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Inositol cyclic phosphates are produced by cleavage of phosphatidylphosphoinositols (polyphosphoinositides) with purified sheep seminal vesicle phospholipase C enzymes

(phosphatidylinositol phosphodiesterase/^{18}O labeling/mass spectrometry)

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ABSTRACT **Previous studies have shown that metabolism of phosphatidylinositol by phospholipase C produces a mixture of two water-soluble products: inositol 1-phosphate and inositol 1,2-(cyclic)phosphate. In the present study, we demonstrate that the water-soluble products of phosphatidylphosphoinositol (polyphosphoinositide) cleavage by purified ram seminal vesicle phospholipase C enzymes also contain cyclic phosphates. Inositol cyclic phosphates were detected by ^{18}O labeling. In the presence of acid, cyclic phosphates are rapidly hydrolyzed to phosphomonoesters, and when the hydrolysis is carried out in $H_2{}^{18}O$, the resultant phosphomonoesters will contain ^{18}O. The ^{18}O content of the phosphomonoesters was measured following alkaline phosphatase treatment and conversion of the inorganic phosphate to a volatile derivative for gas chromatography/mass spectrometry. Inositol cyclic phosphates were found in the phospholipase C cleavage products of all three phosphoinositides, but the ratio of cyclic to noncyclic product was found to decrease in the order phosphatidylinositol > phosphatidylinositol 4-phosphate > phosphatidylinositol 4,5-bisphosphate. The formation of *myo*-inositol 1,2(cyclic)-4-bisphosphate was further substantiated by anion-exchange HPLC of the water-soluble products of [^{32}P]phosphatidylinositol 4-phosphate metabolism by phospholipase C. Two peaks were detected, one of which, on acid treatment, incorporated ^{18}O from $H_2{}^{18}O$ into phosphate groups, consistent with this peak containing the cyclic phosphate product. These results suggest that polyphosphoinositide breakdown in stimulated cells may occur via a cyclic phosphate intermediate, as has been described for phosphatidylinositol. These cyclic phosphates contain a reactive bond that may play a role in phosphoinositide-derived signal transduction.**

Stimulation of platelets and many other cell types with agonists results in the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol (PtdIns) and the polyphosphoinositides phosphatidylinositol 4-phosphate (PtdIns-4-*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) (for reviews, see refs. 1–3). Agonist-induced phosphoinositide degradation produces a variety of cellular messenger molecules. Phosphoinositide-derived diacylglycerol can serve to activate protein kinase C (4) and can be further metabolized by diacylglycerol and monoacylglycerol lipase activities to yield free arachidonic acid for icosanoid synthesis (5, 6). Inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3), the product of PtdIns-4,5-P_2 hydrolysis by PLC, has been implicated as a Ca^{2+}-mobilizing agent in a number of experimental systems (7–16).

Dawson *et al.* (17) have shown that the product of PtdIns metabolism by PLC consists of a mixture of inositol 1-phosphate (Ins-1-*P*) and inositol 1,2-(cyclic)phosphate (Ins>*P*). These investigators concluded that during the PLC reaction either the 2-hydroxyl of inositol or a free hydroxide ion can attack the phosphorus atom of PtdIns, leading to the cyclic and noncyclic product, respectively. By analogy, the formation of a 1,2-cyclic phosphate might be anticipated in the PLC cleavage products of PtdIns-4-*P* and PtdIns-4,5-P_2 (see Fig. 1). Recently, it was shown that two purified sheep seminal vesicle PLC enzymes, PLC-I and PLC-II, are capable of hydrolyzing all three of the phosphoinositides (18, 19). The products of metabolism of PtdIns by these enzymes include both Ins>*P* and Ins-1-*P* (18). In the present study, we employ ^{18}O labeling of phosphate to show that cyclic phosphate esters are present in the water-soluble products of PtdIns-4-*P* and PtdIns-4,5-P_2 metabolism by the purified seminal vesicle PLC enzymes.

MATERIALS AND METHODS

Materials. Phospholipids, inositol-2-phosphate (Ins-2-*P*), and *Escherichia coli* alkaline phosphatase (ammonium sulfate suspension) were purchased from Sigma. The cyclohexylamine salt of Ins>*P*, prepared by the method of Pizer and Ballou (20), was supplied by Merck Sharp & Dohme. $H_2{}^{18}O$ (>95% enriched) was obtained from Monsanto/Mound, Miamisburg, OH. [^{32}P]Orthophosphoric acid was purchased from New England Nuclear. Inositol 1,4-bis[^{32}P]phosphate, inositol 1,4,5-tris[^{32}P]phosphate, and their unlabeled counterparts were prepared from erythrocyte membranes by the method of Downes *et al.* (21). [^{32}P]PtdIns-4-*P* and [^{32}P]PtdIns-4,5-P_2 were isolated from labeled erythrocytes (22) by chromatography on neomycin-glass beads (23, 24). Ram seminal vesicle PLC-I and PLC-II were purified as described (18, 19). These two antigenically distinct enzymes have similar substrate specificities and Ca^{2+} dependence but differ greatly in tissue distribution (18, 19). For example, the majority of PLC activity in liver is due to PLC-I, whereas PLC-II makes up most of the activity in platelets and brain. PLC-II was dialyzed against 50 mM Hepes, pH 7.0/100 mM NaCl prior to use to remove free phosphate. Small unilamellar phospholipid vesicles containing phosphoinositide

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Abbreviations: PtdIns, phosphatidylinositol; PtdIns-4-*P*, phosphatidylinositol 4-phosphate; PtdIns-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; Ins-1-*P*, *myo*-inositol 1-phosphate; Ins-1,4-P_2, *myo*-inositol 1,4-bisphosphate; Ins-1,4,5-P_3, *myo*-inositol 1,4,5-trisphosphate; Ins>*P*, *myo*-inositol 1,2(cyclic)phosphate; Ins(>*P*)*P*, inositol 1,2(cyclic)-4-bisphosphate; Ins(>*P*)P_2, inositol 1,2(cyclic)-4,5-trisphosphate. Phosphoinositides include PtdIns, PtdIns-4-*P*, and PtdIns-4,5-P_2; polyphosphoinositides include PtdIns-4-*P* and PtdIns-4,5-P_2.

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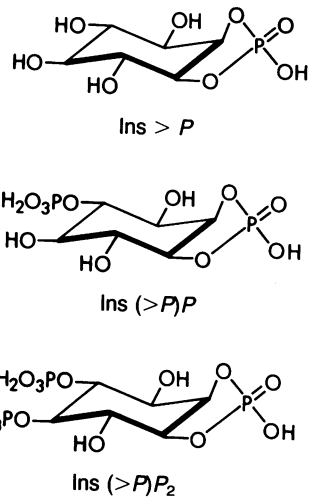

FIG. 1. Structures of inositol 1,2-(cyclic)phosphate (Ins>*P*) inositol 1,2(cyclic)-4-bisphosphate [Ins(>*P*)*P*], and inositol 1,2(cyclic)-4,5-trisphosphate [Ins(>*P*)P_2].

(PtdIns, PtdIns-4-*P*, or PtdIns-4,5-P_2) and phosphatidylethanolamine (1:0.4, mol/mol) were prepared as described (25).

^{18}O Incorporation into Cyclic Phosphates. PLC reaction mixtures contained phosphoinositide/phosphatidylethanolamine vesicles (300 μM phosphoinositide), 10 mM Tris acetate (pH 5.5), 20 mM NaCl, 0.75 mM $CaCl_2$ and 300 ng of PLC-I or PLC-II in a total volume of 100 μl. Reaction mixtures were incubated for 10 min at 37°C in Eppendorf tubes. To measure inositol cyclic phosphate content, the PLC incubations were terminated by the simultaneous addition of $H_2{}^{18}O$ (to a final concentration of 43%) and HCl (to a final concentration of 1 M). After a 1-min incubation at room temperature, the acidified reaction mixtures were frozen in liquid N_2 and then lyophilized. To control for acid-catalyzed exchange of $H_2{}^{18}O$ into phosphomonoesters, parallel PLC incubations were treated with 1 M HCl for 1 min at room temperature; $H_2{}^{18}O$ containing 1 M HCl then was added to a final concentration of 43% $H_2{}^{18}O$. After 1 min at room temperature, the acidified reaction mixtures were frozen in liquid N_2 and lyophilized. The lyophilized material from test or control samples was taken up in 0.5 ml of H_2O, transferred to glass tubes, and extracted by the addition of 1 ml of chloroform/methanol (1:1, vol/vol). The upper, aqueous phases of the extraction mixtures were lyophilized and then dissolved in 300 μl of 80 mM $NaHCO_3$. Phosphomonoesters were converted to inorganic phosphate by addition of 3.5 μg of *E. coli* alkaline phosphatase and incubation at 37°C for 10 min. The reaction mixtures were frozen and lyophilized in Reactivials (Pierce).

Derivatization of Inorganic Phosphate. Derivatization reagent was prepared by mixing 1 volume of *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide containing 1% *N*-methyl-*N*-(*t*-butyldimethyl)chlorosilane (Regis Chemical, Morton Grove, IL) with 2 volumes of dimethylformamide (Regis). A 300-μl portion of this reagent was added to the lyophilized products of the alkaline phosphatase reaction, and the samples were left overnight at room temperature, without stirring, before analysis.

Gas Chromatography/Mass Spectrometry. Mass spectral analysis of the derivatized phosphates was performed on a Finnigan model 3200 electron-ionization gas chromatograph/mass spectrometer equipped with a 1.3 m × 4 mm column of 3% OV-17 on Chromosorb W-HP 80/100 mesh (Ohio Valley Specialty, Marietta, OH). Instrument conditions were as follows: GC column, 180°C isothermal with helium carrier gas at 30 ml/min; injector, 200°C; separator, 250°C; ionization potential, 70 eV. To estimate recoveries of inorganic phosphate obtained from hydrolysis of phosphorylated inositols, a standard curve was constructed for derivatized $(NH_4)_2HPO_4$. Values obtained from the standard curve were compared to values obtained with phosphorous assays on the phosphorylated inositols (26).

HPLC of Inositol Phosphates. Phosphorylated inositols were resolved on an Alltech/Applied Science (Deerfield, IL) Econosphere-NH_2 anion-exchange column (25 cm × 4.6 mm, 5-μm). The elution scheme consisted of isocratic elution with 75 mM ammonium formate (pH 4.3) for 10 min, followed by a linear gradient from 75 mM to 300 mM ammonium formate (pH 4.3) over the next 10 min and then isocratic elution with 300 mM ammonium formate (pH 4.3) for 15 min. The flow rate was 1 ml/min. Under these conditions the following retention times were obtained: Ins-1-*P*, 4 min; Ins-2-*P*, 5 min; Ins>*P*, 3 min; Ins-1,4-P_2, 21 min; Ins-1,4,5-P_3, 26 min.

Other Methods. Trimethylsilyl (Me_3Si) derivatives of Ins>*P*, Ins-1-*P*, and Ins-2-*P* were prepared by adding 50 μl of a 1:1 mixture of pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide/10% (vol/vol) trimethylchlorosilane (Pierce) to dry samples. The reaction mixtures were left overnight at room temperature and resolved by gas chromatography at 190°C on OV-17, as described by Leavitt and Sherman (27).

RESULTS

We have employed ^{18}O labeling of phosphate to detect cyclic phosphate esters in the products of phosphoinositide cleavage by PLC. In the presence of acid, cyclic phosphates are rapidly hydrolyzed to phosphomonoesters. Incubation of Ins>*P* in 1 M HCl for 1 min at room temperature resulted in a quantitative conversion of the cyclic compound to a mixture of Ins-1-*P* (85%) and Ins-2-*P* (15%) as documented by both anion-exchange HPLC of these compounds and by gas chromatography of the Me_3Si derivatives (data not shown). When acid-catalyzed hydrolysis of a cyclic phosphate is carried out in the presence of $H_2{}^{18}O$, the resultant phosphomonoester contains a single atom of ^{18}O. After removal of unreacted $H_2{}^{18}O$ by lyophilization, ^{18}O enrichment of phosphate groups can then be measured by treatment of the inositol phosphomonoesters with alkaline phosphatase, followed by conversion of the inorganic phosphate to the volatile *t*-butyldimethylsilyl (tBuMe$_2$Si) derivatives for GC/MS. Mawhinney (28) has shown that tBuMe$_2$Si derivatives of oxyanions have properties that make them suitable for GC/MS. However, Mawhinney observed that the sodium salts of oxyanions, including phosphate, were poorly derivatized by $CF_3C(O)N(Me)(t$BuMe$_2$Si) in dimethylformamide. We found that the sodium salt of inorganic phosphate was quantitatively converted to the tBuMe$_2$Si derivative when incubated overnight as described in *Materials and Methods*. The mass spectrum of the tBuMe$_2$Si derivative of phosphate is shown in Fig. 2. The base-peak fragment ion at m/z 383 results from the loss of a single *t*-butyl group from the molecular ion (28). The ^{18}O enrichment of phosphate groups was measured by using selected-ion monitoring in which the ratio of the m/z 385 peak height to the m/z 383 peak height (385/383 ratio) was monitored. In the absence of enrichment, the experimental value for the 385/383 ratio was 0.1553 ± 0.0005 (n = 10) and was invariant from experiment to experiment. This value mainly reflects the natural abundance of high-mass isotopes of silicon in the tBuMe$_2$Si derivatives. Therefore, 385/383 ratios greater than 0.1553 are a measure of the ^{18}O enrichment.

Selected-ion monitoring traces from a typical ^{18}O-enrichment experiment in which the products of PtdIns hydrolysis by PLC were treated two different ways are shown in Fig. 3. For the analysis shown in Fig. 3*A*, the products of the reaction were treated for 1 min with 1 M HCl in the presence

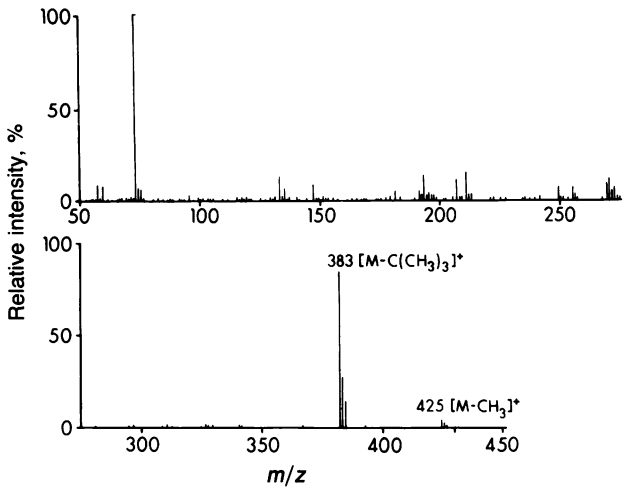

FIG. 2. Mass spectrum of tris(*t*BuMe$_2$Si)phosphate. The base peak at *m/z* 383 arises from elimination of a single *t*-butyl group from the parent ion. The peak at *m/z* 425 arises from elimination of a single methyl group.

of $H_2{}^{18}O$ (conditions that convert cyclic inositol phosphates to noncyclic inositol phosphates). The phosphate groups were then removed and derivatized. For the analysis shown in Fig. 3*B*, the products of the PLC reaction were treated for 1 min with 1 M HCl in the absence of $H_2{}^{18}O$; $H_2{}^{18}O$ containing 1 M HCl was then added and the incubation was continued for an additional minute. This control ensured that acid hydrolysis of the cyclic ester was complete in 1 min and that there was no acid-catalyzed exchange of ^{18}O into phosphomonoesters during this period. An increase in the *m/z* 385 peak and a decrease in the *m/z* 383 peak were observed when the acid hydrolysis was carried out in $H_2{}^{18}O$ rather than $H_2{}^{16}O$ (Fig. 3 *A* versus *B*). The ratio of peak heights (385/383) in Fig. 3*A* is 0.3201, whereas in Fig. 3*B* it is 0.1562, essentially the same as unenriched phosphate. When chemically synthesized Ins>*P* (of 60% purity, the balance being Ins-1-*P* and Ins-2-*P*, as determined by GC and ^{31}P NMR) was treated the same way as the products of the PtdIns reaction, ratios of 0.3602 for the test sample and 0.1562 for the control were obtained.

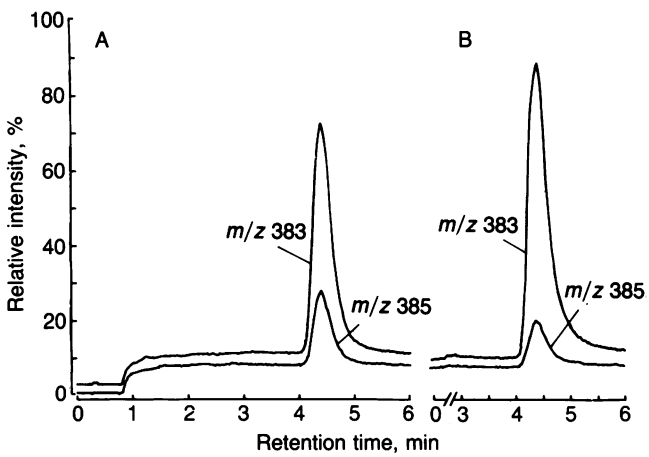

FIG. 3. GC/MS of the tris(*t*BuMe$_2$Si)phosphate from a typical ^{18}O-enrichment experiment. The products of PtdIns hydrolysis by PLC-I were treated with HCl and $H_2{}^{18}O$ for 1 min (*A*) or HCl and $H_2{}^{16}O$ for 1 min followed by HCl and $H_2{}^{18}O$ for an additional minute (*B*). After lyophilization, phosphate groups were cleaved from the inositol ring with alkaline phosphatase and converted to the *t*BuMe$_2$Si derivatives. Gas chromatography with selected-ion monitoring of *m/z* 383 and *m/z* 385 was performed as described in *Materials and Methods*.

An experiment showing the detection of cyclic phosphates in the water-soluble products of PLC-I cleavage of PtdIns, PtdIns-4-*P*, and PtdIns-4,5-P_2 is presented in Table 1. For each of the substrates, parallel reaction mixtures were treated in the following three ways prior to alkaline phosphatase treatment and derivatization: (*Procedure 1*) The PLC reaction occurred in the presence of $H_2{}^{18}O$, and the sample then was treated with 1 M HCl for 1 min. Whether the product of the enzyme reaction is cyclic or noncyclic, the 1-phosphate group will contain a single atom of ^{18}O. This 385/383 ratio is defined as 100% enrichment. (*Procedure 2*) The PLC reaction occurred in $H_2{}^{16}O$ and the sample then was treated for 1 min with acid in the presence of $H_2{}^{18}O$. The percentage of phosphorylated inositols with 1,2-cyclic phosphates can be calculated by comparing this 385/383 ratio with the ratio obtained by procedure 1. (*Procedure 3*) The PLC reaction occurred in $H_2{}^{16}O$ and the sample then was treated with acid for 1 min, followed by the addition of $H_2{}^{18}O$ for 1 min. This control is identical to that described in Fig. 3*B*.

A number of observations can be made about the data in Table 1. Proceeding from PtdIns to PtdIns-4-*P* to PtdIns-4,5-P_2, one observes a decrease in the 385/383 ratio for the PLC reactions that occurred in $H_2{}^{18}O$. The ratios for the polyphosphoinositide-derived products are probably reduced by dilution with unlabeled inorganic phosphate released from positions 4 and 5 by alkaline phosphatase.

The products of PLC-mediated hydrolysis of all three phosphoinositides contained cyclic phosphates, but the percentage of phosphorylated inositols with cyclic phosphates differs for the three substrates. The ratio of cyclic to noncyclic product decreases in the order PtdIns > PtdIns-4-*P* > PtdIns-4,5-P_2. The control reactions for each of the three substrates gave ratios near that of unenriched phosphate (i.e., 0.1553), indicating that there is no acid-catalyzed exchange of ^{18}O into phosphomonoesters under the conditions used. When Ins-1,4-P_2 and Ins-1,4,5-P_3 (prepared from erythrocyte ghosts) and Ins-2-*P* were tested for ^{18}O incorporation, ratios similar to that of unenriched phosphate were obtained. Selected-ion monitoring of *m/z* 387 indicated that only one ^{18}O atom was incorporated into the cyclic phosphates during acid hydrolysis (data not shown).

The cyclic phosphate content in the products of PLC-I- and PLC-II-mediated cleavage of the phosphoinositides was measured in several additional experiments. These results are summarized in Table 2. Similar results were obtained with PLC-I and PLC-II: inositol cyclic phosphates were present in the products of PLC-I and PLC-II cleavage of all three substrates, but the ratio of cyclic to noncyclic product decreased in the order PtdIns > PtdIns-4-*P* > PtdIns-4,5-P_2.

To verify that Ins(>*P*)*P* was formed by the action of PLC on PtdIns-4-*P*, the water-soluble products of [^{32}P]PtdIns-4-*P* cleavage by PLC-I were subjected to anion-exchange HPLC. As shown in Fig. 4*B*, two peaks were present in the chromatogram of the reaction products. Neither of these peaks was formed in the absence of PLC (Fig. 4*D*). The second of the two peaks coincided with that of Ins-1,4-P_2 prepared from erythrocyte ghosts (Fig. 4*A*), and the first peak was converted into the second by acid treatment (Fig. 4*C*). The fractions containing products (Fig. 4*B*) were treated with acidified $H_2{}^{18}O$ under the conditions described in Table 1. As shown in Fig. 4*B*, the first peak incorporated ^{18}O into phosphate moieties, suggesting that this peak contained cyclic phosphate.

DISCUSSION

Inositol cyclic phosphates have been identified in the products of PtdIns cleavage by mammalian (17) and bacterial (29, 30) PLC. Since PLC-mediated degradation of PtdIns and the polyphosphoinositides would be expected to proceed by a

Table 1. Detection of inositol cyclic phosphates from the PLC-I reaction mixture

Substrate	Ratio, m/z 385 to m/z 383			% phosphorylated inositols with cyclic phosphates
	Procedure 1 (PLC-I in $H_2{}^{18}O$)	Procedure 2 (Acid plus $H_2{}^{18}O$)	Procedure 3 (Acid, then $H_2{}^{18}O$)	
PtdIns	0.3940 ± 0.0073	0.3296 ± 0.0021	0.1562 ± 0.0009	73
PtdIns-4-*P*	0.3054 ± 0.0037	0.2115 ± 0.0010	0.1546 ± 0.0004	37
PtdIns-4,5-*P*	0.2295 ± 0.0009	0.1658 ± 0.0005	0.1555 ± 0.0002	14

Cyclic phosphates were detected in the products of PtdIns, PtdIns-4-*P*, and PtdIns-4,5-P_2 cleavage by PLC-I by using ^{18}O labeling as described in *Materials and Methods* and the accompanying text (procedures 1–3). Triplicate GC/MS measurements were made on each sample of derivatized phosphate. Each value represents the mean ± SEM of the ratio of peak heights. The percentage of phosphorylated inositols with cyclic phosphates was calculated from the relation

$$\frac{A - 0.1553}{B - 0.1553} \times 100\%,$$

where A = the value obtained for procedure 2, B = the value obtained for procedure 1, and 0.1553 = the m/e 385 to m/e 383 ratio for unenriched inorganic phosphate.

common mechanism, cyclic phosphates would be anticipated in the products of PtdIns-4-*P* and PtdIns-4,5-P_2 cleavage by mammalian PLC. We have now shown by ^{18}O labeling that cyclic phosphates are indeed present in the products of phosphoinositide degradation by PLC. The ratio of cyclic to noncyclic product decreases as one proceeds from PtdIns to PtdIns-4-*P* to PtdIns-4,5-P_2. These lower ratios may reflect greater instability of the cyclic phosphates derived from polyphosphoinositides. Alternatively, the low ratio of cyclic phosphate in the product of phosphoinositide cleavage by PLC may reflect the local hydroxide ion concentration present at the active site of the enzyme reaction. Dawson *et al.* (17) have shown that as the pH of a PLC reaction mixture is increased, the ratio of Ins>*P* to Ins-1-*P* formed from cleavage of PtdIns decreases. These investigators have suggested that the increased concentration of hydroxide ions that accompanies a rise in pH competes with the 2-position hydroxyl of inositol for nucleophilic attack on the phosphorus atom. The additional phosphate groups on PtdIns-4-*P* and PtdIns-4,5-P_2 may create local charge effects that also increase the local concentration of hydroxide ions, leading to preferential production of the noncyclic product.

Lapetina *et al.* (31) attempted to detect a cyclic phosphate in the product of PtdIns-4,5-P_2 cleavage by a crude preparation of kidney PLC by using high voltage paper electrophoresis. These investigators prepared a standard of [Ins(>*P*)P_2] by treating Ins-1,4,5-[^{32}P]P_3 with dicyclohexylcarbodiimide. A product of this reaction moved faster than Ins-1,4,5-P_3 on paper electrophoresis and was converted back to Ins-1,4,5-P_3 by heating at 100°C in 1 M HCl. When the product of [^{32}P]PtdIns-4,5-P_2 hydrolysis was run on paper electrophoresis with this standard, no Ins(>*P*)P_2 was detected. There are several possible explanations for the disparity between that study and ours. We detect only 15% Ins(>*P*)P_2, which

Table 2. Percentage of phosphorylated inositols with cyclic phosphates in the products of PLC-I- and PLC-II-mediated cleavage of the phosphoinositides: A summary of multiple experiments

Substrate	% cyclic phosphates					
	PLC-I			PLC-II		
	Mean	Range	No. of expts.	Mean	Range	No. of expts.
PtdIns	68	63–73	2	45	29–61	4
PtdIns-4-*P*	35	32–37	2	32	21–41	3
PtdIns-4,5-P_2	14	12–15	3	6	0–15	5

Cyclic phosphates were detected in the products of PtdIns, PtdIns-4-*P*, and PtdIns-4,5-P_2 cleavage by PLC-I and PLC-II by use of ^{18}O labeling as described in the legend to Table 1 and *Materials and Methods*. In every experiment listed, controls (procedure 3, see text and Table 1) gave m/z 385 to m/z 383 ratios that were the same, within experimental error, as that for unenriched phosphate.

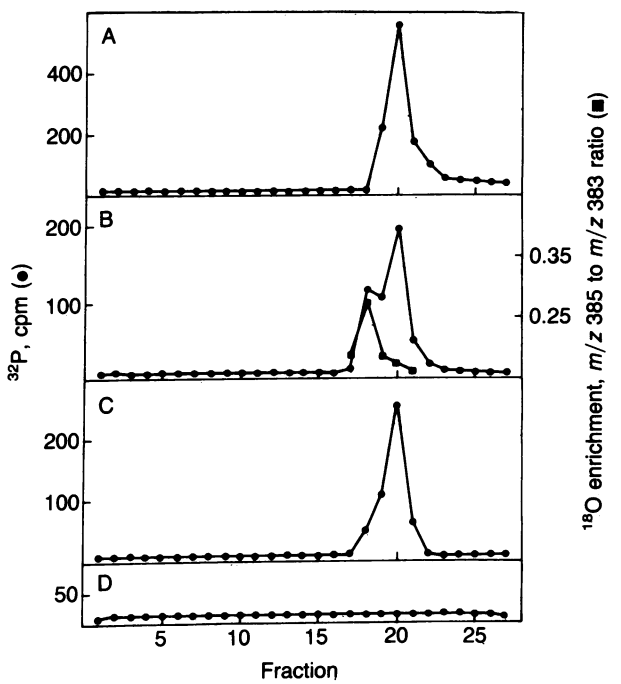

FIG. 4. Anion-exchange HPLC of the products of PLC-I mediated cleavage of [^{32}P]PtdIns-4-*P*. The PLC reaction mixtures contained [^{32}P]PtdIns-4-*P*/phosphatidylethanolamine vesicles (300 μM phosphoinositide, 200 cpm/nmol), 10 mM Tris acetate (pH 5.5), 35 mM NaCl, and 0.75 mM $CaCl_2$ in the presence or absence of 400 ng of PLC-I (total volume of 200 μl). Reaction mixtures were incubated for 10 min at 37°C and then were extracted by the addition of 0.5 ml of chloroform, 0.5 ml of methanol, and 0.3 ml of water. The upper phase of the extraction mixture was lyophilized and then dissolved in 200 μl of water. A parallel sample of the lyophilized PLC reaction products was taken up in 200 μl of 1 M HCl, incubated for 1 min at room temperature, lyophilized, and resuspended in 200 μl of water. Samples were chromatographed on an Econosphere-NH_2 column as described in *Materials and Methods*. One-milliliter fractions were collected and Cerenkov radioactivity was measured in a scintillation counter. (*A*) [^{32}P]Ins-1,4-P_2 from erythrocyte ghosts. (*B*) Water-soluble products from PLC-I reaction. (*C*) Acid-treated, water-soluble products from PLC-I reaction. (*D*) Water-soluble products from a reaction in the absence of PLC-I. Fractions from *B* were lyophilized and acid-catalyzed incorporation of $H_2{}^{18}O$ (■ in *B*) was determined under the same conditions described in Tables 1 and 2.

might have been overlooked by Lapetina *et al.* (31). The crude preparation of PLC employed by these investigators may have contained hydrolases that converted Ins(>*P*)P_2 to Ins-1,4,5-P_3. The Ins(>*P*)P_2 standard synthesized by Lapetina *et al.* (31) may not have been Ins(>*P*)P_2 but rather another compound such as a carbodiimide-induced dimer of two Ins-1,4,5-P_3 molecules. We have observed such a dimer in preparations of Ins>*P* (32).

Our findings are not at odds with recent proposals that breakdown of PtdIns-4,5-P_2 with release of Ins-1,4,5-P_3 is responsible for Ca^{2+} mobilization (3). Assuming our *in vitro* experiments reflect the *in vivo* situation, we would predict that >85% of the InsP_3 produced during PLC-mediated breakdown of PtdIns-4,5-P_2 would be noncyclic. It is possible that the inositol cyclic polyphosphates are important as second messengers in that hydrolysis of the labile reactive cyclic phosphate (33) is part of the mechanism of producing a transient signal. The Ca^{2+} flux that occurs when cells are activated may be extremely short-lived; in *Limulus* photoreceptors it lasts <2 sec (34). Such a time course is much more rapid than the degradation of Ins-1,4,5-P_3, which appears to take minutes in those cases where it has been measured (32, 35–38).

^{18}O labeling is a useful technique for the identification of cyclic phosphates in the water-soluble products of phosphoinositide degradation. Because of the polarity of Ins>*P*, its analysis by GC or GC/MS is extremely difficult. The cyclic esters of higher inositol phosphates are even less amenable to these techniques. Thus, an indirect method that reveals the presence of cyclic phosphates by ^{18}O labeling has proven to be useful. Moreover, this technique is extremely sensitive. Only 30 pmol of derivatized phosphate is required to make a single GC/MS measurement. In future experiments, we hope to adapt the technique to permit detection of cyclic phosphates in stimulated cells. If Ins(>*P*)*P* and Ins(>*P*)P_2 are documented in cells, enzyme activities responsible for degradation of these cyclic compounds will have to be sought. Dawson and Clarke (39) have identified a hydrolase activity in extracts of kidney and other organs that converts Ins>*P* to Ins-1-*P*. Analogous enzyme activities may exist for Ins(>*P*)*P* and Ins(>*P*)P_2.

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