Metabolism of inositol bis-, tris-, tetrakis- and pentakis-phosphates in GH₃ cells

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Previous studies demonstrated a multiplicity of isomers of inositol phosphates in GH₃ rat pituitary tumour cells. In order to determine their origin, we have investigated the metabolism of radiolabelled inositol phosphates (IP_n) in GH₃ cell homogenates by using h.p.l.c. I(1,4,5)P₃ is either phosphorylated to I(1,3,4,5)P₄ (in the presence of ATP) or dephosphorylated to I(1,4)P₂ (in the absence of ATP). I(1,4)P₂ is dephosphorylated to I(4)P (> 95 %). I(1,3,4,5)P₄ hydrolysis yields two products. By using dual-labelled [³²P, ³H]I(1,3,4,5)P₄ with ³²P in either the 3 or the 4/5 position, we have identified the probable configuration of these isomers. The predominant (> 97 %) IP₃ formed is I(1,3,4)P₃, with a minor I(1,4,5)P₃ peak. Subsequent I(1,3,4)P₃ hydrolysis yields two IP₂ isomers [the major (≈ 85 %) is I(3,4)P₂; the minor (≈ 15 %) is I(1,3)P₂] and two IP isomers [the major (≈ 90 %) is I(3)P [L-I(1)P], the minor I(4)P]. IP₅ is very slowly dephosphorylated to an IP₄ of undetermined isomeric configuration. We have also examined GH₃ cell lipids for the presence of phosphoinositides either more highly phosphorylated than PIP₂ (as potential sources of the IP₄/IP₅ and IP₆ in these cells) or phosphorylated in positions other than 1, 4 and 5, and have been unable to find evidence of either. These data suggest two main routes of metabolism for I(1,4,5)P₃ in GH₃ cells: either (1) phosphorylation to I(1,3,4,5)P₄, and the subsequent consecutive dephosphorylation to I(1,3,4)P₃, I(3,4)P₂ and finally L-I(1)P [D-I(3)P]; or (2) dephosphorylation to I(1,4)P₂ and, subsequently, I(4)P.

INTRODUCTION

The hydrolysis of PIP₂ by phospholipase C yields two second messengers, I(1,4,5)P₃ and DAG. I(1,4,5)P₃ releases Ca²⁺ from intracellular stores and DAG activates protein kinase C (Berridge & Irvine, 1984; Nishizuka, 1984). The subsequent metabolism of I(1,4,5)P₃ is of considerable interest because of the potential role of other inositol phosphates in regulating intracellular Ca²⁺ (Irvine & Moor, 1986; Irvine *et al.*, 1986b). We have previously investigated the changes which occur in levels of individual radiolabelled inositol phosphate isomers in the GH₃ rat pituitary tumour cell line in response to thyrotropin-releasing hormone (TRH) (Dean & Moyer, 1987). These studies revealed a complex pattern of inositol phosphates, some of which remained unidentified, with different kinetic patterns.

Consequently we have addressed a number of specific points raised by these studies: the source of I(4)P, the identity of the unknown IP₂ isomers, and the pathways by which I(1,3,4,5)P₄ is both generated and metabolized. We have also investigated GH₃ cells for the presence of more highly phosphorylated inositol phosphates (IP₅ and IP₆).

EXPERIMENTAL

Materials and h.p.l.c.

Radiolabelled phosphoinositol lipids and inositol phosphates were obtained from Amersham International,

Des Plaines, IL, U.S.A., and du Pont/NEN Products, Boston, MA, U.S.A. ³H-labelled inositol phosphates for use as h.p.l.c. standards were prepared as described previously (Dean & Moyer, 1987). H.p.l.c. separations were performed on a Whatman Partisil 10 SAX column (25 cm × 0.46 cm) with a pre-column containing Whatman pellicular anion-exchange resin. Elution was by ammonium phosphate (pH 3.8) gradients at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. The gradients used are described in their respective experiments. Fractions were mixed with 6 ml of Aqueous Counting Scintillant (Amersham International) containing 5 % (v/v) water, and radioactivity was determined by liquid-scintillation counting.

GH₃ cells

GH₃ cells were grown as described previously (Dean & Moyer, 1987).

Hydrolysis of inositol phosphates

GH₃ cells were removed from two 150 cm³ flasks by use of a cell scraper and suspended in 0.9 % NaCl with 0.02 % EDTA, then collected by centrifugation (1000 g, 2 min). The cells were suspended in 0.7 ml of 0.2 M-Tris/HCl, pH 7.6, with 2 mM-EDTA and allowed to swell on ice for 10 min. Homogenates were then prepared by 10–20 strokes of a glass/Teflon homogenizer. The protein concentration in these homogenates was 6–10 mg/ml as determined by the method of Smith *et al.* (1985). Incubations were performed at 37 °C in a total volume of

Abbreviations used: DAG, diacylglycerol; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositol phosphate (similarly GPIP₂, GPIP₃, GPIP₄ and GPIP₅); IP, *myo*-inositol monophosphate; IP₂, *myo*-inositol bisphosphate (similarly IP₃, IP₄, IP₅ and IP₆, with locants designating specific isomers where appropriate and assumed to be D-enantiomers unless otherwise stated); PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate (similarly PIP₂ and PIP₃); TRH, thyrotropin-releasing hormone.

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0.375 ml consisting of 0.3 ml of buffer (10 mM-Hepes/4 mM-MgCl₂/10 mM-KCl/1 mM-EGTA, pH 7.2), 0.05 ml of cell homogenate and 25 μ l of radiolabelled substrate at 1 μ Ci/100 μ l. Portions of the reaction mixture (75 μ l) were removed at the indicated times and mixed with 5 μ l of 15% trichloroacetic acid to terminate the reaction. The trichloroacetic acid was removed by three extractions with water-saturated diethyl ether. Samples were stored at -20 °C until analysed.

This technique was used to examine the hydrolysis with time of I(1,4)P₂, I(1,4,5)P₃, I(1,3,4,5)P₄ (all at 0.7 nM) and IP₅ (extracted from GH₃ cells).

In order to determine whether GH₃ cells contain an IP₃ kinase, cell homogenates were prepared as described, and 50 μ l added to 250 nCi of [³H]I(1,4,5)P₃ in 325 μ l buffer (10 mM-NaCl/70 mM-potassium glutamate/30 mM-KCl/4 mM-MgCl₂/10 mM-Hepes/1 mM-EGTA/5 or 10 mM-ATP, pH 7.4). Portions of the reaction mixture (75 μ l) were removed at different times (up to 30 min) and the reaction terminated with 5 μ l of 15% (w/v) trichloroacetic acid. The supernatant was extracted three times with water-saturated diethyl ether, and the products formed were examined by h.p.l.c.

We have also examined the hydrolysis of [³H]I(1,4)P₂ by rat liver homogenates. Rat liver was homogenized in 0.5 M-sucrose, and incubations with [³H]I(1,4)P₂ were performed with portions of the homogenate (25 mg of liver/ml of 0.5 M-sucrose) exactly as described by Storey *et al.* (1984).

Preparation of [³H]I(1,3,4,5)P₄/I[4,5-³²P](1,3,4,5)P₄ and [³H]I[3-³²P](1,3,4,5)P₄

We have used rat brain as a source of the I(1,4,5)P₃ 3-kinase characterized by Irvine *et al.* (1986a) to generate I(1,3,4,5)P₄ from I(1,4,5)P₃. A mixture of [³H]I(1,3,4,5)P₄ and I[4,5-³²P](1,3,4,5)P₄ ([4,5-³²P]IP₄) was prepared by the phosphorylation of [³H]I(1,4,5)P₃ and I[4,5-³²P]-(1,4,5)P₃. A 400 nCi portion of [³H]I(1,4,5)P₃ (sp. radioactivity 1 Ci/mmol) (Amersham International) and a 400 nCi portion of [4,5-³²P]I(1,4,5)P₃ (sp. radioactivity 20–200 Ci/mmol) (du Pont/NEN) were combined and dried under N₂. These were reconstituted in 1 ml of 100 mM-Tricine, pH 8, containing 10 mM-MgCl₂, 3 mM-2,3-diphosphoglycerate and 10 mM-ATP (disodium salt) (Sigma). To this was added 50 μ l of a 4% (w/v) rat brain homogenate (in 0.15 M-sucrose), the mixture was vortex-mixed and incubated for 15 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of 30% trichloroacetic acid (4 °C). The supernatant, containing IP₄, was collected, trichloroacetic acid removed by five extractions with water-saturated diethyl ether, and stored at -20 °C before purification by h.p.l.c.

A dual-labelled [³H,3-³²P]I(1,3,4,5)P₄ was prepared by the phosphorylation of [³H]I(1,4,5)P₃ with high-specific-radioactivity [γ -³²P]ATP as a phosphate donor. A 2 μ Ci portion of [³H]I(1,4,5)P₃ (specific radioactivity 1 Ci/mmol) (Amersham International) was dried under N₂ and reconstituted in 250 μ l of 100 mM-Tricine, pH 8, containing 40 mM-MgCl₂, 6 mM-2,3-diphosphoglycerate and 4 mM-ATP (disodium salt) (Sigma). To this was added 1.0 mCi of [γ -³²P]ATP (in 100 μ l of 10 mM-Tricine) (specific radioactivity 6000 Ci/mmol) (du Pont/NEN). A 150 μ l portion of a 4% rat brain homogenate (in 0.15 M-sucrose) was then added, the mixture was vortex-mixed and incubated at 37 °C for 15 min. The reaction was terminated by the addition of 0.5 ml of 30%

trichloroacetic acid (4 °C) and the supernatant processed and stored as previously described.

The radiolabelled I(1,3,4,5)P₄ from each preparation was purified by h.p.l.c. of the neutralized supernatant. The gradient used was 0.01–0.6 M-ammonium phosphate (over 15 min), followed by 30 min at 0.6 M to elute IP₃ and ATP. I(1,3,4,5)P₄ was eluted by increasing the ammonium phosphate concentration to 1.0 M. The radiolabelled I(1,3,4,5)P₄ fraction was recovered from the column, diluted 5-fold with distilled water and applied to 5 ml of AG 1-X8 (100–200 mesh; formate form) anion-exchange resin (Bio-Rad). P_i was eluted with 30 ml of 0.2 M-ammonium formate/0.1 M-formic acid and I(1,3,4,5)P₄ was eluted with a subsequent wash of 20 ml of 1.4 M-ammonium formate/0.1 M-formic acid. The I(1,3,4,5)P₄ fractions were passed over 25 ml of acid-washed Dowex-HCR-W2 cation-exchange resin (H⁺ form) (Sigma) to remove NH₄⁺, frozen and freeze-dried. The purified I(1,3,4,5)P₄ preparations were reconstituted in 500 μ l of distilled water and stored at -20 °C.

Subsequent h.p.l.c. analysis revealed the [³H]I(1,3,4,5)-P₄/I[4,5-³²P](1,3,4,5)P₄ mixture to be > 99% radiochemically pure, and the [³H]I[3-³²P](1,3,4,5)P₄ to be > 95% radiochemically pure (approx. 3% of the ³²P radioactivity was eluted as a broad peak between I(1,4,5)P₃ and I(1,3,4,5)P₄, and [³²P]P_i was present to the extent of approx. 1%).

Purification and identification of IP₅ and IP₆

GH₃ cells growing in 150 cm³ flasks were labelled for 72 h with 100 μ Ci of *myo*-[³H]inositol/flask. A trichloroacetic acid extract of the [³H]inositol-labelled water-soluble metabolites from ten flasks was prepared (Dean & Moyer, 1987). The [³H]inositol phosphates of the extract were eluted from a SAX h.p.l.c. column with a gradient of 0.01–1.9 M-ammonium phosphate (30 min), followed by isocratic 1.9 M-ammonium phosphate for a further 30 min, and 0.5 ml fractions were collected. This separation revealed two more polar [³H]inositol-labelled compounds eluted after I(1,3,4,5)P₄ (Fig. 1). These were tentatively identified as IP₅ and IP₆ respectively. In order

Fig 1. Separation by h.p.l.c. of [³H]inositol-labelled compounds formed in GH₃ cells

Labelling and extraction of cells and h.p.l.c. methods are described in the Experimental section. Elution was with a linear gradient of ammonium phosphate, pH 3.8, from 0.01 M to 1.9 M over 30 min, followed by 30 min of isocratic 1.9 M-ammonium phosphate. The experiments to characterize IP₅ and IP₆ are described in the text.

to confirm this designation, the remaining pooled [³H]-inositol-labelled extract was separated as described above and the putative IP₅ and IP₆ recovered from the column. In order to desalt the compounds, each was diluted to 40 ml with distilled water and applied to 5 ml of Dowex-1 (Cl⁻) anion-exchange resin (Sigma) and eluted by a modification of the method of Cosgrove (1969). First, the columns were washed with distilled water (10 ml) and then 0.05 M-HCl (20 ml) to remove P_i. IP₅ was eluted with 20 ml of 0.6 M-HCl and IP₆ with 20 ml of 0.75 M-HCl. The desalted samples were freeze-dried and reconstituted in 0.5 ml of distilled water.

Confirmation of the identity of IP₅ and IP₆ was obtained by co-migration with unlabelled IP₅ or IP₆ (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) on electrophoresis plates (20 cm × 20 cm t.l.c. cellulose plates; Eastman-Kodak; 400 V/150 mA in 0.1 M-oxalic acid). The unlabelled standards were located with a phosphate-detecting acid-molybdate spray reagent (Sigma), and the labelled compounds from GH₃ cells were located by scraping the plates into strips and counting the radioactivity. Further confirmation of the identity of IP₅ and IP₆ from GH₃ cells was obtained by treating the desalted samples with phytase (Sigma). A 300 μl portion of either [³H]IP₅ or [³H]IP₆ was incubated at 37 °C with 6 mg of enzyme (0.24 unit) in 25 mM-sodium acetate buffer, pH 5.1, containing 0.2 mM-MgSO₄, in a final volume of 900 μl (Tomlinson & Ballou, 1962). Aliquots (300 μl) were removed at different times up to 90 min, and the reaction was terminated by the addition of 600 μl of 0.6 M-HCl. Examination of the water-soluble products by h.p.l.c. revealed the time-dependent accumulation of IP, IP₂ and IP₃ (results not shown).

Identification of inositol lipids of GH₃ cells

We have examined GH₃ cells for the presence of inositol lipids more highly phosphorylated than PIP₂ (as potential sources of IP₄, IP₅ and IP₆), and for the presence of phosphoinositol lipids phosphorylated in positions other than 1, 4, or 5 [as potential sources of I(1,3,4)P₃ or I(4)P]. [³H]Inositol-labelled lipids were deacylated (Hawkins *et al.*, 1986), and the extract was separated by h.p.l.c. with the gradient described in Fig. 1; however, no peaks corresponding to GPIIP₃, GPIIP₄ or GPIIP₅ were found, indicating that GH₃ cells do not contain PIP₃, PIP₄ or PIP₅. A similar preparation of deacylated [³H]inositol-labelled lipid was treated with 1 mM-sodium periodate to produce inositol phosphate isomers with the same phosphate configuration as the parent lipids (Brown & Stewart, 1966; Heslop *et al.*, 1986). After separation by h.p.l.c., peaks of radioactivity corresponding to I(1)P, I(1,4)P₂ and I(1,4,5)P₃ were found, with no peaks corresponding to I(4)P or I(1,3,4)P₃, confirming the conventional isomeric configuration for inositol lipids in these cells.

RESULTS AND DISCUSSION

I(1,4)P₂ metabolism

Incubation of I(1,4)P₂ with GH₃ cell homogenates results in the formation of I(4)P, with no appreciable accumulation of I(1)P and a much slower accumulation of inositol (Fig. 2); thus the dephosphorylation of I(1,4)P₂ is highly specific and proceeds via the action of an I(1,4)P₂ 1-phosphatase.

Fig. 2. Production of [³H]I(4)P by the hydrolysis of [³H]I(1,4)P₂ with GH₃-cell homogenates

The incubation conditions and h.p.l.c. methods were as described in the Experimental section. The gradient used was ammonium phosphate, pH 3.8, 0.01–0.06 M over 30 min, followed by 0.06 M–1.0 M over 10 min. —, Zero time; ----, 5 min incubation.

A number of previous studies have investigated the dephosphorylation of I(1,4)P₂ in different tissue and have drawn differing conclusions. In an early study, Berridge *et al.* (1983) added I[4-³²P](1,4)P₂ to homogenized blowfly salivary glands and were unable to find evidence for the formation of I[4-³²P](4)P, only [³²P]P_i, and so have assumed that I(1)P is exclusively formed from I(1,4)P₂. In a second study, Storey *et al.* (1984) have described the hydrolysis of I(1,4)P₂ by rat liver homogenates. These workers also used I[4-³²P](1,4)P₂ as a substrate and concluded that the 1- and 4-phosphate groups of I(1,4)P₂ are removed at approximately the same rate, presumably yielding equal amounts of I(1)P and I(4)P. Because of our results in GH₃ cells, we have re-examined the hydrolysis of I(1,4)P₂ by rat liver homogenates using [³H]I(1,4)₂ under conditions identical with those used by Storey *et al.* (1984) and have separated the ³H-labelled metabolic products by h.p.l.c. I(4)P was found to be formed almost exclusively (~98%), with only a trace of I(1)P. The I(1,4)P₂ 1-phosphatase activity from rat liver has been partially purified and characterized (Moyer *et al.*, 1987). It seems likely that the direct separation of I(1)P from I(4)P by h.p.l.c. is a more accurate method of determining the products of hydrolysis of I(1,4)P₂ than resolving [³²P]P_i from I[4-³²P](4)P, which was required in the previous studies.

In GH₃ cells treated with TRH and LiCl, I(4)P is the predominant monophosphate formed, and increases rapidly (~10 s) after stimulation, whereas I(1)P does not increase until 60–90 s after TRH treatment (Dean & Moyer, 1987). As we have demonstrated that I(4)P is the major (or exclusive) product of I(1,4)P₂ hydrolysis, these kinetic studies reported previously indicate that no change in PI hydrolysis is occurring in these cells over the first 60–90 s of stimulation. The subsequent increase in I(1)P could result from either I(1,3,4)P₃ metabolism or increased PI hydrolysis by phospholipase C. The sustained increase in I(4)P in GH₃ cells for up to 30 min

Fig. 3. Separation by h.p.l.c. of the [^3H]inositol phosphates formed by the hydrolysis of [^3H]I(1,3,4,5) P_4 by GH_3 -cell homogenates

The incubation conditions and h.p.l.c. methods were as described in the Experimental section. The gradients used were sequential steps of ammonium phosphate, pH 3.8, 0.01–0.06 M (30 min), 0.22–0.26 M (30 min), 0.53–0.55 M (30 min) and isocratic 1.0 M (30 min). The identification of peaks was by comparison of retention times with those of standards and the subsequent use of $^{32}\text{P}/^3\text{H}$ -labelled preparations of I(1,3,4,5) P_4 . The rationale for identification of I(1,3) P_2 , I(3,4) P_2 and I(1,4,5) P_3 is described in the Results and discussion section. This particular commercial preparation of [^3H]I(1,3,4,5) P_4 contained $\approx 15\%$ contaminant (presumably [^3H]inositol), which was eluted in the void volume and did not increase appreciably with time. Incubation times: (a) 0 min; (b) 1 min; (c) 10 min.

after stimulation (Dean & Moyer, 1987) indicates a continuous hydrolysis of PIP/PIP_2 .

Recent studies confirm that I(4)P is the major (or exclusive) product of I(1,4) P_2 dephosphorylation in a variety of tissues. Morgan *et al.* (1987) incubated [^3H]-

I(1,4,5) P_3 with saponin-permeabilized rat anterior-pituitary cells and demonstrated the exclusive accumulation of I(4)P rather than I(1)P. Similarly, Ackermann *et al.* (1987) hydrolysed I(1,4) P_2 with a dialysed rat brain preparation and showed that both I(4)P and I(1)P are formed, with I(4)P predominating. Subsequent to the completion of the present study, Inhorn *et al.* (1987) have described similar results to our own using calf brain as an enzyme source. They have partially purified an enzyme which specifically hydrolyses the 1-phosphate from both I(1,4) P_2 and I(1,3,4) P_3 , and they conclude that the action of this enzyme on I(1,4) P_2 specifically and exclusively yields I(4)P, in agreement with our own observations in GH_3 cells and liver extract.

I(1,4,5) P_3 metabolism

Incubation of I(1,4,5) P_3 with GH_3 cell homogenates in the absence of ATP results in the rapid, time-dependent, accumulation of I(1,4) P_2 , which represents $> 99\%$ of the IP_2 formed. When 5 or 10 mM-ATP is included in the incubation medium, there is an inhibition of I(1,4,5) P_3 hydrolysis ($> 90\%$) and an accumulation of I(1,3,4,5) P_4 (results not shown). These results are consistent with the hydrolysis of I(1,4,5) P_3 in GH_3 cells by a highly specific, well-characterized, 5-phosphatase (Downes *et al.*, 1982; Seyfred *et al.*, 1984; Storey *et al.*, 1984) or phosphorylation by the recently characterized I(1,4,5) P_3 3-kinase (Irvine *et al.*, 1986a) to produce I(1,3,4,5) P_4 in an ATP-dependent reaction.

Although I(1,4) P_2 represents $> 99\%$ of the IP_2 hydrolysis products formed from I(1,4,5) P_3 , trace ($< 1\%$) amounts of I(4,5) P_2 are also generated, which may account for the very low levels of this isomer found in GH_3 cells (Dean & Moyer, 1987).

I(1,3,4,5) P_4 metabolism

Hydrolysis of I(1,3,4,5) P_4 by GH_3 cell homogenates yields a complex pattern of products (Fig. 3). The major ($\approx 98\%$) IP_3 produced has a retention time identical with that of I(1,3,4) P_3 . Prolonged incubation results in the hydrolysis of this compound to generate two novel IP_2 isomers [the minor one ($\approx 15\%$) being eluted ahead of I(1,4) P_2 , the major one ($\approx 85\%$) being eluted after I(1,4) P_2] and two IP isomers, identified by their retention times as I(1)P and I(4)P. The accumulation of these isomers with time is shown in Fig. 4.

The commercial preparation of [^3H]I(1,3,4,5) P_4 used in the present study contains a contaminating component ($\approx 10\%$ total radioactivity) which is eluted in the void volume (presumably [^3H]inositol) and does not change appreciably during the time of incubation. There is also a minor ($< 1\%$ total radioactivity) contaminant that is eluted in the position of I(1,4,5) P_3 and approximately doubles in amount during the course of the incubation.

To enable full characterization of the isomers of inositol phosphate produced (particularly the unknown IP_2 isomers), we have synthesized two different preparations of I(1,3,4,5) P_4 . One is a mixture of ^3H -labelled I(1,3,4,5) P_4 and 4,5- ^{32}P -labelled I(1,3,4,5) P_4 , and the other is dual-labelled [^3H , 3- ^{32}P]I(1,3,4,5) P_4 . Hydrolysis of each of these I(1,3,4,5) P_4 preparations will yield different profiles of dual-labelled or ^3H -labelled inositol phosphates, the ratios of $^{32}\text{P}/^3\text{H}$ in each product depending on the ^{32}P -labelled phosphate groups removed.

Hydrolysis of [^3H , 4,5- ^{32}P]I(1,3,4,5) P_4 yields the profile

Fig. 4. Accumulation of [³H]inositol phosphates from the hydrolysis of [³H]I(1,3,4,5)P₄ by GH₃-cell homogenates

Isomers were separated as described in the legend to Fig. 3. The major components are shown in the main Figure, the minor components being shown in the inset. Each point represents the mean for two separate samples in a single typical experiment.

of inositol phosphates shown in Fig. 5(a). The ratios of ³²P/³H labelling of radioactive peaks in a single typical experiment are shown in Table 1 (the experiment was repeated three times with similar results). We have used this preparation to identify the unknown IP₂ isomers and to confirm the identity of the putative I(1,3,4)P₃. The major IP₃ produced has a ³²P/³H ratio which is approximately one-third that of the original I(1,3,4,5)P₄, presumably owing to the removal of the ³²P-labelled 5-phosphate group. Similar preparations of [4,5-³²P]-I(1,4,5)P₃ to those used to generate our I(1,3,4,5)P₄ have been reported to be labelled, with 69% ³²P in the 5-position and 31% in the 4-position (Hawkins *et al.*, 1984); consequently, a ³²P/³H ratio of approximately one-third rather than one-half that of the original I(1,3,4,5)P₄ would be expected for any I(1,3,4)P₃ generated, consistent with our observation. The only other possible hydrolysis product of I(1,3,4,5)P₄ which could yield an IP₃ with a similar ratio [i.e. less than the original I(1,3,4,5)P₄] would be I(1,3,5)P₃. This metabolite is highly unlikely to be formed, because of the presence of a highly active specific phosphomonoesterase that hydrolyses the 5-phosphate IP₃ present in these cells and has also been shown to hydrolyse I(1,3,4,5)P₄ (Connolly *et al.*, 1987). Consequently the major IP₃ product of I(1,3,4,5)P₄ hydrolysis in GH₃ cells is I(1,3,4)P₃. A second IP₃ peak eluted in the position of I(1,4,5)P₃ is also generated, but to a much lesser extent (≈ 2–3% of total [³H]IP₃ radioactivity), with a ³²P/³H ratio similar to the

original I(1,3,4,5)P₄. We have assumed, therefore, that the production of IP₂ isomers in this preparation results from the subsequent hydrolysis of I(1,3,4)P₃. [This has been confirmed by incubating [4-³²P](1,3,4)P₃ with GH₃ homogenates; N. M. Dean & J. D. Moyer, unpublished work]. The three possible IP₂ isomers formed by dephosphorylation of I(1,3,4)P₃ are I(1,3)P₂, I(1,4)P₂ and I(3,4)P₂. Chromatography revealed that no I(1,4)P₂ is generated, and so our two unknown IP₂ isomers must be I(1,3)P₂ and I(3,4)P₂. Fig. 5(a) demonstrates that the largest of the two components [eluted after I(1,4)P₂] is ³²P-labelled in the 4-position and consequently is identified as I(3,4)P₂. By deduction the lesser IP₂ component generated, which is eluted ahead of I(1,4)P₂, must be I(1,3)P₂.

Hydrolysis of [³H,3-³²P]I(1,3,4,5)P₄ for 10 min yielded the profile of inositol phosphates shown in Fig. 5(b), and the ratios of ³²P/³H labelling are shown in Table 1. As expected, I(1,3,4)P₃ has a similar ³²P/³H ratio to IP₄. The second IP₃ peak generated is ³H-labelled and not ³²P-labelled, confirming its identity as I(1,4,5)P₃ [and not I(3,4,5)P₃ or I(1,3,5)P₃]. Both I(3,4)P₂ and I(1,3)P₂ are dual-labelled, again with ratios similar to that of I(1,3,4,5)P₄, confirming the presence of ³²P label in the 3-phosphate position of both isomers. Unexpectedly the peak of radioactivity corresponding to I(1)P contains ³²P labelling also, in a ratio (³²P/³H) only slightly less than that of the precursor, I(1,3,4,5)P₄. This means that the I(1)P formed must be predominately L-I(1)P [or D-I(3)P] generated by the removal of the 4-phosphate from I(3,4)P₂ or the 1-phosphate from I(1,3)P₂. As anion-exchange chromatography achieves separation of different compounds by virtue of their different charge, enantiomers (D/L forms) will not be resolved. The ³H radioactivity in the [³²P]P₁ peak represents spill-over between channels, and only approx. 1% the total ³²P radioactivity will appear as ³H (here a ³²P/³H ratio of ≈ 75), which cannot account for a significant proportion of the ³H radioactivity seen in any inositol phosphate peaks.

The hydrolysis of I(1,3,4,5)P₄ by a 5-phosphomonoesterase yielding I(1,3,4)P₃ has been well documented with semi-purified enzyme (Connolly *et al.*, 1987), red-cell membranes (Batty *et al.*, 1985), rat liver homogenate (Hansen *et al.*, 1986), rat parotid-gland homogenates (Hawkins *et al.*, 1986) and permeabilized hepatocytes (Tennes *et al.*, 1987). Our results with GH₃-cell homogenates are in agreement with these observations and suggest a source for the I(1,3,4)P₃ found in GH₃ cells (Dean & Moyer, 1987). This is consistent with the proposal that I(1,3,4,5)P₄ metabolism in these cells proceeds primarily via the action of the 5-phosphomonoesterase to generate I(1,3,4)P₃. A small amount of I(1,4,5)P₃ formation is consistently observed when I(1,3,4,5)P₄ is incubated with cell homogenates; however, this represents a very minor (≈ 2% of IP₃ radioactivity) route of metabolism relative to I(1,3,4)P₃ formation. This is not, however, necessarily a general rule, as substantial amounts of I(1,4,5)P₃ are generated from I(1,3,4,5)P₄ in the rat basophil (2H3) cell line (J. R. Cunha-Melo, N. M. Dean & M. A. Beaven, unpublished work).

I(1,3,4)P₃ is further dephosphorylated in GH₃ homogenates to two IP₂ isomers which we have identified as I(3,4)P₂ and I(1,3)P₂. Both of these isomers are readily resolved from I(1,4)P₂ by h.p.l.c. and are present in both

Fig. 5. Separation by h.p.l.c. of the [^{32}P]- and [^3H]-inositol phosphates formed by the hydrolysis of either (a) a mixture of [^3H]I(1,3,4,5) P_4 and I(4,5- ^{32}P)(1,3,4,5) P_4 (5 min incubation) or (b) [^3H]I[3- ^{32}P](1,3,4,5) P_4 (10 min incubation)

The incubation conditions, h.p.l.c. methods and preparation of radiolabelled IP_4 are described in the Experimental section. The gradients used are described in the legend to Fig. 3. Fractions were counted for radioactivity by using a dual-label ($^3\text{H}/^{32}\text{P}$) program. —, ^3H radioactivity; ----, ^{32}P radioactivity. The $^{32}\text{P}/^3\text{H}$ ratios of individual isomers are shown in Table 1. Each experiment was repeated at least three times, with identical findings, and the values represent results from a single typical incubation.

GH $_3$ cells (Dean & Moyer, 1987) and 2H3 cells (Cunha-Melo *et al.*, 1987) as well as I(1,4) P_2 . Previous studies by other workers have demonstrated two IP_2 isomers in extracts of cells labelled with [^3H]inositol (Heslop *et al.*, 1985, 1986; Hansen *et al.*, 1986). The earlier-to-be-eluted isomer has been identified as I(1,4) P_2 . Hansen *et al.* (1986) have suggested that the later-to-be-eluted compound is I(1,3) P_2 , whereas Heslop *et al.* (1986) suggest I(3,4) P_2 as the structure of this isomer. Shears *et al.*

(1987) have determined the site of hydrolysis of a dual-labelled [$^{32}\text{P}, ^3\text{H}$]I(1,3,4) P_3 in liver homogenates and demonstrated that the second IP_2 isomer is I(3,4) P_2 , a finding in accord with our own observations. They also observe a small accumulation of I(1,3) P_2 , based upon changes in $^{32}\text{P}/^3\text{H}$ ratios in their preparation. We have been able to confirm the generation of I(1,3) P_2 from I(1,3,4,5) P_4 in GH $_3$ homogenates, where this isomer represents approx. 15 % of the IP_2 formed. Inhorn *et al.*

Table 1. $^{32}\text{P}/^3\text{H}$ ratios of dual-labelled I(1,3,4,5) P_4 and inositol phosphates formed by hydrolysis with GH $_3$ -cell homogenates

Dual-labelled IP_4 ($^{32}\text{P}/^3\text{H}$ ratio)	IP_n formed . . .	I(1,3,4) P_3	I(1,4,5) P_3	I(3,4) P_2	I(1,3) P_2	I(1) P
[$^3\text{H}, 4, 5\text{-}^{32}\text{P}$]I(1,3,4,5) P_4 (0.22)		0.09	0.26	0.11	—	—
[$^3\text{H}, 3\text{-}^{32}\text{P}$]I(1,3,4,5) P_4 (5.0)		4.3	< 1	4.3	5.1	4.2

Broken arrows represent minor routes of metabolism.

We have also found that, in GH_3 cells, $\text{I}(1,3,4)\text{P}_3$ [via $\text{I}(1,3)\text{P}_2$ and $\text{I}(3,4)\text{P}_2$] is hydrolysed mainly to $\text{L-I}(1)\text{P}$ (Fig. 5*b* and Table 1). $\text{L-I}(1)\text{P}$ is known to be formed in tissue by the isomerization of glucose 6-phosphate (Eisenberg & Maeda, 1985). Ackermann *et al.* (1987) considered the possibility that in rat brain levels of $\text{I}(1)\text{P}$ were increased owing to dephosphorylation of $\text{I}(1,3,4)\text{P}_3$, yielding $\text{L-I}(1)\text{P}$, but were able only to find 7–13% of $\text{I}(1)\text{P}$ present in the L form. This gives no indication, however, as to what percentage of $\text{L-I}(1)\text{P}$ originates from glucose 6-phosphate and what originates from $\text{I}(1,3,4)\text{P}_3$. If similar ratios of D/L forms of $\text{I}(1)\text{P}$ exist in other tissues, it would seem unlikely that $\text{I}(1,3,4,5)\text{P}_4$ contributes significantly to the overall pool of $\text{I}(1)\text{P}$ found.

GH₃ cells contains substantial amounts of IP₅ and IP₆, although levels do not change consistently on stimulation with TRH (N. M. Dean & J. D. Moyer: unpublished work). As we have been unable to demonstrate a lipid precursor for IP₅ or IP₆ (see below) in GH₃ cells, it seems likely that the two compounds are formed by the action of (a) kinase(s) on IP₄, as in the chicken erythrocyte (Chakrabarti & Biswas, 1981). Hawkins *et al.* (1986) has suggested the possibility that I(1,3,4,5)P₄ might arise from the hydrolysis of IP₅. When [³H]IP₅ was added to GH₃-cell homogenates, a [³H]IP₄ of unknown configuration was slowly produced [under conditions where > 95% I(1,3,4,5)P₄ is converted into IP₃, only ≈ 10% hydrolysis of IP₅ occurs; results not shown]. Consequently it seems unlikely that, in GH₃ cells at least, IP₅ is a major source of I(1,3,4,5)P₄.

Our study provides information on the metabolic fate of different inositol phosphates in GH₃ cells and suggests routes (Scheme 1) for the metabolism of individual isomers. The important points of this Scheme are that I(1,4)P₂ is exclusively dephosphorylated to I(4)P and that

A similar general scheme has been proposed by Inhorn *et al.* (1987) on the basis of results obtained from enzymes purified from rat brain. Their scheme denotes a major hydrolysis of PI relative to PIP and PIP₂. It seems likely, however, that the relative amounts of each lipid hydrolysed will differ from tissue to tissue. For example, Morgan *et al.* (1987) have demonstrated that, in pituitary gonadotrophs exposed to gonadotropin-releasing hormone, the inositol monophosphate accumulation is almost entirely I(4)P. Our own observations in GH₃ cells are that both I(1)P and I(4)P accumulate, with the latter predominating (Dean & Moyer, 1987). By contrast, in the rat basophilic leukaemia (RBL-2H3) cell stimulated with IgE, I(1)P accumulation is greater than I(4)P accumulation (Cunha-Melo *et al.*, 1987). This probably reflects different rates of hydrolysis of PI relative to PIP and PIP₂. Whether these differences can be correlated with the different functions of individual cell types or the degree of differentiation remains to be seen. It is interesting to note that both cell types where I(4)P predominates are secretory cells (gonadotrophs and mammatrophs).

The present study has answered questions posed by our previous work (Dean & Moyer, 1987). The IP₂ isomer found in GH₃ cells that is eluted ahead of I(1,4)P₂ is probably I(1,3)P₂, and the major isomer that is eluted after I(1,4)P₂ is I(3,4)P₂. We have also shown that I(1,4)P₂ is the source of I(4)P. Whether the metabolic pathways shown in Scheme 1 apply generally to all cell types remains to be determined.

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