these minor fragments contained part of the radiolabeled PH domain missing the epitope recognized by the sequence-specific amino-terminal antibody (amino acid residues 3−16). We

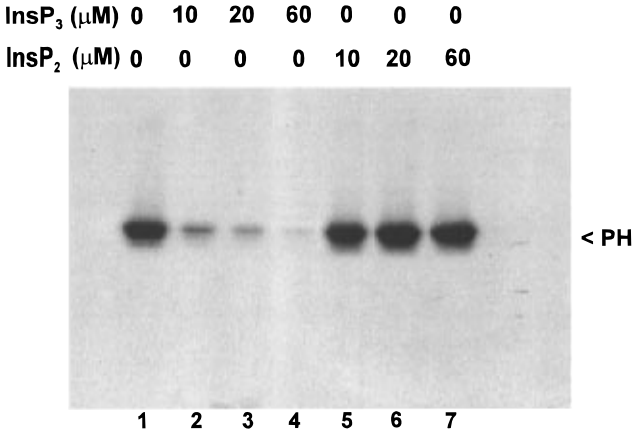


FIGURE 5: Photo-cross-linking of [^{3}H]BZDC-Ins(1,4,5)P_3 to the PH domain of PLC-δ_1. The isolated PH domain of PLC-δ_1 was incubated with [^{3}H]BZDC-Ins(1,4,5)P_3 (0.75 μM) in the absence (lane 1) or presence of Ins(1,4,5)P_3 (lanes 2−4) or Ins(1,4)P_2 (lanes 5−7) and irradiated with 350 nm light. The samples were subjected to SDS−PAGE and autoradiography. Only Ins(1,4,5)P_3 effectively displaced the photoaffinity reagent.

were unable to recover a fragment of PLC-δ_1 that encompassed only the intact PH domain or to identify the radioactive material that migrated with an apparent molecular mass of 20 kDa. These results are consistent with our previous report showing that the amino-terminal 60 amino acids, which form part of this domain, are susceptible to extensive proteolysis (Cifuentes et al., 1993). In contrast, the X and Y boxes were determined to be resistant to digestion.

To test the identity of the labeled region, the PH domain of PLC-δ_1 was photoreacted with [^{3}H]BZDC-Ins(1,4,5)P_3. Like the native enzyme, the isolated PH domain was highly and specifically labeled with this compound (Figure 5). Labeling was effectively displaced by nonradioactive Ins-(1,4,5)P_3, but not by Ins(1,4)P_2. These results are consistent with those obtained with native PLC-δ_1 and identify the PH domain as the principal binding site in this isoform.

Although PLC-β_1 and -β_2 failed to bind to or be photoaffinity labeled by [^{3}H]BZDC-Ins(1,4,5)P_3, they might still possess binding sites for higher-order inositol polyphosphates, such as Ins(1,3,4,5)P_4 and InsP_6. To test this idea, the radiolabeling of PLC-β_1 and -β_2 by [^{3}H]BZDC-Ins-(1,3,4,5)P_4 or [^{3}H]BZDC-InsP_6 was compared with that of PLC-δ_1 and its PH domain (Table 2). Neither PLC-β_1 nor PLC-β_2 was specifically radiolabeled (Table 2, rows 2−8). In contrast, PLC-δ_1 and its PH domain, reacting with [^{3}H]-BZDC-Ins(1,3,4,5)P_4, incorporated significant levels of radioactivity (row 2) that were effectively inhibited by 30 μM Ins(1,4,5)P_3, but not Ins(1,4)P_2 (rows 3 and 4). Labeling of the PLC-δ_1 PH domain by [^{3}H]BZDC-InsP_6 was also observed (rows 5−8).

To confirm the results of the photo-cross-linking experiments, binding of [^{3}H]Ins(1,4,5)P_3 to PLC-δ_1, -β_1, -β_2, and -β_3 was directly measured (Figure 6). Because calcium is known to bind to the active site of PLC and to stimulate PIP_2 hydrolysis (Essen et al., 1996), the measurements were performed in the presence and absence of this ion. The results confirm the presence, in PLC-δ_1, of a single high-affinity binding site for Ins(1,4,5)P_3 that is unaffected by calcium ions. This site has an apparent K_d of ~0.3 μM, similar to that reported for the intact enzyme and its isolated

Table 2: Incorporation of [^{3}H]BZDC-Inositol Polyphosphates and Polyphosphoinositides into PLC-δ_1, -β_1, and -β_2

	[^{3}H]BZDC compound	unlabeled compound (30 μM)	PLC isozyme δ_1	β_1	β_2	δ_1-PH
1	IP_3	−	2.143 ± 0.769[a] (8)[b]	0.009 ± 0.005 (3)	0.004 ± 0.000 (3)	0.192 ± 0.118 (6)
2	IP_4	−	0.857 ± 0.194 (3)	0.061 ± 0.021 (4)	0.079 ± 0.092 (4)	0.025 ± 0.011 (4)
3	IP_4	IP_3	0.090 ± 0.015 (2)	0.043 ± 0.015 (2)	0.070 ± 0.020 (2)	0.005 ± 0.003 (4)
4	IP_4	IP_2	0.921 ± 0.059 (2)	0.059 ± 0.016 (2)	0.051 ± 0.013 (2)	0.034 ± 0.021 (4)
5	IP_6	−	0.484 ± 0.217 (2)	−	0.120 ± 0.000 (2)	0.006 ± 0.003 (2)
6	IP_6	IP_3	0.050 ± 0.024 (2)	−	0.164 ± 0.063 (2)	0.003 ± 0.000 (2)
7	IP_6	IP_2	0.450 ± 0.131 (2)	−	0.140 ± 0.021 (2)	0.007 ± 0.002 (2)
8	IP_6	IP_6	0.091 ± 0.057 (2)	−	0.132 ± 0.006 (2)	0.005 ± 0.001 (2)
9	PIP_2 triester	−	1.498 ± 0.667 (4)	0.040 ± 0.011 (6)	0.045 ± 0.013 (5)	−
10	PIP_2 triester	PIP_2	0.074 ± 0.035 (4)	0.035 ± 0.015 (6)	0.038 ± 0.010 (5)	−
11	PIP_2 triester	PI	1.336 ± 0.373 (4)	0.036 ± 0.013 (6)	0.044 ± 0.015 (5)	−
12	PIP_3 triester	−	0.442 ± 0.220 (4)	0.031 ± 0.007 (4)	0.057 ± 0.014 (4)	−
13	PIP_3 triester	PIP_2	0.019 ± 0.006 (4)	0.028 ± 0.009 (4)	0.056 ± 0.019 (4)	−
14	PIP_3 triester	PI	0.270 ± 0.074 (4)	0.040 ± 0.010 (4)	0.060 ± 0.016 (4)	−
15	PIP_2 (acyl)	−	0.121 ± 0.034 (4)	0.035 ± 0.012 (4)	0.029 ± 0.012 (3)	0.012 ± 0.001 (2)
16	PIP_2 (acyl)	PIP_2	0.038 ± 0.011 (4)	0.037 ± 0.014 (4)	0.032 ± 0.020 (3)	0.002 ± 0.000 (2)
17	PIP_2 (acyl)	PI	0.110 ± 0.008 (4)	0.037 ± 0.016 (4)	0.034 ± 0.024 (3)	0.007 ± 0.001 (2)

[a] Mol % of PLC covalently cross-linked (mean ± standard deviation). [b] *n* equals the number of determinations.

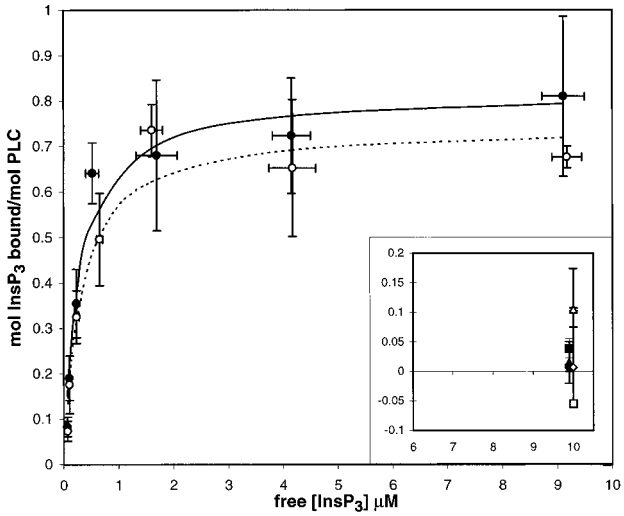


FIGURE 6: Binding of Ins(1,4,5)P_3 to PLC. Binding of [^{3}H]Ins-(1,4,5)P_3 to PLC-δ_1 (●), -β_1 (■), -β_2 (▲), and -β_3 (◆) was determined in the presence (open symbols) or absence (filled symbols) of calcium ions (83 μM). The inset is a magnified view of the binding data obtained with the PLC-β isozymes, which were determined at 10 μM Ins(1,4,5)P_3. Free Ins(1,4,5)P_3 concentrations were calculated from the amounts bound. Binding isotherms predicted by eq 1 were fit to the results obtained in the presence (- - -) or absence (—) of calcium ions from at least two independent experiments. Maximum binding in the presence or absence of calcium was 0.82 ± 0.04 and 0.74 ± 0.04, respectively. The apparent K_d values were 0.28 ± 0.06 and 0.31 ± 0.08 μM. Standard deviations are shown if they are larger than the symbol sizes.

PH domain (Rebecchi et al., 1992; Garcia et al., 1995; Lemmon et al., 1995). Significant binding to PLC-β_1, -β_2, or -β_3 was not detected (Figure 6, inset). At the limit of detectable binding in this assay (30 μM), no further increases in the amounts of Ins(1,4,5)P_3 bound to the PLC isozymes were detected (results not shown).

The possibility that the presence of a lipid moiety contributes to high-affinity binding to polyphosphoinositides was tested. Novel PI(4,5)P_2 and PI(3,4,5)P_3 analogs (see Figure 1b), containing the photoactivatable group [^{3}H]BZDC located in either the polar head group (triester) or acyl chain region (Prestwich, 1996; Gu & Prestwich, 1996; Chen et al., 1996), were used to probe PLC-δ_1, -β_1, and -β_2. Only PLC-δ_1 was specifically labeled in the phospholipid/detergent mixed micelle (Table 2, rows 9, 12, and 15); inclusion of 30 μM PI(4,5)P_2 (rows 10, 13, and 16), but not PI (rows 11, 14, and 17), substantially inhibited photolabeling. Its PH domain was similarly radiolabled by [^{3}H]BZDC-PI(4,5)P_2 acyl ester (rows 15−17). Whether radiolabeled with [^{3}H]-BZDC-Ins(1,4,5)P_3, [^{3}H]BZDC-PI(4,5)P_2 (triester or acyl-linked diester), or [^{3}H]BZDC-PI(3,4,5)P_3 (triester), digestion of PLC-δ_1 with trypsin always yielded the same pattern of radiolabeled peptides (results not shown), demonstrating that the same domain was labeled with each compound. Specific incorporation into either PLC-β_1 or -β_2 of any polyphosphoinositide analog was not observed (rows 9−17).

Table 3: Effect of Ins(1,4,5)P_3 on the Rate of PIP_2 Hydrolysis Catalyzed by PLC-β_1, -β_2, -β_3, or -δ_1

PLC isozyme	% control rate[a]	SD	*n*[c]
β_1	93	10	6
β_2	100	1	6
β_3	123[b]	18	6
δ_1	58[b]	2	6

[a] Percent of PI(4,5)P_2 hydrolysis rate determined in the absence of 30 μM Ins(1,4,5)P_3. Significance was determined by analysis of variance (Tukey's Test). [b] $p < 0.05$. [c] n = number of experiments.

We considered the possibility that the affinities of the PLC-β isozymes for Ins(1,4,5)P_3 may be much higher under catalytic conditions. To test this possibility, the rates of PI-(4,5)P_2 hydrolysis were measured in the presence or absence of a high concentration (30 μM) of Ins(1,4,5)P_3, which had previously been shown to inhibit PLC-δ_1 activity by competing for the binding to PI(4,5)P_2 (Kanematsu et al., 1992; Cifuentes et al., 1994). The bilayer membranes contained a high concentration (75 mol %) of PE that, although unnecessary for PLC-δ_1, was required for measuring the PLC-β activities. Under these conditions, PLC-δ_1 catalytic activity was inhibited nearly 50% by Ins(1,4,5)P_3, whereas the activities of PLC-β_1 and -β_2 were not (Table 3). These results agree with the photo-cross-linking and binding studies. Surprisingly, PLC-β_3 was slightly stimulated. Whether this was a specific effect of Ins(1,4,5)P_3 on the β_3 isozyme was not explored further.

DISCUSSION

We tested for the presence of high-affinity PI(4,5)P_2 and PI(3,4,5)P_3 binding sites in four phospholipase C (PLC) isozymes (δ_1, β_1, β_2, and β_3) by probing these proteins with photoreactive analogs of inositol phosphates, D-Ins(1,4,5)P_3, D-Ins(1,3,4,5)P_4, and InsP_6, and polyphosphoinositides PI(4,5)P_2 and PI(3,4,5)P_3. Only PLC-δ_1 was specifically radiolabeled. More than 90% of the label was found in tryptic and chymotryptic fragments which reacted with antisera against the PH domain, whereas less than 5% was recovered in fragments that encompassed the catalytic core. In separate experiments, the isolated δ_1-PH domain was also specifically labeled. Equilibrium binding of D-Ins(1,4,5)P_3 to PLC-δ_1 indicated the presence of a single, high-affinity binding site; binding of D-Ins(1,4,5)P_3 to PLC-β_1, -β_2, or -β_3 was not detected. The catalytic activity of PLC-δ_1 was inhibited by the product D-Ins(1,4,5)P_3, whereas no inhibition of PLC-β_1, -β_2, or -β_3 activity was observed. These results demonstrate that the PH domain is the sole high-affinity PI(4,5)P_2 binding site of PLC-δ_1 and that a similar site is not present in PLC-β_1, -β_2, or -β_3. The data strongly support the idea that the PH domain of PLC-δ_1, but not of the β isozymes, directs the catalytic core to membranes enriched in PI(4,5)P_2 and is subject to product inhibition.[2]

Neither the [^{3}H]BZDC-labeled inositol phosphates nor polyphosphoinositides exhibited specific photoattachment to the PLC-β_1 or -β_2 isozymes; the lower limit of detection in these experiments was about 2% of the label found in PLC-δ_1. No labeling of PLC-β_1 was observed in the presence of substrate vesicles and/or calcium ions. Furthermore, no specific binding was detected in equilibrium experiments, even at concentrations of InsP_3 as high as 30 μM. PLC-β, isolated from turkey erythrocyte membranes, has been shown to bind PI(4,5)P_2 with an affinity comparable to that of PLC-δ_1 (James et al., 1995). These investigators suggested that, like PLC-δ_1, this isozyme cleaves its substrate processively. Their results were consistent with a model in which PLC bound PI(4,5)P_2 at a minimum of two sites, one of which is catalytic. A similar model, based on approaches developed by other investigators (Gelb et al., 1995; Carman et al., 1995), has been used to explain the catalytic and membrane binding behavior of the δ_1 isozyme (Cifuentes et al., 1993). Our experiments, however, demonstrate that neither PLC-β_1, -β_2, nor -β_3 binds the polar head groups of PI(4,5)P_2 or PI(3,4,5)P_3 with specificity or affinity comparable to that of PLC-δ_1. Recently, Runnels et al. (1996) demonstrated that PLC-β_1 and -β_2 bind to membrane bilayers with high affinity, but without the PI(4,5)P_2 specificity observed for PLC-δ_1. What could account for high-affinity binding is unclear from the sequence of the erythrocyte isozyme. Whatever PI(4,5)P_2 binding sites may be present, they seem to be unique to this avian isoform.

The PH domains of the PLC-β isozymes are missing critical amino acid residues involved in binding the PI(4,5)P_2 polar head group (Ferguson et al., 1995b). In the PLC-β isozymes, the loops connecting predicted β-strands 1 and 2 and 3 and 4, which correspond to the PI(4,5)P_2 binding site of the PLC-δ_1 PH domain, contain none of the important residues involved in ligating Ins(1,4,5)P_3 to δ_1. The corresponding sequences of the PH domains found in PLC-γ_1 and PLC-γ_2 also lack the appropriate residues. Remarkably, key residues present in the loop connecting predicted β-strands 3 and 4 are not well conserved in the other δ isozymes. Therefore, it appears that PLC-δ_1 contains a PH domain that binds PI(4,5)P_2 with uniquely high affinity.

The three-dimensional structure of the PLC-δ_1 PH domain complexed with Ins(1,4,5)P_3 provides a molecular explanation of the energies involved in binding, and the high degree of specificity for the 4- and 5-position phosphates of PI(4,5)P_2 (Ferguson et al., 1995b). Like other PH domains, the overall structure is well conserved and electrically polarized, with a core consisting of two nearly orthogonal antiparallel β-sheets and a long C-terminal α-helix that ties together one end of a pseudo-β-sandwich structure. The binding site lies at the center of the protein's positively charged face. Most of the specific contacts involve hydrogen bonds to the 4- and 5-position phosphates and are contributed by the side chains of nine amino acids extending from the loops connecting β-strands 1 and 2 and 3 and 4. Remarkably, seven of the twelve hydrogen bonds formed in the complex are contributed by six amino acids that participate in contacts with the 5-position phosphomonoester group. The photo-cross-linking data are consistent with this structure and the high degree of specificity for this group. A similar structure was also reported for the complex of Ins(1,4,5)P_3 and the β-spectrin PH domain (Hyvönen et al., 1995), but here, the affinity for Ins(1,4,5)P_3 is nearly 2 orders of magnitude weaker than that of the PH domain of PLC-δ_1. The β-spectrin binding site is a shallow groove in the surface of the protein rather than a binding pocket and involves fewer contacts between the protein side chains and the phosphomonoester groups.

The reaction product, Ins(1,4,5)P_3, inhibits PLC-δ_1 by preventing membrane adsorption (Kanematsu et al., 1992; Cifuentes et al., 1994). This effect could represent a negative feedback pathway in the regulation of the PLC-δ_1 catalytic activity in living cells (Lemmon et al., 1995). Although we could observe product inhibition of PLC-δ_1, neither PLC-β_1, -β_2, nor -β_3 was similarly affected (Table 3). Our results suggest that direct control of PLC-β catalytic activity by the level of cellular Ins(1,4,5)P_3 is unlikely.

Regions, other than the PH domain, may anchor the PLC-β isozymes to the interface but are unlikely to provide sufficient specificity to direct them to membranes enriched in PI(4,5)P_2. A unique region extends approximately 400 amino acids beyond the Y box of the PLC-β isozymes (Lee & Rhee, 1995) and is important for binding acidic phospholipids (Wu et al., 1993; Lee et al., 1993), activation by G-protein α-subunits, and/or nuclear transport (Kim et al., 1996). Our results suggest that this region lacks high-affinity binding sites for PI(4,5)P_2, PI(3,4,5)P_3, and their polar head groups.

It has been proposed that the sequence (K/R)XXXKXK-(K/R), found in the X box region of all eukaryotic PLC isozymes, could serve as a specific high-affinity binding site for PI(4,5)P_2 (Simoes et al., 1993, 1995). Similar suggestions by other investigators (Yu et al., 1992) were based on homology between this peptide and sequences found in

[2] In previous studies, we (Cifuentes et al., 1993) and others (Yagisawa et al., 1994) have shown that the amino-terminal region is required for high-affinity PI(4,5)P_2 and Ins(1,4,5)P_3 binding. These experiments involved truncation of the enzyme that may have produced a conformational change which obscured a high-affinity binding site in the X or Y regions ("catalytic core"). By labeling the intact enzyme and mapping these regions, we appear to exclude this possibility.

profilin, cofilin, gelsolin, and other PI(4,5)P_2 binding proteins (Janmey, 1994). This site is clearly seen in the three-dimensional structure of the PLC-δ_1 catalytic core (Essen et al., 1996). Lys 438 and Lys 440, the invariant lysine residues, and Ser 522 and Arg 549 are situated in the active site where they form hydrogen bonds with the 4- and 5-position phosphates of PI(4,5)P_2. We conclude from our results that this is a low-affinity site ($K_d > 10^{-4}$ M) and is unlikely to serve as a membrane tether for PI(4,5)P_2.

Calcium ions, which stimulate PLC catalytic activity and bind to the catalytic and C-2 domains (Essen et al., 1996), are likely to play multiple roles in regulating catalysis. In the active site of PLC-δ_1, a single calcium ion is ligated by Glu 341, Glu 390, Asp 343, and Asn 312 but only interacts with the inositol ring through the 2-position hydroxyl and the 1-position phosphate (Essen et al., 1996). Calcium also binds to the PLC-δ_1 C-2 domain. Regardless of the mechanism by which calcium affects catalysis, our results (Figure 6) show that this cation does not significantly alter the affinity of the PH domain for the PI(4,5)P_2 polar head group.

The hydrophobic amino acids that surround the entrances to the active sites of PLC-δ_1 and bacterial PI-PLC (Essen et al., 1996; Heinz et al., 1995) are likely to penetrate the membrane surface, a process necessary for catalysis (Rebecchi et al., 1992b). These residues may contact the acyl chain region. Consistent with this idea are the observations that a minimum acyl chain length of six carbons is required for PLC-δ_1 to catalyze hydrolysis and that lyso-PI and glycerol 1-inositolmonophosphate are not substrates (Rebecchi et al., 1993). Labeling of the catalytic core by the photoaffinity analogs of PI(4,5)P_2, however, was not observed. Our negative findings are unlikely to be due to the position of the photoreactive probe; compare the placement of the photoreactive groups of the acyl and triester analogs of PI-(4,5)P_2. Thus, the acyl chains do not enhance the affinity of these lipids for the active site to the extent that photolabeling is promoted. These results suggest a weak and nonspecific association of the protein with the acyl chain region in the absence of calcium ions.

In summary, the PH domain of the PLC-δ_1 isozyme can direct its host protein to membranes enriched in PI(4,5)P_2 and is subject to product inhibition. By contrast, we find no evidence that the mammalian β isozymes possess a comparable binding site for this lipid in their PH domains, catalytic cores, or extended carboxy-terminal sequences or that these isozymes are inhibited by Ins(1,4,5)P_3. On the basis of these observations and comparisons with other PLC sequences, it appears that the δ_1 isozyme is unique in its capacity to bind PI(4,5)P_2. The affinities of the other PLC isoforms for PI(4,5)P_2 may be too weak to tether them to membranes enriched in this phosphoinositide in the absence of additional interactions.

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