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Phosphatidylinositol(4,5)bisphosphate and Phosphatidylinositol(4)phosphate in Plant Tissues[1]

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ABSTRACT

Pea (*Pisum sativum*) leaf discs or swimming suspensions of *Chlamydomonas eugametos* were radiolabeled with [^{3}H]*myo*-inositol or [^{32}P]Pi and the lipids were extracted, deacylated, and their glycerol moieties removed. The resulting inositol trisphosphate and bisphosphate fractions were examined by periodate degradation, reduction and dephosphorylation, or by incubation with human red cell membranes. Their likely structures were identified as D-*myo*-inositol(1,4,5)trisphosphate and D-*myo*-inositol(1,4,)-bisphosphate. It is concluded that plants contain phosphatidylinositol(4)phosphate and phosphatidylinositol(4,5)bisphosphate; no other polyphosphoinositides were detected.

The polyphosphoinositide intracellular signaling system is now well investigated in the animal kingdom, and it is generally agreed that its principal purpose is to generate the second messengers, diacylglycerol (24), Ins(1,4,5) P_3[2] (3), and probably Ins(1,3,4,5) P_4 (18). A number of pieces of experimental evidence suggest that the same signaling system may exist in plants, for example: (a) the release of Ca^{2+} from plant membrane preparations by Ins(1,4,5)P_3 (8, 27), (b) the possible presence in plants of protein kinase C (25, 26), (c) the existence of PtdInsP_2 phosphodiesterase in plant tissues (20, 21), (d) the probable existence of the polyphosphoinositol lipids, PtdIns$P$ and PtdInsP_2, the latter being of particular significance in that it is the common precursor of the second messengers mentioned above (*e.g.* 10, 13), and (e) the evidence for stimulated production of InsP_3 by plant effectors (*e.g.* 10, 23).

The existence of PtdIns(4,5)P_2 in plants has, however, not been unequivocally established, and its identity has been complicated by the presence of other lipids which incorporate [^{3}H]*myo*inositol and [^{32}P]Pi and which show chromatographic behavior similar to Ptd Ins P_2 in some TLC separation systems (9); a further complication is the discovery in animals that there is more than one PtdIns$P$ (31). Thus, we thought it necessary to perform some experiments aimed at confirming the existence of PtdIns$P$ and PtdInsP_2 in plants, and to establish by a number of indirect means that these lipids do indeed contain D-Ins(1,4)P_2 and D-Ins(1,4,5)P_3 as headgroups. Once it is firmly established that lipids with these structures exist in both higher and lower plants, then subsequent studies can address themselves to the physiological details, rather than reestablishing the chemistry each time.

MATERIALS AND METHODS

[3H]Inositol Labeling of Pea (*Pisum sativum*) Leaf Discs

Seeds of *Pisum sativum* were surface-sterilized and imbibed in distilled water for 16 h prior to sowing in moist vermiculite. Germination was in darkness at 25°C. Five d after sowing, the seedlings were transferred to a 12 h day/night light regime and the vermiculite was kept moist with tap-water. On d 12 after sowing, 4 mm diameter leaf discs were cut from mature leaves and transferred (0.35 g fresh weight of tissue/flask) to 25 mL conical flasks containing 8 mL of incubation medium (10 mM Mes [pH: 5.5], 0.33 M sorbitol, 100 μM KH_2PO_4, and 7 μM *myo*-inositol). After vacuum infiltration of the discs, 100 μCi *myo*-[2-^{3}H]inositol (New England Nuclear, specific activity 10–20 Ci/mmol) was added to the flasks. The leaf discs were incubated for 3 h on a rotary shaker (100 rpm) under cool-white light. After rapid removal of the incubation medium by aspiration, 15 mL ice-cold Chl:MeOHCl (concentrate) (100:100:0.75 v/v/v) was added and the lipids were extracted as described by Drøbak *et al.* (6, 9).

Suspension Cultured and [^{32}P]Pi-Labeling of Carrot (*Daucus carota*) Cells

Suspension cultured carrot cells were subcultured weekly and grown in 250 mL conical flasks in 60 mL of standard Murashige and Skoog medium (M&S medium, obtained from Flow Laboratories, Hertfordshire, U.K.) with the addition of 2,4-D (4.5 μM) and kinetin (2.3 μM). Cultures were incubated on an orbital shaker (100 rpm) at 25°C with a 12 h photoperiod. On d 3 after subculturing, 75 μCi [^{32}P]Pi (New England

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[2] Abbreviations: Ins$P$, InsP_2, InsP_3, and Ins$P$4, *myo*-inositol mono-, bis-, tris-, and tetrakisphosphates, respectively, with isomeric numberings as appropriate; PtdIns, PtdIns$P$, and PtdInsP_2, phosphatidylinositol and its mono- and bis-phosphorylated derivatives; Gro$P$Ins, Gro$P$InsP, and Gro$P$InsP_2, glycerophosphoinositol and its mono- and bisphosphorylated derivatives.

Nuclear) was added to each of the cultures. After 24 h incubation the growth medium was rapidly removed by filtration and the cells transferred to 15 mL of ice-cold chloroform:methanol:HCl (concentrate) (180:120:1 v/v/v). Lipids were extracted as described above.

Chlamydomonas eugametos Cultures

The mating type 'plus' strain referred to as 17.17.2 (28) was used throughout this study. It was cultivated in Petri dishes on agar-containing medium in a 12 h light/12 h dark regime as described by Mesland (22). Suspensions of gametes were obtained by flooding 2- to 4-week old cultures with distilled water at the end of the light period. The cells were harvested the next morning as motile, biflagellate, sexually competent gametes. They were concentrated by centrifugation (1000 *g*) and resuspended in the supernatant at a concentration of approximately 10^8 cells/mL. Fifteen mL of suspension were incubated for 2 h with 250 μCi ^{32}P[Pi] or 6 h in 60 μCi *myo*-[2-^{3}H]inositol. Isotope incorporation was stopped by adding 1.5 mL 50% perchloric acid. The lipids were extracted into chloroform/methanol as described by Dawson and Eichberg (6).

HPLC Analysis of Inositol Phosphates

This followed the method of Irvine *et al.* (15), by which most inositol trisphosphate isomers are separated from each other. Deacylation prior to this analysis followed Clarke and Dawson (5) exactly, and subsequent removal of the glycerol moiety was as in Brown and Stewart (4) as adapted by Irvine *et al.* (15). Note that with deacylated lipids from the leaf extract, removal of the glycerol was not possible until non-polar glycerol derivatives had been removed by ion exchange on Sep-Paks (32). Removal of the glycerol involves mild treatment with periodate in the crude lipid deacylate and the galactose ring of galactosyl-glycerols consumed all of this periodate. Consequently, until these galacosyl-glycerols were removed, only minor oxidation of the glycerol moiety was achieved.

RESULTS AND DISCUSSION

The presence of PtdInsP_2 was difficult to establish in suspension cultured tomato cells because of the presence of lipids which incorporated radioactive inositol and [^{32}P]Pi and which chromatographed close to a PtdInsP_2 standard on TLC (9). Similar problems were encountered with suspension-cultured carrot cells, which also contain such lipids (data not shown). However, if the carrot cells were highly labeled with [^{32}P]Pi, and the lipids extracted, deacylated (5) and analyzed by HPLC (15) with an internal standard of ^{3}H-labeled GroPIns(4.5)P_2 prepared from mammalian PtdInsP_2, there were [^{32}P] counts detectable above a very high background which suggested the presence of PtdInsP_2 (Fig. 1), but at an extremely low level (<0.1% of PtdIns). The limited degree to which we could label the polyphosphoinositol of these suspension-cultured cells with [^{3}H]*myo*-inositol made them of limited use for the preparation of sufficient Gro*P*Ins*P* and Gro*P*InsP_2 for further analysis. However, as an alternative tissue we tried discs from

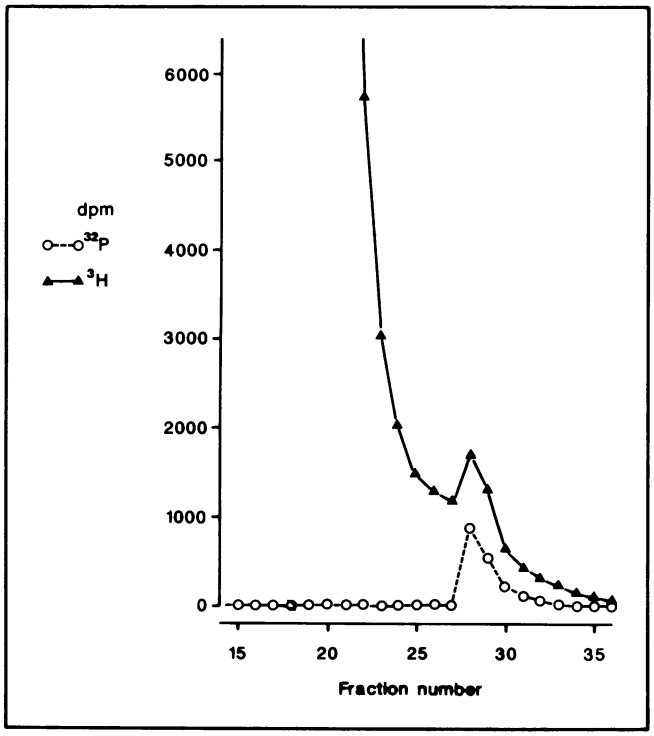

Figure 1. HPLC profile of deacylated phospholipids from ^{32}P-labeled carrot cells, chromatographed with mammalian [^{3}H]Gro*P*Ins(4,5)P_2. HPLC method (15).

pea leaves (see "Materials and Methods"). Here again, the incorporation of [^{3}H]-*myo*-inositol into PtdInsP_2 was only about 0.04% of the total radiolabel incorporated into lipids, but (a) the unknown polar lipids (9) were barely detectable, and (b) a much higher level of radioisotope incorporation was achieved. Thus, we were able to prepare sufficient quantities of Gro*P*Ins*P* and Gro*P*InsP_2 from a deacylate of the lipid extract of these discs for further analysis. These two compounds coincided with their [^{32}P]Pi-labeled mammalian counterparts on HPLC (data not shown), and before removal of their glycerol moieties, contained negligible InsP_2 or InsP_3, respectively. After removal of the glycerol moieties (4, 15), the two compounds were quantitatively converted, respectively, to an InsP_2 fraction and InsP_3 fraction, which were then subjected to further analysis after final purification on Sep-Paks (15) and desalting by freeze drying.

The InsP_3 produced in this way coincided exactly with mammalian [^{32}P]Ins(1,4,5)P_3 on HPLC (Fig. 2A). After periodate oxidation, reduction and dephosphorylation (12, 14, 30) iditol was the only alditol detected (11, 14). This was confirmed independently by L. R. Stephens of Smith, Kline & French, Welwyn, U.K. by using HPLC on a Brownlee polypore Pb^{3+} column (see Ref. 29 for method). The [^{3}H] iditol from the InsP_3 was resistant to L-iditol dehydrogenase even under conditions where an internal marker of L-iditol was entirely oxidized (29), and this shows that it was D-iditol (Fig. 2B). Thus, the structure of the InsP_3 in question must be D-Ins(1,4,5)P_3 or D-Ins(1,4,6)P_3 (12, 30), and the distinction between these two possibilities is reported below.

The InsP_2 fraction derived from pea leaf lipids cochromat-

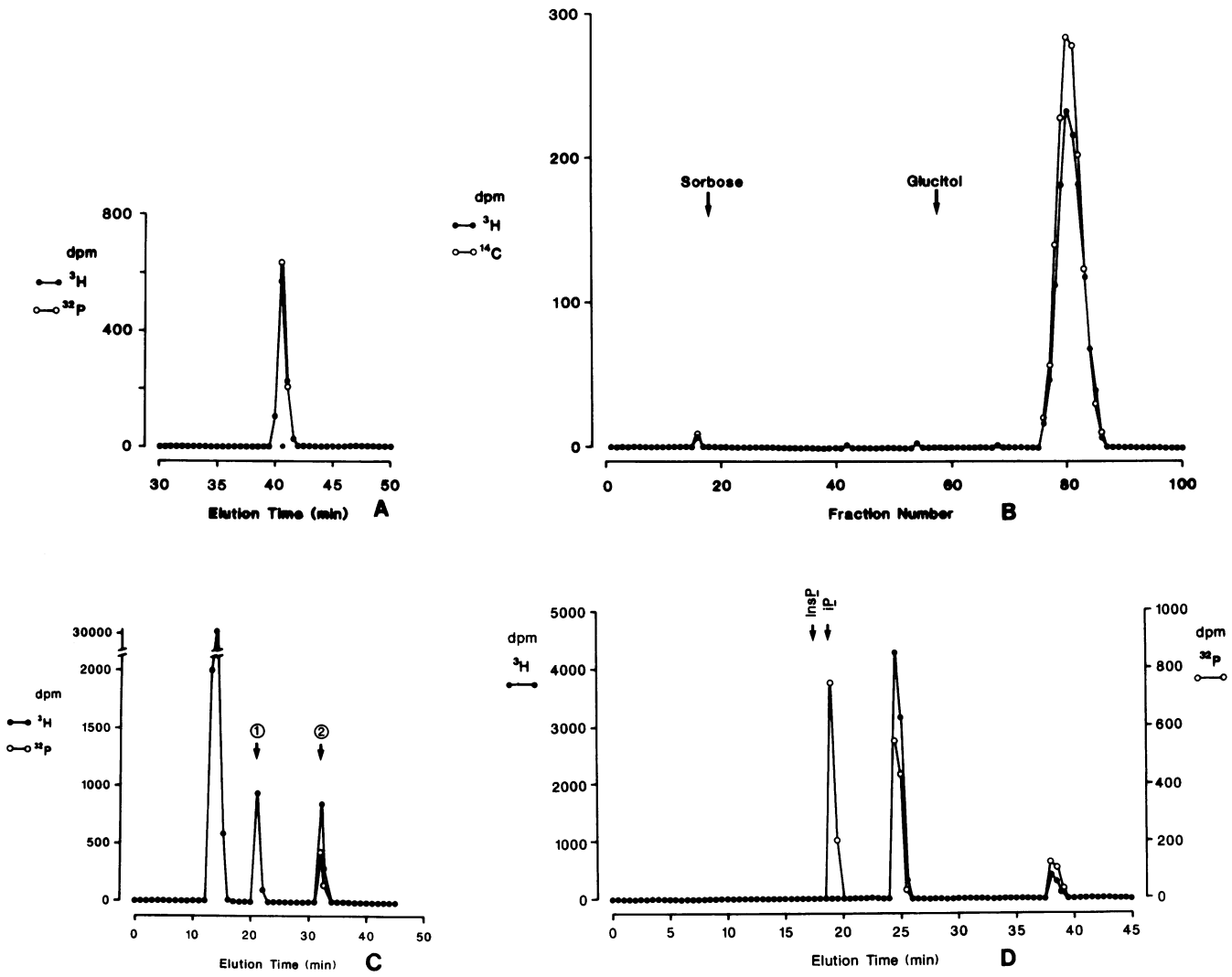

Figure 2. *HPLC profiles of inositol phosphates and alditols*. A, [^{3}H]InsP_3 prepared from a lipid extract of pea leaves, chromatographed with [^{32}P] Ins(1,4,5)P_3 from mammalian cells (Amersham, U.K.). HPLC method (15). B, [^{3}H]Alditols derived from the InsP_3 in A (see text for details). The sample was also spiked with an internal marker of [^{14}C]D-Iditol. This sample was incubated with yeast L-Iditol dehydrogenase until all added L-Iditol was oxidized to L-Sorbose (as judged by the formation to a steady state of NADH in this particular sample, and complete conversion of [^{3}H]L-Iditol to [^{3}H]sorbose in a parallel incubation [see Ref. 29 for details]). The lack of any [^{3}H]sorbose formation shows that all the ^{3}H iditol in this sample is the D-isomer. HPLC method (29). Positions of nonradiolabeled markers of glucitol and sorbase are indicated. C, Deacylated lipid extract from a [^{3}H]*myo*inositol-labeled *C. eugametos* suspension (see text). Also included in this sample was some [^{32}P]Gro*P*Ins(4,5)P_2 prepared from mammalian brain. The elution positions in a preceding run, of the two most polar ^{32}P-labeled compounds from an identical experiment using ^{32}P-labeled *C. eugametos* (see text), are also indicated as (1) and (2). HPLC method (15). D, Products formed by incubation of [^{32}P]InsP_3 prepared from [^{32}P]Pi-labeled *C. eugametos* lipids (see text) mixed with [^{3}H]Ins(1,4,5)P_3 (Amersham, U.K.), and human red cell membranes. This particular profile is the 60 minute incubation from Figure 3. The positions of [^{14}C]Ins(3)*P* (Amersham, U.K.) and [^{32}P]Pi in a preceding run are indicated. Note that three other contaminating peaks of tritium (total <10%) eluting at 3, 15, and 25 min were also detected, but were present at time 0, and did not change during incubation with red cell membranes; they are therefore subtracted as part of the background for this figure. HPLC method (15).

ographed on HPLC with [^{32}P] Ins(1,4)P_2 (not shown) and when subjected to periodate oxidiation for 14 d, reduction and dephosphorylation yielded no noncyclic alditols (<200 cpm from 20,000 cpm) even though 80% of a [^{3}H] Ins(1,3,4,5,)P_4 spike included in it (to check alditol recovery) was recovered as [^{3}H]inositol. This result suggests (a) that the phosphates are *para* (30) and therefore, given the linking of the D-1 phosphate to the glycerol moiety in plant PtdIns (1), they must be in the 1 and 4 positions, and (b) that if there is any PtdIns(3)*P* in plants (31) it was not detectable (*i.e.* is <0.5%) in this particular preparation. (No ribitol was detected, this being the expected breakdown product of Ins(1,3)P_2 by this procedure (17, 31); Ins(1,5)P_2, Ins(1,2)P_2, and Ins(1,6)P_2 would yield xylitol, erythritol, and threitol, respectively.)

To return to the leaf InsP_3, given the exact coincidence of this InsP_3 with Ins(1,4,5)P_3, its structure is most likely to be D-Ins(1,4,5)P_3 rather than Ins(1,4,6)P_3, but the data do not rule out the latter. Unfortunately, insufficient InsP_3 could be obtained for partial dephosphorylation and identification of Ins(5)P as was described by Tomlinson and Ballou (30). However, some evidence against the Ins(1,4,6)P_3 structure can be obtained by showing that the plant InsP_3 behaves exactly the same as Ins(1,4,5)P_3 as a substrate for the specific Ins(1,4,5)P_3-5-phosphatase found in human red cell membranes (7). This membrane preparation very specifically removes the 5-phosphate from Ins(1,4,5)P_3 (7) and Ins (1,3,4,5)P_4 (2), but it is very slow in hydrolyzing Ins(1,3,4,5,6)P_5 (16) and Ins(1,4,6)P_3 (L Stephens, personal communication). We did not have sufficient InsP_3 from these leaf experiments for detailed kinetic experiments, although in one experiment we mixed [^{32}P]Ins(1,4,5)P_3 (Amersham, UK) with duplicate samples of the leaf [^{3}H]InsP_3 and incubated for 30 min with human red cell membranes exactly as described below for the experiment in Figure 3. The [^{3}H] and [^{32}P] in InsP_3 decreased by 65% in both samples, and Pi and a single InsP_2 with the chromatographic properties of Ins(1,4,)P_2 were the only products detected. For a more thorough and kinetic examination we needed a more plentiful source of InsP_3, and for this we used the lipids of swimming suspensions of *Chlamydomonas eugametos* gametes (see "Materials and Methods").

These *Chlamydomonas* suspensions labeled very readily with [^{32}P]Pi, and when the lipids were deacylated, two peaks containing 5 and 15% of the total lipid radioactivity, respectively, were obtained which coincided exactly with [^{3}H] Gro*P*Ins*P* and [^{3}H]Gro*P*InsP_2 from mammalian cells (not shown). The *C. eugametos* suspensions were also labeled with [^{3}H]*myo*inositol for 4 h, and the lipids extracted and deacylated, and peaks containing 2.9 and 3.1% of the radioactivity, respectively, had identical chromatographic properties to these ^{32}P-labeled compounds, confirming that the ^{32}P-labeled compounds are indeed derived from inositol lipids (Fig. 2c; note also the inclusion of mammalian [^{32}P] Gro*P*Ins(4,5)P_2 in this HPLC run). Removal of the glycerol moieties from the ^{32}P-labeled deacylate, and fractionation and desalting on Sep-Paks (as above) gave sufficient [^{32}P]InsP_3 from *Chlamydomonas* lipids to perform the desired kinetic experiments. When mixed with mammalian [^{3}H]Ins(1,4,5)P_3 and presented to human red cell membranes the ^{3}H and ^{32}P were hydrolyzed at apparently identical rates (Fig. 3). The products of the ^{32}P compound coincided exactly with the [^{3}H]Ins(1,4)P_2 formed (Fig. 2D) or with inorganic phosphate, and no other products were detected. No further degradation of the [^{32}P]-InsP_2 was detectable, in keeping with the lack of any [^{3}H]Ins(1,4)P_2 hydrolysis (Fig. 3; and see Ref. 7). Given the known specificity of this human red cell membrane preparation (above), it therefore seems highly likely that the InsP_3 obtained from *C. eugametos* lipids is Ins(1,4,5)P_3 rather than Ins(1,4,6)P_3.

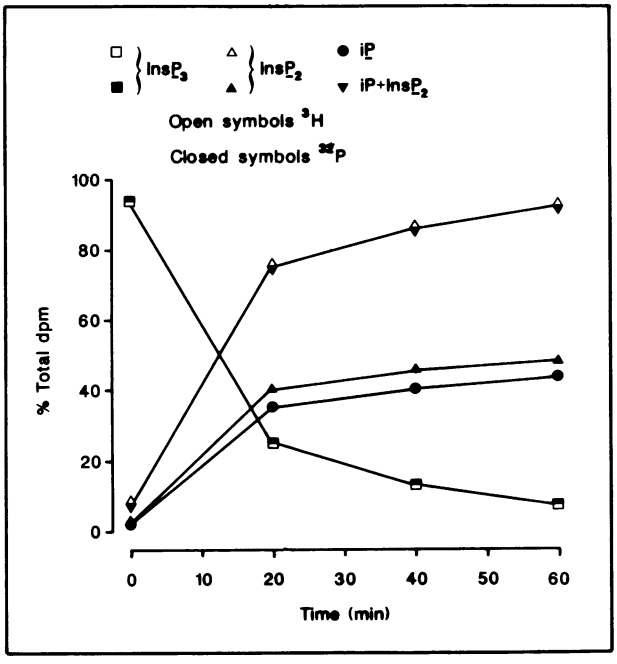

Figure 3. Time course of hydrolysis of [^{32}P]InsP_3, prepared from (^{32}P)-labeled *C. eugametos* lipids, and [^{3}H](1,4,5)InsP_3. [^{32}P]InsP_3 was prepared from *Chlamydomonas* as described in the text, and was mixed with [^{3}H]Ins(1,4,5)P_3 (Amersham, U.K.), and incubated at 37°C for 0 to 60 min at pH 7.0 (Tris/maleate), 5 mM MgAcetate with human red cell membranes (2, 7). After quenching and neutralization (32), the samples were analyzed by HPLC (15). See Figure 2D for the HPLC profile of the 60 min incubation.

CONCLUSIONS

Until sufficient quantities of lipids are obtained for mass-spectroscopic or NMR analysis (*cf.* Ref. 19), indirect evidence will have to remain the only way to identify plant polyphosphoinositides. Here we show that chemical degradation of inositol lipids from *Pisum sativum* or *Chlamydomonas* cultures yields InsP_3 fractions which coincide exactly with D-Ins(1,4,5)P_3 on HPLC. The leaf InsP_3 yields D-iditol on periodate degradation, and the *Chlamydomonas* product behaves as a substrate kinetically identical to D-Ins(1,4,5)P_3 using human red cell membranes as an enzyme source. Together these pieces of evidence establish virtually beyond doubt that the structure of the parent lipid is PtdIns(4,5)P_2. Evidence from HPLC and periodate degradation also suggests that PtdIns(4)P is the only other polyphosphoinositide present, and so we conclude that plants do indeed have the same polyphosphoinositol lipids as animals do, and that they may very well fulfill a similar function.

ACKNOWLEDGMENT

We are very grateful to Len Stephens for confirming for us the identity of the D-Iditol (Fig. 2B).

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