Phosphatidylinositol 4,5-Bisphosphate Binding to the Pleckstrin Homology Domain of Phospholipase C-δ1 Enhances Enzyme Activity*

(Received for publication, March 25, 1996, and in revised form, July 25, 1996)

The pleckstrin homology (PH) domain is a newly recognized protein module believed to play an important role in signal transduction. While the tertiary structures of several PH domains have been determined, some co-complexed with ligands, the function of this domain remains elusive. In this report, the PH domain located in the N terminus of human phospholipase C-δ1 (PLCδ1) was found to regulate enzyme activity. The hydrolysis of phosphatidylinositol (PI) was stimulated by phosphatidylinositol 4,5-bisphosphate (PIP2) in a dose-dependent manner with an EC50 = 1 μM (0.3 mol%), up to 9-fold higher when 5 μM (1.5 mol%) of PIP2 was incorporated into the PI/phosphatidylserine (PS)/phosphatidylcholine (PC) vesicles (30 μM of PI with a molar ratio of PI:PS:PC = 1:5:5). Stimulation was specific for PIP2, since other anionic phospholipids including phosphatidylcholine 4-phosphate had no stimulatory effect. PIP2-mediated stimulation was, however, inhibited by inositol 1,4,5-trisphosphate (IP3) in a dose-dependent manner, suggesting a modulatory role for this isoinosine. When a nested set of PH domain deletions up to 70 amino acids from the N terminus of PLCδ1 were constructed, the deletion mutant enzymes all catalyzed the hydrolysis of the micelle forms of PI and PIP2 with specific activities comparable with those of the wild type enzyme. However, the stimulatory effect of PIP2 was greatly diminished when more than 20 amino acid residues were deleted from the N terminus. To identify the specific residues involved in PIP2-mediated enzyme activation, amino acids with functional side chains between residues 20 and 40 were individually changed to glycine. While all these mutations had little effect on the ability of the enzyme to catalyze the hydrolysis of PI or PIP2 micelles, the catalytic activity of mutants K24G, K30G, K32G, R38G, or W36G was markedly unresponsive to PIP2. Analysis of PIP2-stimulated PI hydrolysis by a dual substrate binding model of catalysis revealed that the micellar dissociation constant (KD) of PLCδ1 for the PI/PS/PC vesicles was reduced from 558 μM to 53 μM, and the interfacial Michaelis constant (Km) was reduced from 0.21 to 0.06 by PIP2. The maximum rate of PI hydrolysis (Vmax) was not affected by PIP2. These results demonstrate that a major function of the PH domain of PLCδ1 is to modulate enzyme activity. Further, our results identify PIP2 as a functional ligand for a PH domain and suggest a general mechanism for the regulation of other proteins by PIP2.

PLCδ1 is a member of a family of inositol phospholipid-specific phospholipase C (PLC)1 isozymes through which a variety of hormones, neurotransmitters, and growth factors elicit intracellular responses (1). All three major PLC isozyme families (β, δ, and γ) are able to recognize phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP2) and carry out the Ca2+-dependent hydrolysis of these inositol phospholipids (2). Comparison of the primary structures of members from each family shows significant amino acid sequence identity with other family members, but little identity exists between members of different families. Three exceptions to this divergence of primary structure among different isoforms are two distinct conserved sequences denoted as the X and Y domains and the newly recognized pleckstrin homology domain. X and Y domains are regions of ~170 and ~260 amino acids, respectively, which share 60–40% amino acid identity among all isoforms (2, 3). The X and Y domains are necessary for catalytic activity of PLC (2–8).

The pleckstrin homology (PH) domain is a newly recognized protein module composed of approximately 120 amino acids. This domain was initially identified at the N and C termini of pleckstrin, a major PKC substrate in platelet (9). PH domains have been found in about 60 signaling proteins, including all three eukaryotic isozyme families of PLC, and are believed to play an important role in signal transduction (10–13). The X and Y domains are necessary for catalytic activity of PLC (14–16) as well as the crystal structures (17–21) of several PH domains have determined that the PH domain is a β-sandwich structure composed of seven orthogonal β-sheets with an α-helix at the C-terminal end of the molecule, which caps the end of the sandwich. Several ligands have been shown to interact with various PH domains. These include inositol 1,4,5-trisphosphate (IP3) (19, 22, 23), PIP2 (19, 22, 24–26), and βγ subunits of heterotrimeric G proteins (27–29). Taken together, these studies suggest that PH domains

* This work was supported by grants from the Pfizer Scholars Program for New Faculty (to J. W. L.), the National Sciences Council of the Republic of China (to K. K.), and the Institute of Biomedical Sciences, Academia Sinica Intramural Funding (to K. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PLC, phosphoinositide-specific phospholipase C; PH domain, pleckstrin homology domain; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate, PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidyl glycerol; BSA, bovine serum albumin; SUV, small unilamellar vesicles.
mediate either protein-protein or protein-lipid interactions and lead to membrane anchorage.

Several partially revealing and sometimes conflicting reports have been published concerning the PH domain of PLCδ1. Both IP3 and PIP2 have been demonstrated to bind with high affinity to the PH domain of PLCδ1 when this domain has been studied as an isolated fusion protein (17, 30). Several groups have reported conflicting functional effects as the result of proteolysis or molecular genetic truncation of the N terminus of PLCδ1 (the PH domain is located in the N terminus of PLCδ1). Biochemical studies utilizing proteolyzed enzyme have indirectly suggested a functional role for the N-terminal PH domain of PLCδ1. Cifuentes et al. (31) demonstrated that the first 60 N-terminal residues of the PLCδ1 sequence (which overlap the putative PH domain) are not essential for Ca2+-dependent catalysis but are required for the enzyme to hydrolyze PIP2 in a processive manner. Conversely, Ellis et al. (4) and Yagisawa et al. (8) found that truncation of the N terminus of PLCδ1 did not affect its catalytic activity.

In addition to serving as substrate for PI-PLC to generate second messengers, PIP2 also plays an important role in several other cellular signalings. PIP2 has been shown to regulate the cell filament activity by interacting with profilin, coflin, and gelsolin (32, 33). The activity of phospholipase D has been shown to be stimulated by PIP2 directly (34) or through a high affinity and specificity to the PH domain of PLCδ1 (35–37). High affinity binding of PIP2 to the PH domain of dynamin leads to activation of the GTPase of the protein (25, 38). However, the structural requirement for these interactions and the mechanism underlying the phenomenon would require further investigation. In particular, given the fact that PIP2 can bind with high affinity and specificity to the PH domain of PLCδ1, it would be of interest to know whether PIP2 might also modulate the activity of PLCδ1. In the present report, we provide the first demonstration of high affinity binding of PIP2 to the PH domain of PLCδ1. As a result of this investigation, we have observed that the structural determinants mediating this activity in the PH domain of PLCδ1. Further, we have demonstrated that this specific protein-phospholipid interaction is crucial for efficient hydrolysis of substrate by PLCδ1, demonstrating an important role for PH domains in regulating enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Materials.—**The expression plasmid pSETA(BR) was from Invitrogen. The resulting expression construct (pSETA(BR)E) was placed under the control of the T7 promoter for expression in the Escherichia coli strain BL21(DE3)pLys (Novagen). Phosphatidylethanolamine (PE), PI, phosphatidic acid (PA), phosphatidyl glycerol (PG), phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Avanti Polar Lipids, Inc. 

**Construction of Mutant PLCδ1.—**A nested set of N-terminal deletion mutants (30-base pair increments) were generated in the human PLCδ1 cDNA using the polymerase chain reaction as described previously (39). Replacement of single amino acid residues in PLCδ1 was accomplished by polymerase chain reaction as described previously (7). All mutants were subsequently confirmed by DNA sequence analysis.

**Expression and Purification of PLCδ1—**Native and mutant enzyme cDNAs were introduced into the BamHI site of pSETA(BR). The resulting recombinant PLCδ1 contained a 34-amino acid residue module at the N terminus of the protein, which included six consecutive histidine residues. The enzymes were expressed in the E. coli strain BL21, solubilized, and purified by sequential Ni2+-nitrilotriacetic acid agarose chromatography as described previously (7). The homogeneity of the purified enzymes was demonstrated by SDS-polyacrylamide gel electrophoresis (data not shown). Functional activities of the enzyme preparations were determined by measuring the hydrolysis of [3H]IP1 in detergent micelles (40). Activities were expressed as specific activities (μmol/mg/min).

**Assay of PLCδ1 Activity—**PLCδ1-catalyzed hydrolysis of PIP2 was determined as described previously (41). Briefly, purified enzyme (0.05 mg/ml) was incubated in an assay volume of 60 μl containing 50 mM Hepes, pH 7.2, 3 mM EGTA, 0.2 mM EDTA, 0.83 mM MgCl2, 20 mM NaCl, 30 mM KCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.16% sodium cholate, 1.5 mM CaCl2, 50 μM [3H]IP1 (16,000 cpm), and 500 μM PE. The reaction was carried out at 30°C for 2–15 min and terminated by the addition of 0.2 ml of 10% ice-cold trichloroacetic acid and 1 ml of bovine serum albumin (10 mg/ml). After incubation on ice for 15 min, the unhydrolyzed [3H]IP1 (pellet) was separated from [3H]IP1 (supernatant) by centrifugation at 2000 × g for 10 min. Radioactivity in the supernatant was measured by liquid scintillation counting. PIP2 hydrolytic activity was expressed as μmol of IP1/mg/min of protein. Determination of PI hydrolytic activity was essentially as described by Hofmann and Majerus (40). Assays using dodecyl maltoside/PIP2 or dodecyl maltoside/PI mixed micelles were performed in a manner similar to those described by Cifuentes et al. (31) with slight modification. In brief, the indicated amounts of PIP2/[3H]PIP2 or PI/[3H]PI (4 × 105 cpm) in chloroform/methanol (19:1) were dried under a stream of N2 and then lyophilized for 30 min. Lipids were then solubilized by probe sonication in 0.95 ml of a solution containing 300 μM of dodecyl maltoside, 50 mM HEPES, pH 7.0, 100 mM NaCl and 2 mM EGTA. Fifty μl of bovine serum albumin in the same buffer was added to yield a final concentration of 500 μg/ml. To assay the PIP2 hydrolytic activity, 50 μl of dodecyl maltoside/PI or PIP2 mixed micelles was preincubated at 30°C for 5 min with 0.1–10 ng of enzyme. The reaction was initiated by adding 2.5 μl of 40 mM of CaCl2 and incubated at 30°C for another 1–15 min. For micelles containing PI reactions, the addition of 250 μl of chloroform/methanol/HCI (100:100:0.6) followed by 75 μl of 1 N HCI containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 100-μl portion of the upper aqueous phase was counted by liquid scintillation. For micelles containing PIP2, reactions were stopped by adding 0.17 ml of 10% ice-cold trichloroacetic acid and 0.85 μl of bovine serum albumin (10 mg/ml). After incubation on ice for 15 min, the unhydrolyzed [3H]PIP2 (pellet) was separated from [3H]IP1 (supernatant) by centrifugation at 2000 × g for 10 min.

**Hydrolysis of PI in Phospholipid Vesicles—**Hydrolysis of 30 μM PI was carried out in PI/PS/PC (molar ratio of 1:5:5) small unilamellar vesicles (SUV) incorporated with varying amounts of PIP2. Single bilayer vesicles were prepared as described previously (42) with slight modification. In brief, a stock solution of lipids, 30 nmol of H-bonded PI (4,000,000 cpm), 150 nmol of PS, 150 nmol of PC, and the indicated amount of PIP2 (0–7.5 nmol) were mixed and dried under a stream of nitrogen. The dried lipids were resuspended in 0.95 ml of 50 mM HEPES, pH 7.0, 100 mM NaCl, and 1 mM EGTA. The mixture was vortexed and followed by twofold cycles of ultracentrifugation on a Heat Systems Sonicator (Heat Systems, Farmingdale, NY) with 45-s cooling intervals. Samples were then centrifuged at 120,000 × g for 60 min, and the clear supernatant was carefully removed. Fifty μl of BSA was added to give a final concentration of 200 μg/ml. Greater than 90% of the phospholipid was routinely recovered in the supernatant. Samples were then bubbled with nitrogen and stored at room temperature. The final concentration of PI, PS, and PC was 30, 150, and 150 μM, respectively plus the indicated concentration of PIP2. Lipids were used within 24 h. To assay the PI-PLC activity, 50 μl of SUV containing 30 μM PI (20,000 cpm) was preincubated at 37°C for 5 min with 0.01–10 ng of enzyme. The reaction was initiated by adding 2.5 μl of 60 mM CaCl2 and incubated at 37°C for another 1–15 min. The reaction was terminated by adding 0.2 ml of 300 mM of chloroform/methanol/HCl (100:100:0.6), followed by 0.06 ml of 1 N HCl containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 100-μl portion of the upper aqueous phase was counted by liquid scintillation.

**Analysis of Kinetic Data—**PI hydrolysis catalyzed by native PLCδ1 and N-terminal deletion mutant PLCδ1 was measured as a function of total substrate concentration under case III conditions previously described for phospholipase A2 and PLCδ1 (43, 44). In case III, the total concentration of diluent detergent (dodecyl maltoside) was fixed, and the PLC activities were measured with increasing concentrations of the substrate. A dual phospholipid binding model of catalysis (Equations 1 and 2) (43) was used to analyze the kinetic data.

**Equation 1**

\[ E + 3S \overset{k_{1}}{\overset{k_{-1}}{\rightleftharpoons}} ES + S \]

**Equation 2**

\[ ES + S \overset{k_{2}}{\overset{k_{-2}}{\rightleftharpoons}} ESS \overset{k_{3}}{\rightleftharpoons} ES + P \]
TABLE I  

| Vesicle compositiona (molar fraction) | PI hydrolysis activityb (μmol/min/mg) |
|--------------------------------------|--------------------------------------|
| PC (0.9) + PI (0.1)                  | 0.20 ± 0.03                           |
| PC (0.455) + PS (0.455) + PI (0.09)  | 3.4 ± 0.4                             |
| PC (0.455) + PA (0.455) + PI (0.09)  | 4.1 ± 0.1                             |
| PC (0.455) + PG (0.455) + PI (0.09)  | 2.3 ± 0.3                             |
| PC (0.455) + PE (0.455) + PI (0.09)  | 0.4 ± 0.05                            |

a Nitrogen-dried phospholipid mixture containing 30 nmol of 3H-labeled inositol phospholipids (4 × 10⁵ cpm) and the indicated molar fraction of diluent phospholipids were dissolved by sonication into 1 ml of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, and 500 μg/ml bovine serum albumin. 

b Catalytic hydrolysis of PI in vesicles containing indicated diluent phospholipid was carried out in 50 μl of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 30 μM [3H]PI (20,000 cpm), 150 μM PS and 150 μM PC, 500 μg/ml bovine serum albumin, and 3 mM CaCl₂. After 5–15 min of incubation at 37 °C, reactions were terminated as described (40).

This model takes into account the fact that the reaction catalyzed by PLCβ1 occurs at the water-lipid interface of the phosphoinositide/dodecyl maltose mixed micelles. Initial binding of the enzyme to the water-lipid interface of the micelles is described by the micellar dissociation constant, K₆ = k₆₋₁,₆ (molar unit). This constant is dependent on both the enzyme concentration and the total substrate concentration. Once attached to the surface of mixed micelles, the enzyme searches for and binds to a second lipid molecule via the catalytic site. The binding of the second lipid molecule and the subsequent catalysis by PLCβ1 is described by the interfercal Michaelis constant, K₅ = k₅₋₁,₅ (molar unit). Initial rates of catalysis (v) as a function of total concentration of PI in the vesicles with a fixed concentration of diluent nonsubstrate phospholipids (T₄) measured under case III conditions were fitted using Equation 3 (43) to obtain the values of Vₘ₅ₙ₅, K₅, and K₆. 

\[ v = \frac{V_{max}^2}{K_vT_v + (T_v + K_vS_v + (K_v + 1)S_v)} \]  

where the absolute rate (Vₘ₅ₙ₅) occurs at an infinite substrate concentration, and the saturated substrate molar fraction, Sₐ is the total substrate concentration.

Centrifugation/IP₃ Binding Assays—Dose-dependent binding of enzyme to PI/PC/IP₃ vesicles (molar ratio of 4:1:0.25) was demonstrated using ultracentrifugation as described previously (7). In brief, 0.5–1 μg of enzyme was incubated with vesicles of defined lipid composition in a 0.2-mL volume for 15 min at 30 °C. Ultracentrifugation (400,000 × g) was carried out in a Beckman TL-100 table top ultracentrifuge and TLA-100 rotor for 40 min. Enzyme remaining in the unbound fractions (supernatant) was quantified by measuring catalysis of [3H]PI and by immunoblotting using mixed monoclonal antibodies specific for PLCβ1. The bound enzyme fractions (pellets) were quantitated by dissolving the pellet in 0.05 M of phosphate-buffered saline buffer and performing Western blot analysis.

RESULTS

Influence of Diluent Phospholipids on the Hydrolysis of PI in Small Unilamellar Vesicles—Our laboratory has recently found that certain lipids when used as diluents can have profound effects on the PI-hydrolyzing activity of PLCβ1. PI is hydrolyzed faster in anionic phospholipid vesicles than in zwitterionic phospholipid vesicles. To study the effect of anionic diluent phospholipids on the ability of PLCβ1 to catalyze the hydrolysis of PI, we compared the hydrolysis of [3H]PI in PI/PC and PI/PC/PE vesicles that do not contain anionic phospholipids other than the substrate with those in which the anionic phospholipid PA, PS, or PG were added. To minimize the effect of substrate phospholipid (PI) on the phase structure of vesicles, we limited the molar fraction of PI to no more than 0.05 of total lipid in the SUV. As shown in Table I, when only PC was used as a diluent phospholipid, the rate of hydrolysis of PI was 0.2 μmol/min/mg of enzyme. Adding the nonanionic phospholipid PE increased hydrolysis moderately to 0.4 μmol/min/mg of enzyme. Activity was greatly enhanced by including the anionic phospholipids such as PA, PG, or PS into the vesicles; the rate of PI hydrolysis was 1.1, 2.3, and 3.4 μmol/min/mg, respectively, which was a 5–15-fold increase over SUV containing PC only. These results demonstrated that the rate of PI hydrolysis catalyzed by PLCβ1 was significantly enhanced in SUV that contain a combination of PC (0.45 molar fraction) and anionic phospholipid (0.45 molar fraction).

The Anionic Phospholipid IP₃ Specifically Stimulates PI Hydrolysis in SUV—The enhancing effect of PA, PS, and PG on the hydrolysis of PI catalyzed by PLCβ1 could be due to specific lipid-protein interactions or due to nonspecific electrostatic interactions. To distinguish between these two possibilities, we tested the effects of anionic phospholipids on the rate of catalysis by including in SUV that either carry negatively charged phospholipids (PI/PC/PS and PI/PC/PA) or contain no anionic phospholipid (PI/PE and PI/PC). To minimize the effect of the added anionic phospholipids on the structure of SUV, we limited the PI to 0.09 molar fraction and the added anionic phospholipids to 0.03 molar fraction of the total lipid. The rate of PI hydrolysis in SUV catalyzed by PLCβ1 and the extent of its enhancement by anionic phospholipids were highly dependent on the composition of the diluent phospholipids used to make the SUV. All of the anionic phospholipids examined can markedly stimulate PLCβ1 to catalyze the hydrolysis of PI in PI/PC and PI/PE vesicles that do not contain negatively charged phospholipid constituents (from 7- to 20-fold). As shown in Fig. 1A, the ability of PLCβ1 to catalyze the hydrolysis of PI in PI/PC (molar ratio = 1:10) or PI/PE (molar ratio = 1:10) SUV was 0.20 and 0.42 μmol/min/mg, respectively. Catalytic activity increased to 2.7, 7.8, 5.7, or 8.9 μmol/min/mg by including in the PI/PE vesicles 3% PA, PS, PG, or IP₃, respectively. The hydrolysis of PI in PI/PE vesicles was increased from 0.2 μmol/min/mg to 1.4, 2.9, 2.0, or 3.3 μmol/min/mg by 3% PA, PS, PG, or IP₃, respectively. These results demonstrated that the negative charge in FA, PS, PG, and IP₃ is important to enhance the hydrolysis of PI in PI/PE or PI/PC vesicles. In contrast to the enhancement of catalysis by the addition of negatively charged phospholipids to PI/PC (molar ratio = 1:10) or PI/PE (molar ratio = 1:10) SUV that contain a relatively small fraction of total anionic phospholipids (9%) the addition of most negatively charged phospholipids to SUV already containing a high fraction (45%) of anionic phospholipid failed to stimulate PI hydrolysis. As shown in Fig. 1B, further incorporation of 3% of PA, PS, or PG into vesicles already containing 45% anionic phospholipids did not affect the rate of hydrolysis of PI. The notable exception was IP₃, where the activity of PI hydrolysis was significantly increased (8-fold) by incorporation of 3% IP₃ into vesicles that already contain 45% PS, PA, or PG. These results demonstrated that while PS, PA, or PG can enhance the catalysis of PI by PLCβ1 in vesicles that contain a small molar fraction of anionic phospholipids (<9.10), IP₃ could stimulate catalysis even in vesicles containing a high molar fraction (0.45) of anionic phospholipids. This result suggested that the IP₃-stimulatory process was due to a specific interaction with PLCβ1 rather than the presumed electrostatic interactions seen with PS, PA, or PG.

The effect of IP₃ on PI hydrolysis was also examined using PI/dodecyl maltose mixed micelles. In this system the PI-hydrolyzing activity was only slightly increased by IP₃ (data not shown). No IP₃ stimulation occurred when deoxycholate was used as the solubilizing detergent. For these reasons, the remaining experiments reported in the present communication employed PI/PS/PC (molar ratio = 1:5:5) SUV as substrate.

Dose-dependent Effects of IP₃, IP₃, and IP₃ on PI Hydrolysis—To further assess the specificity of IP₃ as a ligand to
stimulate PLC\(\delta1\) and to determine the necessary structural requirements of the lipid molecule required for enzyme activation, the dose-dependent effects of PIP2 and PIP on PI hydrolysis were assessed. Since PIP differs structurally from PIP2 only through the lack of a phosphate at the 5-position of the inositol ring, this allowed us to examine the importance of this phosphate group for the PIP2-mediated stimulatory effect. Fig. 2 demonstrates that relatively low concentrations of PIP2 (0.2 \(\mu\)M) were able to stimulate PLC\(\delta1\)-catalyzed hydrolysis of a fixed concentration (30 \(\mu\)M) of \([3H]\)PI in SUV (PI/PC molar ratio = 1:5:5). The activity increased quickly as a function of PIP2 concentration. Maximal 8–9-fold stimulation was seen with 5 \(\mu\)M PIP2, and half-maximal activity (4.5-fold stimulation) was seen with 1 \(\mu\)M PIP2. The dose response curve clearly demonstrates overall stimulation of PI hydrolysis at low concentrations of PIP2; however, as the concentration of PIP2 increased to 30 \(\mu\)M, the rate of PI hydrolysis began to decrease, presumably due to the competition of \([3H]\)PI and PIP2 for the catalytic site. Although a low level of PIP2 hydrolysis was seen in the present system (PIP2/PC/PS), this level was significantly reduced if the vesicles contained 0.07 or more molar fraction of PI (data not shown). Thus, inclusion of micromolar or submicromolar concentrations of PIP2 into vesicles containing PI, PS, and PC resulted in stimulation of PI hydrolysis rather than inhibition. In contrast, PIP did not stimulate PI hydrolysis. Instead, under the present conditions this phospholipid inhibited PLC\(\delta1\)-catalyzed hydrolysis of PI (Fig. 2B). These results suggested that the phosphorylation state of inositol ring is critical for inositol phospholipids to enhance the PI-hydrolyzing activity of PLC\(\delta1\). To further probe this, the effect of IP3 on the

**FIG. 1.** Effect of anionic phospholipids on the hydrolysis of PI by PLC\(\delta1\). A, the hydrolysis of PI in PI/PC (PI/PC molar ratio = 1:10) or in PI/PE (PI/PE molar ratio 1:10) vesicles (basal) and after incorporation of 3% PA, PS, PG, or PIP2. B, the hydrolysis of PI (basal activities) in vesicles of PI/PA/PC (molar ratio = 1:5:5), PI/PS/PC (molar ratio = 1:5:5), or PI/PG/PC (molar ratio = 1:5:5) and after incorporation of 3% PA, PS, or PG. Hydrolysis of \([3H]\)PI (30 \(\mu\)M) in the indicated phospholipid vesicles by PLC\(\delta1\) at 37 °C was carried out in 50 \(\mu\)l of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 3 mM CaCl2, and 500 \(\mu\)g/ml BSA for 5–15 min. The reaction was terminated, and the cleavage of PI was quantitated as described under “Experimental Procedures.” Data shown are the average of three experiments.
stimulatory activity of PIP₂ was examined. As shown in Fig. 3, IP₃ (0–5 μM) alone did not affect the ability of PLCδ₁ to catalyze the hydrolysis of PI in PI/PS/PC vesicles, suggesting that IP₃ at these concentrations did not compete with PI for the catalytic site of PLCδ₁. However, IP₃ did reduce the stimulatory effect of PIP₂ on the hydrolysis of PI in a dose-dependent manner with an IC₅₀ of <1 μM. Taken together, these observations imply that stimulation of PLCδ₁ to catalyze the hydrolysis of PI is highly specific for PIP₂, and both phosphates at D-4 and D-5 positions of the inositol ring are essential for the stimulation of hydrolysis. Further, IP₃ can inhibit PIP₂-mediated activation presumably by competing for PIP₂ at a site of interaction on PLCδ₁ that is distinct from the catalytic site.

**Elucidation of Structural Determinants of PIP₂-mediated Enzyme Activation**—The specificity of PIP₂ for stimulation of PI hydrolysis and IP₃ for inhibition suggested that the phosphate groups on the inositol ring may be recognized by particulate activation presumably by competing for PIP₂ at a site of PIP₂ interaction. Positive charges from the conserved lysine residues were neutralized by mutation to glycine. Mutant enzymes K434G (Lys⁴³⁴ → Gly), K440G (Lys⁴⁴⁰ → Gly), and K441G (Lys⁴⁴¹ → Gly) were stimulated by PIP₂; like the native enzyme (Fig. 4). Deleting the first 80 residues from the N terminus of PLCδ₁ ablated PIP₂-mediated stimulation (Fig. 4), while the basal PI-hydrolyzing activity was not affected by the truncation. This result revealed that structural determinants involved in the specific stimulatory effect of PIP₂ lie in the N-terminal region of PLCδ₁.

**The PH Domain of PLCδ₁ Mediates PIP₂ Stimulation of Catalysis**—Residues 16–134 in the N terminus of PLCδ₁ are homologous with the PH domains from pleckstrin and spectrin (11, 46), which have been shown to bind PIP₂ or IP₃ with high affinity and specificity (19, 26). In order to map structural determinants in the PH domain essential for PIP₂ activation of PLCδ₁, a nested set of N-terminal deletion mutant enzymes was constructed (Fig. 5). Deleting amino acids up to 70 residues from the N terminus of PLCδ₁ had little effect on the ability to catalyze the hydrolysis of PI and a modest effect on PIP₂ micelles (Table II). Like the native enzyme, the abilities of these truncated mutant enzymes to catalyze the hydrolysis of PI or PIP₂ were highly dependent on substrate concentration (data not shown). The ability to hydrolyze PI or PIP₂ increased in a manner comparable with those of the native enzyme as the concentration of substrates increased. These analyses demonstrate that deletion of the first 70 N-terminal amino acid residues of PLCδ₁ does not drastically affect the tertiary structure of the enzyme.

Conversely, when the enzymes were assayed in SUV, almost all deletion mutants were severely defective in PIP₂-mediated stimulation of PI hydrolysis (Fig. 6). Mutant enzymes such as del-20 PLCδ₁ required 10-fold higher concentrations of PIP₂ to enhance the activity 4-fold and never attained the high specific activity obtained with the native enzyme (23 μmol/min/mg versus 16 μmol/min/mg for the mutant). As more residues were deleted from the N terminus, the enzyme became more severely
impaired. The PI hydrolysis activities of the mutant enzymes del-50 through del-70 PLC\(_d1\) were scarcely enhanced even in the presence of 15 \(\mu\)M PIP2 (data not shown). As would be expected, del-10 PLC\(_d1\) was comparable with native enzyme (the initial 16 amino acids of PLC\(_d1\) are not thought to encode for the PH domain). Although the native enzyme and all of the deletion mutants display similar abilities to catalyze the hydrolysis of 30 \(\mu\)M of [3H]PI in vesicles composed of PI:PC:PS (molar ratio of PI:PC:PS = 1:5:5), only significant stimulation of the native and the del-10 PLC\(_d1\) enzyme was observed at relatively low concentrations of PIP2 (0.2 \(\mu\)M).

Partial deletion of a protein can have subtle but adverse effects on overall protein structure. Sometimes the structural changes can be in domains of the protein that are far removed from the area of deletion. Although we could not demonstrate that the PLC\(_d1\) deletion mutants reported here evidenced any change in overall protein structure that differed from wildtype enzyme (PI hydrolysis in micelles was identical for mutant and native enzymes), subtle adverse changes in structure cannot be completely ruled out. Therefore, to reduce the possibility that the impairment of the PIP2-stimulatory effect seen with the PLC\(_d1\) deletion mutants could arise from subtle structural changes that may indirectly perturb PIP2 interaction, mutant proteins were constructed by single amino acid substitution that should have minimal disturbance in structure. Specifically, amino acids with functional side chains between residues 20 and 40 of PLC\(_d1\) were replaced with glycine. This region of the PH domain is highly charged and has been shown to be important for IP3 and PIP2 binding to the PH domains of pleckstrin and spectrin (19, 24, 26). The conversion of Lys24 (K24G), Lys30 (K30G), Lys32 (K32G), Arg37 (R37G), or Trp36 (W36G) of PLC\(_d1\) to glycine did not affect the hydrolysis of PI; however, the hydrolysis of PIP2 in phospholipid vesicles was
Hydrolysis of PI and PIP₂ in phospholipid vesicles and dodecylmaltoside mixed micelles by the wild type and the deletion mutant PLCδ1

| Type of enzyme | PS/PC phospholipid vesicle activity | Dodecyl maltoside mixed micelle activity |
|---------------|------------------------------------|----------------------------------------|
|               | PI | PIP₂ | PI | PIP₂ |
| Wild type     | 3.2 ± 0.29 | 11 ± 1 | 36 ± 4 | 78 ± 4 |
| del-10        | 2.85 ± 0.3 | 9.8 ± 0.50 | 34 ± 3 | 77 ± 8 |
| del-20        | 2.81 ± 0.3 | 7.2 ± 0.80 | 36 ± 3 | 45 ± 5 |
| del-30        | 2.74 ± 0.25 | 2.58 ± 0.27 | 35 ± 4 | 32 ± 3 |
| del-40        | 2.85 ± 0.32 | 1.96 ± 0.19 | 33 ± 2 | 30 ± 2 |
| del-50        | 2.55 ± 0.23 | 2.05 ± 0.19 | 34 ± 3 | 33 ± 2 |
| del-70        | 2.63 ± 0.27 | 2.20 ± 0.22 | 35 ± 3 | 32 ± 3 |

* Hydrolysis of 30 μM [³H]PI (20,000 cpm) or 30 μM [³H]PIP₂ (16,000 cpm) at 37°C in phospholipid vesicles (PI/PS/PC; molar ratio = 1:5:5) or (PIP₂/PS/PC; molar ratio = 1:5:5) were carried in 50 μl of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl₂, and 500 μg/ml BSA. Reaction of PI hydrolysis was stopped and quantitated as described under “Experimental Procedures.” Reaction of PIP₂ hydrolysis was stopped by adding 0.17 ml of 10% ice-cold trichloroacetic acid and 0.85 ml of bovine serum albumin (10 mg/ml). After incubating on ice for 15 min, the unhydrolyzed [³H]PIP₂ (pellet) was separated from [³H]IP₃ (supernatant) by centrifugation at 2000 × g for 10 min. See “Experimental Procedures.”

Hydrolysis of 44 μM [³H]PI (20,000 cpm) or 46 μM [³H]PIP₂ (16,000 cpm), 0.3 mM dodecyl maltoside mixed micelles at 37°C in 50 μl of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl₂, and 500 μg/ml BSA. See “Experimental Procedures.”

Table II

Table III

Hydrolysis of PI and PIP₂ in phospholipid vesicles and dodecylmaltoside mixed micelles by the wild type and the deletion mutant PLCδ1

| Type of enzyme | PI hydrolysis | PIP₂ hydrolysis |
|---------------|---------------|-----------------|
|               | μmol/min/mg   | μmol/min/mg     |
| Wild type     | 3.3 ± 0.3     | 11 ± 1          |
| K24G          | 3.2 ± 0.2     | 8.1 ± 0.7       |
| K30G          | 3.2 ± 0.2     | 5.5 ± 0.6       |
| K32G          | 2.9 ± 0.2     | 4.7 ± 0.5       |
| W36G          | 3.2 ± 0.2     | 4.3 ± 0.4       |
| R37G          | 3.1 ± 0.3     | 4.1 ± 0.4       |

* Hydrolysis of 30 μM [³H]PI at 37°C in phospholipid vesicles (PI/PS/PC; molar ratio = 1:5:5). See “Experimental Procedures.”

* Hydrolysis of 30 μM [³H]PIP₂ at 37°C in phospholipid vesicles (PI/PS/PC/PIP₂; molar ratio = 1:5:5). See “Experimental Procedures.”

Fig. 6. Effect of PIP₂ on hydrolysis of PI by the wild type and N terminus-truncated PLCδ1. Catalytic hydrolysis of 30 μM PI in PI/PS/PC (molar ratio of 1:5:5) vesicles incorporated with the indicated amount of PIP₂ (0–15 μM of total PIP₂ corresponding to 0–4.4 mol%) by the wild type (●), del-10 (■), del-20 (▲), del-30 (○), and del-40 (□) mutant enzymes is shown. Data are representative of three independent duplicated experiments.

Consistently reduced (Table III). Furthermore, the stimulatory effect of PIP₂ on PI hydrolysis for all these single amino acid substitution mutants was remarkably reduced (Fig. 7). In contrast to the wild type enzyme, for which co-incorporation of 5 μM PIP₂ would maximally stimulate the enzyme (8-fold), 15 μM PIP₂ barely stimulated (1.5–3-fold) the hydrolysis of PI by these mutant enzymes.

Mutants Defective in PIP₂ Stimulation Are Also Unable to Bind PIP₂ with High Affinity—It has been shown that the isolated PH domain from PLCδ1 can recognize and bind PIP₂ with high affinity (22, 30). If PIP₂-dependent enzyme activation is mediated through the PH domain, then mutants defective in PIP₂ mediated stimulation should also be deficient in high affinity PIP₂ binding. To test this hypothesis, an equilibrium binding centrifugation assay using vesicles of defined phospholipid composition was employed to examine the physical interaction between these mutant enzymes and PIP₂. As shown in Fig. 8A, wild type PLCδ1 or del-10 mutant enzyme with its first 10 N-terminal amino acid residues truncated can bind with high affinity and specificity to PIP₂ in phosphatidylethanolamine and phosphatidylcholine vesicles. Fifty percent of the protein was bound when incubated with 2 μM PIP₂, and the binding was saturated at 8 μM. In contrast, the truncated mutant enzymes with 20 amino acid residues or more deleted from the N terminus displayed impaired PIP₂ binding. Even deleting a few residues from the putative PH domain in PLCδ1 resulted in a strongly impaired phenotype. PLCδ1 (del-20) exhibited a marked reduction in PIP₂ binding (Fig. 8A); only 50% of the del-20 mutant enzyme bound at relatively high concentrations of PIP₂ (20 μM). The most impaired mutant enzyme was one in which 40 or more amino acid residues from the N terminus were truncated; less than 20% of the protein...
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Fig. 8. Centrifugation binding assay to assess binding of wild type and mutant PLCδ1 to PIP2-containing vesicles. A, dose-dependent binding of PE/PC/PIP2 (4:1:0.25) lipid vesicles to the wild type (●), del-10 (■), del-20 (▲), del-30 (○), del-40 (□), del-50 (×), and del-70 (△) enzymes. B, dose-dependent binding of PE/PC/PIP2 (4:1:0.25) lipid vesicles to the wild type (●) or K24G (▲), K30G (○), W36G (■), K32G (□), and R32G (△) mutant PLCδ1. Data shown are representative of three independent duplicated experiments.

was bound at 20 μM PIP2. Likewise, the single point mutants K24G, K30G, K32G, R36G, R37G, and W36G were impaired in PIP2 binding (Fig. 8B). At least 10-fold more PIP2 was required to achieve 50% binding of mutant protein. These results are consistent with the conclusion that the N-terminal residues of the PH domain from PLCδ1 are essential for high affinity binding of PIP2 (17, 22, 30). Furthermore, the present results demonstrate that mutants defective in high affinity binding of PIP2 are also defective in PIP2-mediated enzyme stimulation, indicating that stimulation of PLCδ1 by PIP2 is mediated by high affinity binding of PIP2 to the PH domain.

PIP2 Binding to the PH Domain of PLCδ1 Affects \( K_s \) and \( K_m \) but Not \( V_{max} \)—In an attempt to understand the enzymatic mechanism by which PIP2 enhances enzyme activity, a dual phospholipid binding model was used to analyze the kinetics of PIP2-stimulated PI hydrolysis. According to this model (Equations 1 and 2), the enzyme binds to phospholipid at the surface of the mixed micelles through a noncatalytic site that serves to anchor the enzyme during catalysis. This interaction depends on the total concentration of both the substrate and the enzyme and is governed by the micellar dissociation constant, \( K_s \). Once bound to the micellar surface, the enzyme binds and cleaves a second molecule of phospholipid through a separate catalytic site, an interaction that is described by the interfacial Michaelis constant, \( K_m \). Thus, the dual substrate binding model of catalysis predicts that anchoring the enzyme through a noncatalytic site could allow PLCδ1 to catalyze the hydrolysis of numerous molecules of substrate during a single binding to the micellar surface.

We have observed, as have other groups, that the reduction in hydrolysis of PI in mixed phospholipid vesicles by PLCδ1 is disproportionately large with respect to both molar fraction and total concentration of nonsubstrate lipid added, indicating that the nonsubstrate phospholipids exert an additional inhibitory effect on PLCδ1 (44). Therefore, in our kinetic experiments, the bulk concentration of nonsubstrate lipid was kept constant at 150 μM each (PS and PC); the total nonsubstrate lipid would be 300 μM, and the hydrolysis of PI was measured at different substrate concentrations. The rate of PI hydrolysis obtained under this condition could be plotted as a function of PI concentration and could be fit to Equation 3 as described by Hendrickson and Dennis (43). As the total concentration of PI was increased from 22 μM to 810 μM, the rate of PI hydrolysis catalyzed by the native enzyme increased from 2.1 to 49 μmol/min/mg in the absence of PIP2 and from 15.6 to 60 μmol/min/mg in the presence of 5 μM of PIP2 (Fig. 9A). The most dramatic stimulation by PIP2 (7–9-fold) was seen at low concentrations of PI, which were below saturation concentrations for the catalytic site. As the PI concentration reached saturation, the stimulatory effect of PIP2 diminished. Although the rate of PI hydrolysis catalyzed by del-80 PLCδ1 exhibited similar dependence on the substrate concentration as the native enzyme, we were not able to detect any stimulation by PIP2 within the range of PI between 22 and 810 μM (Fig. 9B). Computer fitting of the kinetic data obtained in the presence or absence of PIP2 to Equation 3 showed that the catalysis of PI by the native enzyme in the present system followed the prediction of the dual substrate binding model (Fig. 9, A and B). When compared with these kinetic parameters (Table IV), it can be shown that the effect of PIP2 on the catalysis of PI hydrolysis by PLCδ1 was primarily on the vesicle dissociation constant \( K_s \), which governs the binding of PLCδ1 to the surface of phospholipid vesicles. \( K_s \) was reduced from 550 to 53 μM by 5 μM PIP2. The interfacial Michaelis constant was also reduced from 0.21 to 0.06 by 5 μM PIP2. Conversely, PIP2 had little effect on the \( V_{max} \) raising it from 62 to 66 μmol/min/mg. These data suggest that the mechanism of PLCδ1 activation by PIP2 involves enhancement of interfacial catalysis by increasing the affinity of the enzyme for vesicles as well as for substrate. This activation mechanism requires an intact PH domain in the N terminus of PLCδ1. Although the \( V_{max} \) of del-80 PLCδ1 is similar to that of the native enzyme, its \( K_m \) and \( K_s \) was not affected by PIP2. Furthermore, the rates of PI hydrolysis (\( V_{max} \)) at saturated molar fractions of substrate and infinite substrate concentrations of all the deletion mutants were comparable to that of the native enzyme, demonstrating that the N-terminal deletion did not interfere with the catalytic center of the enzyme. This finding showed that the primary effect of PIP2-PH domain interaction is not on the rate of catalysis at saturated substrate molar fractions or infinite substrate concentration; rather, it affects the binding affinity of the enzyme for the vesicles and substrates.

**DISCUSSION**

Lipid headgroups have been shown to modulate the activity of proteins such as transporters, receptors, cytoskeletal elements, and effector molecules (47). In some cases, there is extreme specificity for lipid-protein interaction. For example, protein kinase C, an important regulator of phospholipid hydrolysis and hence signal transduction, requires PS for activity. Other acidic phospholipids have no effect (48, 49). While all isozymes of phospholipases C, D, and A require PIP2 for activity, the functional site of phospholipid-protein interaction is unknown. In this report, we use an isozyme of PLC (PLCδ1) to
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shown to modulate the function of phospholipase D (34, 36) and ARF (35) in a specific manner. In both cases, PIP₂ was required for optimal stimulation of phospholipase D and ARF activities, and the effects of other anionic phospholipids were negligible. To our knowledge, this PIP₂ stimulation of PLC hydrolytic activity has never been described. In fact, the opposite result might have been predicted, that PIP₂ would inhibit PI or PIP₂ hydrolysis by competing for substrate at the catalytic site. Although competition does occur at high (30 μM) PIP₂ concentrations, at micromolar or submicromolar concentrations (EC₅₀ = 1 μM or 0.3 mol% of total lipids), which are thought to be close to physiologic (approximately 0.1–0.8 mol%, depending on the type of plasma membrane) (51), the net effect is to stimulate activity. Phosphoinositides constitute 2–8% of the lipid in cell membrane; 2–10% of the membrane phosphoinositides are PIP₂. Whether PIP₂ would stimulate PI hydrolysis in situ will require further investigation. However, this comparison is relevant to the present vesicle constitution of PIP₂ and PI when half-maximal activity to hydrolyze PI is stimulated by 1 μM PIP₂, which corresponds to 3.3% of the total inositol phospholipids or 0.3 mol% of the total phospholipids in the vesicles.

In agreement with previous studies, we could demonstrate high affinity binding of the holoenzyme to PIP₂-containing vesicles (52, 53). The high affinity binding of PIP₂ to PLCδ1 correlated with the ability of PIP₂ to stimulate PLCδ1; the tighter the binding between PIP₂ and PLCδ1, the more potent PIP₂ was in its ability to stimulate the enzyme. The structural determinants mediating PIP₂ activation and high affinity PIP₂ binding were found to lie in the N-terminal PH domain. Several reports have described the structure and putative ligands for the PH domains from various molecules (19, 22, 26). These reports have studied the domain as an isolated fusion protein and therefore could only speculate about function. The present report has studied the PH domain as it exists in nature; as part of a macromolecule. This approach has allowed the delineation of a functional role for the PH domain in the N terminus of PLCδ1, which was to modulate enzyme activity.

The PH domain of PLCδ1 appears essential for the enzyme to perform efficient catalysis, since the structural integrity of the PH domain of this enzyme is indispensable for PIP₂-mediated activation of enzyme activity. Since low molar concentrations of PIP₂ can significantly increase PI hydrolysis by PLCδ1, this may be a mechanism by which PLCδ1 is able to utilize efficiently the large molar excess of PI relative to PIP₂ in the cell membrane. The PIP₂-hydrolyzing activities of mutants defective in high affinity PIP₂ binding were significantly reduced when PIP₂/PS/PC vesicles were used as substrates. This is consistent with the present conclusion that high affinity binding of PIP₂ is essential for the stimulation of PLCδ1 and agrees with the observations that blocking high affinity PIP₂ binding to the PH domain with IP₃ leads to inhibition of PIP₂ hydrolysis (54, 55). PI is not able to bind PLCδ1 with an affinity comparable with that of PIP₂ (7, 22, 30); thus, PI is not able to enhance the catalytic function of PLCδ1. This may be the reason why all the PLCδ1 mutants that were defective in high affinity PIP₂-binding could not catalyze the hydrolysis of PI at a rate comparable with that of wild type enzyme. In general, our results are consistent with several other reports showing that N-terminal sequences of PLCδ1 are not involved in substrate hydrolysis (4, 8, 31) but are required for specific high affinity binding to PIP₂. Furthermore, the present work demonstrated that binding of PIP₂ leads to enhanced enzyme catalysis and thus provides an alternative and direct assay system to study ligand-PH domain interactions.

The present results also revealed that the hydrolysis of PIP₂ by the mutant enzymes was highly dependent on the assay
conditions. Truncation of the N-terminal sequence of PLCδ1 did not affect the PIP2-hydrolyzing activities when sodium cholate was used to solubilize the phospholipids. Similar results were also found by Ellis et al. (4, 5), who showed that the activity of PLCδ1 is not affected by truncation of its first 58 N-terminal residues. This may simply reflect weak PIP2-binding in the sodium cholate detergent micelles, because the PI-hydrolyzing activity of PLCδ1 did not respond to the stimulatory effect of PIP2 at all if PI/sodium cholate mixed micelles were used as substrate (data not shown).

The PLCδ1 single point mutants K24G, K30G, K32G, R37G, and W36G were impaired in PIP2 binding and in PIP2-stimulated catalysis. N-terminal residues from 24 to 40 correspond to residues 7–30 of human pleckstrin or residues 2198–2225 of mouse brain spectrin, which form the first β-strand variable loop and the N-terminal portion of the second β-strand of the PH domain. These regions play important roles in mediating specific interactions with the PIP2 molecule (19, 23, 24). The co-crystal structure of the isolated PH domain from rat PLCδ1 complexed with IP3 revealed that Lys30 and Lys32 were found to hydrogen bond either directly or via their side chains to the 4- and 5-phosphate groups of IP3. The side chain of Trp36 was found to hydrogen bond with the 1-phosphate of IP3 as well as to hydrogen bond to the 4-phosphate of IP3. Once bound to the micellar surface, the enzyme binds to phospholipid through the catalytic site, an interaction that is described by the interfacial Michaelis constant, $K_m$. Our kinetic experiments have demonstrated that PIP2 greatly enhances the affinity of the enzyme for vesicles. $K_m$ was reduced from 558 to 53 μM by 5 μM of PIP2. Once bound to the micellar surface, the enzyme binds a second molecule of phospholipid through the catalytic site, an interaction that is described by the interfacial Michaelis constant, $K_m$. The $K_m$ was also reduced from 0.21 to 0.06 by 5 μM of PIP2 (0.15 mol%), suggesting that ligand binding to the PH domain enhances both micellar and substrate binding. This is consistent with a recent study of dynamin by Zheng et al. (25), which suggests that binding of PIP2 to the PH domain may lead to activation of its GTPase activity. Hydrolysis of GTP by dynamin is not involved in processive catalysis (38); therefore, PIP2 binding to the PH domain may allosterically alter the structure of this enzyme.

It seems unlikely that the binding of PIP2 also contributes directly to PLCδ1 stimulation, since the $V_{max}$ for the enzyme is unchanged. Similar results have also been found upon lipid binding to the PH domain of the serine/threonine kinase Akt (60). Further detailed kinetic analyses and identification of the site of lipid-protein interaction in dynamin and Akt will be required to provide a fuller understanding of the mechanism by which ligand binding to the PH domain modulates enzyme activity.

Our results clearly indicate that PIP2 may in fact be the major ligand for the PH domain in PLCδ1. While it is unlikely that PIP2 would be the functional ligand for many PH domains, it is probable that PH domains in many molecules serve to regulate the wide variety of proteins that contain PH domains and suggest a potential new target for the develop-
ment of therapeutics. Whether PIP$_2$ regulates the function of other proteins by high affinity and specific binding to the PH domain remains to be determined.

Acknowledgments—We thank Dr. Y.-S. Lin, Dr. Marlene Hosey, and H.-N. Yeung for critical discussions and for reading the manuscript.

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