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3- and 4-phosphorylated phosphatidylinositols in the aquatic plant *Spirodela polyrhiza* L.

Charles A. BREARLEY* and David E. HANKE
Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

Labelling of *Spirodela polyrhiza* L. plants with [^{3}H]inositol and [^{32}P]P_i yielded a series of phosphoinositides which were identified as PtdIns, PtdIns4*P* and PtdIns(4,5)P_2. In addition, systematic degradation of a phospholipid extract identified PtdIns3P. Analysis of the distribution of ^{32}P label between the monoester and diester phosphate groups of PtdIns3*P* and PtdIns4*P* revealed differences in the labelling of the monoester phosphate, suggesting that the two PtdIns*P* species are not synthesized or metabolized in a co-ordinate manner.

INTRODUCTION

Recent evidence suggests that the 1D-*myo*-inositol 1,4,5-trisphosphate second messenger of animal cells is also present in plants and that its cytoplasmic level is affected by physiological stimuli such as light (Morse *et al.*, 1987), osmotic stress (Ettlinger & Lehle, 1988) or auxin (Zbell & Walter-Beck, 1988). Moreover, the recent demonstration that Ins(1,4,5)P_3 transients affect K^+ efflux via K^+ channels (Blatt *et al.*, 1990), which may themselves be controlled by cytoplasmic Ca^{2+} (McAinsh *et al.*, 1990), strongly implicates Ins(1,4,5)P_3 as a second messenger in abscisic acid-induced stomatal closure, which is mediated by K^+ fluxes (Schroeder & Hagiwara, 1989).

That the InsP_3 detected in plants is the 1D-1,4,5 isomer and is derived from phosphoinositides have yet to be unequivocally demonstrated, a matter of some complexity, not least considering the ubiquitous nature of *myo*-inositol hexakisphosphate (phytate), the as yet unidentified synthetic pathway to phytate and the widespread occurrence of phytase activities in plants.

The recent identification of 3-phosphorylated analogues of PtdIns4*P* in yeast (Auger *et al.*, 1989), in fibroblasts, *in vitro* at least (Whitman *et al.*, 1988), and in cultured astrocytoma cells (Stephens *et al.*, 1989*a*), and in addition of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Stephens *et al.*, 1991), adds a further degree of complexity to the picture of phosphoinositide-derived second messengers and raises the possibility of a novel signalling pathway. The presence of 3-phosphorylated lipids in higher plants thus becomes a matter of some speculation. There are, however, to date very few rigorous identifications of even PtdIns4*P* or PtdIns(4,5)P_2 in plant tissues (Irvine *et al.*, 1989; Coté *et al.*, 1989), a situation arising in part due to the occurrence in some tissues of other lipids which not only incorporate inositol and orthophosphate but co-chromatograph with the polyphosphoinositides on t.l.c. (Drøbak *et al.*, 1988; Hanke *et al.*, 1990) and also on h.p.l.c. (C. A. Brearley & D. E. Hanke, unpublished work).

We have set out to characterize the inositol phospholipids in ^{3}H- and ^{32}P-labelled plant tissue (*Spirodela polyrhiza* L.) and in particular to explore the possibility that plants also possess 3-phosphorylated phosphoinositides.

MATERIALS AND METHODS

Reagents

myo-[2-^{3}H]Inositol, D-[1-^{14}C]ribose and [^{32}P]P_i (carrier-free) were obtained from Amersham International (Amersham, Bucks., U.K.). D-[1-^{3}H]Ins(1,4,5)P_3 was obtained from New England Nuclear/DuPont (Stevenage, Herts., U.K.). Periodic acid, $NaBH_4$ and alkaline phosphatase (bovine intestinal, type 5521) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Methylamine (40% aq. solution) was obtained from Aldrich Chemical Co. (Poole, Dorset, U.K.).

Tissue

The aquatic monocotyledonous plant *Spirodela polyrhiza* L. was maintained in axenic culture as described by Smart & Trewavas (1983).

Radiolabelling

Two plants were inoculated into media containing 20 μCi of [^{3}H]inositol (Amersham)/ml or 0.5 mCi of [^{32}P]P_i (carrier-free; Amersham)/ml. For phosphate labelling, the phosphate concentration of the labelling medium was decreased from the 1.15 mM value of the culture medium to 10 μM without any visible impairment of growth or development during the radiolabelling incubation.

Tissue extraction

After 4 days (^{3}H labelling) or 3 days (^{32}P labelling) the plants (approx. 30 mg fresh wt.) were washed with water, cooled in liquid nitrogen, ground in a liquid-nitrogen-cooled mortar and pestle and extracted with 1.1 ml of 3.5% (w/v) $HClO_4$. The cell debris was pelleted by centrifugation for 5 min at 3000 $g_{max.}$ in a refrigerated centrifuge and the lipids were extracted by the method of Boss (1989). To the cell debris was added 1.5 ml of chloroform/methanol (1:2, v/v) and the sample was mixed vigorously. Further additions to the sample, each with vigorous mixing, were in turn 2.4 M-HCl (0.5 ml), 1 mM-EDTA (0.5 ml), and chloroform (0.5 ml). The sample was centrifuged for 5 min at 3000 $g_{max.}$ and the lower organic phase was removed. The upper aqueous phase was extracted with 2 × 0.5 ml of chloroform, and the lower organic phases obtained were pooled with the original organic phase. The pooled organic phase was washed with 2 × 2 ml of 1 M-HCl/methanol (1:1, v/v).

Lipid deacylation

Lipids were deacylated by the method of Hawkins *et al.* (1986).

Abbreviations used: Gro*P*Ins, Gro*P*Ins3*P*, Gro*P*Ins4*P* and Gro*P*Ins(4,5)P_2, glycerophosphoinositol, glycerophosphoinositol 3-monophosphate, glycerophosphoinositol 4-monophosphate, glycerophosphoinositol 4,5-bisphosphate.

* To whom correspondence and reprint requests should be addressed.

Deglyceration of glycerophosphoinositol phosphates

Glycerol was cleaved from Gro*P*Ins*P* and Gro*P*InsP_2 species by mild treatment with sodium periodate as described by Whitman *et al.* (1988).

H.p.l.c. of inositol phosphates

Water-soluble products of deacylation and the InsP_2 products of deglyceration were resolved on a 25 cm Partisphere SAX h.p.l.c. column (Whatman) by using the gradient of Stephens *et al.* (1989*a*). The column was eluted with a gradient derived from buffers A (water) and B [1.25 M-$(NH_4)_2$ HPO_4 adjusted to pH 3.8 with H_3PO_4] at a flow rate of 1 ml/min: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 52 min, 20% B, 64 min, 100% B; 70 min, 100% B.

The InsP_3 product of Gro*P*InsP_2 deglyceration was run on a weak anion-exchange column (10 cm Partisphere WAX; Whatman) eluted with the following gradient: A (water)/B 0.5 M-$(NH_4)_2HPO_4$ adjusted to pH 3.2 with H_3PO_4, at a flow rate of 1 ml/min: 0 min, 0% B; 10 min, 10% B; 60 min, 15% B; 70 min, 15% B.

Desalting of h.p.l.c. fractions

Fractions containing phosphate were diluted 5–10-fold with water adjusted to pH 6–7 with triethylamine and desalted as described by Stephens *et al.* (1988*c*).

Periodate oxidation of InsP_2

A ^{3}H-labelled InsP_2 derived from a Gro*P*Ins*P* was oxidized with periodic acid as described by Stephens *et al.* (1988*b*).

Dephosphorylation of products of periodate oxidation

The desalted and freeze-dried product of vigorous periodate oxidation of ^{3}H-labelled Gro*P*Ins*P* was dephosphorylated by incubation with alkaline phosphatase (method modified from Stephens *et al.*, 1989*a*).

H.p.l.c. of polyols

Resolution of polyols was achieved on a cation-exchange column, Polypore Pb^{2+} (22 cm × 0.46 cm, with a 3 cm guard cartridge of the same material), purchased from Owens Polyscience, Macclesfield, Cheshire, U.K. The mobile phase was deionized water run at ambient temperature and at a flow rate of 0.2 ml/min. The sample loop volume was 20 μl. Detection of 50 μg of unlabelled polyol standards was achieved in a run parallel to that used to separate radiolabelled polyols by monitoring the u.v. absorbance (200 nm) on a Spectra Physics 200 u.v. detector. Radiolabelled polyols were detected in fractions by measuring the radioactivity after addition of 4 ml of Hionic Fluor scintillation fluid (Canberra Packard). It is possible to separate on Polypore Pb^{2+}, except for erythritol and ribitol (adonitol), all of the polyols that would be derived from all of the possible non-cyclic *myo*-inositol bisphosphates (Whitman *et al.*, 1988; Stephens *et al.*, 1989*b*). We were unable to resolve completely ^{14}C-labelled standards of inositol and ribitol. Similarly, ribitol was not resolved from erythritol (see also Whitman *et al.*, 1988; King *et al.*, 1990).

The successful resolution of inositol, ribitol and erythritol was achieved, however, on an amino column (25 cm × 0.46 cm main column and 3 cm × 0.46 cm guard column of the same material) from Rainin Instrument Co., obtained from Anachem, Luton, Beds., U.K. The column was eluted isocratically with acetonitrile/water (13:7, v/v) at a flow rate of 0.5 ml/min. The sample taken up in acetonitrile/water was loaded in a 50 μl loop. Unlabelled standards (20 μg) were detected by A_{200} in a parallel run.

Dephosphorylation of Gro*P*Ins*P* species

Desalted preparations of both ^{3}H- and ^{32}P-labelled Gro*P*Ins*P* species were dephosphorylated with alkaline phosphatase (method modified from Stephens *et al.*, 1989*a*).

Polyols

myo-Inositol and erythritol were purchased from Sigma. D-Iditol and D-ribitol were prepared from D-idose and D-ribose (purchased from Sigma) by reduction with $NaBH_4$ (Stephens *et al.*, 1988*a*). Similarly, [^{14}C]ribitol was prepared from D-[1-^{14}C]ribose (Amersham). D-[^{14}C]Inositol and D-[^{14}C]glucitol and other standards where indicated in the text were generously given by Dr. L. R. Stephens (Biochemistry Department, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.).

RESULTS

Deacylation of phospholipid extracts from [^{3}H]Ins-labelled *Spirodela polyrhiza* L. gave products with the chromatographic profile detailed in Fig. 1. As the gradient employed was identical with that of Stephens *et al.* (1989*a*), we have tentatively assigned the peaks as Gro*P*Ins, Gro*P*Ins3*P*, Gro*P*Ins4*P* and Gro*P*Ins(4,5)P_2. Of particular interest is the peak eluted 2–3 min before that assigned as Gro*P*Ins4*P* and which contained typically 10% of the ^{3}H in Gro*P*Ins4*P*. H.p.l.c. of ^{32}P-labelled deacylated lipid gave a chromatographic profile (not shown) which in the Gro*P*Ins3*P*/Gro*P*Ins(4,5)P_2 region showed components which were eluted in the same positions as those observed in Fig. 1. However, the early part of the gradient, up to Gro*P*Ins, showed various additional peaks which are likely to be the deacylation products of zwitterionic lipids such as phosphatidylcholine and phosphatidylethanolamine or other acidic phospholipids, eg. phosphatidylglycerol, phosphatidylserine and phosphatidic acid.

Analysis of ^{3}H-labelled lipid extracts by the h.p.l.c. method of Coté *et al.* (1989) revealed (results not shown) a variety of phospholipids, some of which were resistant to deacylation and were assumed to be sphingolipids, as reported in other plant tissues. Of the remaining peaks, the most strongly retarded compound co-eluted with a standard of ^{32}P-labelled PtdIns(4,5)P_2 (generously given by Dr. L. R. Stephens). The earliest-eluted and most heavily labelled peak, and a minor peak eluted shortly thereafter, both gave products on deacylation and h.p.l.c. which were eluted in a position expected for Gro*P*Ins. These lipids were tentatively identified as PtdIns and lysoPtdIns respectively.

Intermediate to the putative lysoPtdIns and PtdInsP_2 peaks was a single peak of radioactivity which on deacylation and subsequent h.p.l.c. was quantitatively recovered as two peaks with the same elution times as the peaks tentatively identified as Gro*P*Ins3*P* and Gro*P*Ins4*P* in the deacylated total-lipid extract of Fig. 1.

Having preliminarily identified various deacylation products, a more rigorous characterization of some of these components was undertaken. Individual peaks of Gro*P*Ins*P* species from h.p.l.c. gradients were desalted as described in the Materials and methods section.

Identification of Gro*P*Ins4*P* in deacylated preparations

A sample of ^{3}H-labelled putative Gro*P*Ins4*P* was mixed with a sample of authentic Gro*P*[^{14}C]Ins4*P* (given by Dr. L. R. Stephens) and rechromatographed on SAX h.p.l.c. The results of this experiment are detailed in Fig. 2. The desalted

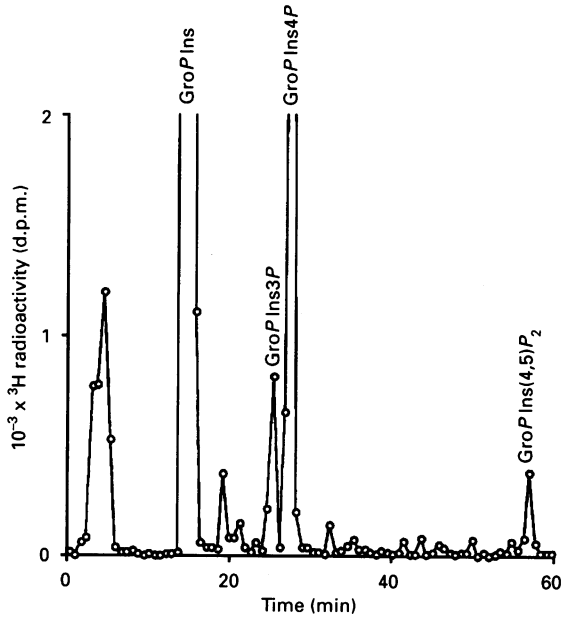

Fig. 1. Anion-exchange separation of the water-soluble products of deacylation of [^{3}H]inositol-labelled lipids

A lipid extract from [^{3}H]inositol-labelled *Spirodela polyrhiza* L. tissue was deacylated. The water-soluble products were applied to a Partisphere SAX h.p.l.c. column and eluted (all steps as described in the Materials and methods section). Fractions (0.7 min) were collected, and a sample was used for measurement of radioactivity. Peaks labelled represent tentative identifications of the components, subsequently confirmed.

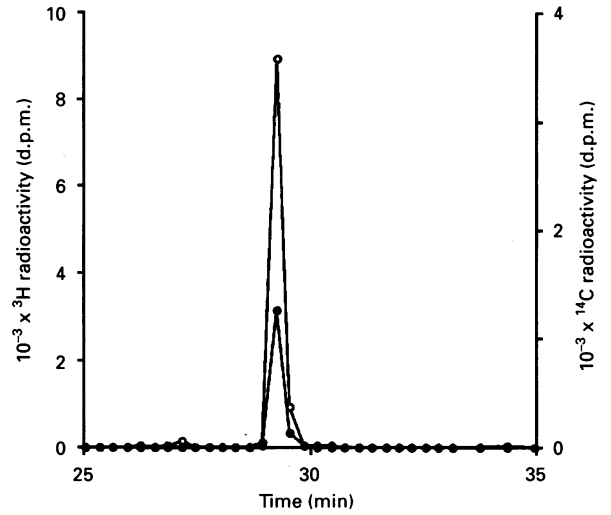

Fig. 2. Anion-exchange separation of h.p.l.c.-purified and desalted ^{3}H-labelled putative Gro*P*Ins4*P*

A sample of h.p.l.c-purified and desalted [^{3}H]inositol-labelled putative Gro*P*Ins4*P* was mixed with authentic Gro*P*[^{14}C]Ins4*P*, re-applied to an anion-exchange column and eluted under the same conditions (all steps as described in the Materials and methods section). Fractions (0.3 min) were collected and their radioactivity was measured. The region of the gradient shown accounted for > 95% of the ^{3}H radioactivity recovered on the gradient. Symbols: ○, ^{3}H; ●, ^{14}C.

sample of ^{3}H-labelled putative Gro*P*Ins4*P* co-chromatographed precisely with the authentic ^{14}C-labelled standard. No other peaks were observed.

Further confirmation of the identity of the putative Gro*P*Ins4*P* and the putative Gro*P*Ins3*P* species was obtained by enzymic dephosphorylation of the ^{3}H-labelled compounds with alkaline phosphatase. Both compounds gave a single ^{3}H-labelled product (confirmed by control incubation in the absence of enzyme) which was eluted in the position expected of Gro*P*Ins, as confirmed by mixing the reaction products with a sample of ^{32}P-labelled putative Gro*P*Ins (results not shown). A similar experiment which employed ^{32}P-labelled Gro*P*Ins*P* species as substrates gave in both cases only two products, these being Gro*P*Ins and P_i (results not shown). The results of both experiments confirm the presence of two distinct Gro*P*Ins*P* species in deacylated lipid extracts of *Spirodela polyrhiza* L.

Identification of Gro*P*Ins3*P* in deacylated preparations

The Gro*P*Ins*P* species obtained above were analysed further. Desalted peaks were treated with 10 mM-periodate to cleave the glycerol moiety, leaving InsP_2. The desalted and freeze-dried products of this mild periodate treatment were mixed with ^{32}P-labelled putative Gro*P*Ins and Gro*P*Ins4*P* and rechromatographed on the gradient used to resolve deacylation products. Using the ^{32}P-labelled putative Gro*P*Ins and Gro*P*Ins4*P* as markers, justified by the fact that they were both eluted from the column at identical times in these two h.p.l.c. runs, the deglyceration products of each of the two Gro*P*Ins*P* species gave different retention times (Fig. 3). That the retention times were different and were typical of InsP_2 species, i.e. they were both eluted after the parent compound, is evidence that the two compounds are different isomers of InsP_2.

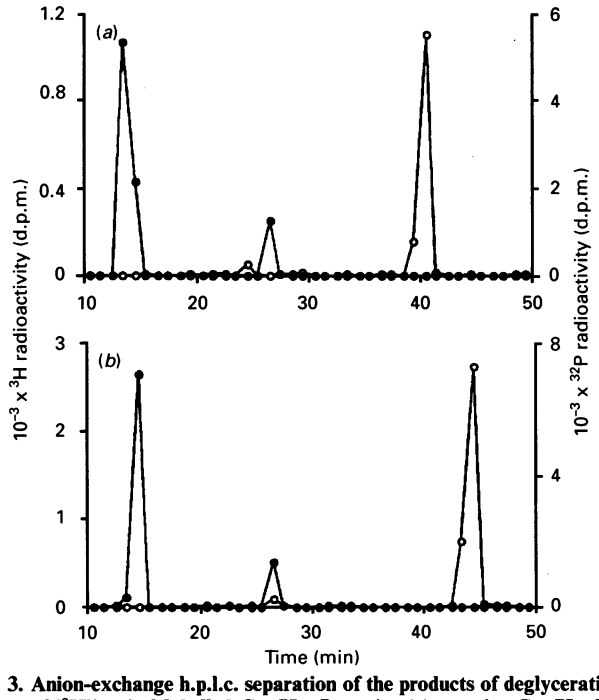

Fig. 3. Anion-exchange h.p.l.c. separation of the products of deglyceration of [^{3}H]inositol-labelled Gro*P*Ins*P* species (*a*) putative Gro*P*Ins3*P*, (*b*) putative Gro*P*Ins4*P*

Samples of h.p.l.c-purified and desalted [^{3}H]inositol-labelled putative Gro*P*Ins*P* species were subjected to mild periodate oxidation, desalted, mixed with h.p.l.c.-purified and desalted ^{32}P-labelled putative Gro*P*Ins and Gro*P*Ins4*P*, re-applied to an anion-exchange h.p.l.c. column and eluted under the same conditions (all steps as described in the Materials and methods section). Fractions were collected [one per min up to 30 min (*a*) and 36 min (*b*), and one every 0.5 min thereafter] and their radioactivity was measured. Symbols: ○, ^{3}H; ●, ^{32}P.

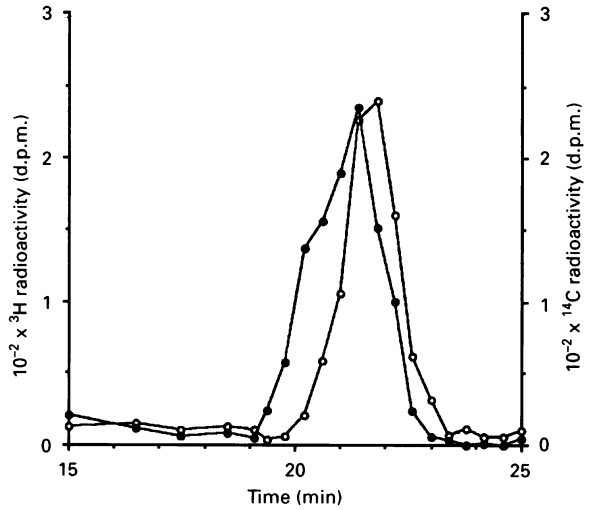

Fig. 4. Cation-exchange (Polypore-Pb^{2+}) separation of polyols derived from ^{3}H-labelled putative Gro*P*Ins3*P*

A sample of h.p.l.c.-purified and desalted [^{3}H]inositol-labelled putative Gro*P*Ins3*P* was oxidized with 0.1 M-periodic acid, pH 2.0, for 36 h at 25 °C and reduced with $NaBH_4$. The sample was desalted, mixed with [^{14}C]inositol and [^{14}C]ribitol and treated with alkaline phosphatase. The products were desalted on Amberlite MB-3 resin. The polyols obtained were applied to a Brownlee Polypore-Pb^{2+} column eluted isocratically with water at a flow rate of 0.2 ml/min (all steps as described in the Materials and methods section). Fractions (every 1 min up to 19 min, and 0.4 min thereafter) were collected and their radioactivity was measured. The region of the chromatograph shown accounted for >95 % of the ^{3}H radioactivity recovered. Symbols: ○, ^{3}H, ●, ^{14}C.

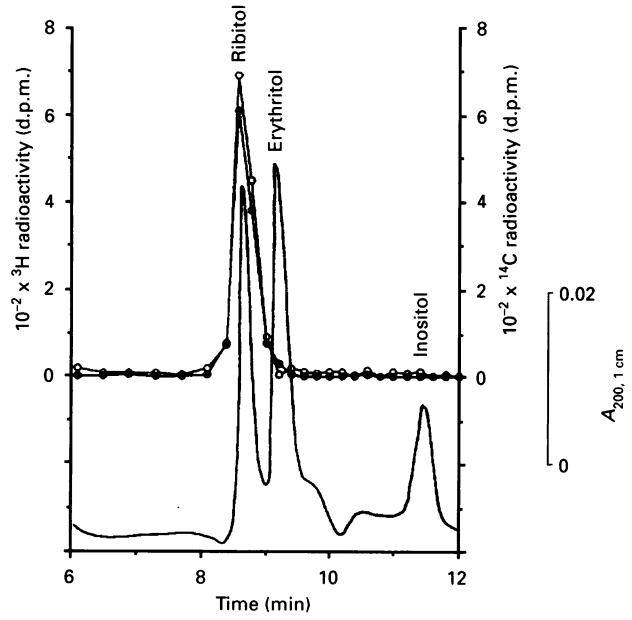

Fig. 5. H.p.l.c. separation of polyols on Microsorb-NH_2 column

A ^{3}H-labelled polyol derived from putative Gro*P*Ins3*P* was mixed with [^{14}C]ribitol and approx. 50 μg of ribitol, erythritol and inositol in acetonitrile/water (13:7, v/v). The sample was applied to a Microsorb-NH_2 column (Rainin Instrument Co., obtained from Anachem, Luton, Beds., U.K.) and eluted isocratically with acetonitrile/water (13:7, v/v) at a flow rate of 0.5 ml/min as described in the Materials and methods section. Fractions (0.4 min up to 8.4 min, and 0.2 min thereafter) were collected and their radioactivity was measured. Symbols: ○, ^{3}H; ●, ^{14}C. Unlabelled polyols were detected by u.v. absorbance at 200 nm in a parallel h.p.l.c. run.

The generation of Ins*P*$_2$ products is again consistent with a Gro*P*Ins*P* structure for the parent compound. A rigorous identification of the Ins*P*$_2$ product of deglyceration of the putative Gro*P*Ins3*P*, and hence of the parent phospholipid, rests on the stereochemical identification of the Ins*P*$_2$ species generated. This was achieved by vigorous oxidation of the ^{3}H-labelled putative Gro*P*Ins3*P* with sodium periodate, reduction with $NaBH_4$ and subsequent alkaline phosphatase treatment to yield a ^{3}H-labelled polyol (see the Materials and methods section). The polyol(s) obtained was spiked with standards of [^{14}C]inositol, [^{14}C]ribitol and [^{14}C]glucitol, and resolved on a Polypore Pb^{2+} cation-exchange column. The results of this experiment (Fig. 4) show that the ^{3}H counts recovered were eluted under the tail of a peak comprising the [^{14}C]inositol and [^{14}C]ribitol standards, which were poorly resolved under these experimental conditions (see also King *et al.*, 1990). That the ^{3}H counts were eluted with the tail of the ^{14}C peak is consistent with the reported elution of ribitol shortly after inositol on the same column (Stephens *et al.*, 1989*b*). The poor resolution of inositol and ribitol was confirmed in parallel h.p.l.c. runs by detection (A_{200}) of 50 μg of unlabelled standard compounds (results not shown).

One remaining possibility is that the ^{3}H label in the polyol prepared from putative Gro*P*Ins3*P* is in fact associated with erythritol, and hence is derived from a different phospholipid. Erythritol was poorly resolved from ribitol on Polypore Pb^{2+} h.p.l.c. (results not shown). Erythritol is the polyol obtained on complete periodate oxidation of Ins(1,2)*P*$_2$ or Ins(2,3)*P*$_2$ (Stephens, 1990). Ins(1,2)*P*$_2$ would be the product of deacylation and subsequent deglyceration of a phospholipid, as yet unidentified, which would have the structure PtdIns2*P*. The possibility that Ins(1,2)*P*$_2$ or its enantiomer Ins(2,3)*P*$_2$ is the parent of the ^{3}H-labelled polyol resolved on Polypore Pb^{2+} h.p.l.c. can be discounted, however, as Ins(1,2)*P*$_2$ and Ins(2,3)*P*$_2$ were eluted after Ins(1,4)*P*$_2$ on strong anion-exchange h.p.l.c. (Stephens *et al.*, 1989*a*), whereas the Ins*P*$_2$ product of deglyceration of ^{3}H-labelled putative Gro*P*Ins3*P* ran before the Ins(1,4)*P*$_2$ product of [^{3}H]Gro*P*Ins4*P* deglyceration (Fig. 3), albeit in a parallel h.p.l.c. run but nevertheless in a run in which internal standards of ^{32}P-labelled putative Gro*P*Ins and Gro*P*Ins4*P* ran in identical positions.

Ins(2,3)*P*$_2$, the enantiomer of Ins(1,2)*P*$_2$ and the only other Ins*P*$_2$ giving erythritol on complete periodate cleavage, could only arise from an as yet unidentified phospholipid with a phosphodiester linkage to the 2- or 3-position of the *myo*-inositol-containing head-group.

Further confirmation of the identity of the ^{3}H-labelled polyol obtained as ribitol, and hence of the parent phospholipid as PtdIns3*P*, was achieved by the separation of ribitol, erythritol and inositol on an amino column eluted isocratically with acetonitrile/water (13:7, v/v). Fig. 5 illustrates the resolution of ribitol, erythritol and inositol and the precise co-chromatography of the ^{3}H-labelled polyol and the [^{14}C]ribitol standard.

The above identification of the parent phospholipid as PtdIns3*P* does not distinguish (Stephens *et al.*, 1989*a*) between the two possible structures of the parent phospholipid, that is 1D-PtdIns3*P* and 1L-PtdIns3*P*.

Identification of Gro*P*Ins(4,5)*P*$_2$ in deacylated preparations

A sample of ^{3}H-labelled putative Gro*P*Ins*P*$_2$ when mixed with a standard of ^{32}P-labelled Gro*P*Ins(4,5)*P*$_2$ prepared by deacylation of authentic ^{32}P-labelled PtdIns(4,5)*P*$_2$ (given by Dr. L. R. Stephens) and rechromatographed on the SAX h.p.l.c.

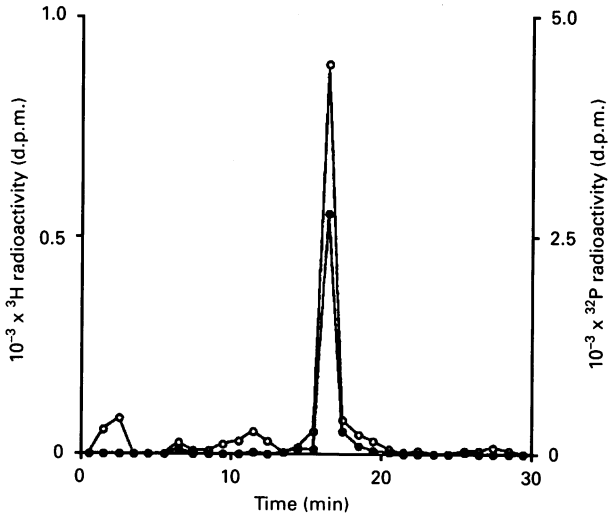

Fig. 6. Anion-exchange separation of products of deglyceration of [^{3}H]inositol-labelled putative Gro*P*InsP_2

A sample of h.p.l.c.-purified and desalted [^{3}H]inositol-labelled putative Gro*P*InsP_2 was subjected to mild periodate oxidation, desalted, mixed with authentic [^{32}P]Ins(1,4,5)P_3, applied to a weak anion-exchange column and eluted (all steps as described in the Materials and methods section). Fractions (1 min) were collected and their radioactivity was measured. Symbols: ○, ^{3}H; ●, ^{32}P.

system described above yielded a single ^{3}H-labelled peak which co-eluted precisely with the ^{32}P-labelled Gro*P*Ins(4,5)P_2 standard (results not shown). On deglyceration (Fig. 6), a sample of the ^{32}P-labelled putative Gro*P*Ins(4,5)P_2 yielded a product which when mixed with an authentic standard of [^{3}H]Ins(1,4,5)P_3 co-eluted precisely with that standard on a WAX column eluted with a gradient adapted from Stephens *et al.* (1989*b*). This result is consistent with the parent Gro*P*InsP_2 species being Gro*P*Ins(4,5)P_2 and the parent phospholipid being PtdIns(4,5)P_2, but is not an entirely rigorous identification, owing to the number of InsP_3 species which co-elute on this h.p.l.c. system (Stephens *et al.*, 1989*b*).

^{32}P-labelling of monoester and diester phosphates of Gro*P*Ins*P*s

In order to ascertain whether there are any differences in the metabolism of PtdIns3*P* and PtdIns4*P* species identified above, an experiment was undertaken to determine the distribution of ^{32}P label between the monoester and diester phosphates of the Gro*P*Ins3*P* and Gro*P*Ins4*P* products of lipid deacylation.

Samples of desalted Gro*P*Ins3*P* and Gro*P*Ins4*P* deacylation products were treated with alkaline phosphatase (as detailed in the Materials and methods section) to liberate the phosphate in the 3- and 4-positions respectively as [^{32}P]P_i. The products of alkaline phosphatase treatment were resolved on the gradient used to resolve the deacylation products. Both Gro*P*Ins*P* species yielded two ^{32}P-labelled products, one which was eluted precisely in the position expected of P_i and an earlier one which co-chromatographed precisely with ^{3}H-labelled putative Gro*P*Ins added after alkaline phosphatase treatment but before h.p.l.c. (results not shown). Analysis of the distribution of ^{32}P label between the P_i and Gro*P*Ins peaks gives the labelling of the 3- and 4-monoester phosphates and the diester phosphate, which is resistant to attack by alkaline phosphatase, respectively. Such an analysis revealed that the monoester phosphate of Gro*P*Ins3*P* was approximately 4 times more heavily labelled than its diester counterpart, whereas for Gro*P*Ins4*P* the monoester and diester phosphates were labelled to approximately similar extents.

Irrespective of possible differences in the turnover of monoester and diester phosphates in the individual phospholipids, the differences between Gro*P*Ins3*P* and Gro*P*Ins4*P* in the labelling of the monoester phosphate group relative to the diester phosphate indicate that the two parent lipids differ from each other in the pattern of synthesis or metabolic turnover.

DISCUSSION

This paper confirms previous reports (Drøbak *et al.*, 1988; Coté *et al.*, 1989) of phosphoinositide components of the well-established Ins(1,4,5)P_3-mediated signal-transduction pathway of animal cells in members of the plant kingdom and extends those observations to monocotyledonous plants. The rigorous identification of PtdIns3*P*, previously unreported in plants, raises the possibility that other phosphoinositide components, namely PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, recently identified in human neutrophils (Stephens *et al.*, 1991) may also be found in plants. In the absence of an identification in the present study of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, the origin of the PtdIns3*P* observed becomes a matter of speculation.

The differences in the labelling of PtdIns3*P* and PtdIns4*P* noted above imply that the two phospholipids have different metabolic and perhaps physiological functions in plants, as has been suggested for these compounds in human neutrophils (Stephens *et al.*, 1991). However, physiological functions for phosphoinositides and their cleavage products in plant tissues have not yet been established. The most convincing evidence of a physiological role for Ins(1,4,5)P_3 is that provided by microinjection of 'caged' Ins(1,4,5)P_3 into guard cells of *Commelina communis* (Blatt *et al.*, 1990) or *Vicia faba* (Gilroy *et al.*, 1990), which on photolytic liberation of Ins(1,4,5)P_3 brought about the physiological response of stomatal closure.

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