Positive Charge at Position 549 Is Essential for Phosphatidylinositol 4,5-Bisphosphate-hydrolyzing but Not Phosphatidylinositol-hydrolyzing Activities of Human Phospholipase C δ1

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Point mutagenesis, phosphatidylinositol (PI), and phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis assays and equilibrium centrifugation PIP₂ assays were used to study the functional roles of four highly conserved arginine residues in the Y region of human phospholipase C δ1 (PLCδ1) (Arg-527, -549, -556, -701). Most of the mutant enzymes were either partially defective or fully active in their abilities to catalyze the hydrolysis of PI or PIP₂. However, upon substitution of Arg-549 by glycine or histidine, the mutant enzyme was defective in its ability to catalyze the hydrolysis of PIP₂, but it is still able to hydrolyze PI. Replacing Arg-549 with lysine had little effect on the level of PI and PIP₂ hydrolytic activities of the mutant enzyme. The residual PIP₂ hydrolyzing activity of R549H is highly dependent on pH. R549H showed 5–10% of the PIP₂-hydrolyzing activity of the native enzyme between pH 5 and 7 and nondeectable PIP₂-hydrolyzing activity at pH 8. The PIP₂-hydrolyzing activity of R549G was not detectable at all pH values. Kinetic analysis of PLCδ1-catalyzed PIP₂ hydrolysis revealed that the micellar dissociation constant \( K_d \) and interfacial Michaelis constant \( K_m \) were similar in the native, R549K, and R549H enzymes; but the specific activity at the saturated substrate mole fraction and infinite level of substrate \( (V_{\text{max}}) \) of the R549H mutant were reduced by a factor of 15. PIP₂ competitively inhibits the native enzyme to hydrolyze PI at both pH 7 and 8. However, PIP₂ inhibits R549H only at pH 7.0 and does not inhibit R549G at either pH. Taken together, these results suggest that positive charge at position 549 of PLCδ1 protein is essential for the enzyme to recognize and catalyze the hydrolysis of PIP₂ but not PI.

Phosphatidylinositol-specific phospholipase C (PI-PLC) hydrolyzes inositol phospholipids into diacylglycerol and inositol 1,4,5-trisphosphate, which function as important second messengers to activate protein kinase C and mobilize intracellular Ca²⁺, respectively (1). These trigger multiple enzymatic cascades to regulate cellular activities including cell growth and neuronal activities. Three isoforms of PI-PLC have been identified and designated as β, γ, and δ (2). Mechanisms of activation have been demonstrated only for the β and γ isoforms. PLCγ has been shown to be activated following phosphorylation by nonreceptor or receptor protein tyrosine kinase activities (3, 4), whereas PLCβ has been demonstrated to be regulated by α subunits of G proteins (5–7) or by βγ subunits (8–10).

Although each isoform of PI-PLC has its distinct mode of cellular regulation, all eucaryotic PI-PLC isozymes can catalyze the hydrolysis of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP₁), and phosphatidylinositol 4,5-bisphosphate (PIP₂) in vitro (2, 11). Structural similarity among each isoform of PI-PLC has also been identified. Two highly homologous regions designated as X and Y have been found in all PI-PLCs isolated from eucaryotic sources (2). These two regions are important for the enzyme to catalyze the hydrolysis of inositol phospholipids, since partial deletion of either X or Y region has been shown to inactivate the catalytic function of the enzyme (12–15). Since only X region homologous sequences have been identified in bacterial enzymes (16), it has been suggested that residues directly involved in catalytic activity are located in the X region (17). Part of this prediction has recently been confirmed by site-directed mutagenesis of X region amino acid residues that are invariant in all PI-PLCs from human to bacteria (18). Although most of the mutations have little effect on the catalytic function of the enzyme, substitution of amino acid residues at three positions (Arg-338, Gly-341, and His-356) does render the enzyme completely incapable of hydrolyzing PI and PIP₂. Since these mutant enzymes can still interact with PIP₂ in a manner comparable with that of the native enzyme, it was concluded that these amino acids are involved in the cleavage step. Some residues in the X region, which are only conserved in eucaryotic PI-PLCs, have also been identified to be involved in the catalytic process (19).

In addition to the X homologous region, eucaryotic enzymes possess a conserved Y region that is missing in bacterial enzymes (16, 17). Functions of the Y region and its conserved residues are unknown except for the hypothesis derived from the comparison of PI-PLC from eucaryotic cells and procaryotic cells. Bacterial PI-PLCs differ from eucaryotic enzymes in at least two aspects (17). 1) The bacterial enzyme does not catalyze the hydrolysis of the higher order phosphorylated forms of inositol phospholipids, and 2) its ability to catalyze the hydrolysis of PI does not require calcium as a cofactor. This implies that the Y region of eucaryotic PI-PLC may be involved in Ca²⁺ binding and the specific catalytic hydrolysis of the higher phosphorylated form of inositol phospholipids. To verify this implication and to gain further insight into the functions of the Y region, conserved residues with positively charged side chains in the Y region were subjected to base substitution mutagene-
sis in the present study. The mutant PLCδ1 constructs were expressed in Escherichia coli, and the mutant proteins were purified by two-step chromatography. To determine the functional consequence of these residue substitutions, we characterized the mutant proteins by their ability to bindPIP2 and to catalyze the hydrolysis of both PIP2 and PI. We also analyzed their dependence on pH, substrate, and calcium ion concentrations. Using these approaches, we are able to demonstrate that the positive charge at position 549 in human PLCδ1 is required for the enzyme to catalyze the hydrolysis of PIP2 but not PI.

**EXPERIMENTAL PROCEDURES**

**Plasmid, E. coli Strain, and Chemicals—**Full-length human PLCδ1 cDNA was inserted into the BamHI site of pSRET(A)R, whose EcoRI site in the polylinker region was destroyed by filling-in reaction. The resulting expression construct (pSRETApelc(R)) was used in the present investigation for mutagenesis and for expression of the enzyme under the control of T7 promoter in E. coli strain BL21(DE3)/pLyS. Phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylinositol 4-phosphate (PI4P), and phosphatidylinositol 4,5-bisphosphate (PI(2,4,5)) were obtained from Avanti Polar Lipids, Inc. Phosphatidylinositol 4,5-bisphosphate (PI(2,4,5)) was obtained from Calbiochem.

**Site-directed Mutagenesis—**Site-directed point mutagenesis by a two-stage PCR method was used (20) with slight modification. Primary PCR steps consisted of two separate PCR reactions for each point mutation. One of the PCR reactions was initiated using a 5′ primer and the other an outer primer. The template DNA and all primer and outer primers; these second was initiated by using an internal primer and the other an outer primer. The template DNA and all expression constructs used in the present investigation were pSRETApelc(R), in which the codon for Arg-527, -549, and -556 was flanked by two unique restriction endonuclease sites (Sphe/EcoRI). The codon for Arg-701 was flanked by an EcoRI site in the PLCδ1 gene, and a HindIII site was located at the 3′-end of the polylinker region of pSRETApelc(R). The PCR took place in a reaction volume of 100 μl containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 0.01% gelatin (w/v), 1.5 mM MgCl2, 200 μM dNTPs, 10 μM template (pSRETApelc(R)), 1 μM primer, and 2.5 units of Taq polymerase. The reaction mixture was prewarmed to 55°C for 5 min; the 30-cycle 3-step PCR (94°C for 40 s, 55°C for 1 min, and 72°C for 2 min) was initiated by adding 0.5 μl (2.5 units) of Taq polymerase. The products of primary PCR reactions were separated electrophoretically and isolated in a low melting point agarose gel. The purified primary PCR products in low melting agarose were incubated at 70°C for 15 min and subsequently diluted with distilled H2O to a final concentration of 5 μg/ml. Equal volumes (20 μl) of two diluted primary PCR products were mixed, boiled, cooled to 55°C, and slow cooled to 30°C. The secondary PCR reaction was carried out in a volume of 100 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin (w/v), 1.5 mM MgCl2, 200 μM dNTPs, 50 ng of template (the mixed and annealed primary PCR products), 1 μM of two outer primers, and 2.5 units of Taq polymerase. Amplified mutant DNA fragments from the secondary PCR reaction were electrophoretically purified in low melting agarose gel, followed by phenol/chloroform extraction and ethanol precipitation. The purified mutant DNA fragment was then digested with EcoRI and HindIII restriction endonuclease and used to replace the corresponding restriction fragment of the wild type pSRETApelc(R). The desired point mutation (Arg-701 to Gly) and sequence flanking by corresponding restriction fragment of the wild type pRSETAplc(R). The EcoRI site in the polylinker region had been destroyed by T4 DNA polymerase filling-in and the subsequent self-ligation.

**Protein Expression and Purification, E. coli—**BL21(DE3) pLyS cells harboring pSRETApelc plasmid were grown at 30°C in 1.5 liters of LB medium containing 100 μg/ml ampicillin. When the A600 of the culture reached 1.0, 20 ml of 0.1% isopropl-1-thio-β-d-galactopyranoside was added, and the culture was incubated at 18°C for an additional 8 h. Cells were harvested by centrifugation and resuspended in 50 ml of ice-cold lysis buffer containing 50 mM sodium phosphate, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Tween 20. All subsequent steps were performed at 4°C. Cells were lysed by 2 cycles of microfluidizer. The cell lysate was cleared by centrifugation at 15,000 × g for 30 min, and the supernatant fraction (120 ml of 3.2 mg/ml protein) was directly applied to a column of 5 ml of Ni2+-nitrilotriacetic acid-agarose (Qiagen) equilibrated with a buffer of 50 mM sodium phosphate, pH 8.0, 0.1 mM KCl, 0.1% Tween 20, and 10 μM phenylmethylsulfonyl fluoride (buffer A). The column was washed with 100 ml of buffer A containing 15 mM imidazole and eluted with 50 ml of the same buffer containing 100 mM imidazole; the eluent was collected as 1.5 ml per fraction. The active fractions were identified by PI hydrolysis assay and then pooled. The partially purified PLCδ1 from the Ni2+-nitrilotriacetic acid column was concentrated in an Amicon Centriprep-30 concentrator, diluted 10-fold with lysis buffer, and then directly applied to 1 ml of heparin-Sepharose CL-6B column (Pharmacia Biotech, Inc.) prequiolibrated with lysis buffer. After being washed with 10 ml of the same buffer, PLCδ1 was eluted by a 50-ml linear salt gradient from 0.1 to 0.5 mM KCl in 50 mM sodium phosphate, pH 8.0, 0.1% Tween 20, 5 mM EDTA, and 5 mM EGTA. The active fractions were identified by PI hydrolysis activity or Western blotting and were pooled.

**Assay of PI-specific PLCδ1 Activity—**Hydrolysis of PIP2 by PLCδ1 was determined as described previously (5) in an assay volume of 60 μl of 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 μg/ml bovine serum albumin, 0.16% sodium cholate, 1.5 mM CaCl2 containing 50 μM PIP2 (8000 cpm), and 500 μM PE. The reaction was carried out at 30°C for 2–15 min and terminated by adding 0.2 ml of 10% ice-cold trichloroacetic acid and 0.1 ml of bovine serum albumin (10 mg/ml). Subsequent to incubation on ice for 15 min, the unhydrolyzed [3H]PIP2 (pellet) was separated from [3H]IP3 (supernatant) by centrifugation at 4°C, 2000 × g for 10 min. Radioactivity in the supernatant was measured by liquid scintillation counting. The activity of PIP2 hydrolysis is expressed as μmol of IP3/min/mg protein; 0.1–100 ng of purified recombinant PLCδ1 was used per assay. Determination of PI hydrolysis activity was essentially the same as described by Hofmann and Majerus (21). The reaction was carried out in a volume of 200 μl of 50 mM HEPES, 1 mM CaCl2, 1 mM EGTA. 0.1% sodium cholate containing 30,000 cpm of [3H]PI (300 μM). After incubation at 37°C for 5–15 min, the reaction was terminated by adding 1 ml of chloroform/methanol/HCl (100:100:0.6), followed by 0.3 ml of 1 N HCl containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 400-μl portion of upper aqueous phase was counted by liquid scintillation.

**Assays using dodecyl maltoside (DM) or Triton X-100** mixed micelles as substrate were performed in a manner similar to those described by Cifuentes et al. (22) with slight modification. In brief, indicated amount of PIP2/[3H]PIP2 or PI/[3H]PI (4 × 105 cpm) in chloroform/methanol (19:1) were flushed-dried under a stream of N2, and followed by lyophilization for 30 min. Lipids were solubilized by probe sonication in 0.95 ml of 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. When measuring the PLC activity as a function of mole fraction of substrate, the total concentrations of PIP2 were kept constant, and the variation of mole fraction of PIP2 was achieved by varying the total concentration of dodecyl maltoside. To assess activity as a function of total concentration of substrate, the mole fraction of PIP2, the variation of PIP2 concentration was achieved by proportionally varying the total PIP2 and the total dodecyl maltoside in the mixed micelles. To assay the activity, 50 μl of dodecyl maltoside (DM) or PIP2 mixed micelles was preincubated at 30°C for 5 min, and 0.1–100 ng of enzyme was added in a volume of 1 μl. The reaction was initiated by adding 2.5 μl of 40 mg/ml of CaCl2, and incubated at 30°C for another 1–15 min. For the analysis of the hydrolysis of PIP2, the reactions were terminated by adding 250 μl of chloroform/methanol/HCl (100:100:0.6), followed by 75 μl of 1 N HCl containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 100-μl portion of upper aqueous phase was counted by liquid scintillation.

**Analysis of Kinetic Data—**Assays of PLC hydrolysis were monitored as a function of total substrate concentration and mole fraction in dodecyl maltoside mixed micelles under Case I, Case II, and Case III conditions previously described for phospholipase A2 and PLCδ1 (23, 24). Case I involves determination of PLC activities with increasing mole fraction of substrate while keep the total substrate concentration constant. In Case II, the total concentration of diluent detergent (dodecyl maltoside) was fixed, and the PLC activities were measured with increasing concentration of the substrate. Dual phospholipid binding model of catalysis (Equation 1a and 1b) (23) was used to analyze the kinetic data.
This model takes into account the fact that the reaction catalyzed by PLCδ1 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, \( K_d = k_{-2}/k_{-1} \) (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 reaction of the lipid molecule and the subsequent catalysis by PLCδ1 is described by the interfacial Michaelis constant, \( K_m = k_{-3}/k_{-2} + k_{-1}/k_{-2} \) (mole fraction, unitless). Initial rates of catalysis \( v \) as a function of mole fraction of substrate in the mixed micelles measured under Case I and II conditions were fitted using Equation 2 (23) to obtain values of \( V_{max}, K_m \), and \( K_n \).

\[
V_{max} = \frac{v}{K_m + K_n + S_n} (Eq. 2)
\]

The absolute rate \( V_{max} \) occurs at an infinite substrate concentration, and the saturated substrate mole fraction, \( S_n \), is the total substrate concentration, and \( K_n \) is the mole fraction of the substrate on the surface of the mixed micelles. When the diluent nonsubstrate phospholipids or detergents exhibit modulatory function on the catalytic reactions, then the initial rates of substrate hydrolysis \( v \) as a function of total concentration of substrate at a fixed concentration of diluent dodecyl maltoside \( (T_n) \) fitted to Equation 3 (23).

\[
V_{max} = \frac{v}{K_m + K_n + S_n + S_n} (Eq. 3)
\]

Preparation of Phospholipid Vesicles—Phospholipid vesicles \( \text{PE/PC (4:1 molar ratio) containing indicated concentration of PIP}_2 \) were prepared as described by Mueller et al. (25) with slight modifications. A dry phospholipid film was formed by slowly blowing 0.25-ml solution of chloroform/methanol (2:1, \( v/v \)) containing mixed lipids (320 nmol of PE, 80 nmol of PC, and indicated amount of PIP2 or PI) under a stream of nitrogen for 18 h at 4 °C followed by mixing with an equal volume of 100 mM HEPES, pH 7.0, 200 mM KCl, and 10 mM EGTA. Vesicles were isolated from the pellet by centrifugation at 120,000 \( \times g \) for 1 h and stored at -20 °C until use.

Results

Mutagenesis of Highly Conserved Arg Residues in the Y Region of PLCδ1—Four highly conserved positively charged amino acid residues (Arg-527, -549, -556, and -701) were identified by sequence alignment in the Y region of eucaryotic PI-PLC (18). To investigate the role of these residues in enzyme catalysis, they were individually mutated by site-directed mutagenesis to Gly. All mutations were introduced into recombinant PLCδ1 with 34-amino acid residues including 6 consecutive histidine residues fused to its N terminus (18). The mutant enzymes were expressed in E. coli strain BL21, purified by Ni\(^{2+}\)-NAT-agarose followed by heparin-Sepharose column chromatography. The homogeneity of the mutant protein was examined by SDS-polyacrylamide gel electrophoresis and Western blotting analysis (data not shown). Preliminary enzymatic characterization of mutant enzymes by PI- and PIP2-hydrolysis assays allowed us to categorize these mutants into three classes (Table I). The first class, Arg-527 → Gly, had little effect on either PI or PIP2 hydrolysis activity. The second class of mutants, in which Arg-556 or Arg-701 was replaced by Gly, was partially defective in catalyzing both the hydrolysis of PIP2 and PI. In the third class of mutant enzyme, Arg-549 was replaced by Gly; its PIP2 hydrolysis activity was not detectable, but it was able to catalyze the hydrolysis of PI at 15% efficiency compared with that of the native enzyme. This suggests that although all four Arg residues are highly conserved or invariant in all eucaryotic PI-PLCs, they may play different roles in either maintaining the structure of the enzyme or in its catalytic function. To focus the scope of the present investigation on the molecular mechanism of substrate selectivity by PLCδ1, only the R549G mutant, whose ability to hydrolyze PIP2 was selectively eliminated, was subjected to further structural and functional characterization.
interactions between the enzyme and PIP2. To address this concern, we further examined the effect of the chemical and physical compositions of the side chain at position 549 on the cleavage activity of PLCζ1 by making a more conservative substitution at position 549; the arginine residue was replaced individually with lysine or histidine. For mutant enzyme in which Arg-549 was converted to His (R549H), its ability to hydrolyze PIP2 (4 μmol/min/mg) was selectively reduced to 10% of that of the native enzyme (37 μmol/min/mg), while its ability to hydrolyze PI (58 μmol/min/mg) was comparable with that of native enzyme (64 μmol/min/mg) (Table I). These results indicate that replacing Arg-549 with histidine results in selective loss of PIP2-hydrolyzing activity but has negligible effect on PI hydrolysis. When the positive charge at position 549 was preserved, as in mutant R549K, the mutation had a minimum effect on the ability of the enzyme to catalyze the hydrolysis of PIP2 or PI (Table I). These results suggest that the positive charge of Arg-549 may be essential for catalyzing the hydrolysis of PIP2 but not PI.

Dependence of PI and PIP2 Hydrolysis Activities on Total Substrate Concentration—Since the ability of PLCζ1 to catalyze the hydrolysis of PI or PIP2 is highly dependent on the total substrate concentration, thus on the affinity of the substrate for the catalytic center of PLCζ1, the selective deficiency in catalyzing the hydrolysis of PIP2 by mutant R549H may be caused by a subtle structural change that alters affinity of PIP2 for the catalytic site of PLCζ1. To test this possibility, we examined the dependence of the cleavage activity on the total substrate concentration. Fig. 1A shows that in a standard PIP2 cleavage assay using PIP2/PE/sodium cholate mixed micelles as a substrate (5), the activity of the native or mutant R549K increased by at least 10-fold (from 17 to 180 μmol/min/mg) as the concentration of PIP2 was increased from 25 to 290 μM and almost attained saturation as the total PIP2 concentration reached 290 μM. On the other hand, the PIP2-hydrolyzing activity of R549H mutant increased no more than 4-fold over this range of substrate concentration and never exceeded 10% of that of the native enzyme. In sharp contrast, the substrate dependence of mutant R549H to hydrolyze PI was comparable with that of native enzyme. As shown in Fig. 1B, the PI-hydrolyzing activities of all three mutants increased in a parallel manner as the substrate concentration was increased from 25 to 300 μM, and the activity approached a plateau as the concentration of PI reached 475 μM. These results clearly show that R549H mutant enzyme is deficient in PIP2-hydrolyzing activity, at least within the substrate concentration range from 25 to 290 μM, while its substrate dependence of PI-hydrolyzing activity is comparable with that of native enzyme.

Quantitative Comparison of PI- and PIP2-Hydrolyzing Activities of the Native and Arg-549 Mutant Enzymes—The standard PI and PIP2 hydrolysis assays we used, as described by Hofmann and Majerus (21) and Hepler et al. (5), have been widely used to measure the PI- and PIP2-hydrolyzing activities of PLC. However, these two systems used different concentrations of Ca2+ ion, different detergent systems, and different phospholipid systems to assay the PI- and PIP2-hydrolyzing activities. To avoid the ambiguity that may arise from these differences in measuring the PI- and PIP2-hydrolyzing activities, we also examined substrate dependences of these activities in inositol phospholipid/dodecyl maltoside mixed micelles system. Dodecyl maltoside under the present condition serves as a neutral detergent for the target substrates and does not bind to or inhibit PLC activity (24), thus allowing a more accurate determination of the activities as the mole fraction or total concentration of the substrates changes and an examination of the hydrolysis of PI and PIP2 under identical conditions. Results similar to those obtained with standard PI and PIP2 hydrolysis assays were also found when using inositol phospholipid/dodecyl maltoside mixed micelles as substrate. As shown in Fig. 2A, the PIP2-hydrolyzing activities of the native enzyme and R549K rose by at least 6-fold (from 24 to 125 μmol/min/mg) as the total concentration of PIP2 was increased from 9 to 360 μM and reached saturation near 100 μM of total PIP2. As in the standard assay, the PIP2-hydrolyzing activity of R549H was lower than that of the native enzyme by a factor of at least 10 and only slightly increased within this range of PIP2 concentrations. The PI-hydrolyzing activities of the native and R549H mutant were increased approximately by a factor of 14 when the substrate concentration was increased from 20 to 275 μM (Fig. 2B). Since the PI and PIP2 hydrolyses were measured under identical conditions except using different phospholipid substrates, the selective reduction of PIP2-hydrolyzing activity of R549H is not caused by variation of the levels of free calcium or detergents used in the standard assays.

The selective reduction in PIP2-hydrolyzing activity of
R549H mutant can also be demonstrated by allowing it to hydrolyze PI and PIP₂ simultaneously. This was achieved by using dodecyl maltoside mixed micelles containing both PI and PIP₂ as substrates for the enzyme and separating the products later. As shown in Table II, the specific activity of the native enzyme (38 μmol/min/mg) was 2.5-fold higher than that to catalyze PI (17 μmol/min/mg). The PIP₂-hydrolyzing activity of R549K is comparable with that of the native enzyme (38 μmol/min/mg) and about 4-fold higher than its PI-hydrolyzing activity. In contrast, the PIP₂-hydrolyzing activity of R549H (3.5 μmol/min/mg) was lower than its PI-hydrolyzing activity (29 μmol/min/mg) by a factor of 8. The PIP₂-hydrolyzing activity of R549K was hardly detectable under the present assay condition. These findings reveal that the selective deficiency of PIP₂ hydrolysis by mutant R549G and R549H does not stem from variations in assay conditions initially used to identify the phenotype of R549G.

**pH Dependence of PLC81 Activities of the Native, R549H, R549K, and R549G Mutant Enzymes**—Selective deficiency in the PIP₂-hydrolyzing activity of R549H and R549G but not R549K suggested that the positive charge of Arg-549 in the native enzyme is correlated with PIP₂ hydrolysis. The fact that R549H exhibited 10% residual PIP₂-hydrolyzing activity at pH 7 may reflect the incomplete deprotonation of the imidazole side chain of histidine 549. This possibility is consistent with the finding that replacing the positively charged guanidinium group with a proton (R549G) abolished PIP₂ hydrolysis. To test the possibility that the positive charge at position 549 is involved in the hydrolysis of PIP₂, the effect of pH on the PIP₂-hydrolyzing activity of the native and mutant enzymes was determined by using PIP₂/dodecyl maltoside mixed micelles as substrate. To correlate the protonation state of the imidazole ring in R549H with the PIP₂-hydrolyzing activities of the mutant enzymes, the activities of the native and the mutant PLC81 were assayed between pH 5 and 8. As shown in Fig. 3, A and B, although R549H displayed 5–10% residual PIP₂-hydrolyzing activity when measured in the pH range 5–7, all three enzymes exhibited pH optima at 5.5 and decreased in a similar rate as pH increased to 7. When the pH was increased from 5.5 to 7, the PIP₂-hydrolyzing activity of the native PLC81 decreased from 105 to 72 μmol/min/mg and that of R549H decreased from 6.7 to 5.1 μmol/min/mg. When the pH was increased from 7 to 8, the activity of the native and mutant R549K decreased by a factor of 1.6 (from 72 to 45), whereas that of R549H decreased from 5.1 μmol/min/mg to basal levels. This mutant enzyme was not able to catalyze the hydrolysis of PIP₂ at pH 8.0 even when 20 μg of R549H was used to digest PIP₂ in 50 μl of assay mixture or when the substrate concentration was raised to 300 μM (the saturation level of PIP₂ hydrolysis by the native and R549K enzyme) (Fig. 4). This implies that at pH 8, the mutant R549H is either severely defective in PIP₂ cleavage or has lost its affinity for PIP₂. This rapid reduction in PIP₂-hydrolyzing activity from pH 7 to 8 is not due to a disturbance of the overall structure of mutant R549H, because a similar sharp reduction in hydrolyzing activity of R549H was not observed when PI was used as substrate: the activity decreases to 40% as pH increased from 7 to 8 (Fig. 3C). This analysis shows that the residual PIP₂-hydrolyzing activity of R549H is much more sensitive to pH change from 7 to 8 than that of the native or the R549K mutant enzyme. The results are consistent with the prediction that the ionization state of imidazole in R549H is

**TABLE II**

| Enzyme          | PI hydrolysis* | PIP₂ hydrolysis* |
|-----------------|----------------|------------------|
| Native          | 17 ± 3         | 42 ± 4           |
| R549G           | 7 ± 0.6        | <0.5             |
| R549H           | 29 ± 3         | 3.5 ± 0.4        |
| R549K           | 10 ± 2         | 38 ± 4           |

* Catalytic hydrolysis of PI with PI in dodecyl maltoside mixed micelles at 30 °C in 50 μl of 20 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl₂, and 0.5 mg/ml BSA, containing 44 μM of [3H]PI (10,000–15,000 cpm), 46 μM of PIP₂, 0.3 mM dodecyl maltoside. Reactions were carried at 30 °C for 2–15 min, terminated, and the [3H]IP were extract and separated as described (5).
Kinetic Analysis of PIP2 Hydrolysis Catalyzed by Native, R549H, and R549K Enzymes—It has been shown that the hydrolysis of PIP2 catalyzed by PI-PLC follows the dual phospholipid binding model of enzyme kinetics as described by Dennis and co-workers (23). According to this model (Equations 1a and 1b), 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 would allow PLC$_{d1}$ to catalyze the hydrolysis of numerous molecules of substrate during a single binding step to the micellar surface. Since the binding of a second substrate molecule and the subsequent hydrolysis occurs on the surface of mixed micelles, the apparent rate of catalysis depends on both the total concentration of the substrate as well as on the concentration of substrate on the surface of micelles (mole fraction). To further investigate the effect of the functional side chain at position 549 on the catalytic mechanism of PLC$_{d1}$, we used a PIP$_2$/dodecyl maltoside mixed micelles assay system previously described by Rebecchi and co-workers (22) to measure the rate of PIP$_2$ hydrolysis either as a function of mole fraction or as a function of total PIP$_2$ concentration. The data were fitted to Equation 2, and the effects of mutation of Arg-

determined using dodecyl maltoside/PIP$_2$ or PI mixed micelles as described under “Experimental Procedures,” except 50 mM Tris/HCl, pH 8.0, was used to buffer the reaction mixtures.

Correlated with its ability to catalyze the hydrolysis of PIP$_2$, PLC$_{d1}$ Substrate Recognition

FIG. 3. Effect of pH on the native and mutant PLC$_{d1}$ activities. Hydrolysis of 50 $\mu$M of PIP$_2$ in PIP$_2$/dodecyl maltoside mixed micelles as a function of pH values by native (●) enzyme and R549H (○) (A) or by R549K (□) (B). Hydrolysis of 50 $\mu$M of PI in PI/dodecyl maltoside mixed micelles by native (○) PLC$_{d1}$, R549K (□), or R549H (●) (C). PI- and PIP$_2$-hydrolyzing activities of the native and the mutant enzymes were correlated with its ability to catalyze the hydrolysis of PIP$_2$.
549 on $V_{\text{max}}$, $K_m$, and $K_s$ of the enzyme were examined. When the total PIP$_2$ concentration was increased at constant mole fraction by proportionally increasing both PIP$_2$ and the detergent, rates of PIP$_2$ hydrolyzed by the native, R549K, and R549H were highly dependent on the total concentration of PIP$_2$. As shown in Fig. 5, when the total concentration of PIP$_2$ in PIP$_2$/dodecyl maltoside mixed micelles (mole fraction = 0.01) was increased from 10 to 200 μM, the PIP$_2$-hydrolyzing activity of native and mutant R549K increased from 1.48 and 0.93 to 11.2 and 7.6 μmol/min/mg, respectively, whereas that of mutant R549H increased from 0.12 to 2.5 μmol/min/mg, much lower than those of the native and the mutant R549K enzymes. Fig. 6 shows the dependence of PIP$_2$-hydrolyzing activities of the native, R549H, and R549K mutant enzymes on the mole fraction of PIP$_2$ in the dodecyl maltoside mixed micelles when the total concentration of PIP$_2$ was held constant at 100 μM. As the mole fraction of PIP$_2$ was increased from 0.01 to 0.24, the rate of PIP$_2$ hydrolysis catalyzed by the native and mutant R549K enzymes increased from 12.3 and 9.2 μmol/min/mg to 84 and 63 μmol/min/mg, respectively. The rate of PIP$_2$ hydrolysis catalyzed by R549H mutant was also increased from 1.2 to 6.3 μmol/min/mg, but the values were lower than that of the native enzyme by a factor of 10. Computer fitting of the kinetic data obtained with the native and mutant enzymes to Equation 2 showed that enzyme catalysis of PIP$_2$ hydrolysis in the present system followed the prediction of the dual substrate binding model (Fig. 5 and Fig. 6). Compared with the derived kinetic parameters obtained with the native enzyme (Table III), it is evident that the effect of mutation of Arg-549 to histidine is primarily on the rate of PIP$_2$ hydrolysis. The $V_{\text{max}}$ of R549H was reduced by a factor of 15 (7.9 relative to 123 of the native enzyme in μmol/min/mg). This mutation had little effect on the micellar dissociation constant ($K_s$), which governs the association of the enzyme with the water-lipid interface of the mixed micelles. R549K whose positive charge was preserved displayed comparable kinetic parameters with those of native enzyme. The present results also show that Arg-549 plays a minimum role in hydrolyzing PI, as mutation of Arg-549 to histidine or lysine has minimal effect on the kinetic parameters $V_{\text{max}}$, $K_m$, or $K_s$.

**Calcium Dependence of the Mutant R549H Activity**—It has been shown that the PIP$_2$ hydrolysis activity of mammalian PI-PLC is highly dependent on the free calcium concentration (5, 18). In particular, a rapid increase in the PIP$_2$ hydrolysis activity catalyzed by PLCδ1 has been observed as the free calcium concentration increased from 0.1 to 5.6 μM. If there is
a shift in calcium ion dependence to the right by 1 order of magnitude in hydrolyzing PIP$_2$, then the ability to hydrolyze PIP$_2$ would be reduced at least by a factor of 10. To address these possibilities, we compared the dependence of the PIP$_2$ hydrolysis reaction on free calcium concentration. The specific activity of the native PLC81 has been found to be highly dependent on free calcium concentration. A similar dependence of PIP$_2$ hydrolytic activity on free calcium was also found for the R549H mutant enzyme, as shown in Fig. 7. The specific activity increased 27-fold as the free calcium concentration increased from 0.1 to 5.6 μM. This result demonstrates that the selective reduction in PIP$_2$ hydrolysis by changing Arg-549 to His is not caused by an altered Ca$^{2+}$ dependence of the catalysis reaction.

### Table III

| Type of enzyme | $V_{\text{max}}$ (μmol/min/mg) | $K_m$ (μM) | $K_i$ (μM) | $V_{\text{max}}$ (μmol/min/mg) | $K_m$ (μM) | $K_i$ (μM) |
|---------------|-------------------------------|-----------|-----------|-------------------------------|-----------|-----------|
| Native        | 123 ± 24                      | 0.050 ± 0.010 | 79 ± 30  | 157 ± 31                      | 0.20 ± 0.035 | 650 ± 23 |
| R549H         | 7.9 ± 1.4                     | 0.018 ± 0.003 | 83 ± 33  | 144 ± 30                      | 0.18 ± 0.033 | 568 ± 17 |
| R549K         | 102 ± 19                      | 0.063 ± 0.011 | 65 ± 27  | 98 ± 21                       | 0.22 ± 0.042 | 600 ± 19 |

$^a$ Hydrolysis of PIP$_2$ was measured in PIP$_2$/dodecyl maltoside mixed micelles as a function of mol fraction (total 100 μM of PIP$_2$) or as a function of total PIP$_2$ concentration when the mole fraction of PIP$_2$ was held at 0.01. The $V_{\text{max}}$, $K_m$, and $K_i$ correspond to the constants defined in the dual substrate binding model (Equations 1 and 2); values of $V_{\text{max}}$, $K_m$, and $K_i$ were calculated by fitting the data to Equation 2.

$^b$ PI hydrolysis was measured in PI/dodecyl maltoside as a function of total PI concentration, with fixed total concentration of dodecyl maltoside of 300 μM. The $V_{\text{max}}$, $K_m$, and $K_i$ correspond to the constants defined in the dual substrate binding model (Equations 1 and 3); values of $V_{\text{max}}$, $K_m$, and $K_i$ were calculated by fitting the data to Equation 3.

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**Fig. 7. Ca$^{2+}$ dependence of R549H mutant PLC81.** The PIP$_2$ hydrolytic activity of the native (●) and R549H mutant (○) enzymes was determined according to Hepler et al. (5), and the free calcium concentrations were calculated according to Fabiato and Fabiato (36). The values are expressed relative to the activity at free Ca$^{2+}$ concentration of 95 mM.

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2. J. W. Lomasney, H.-F. Cheng, L.-P. Wang, Y.-S. Kunn, S.-M. Liu, S. W. Pesik, and K. King, manuscript in preparation.
The concentration of PIP2 in inhibition of the native and mutant PLC
Dose-dependent binding of PE/PC/PIP2 (4:1:0.25) lipid vesicles to the
hydrolysis by this mutant enzyme. The concentration of PIP2 (μM) was a fraction of phospholipid vesicle containing PE/PC/PIP2 with a molar ratio 4:1:0.25. All centrifugation assays were carried out in 0.2 ml of total volume using a Beckman TL-100 tabletop ultracentrifuge and TLA-100 rotor (see "Experimental Procedures"). The unbound enzyme fractions (supernatant) were quantified by a PI hydrolysis assay and immunoblotting using mixed monoclonal antibodies. The bound enzyme fractions (pellets) were dissolved in 0.05 ml of phosphate-buffered saline and then quantified by Western blotting analysis.

Two independent observations indicated that the positive charge at position 549 may be involved in binding PIP2 to the catalytic site of PLCδ1. First, the present result shows that removing the positive charge at position 549 by Arg-549 → Gly mutation in PLCδ1 renders the enzyme insensitive to the inhibitory effect of PIP2. Although R549H exhibits 5–10% residual PI-hydrolyzing activity at pH 7.0, the mutant enzyme was inactive when the pH was raised to 8.0, presumably due to deprotonation of the imidazole side chain of histidine 549 at pH 8.0. To test the possibility that the positive charge at position 549 in PLCδ1 is required for binding PIP2, we further investigated the pH dependence of PIP2 inhibition of the native and mutant R549H to hydrolyze PI. If the positive charge is involved in binding PIP2, deprotonation of the imidazole side chain of histidine 549 at pH 8.0 at high pH would reduce its affinity for PIP2. As shown in Fig. 10B, similar to the results obtained at pH 7.0, the rate of PI hydrolysis by the native enzyme and R549H was initially increased and then decreased as the concentration of PIP2 was raised; the activity was reduced to 20% by 100 μM PIP2. Although R549H was also slightly stimulated by PIP2, increasing PIP2 concentration was not able to inhibit R549H to catalyze the hydrolysis of PI. Instead PI hydrolysis by R549H at pH 8.0 was higher than the original level even in the presence of 100 μM PIP2. In fact PIP2 slightly activates R549H to catalyze the hydrolysis of PI. The inhibition of PI hydrolysis by R549H by PIP2 at pH 8.0 within the present range of concentrations. In summary the present results show that PIP2 competitively inhibits the native enzyme to catalyze the hydrolysis...
of PI at both pH 7 and 8. In contrast, PIP2 stimulates R549G to catalyze the hydrolysis of PI at both pH values. On the other hand, the effect of PIP2 on R549H is dependent on pH, and PIP2 is inhibitory at pH 7 and slightly stimulatory at pH 8.0.

**DISCUSSION**

To explore the function of highly conserved Y region residues in eucaryotic PI-PLC, we mutated the conserved Arg residues in this region of human PLCδ1. Cleavage assays of the purified enzyme revealed that mutation of Arg at position 527 to Gly has little effect on the catalytic activity. Replacing the Arg residues at position 556 and 701 with Gly caused a parallel reduction in both PI- and PIP2-hydrolyzing activities of the enzyme, whereas substitution of arginine 549 with glycine results in a mutant enzyme selectively defective in hydrolyzing PIP2. This result is consistent with the finding that positively charged side chains at position 549, 556, and 701 are invariant in PI-PLC from eucaryotic sources, whereas that of Arg-527 is highly conserved but not invariant (28).

In the eucaryotic PLCδ1 catalysis, the simplest interpretation of the present data is that the positive charge at position 549 is essential for hydrolyzing PIP2.

When Arg-549 is replaced with glycine, the enzyme loses its ability to bind PIP2 via its catalytic site, whereas maintaining the positive charge, as in R549K, preserves PIP2 binding to the catalytic site. A plausible interpretation is that the positive charge at position 549 is essential for the specific binding of PIP2. This is consistent with results on the pH-dependent inhibition of mutant R549H by PIP2. Although PIP2 inhibits R549H to hydrolyze PI in a manner similar to that of native enzyme at pH 7.0, PIP2 is not able to inhibit R549H at pH 8.0, presumably due to deprotonation of the 549 imidazole side chain with increasing pH. This pH-dependent loss of PIP2 binding to the catalytic site of R549H mutant may explain why R549H mutant is inactive in hydrolyzing PIP2 at pH 8.0 but retains 5–10% residual activity between pH 5.5 and 7. Since this positive charge has minimal effect on the hydrolysis of PI, these analyses also provide evidence that the positive charge at position 549 is essential for the binding of PIP2 but not PI.

Which structural differences between the PIP2 and the PI molecules would cause the enzyme to discriminate against PIP2 as a substrate when Arg-549 is changed to Gly or His? Since the diacylglycerol moeity is probably not involved in the recognition by the enzyme (29), the obvious and important features lacking in the PI molecules are the vicinal phosphates at the 4 and 5 positions of the inositol ring in the PIP2 molecule. To accommodate the phosphate groups of PIP2 into the catalytic site, positively charged residues in the cleavage center may be required to form salt bridges with the negatively charged phosphate group in PIP2 or both would dramatically diminish the ability of the enzyme to recognize PIP2 but not PI.

The important role of the positive charge at position 549 in recognizing PIP2 by PLCδ1 is further supported by the observation that preserving the positive charge at position 549 by changing Arg-549 to lysine has minimal effect on the ability of the enzyme to catalyze the hydrolysis of PIP2, whereas abolishing the positive charge by mutating Arg-549 to glycine or by deprotonating the side chain of His-549 in R549H results in a loss of PIP2 binding. This interpretation is consistent with the recent report of a 2.4 Å resolution crystal structure of rat PLCδ1 (30), showing Arg-549 at the bottom of the catalytic site.
with its positively charged guanidinium group forming a salt bridge with the negatively charged phosphate at position 4 and the 3'-hydroxyl group of the inositol ring.

When the dual substrate binding model was used to analyze the kinetic properties of the native and the mutant enzymes, it was found that R549H mutant is defective primarily in the rate of PIP₂ hydrolysis at saturated substrate mole fraction and infinite substrate concentration (V_max). The mutation had minimal effect on the ability of PLCδ₁ to anchor itself on the surface of the mixed micelles. This observation is consistent with the findings that the interaction of enzyme with PIP₂ via the noncatalytic site of PLCδ₁ was not affected by mutation of Arg-549 to histidine or to glycine. The positive charge is important for the V_max of PLCδ₁, because we are not able to detect PIP₂-hydrolyzing activity by R549G or by R549H at pH 8.0, whereas there is little effect on the value of V_max when the positive charge of Arg-549 is preserved in R549K.

Although the positive charge at position 549 is required to bind PIP₂ and to hydrolyze PIP₂, it is not sufficient for PLCδ₁ to catalyze the hydrolysis of PIP₂. This is evident by the fact that the native and mutant R549H can bind to PIP₂ via the catalytic site in a similar manner at pH 7 (Fig. 10), but the V_max of PIP₂ hydrolysis is reduced by a factor of 15 compared with that of the native enzyme, indicating that binding is essential but not sufficient for PLCδ₁ to hydrolyze PIP₂. If the activity reduction of a mutant enzyme is simply due to a decrease in substrate affinity, increasing the concentration of substrate should restore the activity of the mutant enzyme. However, increasing the concentration and mole fraction of PIP₂ did not increase PIP₂-hydrolyzing activity of R549H to a level compa-

**FIG. 11.** PIP₂ inhibits PI hydrolysis competitively in P/IP/PIP₂/dodecyl maltoside mixed micelles. Hydrolysis of increasing concentrations of PI in dodecyl maltoside in the presence of 50 μM PIP₂ (●) or PG catalyzed by the native PLCδ₁ (○) (A, pH 7.0 or C, pH 8.0) or by R549H mutant enzyme (B, pH 7.0 or D, pH 8.0). Reaction (50 μl) contains indicated concentrations of PI and 600 μM dodecyl maltoside, 50 μM PIP₂, or 50 μM PG in 50 mM HEPES, pH 7.0 or 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl₂, 0.5 mg/ml BSA, and 0.1–10 ng of enzyme. After incubation at 30°C for 2–15 min, reactions were stopped, and the [³H]IPs were separated and quantitated as described by Hofmann and Majerus (21).
rable with that of native enzyme. These analyses suggest that factors other than the physical binding of PIP$_2$ are also required for PIP$_2$ cleavage. In particular, if both R549 and H549 are positively charged at pH 7.0, the key differences between arginine and histidine are the length of their side chains and their solvation properties. Although the latter may not be so critical for ground state substrate binding, these factors may be required to stabilize the transition state optimally for PIP$_2$ cleavage.

Specific recognition is one of the fundamental functions of the molecular interaction in biological systems. Specificity often allows biologically active molecules to discriminate between stereoisomers and between substrates that differ by a single functional group, etc. Several single point mutant forms of PI-PLC defective in cleavage activity have been used to correlate the phosphatidylinositide breakdown and subsequent cellular physiological responses (31, 32). By analogy, similar experiments using mutant PI-PLC selectively defective in the ability to catalyze the hydrolysis of PIP$_2$ can be performed to analyze the physiological consequences related to the order and sequence of phosphatidylinositol turnover by PI-PLC. For example, the major cellular event in phosphatidylinositol turnover upon agonist stimulation is breakdown of PIP$_2$, but direct hydrolysis of PI and PIP allowing the separate regulation of the production of inositol 1,4,5-trisphosphate and diacylglycerol has been also reported in a number of systems (33–35). Arg-549 in PLC$_b1$ is invariant in all eucaryotic PI-PL. Thus, studying mutant enzymes like R549H cannot only help to better understand the molecular mechanism of how different phosphorylated forms of inositol phospholipids are recognized by the enzyme but may also provide a useful tool with which to analyze the PI-PLC-mediated signal transduction cascade. Replacing similar residues in other isoforms should also cause the enzyme to be selectively defective in PIP$_2$ but not in PI hydrolysis. Thus, this kind of mutant enzyme can provide a useful tool for dissecting PI-PLC-mediated signal transduction in vivo.

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