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New Aspects of Formation of 1,2-Cyclic Phosphates by Phospholipase C-δ1

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Abstract—Phosphoinositide-specific phospholipase C-δ1 (PI-PLC-δ1) cleaves phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2, **1**), 5-phosphate (PI-5-P, **2**) and 4-phosphate (PI-4-P, **3**) to form the mixture of the corresponding 4,5-, 5- and 4-phosphorylated inositol 1,2-cyclic phosphate (IcP) and 1-phosphate (IP) (**4**–**6** and **7**–**9**, respectively). In this work, we have studied the rates of the cleavage and the ratios of the cyclic-to-acyclic phosphate products under various pH and Ca^{2+} concentration conditions using ^{31}P NMR to monitor the reactions. In agreement with the previous report (Kim et al. *Biochim. Biophys. Acta* **1989**, *163*, 177), our results indicate that the IcP/IP ratios strongly depend on the reaction conditions, with the cyclic phosphate products formed predominantly at low pH (pH 5.0) and high calcium concentration (5 mM). Surprisingly, however, we have found that at pH 8.0 and 5 mM Ca^{2+}, PI-5-P rather than PI-4,5-P_2 is the most preferred substrate with the highest V_{max}. The cleavage of PI-5-P generated also more cyclic phosphate product than the other two substrates. In addition, we have studied the analogous reaction of phosphorothioate analogues of **1** with the sulfur placed in the nonbridging (**10**) or bridging (**13**) positions. We have found that the phosphorothioate analogue **10** produced exclusively the cyclic product **11**, whereas the analogue **13** afforded exlusively the acyclic product **7**. These results are discussed in terms of the mechanism of PI-PLC, where the cyclic product is formed by 'leaking' from the active site before its subsequent hydrolysis. The potential significance of the cyclic products in the signaling pathways is also discussed.

Hydrolysis of inositol phospholipids by phosphoinositide-specific phospholipases C producing inositol 1,4,5-trisphosphate and diacylglycerol second messengers is one of the most important steps in the overall cellular signaling.[1–4] Both the functional[4–6] and mechanistic aspects[1–3,5,7] of this reaction have been thoroughly investigated in the past, and have been subject to numerous original reports and reviews. Mechanistic studies performed so far indicate that PI-PLC-catalyzed cleavage of phosphatidylinositol 4,5-bisphosphate (**1**) results first in formation of the cyclic intermediate, inositol 1,2-cyclic-4,5-trisphosphate (**4**, Scheme 1), which is next hydrolyzed to afford inositol 1,4,5-trisphosphate (IP_3, **7**) second messenger.[8–10] In most PI-PLC catalyzed reactions, formation of small amounts of the cyclic phosphate accompanies generation of IP_3, and the cyclic phosphate can be detected in the cells with vigorous phosphoinositide turnover.[11–15] In contrast to IP_3, however, this cyclic intermediate has not received much attention, predominantly because it has been considered a mere by-product of the second-messenger generation, and because its cellular levels are usually low and its turnover is slow. In our view, the functional significance of the cyclic inositol phosphate could be greater than the current state of knowledge might indicate. For example, in certain cells, the cyclic phosphate is the major product of the PIPn cleavage.[11–13] In addition, several of the putative inositol-containing second messengers, including inositolphosphoglycans[16] and prostaglandin–inositol conjugate[17] do contain the 1,2-cyclic phosphate residue. The latter molecule is biosynthesized from inositol 1,2-cyclic-4-bisphosphate **6** or the corresponding 5-phosphate **5**,[17] both most likely the products of PI-PLC reaction. These results raise the possibility that the cyclic inositol phosphates could have an important physiological function, although it has yet to be firmly established.

Despite much work in the area of PI-PLC mechanism, certain aspects of formation of the cyclic inositol

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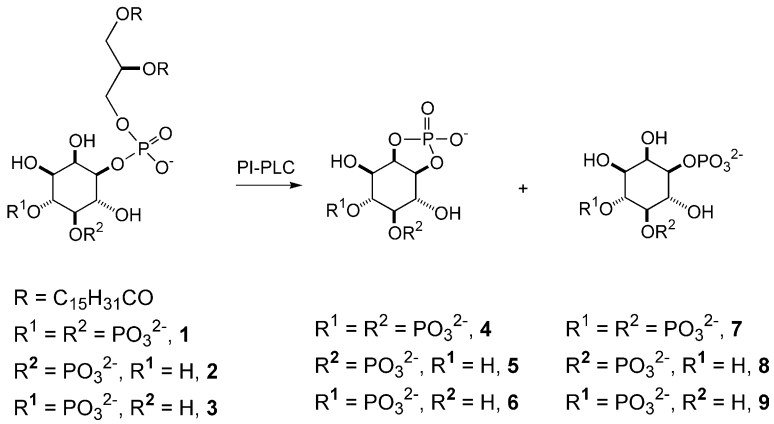


Scheme 1.

phosphates remain unclear. It is generally accepted that the first chemical step of PI cleavage (k_2, Fig. 1) produces IcP-intermediate; this step is followed by the hydrolysis (k_3) to form the acyclic phosphate product.[1–3,7–10] The IcP-intermediate can, however, also 'leak out' from the active site (k_4) prior to its hydrolysis giving the IcP product. The ratio of IcP to IP products is then decided by the magnitude of the partition ratio (k_4/k_3). In an apparent contradiction to this mechanism, the direct hydrolysis of the cyclic trisphosphate **4** by PI-PLC could not be achieved. This observation is in contrast to the behavior of the bacterial PI-PLC for which the cyclic phosphodiesterase activity is well documented.[1,18,19] Only recently, Wu et al.[9] reported hydrolysis of IcP by PI-PLC-δ1 with very low activity, however, the analogous cyclic phosphodiesterase activity with the more interesting polyphosphorylated inositol 1,2-cyclic phosphate such as **4**–**6**, has not been reported so far. The low cyclic phosphodiesterase activity in the absence of the phospholipid substrate could be due to several factors: (i) the nonhydrophobic cyclic phosphate product is unable to trigger the conformational change of PI-PLC necessary to obtain active enzyme;[9,10] (ii) the protonation status of the general acid and general base in the active site suitable for IcP hydrolysis could only be achieved after the first cyclization step. Diffusion of IcP from the active site could enable enzyme re-protonation to the state suitable for catalysis of the first step only. In either case, the diffusion of the cyclic intermediate from the active site leaves the enzyme in the kinetically incompetent state with regard to catalysis of the second step. If the conformational change is responsible, it is unclear which of the two processes: 'leakage' of IcP or the conformational change is the cause and result. Slowing down the leakage process, for example by providing additional binding interaction to the enzyme such as in PI-4-P and PI-4,5-P_2 as compared to PI,[20,21] should result in smaller amount of the cyclic phosphate product. Consistently, the ratios of IcP to IP were found to decrease in the order PI > PI-4-P > PI-4,5-P_2.[14] In this work, we provide further mechanistic evidence supporting the 'leakage' mechanism by alteration of cIP/IP ratios through selective variations of the k_2 and k_3 steps versus k_4. Although PI-PLC activity was determined with the most abundant, naturally occurring phosphatidylinositols such as PI, PI-4-P and PI-4,5-P_2, the cleavage of the recently discovered phosphatidylinositol 5-phosphate (**2**)[22] has not yet been investigated. This potential substrate is of interest since the crystallographic structure of PI-PLC complex with inositol 1,4,5-trisphosphate indicates different modes of enzyme interactions with the 4-phosphate and 5-phosphate groups.[20,21] The results of the current work indicate a different substrate behavior of PI-5-P from those of other phosphatidylinositols.

Results and Discussion

The dispersions of phosphatidylinositol 4,5-bisphosphate, 5-phosphate and 4-phosphate and dihexanoylphosphatidylcholine (1:3 molar ratio) have been treated with PI-PLC-δ1 under four different conditions: (i) pH 8.0 (Tris buffer), 5 mM Ca^{2+}; (ii) pH 8.0, 50 μM Ca^{2+}; (iii) pH 5.0 (acetate buffer), 5 mM Ca^{2+}; and (iv) pH 5.0, 50 μM Ca^{2+}. The concentration of substrates was 6 mM, and the solutions contained 0.5 mM and 10 μM EDTA in high and low calcium buffers, respectively. The reactions were followed by ^{31}P NMR allowing

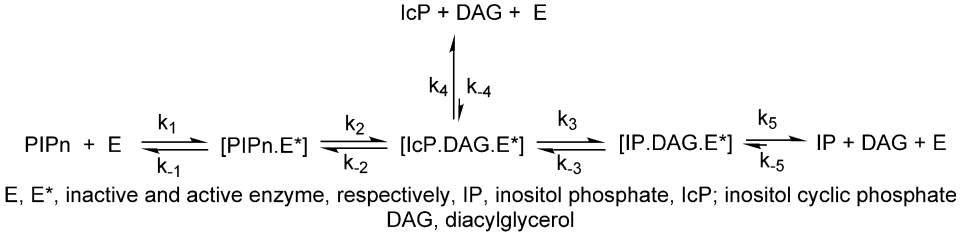


Figure 1. Simplified kinetic scheme of the PI-PLC reaction. The physical steps of enzyme binding to a lipid–water interface are omitted for simplicity.

simultaneous determination of V_{max} values and the IcP/IP ratio, and the results are listed in Table 1.

The inspection of Table 1 indicates that at pH 8.0 and 50 μM Ca^{2+}, DPPI-4,5-P_2 (**1**) was the best substrate, as expected, and both DPPI-5-P (**2**) and DPPI-4-P (**3**) had several-fold lower activities. The reactions at pH 5.0 and 50 μM Ca^{2+} were too slow to give reliable kinetic data using ^{31}P NMR assay, and are omitted from Table 1. Highly unexpectedly, PI-PLC had twice as high activity with DPPI-5-P than with DPPI-4,5-P_2 at 5 mM calcium concentration. In addition, DPPI-5-P consistently produced more cyclic phosphate than the other two substrates, under any conditions that we tested. This result is significant since PI-PLC-δ1 has several calcium binding sites,[9,10,20,21] including the catalytic and calmodulin-like EF-hands domains, and could be regulated by the calcium levels.[23] Thus, the origin of the high activity with DPPI-5-P at high Ca^{2+} concentration could be due to saturation of these multiple binding sites and their influence on the interactions of the 5-phosphate group with the catalytic domain. These interactions could make the active site more open facilitating the 'leakage' process.

Following the complete disappearance of the phospholipid substrate at pH 5.0 (giving the highest quantities of the cyclic phosphate products), the pH of the reaction mixtures was adjusted to 8.0 (to attain higher activity), additional amounts of enzyme were added, and the hydrolyses of the cyclic product to the acyclic phosphates were followed by ^{31}P NMR. The results are shown in Figures 2 and 3. The hydrolysis of the cyclic trisphosphate **4** and bisphosphate **5** was observed with the estimated initial rates of 0.25 μmol mg^{-1} min^{-1} and 0.53 μmol/mg^{-1} min^{-1}, respectively, some 150 and 300 times slower than those of the cleavage of the corresponding phospholipids. Incidentally, these rates of hydrolysis are also similar to that of inositol 1,2-cyclic phosphate reported earlier.[9] The difference in phosphotransferase and cyclic phosphodiesterase activities of PI-PLC-δ1 appears also to be in the similar range (ca. 100) as that reported for bacterial PI-PLC.[7,18] The reactions could be brought into completion only after several days, most likely due to combination of several effects such as product inhibition and loss of enzyme activity over the extended period of time. In the control experiments, the cyclic phosphates appeared to be completely stable within several weeks at pH 5.0, therefore the observed hydrolysis of the cyclic phosphate is due to enzyme activity and not due to acid-catalyzed chemical hydrolysis. The above result indicates that the cyclic phosphate is the substrate of PI-PLC, although in the absence of the hydrophobic group in the substrate the catalysis is severely impaired. The hydrolysis of 1:2-cyclic-4-phosphate **6** was not observed to any significant extent. Likewise, no enzymatic hydrolysis of any cyclic phosphate was observed at pH 5.0.

Table 1. Specific activities of PI-PLC-δ1 and IcP/IP ratios under various conditions

Substrate/conditions	DPPI-4-P		DPPI-5-P		DPPI-4,5-P_2	
	V_{max}	IcP/IP	V_{max}	IcP/IP	V_{max}	IcP/IP
pH 8.0, 50 μM Ca^{2+}	16.9	0.055	17.4	0.21	82.0	0.071
pH 8.0, 5 mM Ca^{2+}	10.9	0.029	153.3	0.167	38.5	0.037
pH 5.0, 5 mM Ca^{2+}	3.9	0.77	7.3	1.18	13.8	0.85

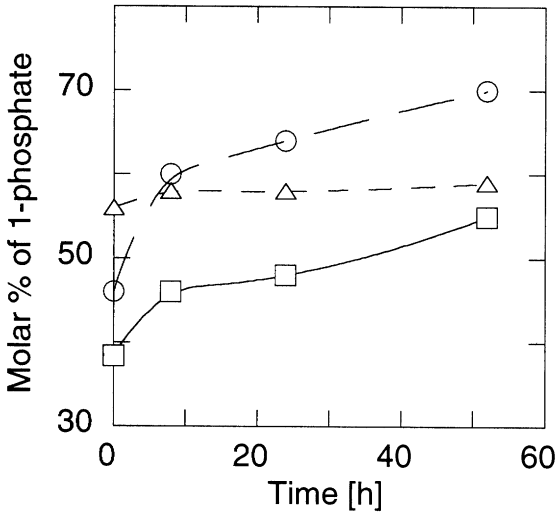


Figure 2. Time course of PI-PLC-catalyzed hydrolysis of 1:2-cyclic phosphates derived from phospholipids **1**–**3**. □, hydrolysis of IcP-4,5-P_2; ○, hydrolysis of IcP-5-P, △, hydrolysis of IcP-4-P.

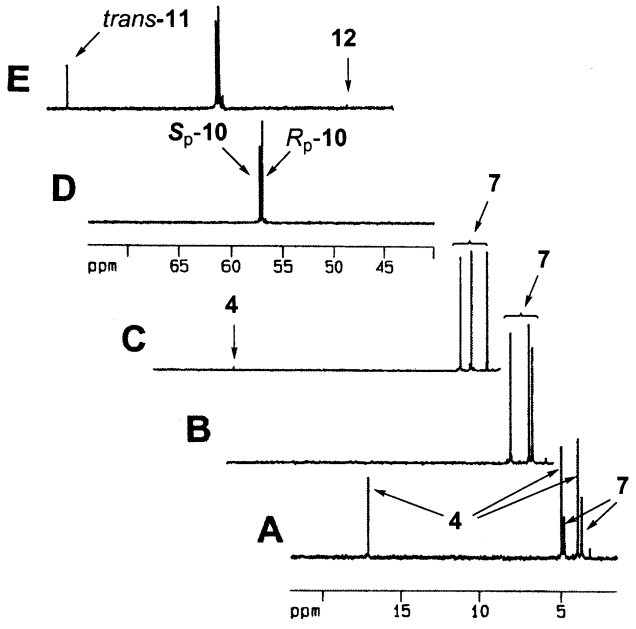


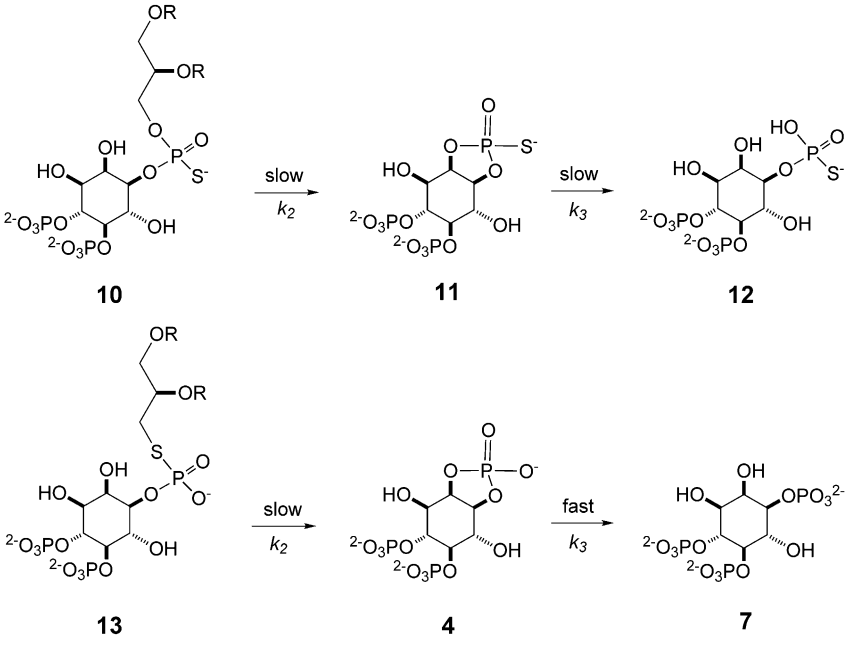
Figure 3. The time courses of PI-PLC reactions monitored by ^{31}P NMR. The spectra A–C and D–E feature the same horizontal expansion as those indicated by the corresponding chemical shift axes: (A) cleavage of DPPI-4,5-P_2 (**1**) at pH 5.0 and 5 mM Ca^{2+} showing formation of product **4** and **7** at 1:1 ratio (by integrated intensity); (B) the above reaction mixture 96 h after adjusting pH to 8.0 and adding an additional amount of enzyme showing complete conversion of compound **4** into **7**; (C) cleavage of the substrate **13** by PI-PLC-δ1 showing almost exclusive formation of **7**; (D) the phosphorothioate region of the spectrum of the substrate **10** prior to adding PI-PLC, showing the presence of *R*p and *S*p-diastereomers The phosphate region of the spectrum is not shown; (E) product of the cleavage of the analogue **10** by PI-PLC at pH 8.0. Note, only diastereomer (*R*p) is cleaved by the enzyme to produce a single product *trans*-**11**.

As we have noted above, the reduction in k_3 relative to k_4 (Fig. 1) should result in generation of more cyclic phosphate. We have tested this hypotheses using the phosphorothioate analogue of PI-4,5-P_2 with the sulfur placed in the nonbridging position (**10**) (Scheme 2). Our earlier work on the magnitude of thio-effects in the reactions catalyzed by bacterial PI-PLC indicated that oxygen-sulfur substitution causes reduction of the turnover rates due to impaired enzyme catalysis.[26,27] With analogue **10** as substrate both steps k_2 and k_3 should be slower (as both the substrate and the cyclic intermediate contain sulfur) as compared to the natural substrate, whereas the rate of the physical step, the diffusion of the intermediate from the active site (k_4) is not expected to be significantly affected by the phosphorothioate modification. As a result, the partition ratio k_4/k_3 should be higher, and hence the cleavage of **10** should produce more cyclic phosphate. As shown in Figure 3, the results are fully consistent with our working hypothesis. The treatment of **10** with PI-PLC-δ1 at pH 8.0 and 50 μM Ca^{2+} (the conditions which produced very little cyclic phosphate with the substrate **1**) afforded almost exclusively the cyclic product **11** (δ 72 ppm); no formation of the acyclic product **12** with chemical shift at ca. 45 ppm was observed. The reaction was ca. 10^3-times slower than that of **1**. We also expect that cleavage of **1** by any low activity PI-PLC mutant should also produce IcP as an only product, however, despite extensive site-directed mutagenesis studies of PI-PLC-δ1, the type of product produced by mutants was not reported.[28] In contrast, the cleavage of the analogue **13**, for which only the k_2 step is expected to be slower than that of **1**, and both k_3 and k_4 should remain unchanged (since the intermediate is the same as that in the cleavage of **1**), afforded solely the acyclic product **7**, even under the conditions that produced most cyclic product with the substrate **1**. The interpretation of this result is unclear at the moment. It is possible that the fact that the enzyme produces the cyclic intermediate **4** at a lower rate (diminished k_2) could cause its more efficient conversion to the acyclic phosphate thus minimizing the 'leakage' process.

In summary, in this work we have shown that the minor membrane constituent PI-5-P could be a more preferred substrate than the generally accepted PI-4,5-P_2 under high calcium concentration. While Ca^{2+} concentration at which this preference is observed is significantly higher than the physiological levels, it is possible that Ca^{2+} fluxes through the plasma membrane could generate gradients in the cell with high local transient concentrations. In addition, it is also possible that the activity increase with PI-5-P that we observe as a result of high calcium could be achieved in the natural environment due to interactions of the enzyme with other factors. More work is currently underway to explore such possibilities. PI-5-P produces much more of the cyclic product than any other inositol phospholipid. This result is important in view of the recent finding that inositol 1:2-cyclic-phospho-5-bisphosphate is incorporated into biosynthesis of prostaglandylinositol cyclic phosphate.[29] Finally, by selective manipulation of various rate constants in the kinetic scheme of PI-PLC we have provided additional evidence in support of the 'leakage' hypothesis to explain formation of the cyclic product by PI-PLC.

Experimental

Synthetic dipalmitoylphosphatidylinositol 4,5-bisphosphate (**1**), 5-phosphate (**2**), 4-phosphate (**3**) and 1-(1,2-diplamitoyl-*sn*-3-glycerophosphothio)-*myo*-inositol-4,5-bisphosphate (**10**) were synthesized as described.[24,25] Synthesis of 1-(1,2-dihexanoyloxypropane-3-thiophospho)-*myo*-inositol-4,5-bisphosphate (**13**) PI-PLC-δ1 will be described elsewhere. ^{31}P NMR assays of PI-PLC



Scheme 2.

were performed on Bruker DPX-360 NMR spectrometer operating at 145.78 MHz frequency using 5400 Hz sweep width, 9.5 ms pulse width, 0.7 s acquisition time and 0.3 s recycle delay time. Spectra were processed using 1 Hz line broadening, the signals were integrated and their intensities were normalized using the diC_6PC signal as the internal standard. Measurements were performed in duplicate and the obtained rate constants were averaged. The obtained kinetic data were processed using GraFit software vsn. 4.0 to obtained initial rates and V_{max} values.

The appropriate amount of phosphatidylinositol phosphate (3–4 μmol) was added with 100 μL of the detergent stock solution containing 100 mM diC_6PC in 2 mL D_2O and 100 μL of buffer solution (buffer A: pH 8.0, 100 mM Tris–HCl, 25 mM $CaCl_2$, 2.5 mM EDTA; buffer B: pH 5.0, 100 mM sodium acetate, 25 mM $CaCl_2$, 2.5 mM EDTA; buffer C: pH 8.0, 100 mM Tris–HCl, 250 μmol $CaCl_2$, 50 μmol EDTA; buffer D: pH 5.0, 100 mM sodium acetate, 250 μmol $CaCl_2$, 50 μmol EDTA and 300 μL of D_2O). The samples were homogenized by sonication for 5 min at room temperature; clear sample were obtained at low calcium and slightly opaque samples were obtained at high calcium concentration. It is assumed that the resulting high PIPn concentrations (5–10 mM) ensure saturation kinetics. The reference ^{31}P NMR spectrum was obtained before the aliquot of PI-PLC-δ1 was added, the reactions were monitored by recording ^{31}P NMR spectra at several hours or days intervals as necessary, and the data were quantitated as described above.

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