Identification of Multiple Phosphoinositide-specific Phospholipases D as New Regulatory Enzymes for Phosphatidylinositol 3,4,5-Trisphosphate*

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Tsui-Ting Ching, Da-Sheng Wang, Ao-Lin Hsu, Pei-Jung Lu, and Ching-Shih Chen‡

From the Division of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082

In the course of delineating the regulatory mechanism underlying phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) metabolism, we have discovered three distinct phosphoinositide-specific phospholipase D (PI-PLD) isozymes from rat brain, tentatively designated as PI-PLDα, PI-PLDβ, and PI-PLDγ. These enzymes convert [3H]PI(3,4,5)P3 to generate a novel inositol phosphate, [3H]Ins(3,4,5)P3, and phosphatidic acid. These isozymes are predominantly associated with the cytosol, a notable difference from phosphatidylinositol PLDs. They are partially purified by a three-step procedure consisting of DEAE, heparin, and Sephacryl S-200 chromatography. PI-PLDα and PI-PLDβ display a high degree of substrate specificity for PI(3,4,5)P3, with a relative potency of PI(3,4,5)P3  >> phosphatidylinositol 3-phosphate (PI(3)P) or phosphatidylinositol 4-phosphate (PI(4)P) > phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) > phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2). In contrast, PI-PLDγ preferentially utilizes PI(3)P as substrate, followed by, in sequence, PI(3,4,5)P3, PI(4)P, PI(3,4)P2, and PI(4,5)P2. Both PI(3,4)P2 and PI(4,5)P2 are poor substrates for all three isozymes, indicating that the regulatory mechanisms underlying these phosphoinositides are different from that of PI(3,4,5)P3. None of these enzymes reacts with phosphatidylycholine, phosphatidylserine, or phosphatidylethanolamine. All three PI-PLDs are Ca2+-dependent. Among them, PI-PLDβ and PI-PLDγ show maximum activities within a sub-μM range (0.3 and 0.9 μM Ca2+, respectively), whereas PI-PLDα exhibits an optimal [Ca2+] of 20 μM. In contrast to PC-PLD, Mg2+ has no significant effect on the enzyme activity. All three enzymes require sodium deoxycholate for optimal activities; other detergents examined including Triton X-100 and Nonidet P-40 are, however, inhibitory. In addition, PI(4,5)P2 stimulates these isozymes in a dose-dependent manner. Enhancement in the enzyme activity is noted only when the molar ratio of PI(4,5)P2 to PI(3,4,5)P3 is between 1:1 and 2:1.

PI(3,4,5)P3 and PI(3,4)P2 are produced by PI 3-kinase in response to a wide array of external stimuli (1, 2). These two phosphoinositides and their downstream effector Akt constitute the key component of a major signaling pathway that acts both to stimulate cell growth and to prevent apoptosis (3–5). In view of their physiological importance, the metabolism of these lipid second messengers has been the focus of many recent investigations. Evidence indicates that they are not susceptible to hydrolysis by any known phospholipase C (6) and that different types of phosphatases mediate the major degradative pathway via dephosphorylation. For example, there exist multiple inositol polyphosphate 5-phosphatases that transform PI(3,4,5)P3 to PI(3,4)P2, through which the ratio of these two lipid second messengers is controlled. These enzymes include PI(3,4,5)P3 5-phosphatases (7, 8), SHIP (SH2-containing inositol 5-phosphatase), (9–11) or SIP (signaling inositol polyphosphate 5-phosphatase) (12), and synaptojanin (13). Especially noteworthy is the discovery that the PTEN tumor suppressor displays a PI(3,4,5)P3 3-phosphatase activity (14), thus terminating the second messenger activities of PI(3,4,5)P3 by converting it to PI(4,5)P2. This finding provides an intricate link between PI(3,4,5)P3 regulation and tumorigenesis (15). Tumor cells with mutant forms of PTEN lack such an off-switch mechanism for PI 3-kinase, thereby containing high levels of PI(3,4,5)P3 and PI(3,4)P2 and high endogenous Akt activity (16, 17). Loss of PTEN function has been found in a variety of common human cancers, including breast, prostate, and brain cancer (18), and may attribute to the inability of cancer cells to undergo apoptosis (17).

In an effort to gain insight into the complex machinery that regulates PI(3,4,5)P3, we have synthesized [3H]PI(3,4,5)P3 to examine its metabolite fate in rat brain extracts. Here we report the identification of three distinct cytosolic PI-PLD isozymes that convert [3H]PI(3,4,5)P3 to a novel inositol phosphate [3H]Ins(3,4,5)P3 and PA. The present data raise a possibility that these PI-PLDs act as a regulator of PI(3,4,5)P3 in vivo. This premise connotes physiological implications conforming to that of the PTEN tumor suppressor. Furthermore, because PA is generated, these isozymes may provide a putative link between PI 3-kinase and other signaling pathways mediated by PA or its metabolites.
For D2, fractions 125–139 (designated as D2/H) were pooled and concentrated by ultrafiltration.

**Step 3. Sephacryl S-200-HR Chromatography—**The concentrated D1/H (4.5 mg; 2.3 nmol of PIP2/min specific activity), D1/H2 (2.3 mg; 9.1 nmol of PIP2/min specific activity), and D2/H (3.84 mg; 2.6 nmol of PIP2/min specific activity) from step 2 were loaded individually onto a Sepharose S-200-HR column (1 × 100 cm). The equilibration and eluting buffer was buffer A, and fractions of 2 ml were collected (see Fig. 3, A–C). The collected fractions were: the D1/H1 column, fractions 76–79 (designated as D1/H1/S or PI-PLDa; 0.18 mg; 13.7 nmol of PIP2/min specific activity); the D1/H2 column, fractions 71–78 (designated as D1/H2/S or PI-PLDb; 0.077 mg; 0.0072 mmol of PIP2/min specific activity); and the D2/H column, fractions 71–77 (designated as D2/H2/S or PI-PLDc; 0.24 mg; 39.8 nmol PIP2/min specific activity).

**Identification and Quantitation of the Radiolabeled Inositol Phosphates by HPLC**

For the substrate specificity study, the identity and quantity of the radioactive phosphoinositides liberated by the enzymatic hydrolysis of [3H]Ins(1,3,4,5)P4, [3H]Ins(1,4,5)P3, and [3H]Ins(3,4,5)P3 were determined by HPLC. The enzyme incubation, under the same conditions as described above, was quenched by adding 100 μl of HCO3−/CH3CN ([v/v] 10.33) followed by 100 μl of 10 mg/ml bovine serum albumin. The mixture was centrifuged at 13,000 × g for 10 min. The supernatant was collected and was extracted immediately with 300 μl of tri-n-octylamine/Freon ([v/v] 1:1) twice to remove HCO3−. The neutralized supernatant was filtered and transferred to the reaction vial, and the phosphoinositide radiolabeled phosphate was analyzed by HPLC on an Adsorbosphere Sph column (5 μm; 4.6 × 200 mm) equilibrated with H2O. The phosphoinositol was eluted with a linear gradient of 0–0.9 M NH4H2PO4 in 60 min at a flow rate of 1 ml/min. Fractions were collected every 1 ml, and their radioactivity was measured by liquid scintillation. Synthetic [3H]Ins(1,3,4,5)P4, [3H]Ins(1,4,5)P3, [3H]Ins(3,4,5)P3, and [3H]Ins(3,4,5)P3 were used as standards. The respective retention times were 60, 50, 43, 41, and 39, respectively.

**Stoichiometric Formation of Ins(3,4,5)P3 and PA**

A mixture of [1-3H]PIP2 (3,4,5)P3 (15 μM; 20,000 cpn) and [palmitoyl-1-14C]PIP2 (3,4,5)P3 (117 μM; 35,000 cpn) was exposed to fraction D1/H2/S (PI-PLDb), in a final volume of 200 μl, in the presence of 0.3 μM Ca2+. Reaction was stopped at the indicated times 1, 5, 20, 40, and 60 min by adding the mixture with 300 μl of HCO3−/CH3CN ([v/v] 10.33). A brief centrifugation, the two phases were separated. The aqueous phase was treated as described above for the HPLC analysis of Ins(3,4,5)P3. The organic phase was transferred to a new vial and dried by a stream of N2. The residue was dissolved in CHCl3, spotted onto 1% oxalic acid-treated TLC plate, and developed with n-propyl alcohol and 2 M acetic acid (15:7) overnight. After drying, spots were located by autoradiography and compared with standards. The autoradiograms were scanned by a photodyne image system. The spots corresponding to [3H]CIP and [3H]CIP(3,4,5)P3 were scraped off the plate, and the associated radioactivity was measured by liquid scintillation.

**Periodate Oxidation of [1-3H]Ins(3,4,5)P3**

The HPLC-purified [1-3H]Ins(3,4,5)P3 with a total radioactivity of approximately 20,000 cpn was oxidized with 0.1 M periodic acid, pH 2.0, and reduced with 1 M NaBH4 as described (21). Such treatments would oxidize inositol phosphates at vicinal hydroxyls. It was found that the resulting polyphosphate lost all radioactivity, indicating the adjacent 1-, 2-, and 6-hydroxyls were unsubstituted.

**RESULTS**

To investigate the metabolic fate of D-3 phosphoinositides, [1-3H]PIP2 (3,4,5)P3 was synthesized and exposed to the cell lysate of rat brain. The incubation mixture was extracted with CHCl3/CH3OH to isolate the phosphoinositide metabolites. However, substantial radioactivity appeared in the aqueous phase in a time- and protein concentration-dependent manner, suggesting that the “3H”-labeled head group was decomposed from [1-3H]PIP2 (3,4,5)P3 via phopholipase hydrolysis. HPLC analysis of the aqueous fraction revealed that the liberated radioactivity was associated with free inositol and trace amounts of inositol mono- and bisphosphates (data not shown). This result showed that the inositol phosphate generated from PIP2 (3,4,5)P3 was rapidly metabolized in the crude extract. Other rat tissues
examined including the liver, the kidney, and platelets also contained such PI(3,4,5)P3-metabolizing activity.

As part of our effort to verify the identity of the PI(3,4,5)P3-metabolizing enzyme(s), purified phospholipase preparations from different sources were examined for the activity toward [1-3H]PI(3,4,5)P3. These included porcine pancreas PLA2, recombinant PLC-γ1, recombinant PLC-δ1, B. cerus PI-specific PLC, recombinant PLD1, and cabbage PLD. However, none of these enzymes showed appreciable hydrolysis of [1-3H]PI(3,4,5)P3. With regard to enzyme inhibition, neomycin (1 mM), which binds phosphoinositides with high affinity (22), completely blocked the hydrolysis by rat brain extracts. Other known phospholipase inhibitors such as aristolochic acid for PLA2, ET-18-OCH3 for PI-PLC, and dihydroerythro-sphingosine for PLD gave no or only partial inhibition of the enzyme activity even at concentrations 20 times over the corresponding IC50 values (data not shown).

These data prompted us to identify the enzyme(s) responsible for [3H]PI(3,4,5)P3 hydrolysis. Subcellular fractionation indicated that more than 85% of the [3H]PI(3,4,5)P3-metabolizing activity resided in the cytosolic fraction (100,000 × g supernatant). The rest of the activity was associated with various subcellular fractions, represented by 0.3 and 20 μM Ca2+ requirements, PI(3,4,5)P3-metabolizing enzyme(s), purified phospholipase preparations from different sources were examined for the activity toward [1-3H]PI(3,4,5)P3. As shown, fraction D1 gave two well resolved activity peaks between fractions 150 and 210 (Fig. 2A). Among these active fractions, the enzyme activity of the first peak increased with higher [Ca2+] (0.3 μM, closed triangles; 20 μM, open squares), whereas the activity of the second peak was unaffected by Ca2+ change. Accordingly, fractions 145–160 and 177–192 were pooled separately and were designated as D1/H1 and D1/H2, respectively. Similarly, two PI(3,4,5)P3-metabolizing activity peaks were also noted for fraction D2 after the heparin column (Fig. 2B; 0.3 μM, closed triangles; 20 μM, open squares). In contrast, the first peak exhibited a higher activity at low Ca2+ compared with high Ca2+, whereas the second, smaller peak showed no significant difference in the Ca2+ requirement. Considering that fractions D1 and D2 were juxtaposed in the DEAE elution profile, it was possible that the second peak from fraction D2 was identical to fraction D1/H2 in light of their Ca2+ requirement. The resulting active fractions were designated as D1/H1/S, D1/H2/S, and D2/H/S for further discussion. This three-step procedure resulted in 354-, 1,056- and 1,027-fold purification with specific activities of 13.7, 40.9, and 39.8 nmol of PI(3,4,5)P3/mg of protein/min ([Ca2+] = 2 μM) for fractions D1/H1/S, D1/H2/S, and D2/H/S, respectively. Concerning PI(3,4,5)P3-metabolizing activity, fraction D2/H/S was highly unstable. Up to 80% of the enzyme activity was lost after storing at 0 °C for 2 days.
The Ca\(^{2+}\) dependence of these enzyme preparations was examined. Fig. 4 indicates that the PI(3,4,5)P\(_3\)-metabolizing activity of these enzymes was inhibited by EDTA, and the inhibition could be overcome by adding Ca\(^{2+}\) in a concentration-dependent manner. Both D1/H2/S and D2/H/S showed a similar Ca\(^{2+}\) requirement, with maximum PI(3,4,5)P\(_3\)-metabolizing activities at a sub-\(\mu\)M range (0.3 and 0.9 \(\mu\)M, respectively), whereas D1/H1/S displayed an optimal [Ca\(^{2+}\)] at 0.3 \(\mu\)M. The difference in the Ca\(^{2+}\) requirement underscored a potential distinction in the roles of these enzymes in PI(3,4,5)P\(_3\) metabolism. Moreover, Mg\(^{2+}\) had no appreciable effect by itself on the activity of or on the Ca\(^{2+}\) dependence for any of these enzymes (data not shown).

In an effort to gain insight into the catalytic behaviors of these enzyme preparations, we also synthesized [1-\(^3\)H]PI(3,4,5)P\(_3\), [1-\(^3\)H]PI(3,4)P\(_2\), [1-\(^3\)H]PI(4,5)P\(_2\), [1-\(^3\)H]PI(3)P, and [1-\(^3\)H]PI(4)P for examinations. These phospholipids were exposed to individual enzymes, and the released water-soluble products were analyzed by reverse-phase HPLC, aiming at both product identification and substrate specificity determination. Representative HPLC profiles of the [1-\(^3\)H]phosphoinositol products from incubations of the respective substrates with fraction D1/H2/S are shown in Fig. 5.

These HPLC profiles revealed two important findings. First, D1/H2/S displayed a high degree of substrate specificity for PI(3,4,5)P\(_3\). The relative potency for the substrates examined was PI(3,4,5)P\(_3\) >> PI(4)P > PI(3)P >> PI(4,5)P\(_2\) and PI(3,4)P\(_2\). The utilization of the latter two, especially PI(3,4)P\(_2\), accounted for less than 5% of that of PI(3,4,5)P\(_3\) (Fig. 6, panel A). These HPLC results were consistent with those obtained by measuring [\(^3\)H]phosphoinositol release from the respective substrates by liquid scintillation.
Second, the retention times of the hydrolysis products from \[^{13}H\]PI(3,4,5)P_3, \[^{13}H\]PI(3,4)P_2, \[^{13}H\]PI(4,5)P_2, \[^{13}H\]PI(3)P, and \[^{13}H\]PI(4)P coincided with those of \[^{3}H\]Ins(3,4,5)P_3, \[^{3}H\]Ins(3,4)P_2, \[^{3}H\]Ins(4,5)P_2, \[^{3}H\]Ins(3)P, and \[^{3}H\]Ins(4)P, respectively. These data clearly indicated that D1/H2/S displayed a PLD activity. In principle, should it be a PLC, the respective degradative products would have been \[^{3}H\]Ins(1,3,4,5)P_4, \[^{3}H\]Ins(1,3,4)P_3, \[^{3}H\]Ins(1,4,5)P_3, \[^{3}H\]Ins(1,3)P_2, and \[^{3}H\]Ins(1,4)P_2, of which the retention times would differ from the respective experimental data by almost 10 min because of an additional phosphate moiety in the PLC products. Moreover, it is worthy to note that after periodate oxidation/NaBH₄ reduction, the radioactivity associated with the hydrolysis product of PI(3,4,5)P_3 was completely lost, confirming that the adjacent 1-, 2-, and 6-hydroxyls were unsubstituted (data not shown).

Additional evidence that the responsible enzyme was a PLD was obtained from the stochiometry of product formation. A mixture of palmitoyl-[²⁵⁴C(U)]PI(3,4,5)P_3 and [¹-³H]PI(3,4,5)P_3 was exposed to fraction D1/H2/S, and the formation of [³H]PA and [³H]Ins(3,4,5)P_3 was analyzed by TLC and HPLC, respectively, at different time intervals (Fig. 7). The amounts of [³H]Ins(3,4,5)P_3 formed at the indicated times were consistent with those of [³H]PA.

Similar analytical data from HPLC and TLC were obtained with fractions D1/H1/S and D2/H/S, confirming that these two enzymes were also of the type of PLD. However, their substrate specificities were slightly different from that of D1/H2/S (Fig. 6). For further discussion, D1/H1/S, D1/H2/S, and D2/H/S were tentatively designated as PI-PLDa, PI-PLDb, and PI-PLDc. As shown, PI-PLDa (Fig. 6, panel B) and PI-PLDb (panel A) displayed high degree of specificity for PI(3,4,5)P_3. The activities toward other substrates examined accounted for 1% (PI(3,4)P_2) to 30% (PI(3)P or PI(4)P) of that of PI(3,4,5)P_3. In contrast, PI-PLDc preferentially hydrolyzed PI(3)P (relative activity, 100%), followed by, in sequence, PI(3,4,5)P_3 (60%), PI(4)P (37%), PI(3,4)P_2 (12%), and PI(4,5)P_2 (3%). Evidence showed that none of these PI-PLDs utilized PC, PS, or PE as substrates. First, prolonged exposure of N-[methyl-³H]PC to any of these isozymes did not give rise to appreciable release of radioactivity into the milieu. Second, the rate of [³H]PI(3,4,5)P_3 hydrolysis was not affected by the addition of excess amounts of PE or PS to the incubation mixture.

The effect of detergents on the PI-PLD activity was investigated. In all of the aforementioned assays, the reaction mixture contained 0.8 mM sodium deoxycholate. Removal of the detergent or replacement with 0.1–1% Nonidet P-40 or Triton X-100 resulted in substantial loss of enzyme activity for all three isozymes, indicating the stringent requirement of sodium deoxycholate for PI-PLD activity. This dependence might be attributable to the effect of detergent on PI(3,4,5)P_3 packaging in lipid vesicles, which affected the substrate availability and/or enzyme accessibility.

Earlier studies have shown that PC-PLDs were strongly stimulated by PI(4,5)P_2 and PI(3,4,5)P_3 with an equal potency (23, 24). Fig. 8 depicts the effect of PI(4,5)P_2 on the PI(3,4,5)P_3-metabolizing activity of three PI-PLD isozymes. The individual enzymes were incubated with [³H]PI(3,4,5)P_3 in the presence of increasing amounts of PI(4,5)P_2 without sodium deoxycholate. As shown, PI(4,5)P_2 enhanced the basal enzyme activity up to 2.5-fold. However, this stimulating effect occurred only within a narrow range of PI(4,5)P_2/PI(3,4,5)P_3 molar ratios between 1:1 and 2:1. Excess amounts of PI(4,5)P_2 either inhibited or had no effect on the PI(3,4,5)P_3-metabolizing activity. It is plausible that because PI(4,5)P_2 was a poor substrate, it might compete with PI(3,4,5)P_3 for enzyme binding, thereby counteracting its stimulating effect.

**DISCUSSION**

PI 3-kinase activation leads to a transient accumulation of PI(3,4,5)P_3 and PI(3,4)P_2, of which the concentrations rise from 0.05–0.2 μM at resting states to 1–2 μM upon agonist stimulation (25). The prevailing levels of these PI 3-kinase lipid products are regulated by a delicate balance between its rates of synthesis and metabolism. This study presents the first evidence that there exist at least three distinct cytosolic Ca²⁺-dependent PLD isozymes that may take part in PI(3,4,5)P_3 regulation in vivo. Taken together, the metabolism of PI(3,4,5)P_3...
activity toward \([3H]\)phosphatidylinositol has been detected in enzymes, however, have not met with success. Moreover, PLD reported in the literature (32–34). Attempts to purify these PLD activities with varying substrate preference have been predominantly membrane-associated enzymes (31), PI-PLD trast to mammalian PC-PLDs (PLD1 and PLD2) which are subtypes in different subcellular compartments of mammalian PI-PLDs.

Among these products, PI(3,4)P2 and PI(4,5)P2, are poor substrates for all three PI-PLD isozymes to form Ins(3,4,5)P3. Among these products, PI(3,4)P2 stimulation was noted only within a narrow dose-dependent manner. However, PI(4,5)P2 stimulation was noted only within a narrow molar ratio range. It is unclear whether this stimulatory effect is mediated by direct enzyme activation or by affecting the PI(3,4,5)P3 packaging in lipid vesicles, which remains to be investigated.

The biological utility of PI-PLD may be multifaceted. As demonstrated by the tumorigenic consequence of PTEN mutations, modifications to any of these PI(3,4,5)P3-metabolizing activities may also lead to pathological conditions. In light of the formation of PA, PI-PLD may provide a putative link between D-3 phosphoinositides and signaling pathways mediated by these PLD products. The physiological function of PC-PLD has been largely attributed to the rapid and transient increases in PA (27–30). Evidence indicates that PA and its metabolites such as diacylglycerol and lysophosphatidic acid serve as regulators of key cellular processes including vesicle trafficking and other membrane-associated events. The premise that PI-PLD may contribute to transient PA increase in a manner distinct from that of PC-PLD suggests a potential mechanism for cross-communication between PI 3-kinase and PA-dependent processes. With regard to Ins(3,4,5)P3, it is a novel inositol phosphate whose physiological function remains unknown. Recent evidence indicates that its structural analog Ins(3,4,5,6)P4 is a potent activator of Ca2⁺-dependent chloride channels (36).

In summary, this study provides definite evidence that there exist at least three cytosolic PI-PLD isozymes. Although these isozymes need further characterization, the present data suggest that they represent new regulators of PI(3,4,5)P3 in vivo. It is conceivable that additional regulating and membrane localization mechanisms are involved in the regulation of these PI-PLD activities, which is currently under investigation.

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