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Novel Phosphoinositides in Barley Aleurone Cells

Additional Evidence for the Presence of Phosphatidyl-*scyllo*-Inositol[1]

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A novel isomer of phosphatidylinositol that differs in the structure of the head group was detected in barley (*Hordeum vulgare* cv Himalaya) seeds. In this paper we describe our efforts to elucidate the structure of the novel isomer. Evidence from a variety of techniques, including chemical modification of in vivo ^{32}Pi- and *myo*-[^{3}H]inositol-labeled compounds, gas chromatography-mass spectrometry analysis, in vivo incorporation of *scyllo*-[^{3}H]inositol, and enzymatic studies that suggest that the structure is phosphatidyl-*scyllo*-inositol (*scyllo*-PI), is presented. The use of microwave energy to significantly enhance the slow rate of hydrolysis of phosphoinositides is described. The presence of *scyllo*-PI can be easily overlooked by the methods commonly employed; therefore, experimental considerations important for the detection of *scyllo*-PI are discussed.

The central role that plasma membrane phosphoinositides play in signal transduction in animal cells is now well established (Berridge, 1993). Numerous investigations aimed at delineating the role of phosphoinositides in plant cells have recently been published (reviewed by Drøbak, 1993; Cho et al., 1995; Lee et al., 1996). In recent papers from this laboratory, we have described our efforts to characterize phosphoinositides in the aleurone tissue of barley (*Hordeum vulgare* cv Himalaya) seeds (Murthy et al., 1989, 1992; Kinnard et al., 1995). To obtain structural information, we applied the general methods developed by Brockerhoff and Ballou (1961) and Tomlinson and Ballou (1961), which involve modification of specific parts of the molecule by deacylation and deglyceration and identifying the products of such reactions by co-migration with standards. Modification of ^{32}Pi- and *myo*-[2-^{3}H(N)]inositol-labeled compounds suggested the presence of isomeric PI that differs in the structure of the head group (Murthy et al., 1992). More recent data suggested that the compound contains *scyllo*-inositol (Kinnard et al., 1995). Brearley and Hanke (1994) reported on their investigation regarding the structure of phosphoinositides in aleurone tissue of barley seeds and concluded that their data did not provide evidence for the presence of isomeric PI that differs in the structure of the head group.

In this paper we provide new evidence that confirms the presence of an additional isomer of PI and indicates that the structure of the head group is *scyllo*-inositol, we clarify our rationale and develop arguments that the experimental methods employed justify the conclusions drawn, and we discuss the experimental considerations necessary to detect *scyllo*-PI.

MATERIALS AND METHODS

Labeling with Radioactive Precursor

The barley (*Hordeum vulgare* cv Himalaya, 1979, 1985, and 1991 harvests) seeds were obtained from Seed Technology (Department of Agronomy, Washington State University, Pullman), and the aleurone layers were isolated from barley half-seeds as described previously (Murthy et al., 1989). To label Pi-derived compounds with ^{32}P, the aleurone layers were incubated in solution (1 mL per 10 layers) containing succinate buffer (20 mM, pH 5.0), $CaCl_2$ (20 mM), chloramphenicol (30 μM), and ^{32}Pi (50 μCi per 10 layers; ^{32}Pi in 0.02 N HCl [DuPont-NEN] was neutralized with 0.02 N NaOH just before use). To label *scyllo*-inositol-derived compounds with tritium, *scyllo*-[^{3}H(N)]inositol (American Radiolabeled Chemicals, St. Louis, MO) (70 μCi per 10 layers) was added to the incubation medium instead of ^{32}Pi. After 24 h, the radioactive medium was removed

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Abbreviations: *chiro*-PI, phosphatidyl-*chiro*-inositol; GPC, glycerophosphocholine; GPI, GPIP, and $GPIP_2$, glycerophospho-*myo*-inositol and its mono- and bisphosphorylated derivatives; GPIx, deacylated phospholipid from PIx; HPTLC, high-performance TLC; HVE, high-voltage paper electrophoresis; $Ins(1)P_1$, *myo*-inositol-1-phosphate; IPS, inositolphosphosphingolipid; PI, PIP, and PIP_2, phosphatidyl-*myo*-inositol and its mono- and bisphosphorylated derivatives; PI-PLC, phosphatidylinositol-specific phospholipase C; PIx, phosphatidylinositol of unknown structure; *scyllo*-PI, phosphatidyl-*scyllo*-inositol; TMS, trimethylsilyl.

and washed free of isotopes (three times with an equal volume of incubation medium minus radioisotopes).

Extraction and Separation of Phospholipids

The incubation medium was removed and the tissue was ground with a small amount of sand in a glass homogenizer and centrifuged. Phospholipids were extracted from the pellet by a modified acidic Bligh-Dyer method (Murthy et al., 1992; Kinnard et al., 1995). Unlabeled phosphoinositides (0.075 mg) from soybean were added to the organic extract. The phospholipids were separated on a silica-gel HPTLC plate (EM Separations, Cherry Hill, NJ, 10 cm × 20 cm) in a solvent system consisting of $CHCl_3$:CH_3OH:30% aqueous NH_4OH (90:90:20, v/v) for 3 h. Labeled phospholipids were visualized by autoradiography. The silica gel corresponding to the phosphoinositides of interest was scraped and the phospholipids were extracted with $CHCl_3$:CH_3OH (2:1, v/v) acidified to pH 1.5 with concentrated HCl.

Deacylation of Phospholipids

The radiolabeled phospholipids were combined with 0.5 mg of a mixture of phosphoinositides (from soybean, Sigma) and subjected to deacylation conditions as described previously (Clark and Dawson, 1981; Murthy et al., 1989). The glycerophospholipids produced were analyzed by HPLC or HVE as described below.

HPLC Analysis

The glycerophosphoinositides and inositol phosphates were separated on a Whatman Partisil 10 SAX column (25 cm × 0.46 cm) or a Whatman PartiSphere 10 SAX column (12.5 cm × 4.6 cm) using one of the following ammonium phosphate elution protocols (pH 3.8, flow rate 1 mL/min) (Dean and Moyer, 1987; Murthy et al., 1992). Protocol A: 0.0001 M for 5 min, 0.0001 to 0.06 M over 40 min, 0.06 to 0.2 M over 5 min, 0.2 to 0.29 M over 30 min, 0.29 to 0.48 M over 5 min, 0.48 to 0.53 M over 30 min, and 0.53 to 0.6 M over 10 min. Protocol B: 0.0001 M for 5 min, 0.0001 to 0.18 M over 40 min, 0.18 to 0.6 M over 5 min, and 0.6 M for 20 min. Fractions (1-mL volume for protocol A and 0.5 mL for protocol B) were collected, mixed with 5 mL of scintillant, and counted in a liquid scintillation counter. To calibrate and monitor the performance of the column, a mixture of AMP, ADP, and ATP was co-injected with all samples and detected with a UV detector (248 nm).

HVE Analysis

Glycerophosphoinositides were separated by HVE in 0.006 M sodium oxalate buffer (Murthy et al., 1989). The paper was dried and ^{32}Pi-labeled glycerophospholipids were detected by autoradiography. Areas corresponding to glycerophospholipids were cut out, minced, and counted by liquid scintillation counting. Nonlabeled standards (Sigma) were visualized with a phosphate spray (Clarke and Dawson, 1981). In our experience, separation by HVE is influenced by the concentration of dipping buffer, the duration of the separation process, and the temperature of the cooling water.

Reaction of Phospholipids with PI-PLC

In vivo ^{32}Pi-labeled phospholipids were separated on HPTLC plates and visualized by autoradiography. Silica gel corresponding to PI and PIx was scraped and the phospholipids were extracted. Nonlabeled PI (0.2 mg) from soybean was added to the labeled PI and PIx from barley seeds, and the solution was evaporated to dryness. Sodium deoxycholate (0.1 mL of 0.8% solution) was added, and the mixture was sonicated for 3 min to form mixed micelles. To the mixed micelles was added Hepes-KOH (0.2 mL, pH 7.5) and PI-PLC (10 units of PI-PLC from *Baccilus cereus* [EC 3.1.4.10] from Sigma, in Hepes-KOH, pH 7.5) containing 0.1% BSA, and the reaction mixture was incubated at 37°C for 4 h (Sundler et al., 1978; Griffith et al., 1991). The reaction was terminated by the addition of 2.5 mL of chloroform:methanol (2:1, v/v), mixed and centrifuged, and the aqueous and organic layers were separated. Radioactivity in the organic layer was determined by liquid scintillation counting. To obtain structural information regarding the radiolabeled compound in the aqueous layer, the aqueous solution was concentrated, mixed with AMP, and separated by HPLC. To obtain structural information regarding the radiolabeled compounds in the organic layer, the compounds were deacylated and the product glycerophospholipids were analyzed by HPLC.

Acid Hydrolysis and GC-MS Analysis of TMS Derivatives

Phospholipids were mixed with 6 N HCl, heated in a sealed vial at 110°C to dissolve the sample, if necessary, and then transferred to the Teflon beaker (45 mL) of a microwave acid-digestion bomb (model 4782, Parr Instruments Co., Moline, IL) (Kingston and Jassie, 1986; Nicholson et al., 1989). The sample was heated in a microwave oven (model 4A56, Sharp Electronics Co., Mahway, NJ) operating at 2450 MHz and equipped with a turntable for even heating, at 450 W power for various periods of time. A duration of 30 s was sufficient to hydrolyze phosphoinositides. After acid hydrolysis the samples were transferred to clean vials and concentrated in a stream of N_2 gas. To dry the samples, the solution was lyophilized successively with benzene:ethanol (1:1, v/v) and benzene and dried in a vacuum centrifuge (Savant, Farmingdale, NY). Samples to be analyzed by GC-MS were converted to hexaTMS derivatives as follows (Mato et al., 1987a, 1987b; Larner et al., 1988; Pak et al., 1992): The hydrolyzed products were placed in a glass vial, dried, dissolved in dry pyridine (0.1 mL), and mixed with *N-O*-bis(TMS)-trifluoroacetamide containing 1% TMS chloride (0.1 mL) in a N_2 atmosphere. The vial was sealed, heated in a 70°C oven for 2 h, and left at room temperature overnight. The resulting hexaTMS derivatives were separated on a gas chromatograph (Hewlett-Packard 5890 model A) equipped

with a 0.25 mm × 30 m DB 17 (J&W Scientific, Folsom, CA) capillary column and flame-ionization detector. The initial temperature was held at 155°C for 2 min and then increased linearly at the rate of 2°C/min to 192°C. GC-MS analysis was performed on a Hewlett-Packard 5970 quadrupole instrument. Samples were ionized by electron impact (70 eV) with the source temperature of 160°C. The retention times and fragmentation patterns were compared with those of standard inositols under similar conditions (Sherman et al., 1970; Mato et al., 1987a, 1987b; Larner et al., 1988; Pak et al., 1992).

RESULTS

Evidence That PIx Is Not a Sphingolipid or an Ether Lipid

Separation of phospholipids from adventitious contamination by sphingolipids (such as IPS) can be readily accomplished by subjecting the phospholipid extract to mild deacylation conditions that take advantage of the fact that carboxylate esters undergo hydrolysis faster than amides because the nitrogen atom imparts ground-state stabilization to the carbonyl group (O'Connor, 1970; Carey and Sundberg, 1990). The partitioning of the reaction mixture between aqueous and organic phases readily separates the unreacted lipids such as sphingolipids, ether phospholipids, and plasmalogens (Fig. 1), which partition into the organic phase, from the aqueous-soluble glycerophospholipids. Clark and Dawson (1981) clearly demonstrate that under the mild methylamine conditions described, sphingomyelin does not undergo N-deacylation and consequently partitions into the organic phase during work-up. When deacylation conditions were optimized by subjecting in vivo ^{32}Pi-labeled phospholipids to varying methylamine concentrations and reaction durations (Fig. 2) (Clark and Dawson, 1981), >95% of radioactivity in the reaction mixture consistently partitioned into the aqueous phase, suggesting the presence of little (<5%), if any, labeled sphingolipids in the phospholipid extract. The recovery of little radioactivity in the organic phase suggests that the lipophilic groups in the parent phospholipids are connected by carboxylate ester linkages and not amide linkages as in IPS or ether linkages as in plasmalogens. Therefore, PIx is not a sphingolipid or an ether lipid.

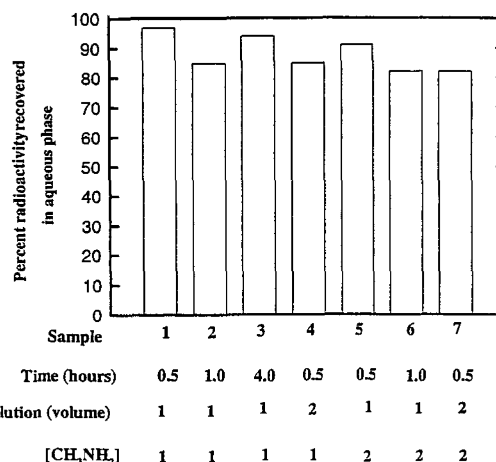

Figure 2. Optimization of conditions for deacylation of ^{32}Pi-labeled phospholipids from barley seeds. ^{32}Pi-labeled phospholipids were deacylated at 53°C under the conditions described below. The reaction mixture was worked up as described in "Materials and Methods," and the radioactive content in the aqueous phase was determined. Samples 1 to 3 received 1 mL of reagent solution A (methylamine [40%, w/w in water]:methanol:water:*n*-butanol [5:4:3:1, v/v]), and the duration of reaction is indicated (0.5, 1, and 4.0 h). Sample 4 received 2 mL of reagent solution A. Samples 5 to 7 received 2 mL of reagent solution B, which contained twice the concentration of methylamine (methylamine [40%, w/w in water]: methanol:water:*n*-butanol [10:4:3:1, v/v]). The duration of the reaction is indicated.

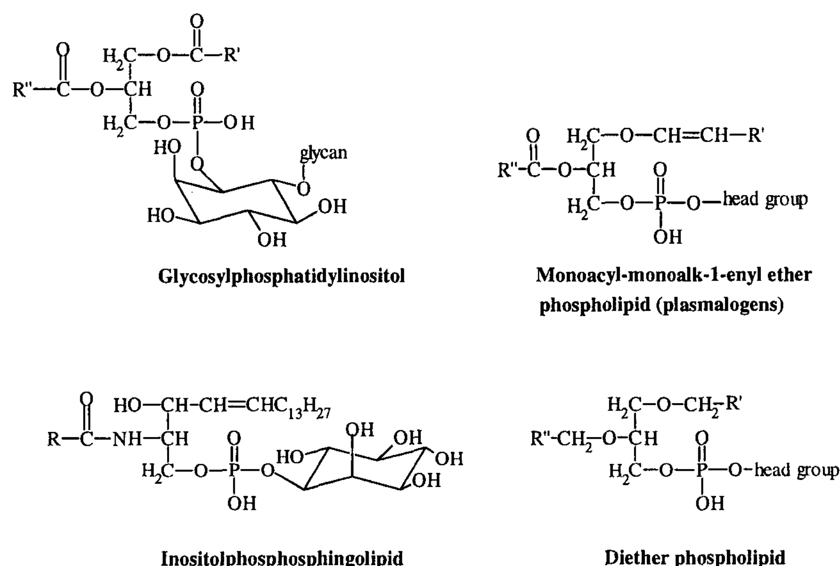

Figure 1. Structures of phospholipids.

Evidence That the Deacylated Product of PIx, GPIx, Is Not $Ins(1)P_1$ That Could Be Formed by Hydrolysis at the Phosphate Ester under the Deacylation Conditions Employed

To obtain structural information regarding the deacylated products, the mixture was separated by HVE and HPLC and the migratory characteristics of products were compared with standards. Figure 3 illustrates the separation of ^{32}Pi-labeled phospholipids by HVE. Three major bands contained about 90% of the total radioactivity in the aqueous layer. Compounds corresponding to bands 1, 3, 5, and 7 had migratory properties similar to the standards GPC, GPI, GPIP, and $GPIP_2$, respectively. The compound in band 2 (GPIx) consistently contained as much or more radioactivity as that in band 3. That the compound in band 2, labeled GPIx, is not $Ins(1)P_1$ that could be formed by further hydrolysis at the phosphate ester under the deacylation conditions employed was indicated by the fact that (a) when the deacylation products were separated by HVE in parallel with the ^{3}H-labeled standards $Ins(1)P_1$, $Ins(1,4)P_2$, and $Ins(1,4,5)P_3$, GPIx was clearly resolved from $Ins(1)P_1$ (Fig. 3), and (b) evidence presented previously that when standard ^{3}H-labeled PI was subjected to the same reaction conditions, no detectable $Ins(1)P_1$ was formed (Murthy et al., 1992).

Consistent with our previous observations, HPLC separation of the deacylated products using Partisil 10 SAX columns showed the presence of two radiolabeled compounds in the GPI-$InsP_1$ region (Murthy et al., 1992). However, in contrast to our previous observations, GPIx, AMP, and $Ins(1)P_1$ co-eluted (Murthy et al., 1992). Attempts to separate GPIx, AMP, and $Ins(1)P_1$ with PartiSphere 10 SAX columns were also unsuccessful. Separation by HPLC is influenced by a number of factors, including the packing material of the column, the age of the column, and the buffer gradient. HPLC column characteristics, such as the number of theoretical plates and asymmetry value, did not influence the separation of GPIx and $Ins(1)P_1$. Variability in the separation afforded by HPLC columns bought in 1991 and 1994 could be due to differences in the base silica gel, such as carbon content, surface area, and pore volume. Consultations with Dr. Elaine Heilweil (Whatman) revealed that the carbon content of the silica gel used in HPLC columns had increased over the 3-year period from 1991 to 1994. Over the same 3-year period, the resolution of AMP, $Ins(1)P_1$, and GPIx had progressively deteriorated in our hands. Compounds in bands 4 and 6 have not been identified.

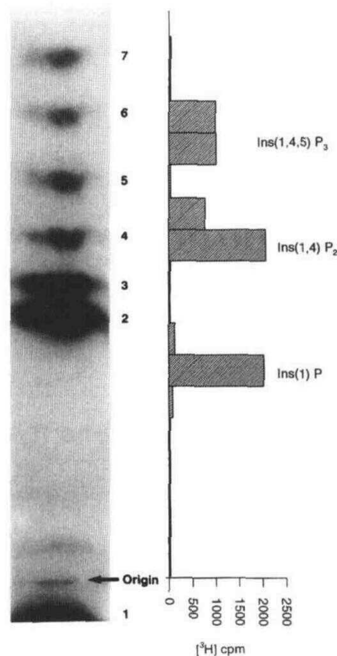

Figure 3. Separation of deacylated, ^{32}P-labeled anionic phospholipids, standard glycerophosphoinositides, and ^{3}H-labeled inositol phosphate standards by HVE. In vivo ^{32}Pi-labeled phospholipids were converted to glycerophospholipids and separated by HVE in parallel with the tritium-labeled standards $Ins(1)P_1$, $Ins(1,4)P_2$, and $Ins(1,4,5)P_3$ and the unlabeled standards GPI, GPIP, and $GPIP_2$. ^{32}Pi-labeled glycerophospholipids were visualized by autoradiography; unlabeled standards GPC (1), GPI (3), GPIP (5), and $GPIP_2$ (7) were visualized by molybdate spray reagent, and ^{3}H-labeled standards $Ins(1)P_1$, $Ins(1,4)P_2$, and $Ins(1,4,5)P_3$ (Amersham) were localized by cutting the paper into 1-cm sections and determining the radioactive content by liquid scintillation counting.

Evidence That the Inositol-Derived Head Group Is *scyllo*-Inositol

In plant tissues, the conversion of *myo*-inositol to other isomers of inositol, to methyl ethers, and to other sugars such as glucuronic acid, galacturonic acid, Xyl, Ara, and galactinol is well documented (Hoffman-Ostenhoff and Pittner, 1982; Loewus, 1990). Therefore, the head group could be any one of these inositol-derived compounds. A general method to analyze the structure of the head group of phospholipids, including PI and glycosylated PI, is to release the head group moiety by acid hydrolysis and then obtain structural information by paper chromatography, GC-MS, or HPLC analysis. Complete hydrolysis of PI yields glycerol, phosphoric acid, and inositol.

Previous work in our laboratory has shown that the tritium-labeled product obtained after acid (6 N HCl) hydrolysis of in vivo *myo*-[2-^{3}H(N)]inositol-labeled PI and PIx exhibited the same migratory characteristics as *myo*- and *scyllo*-inositol, respectively, in descending paper chromatography (Kinnard et al., 1995). Additional confirmation that the hydrolyzed product was *scyllo*-inositol was obtained by GC-MS analysis, namely, by comparison of GC retention times and mass spectra of the TMS derivatives with those of standard inositols (Sherman et al., 1970; Mato et al., 1987a, 1987b; Larner et al., 1988; Pak et al., 1992). The TMS derivative of *myo*- and *scyllo*-inositol can be readily separated on GC columns (Table I). Previous research by Sherman et al. (1970) has shown that although derivatized inositols yield the same characteristic ions (m/z = 73, 147, 217, 265, 305, 318, 432, and the molecular ion, 612), each isomer yields a unique mass spectrum due to differences in the relative abundance of the ions. Although the effect of stereoisomerism on the fragmentation pattern of inositol

Table I. *GC-MS data of TMS derivatives of inositols TMS derivatives of inositol standards (*myo *and* scyllo*) and hydrolytic products of phospholipids (PI and PIx) were synthesized and analyzed as described in "Materials and Methods"*

HexaTMS Derivatives	GC Retention Time	Characteristic Ions	Ratio of Ions, 305/318
	min	*m/z*	*m/z*
myo-Inositol	16.76	73, 147, 191, 217, 265, 305, 318, 432, 507, 612	1.55
scyllo-Inositol	15.35	Same as above	0.59
Hydrolyzed product from PI	16.75	Same as above	1.65
Hydrolyzed product from PIx	15.39	Same as above	0.42

derivatives is not well understood, comparison of the relative abundance of characteristic ions with those of standard compounds provides important structural information (Sherman et al., 1970; Mato et al., 1987a, 1987b; Larner et al., 1988; Pak et al., 1992). Of particular interest is the relative abundance of ions *m/z* 318, not found in the TMS derivatives of hexoses, and *m/z* 305. Consistent with previous observations by Sherman et al. (1970), the *m/z* 305 ion is more abundant than the 318 ion (305/308 = 1.5) in the case of *myo*-inositol, whereas in *scyllo*-inositol, the 318 ion is more abundant (305/318 = 0.6) (Table I) (Sherman et al., 1970). As documented in Table I, when PI and PIx were hydrolyzed and the products converted to the hexaTMS derivatives, the retention times of the TMS derivatives were the same as those of *myo*- and *scyllo*-inositol, respectively. In addition, the mass spectra and relative abundance of the ions produced by the TMS derivatives with retention times of 15.39 and 16.75 min were similar to those of *scyllo*- and *myo*-inositol, respectively (Table I). Furthermore, selected ion monitoring of ions *m/z* 73, 147, 265, 305, 318, and 612 indicated the presence of these ions only in compounds with retention times of 15.39 and 16.76 min.

Previous results (Kinnard et al., 1995) had indicated that acid hydrolyses of PI and PIx are very slow, requiring 24 to 60 h (at 110°C in a sealed tube), respectively, for completion. The excessive time required for hydrolysis was inconvenient for routine use; therefore, the use of microwave energy in a sealed, microwave-transparent vessel was investigated. Optimization of conditions (power and time) in a sealed Parr microwave acid-digestion bomb (Kingston and Jassie, 1986; Nicholson et al., 1989) led to significant enhancement of rates due to rapid increases in temperature and pressure; hydrolysis of both PI and PIx was complete in 30 s.

In Vivo Incorporation of *scyllo*-[^{3}H]Inositol into Phospholipids

To provide additional structural information, the incorporation of *scyllo*-[^{3}H]inositol into phospholipids was investigated. When aleurone layers were incubated with *scyllo*-[^{3}H]inositol, maximum incorporation of radiolabel into phospholipids occurred after 12 h of incubation. When in vivo *scyllo*-[^{3}H]inositol-labeled phospholipids were separated by HPTLC, radioactivity ^{3}H was detected only in the region corresponding to PIx. About 80% of the radioactivity loaded on the plate was recovered in the region corresponding to PIx (Fig. 4); no radioactivity above background was detected in areas corresponding to PI.

Structural Information from PI-PLC-Catalyzed Reaction

Substrate specificity of PI-PLC has been successfully used to characterize the head group of phosphoinositides and glycosyl PI (also called phosphatidylinositol glycans; Fig. 1) (Sundler et al., 1978; Futerman et al., 1985; Low et al., 1987; Mato et al., 1987a, 1987b). In general, PI-PLC from bacterial sources, including *Bacillus cereus*, hydrolyze PI, *chiro*-inositol-containing PI, and glycosyl PI-containing *myo*- or *chiro*-inositol in the presence of detergents; the specific activity toward PI and glycosyl PI is about the same; however, enzymatic activity toward *chiro*-PI is 1000-fold lower (Bruzik and Tsai, 1994). PI-PLC from bacterial sources do not hydrolyze the phosphorylated derivatives PIP and PIP_2, whereas PI-PLC from mammalian sources do (Bruzik and Tsai, 1994). PI yields cyclic Ins(1,2)P_1 as the initial product; however, further hydrolysis to Ins(1)P_1 can occur at low rates. In vivo ^{32}Pi-labeled PI and PIx were separated on HPTLC plates, extracted from silica gel, mixed with nonradioactive PI and detergents, and treated with PI-PLC from *B. cereus* (EC 3.1.4.10 from Sigma). The reactions were stopped by the addition of chloroform: methanol (2:1, v/v). After the reaction, about 58% of the radioactivity in PI (51% above control) partitioned into the aqueous phase, whereas 7% of radioactivity from PIx (3% above control) partitioned into the aqueous phase (Table II), suggesting that the PI was a substrate for PI-PLC but PIx is not.

When the aqueous-soluble product from PI was analyzed by HPLC (Fig. 5), a single peak of radioactivity

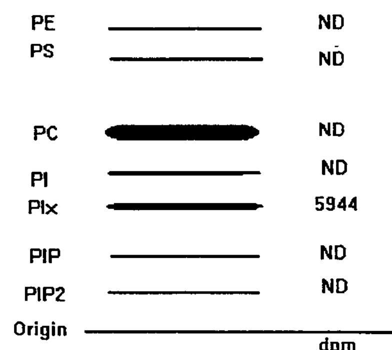

Figure 4. Schematic representation of the separation of in vivo [^{3}H]*scyllo*-inositol-labeled phospholipids. Barley aleurone layers were labeled with [^{3}H]*scyllo*-inositol for 12 h, and the labeled phospholipids were extracted, mixed with [^{32}Pi]-labeled phospolipids, and separated on HPTLC plates. Silica gel corresponding to PI, PIx, phosphatidylcholine, and other regions of the plate were scraped, and the [^{3}H] content was determined by liquid scintillation counting. Number indicates [^{3}H] values above background; ND, no [^{3}H] above background was detected.

Table II. *Distribution of radioactivity in the aqueous and organic phases after treatment of PI and PIx with PI-PLC*

In vivo ^{32}Pi-labeled PI and PIx were treated with PI-PLC as described in "Materials and Methods." The aqueous and organic phases were separated and concentrated, and the radioactive content was determined by liquid scintillation counting.

^{32}P-Labeled Compound	Control (No PI-PLC Treatment)		PI-PLC Treated Sample	
	Percent of ^{32}P in organic phase	Percent of ^{32}P in aqueous phase	Percent of ^{32}P in organic phase	Percent of ^{32}P in aqueous phase
PI	93 (1576 cpm)	7 (120 cpm)	42 (2227 cpm)	58 (3100 cpm)
PIx	96 (1217 cpm)	4 (46 cpm)	93 (2502 cpm)	7 (190 cpm)

eluted about 7 min before AMP (Fig. 5A), as did the product obtained with standard L-3-phosphatidyl[2-^{3}H]*myo*-inositol (Amersham) (Fig. 5B). The elution time is similar to that of cyclic Ins(1,2)P_1, the expected product, which elutes before Ins(1)P_1 (Dean and Moyer, 1987). The elution time is clearly different from that of Ins(1)P_1, which co-elutes with AMP. To establish the identity of the compound remaining in the organic phase, the solution was concentrated and subjected to deacylation conditions using methylamine. Analysis of the deacylated products by HPLC indicated the formation of GPI from PI (Fig. 5C) and GPIx from PIx (Fig. 5D), thus providing evidence that radioactivity in the organic phase is due to unreacted starting material. In summary, these data indicate that although PI-PLC from *B. cereus* hydrolyzed TLC-purified PI from barley aleurone cells to yield a water-soluble compound with elution characteristics (HPLC) similar to those of cyclic Ins(1,2)P_1, in a parallel experiment conducted simultaneously, PIx was not a substrate for PI-PLC; PIx was recovered unchanged. Therefore, these data suggest that PIx is not glycosyl PI; however, the PI-PLC data do not exclude the possibility that PIx contains *chiro*-inositol, since the rate of hydrolysis of *chiro*-PI is considerably lower (Bruzik and Tsai, 1994).

Evidence of PIx in Other Harvests of Barley

To ensure that the presence of PIx in the 1979 harvest was not anomalous, aleurone layers were isolated from seeds of 1985, 1991, and 1995 harvests and incubated with ^{32}Pi. Radiolabeled phosphoinositides were deacylated and the glycerophospholipids were separated by HVE. The autoradiograph of the anionic glycerophospholipids of all harvests studied were very similar to that shown in Figure 3 and clearly showed the presence of GPIx, thereby indicating that PIx is not unique to a particular harvest.

DISCUSSION

The low endogenous concentration of phosphoinositides often precludes the use of techniques such as NMR spectroscopy for structure determination during initial investigations. Therefore, taking advantage of the fact that phospholipids incorporate radiolabeled precursors such as ^{32}Pi and [^{3}H]inositol, a series of reactions that can provide structural information have been routinely used (Brockerhoff and Ballou, 1961; Tomlinson and Ballou, 1961). Other classes of lipids that contain inositol and phosphate moieties and display chromatographic properties similar to phosphoinositides include IPS (Carter et al., 1969; Smith and Lester, 1974; Kates, 1986; Laine and Hsieh, 1987) and glycosyl PI (Low et al., 1987; Pak and Larner, 1992; Saltiel, 1996) (Fig. 1). The data pertaining to the structure of PIx can be summarized as follows: (a) The phospholipid PIx incorporates both *myo*-[2-^{3}H(N)]inositol and ^{32}Pi and therefore must contain *myo*-[^{3}H]inositol or a *myo*-[^{3}H]inositol-derived moiety. (b) Ready hydrolysis of ^{32}Pi-labeled PIx under mild deacylation conditions yields an aqueous-soluble product, thus suggesting the presence of ester linkages in the parent molecule and the absence of lipophilic groups in the deacylated product. These data exclude the possibility that the parent compound, PIx, is a sphingolipid or an ether lipid. (c) Paper chromatography and GC-MS analysis indicate that acid hydrolysis of PIx yields a compound with the migratory properties, GC retention time, and mass spectrum characteristics of *scyllo*-inositol. (d) Upon incubation of aleurone layers with *scyllo*-[^{3}H]inositol, PIx is the only compound that incorporates detectable levels of radioactivity. (e) PIx is not a substrate for PI-PLC from *B. cereus*, suggesting that it is not a *myo*-inositol-containing glycosyl PI. The structure consistent with these data is *scyllo*-PI (Fig. 6). Although all the hydroxyl groups of *scyllo*-PI are shown in equatorial orientations, it is possible that the hydroxyl groups are indeed in axial orientations, since the conformational preferences of inositol phosphates is dependent on multiple factors of the solvent medium such as pH, ionic strength, and counter ions (Barrientos and Murthy, 1996).

The proposed structure of PIx also explains the observed chemical reactivity. Under the reaction conditions of deacylation and deglyceration (which involves iodate ester formation followed by a modified Wolff-Kischer reaction [House, 1972]), *myo*-PI and *scyllo*-PI exhibit similar chemical reactivity. In both cases, bond breaking and bond making occur on the glycerol moiety, which is separated from the head group. Therefore, the reactions are not influenced by the head group. Consequently, phospholipids with widely differing head groups, such as phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, are equally susceptible to these reactions, and hence, the similarity in the chemical rates of the stereoisomers of inositol in PI and PIx is understandable. On the other hand, acid hydrolyses of PI and PIx, reactions in which the head groups are involved, occur at significantly different rates; complete hydrolysis of PIx consistently required significantly more time (60 h) than did hydrolysis of PI (24 h). The

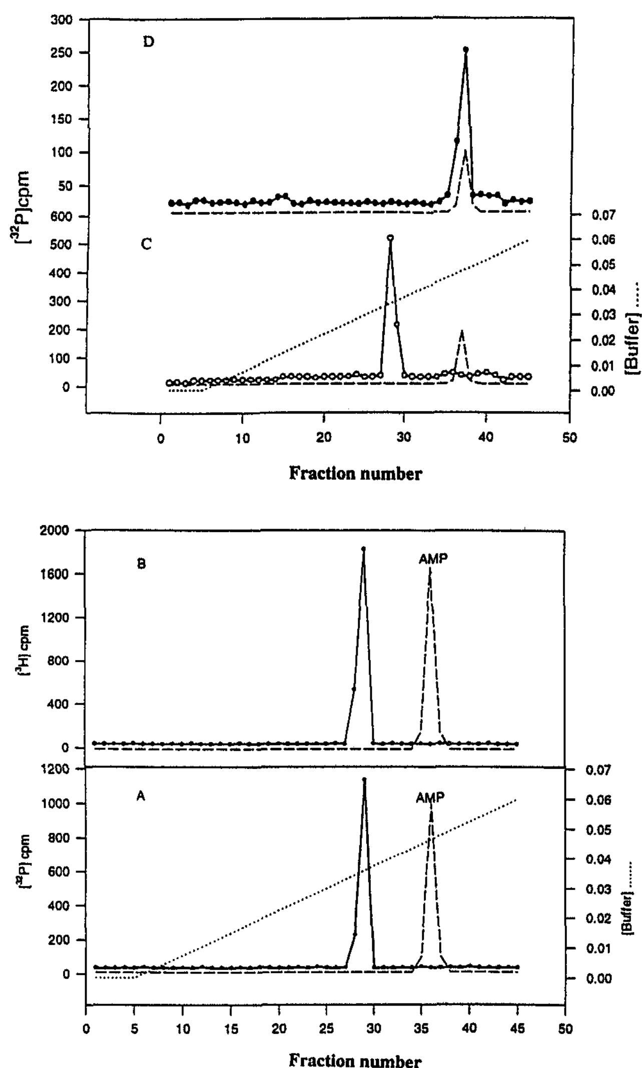

Figure 5. HPLC separation of the aqueous and organic phases of PI-PLC-treated samples. A, In vivo ^{32}Pi-labeled phospholipids were separated on a HPTLC silica gel plate, and PI was extracted from the silica gel and treated with PI-PLC for 4 h. The reaction was terminated by the addition of $CHCl_3$:CH_3OH, and the aqueous layer was concentrated and separated by HPLC using protocol B. B, Standard L-3-phosphatidyl[2-^{3}H]*myo*-inositol was the substrate for PI-PLC. After the reaction the aqueous layer was separated by HPLC. C, ^{32}Pi-labeled phospholipids were separated on a HPTLC silica gel plate, and PI was extracted from the silica gel and treated with PI-PLC. The reaction was quenched and the organic phase of reaction from A below was deacylated and separated by HPLC. D, ^{32}Pi-labeled phospholipids were separated on a HPTLC silica gel plate, and PIx was the substrate for PI-PLC. The reaction was terminated and the organic phase of the reaction was deacylated and separated by HPLC. The elution profile of AMP, which was used as an internal standard and detected by UV A_{248}, is indicated by dashed lines.

faster reaction of *myo*-PI could be due to the facile formation of an unstable cyclic phosphate intermediate (Fig. 6I) by the *cis*-axial hydroxyl in the D-2 position of *myo*-inositol, whereas formation of such a cyclic intermediate by the *trans*-equatorial hydroxyls of *scyllo*-inositol in *scyllo*-PI is much slower. In general, enzymes discriminate between subtle structural differences and therefore exhibit more stringent substrate specificity; thus, the inability of PI-PLC to hydrolyze PIx under the conditions that resulted in hydrolysis of PI is understandable.

The isomerization of *myo*-inositol to *scyllo*-inositol involves the epimerization of the C-2 carbon of *myo*-inositol, possibly via the intermediate *scyllo*-inosose (Hipps et al., 1973, 1977). Hipps et al. (1973) observed that when a partially purified epimerase from the American cockroach was used to catalyze the deuterium-labeled substrate *myo*-[2-^{2}H]inositol, 81% of the ^{2}H label was lost in the product *scyllo*-inositol. Why, then, did *scyllo*-PI contain the ^{3}H label when *myo*-[2-^{3}H(N)]inositol was used as the radioactive precursor in our experiments? The answer may be due to one or both of the following: (a) The ^{3}H label in the nominally labeled *myo*-inositol is predominantly but not exclusively in the D-2 position. Conversations with Dr. Gupta (American Radiolabeled Chemicals) revealed that about 10 to 15% of the ^{3}H label is located on carbons other than the D-2 carbon. Therefore, the ^{3}H retained in *scyllo*-inositol may be from positions other than the D-2 position of *myo*-inositol. (b) The hydrogen that is removed from the D-2 carbon (to form inosose) and transferred to a pyridine nucleotide may be transferred back to the carbonyl from the side opposite that from which it was removed, if the pyridine nucleotide is tightly bound at the enzyme active site and does not dissociate from the enzyme. This question will be best answered in future studies.

The presence of *scyllo*-PI can be easily overlooked in the common experimental protocols used in the phospholipid field for the following: (a) In vivo labeling experiments that employ *myo*-inositol with ^{3}H at the D-2 position exclusively may not label *scyllo*-PI. (b) In our experience the only TLC plates that separate *myo*-PI and *scyllo*-PI are HPTLC plates from Merck. Other TLC plates and oxalate-coated TLC plates have been unsuccessful in our hands. (c) Phospholipids are often converted to glycerophospholipids because they are more amenable to structural analysis. Although HVE can separate *myo*-GPI, *scyllo*-GPI, and Ins(1)P_1, the separation by HPLC is inconsistent. That the investigation of receptor-activated changes in in vivo ra-

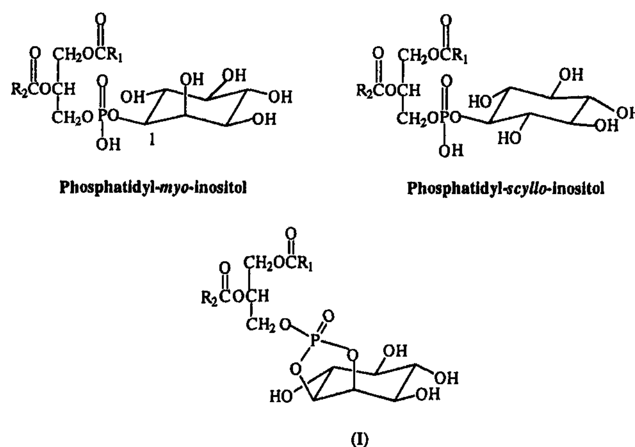

Figure 6. Structures of phospholipids and putative cyclic phosphate intermediate (I).

diolabeled phosphoinositides in plant cells is more complicated than in animal cells has been discussed in numerous papers (Loewus, 1990; Rincón and Boss, 1990; Drøbak, 1992; Coté and Crain, 1993). The challenge is partly due to the low levels of incorporation of *myo*-[^{3}H]inositol and ^{32}Pi into phosphoinositides, the complexity of inositol metabolism in plant cells, and the difficulty of separating phosphoinositides and inositol phosphates from numerous, closely related compounds. The discovery of *scyllo*-PI in plant cells adds another complication to the analysis.

The failure of Brearley and Hanke (1994) to detect the presence of *scyllo*-PI could be due to the following: (a) Their data show the presence of a ^{3}H-labeled peak that elutes just after the glycerophosphoinositol when separated by HPLC (PartiSphere SAX column). The authors presumed that the peak was due to $InsP_1$ on the basis of its elution relative to AMP. In our hands, *scyllo*-inositol-monophosphate, AMP, and $InsP_1$ co-migrate both in PartiSphere and Partisil HPLC columns bought after 1993, as indicated in "Results." Therefore, co-migration of deacylated lipids in HPLC is an inadequate basis for the conclusion. (b) As discussed above, it is possible that the ^{3}H label at the D-2 position is lost during the isomerization of *myo*- to *scyllo*-inositol. If the *myo*-[2-^{3}H]inositol precursor used by Brearley and Hanke for the radiolabeled studies were labeled exclusively at the D-2 position, it is possible that *scyllo*-PI biosynthesized by the aleurone cells would not be labeled. (c) Like other researchers in the field, we used barley seeds (cv Himalaya) grown at Washington State University (Pullman) that are past the postmaturation period. Cereal grains are all dormant to some degree (Bewley and Black, 1983a); barley seeds take about 4 weeks (J.D. Maquire, Seed Technology Laboratory, Washington State University, personal communication) to complete the physiological processes associated with postharvest maturation, including decrease in water content (from 12% to about 9%) and changes in lipid and fatty acid composition (Bewley and Black, 1983a, 1983b). The seeds used by Brearley and Hanke were grown at the Cambridge Botanical Gardens (Cambridge, UK), and may not have undergone the physiological processes associated with postharvest maturation before the experiments were conducted. These factors could have contributed to the observed differences.

In summary, the data presented in this paper provide additional evidence for the presence of *scyllo*-PI in plant cells. Phosphorylated esters of *scyllo*-inositol have previously been identified (Posternak, 1965), and this work indicates that phospholipids containing *scyllo*-inositol as the head group are also present. Preliminary evidence in our laboratory indicates the presence of *scyllo*-PI in other seeds such as oats, wheat, corn, and alfalfa, which suggests that *scyllo*-PI may be widely distributed in plant cells. An understanding of the role of *scyllo*-PI in cell membranes needs further study.

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