Metabolic and structural evidence for the existence of a third species of polyphosphoinositide in cells: D-phosphatidyl-*myo*-inositol 3-phosphate

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When human 1321 N1 astrocytoma cells were labelled to steady state with [³H]inositol and briefly with [³²P]orthophosphate, a compound which contained both radiotracers and which co-migrated with phosphatidylinositol-*myo*-inositol 4-phosphate during t.l.c. could be extracted in acidic chloroform/methanol. Treatment with methylamine under conditions which lead to deacylation of conventional glycerophospholipids yielded a water-soluble moiety which was labelled with both radioisotopes and was eluted from an anion-exchange h.p.l.c. column with a retention time similar to, but distinct from, that of glycerophosphoinositol 4-phosphate. Experiments using sodium periodate and selective phosphatase enzymes to degrade this compound systematically generated a series of products which suggested the structure of the parent phospholipid was phosphatidyl-*myo*-inositol 3-phosphate (PtdIns3P). PtdIns3P is metabolically closely related to the pool(s) of inositol phospholipid(s) that serves as substrate(s) for an agonist-sensitive phosphoinositidase C, as the levels of PtdIns3P fell significantly when 1321 N1 cells were stimulated with carbachol. The relative rate of turnover of the inositol moiety of PtdIns3P is similar to that of both of the major polyphosphoinositides and significantly higher than that of total cellular phosphatidyl-*myo*-inositol. This suggests that all three polyphosphoinositides are synthesized from a common, rapidly metabolized, pool of phosphatidyl-*myo*-inositol.

INTRODUCTION

An inositol tetrakisphosphate that lacks a D-1-phosphate has been identified in both mammalian and avian cells (Stephens *et al.*, 1988a). The metabolic origin of this compound is unknown. Possible sources include inositol phospholipids that either lack a D-1-phosphate or are hydrolysed by a phospholipase D activity. The phospholipids demanded by either of these possibilities have not been described, although their existence has been speculated upon a number of times (Batty *et al.*, 1985; Heslop *et al.*, 1985). These considerations, combined with the recent description of a PtdIns kinase which exclusively makes PtdIns3P (Whitman *et al.*, 1988), at least *in vitro*, led us to investigate the identity of the inositol phospholipids found in [³H]Ins-prelabelled human astrocytoma cells. The experiments reported here characterize a cellular inositol phospholipid whose properties are consistent with it possessing the structure PtdIns3P.

MATERIALS AND METHODS

Radiotracer labelling and extraction of astrocytoma-cell phospholipids

Human astrocytoma cells (1321 N1) were cultured

as described previously (Meeker & Harden, 1982). [³H]Inositol labelling was carried out using inositol-depleted cells according to the following protocol. Confluent cell monolayers were washed once with inositol-free Dulbecco's modified Eagle's medium. The cells were dissociated with trypsin/EDTA (Flow Laboratories), diluted into inositol-free Dulbecco's modified Eagle's medium containing dialysed 5% (v/v) foetal-calf serum (Gibco) and plated out at 20% of their original density in either 3 cm- or 10 cm-diameter Petri dishes. After 12 h the medium was aspirated and replaced with fresh medium. After 5 days the inositol-free medium was replaced with a similar solution containing 2–25 μ Ci of [³H]inositol (New England Nuclear)/ml or 1 μ Ci of [¹⁴C]Ins (Amersham International)/ml, and the cells were used after 48–60 h incubation with tracer.

During this labelling period, both 'inositol-depleted' and 'normal' cultures of 1321 N1 cells expanded approximately linearly with time, although 'inositol-depleted' cells did so at only 60 $\pm$ 6% of the rate for 'normal' cells. After 48–60 h, the incorporation of label into phospholipids of inositol-depleted cells reached a constant value approx. 20–22 times (per culture plate) greater than that in normal cells at the same time. Moreover, at this time the incorporation of tracer into inositol phospholipids of normal cells was still rising. For experiments involving hormonal stimulation the labelling medium was removed

Abbreviations used: Ins, *myo*-inositol; InsP, InsP₂, InsP₃, InsP₄ and InsP₅, *myo*-inositol mono-, bis, tris, tetrakis and pentakis-phosphates; the positions of the phosphates on a given inositol phosphate are denoted by numbering from the position of the phosphate in D-InsP; PtdIns, phosphatidyl-*myo*-inositol; PtdInsP and PtdInsP₂, phosphatidyl-*myo*-inositol phosphate and phosphatidyl-*myo*-inositol bisphosphate; PtdOH, phosphatidic acid; GroPIns, glycerophosphoinositol; GroPInsP, glycerophosphoinositol phosphate; GroPInsP₂, glycerophosphoinositol bisphosphate; BSA, bovine serum albumin; Cho, choline; PDGF, platelet-derived growth factor.

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and replaced with Dulbecco's modified Eagles medium containing 0.5% (w/v) bovine serum albumin (BSA; recrystallized fraction V; Sigma), NaHCO_3 (3 g/l) and 25 mM-Hepes (pH approx. 7.4 at 37°C in air). Agonist additions were made in 0.5 ml of prewarmed medium. Reactions were terminated by rapidly aspirating the medium and replacing it with 1.5 ml of ice-cold 20% (w/v) trichloroacetic acid.

For [^{32}P]P_i labelling experiments, astrocytoma cells were first labelled with [^3H]inositol as described above for 2 days in 10 cm-diameter Petri dishes with a final concentration of [^3H]Ins of 2 $\mu\text{Ci}/\text{ml}$ in a total volume of 20 ml. The cells were then washed twice with phosphate and inositol-free Dulbecco's-modified Eagle's medium containing recrystallized 0.5% (w/v) BSA, 25 mM-Hepes and 1% (v/v) antibiotics (penicillin/streptomycin; Flow Laboratories) which had been equilibrated with 5% CO_2 at 37°C. After 1 h the cells were washed once more with the above medium before recommencing incubation in 10 ml of the same medium containing 1 mCi of [^{32}P]P_i (Amersham). The medium was then rapidly aspirated and the cells were quenched with 5 ml of ice-cold 20% (w/v) trichloroacetic acid.

Phospholipid extraction was carried out by quantitatively transferring the cell debris and supernatants from trichloroacetic acid-precipitated cell lysates to polypropylene test tubes and sedimenting the precipitates in a refrigerated bench-top centrifuge. The supernatants were removed and the pellets washed once with 5% trichloroacetic acid/2 mM-EDTA and then solubilized by vigorous mixing in 1 ml of (0.1 M-HCl/0.1 mM-inositol/10 mM-EDTA)/methanol/chloroform (4:9:5, by vol.). Two phases were obtained by the addition of 370 μl of chloroform and 370 μl of a solution containing 0.1 M-HCl, 0.1 mM-inositol and 10 mM-EDTA. After mixing and centrifugation the upper phases were removed and the lower and interphases were washed with 1.33 ml of 'synthetic' upper phase. The remaining cell debris and lower phase were dried under vacuum and either resuspended in chloroform (if they were to be subjected to t.l.c.) or deacylated (see below).

Preparation of rat brain cytosol fractions

Rat brain homogenates were prepared in 0.25 M-sucrose/50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol/50 μM -phenylmethanesulphonyl fluoride/ethanpain (1 $\mu\text{g}/\text{ml}$)/leupeptin (1 $\mu\text{g}/\text{ml}$)/pepstatin A (1 $\mu\text{g}/\text{ml}$), pH 7.0 at 4°C, at a tissue density of one brain + 5 ml of homogenization buffer. The homogenates were centrifuged at 60000 g for 20 min, and the supernatant was used immediately as a source of Ins(1,3)P₂ 3-phosphate phosphatase, Ins(1,4,5)P₃ 3-hydroxykinase or Ins(1,3,4)P₃ 4-phosphate phosphatase (see below).

Separation techniques

T.l.c. was performed using 10 cm × 10 cm plates (Kieselgel-60; Merck 5631) with the solvent system described by Schacht & Agranoff (1974): chloroform/methanol/28% NH_3 /water (45:35:11:3, by vol.). Ptd [^{14}C]Ins (0.1 μCi , Amersham International; mixed with 25 nmol of carrier PtdIns) and aliquots of ^{32}P -labelled human erythrocyte phospholipid extracts [containing 50 nmol of phospholipid phosphorus and prepared as described by Hawkins *et al.* (1986)] were used as standards (see below). Once the chromatograms had been fully developed, the silica layer was dried and the ^{14}C - and

^{32}P -labelled phospholipids were localized autoradiographically using Kodak X-Omat RP film.

Glycerophosphoinositol phosphates were separated using a Partisphere SAX anion-exchange h.p.l.c. column (Whatman), which was eluted with a gradient based on buffers A (water)/B [1.25 M-(NH_4)₂HPO₄ (adjusted to pH 3.8 with H_3PO_4 at 25°C)] at a flow rate of 1.0 ml·min⁻¹: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 52 min, 20% B; 64 min, 100% B; 70 min, 100% B; 71 min, 0% B.

Inositol biphosphates were separated by using the same column, buffers and flow rate, with the following gradient: 0 min, 0% B; 5 min, 0% B; 6 min, 10% B; 60 min, 10% B; 61 min, 100% B; 70 min, 100% B; 71 min, 0% B.

In some preparative situations a weak anion-exchange h.p.l.c. column (Partisphere WAX, Whatman) was utilized (see below). It was eluted with the following gradient based on A (water) and B [2.0 M-triethylamine (pH 3.8 with formic acid at 25°C)], at a flow rate of 1 ml·min⁻¹: 0 min, 0% B; 5 min, 0% B; 45 min, 100% B; 50 min, 100% B; 51 min, 0% B. This procedure had the advantage that the eluate could be freeze-dried directly.

The dephosphorylation products of GroPInsP, GroPInsP₂ and InsP₂ species (see below) were separated by using a Partisil 10-SAX anion-exchange h.p.l.c. column which was eluted with a gradient based on buffer A (water) and B [1.7 M-ammonium formate (pH 3.7 with H_3PO_4 at 25°C)] at a flow rate of 1.25 ml·min⁻¹: 0 min, 0% B; 5 min, 0% B; 45 min, 29% B; 46 min, 70% B; 48 min, 70% B; 50 min, 0% B.

Polyols contained in 10 μl aliquots of water were separated with a Brownlee-Polypore cation-exchange h.p.l.c. column in the Pb²⁺ mode (Anachem), maintained at 25°C by a thermostatically controlled jacket and eluted with water at a flow rate of 0.2 ml·min⁻¹. Polyols were detected in the eluate from the column by liquid-scintillation counting of individual fractions or by an on-line differential refractometer (Waters model 410).

Desalting fractions of the h.p.l.c. eluate

Compounds in triethylammonium formate or ammonium formate were dried under vacuum. Fractions containing H_3PO_4 were neutralized with triethylamine, diluted 10-fold with water, and applied to a 2 cm × 0.6 cm column of Bio-Rad AG 1X8 (200–400 mesh, formate form) resin. P_i was eluted with 9 ml of 0.15 M-ammonium formate/0.1 M-formic acid. GroPInsP, InsP₂, GroPInsP, InsP₃ and InsP₄ species were eluted as described previously (Downes *et al.*, 1986) in ammonium formate/formic acid mixtures.

Preparation of standards

^{32}P -labelled PtdOH, PtdIns4P and PtdIns(4,5)P₂ were prepared from human erythrocytes as described previously (Hawkins *et al.*, 1986). Aliquots of these preparations were deacylated with methylamine as described previously (Clarke & Dawson, 1981) to yield the corresponding mixture of [^{32}P]glycerophosphoinositol phosphate esters, which were purified and desalted as described above. GroP[^3H]Ins(4,5)P₂ was prepared from [^3H]Ins-prelabelled primary-cultured macrophages as described previously (Stephens *et al.*, 1988a).

[3-phosphate- ^{32}P]Ins(1,3,4,5)P₄ was prepared by incubating 2.0 nmol of Ins(1,4,5)P₃ (Amersham International) with 57.2 μCi of [γ - ^{32}P]ATP (tetraethyl-

ammonium 'stabilized' salt; Amersham International), which had been purified by anion-exchange h.p.l.c. as described previously (Stephens *et al.*, 1988b) before being used as a substrate, and 200 μ l of rat brain cytosol (prepared as described above) in 50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol/0.5 mM-MgNa₂ATP/2 mM-MgCl₂ (pH 7.0, 37 °C) in a total volume of 1 ml for 2.5 min. The reaction was terminated with HClO₄, treated with charcoal, neutralized, and purified by h.p.l.c. as described previously (Stephens *et al.*, 1988b). The fractions (containing Ins[³²P]₄ species) were neutralized with triethylamine and desalted as described above (the yield was 38 % of the starting material, i.e. 0.76 mol of [3-phosphate-³²P]Ins(1,3,4,5)P₄). In order to assess the homogeneity of the [3-phosphate-³²P]-Ins(1,3,4,5)P₄ preparation, an aliquot was mixed with [³H]Ins(1,3,4,6)P₄ and applied to a Partisphere WAX anion-exchange column and eluted as described previously (Stephens *et al.*, 1988c). The ³²P-labelled compound was eluted as a single peak with baseline separation from the [³H]Ins(1,3,4,6)P₄ (results not shown).

[³H]Ins(1,3,4)P₃ was prepared as described previously (Stephens *et al.*, 1988c). [3-phosphate-³²P]Ins(1,3,4)P₃ was prepared by incubating 0.15 nmol of [3-phosphate-³²P]Ins(1,3,4,5)P₄ with 200 μ l of packed human erythrocyte ghosts [prepared as described previously (Stephens *et al.*, 1988c)] in 50 mM-Hepes/2 mM-EGTA/2 mM-MgCl₂/BSA (1 mg/ml), pH 7.0 (final volume 1 ml) at 37 °C for 60 min. The reaction was quenched with HClO₄, neutralized with tri-*n*-octylamine and the products resolved on a Partisil 10-SAX h.p.l.c. column as described previously (Stephens *et al.*, 1988a). Of the starting Ins[³²P]P₄, 87 % was recovered as [3-phosphate-³²P]Ins(1,3,4)P₃.

Ins[³²P](4,5)P₂ was prepared by boiling GroPIns[³²P](4,5)P₂ (prepared as described above) with 2 M-KOH. After 1 h the reaction mixture was cooled and neutralized with HClO₄. The KClO₄ formed was pelleted by centrifugation and the ³²P-labelled reaction products were resolved on a Partisphere SAX column (see above). The fractions containing the major Ins[³²P]P₂ product (approx. 50 % of the recovered radioactivity) were pooled and desalted as described above. The identity of this peak was confirmed by treating an aliquot of GroP[³H]Ins[³²P](4,5)P₂ (prepared as described above) in an identical manner. The major product had a ³H/³²P ratio identical with that of the starting material (results not shown).

Ins[³²P](1,4)P₂ was prepared from [³²P]P_i-prelabelled human erythrocytes and h.p.l.c.-purified as described previously (Downes *et al.*, 1986). [3-phosphate-³²P]-Ins(1,3)P₂ and [3-phosphate-³²P]Ins(3,4)P₂ were prepared by incubating [3-phosphate-³²P]Ins(1,3,4,5)P₄ with rat brain homogenate (300 μ l total assay volume, containing 50 mM-Hepes, 2 mM-EGTA, 1 mM-MgCl₂, 10 mM-LiCl and 40 μ l of rat brain homogenate, pH 7.0 at 37 °C) for 15 min. The products of this reaction which have been established to be Ins(1,3)P₂ and Ins(3,4)P₂ (Stephens *et al.*, 1988c) were obtained in 22 % and 23 % yields and were resolved by h.p.l.c. on a Partisphere SAX column, neutralized and desalted as described above.

[³H,3-phosphate-³²P]Ins(1,3)P₂ was prepared from a mixture of [³H]Ins(1,3,4)P₃ and [3-phosphate-³²P]-Ins(1,3,4)P₃ (prepared as described above) by incubating the trisphosphate substrates with rat brain cytosol in the presence of EDTA for 10 min (the final assay volume

was 1 ml and contained 50 mM-Hepes, 2 mM-EDTA, 80 mM-KCl, 250 μ l of rat brain cytosol fraction and 0.7 nmol of substrate. The incubation was terminated with HClO₄, neutralized, applied to a Partisphere SAX h.p.l.c. column and eluted as described above; 60 % of the starting substrate was recovered as [³H,3-phosphate-³²P]Ins(1,3)P₂.

DL-Ins[³²P](1,2)P₂ was prepared by boiling an aliquot of [3-phosphate-³²P]Ins(1,3)P₂ in 50 μ l of 1.0 M-HCl for 8 min. Under these conditions, phosphate migration across *cis*-related hydroxy groups in *myo*-inositol (i.e. the 1 and 2 and/or 2 and 3 positions) occurs without significant migration between *trans*-related neighbouring hydroxy groups (Pizer & Ballou, 1959; L. Stephens, P. T. Hawkins & C. P. Downes, unpublished work). The sample was then cooled, neutralized precisely with 2 M-KOH and 0.2 M-Hepes, diluted 10-fold with water and desalted as described above. Two Ins[³²P]P₂ species could be resolved by a Partisphere SAX column. One possessed the retention time expected for Ins(1,3)P₂ and the second peak, comprising 50 % of the recovered ³²P, was eluted after [³H]Ins(1,4)P₂ and was presumed to be Ins[³²P](1,2)P₂. This was confirmed by processing a ³H-labelled sample in parallel, which yielded a bisphosphate that gave [³H]erythritol upon periodate oxidation, reduction and dephosphorylation.

[¹⁴C]Ins4P was prepared as described previously (Stephens *et al.*, 1988c). [¹⁴C]Ins3P was purchased from Amersham International. Unlabelled and ¹⁴C-labelled polyols were obtained from previously described sources or prepared as described by Stephens *et al.* (1988a,c).

Dephosphorylation of inositol bisphosphates by 'inositol polyphosphate 3-phosphate phosphomonoesterase'

Rat brain cytosol (prepared as described above) was incubated with 0.2 nmol of [³H]3-phosphate-³²P]-Ins(1,3)P₂ in a buffer containing 250 μ l of rat brain cytosol preparation, 2 mM-EDTA, 20 mM-KCl and 50 mM-Hepes, pH 7.0 at 37 °C, in a final volume of 1 ml for 10 min. Samples were quenched with HClO₄ and processed for application to an anion-exchange h.p.l.c. column as described above.

Dephosphorylation of glycerophosphoinositol phosphates

GroP[³H]Ins[³²P]P and GroP[³H]Ins[³²P]P₂ were hydrolysed to GroP[³H]Ins and [³²P]P_i by incubation with alkaline phosphatase (Sigma). The substrates (in their acid forms) were dissolved in 250 μ l of water and mixed with 750 μ l of buffer containing 20 mM-ethanolamine, 2 mM-MgCl₂, 0.1 % (w/v) BSA and alkaline phosphatase (1 unit/ml; units defined in glycine buffer; Sigma), pH 9.5 at 30 °C. Samples were quenched after 0 or 60 min with HClO₄, neutralized, and processed for anion-exchange h.p.l.c. as described previously (Stephens *et al.*, 1988a).

RESULTS

[³H]Ins-labelled phospholipids in 1321 N1 cells

[³H]Ins-prelabelled 1321 N1 cells were quenched with trichloroacetic acid and extracted with chloroform/methanol/0.1 M-HCl (5:9:4, by vol.). The phospholipids in this extract were deacylated and the water-soluble products were mixed with ³²P- and ¹⁴C-labelled standards and applied to an anion-exchange column (as described above). Major peaks of ³H were eluted with GroPIns-

Fig. 1. Anion-exchange h.p.l.c. separation of the water-soluble deacylation products from [^3H]Ins-labelled 1321 N1 cells

A phospholipid extract from [^3H]Ins-prelabelled 1321 N1 cells was prepared and deacylated as described in the Materials and methods section. The water-soluble products were mixed with various combinations of the following: GroPIns[^{32}P]4P, GroPIns[^{32}P](4,5) P_2 , GroPIns[^{32}P]3P, GroP[^{14}C]Ins, [^{14}C]Ins3P, Ins[^{32}P](1,4) P_2 , Ins[^{32}P](1,4,5) P_3 , Ins[^{32}P](1,3,4,5) P_4 and Ins[^{32}P](1,3,4,5,6) P_5 , and applied to an anion-exchange h.p.l.c. column. The column was eluted as described in the Materials and methods section; 0.4 min (from 0–16 min) and 0.3 min (16 min onwards) fractions were collected and counted for radioactivity using standard dual-label liquid-scintillation counting techniques.

[^{32}P](4,5) P_2 , GroP[^{14}C]Ins (results not shown) and GroP Ins[^{32}P]4P (see Fig. 1). No ^3H -labelled compounds that could be derived from putative PtdIns P_3 or PtdIns P_4 species were detected. However, an unidentified peak of ^3H was eluted just prior to GroPIns[^{32}P]4P. In six independent cell preparations this peak contained between 3 and 15% of the ^3H found in the compound that co-migrated with GroPIns[^{32}P]4P. An analysis of the structure and metabolism of the unidentified inositol phospholipid that yielded the above compound after deacylation was therefore undertaken.

Structural analysis of the unidentified inositol phospholipid in 1321 N1 cells

Cultures of 1321 N1 cells were labelled with [^3H]Ins and sometimes also with [^{32}P]P $_i$ as described in the Materials and methods section. The cells were quenched with acid and a phospholipid extract prepared as described above.

Aliquots of these extracts were resolved by t.l.c. (see the Materials and methods section for details). The distribution of ^{32}P among the phospholipids in the extract was similar to that seen for numerous other cell types labelled briefly with [^{32}P]P $_i$, with most of the radioactivity located in lipids co-chromatographing with PtdIns(4,5) P_2 , PtdIns4P, PtdOH and PtdCho. No [^{32}P]P $_i$ could be detected in PtdIns.

The [^{32}P]phospholipids were scraped from the plates, deacylated and applied to an anion-exchange h.p.l.c. column that was eluted as described above. The PtdIns P_2 spots derived from 1321 N1 cells yielded a single $^{32}\text{P}/^3\text{H}$ -labelled water-soluble compound as resolved by h.p.l.c. (in 92% yield from the parent lipid)

which had a $^3\text{H}/^{32}\text{P}$ ratio of 50 after 20 min and 8.9 after 50 min labelling with [^{32}P]P $_i$. The retention time of the Ptd[^3H]Ins[^{32}P] P_2 deacylation product was similar to that of GroPIns[^{32}P](4,5) P_2 . When total phospholipid extracts from cultures of 1321 N1 cells, which had only been labelled with [^3H]Ins, were deacylated with methylamine they yielded a ^3H -labelled compound which migrated with GroPIns[^{32}P](4,5) P_2 during anion-exchange h.p.l.c. (see above).

When the [^3H]Ins[^{32}P]P-labelled material co-migrating with PtdIns4P during t.l.c. was applied to an anion-exchange column, 93–94% of the ^{32}P in the original t.l.c. scrapings was recovered in three peaks. The first peak, which was eluted as a leading shoulder to the ^3H -labelled metabolite (see Fig. 2), was not labelled with ^3H and was apparently derived from an unidentified lipid that was slightly more mobile than PtdInsP during t.l.c. (results not shown). This peak was not analysed further. The first dual-labelled peak of material to be eluted from the column possessed a $^3\text{H}/^{32}\text{P}$ ratio of 48 after 20 min and 14 after 50 min labelling with [^{32}P]P $_i$ and would appear to be the unidentified [^3H]Ins-labelled deacylation product noted above. The peak that was eluted second possessed a $^3\text{H}/^{32}\text{P}$ ratio of 48 after 20 min and 13.5 after 50 min labelling with [^{32}P]P $_i$. The deacylated phospholipid extracts derived from cultures labelled only with [^3H]Ins yielded two ^3H -labelled metabolites, which were eluted in this part of the h.p.l.c. gradient. The major peak, with the longer retention time, co-migrated with GroPIns[^{32}P]4P.

Samples of the water-soluble, $^3\text{H}/^{32}\text{P}$ -labelled and [^3H]Ins-labelled deacylation products isolated by h.p.l.c. were desalted, and a portion of each of these preparations

Fig. 2. Anion-exchange h.p.l.c. separation of the water-soluble deacylation products of t.l.c.-purified 132I-N1-cell Ptd[^3H]Ins[^{32}P]P species

An aliquot of Ptd[^3H]Ins[^{32}P]P species purified from a phospholipid extract of [^3H]Ins- and [^{32}P]P_i-prelabelled 132I N1 cells by t.l.c. was deacylated, and the water-soluble deacylation products were applied to an anion-exchange h.p.l.c. column and eluted; 0.4 min fractions were collected and counted for radioactivity. About 93–94% of the ^{32}P in the original t.l.c. scrapings was recovered in the region of the chromatogram shown.

was treated with alkaline phosphatase for 0 or 60 min. The products were separated by anion-exchange h.p.l.c. (see the Materials and methods section and Fig. 3 and its legend for details; the data for the PtdInsP₂-derived deacylation product are not shown). The three ^3H -labelled deacylation products yielded a single peak of radioactivity which was co-eluted with internal GroP[^{14}C]Ins (results not shown). The three $^{32}\text{P}/^3\text{H}$ -labelled water-soluble deacylation products yielded two radioactively labelled products. One peak, containing only ^{32}P , was eluted at precisely the time expected for P_i. The other products, which were eluted at the time expected for GroPIns, each possessed $^3\text{H}/^{32}\text{P}$ ratios of greater than 200.

These strongly suggest that all three water-soluble $^3\text{H}/^{32}\text{P}$ -labelled deacylation products are glycerophosphoinositol phosphates and, moreover, that > 98% of the [^{32}P] is located in their monoester phosphate moieties (this being consistent with the observation that, under the serum-free conditions in which the cells were labelled, no [^{32}P]P_i could be detected in PtdIns).

Configuration of the inositol phosphate headgroups of PtdInsP species

Further portions of the h.p.l.c.-purified desalted, $^{32}\text{P}/^3\text{H}$ - and ^3H -labelled glycerophosphoinositol phosphates (the same samples that were treated with alkaline phosphatase above) were converted into their corresponding bisphosphates by oxidation with sodium periodate, under conditions designed to restrict oxidation to the glycerol moiety (Brown & Stewart, 1966; see the Materials and methods section). The residual aldehyde moieties remaining after periodate oxidation were

Fig. 3. Anion-exchange h.p.l.c. separation of the products of the action of alkaline phosphatase on the unidentified GroP[^3H]Ins[^{32}P]P

An aliquot of the h.p.l.c.-purified, desalted, unidentified GroP[^3H]Ins[^{32}P]P (see Fig. 2) was incubated with alkaline phosphatase for 0 min (a) or 60 min (b) as described in the Materials and methods section. The products of the reaction were applied to an anion-exchange h.p.l.c. column, eluted and collected into 0.3 min fractions, which were counted for radioactivity utilizing standard dual-label liquid-scintillation counting techniques. In parallel samples the ^3H -labelled product co-chromatographed with internal GroP[^{14}C]Ins (results not shown).

eliminated with 1,1-dimethylhydrazine and the resulting ^3H - and $^3\text{H}/^{32}\text{P}$ -labelled products were applied to a Partisphere WAX column and eluted with a triethylamine/formic acid gradient (see the Materials and methods section). All three glycerophosphoinositol phosphates (whether dual- or single-labelled) were quantitatively recovered as single peaks of radioactivity which were eluted later than their respective starting materials. The peak fractions were desalted by freeze-drying. Aliquots of these samples were mixed with various ^{32}P -labelled inositol bisphosphates and separated by anion-exchange h.p.l.c. (see Fig. 4 and its legend and the Materials and methods section for details), or subjected to more vigorous periodate oxidation designed to cleave accessible carbon-carbon bonds within the inositol moiety (see the Materials and methods section).

The [^3H]glycerophosphoinositol monophosphate derived from the predominant PtdInsP of astrocytoma cells yielded a ^3H -labelled compound which was co-eluted with Ins[^{32}P](1,4)P₂ and, upon vigorous periodate oxidation, eventually (5 days) yielded volatile ^3H via intermediates which upon reduction and dephosphorylation yielded L-[^3H]altritol and D-[^3H]iditol in a ratio of 1:7 (results not shown; see Stephens *et al.*, 1988c). These results suggest that the original GroPInsP had the

Fig. 4. Anion-exchange h.p.l.c. separation of the [^3H]inositol phosphate head groups from the unidentified [^3H]Ins-labelled phospholipid in 1321 N1 cells

Portions of h.p.l.c.-purified desalted unidentified GroP[^3H]InsP (prepared exactly as described in Fig. 3 and in the Materials and methods section, except that the original cultures of 1321 N1 cells were labelled only with [^3H]Ins) were subjected to mild periodate oxidation (see the Materials and methods section). The ^3H -labelled products were h.p.l.c. purified, desalted, mixed with the ^{32}P -labelled InsP₂ species indicated and separated by anion-exchange h.p.l.c. as described in the text. The column eluate was collected into fractions which were individually counted for radioactivity utilizing standard dual-label liquid-scintillation counting techniques; 92% of the ^3H radioactivity applied to the column was recovered in the region of the salt gradient displayed.

structure D-glycerophosphoinositol 4-phosphate and its parent lipid that of PtdIns4P, as has been described previously (Tomlinson & Ballou, 1960).

That the compound possesses this structure and is not Gro4PIns1P (i.e. the compound in which the 4-phosphate is the site of the phosphodiester bond) is based on the assumption that Gro4PIns4P would not co-migrate precisely with GroPIns4P on an anion-exchange h.p.l.c. column. As these compounds are not stereoisomers, this assumption is very likely to be correct.

The quantitatively minor [^3H]glycerophosphoinositol monophosphate (which was eluted first when subjected to anion-exchange h.p.l.c.) yielded a ^3H -labelled compound which co-chromatographed with Ins[^{32}P](1,3)P₂ during anion-exchange h.p.l.c. (see Fig. 4) and yielded [^3H]adonitol (91% overall recovery of ^3H) upon periodate oxidation, reduction and dephosphorylation (data not shown). These observations are only consistent with the structure of the product of mild periodate oxidation of the minor glycerophosphoinositol monophosphate being [^3H]Ins(1,3)P₂ (note the plane of symmetry in this molecule) and therefore the structure of the parent GroPInsP is D- or L-GroPIns3P and that of the original lipid D- or L-PtdIns3P (the alternative locations for the glycerol in these compounds yield stereoisomers that would not be resolved by h.p.l.c.).

The stereochemical assignment of this series was resolved by incubating the dual-labelled Ins(1,3)P₂ prepared from DL-GroPIns3P (which contained > 98% of its ^{32}P in the monoester phosphate group; see above) in

Fig. 5. Anion-exchange h.p.l.c. separation of the products of [^3H ,3-phosphate- ^{32}P]Ins(1,3)P₂ metabolism by rat brain cytosol in the presence of EDTA

[^3H ,3-phosphate- ^{32}P]Ins(1,3)P₂ (prepared as described in the Materials and methods section) was incubated with rat brain cytosol in the presence of 5 mM-EDTA for 0 min (a) or 10 min (b). The reactions were quenched, and samples were applied to an anion-exchange column, eluted, and collected into fractions. Fractions were individually counted for radioactivity utilizing standard dual-label liquid-scintillation counting techniques.

parallel with a sample of [^3H ,3-phosphate- ^{32}P]Ins(1,3)P₂ (prepared as described above) with rat brain cytosol in the presence of 5 mM-EDTA. Bansal *et al.* (1987), have demonstrated that calf brain cytosol contains an Mg²⁺-independent inositol polyphosphate 3-phosphate phosphatase which converts Ins(1,3)P₂ into Ins1P and P_i. That rat brain cytosol contains a similar activity is shown in Fig. 5. The [^3H ,3-phosphate- ^{32}P]Ins(1,3)P₂ standard yielded [^3H]InsP and [^{32}P]P_i. As there was no [^3H]Ins produced, the InsP cannot have been further dephosphorylated under these conditions and hence the [^{32}P]P_i must be related during the hydrolysis of the [^3H ,3-phosphate- ^{32}P]Ins(1,3)P₂ by an inositol 1,3-bisphosphate 3-phosphate phosphomonoesterase. The sample of [^3H]Ins[^{32}P](1,3)P₂ derived from D- or L-Ptd[^3H]Ins[^{32}P]3P was dephosphorylated by this preparation to yield an inositol monophosphate containing < 2% of the ^{32}P recovered in the products (see Fig. 6). Greater than 98% of the ^{32}P recovered in the products of the dephosphorylation reaction was in P_i; as no [^3H]Ins was produced during the progress of the assay, this [^{32}P]P_i must have been derived from the original substrate. This means that the D-3-phosphate of the [^3H]Ins[^{32}P](1,3)P₂, derived from D- or L-Ptd[^3H]Ins[^{32}P]3P, contained > 98% of the ^{32}P . The D-3 phosphate group must therefore be the monoester phosphate group of the parent phospholipid, which is consequently identified as D-PtdIns3P.

Fig. 6. Metabolism of the head group of the unknown phospholipid by rat brain cytosol in the presence of EDTA: separation of products by anion-exchange h.p.l.c.

An aliquot of the [^3H]Ins[^{32}P] P_2 prepared from the unidentified GroP[^3H]Ins[^{32}P] P was incubated with rat brain cytosol in the presence of 5 mM-EDTA for 0 min (a) or 10 min (b) and the samples were processed exactly as described in the legend to Fig. 5.

Basal and stimulated metabolism of PtdIns3P

The metabolic relationship between PtdIns3P and the other identified inositol phospholipids in 1321 N1 cells was investigated by measuring the relative rates of turnover of the inositol moieties in PtdIns, PtdIns3P, PtdIns4P and PtdIns(4,5) P_2 and [^{14}C , ^3H]inositol dual-labelling as described in the Materials and methods section and by measuring the changes in levels of these lipids upon agonist stimulation. Astrocytoma cells were dual-labelled with [^{14}C]Ins (1 $\mu\text{Ci}/\text{ml}$ for 2.5 days; the initial concentration of Ins in the medium of these cultures was approx. 4 μM) and [^3H]Ins (20 $\mu\text{Ci}/\text{ml}$ for the final 90–150 min of the [^{14}C]Ins prelabelling period defined above). Phospholipids from cells labelled to steady state with [^{14}C]Ins and briefly with [^3H]Ins were extracted, deacylated and separated on an anion-exchange h.p.l.c. column as described above. The $^3\text{H}/^{14}\text{C}$ ratio of GroPIns was significantly lower than those of GroPIns3P, GroPIns4P and GroPIns(4,5) P_2 (see Table 1).

To quantify the changes in the levels of PtdIns3P during agonist stimulation, astrocytoma cells were prelabelled with [^3H]Ins and incubated with carbachol or vehicle for various times. After terminating the incubations with trichloroacetic acid the residual tissue pellet was deacylated with methylamine, and the resulting

Table 1. Relative rates of turnover of the inositol moiety in the inositol phospholipids of 1321 N1 cells

1321 N1 cells were labelled for 2.5 days with [^{14}C]Ins (1 $\mu\text{Ci}/\text{ml}$ final concn.) and, for the last 90 min of this period, [^3H]Ins (20 $\mu\text{Ci}/\text{ml}$ final concn.). Reactions were terminated with ice-cold 10% (w/v) trichloroacetic acid and a phospholipid extract was prepared and deacylated as described in the text. The water-soluble deacylation products were applied to an anion-exchange h.p.l.c. column and eluted as described in the text. The ^3H and ^{14}C content of each inositol phospholipid headgroup was measured by counting individual fractions of column eluate for radioactivity using standard dual-label liquid-scintillation counting techniques. All three polyphosphoinositides possessed significantly higher $^3\text{H}/^{14}\text{C}$ ratios than did PtdIns ($P < 0.05$).

Inositol phospholipid	Radioactivity (d.p.m.) (mean, $n = 3$)		$^3\text{H}/^{14}\text{C}$ ratio (mean $\pm$ S.E.M. $n = 3$)
	^3H	^{14}C	
PtdIns	8035622	4083761	1.925 ± 0.077
PtdIns3P	18499	8494	2.18 ± 0.036
PtdIns4P	350901	148541	2.36 ± 0.024
PtdIns(4,5) P_2	621868	272954	2.28 ± 0.046

water-soluble deacylation products were mixed with ^{32}P -labelled standards and separated by anion-exchange h.p.l.c. (see Fig. 1 and the Materials and methods section). The levels of Ptd[^3H]Ins(4,5) P_2 fell rapidly upon stimulation (see Fig. 7) and followed a pattern of change with time that has been described on numerous occasions in other cell types and with different agonists. Its level declined to a minimum after 0.5–3 min stimulation, then returned towards control levels, despite the continued presence of agonist. In contrast, the levels of Ptd[^3H]Ins4P rose dramatically upon stimulation before paralleling the changes in Ptd[^3H]Ins(4,5) P_2 . Ptd[^3H]Ins levels only fell significantly after prolonged stimulation. Ptd[^3H]Ins3P levels fell significantly in the presence of carbachol, although more slowly than the accompanying changes in Ptd[^3H]Ins(4,5) P_2 .

DISCUSSION

Despite the repeated observation of highly polar inositol phosphates in numerous varieties of cultured cells and tissues (Rapoport & Guest, 1941; Hawkins *et al.*, 1986; Morgan *et al.*, 1987; Stephens *et al.*, 1988a) their origin is unclear. It has been suggested, without any evidence, that they may be derived from highly phosphorylated inositol lipids (Batty *et al.*, 1985; Heslop *et al.*, 1985). Several attempts to detect the presence of a putative [^3H]PtdIns P_3 and/or [^3H]PtdIns P_4 in tissues briefly labelled (< 90 min) with [^3H]Ins (Batty *et al.*, 1985; Hawkins *et al.*, 1986) have proved negative, but the possibility remained that the inositol moiety of such compounds might turn over relatively slowly. We have investigated a line of continuously dividing cultured cells (1321 N1, human-derived 'astrocytoma' cells), which contain substantial quantities of Ins P_3 and Ins P_4 , that were labelled to steady state with [^3H]Ins and relatively briefly with [^{32}P] P_i under a variety of conditions of cell density, medium inositol concentration and hormonal

Fig. 7. Effect of a maximal dose of carbachol on the steady-state levels of inositol phospholipids in 1321 N1 cells

[³H]Ins-prelabelled 1321 N1 cells were labelled to steady state with [³H]Ins as described in the Materials and methods section. Vehicle (○) or vehicle plus carbachol (●) was added to the cells. After various times the cells were quenched with trichloroacetic acid. Three of the control cultures were stopped at $t = 0$ min; three were stopped at $t = 15$ min. The data from the two sets were not significantly different and hence were pooled and meaned for the purposes of presenting the results to yield the 100% values shown for each metabolite ($\pm$ the associated S.E.M.). Phospholipids were extracted, deacylated and separated on an anion-exchange h.p.l.c. column as described in the text. The radioactivity in each peak is presented as a percentage of the control value ($\pm$ S.E.M., $n = 3$) for that compound. Control values were: Ptd[³H]Ins, 86544859 d.p.m.; Ptd[³H]Ins3P, 323272 d.p.m.; Ptd[³H]Ins4P, 4371666 d.p.m.; Ptd[³H]Ins(4,5)P₂, 8835392 d.p.m. An asterisk indicates significantly different from control ($P < 0.05$).

stimulation. Under no circumstances could a phospholipid be detected (either as a water-soluble deacylation product or as a native lipid) that possessed properties consistent with the structure PtdInsP₃ and/or PtdInsP₄ at detection limits of 0.3% (as water-soluble deacylation products resolved by h.p.l.c.) to 3.0% (as native lipids resolved to t.l.c. plates) of the cellular levels of PtdIns(4,5)P₂. Similar experiments with detection limits of 1% of cellular PtdIns(4,5)P₂ did not reveal evidence for these compounds in A431 cells (C. Macphee &

L. Stephens, unpublished work). Detailed analysis of the [³H]Ins-labelled deacylation products obtained from a phospholipid extract of 1321 N1 cells, however, revealed a small peak of radioactivity that was eluted from an anion-exchange h.p.l.c. column slightly earlier than GroPIns4P (and amounting to 3–15% of the quantity of GroP[³H]Ins4P found in the same extracts). The work described here suggests this compound is GroPIns3P and that it is derived from a phospholipid with the structure PtdIns3P.

Previous workers may have failed to detect PtdIns3P for a number of reasons. Ballou & co-workers (Brockerhoff & Ballou, 1962; Grado & Ballou, 1961; Tomlinson & Ballou, 1961) performed their analytical work on a complex phospholipid mixture derived from a post-mortem extract of bovine brain; we do not know whether such extracts contain PtdIns3P. Furthermore, the relatively low concentrations of PtdIns3P would have made its detection by the qualitative techniques employed difficult. The other cell type in which the polyphosphoinositides have received considerable analytical attention is the human erythrocyte (Santiago-Calvo *et al.*, 1964). We can detect no PtdIns[³²P]3P in [³²P]P_i-labelled human erythrocytes (results not shown), suggesting this reaction does not have an inevitable association with 'normal' polyphosphoinositide metabolism. More recent work has depended upon the apparent universal applicability of the earlier structural and metabolic studies on brain and erythrocytes and has not addressed the possibility that the metabolic map, originally outlined by Brockerhoff & Ballou (1962), may not completely describe the metabolism of polyphosphoinositides in the cells under investigation. Moreover, in those studies where some confirmation of these assumptions was sought the chromatographic systems employed were unlikely to have resolved either PtdIns3P from PtdIns4P or GroPIns3P from GroPIns4P.

The recent description of a PtdIns kinase ('type I' PtdIns kinase; Whitman *et al.*, 1987, 1988) which exclusively makes PtdIns3P *in vitro* implies that the compound we have identified is derived from the phosphorylation of PtdIns by a distinct and specific cellular enzyme. The location of PtdIns3-hydroxykinase in the cell is not yet known, but in fibroblasts prostaglandin provokes a 50-fold increase in the PtdIns 3-hydroxykinase activity co-purifying with a prostaglandin F receptor on a lectin affinity column (Whitman *et al.*, 1987), so presumably the enzyme can be located in the plasma membrane. Whether such an association is only transient, and whether the plasma membrane is the only site at which activity is expressed, remain to be established.

The ¹⁴C/³H dual-labelling experiments reported in the Results section suggest that the inositol moiety of PtdIns3P has a similar metabolic profile to those of PtdIns4P and PtdIns(4,5)P₂. All three lipids possessed a significantly higher ³H/¹⁴C ratio than the Ptd[³H,¹⁴C]Ins present in the same extracts. This suggests that the polyphosphoinositides are being synthesized from a pool of PtdIns in which the inositol moiety is turned over more rapidly than the remaining PtdIns. It is possible that this rapidly metabolized pool of PtdIns is synonymous with the hormone-sensitive pool of PtdIns described previously (Monaco & Woods, 1983) and that both PtdIns3P and PtdIns4P are synthesized from this metabolic compartment of PtdIns.

Knowledge of the relative rates of the two PtdIns kinase pathways and turnover times for the pools of PtdIns3P and PtdIns4P would allow a more precise definition of the metabolic pathways responsible for the above patterns of labelling. Theoretically these rates can be measured by analysing [^{32}P]P_i flux into the precursor of the monoester phosphate groups of PtdIns3P and PtdIns4P (i.e. the γ -phosphate of ATP) and the monoester phosphates themselves. The pattern of [^{32}P]P_i incorporation into PtdIns3P in intact cells is very similar to that of other polyphosphoinositides; essentially the rate of turnover of the monoester phosphate is faster than, or very similar to, the rate of turnover of intracellular P_i (note the close approximation in the $^3\text{H}/^{32}\text{P}$ ratios of PtdIns4P and PtdIns3P detailed in the Results section). Hence the rate of this reaction cannot accurately be assessed by [^{32}P]P_i flux analysis because, during the phase of [^{32}P]P_i labelling during which minimal restraint is replaced on [^{32}P]P_i-incorporation data (the 'dynamic phase', very early after the introduction of tracer; Reich, 1968), very little [^{32}P]P_i can safely be incorporated into PtdIns3P. As is the case for the other polyphosphoinositides, the rate of turnover of the phosphodiester phosphate of PtdIns3P is substantially lower than that of the phosphomonoester, although this information says nothing about whether the monoester phosphate moiety is undergoing substrate cycling *in vivo*.

Further evidence that PtdIns3P is a part of, or closely connected to, a hormone-sensitive pool of inositol phospholipids was obtained by exposure of 1321 N1 cells to carbachol, an agonist known to stimulate phosphoinositide hydrolysis. The level of Ptd[^3H]Ins(4,5)P₂ fell rapidly and in parallel with a rise in the levels of Ins(1,4,5)P₃, Ins(1,3,4,5)P₄, Ins(1,3,4)P₃, inositol bisphosphates and inositol monophosphates (results not shown, but see Nakahata & Harden, 1987), a pattern of events which occurs in numerous cells (Berridge & Irvine, 1984). Ptd[^3H]Ins3P levels also fell significantly after stimulation by carbachol, but, by contrast, the level of Ptd[^3H]Ins4P increased rapidly upon addition of the agonist. As the cells were labelled to steady state with [^3H]Ins, the most probable explanation for these agonist-induced effects on labelled phospholipid levels is a parallel change in their cellular concentrations.

Interpreted in terms of the pathways originally defined by Bockerhoff & Ballou (1962), an increase in the concentration of PtdIns4P could result from either increased flux through PtdIns kinase or PtdIns(4,5)P₂ phosphomonoesterase or, alternatively, from decreased flux through PtdIns4P phosphomonoesterase or PtdIns4P kinase. Which of these potential mechanisms is involved in the response of 1321 N1 cells to carbachol has not been established.

A fall in the concentration of PtdIns3P could indicate that this lipid is a substrate for a hormone-sensitive phosphoinositidase C, but, as the water-soluble product of such a reaction is also a product of the dephosphorylation of Ins(1,3,4,5)P₄ (Shears *et al.*, 1987), this question cannot readily be resolved by the detection of Ins(1,3)P₂ in stimulated cells. Alternatively the decreased concentration of PtdIns3P may simply reflect a loss of precursor from a relatively small, hormone-sensitive, pool of PtdIns, leading to a decreased rate of synthesis of PtdIns3P. Nevertheless, these experiments suggest either that PtdIns3P is an integral part of a hormone-sensitive pool of inositol phospholipids or that

it is synthesized from a precursor that it shares with such a pool.

PtdIns 3-hydroxykinase specifically associates with cellular and viral tyrosine kinase [the stimulated PDGF receptor, pp60^{v-src}, or the pp60^{c-src} middle T complex (Whitman *et al.*, 1985; Kaplan *et al.*, 1986)], suggesting that PtdIns3P may have some function in normal and aberrant cellular proliferation. We have demonstrated that the concentration of PtdIns3P in 1321 N1 cells is sensitive to stimulation by cholinergic agonists, but whether changes in the cellular content of this lipid accompany or precede the proliferative responses of cells exposed to growth factors such as prostaglandin F remains to be established. A second question of obvious importance is whether cellular PtdIns3P can be further phosphorylated to yield, for example, PtdIns(3,4)P₂ or a PtdInsP₃. It is noteworthy that no evidence could be found for the existence of such a lipid in extracts of either 1321 N1 or A431 cells (C. Macphee & L. Stephens, unpublished work), although a very recent report describes the hormone-stimulated production of a PtdInsP₃ in neutrophils (Traynor-Kaplan *et al.*, 1988). The first clues about the metabolic relationship that exist between this uncharacterized PtdInsP₃ and PtdIns3P, PtdIns4P and PtdIns(4,5)P₂ will emerge from a full description of its structure.

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