

## Phosphatidylinositol 4,5-Bisphosphate Binding to the Pleckstrin Homology Domain of Phospholipase C- $\delta$ 1 Enhances Enzyme Activity\*

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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- $\delta$ 1 (PLC $\delta$ 1) was found to regulate enzyme activity. The hydrolysis of phosphatidylinositol (PI) was stimulated by phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) in a dose-dependent manner with an EC<sub>50</sub> = 1  $\mu$ M (0.3 mol%), up to 9-fold higher when 5  $\mu$ M (1.5 mol%) of PIP<sub>2</sub> was incorporated into the PI/phosphatidylserine (PS)/phosphatidylcholine (PC) vesicles (30  $\mu$ M of PI with a molar ratio of PI:PS:PC = 1:5:5). Stimulation was specific for PIP<sub>2</sub>, since other anionic phospholipids including phosphatidylinositol 4-phosphate had no stimulatory effect. PIP<sub>2</sub>-mediated stimulation was, however, inhibited by inositol 1,4,5-triphosphate (IP<sub>3</sub>) in a dose-dependent manner, suggesting a modulatory role for this inositol. When a nested set of PH domain deletions up to 70 amino acids from the N terminus of PLC $\delta$ 1 were constructed, the deletion mutant enzymes all catalyzed the hydrolysis of the micelle forms of PI and PIP<sub>2</sub> with specific activities comparable with those of the wild type enzyme. However, the stimulatory effect of PIP<sub>2</sub> was greatly diminished when more than 20 amino acid residues were deleted from the N terminus. To identify the specific residues involved in PIP<sub>2</sub>-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 PIP<sub>2</sub> micelles, the catalytic activity of mutants K24G, K30G, K32G, R38G, or W36G was markedly unresponsive to PIP<sub>2</sub>. Analysis of PIP<sub>2</sub>-stimulated PI hydrolysis by a dual substrate binding model of catalysis revealed that the micellar dissociation constant ( $K_d$ ) of PLC $\delta$ 1 for the PI/PS/PC vesicles was reduced from 558  $\mu$ M to 53  $\mu$ M, and the interfacial Michaelis constant ( $K_m$ ) was reduced from 0.21 to 0.06 by PIP<sub>2</sub>. The maximum rate of PI hydrolysis ( $V_{max}$ ) was not affected by PIP<sub>2</sub>. These results

demonstrate that a major function of the PH domain of PLC $\delta$ 1 is to modulate enzyme activity. Further, our results identify PIP<sub>2</sub> as a functional ligand for a PH domain and suggest a general mechanism for the regulation of other proteins by PIP<sub>2</sub>.

PLC $\delta$ 1 is a member of a family of inositol phospholipid-specific phospholipase C (PLC)<sup>1</sup> isozymes through which a variety of hormones, neurotransmitters, and growth factors elicit intracellular responses (1). All three major PLC isozyme families ( $\beta$ ,  $\delta$ , and  $\gamma$ ) are able to recognize phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and carry out the Ca<sup>2+</sup>-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 NMR solution structure (14–16) as well as the crystal structures (17–21) of several PH domains have determined that the PH domain is a  $\beta$ -sandwich structure composed of seven orthogonal  $\beta$ -sheets with an  $\alpha$ -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 (IP<sub>3</sub>) (19, 22, 23), PIP<sub>2</sub> (19, 22, 24–26), and  $\beta\gamma$  subunits of heterotrimeric G proteins (27–29). Taken together, these studies suggest that PH domains

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<sup>1</sup> The abbreviations used are: PLC, phosphoinositide-specific phospholipase C; PH domain, pleckstrin homology domain; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PIP<sub>2</sub>, 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 PLC $\delta$ 1. Both IP<sub>3</sub> and PIP<sub>2</sub> have been demonstrated to bind with high affinity to the PH domain of PLC $\delta$ 1 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 PLC $\delta$ 1 (the PH domain is located in the N terminus of PLC $\delta$ 1). Biochemical studies utilizing proteolyzed enzyme have indirectly suggested a functional role for the N-terminal PH domain of PLC $\delta$ 1. Cifuentes *et al.* (31) demonstrated that the first 60 N-terminal residues of the PLC $\delta$ 1 sequence (which overlap the putative PH domain) are not essential for Ca<sup>2+</sup>-dependent catalysis but are required for the enzyme to hydrolyze PIP<sub>2</sub> in a processive manner. Conversely, Ellis *et al.* (4) and Yagisawa *et al.* (8) found that truncation of the N terminus of PLC $\delta$ 1 did not affect its catalytic activity.

In addition to serving as substrate for PI-PLC to generate second messengers, PIP<sub>2</sub> also plays an important role in several other cellular signalings. PIP<sub>2</sub> has been shown to regulate the cell filament activity by interacting with profilin, cofilin, and gelsolin (32, 33). The activity of phospholipase D has been shown to be stimulated by PIP<sub>2</sub> directly (34) or through a GTP-bound ADP-ribosylation factor (35–37). High affinity binding of PIP<sub>2</sub> 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 PIP<sub>2</sub> can bind with high affinity and specificity to the PH domain of PLC $\delta$ 1 (8, 22), it would be of interest to know whether PIP<sub>2</sub> might also modulate the activity of PLC $\delta$ 1. In the present report, we provide the first demonstration that high affinity binding of PIP<sub>2</sub> to the PH domain of PLC $\delta$ 1 leads to enhanced enzyme activity. Additionally, we have established (using site-directed mutagenesis) the structural determinants mediating this activity in the PH domain of PLC $\delta$ 1. Further, we have demonstrated that this specific protein-phospholipid interaction is crucial for efficient hydrolysis of substrate by PLC $\delta$ 1, demonstrating an important role for PH domains in regulating enzyme activity.

#### EXPERIMENTAL PROCEDURES

**Materials**—The expression plasmid pRSETA(R) was from Invitrogen. The resulting expression construct (pRSETAplc(R)) 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. PIP<sub>2</sub> was obtained from Calbiochem. IP<sub>3</sub> and dodecyl maltoside were obtained from Sigma.

**Construction of Mutant PLC $\delta$ 1**—A nested set of N-terminal deletion mutants (30-base pair increments) were generated in the human PLC $\delta$ 1 cDNA using the polymerase chain reaction as described previously (39). Replacement of single amino acid residues in PLC $\delta$ 1 was accomplished by polymerase chain reaction as described previously (7). All mutants were subsequently confirmed by DNA sequence analysis.

**Expression and Purification of PLC $\delta$ 1**—Native and mutant enzyme cDNAs were introduced into the *Bam*HI site of pRSETA(R). The resulting recombinant PLC $\delta$ 1 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 Ni<sup>2+</sup>-nitrilotriacetic acid agarose followed by heparin-Sepharose column 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 [<sup>3</sup>H]PI in detergent micelles (40). Activities were expressed as specific activities ( $\mu$ mol/mg/min).

**Assay of PLC $\delta$ 1 Activity**—PLC $\delta$ 1-catalyzed hydrolysis of PIP<sub>2</sub> was

determined as described previously (41). Briefly, purified enzyme (0.1–10 ng) was incubated in an assay volume of 60  $\mu$ l, which contained 50 mM Hepes, pH 7.2, 3 mM EGTA, 0.2 mM EDTA, 0.83 mM MgCl<sub>2</sub>, 20 mM NaCl, 30 mM KCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.16% sodium cholate, 1.5 mM CaCl<sub>2</sub>, 50  $\mu$ M [<sup>3</sup>H]PIP<sub>2</sub> (16,000 cpm), and 500  $\mu$ M 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 0.1 ml of bovine serum albumin (10 mg/ml). After incubation on ice for 15 min, the unhydrolyzed [<sup>3</sup>H]PIP<sub>2</sub> (pellet) was separated from [<sup>3</sup>H]IP<sub>3</sub> (supernatant) by centrifugation at 2000  $\times$  *g* for 10 min. Radioactivity in the supernatant was measured by liquid scintillation counting. PIP<sub>2</sub> hydrolytic activity was expressed as  $\mu$ mol of IP<sub>3</sub>/min/mg of protein. Determination of PI hydrolytic activity was essentially as described by Hofmann and Majerus (40).

Assays using dodecyl maltoside/PIP<sub>2</sub> 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 PIP<sub>2</sub>/[<sup>3</sup>H]PIP<sub>2</sub> or PI/[<sup>3</sup>H]PI (4  $\times$  10<sup>5</sup> cpm) in chloroform/methanol (19:1) were dried under a stream of N<sub>2</sub> and then lyophilized for 30 min. Lipids were then solubilized by probe sonication in 0.95 ml of a solution containing 300  $\mu$ M of dodecyl maltoside, 50 mM HEPES, pH 7.0, 100 mM NaCl, and 2 mM EGTA. Fifty  $\mu$ l of bovine serum albumin in the same buffer was added to yield a final concentration of 500  $\mu$ g/ml. To assay the PIP<sub>2</sub> hydrolytic activity, 50  $\mu$ l of dodecyl maltoside/PI or PIP<sub>2</sub> 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  $\mu$ l of 40 mM of CaCl<sub>2</sub> and incubated at 30 °C for another 1–15 min. For micelles containing PI, reactions were terminated by the addition of 250  $\mu$ l of chloroform/methanol/HCl (100:100:0.6), followed by 75  $\mu$ l of 1 N HCl containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 100- $\mu$ l portion of the upper aqueous phase was counted by liquid scintillation. For micelles containing PIP<sub>2</sub>, reactions were stopped by adding 0.17 ml of 10% ice-cold trichloroacetic acid and 0.85  $\mu$ l of bovine serum albumin (10 mg/ml). After incubation on ice for 15 min, the unhydrolyzed [<sup>3</sup>H]PIP<sub>2</sub> (pellet) was separated from [<sup>3</sup>H]IP<sub>3</sub> (supernatant) by centrifugation at 2000  $\times$  *g* for 10 min.

**Hydrolysis of PI in Phospholipid Vesicles**—Hydrolysis of 30  $\mu$ M PI was carried out in PI/PS/PC (molar ratio of 1:5:5) small unilamellar vesicles (SUV) incorporated with varying amounts of PIP<sub>2</sub>. Single bilayer vesicles were prepared as described previously (42) with slight modification. In brief, a stock solution of lipids, 30 nmol of <sup>3</sup>H-labeled PI (4,000,000 cpm), 150 nmol of PS, 150 nmol of PC, and the indicated amount of PIP<sub>2</sub> (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 two cycles of ultrasonication for 15 s with a Heat Systems Sonicator (Heat Systems, Farmingdale, NY) with 45-s cooling intervals. Samples were then centrifuged at 120,000  $\times$  *g* for 60 min, and the clear supernatant was carefully removed. Fifty  $\mu$ l of BSA was added to give a final concentration of 200  $\mu$ g/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  $\mu$ M, respectively plus the indicated concentration of PIP<sub>2</sub>. Lipids were used within 24 h. To assay the PI-PLC activity, 50  $\mu$ l of SUV containing 30  $\mu$ M 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  $\mu$ l of 60 mM CaCl<sub>2</sub> and incubated at 37 °C for another 1–15 min. The reaction was terminated by adding 0.2 ml 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- $\mu$ l portion of the upper aqueous phase was counted by liquid scintillation.

**Analysis of Kinetic Data**—PI hydrolysis catalyzed by native PLC $\delta$ 1 and N-terminal deletion mutant PLC $\delta$ 1 was measured as a function of total substrate concentration under case III conditions previously described for phospholipase A<sub>2</sub> and PLC $\beta$  (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.

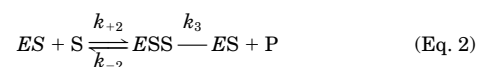


TABLE I  
Effect of diluent phospholipids on the hydrolysis of PI  
by human PLC $\delta$ 1

Vesicle composition <sup>a</sup> (molar fraction)	PI hydrolysis activity <sup>b</sup>
	$\mu\text{mol}/\text{min}/\text{mg}$
PC (0.9) + PI (0.1)	0.20 $\pm$ 0.03
PC (0.455) + PS (0.455) + PI (0.09)	3.4 $\pm$ 0.4
PC (0.455) + PA (0.455) + PI (0.09)	1.1 $\pm$ 0.1
PC (0.455) + PG (0.455) + PI (0.09)	2.3 $\pm$ 0.3
PC (0.455) + PE (0.455) + PI (0.09)	0.4 $\pm$ 0.05

<sup>a</sup> Nitrogen-dried phospholipid mixture containing 30 nmol of <sup>3</sup>H-labeled inositol phospholipids ( $4 \times 10^5$  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  $\mu\text{g}/\text{ml}$  bovine serum albumin.

<sup>b</sup> Catalytic hydrolysis of PI in vesicles containing indicated diluent phospholipid was carried out in 50  $\mu\text{l}$  of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 30  $\mu\text{M}$  [<sup>3</sup>H]PI (20,000 cpm), 150  $\mu\text{M}$  PS and 150  $\mu\text{M}$  PC, 500  $\mu\text{g}/\text{ml}$  bovine serum albumin, and 3 mM CaCl<sub>2</sub>. 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 PLC $\delta$ 1 occurs at the water-lipid interface of the phosphoinositide/dodecyl maltoside mixed micelles. Initial binding of the enzyme to the water-lipid interface of the micelles is described by the micellar dissociation constant,  $K_s = k_{-1}/k_{+1}$  (molar unit). This constant is dependent on both the total enzyme concentration and 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 PLC $\delta$ 1 is described by the interfacial Michaelis constant,  $K_m = (k_{-2} + k_3)/k_{+2}$  (molar fraction, unitless). Initial rates of catalysis ( $v$ ) as a function of total concentration of PI in the vesicles with a fixed concentration of diluent nonsubstrate phospholipids ( $T_o$ ) measured under case III conditions were fitted using Equation 3 (43) to obtain the values of  $V_{\text{max}}$ ,  $K_s$ , and  $K_m$ ,

$$v = \frac{V_{\text{max}}S_o^2}{K_m K_s T_o + (T_o + K_s)K_m S_o + (K_m + 1)S_o^2} \quad (\text{Eq. 3})$$

where the absolute rate ( $V_{\text{max}}$ ) occurs at an infinite substrate concentration and the saturated substrate molar fraction,  $S_o$  is the total substrate concentration.

**Centrifugation/PIP<sub>2</sub> Binding Assays**—Dose-dependent binding of enzyme to PE/PC/PIP<sub>2</sub> vesicles (molar ratio of 4:1:0.25) was demonstrated using ultracentrifugation as described previously (7). In brief, 0.5–1  $\mu\text{g}$  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  $\times g$ ) 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 [<sup>3</sup>H]PI and/or by immunoblotting using mixed monoclonal antibodies specific for PLC $\delta$ 1. The bound enzyme fractions (pellets) were quantitated by dissolving the pellet in 0.05 ml 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 PLC $\delta$ 1. 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 PLC $\delta$ 1 to catalyze the hydrolysis of PI, we compared the hydrolysis of [<sup>3</sup>H]-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 0.091 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  $\mu\text{mol}/\text{min}/\text{mg}$  of enzyme. Adding the nonanionic phospholipid PE increased hydrolysis moderately to 0.4  $\mu\text{mol}/$

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  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively, which was a 5–15-fold increase over SUV containing PC only. These results demonstrated that the rate of PI hydrolysis catalyzed by PLC $\delta$ 1 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 PIP<sub>2</sub> Specifically Stimulates PI Hydrolysis in SUV**—The enhancing effect of PA, PS, and PG on the hydrolysis of PI catalyzed by PLC $\delta$ 1 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 them 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 PLC $\delta$ 1 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 PLC $\delta$ 1 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 PLC $\delta$ 1 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  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively. Catalytic activity increased to 2.7, 7.8, 5.7, or 8.9  $\mu\text{mol}/\text{min}/\text{mg}$  by including in the PI/PE vesicles 3% PA, PS, PG, or PIP<sub>2</sub>, respectively. The hydrolysis of PI in PI/PC vesicles was increased from 0.2  $\mu\text{mol}/\text{min}/\text{mg}$  to 1.4, 2.9, 2.0, or 3.3  $\mu\text{mol}/\text{min}/\text{mg}$  by 3% PA, PS, PG, or PIP<sub>2</sub>, respectively. These results demonstrated that the negative charge in PA, PS, PG, and PIP<sub>2</sub> 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 PIP<sub>2</sub>, where the activity of PI hydrolysis was significantly increased (8-fold) by incorporation of 3% PIP<sub>2</sub> 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 PLC $\delta$ 1 in vesicles that contain a small molar fraction of anionic phospholipids (<0.10), PIP<sub>2</sub> could stimulate catalysis even in vesicles containing a high molar fraction (0.45) of anionic phospholipids. This result suggested that the PIP<sub>2</sub>-stimulatory process was due to a specific interaction with PLC $\delta$ 1 rather than the presumed electrostatic interactions seen with PS, PA, or PG.

The effect of PIP<sub>2</sub> on PI hydrolysis was also examined using PI/dodecyl maltoside mixed micelles. In this system the PI-hydrolyzing activity was only slightly increased by PIP<sub>2</sub> (data not shown). No PIP<sub>2</sub> 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 PIP<sub>2</sub>, PIP, and IP<sub>3</sub> on PI Hydrolysis**—To further assess the specificity of PIP<sub>2</sub> as a ligand to

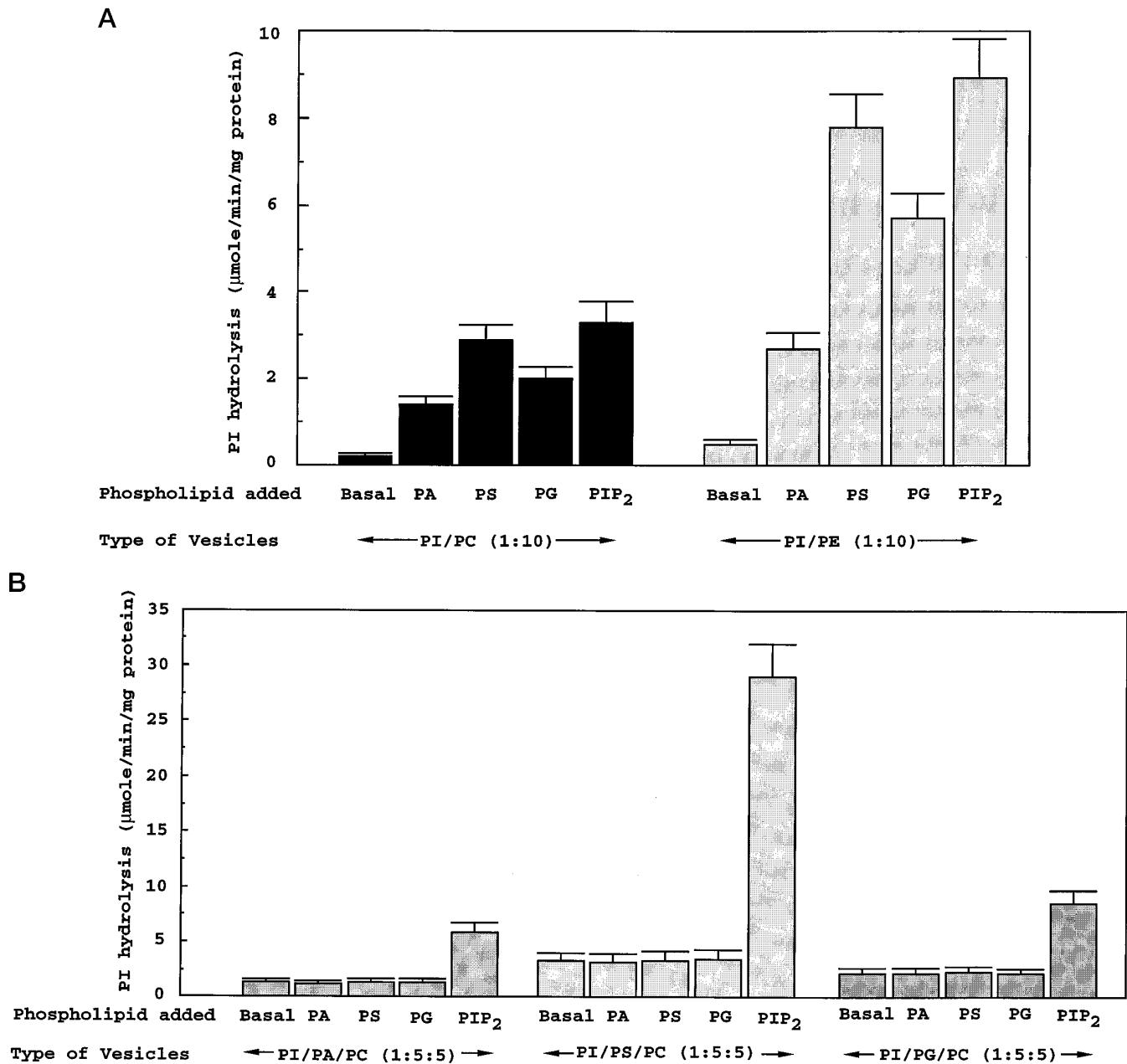
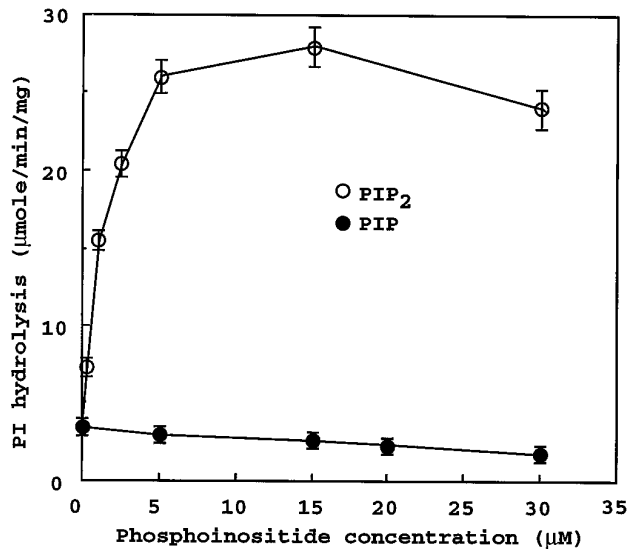


FIG. 1. Effect of anionic phospholipids on the hydrolysis of PI by PLC $\delta$ 1. A, the hydrolysis of PI in PI/PC (PI/PC molar ratio = 1:10) or in PI/PE (PI/PE molar ratio 1:10) vesicles (basal) and after incorporation of 3% PA, PS, PG, or PIP<sub>2</sub>. B, the hydrolysis of PI (basal activities) in vesicles of PI/PA/PC (molar ratio = 1:5:5), PI/PS/PC (molar ratio = 1:5:5), or PI/PK/PC (molar ratio = 1:5:5) and after incorporation of 3% PA, PS, or PG. Hydrolysis of [<sup>3</sup>H]PI (30  $\mu$ M) in the indicated phospholipid vesicles by PLC $\delta$ 1 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 CaCl<sub>2</sub>, 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.

stimulate PLC $\delta$ 1 and to determine the necessary structural requirements of the lipid molecule required for enzyme activation, the dose-dependent effects of PIP<sub>2</sub> and PIP on PI hydrolysis were assessed. Since PIP differs structurally from PIP<sub>2</sub> 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 PIP<sub>2</sub>-mediated stimulatory effect. Fig. 2 demonstrates that relatively low concentrations of PIP<sub>2</sub> (0.2  $\mu$ M) were able to stimulate PLC $\delta$ 1-catalyzed hydrolysis of a fixed concentration (30  $\mu$ M) of [<sup>3</sup>H]-PI in SUV (PI/PS/PC molar ratio = 1:5:5). The activity increased quickly as a function of PIP<sub>2</sub> concentration. Maximal 8–9-fold stimulation was seen with 5  $\mu$ M of PIP<sub>2</sub>, and half-maximal activity (4.5-fold stimulation) was seen with 1  $\mu$ M PIP<sub>2</sub>. The dose response curve clearly demonstrates overall stimulation of PI hydrolysis at low con-

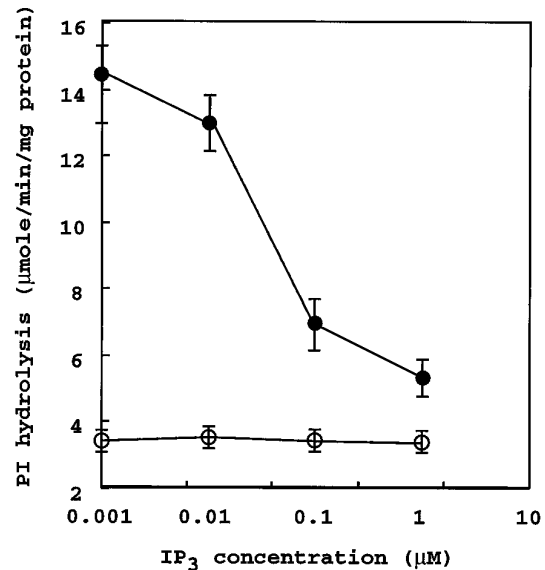
centrations of PIP<sub>2</sub>; however, as the concentration of PIP<sub>2</sub> increased to 30  $\mu$ M, the rate of PI hydrolysis began to decrease, presumably due to the competition of [<sup>3</sup>H]PI and PIP<sub>2</sub> for the catalytic site. Although a low level of PIP<sub>2</sub> hydrolysis was seen in the present system (PIP<sub>2</sub>/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 PIP<sub>2</sub> 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 $\delta$ 1-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 $\delta$ 1. To further probe this, the effect of IP<sub>3</sub> on the



**FIG. 2. Effect of PIP<sub>2</sub> and PIP on PLC $\delta$ 1-catalyzed hydrolysis of PI.** Hydrolysis of [<sup>3</sup>H]PI (30  $\mu$ M) in PI/PS/PC (molar ratio = 1:5:5) vesicles containing increasing amounts of PIP<sub>2</sub> (○) or increasing concentrations of PIP (●) is shown. PIP<sub>2</sub> and PIP are expressed as final concentration in the reaction (0–30  $\mu$ M in total, corresponding to 0–8.6 mol%). The reaction was carried out at 37 °C in 50  $\mu$ l of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, and 3 mM CaCl<sub>2</sub>, 500  $\mu$ g/ml BSA, and 30  $\mu$ M of [<sup>3</sup>H]PI, 150  $\mu$ M of PS, 150  $\mu$ M of PC, and the indicated concentration (0–30  $\mu$ M) of PIP<sub>2</sub> or PIP for 5–15 min. The reaction was terminated, and the [<sup>3</sup>H]IPs were separated and quantitated as described by Hofmann and Majerus (40). Data shown are the average of four experiments.

stimulatory activity of PIP<sub>2</sub> was examined. As shown in Fig. 3, IP<sub>3</sub> (0–5  $\mu$ M) alone did not affect the ability of PLC $\delta$ 1 to catalyze the hydrolysis of PI in PI/PS/PC vesicles, suggesting that IP<sub>3</sub> at these concentrations did not compete with PI for the catalytic site of PLC $\delta$ 1. However, IP<sub>3</sub> did reduce the stimulatory effect of PIP<sub>2</sub> on the hydrolysis of PI in a dose-dependent manner with an IC<sub>50</sub> of <1  $\mu$ M. Taken together, these observations imply that stimulation of PLC $\delta$ 1 to catalyze the hydrolysis of PI is highly specific for PIP<sub>2</sub>, and both phosphates at D-4 and D-5 positions of the inositol ring are essential for the stimulation of hydrolysis. Further, IP<sub>3</sub> can inhibit PIP<sub>2</sub>-mediated activation presumably by competing for PIP<sub>2</sub> at a site of interaction on PLC $\delta$ 1 that is distinct from the catalytic site.

**Elucidation of Structural Determinants of PIP<sub>2</sub>-mediated Enzyme Activation**—The specificity of PIP<sub>2</sub> for stimulation of PI hydrolysis and IP<sub>3</sub> for inhibition suggested that the phosphate groups on the inositol ring may be recognized by particular and perhaps unique structural determinants of PLC $\delta$ 1. It has been shown previously that PLC $\delta$ 1 binds PIP<sub>2</sub> at two distinct sites: a high affinity (1–5  $\mu$ M) site near the N terminus and a low affinity (100  $\mu$ M) site in a C-terminal 67-kDa fragment (7, 8, 31). In addition, a potential phosphoinositide binding sequence originally found in gelsolin was also identified in PLC $\delta$ 1. Therefore, at least three regions in PLC $\delta$ 1 could potentially interact specifically with PIP<sub>2</sub>; the conserved Y region, a PIP<sub>2</sub> binding motif sequence KXXXXKXKK found in residues 434–441 (45) of PLC $\delta$ 1, and the N-terminal PH domain (8, 17, 22, 31). Our laboratory has generated several Y region mutant forms of PLC $\delta$ 1 that are selectively deficient in PIP<sub>2</sub> hydrolysis. Since these mutants have also been shown to be capable of responding to the stimulatory effect of PIP<sub>2</sub>, we thought it unlikely that the Y domain represented the site of PIP<sub>2</sub>-mediated enzyme stimulation; therefore, no further mutagenesis was attempted. Mutagenesis of the potential PIP<sub>2</sub> binding motif (KXXXXKXKK) of PLC $\delta$ 1 suggested that this was also not the site of PIP<sub>2</sub> interaction. Positive charges from the conserved



**FIG. 3. IP<sub>3</sub> competitively inhibits PIP<sub>2</sub>-mediated stimulation of PLC $\delta$ 1.** Hydrolysis of 30  $\mu$ M [<sup>3</sup>H]PI with increasing concentrations of IP<sub>3</sub> by PLC $\delta$ 1 in vesicles containing PIP<sub>2</sub> (●) or in vesicles without PIP<sub>2</sub> (○) is shown. The reaction was carried out at 37 °C for 5–15 min in 50  $\mu$ l of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 3 mM CaCl<sub>2</sub>, 500  $\mu$ g/ml BSA, and 30  $\mu$ M [<sup>3</sup>H]PI in PI/PIP<sub>2</sub>/PS/PC (molar ratio 1:0.033:5:5; with 1  $\mu$ M or 0.03 mol% final concentration of PIP<sub>2</sub>) vesicles or in PI/PS/PC vesicles (molar ratio = 1:5:5; without PIP<sub>2</sub>) and the indicated concentrations of IP<sub>3</sub>. The reaction was terminated, and the [<sup>3</sup>H]IP was quantitated as described under “Experimental Procedures” (40). Data shown are the average of four experiments.

lysine residues were neutralized by mutation to glycine. Mutant enzymes K434G (Lys<sup>434</sup> → Gly), K440G (Lys<sup>440</sup> → Gly), and K441G (Lys<sup>441</sup> → Gly) were stimulated by PIP<sub>2</sub>; like the native enzyme (Fig. 4). Deleting the first 80 residues from the N terminus of PLC $\delta$ 1 ablated PIP<sub>2</sub>-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 PIP<sub>2</sub> lie in the N-terminal region of PLC $\delta$ 1.

**The PH Domain of PLC $\delta$ 1 Mediates PIP<sub>2</sub> Stimulation of Catalysis**—Residues 16–134 in the N terminus of PLC $\delta$ 1 are homologous with the PH domains from pleckstrin and spectrin (11, 46), which have been shown to bind PIP<sub>2</sub> or IP<sub>3</sub> with high affinity and specificity (19, 26). In order to map structural determinants in the PH domain essential for PIP<sub>2</sub> activation of PLC $\delta$ 1, 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 PLC $\delta$ 1 had little effect on the ability to catalyze the hydrolysis of PI and a modest effect on PIP<sub>2</sub> micelles (Table II). Like the native enzyme, the abilities of these truncated mutant enzymes to catalyze the hydrolysis of PI or PIP<sub>2</sub> were highly dependent on substrate concentration (data not shown). The ability to hydrolyze PI or PIP<sub>2</sub> 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 PLC $\delta$ 1 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 PIP<sub>2</sub>-mediated stimulation of PI hydrolysis (Fig. 6). Mutant enzymes such as del-20 PLC $\delta$ 1 required 10-fold higher concentrations of PIP<sub>2</sub> to enhance the activity 4-fold and never attained the high specific activity obtained with the native enzyme (23  $\mu$ mol/min/mg versus 16  $\mu$ mol/min/mg for the mutant). As more residues were deleted from the N terminus, the enzyme became more severely

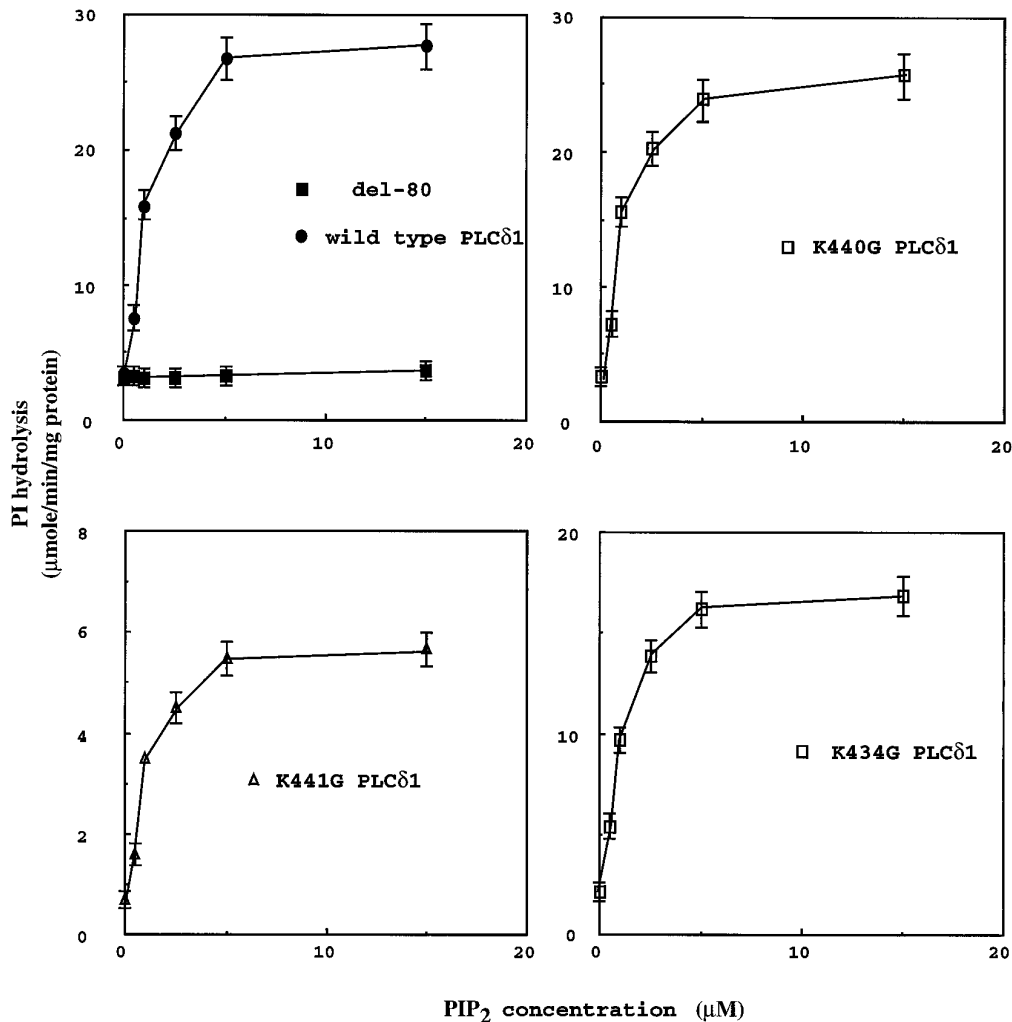


FIG. 4. Effect of PIP<sub>2</sub> on the hydrolysis of PI by wild type and mutant forms of PLC $\delta$ 1. Hydrolysis of [<sup>3</sup>H]PI in PI/PS/PC (molar ratio = 1:5:5) vesicles containing increasing concentrations of PIP<sub>2</sub> (0–15  $\mu$ M of total PIP<sub>2</sub> corresponding to 0–4.4 mol%). The level of co-incorporated PIP<sub>2</sub> was expressed as the final concentration in each reaction. Each reaction was carried out, terminated, and quantitated as described in the legend of Fig. 2. del-80 PLC $\delta$ 1 lacks the first 80 amino acids from the N terminus. K434G, K440G, and K441G are mutants of PLC $\delta$ 1 in which Lys<sup>434</sup>, Lys<sup>440</sup>, and Lys<sup>441</sup> were individually substituted with Gly, respectively.

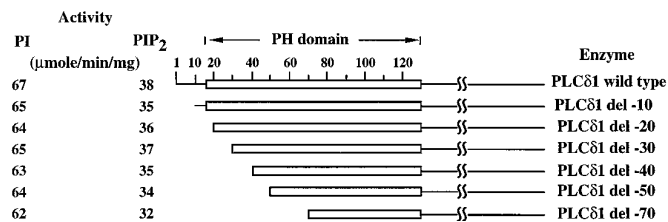


FIG. 5. Schematic representation of the PH domain in PLC $\delta$ 1 and its nested set of deletion mutants. The PH domain is located in the N terminus of human PLC $\delta$ 1 starting from residues 16–124. A nested set of N-terminal deletion mutant enzymes in increments of 10 residues (from del-10 to del-70) were used to map the region important for PIP<sub>2</sub> binding. The PI- and PIP<sub>2</sub> hydrolysis activities of the wild type and the truncated enzymes were determined as described (40, 41) and are listed at the left.

impaired. The PI hydrolysis activities of the mutant enzymes del-50 through del-70 PLC $\delta$ 1 were scarcely enhanced even in the presence of 15  $\mu$ M PIP<sub>2</sub> (data not shown). As would be expected, del-10 PLC $\delta$ 1 was comparable with native enzyme (the initial 16 amino acids of PLC $\delta$ 1 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 [<sup>3</sup>H]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 $\delta$ 1 enzyme was observed at relatively low concentrations of PIP<sub>2</sub> (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 $\delta$ 1 deletion mutants reported here evidenced any change in overall protein structure that differed from wild type 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 PIP<sub>2</sub>-stimulatory effect seen with the PLC $\delta$ 1 deletion mutants could arise from subtle structural changes that may indirectly perturb PIP<sub>2</sub> 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 $\delta$ 1 were replaced with glycine. This region of the PH domain is highly charged and has been shown to be important for IP<sub>3</sub> and PIP<sub>2</sub> binding to the PH domains of pleckstrin and spectrin (19, 24, 26). The conversion of Lys<sup>24</sup> (K24G), Lys<sup>30</sup> (K30G), Lys<sup>32</sup> (K32G), Arg<sup>37</sup> (R37G), or Trp<sup>36</sup> (W36G) of PLC $\delta$ 1 to glycine did not affect the hydrolysis of PI; however, the hydrolysis of PIP<sub>2</sub> in phospholipid vesicles was

TABLE II  
Hydrolysis of PI and PIP<sub>2</sub> in phospholipid vesicles and dodecylmaltoside mixed micelles by the wild type and the deletion mutant PLC $\delta$ 1

Type of enzyme	PS/PC phospholipid vesicle activity <sup>a</sup>		Dodecyl maltoside mixed micelle activity <sup>b</sup>	
	PI	PIP <sub>2</sub>	PI	PIP <sub>2</sub>
	$\mu\text{mol}/\text{min}/\text{mg}$		$\mu\text{mol}/\text{min}/\text{mg}$	
Wild type	3.2 $\pm$ 0.29	11 $\pm$ 1	36 $\pm$ 4	78 $\pm$ 8
del-10	2.95 $\pm$ 0.3	9.9 $\pm$ 0.80	34 $\pm$ 3	77 $\pm$ 8
del-20	2.81 $\pm$ 0.3	7.2 $\pm$ 0.80	36 $\pm$ 3	45 $\pm$ 5
del-30	2.74 $\pm$ 0.25	2.58 $\pm$ 0.27	35 $\pm$ 4	32 $\pm$ 3
del-40	2.85 $\pm$ 0.32	1.96 $\pm$ 0.19	33 $\pm$ 2	30 $\pm$ 2
del-50	2.55 $\pm$ 0.23	2.05 $\pm$ 0.19	34 $\pm$ 3	33 $\pm$ 2
del-70	2.63 $\pm$ 0.27	2.20 $\pm$ 0.22	35 $\pm$ 3	32 $\pm$ 3

<sup>a</sup> Hydrolysis of 30  $\mu\text{M}$  [<sup>3</sup>H]PI (20,000 cpm) or 30  $\mu\text{M}$  [<sup>3</sup>H]PIP<sub>2</sub> (16,000 cpm) at 37 °C in phospholipid vesicles (PI/PS/PC; molar ratio = 1:5:5) or (PIP<sub>2</sub>/PS/PC; molar ratio = 1:5:5) were carried in 50  $\mu\text{l}$  of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 3 mM CaCl<sub>2</sub> and 500  $\mu\text{g}/\text{ml}$  BSA. Reaction of PI hydrolysis was stopped and quantitated as described under "Experimental Procedures." Reaction of PIP<sub>2</sub> hydrolysis was stopped by adding 0.17 ml of 10% ice-cold trichloroacetic acid and 0.85  $\mu\text{l}$  of bovine serum albumin (10 mg/ml). After incubating on ice for 15 min, the unhydrolyzed [<sup>3</sup>H]PIP<sub>2</sub> (pellet) was separated from [<sup>3</sup>H]IP<sub>3</sub> (supernatant) by centrifugation at 2000  $\times$  *g* for 10 min. See "Experimental Procedures."

<sup>b</sup> Hydrolysis of 44  $\mu\text{M}$  [<sup>3</sup>H]PI (20,000 cpm) or 46  $\mu\text{M}$  [<sup>3</sup>H]PIP<sub>2</sub> (16,000 cpm), 0.3 mM dodecyl maltoside mixed micelles at 37 °C in 50  $\mu\text{l}$  of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl<sub>2</sub>, and 500  $\mu\text{g}/\text{ml}$  of BSA. See "Experimental Procedures."

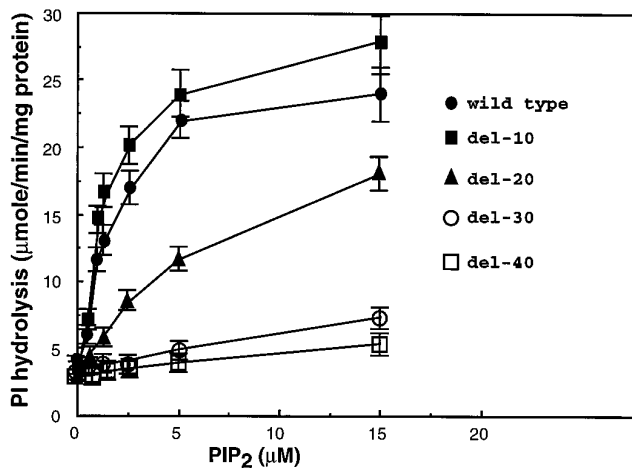


FIG. 6. Effect of PIP<sub>2</sub> on hydrolysis of PI by the wild type and N terminus-truncated PLC $\delta$ 1. Catalytic hydrolysis of 30  $\mu\text{M}$  PI in PI/PS/PC (molar ratio of 1:5:5) vesicles incorporated with the indicated amount of PIP<sub>2</sub> (0–15  $\mu\text{M}$  of total PIP<sub>2</sub> 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 PIP<sub>2</sub> 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  $\mu\text{M}$  PIP<sub>2</sub> would maximally stimulate the enzyme (8-fold), 15  $\mu\text{M}$  PIP<sub>2</sub> barely stimulated (1.5–3-fold) the hydrolysis of PI by these mutant enzymes.

**Mutants Defective in PIP<sub>2</sub> Stimulation Are Also Unable to Bind PIP<sub>2</sub> with High Affinity**—It has been shown that the isolated PH domain from PLC $\delta$ 1 can recognize and bind PIP<sub>2</sub> with high affinity (22, 30). If PIP<sub>2</sub>-dependent enzyme activation is mediated through the PH domain, then mutants defective in PIP<sub>2</sub> mediated stimulation should also be deficient in high affinity PIP<sub>2</sub> 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 PIP<sub>2</sub>. As shown in Fig. 8A, wild type PLC $\delta$ 1 or del-10 mutant enzyme with its first 10 N-terminal amino acid residues truncated can bind with high affinity and specificity to PIP<sub>2</sub> in phosphatidylethanolamine and phosphatidylcholine vesicles. Fifty percent of the protein was bound when incubated with 2  $\mu\text{M}$  PIP<sub>2</sub>, and the binding was saturated at 8  $\mu\text{M}$ . In contrast, the truncated mutant enzymes with 20 amino acid residues or more

TABLE III  
Hydrolysis of PI and PIP<sub>2</sub> in phospholipid vesicles by the wild type and single amino acid-substituted mutant PLC $\delta$ 1

Type of enzyme (PLC $\delta$ 1)	PI hydrolysis <sup>a</sup>	PIP <sub>2</sub> hydrolysis <sup>b</sup>
	$\mu\text{mol}/\text{min}/\text{mg}$	$\mu\text{mol}/\text{min}/\text{mg}$
Wild type	3.3 $\pm$ 0.3	11 $\pm$ 1
K24G	3.2 $\pm$ 0.3	8.1 $\pm$ 0.7
K30G	3.2 $\pm$ 0.2	5.5 $\pm$ 0.6
K32G	2.9 $\pm$ 0.2	4.7 $\pm$ 0.5
W36G	3.2 $\pm$ 0.3	4.3 $\pm$ 0.4
R37G	3.1 $\pm$ 0.3	4.1 $\pm$ 0.4

<sup>a</sup> Hydrolysis of 30  $\mu\text{M}$  [<sup>3</sup>H]PI at 37 °C in phospholipid vesicles (PI/PS/PC, molar ratio = 1:5:5). See "Experimental Procedures."

<sup>b</sup> Hydrolysis of 30  $\mu\text{M}$  [<sup>3</sup>H]PIP<sub>2</sub> at 37 °C in phospholipid vesicles (PIP<sub>2</sub>/PS/PC, molar ratio = 1:5:5). See "Experimental Procedures."

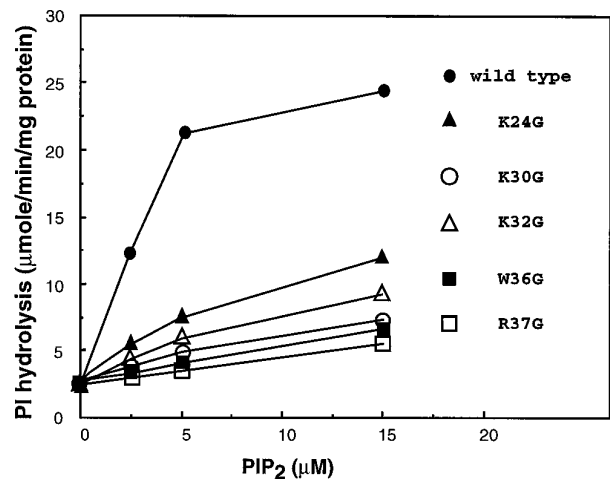


FIG. 7. PIP<sub>2</sub> stimulation of PI hydrolysis catalyzed by wild type and single residue-substituted PLC $\delta$ 1 mutants. The rates of PI hydrolysis catalyzed by the wild type (●), K24G (▲), K30G (○), W36G (■), R37G (□), and K32G (△) mutant enzymes were measured by using PI/PS/PC/PIP<sub>2</sub> vesicles. The substrate concentration of PI and nonsubstrate diluent PS and PC was fixed at 30, 150, and 150  $\mu\text{M}$ , respectively. PIP<sub>2</sub> concentration was varied from 0 to 15  $\mu\text{M}$  (from 0 to 4.4 mol%). Data are representative of two independent duplicated experiments.

deleted from the N terminus displayed impaired PIP<sub>2</sub> binding. Even deleting a few residues from the putative PH domain in PLC $\delta$ 1 resulted in a strongly impaired phenotype. PLC $\delta$ 1 (del-20) exhibited a marked reduction in PIP<sub>2</sub> binding (Fig. 8A); only 50% of the del-20 mutant enzyme bound at relatively high concentrations of PIP<sub>2</sub> (20  $\mu\text{M}$ ). 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

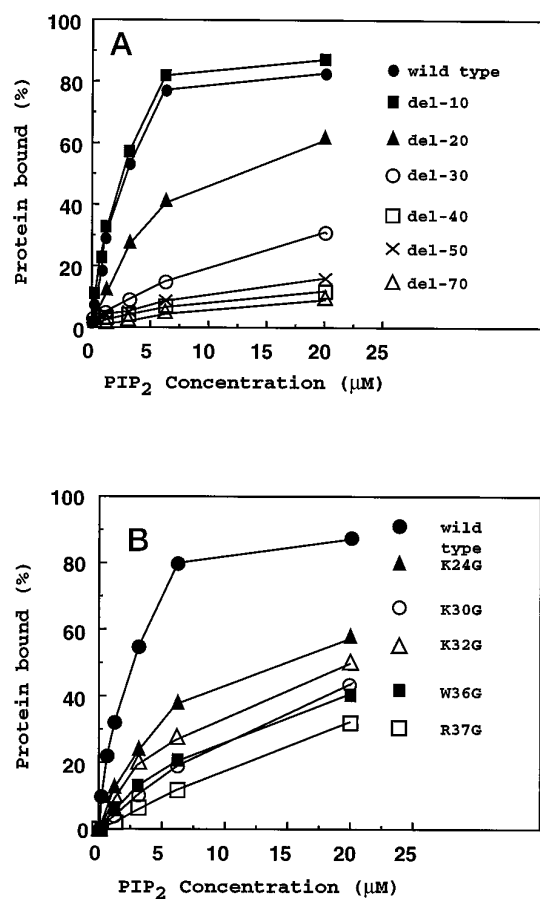


FIG. 8. Centrifugation binding assay to assess binding of wild type and mutant PLC $\delta 1$  to PIP<sub>2</sub>-containing vesicles. A, dose-dependent binding of PE/PC/PIP<sub>2</sub> (4:1:0.25) lipid vesicles to the wild type (●), del-10 (■), del-20 (▲), del-30 (○), del-40 (□), del-50 (×), and del-70 (△) enzymes. B, dose-dependent binding of PE/PC/PIP<sub>2</sub> (4:1:0.25) lipid vesicles to the wild type (●) or K24G (▲), K30G (○), W36G (■), R37G (□), and K32G (△) mutant PLC $\delta 1$ . Data shown are representative of three independent duplicated experiments.

was bound at 20  $\mu\text{M}$  PIP<sub>2</sub>. Likewise, the single point mutants K24G, K30G, K32G, R32G, R37G, and W36G were impaired in PIP<sub>2</sub> binding (Fig. 8B). At least 10-fold more PIP<sub>2</sub> 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 PLC $\delta 1$  are essential for high affinity binding of PIP<sub>2</sub> (17, 22, 30). Furthermore, the present results demonstrate that mutants defective in high affinity binding of PIP<sub>2</sub> are also defective in PIP<sub>2</sub>-mediated enzyme stimulation, indicating that stimulation of PLC $\delta 1$  by PIP<sub>2</sub> is mediated by high affinity binding of PIP<sub>2</sub> to the PH domain.

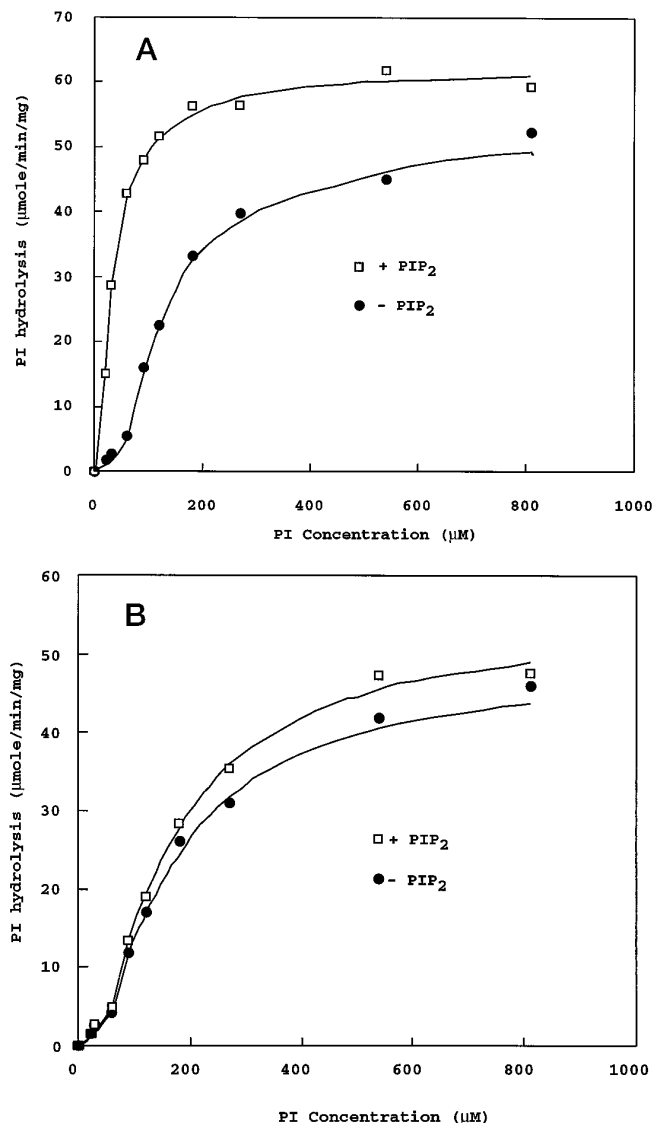
**PIP<sub>2</sub> Binding to the PH Domain of PLC $\delta 1$  Affects  $K_s$  and  $K_m$  but Not  $V_{max}$** —In an attempt to understand the enzymatic mechanism by which PIP<sub>2</sub> enhances enzyme activity, a dual phospholipid binding model was used to analyze the kinetics of PIP<sub>2</sub>-stimulated 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 PLC $\delta 1$  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 PLC 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 PLC $\delta 1$  (44). Therefore, in our kinetic experiments, the bulk concentration of nonsubstrate lipid was kept constant at 150  $\mu\text{M}$  each (PS and PC; the total nonsubstrate lipid would be 300  $\mu\text{M}$ ), 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  $\mu\text{M}$  to 810  $\mu\text{M}$ , the rate of PI hydrolysis catalyzed by the native enzyme increased from 2.1 to 49  $\mu\text{mol}/\text{min}/\text{mg}$  in the absence of PIP<sub>2</sub> and from 15.6 to 60  $\mu\text{mol}/\text{min}/\text{mg}$  in the presence of 5  $\mu\text{M}$  of PIP<sub>2</sub> (Fig. 9A). The most dramatic stimulation by PIP<sub>2</sub> (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 PIP<sub>2</sub> diminished. Although the rate of PI hydrolysis catalyzed by del-80 PLC $\delta 1$  exhibited similar dependence on the substrate concentration as the native enzyme, we were not able to detect any stimulation by PIP<sub>2</sub> within the range of PI between 22 and 810  $\mu\text{M}$  (Fig. 9B). Computer fitting of the kinetic data obtained in the presence or absence of PIP<sub>2</sub> 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 PIP<sub>2</sub> on the catalysis of PI hydrolysis by PLC $\delta 1$  was primarily on the vesicle dissociation constant ( $K_s$ ), which governs the binding of PLC $\delta 1$  to the surface of phospholipid vesicles.  $K_s$  was reduced from 550 to 53  $\mu\text{M}$  by 5  $\mu\text{M}$  PIP<sub>2</sub>. The interfacial Michaelis constant was also reduced from 0.21 to 0.06 by 5  $\mu\text{M}$  PIP<sub>2</sub>. Conversely, PIP<sub>2</sub> had little effect on the  $V_{max}$ , raising it from 62 to 66  $\mu\text{mol}/\text{min}/\text{mg}$ . These data suggest that the mechanism of PLC $\delta 1$  activation by PIP<sub>2</sub> 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 PLC $\delta 1$ . Although the  $V_{max}$  of del-80 PLC $\delta 1$  is similar to that of the native enzyme, its  $K_m$  and  $K_s$  was not affected by PIP<sub>2</sub>. 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 PIP<sub>2</sub>-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 isoforms of phospholipases C, D, and A<sub>2</sub> require PIP<sub>2</sub> for activity, the functional site of phospholipid-protein interaction is unknown. In this report, we use an isoform of PLC (PLC $\delta 1$ ) to



**FIG. 9. Effect of PIP<sub>2</sub> on PI hydrolysis by the native and del-80 PLC $\delta 1$  as a function of total concentration of PIP<sub>2</sub>.** The rates of PI hydrolysis catalyzed by native PLC $\delta 1$  (A) or by del-80 mutant enzyme (B) were determined at increasing concentrations of PI in the presence ( $\square$ ) or absence ( $\bullet$ ) of 5  $\mu\text{M}$  PIP<sub>2</sub> (0.45–1.6 mol% of PIP<sub>2</sub>). The reaction was carried out at 37 °C for 5–15 min in 50  $\mu\text{l}$  of 50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EGTA, 3 mM CaCl<sub>2</sub>, and 500  $\mu\text{g/ml}$  BSA, containing a fixed concentration of PS and PC (150  $\mu\text{M}$  each) and increasing concentration of PI in the presence or absence of 5  $\mu\text{M}$  PIP<sub>2</sub>. Each point represents the mean of three separate experiments performed in triplicate. Solid lines, computer fit to Equation 3.

describe PIP<sub>2</sub> activation of catalysis and to identify the structural determinants mediating phospholipid modulation of enzyme function (3, 50). The determinants for high affinity PIP<sub>2</sub> binding and activation lie in the N-terminal PH domain.

In addition to the classical role of PIP<sub>2</sub> as a substrate for hydrolysis to yield the second messengers IP<sub>3</sub> and DAG, PIP<sub>2</sub> also plays a critical role in the modulation of a number of signaling and cytoskeletal molecules (33). The data presented demonstrate that the activity of native PLC $\delta 1$  is stimulated (up to 9-fold) in a dose-dependent manner by PIP<sub>2</sub>. In order to assess the role of PIP<sub>2</sub> as a regulatory ligand of PLC $\delta 1$  activity, we isolated the modulatory effect of PIP<sub>2</sub> from any concentration-dependent substrate effects by using PI as the substrate for hydrolysis. The specificity of PIP<sub>2</sub> stimulation was demonstrated by the ineffectiveness of other anionic phospholipids, including the very closely related PIP. PIP<sub>2</sub> has also been

shown to modulate the function of phospholipase D (34, 36) and ARF (35) in a specific manner. In both cases, PIP<sub>2</sub> was required for optimal stimulation of phospholipase D and ARF activities, and the effects of other anionic phospholipids were negligible. To our knowledge, this PIP<sub>2</sub> stimulation of PLC hydrolytic activity has never been described. In fact, the opposite result might have been predicted, that PIP<sub>2</sub> would inhibit PI or PIP<sub>2</sub> hydrolysis by competing for substrate at the catalytic site. Although competition does occur at high (30  $\mu\text{M}$ ) PIP<sub>2</sub> concentrations, at micromolar or submicromolar concentrations ( $\text{EC}_{50}$  = 1  $\mu\text{M}$  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 PIP<sub>2</sub>. Whether PIP<sub>2</sub> would stimulate PI hydrolysis *in vivo* will require further investigation. However, this comparison is relevant to the present vesicle constitution of PIP<sub>2</sub> and PI when half-maximal activity to hydrolyze PI is stimulated by 1  $\mu\text{M}$  PIP<sub>2</sub>, 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 PIP<sub>2</sub>-containing vesicles (52, 53). The high affinity binding of PIP<sub>2</sub> to PLC $\delta 1$  correlated with the ability of PIP<sub>2</sub> to stimulate PLC $\delta 1$ ; the tighter the binding between PIP<sub>2</sub> and PLC $\delta 1$ , the more potent PIP<sub>2</sub> was in its ability to stimulate the enzyme. The structural determinants mediating PIP<sub>2</sub> activation and high affinity PIP<sub>2</sub> 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 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 PLC $\delta 1$ , which was to modulate enzyme activity.

The PH domain of PLC $\delta 1$  appears essential for the enzyme to perform efficient catalysis, since the structural integrity of the PH domain of this enzyme is indispensable for PIP<sub>2</sub>-mediated activation of enzyme activity. Since low molar concentrations of PIP<sub>2</sub> can significantly increase PI hydrolysis by PLC $\delta 1$ , this may be a mechanism by which PLC $\delta 1$  is able to utilize efficiently the large molar excess of PI relative to PIP<sub>2</sub> in the cell membrane. The PIP<sub>2</sub>-hydrolyzing activities of mutants defective in high affinity PIP<sub>2</sub> binding were significantly reduced when PIP<sub>2</sub>/PS/PC vesicles were used as substrates. This is consistent with the present conclusion that high affinity binding of PIP<sub>2</sub> is essential for the stimulation of PLC $\delta 1$  and agrees with the observations that blocking high affinity PIP<sub>2</sub> binding to the PH domain with IP<sub>3</sub> leads to inhibition of PIP<sub>2</sub> hydrolysis (54, 55). PI is not able to bind PLC $\delta 1$  with an affinity comparable with that of PIP<sub>2</sub> (7, 22, 30); thus, PI is not able to enhance the catalytic function of PLC $\delta 1$ . This may be the reason why all the PLC $\delta 1$  mutants that were defective in high affinity PIP<sub>2</sub>-binding could 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 PLC $\delta 1$  are not involved in substrate hydrolysis (4, 8, 31) but are required for specific high affinity binding to PIP<sub>2</sub>. Furthermore, the present work demonstrated that binding of PIP<sub>2</sub> 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 PIP<sub>2</sub> by the mutant enzymes was highly dependent on the assay

TABLE IV  
Effect of PIP<sub>2</sub> on kinetic parameters of PLC $\delta$ 1

Type of enzyme	PI/PS/PC vesicles <sup>a</sup>			PI/PIP <sub>2</sub> /PS/PC vesicles (5 $\mu$ M PIP <sub>2</sub> ) <sup>b</sup>		
	V <sub>max</sub>	K <sub>m</sub>	K <sub>s</sub>	V <sub>max</sub>	K <sub>m</sub>	K <sub>s</sub>
	$\mu$ mol/min/mg	mol fraction	$\mu$ M	$\mu$ mol/min/mg	mol fraction	$\mu$ M
Native	62 $\pm$ 8	0.21 $\pm$ 0.07	558 $\pm$ 175	66 $\pm$ 10	0.06 $\pm$ 0.015	53 $\pm$ 20
del-10	71 $\pm$ 14	0.18 $\pm$ 0.07	588 $\pm$ 195	71 $\pm$ 11	0.07 $\pm$ 0.013	56 $\pm$ 17
del-20	55 $\pm$ 7	0.24 $\pm$ 0.11	532 $\pm$ 200	59 $\pm$ 8	0.15 $\pm$ 0.042	300 $\pm$ 110
del-30	60 $\pm$ 9	0.21 $\pm$ 0.07	618 $\pm$ 215	50 $\pm$ 8	0.19 $\pm$ 0.032	350 $\pm$ 150
del-40	57 $\pm$ 8	0.28 $\pm$ 0.10	648 $\pm$ 220	53 $\pm$ 9	0.23 $\pm$ 0.050	500 $\pm$ 149
del-80	51 $\pm$ 4	0.20 $\pm$ 0.09	668 $\pm$ 220	54 $\pm$ 10	0.20 $\pm$ 0.040	540 $\pm$ 146

<sup>a</sup> Hydrolysis of PI in PI/PS/PC vesicles with increasing concentration of PI and fixed concentration of PS/PC (150  $\mu$ M each). The rate of hydrolysis was plotted as a function of total concentration of PI. The V<sub>max</sub>, K<sub>m</sub>, and K<sub>s</sub> correspond to the constants defined in the dual substrate binding model (Equations 1, 2, and 3). V<sub>max</sub>, K<sub>m</sub>, and K<sub>s</sub> were calculated by fitting the data to Equation 3.

<sup>b</sup> PI hydrolysis in PI/PIP<sub>2</sub>/PS/PC vesicles with an increasing concentration of PI and a fixed concentration of 5, 150, and 150  $\mu$ M for PIP<sub>2</sub>, PS, and PC, respectively. Kinetic parameters, V<sub>max</sub>, K<sub>m</sub>, and K<sub>s</sub>, were calculated by fitting the data to Equation 3.

conditions. Truncation of the N-terminal sequence of PLC $\delta$ 1 did not affect the PIP<sub>2</sub>-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 PLC $\delta$ 1 is not affected by truncation of its first 58 N-terminal residues. This may simply reflect weak PIP<sub>2</sub>-binding in the sodium cholate detergent micelles, because the PI-hydrolyzing activity of PLC $\delta$ 1 did not respond to the stimulatory effect of PIP<sub>2</sub> at all if PI/sodium cholate mixed micelles were used as substrate (data not shown).

The PLC $\delta$ 1 single point mutants K24G, K30G, K32G, R37G, and W36G were impaired in PIP<sub>2</sub> binding and in PIP<sub>2</sub>-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  $\beta$ -strand variable loop and the N-terminal portion of the second  $\beta$ -strand of the PH domain. These regions play important roles in mediating specific interactions with the PIP<sub>2</sub> molecule (19, 23, 24). The co-crystal structure of the isolated PH domain from rat PLC $\delta$ 1 complexed with IP<sub>3</sub> revealed that Lys<sup>30</sup> and Lys<sup>32</sup> were found to hydrogen bond either directly or via their side chains to the 4- and 5-phosphate groups of IP<sub>3</sub>. The side chain of Trp<sup>36</sup> was found to hydrogen bond with the 1-phosphate of IP<sub>3</sub> as well as be in van der Waals contact with part of the inositol ring (23). Our functional data clearly demonstrate an important functional role for Trp<sup>36</sup> and Lys<sup>32</sup>, suggesting that interaction of the PH domain of PLC $\delta$ 1 with the inositol ring of PIP<sub>2</sub> is an important determinant for ligand binding and enzyme activation. This is in stark contrast to the interpreted role of the inositol ring of PIP<sub>2</sub> in binding to the PH domain of  $\beta$ -spectrin (19), in which there is virtually no interaction of the inositol ring with the  $\beta$ -spectrin protein. The inositol ring interaction with PLC $\delta$ 1 may help to explain the relatively high affinity binding of PIP<sub>2</sub> to this protein. Furthermore, the present data also identify a functional role for Lys<sup>24</sup> and Arg<sup>37</sup>. The lack of participation of these residues in the binding of IP<sub>3</sub> in the crystal structure may represent an unrecognized role of these residues in the interpreted structure or may indicate a general structural role of these residues in formation of the tertiary structure of the PH domain.

Phosphoinositides such as PIP<sub>2</sub> have been shown to associate with and/or regulate many other proteins including profilin, cofilin, gelsolin, ARF-GAP, phospholipase D, dynamin,  $\beta$ -adrenergic receptor kinase, and cytoplasmic phospholipase A<sub>2</sub> (32, 34, 38, 56–59). Since many of these proteins, whose activities are affected by phospholipids, are known to contain PH domains, it is logical in the context of this report to suggest that PIP<sub>2</sub> acts as a specific ligand to stimulate activity and that the PH domains of these molecules represent the site of action. The present study shows that the stimulatory effect of PIP<sub>2</sub> on PLC $\delta$ 1 is mediated by the binding of PIP<sub>2</sub> to the PH domain

and is very specific for PIP<sub>2</sub>; co-incorporation of other anionic phospholipids into the PI/PS/PC vesicles does not stimulate the enzyme, and IP<sub>3</sub> reduces this stimulatory effect. The effect of anionic phospholipids on the catalysis by these molecules is largely speculative and includes direct effects on membrane packing (phase effects), nonspecific surface charge effects, and stimulation of protein translocation (enzyme anchoring).

The PH domain of PLC $\delta$ 1 has been implicated to serve as an anchor of the enzyme on the PIP<sub>2</sub>-containing membrane (8, 22, 31), thus allowing PLC $\delta$ 1 to catalyze the hydrolysis of PIP<sub>2</sub> in a processive manner. It is very likely that the ability of PLC $\delta$ 1 to catalyze the hydrolysis of PI in PS/PC vesicles may well be greatly enhanced by the anchoring properties of PIP<sub>2</sub> on the vesicle surface, which allow the enzyme to hydrolyze substrate in a processive manner. According to this model (Equations 1 and 2), the enzyme binds to phospholipid at the surface of the mixed micelles through the noncatalytic PH domain, which 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<sub>s</sub>. Our kinetic experiments have demonstrated that PIP<sub>2</sub> greatly enhances the affinity of the enzyme for vesicles. K<sub>s</sub> was reduced from 558 to 53  $\mu$ M by 5  $\mu$ M of PIP<sub>2</sub>. 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<sub>m</sub>. The K<sub>m</sub> was also reduced from 0.21 to 0.06 by 5  $\mu$ M of PIP<sub>2</sub> (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 PIP<sub>2</sub> 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, PIP<sub>2</sub> binding to the PH domain may allosterically alter the structure of this enzyme.

It seems unlikely that the binding of PIP<sub>2</sub> also contributes directly to PLC $\delta$ 1 stimulation, since the V<sub>max</sub> 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 PIP<sub>2</sub> may in fact be the major ligand for the PH domain in PLC $\delta$ 1. While it is unlikely that PIP<sub>2</sub> would be the functional ligand for many PH domains, it is probable that PH domains in many molecules serve to modulate activity. This novel finding has implications for the regulation of the wide variety of proteins that contain PH domains and suggests a potential new target for the develop-

ment of therapeutics. Whether PIP<sub>2</sub> regulates the function of other proteins by high affinity and specific binding to the PH domain remains to be determined.

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