Synthesis of *myo*-inositol 1,3,4,5,6-pentakisphosphate from inositol phosphates generated by receptor activation

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myo-[³H]Inositol 1,3,4,5,6-pentakisphosphate can be made from *myo*-[³H]inositol 1,4,5-trisphosphate in a rat brain homogenate or soluble fraction. Although *D*-*myo*-inositol 3,4,5,6-tetrakisphosphate can be phosphorylated by a soluble rat brain enzyme to give *myo*-inositol 1,3,4,5,6-pentakisphosphate, it is not an intermediate in the pathway from *myo*-inositol 1,4,5-trisphosphate. The intermediates in the above pathway are *myo*-inositol 1,3,4,5-tetrakisphosphate, *myo*-inositol 1,3,4-trisphosphate and *myo*-inositol 1,3,4,6-tetrakisphosphate [Shears, Parry, Tang, Irvine, Michell & Kirk (1987) *Biochem. J.* **246**, 139–147; Balla, Guillemette, Baukal & Catt (1987) *J. Biol. Chem.* **262**, 9952–9955], and it is catalysed by soluble kinase activities of similar anion-exchange mobility and *M_r* value. Compounds with chromatographic and chemical properties consistent with the structures *myo*-inositol 1,3,4,5-tetrakisphosphate, *myo*-inositol 1,3,4,6-tetrakisphosphate and *myo*-inositol 3,4,5,6-tetrakisphosphate are present in avian erythrocytes, human 1321 N1 astrocytoma cells and primary-cultured murine bone-marrow-derived macrophages. The amounts of these inositol tetrakisphosphates rise upon muscarinic cholinergic stimulation of the astrocytoma cells or stimulation of macrophages with platelet-activating factor.

INTRODUCTION

The major species of inositol tetrakisphosphate in avian erythrocytes and murine macrophages is *Ins*(3,4,5,6)*P*₄ (Stephens *et al.*, 1988a). This *InsP*₄ can serve as a substrate for a widely distributed 1-OH kinase, yielding *Ins*(1,3,4,5,6)*P*₅ (Stephens *et al.*, 1988b). Although *Ins*(3,4,5,6)*P*₄ cannot be synthesized from *Ins*(1,4,5)*P*₃ by a single phosphorylation reaction, we observed that addition of [³H]*Ins*(1,4,5)*P*₃ to rat brain homogenates or soluble fractions could yield [³H]*InsP*₅. Presumably, either [³H]*Ins*(3,4,5,6)*P*₄ can be synthesized from [³H]*Ins*(1,4,5)*P*₃ by a currently unknown route, or a second [³H]*InsP*₄ can act also as an intermediate in the formation of [³H]*InsP*₅. The work described below details experiments designed to establish which of these explanations is correct, and describes the existence of a pathway in which *Ins*(1,3,4,6)*P*₄, described by Shears *et al.* (1987b) and by Balla *et al.* (1987), is phosphorylated to *Ins*(1,3,4,5,6)*P*₅. Moreover, *Ins*(1,3,4,6)*P*₄ can be detected, identified and separated from the other cellular [³H]*InsP*₄(s) found in both acidic and neutral aqueous extracts from a number of cell types.

MATERIALS AND METHODS

Cell culture and preparation of homogenates

Primary-cultured murine bone-marrow-derived macrophages were prepared and labelled with [³H]*Ins* (130 μCi/ml for 21 h) as described previously (Stephens *et al.*, 1988a). A human astrocytoma cell line (1321 N1)

was cultured as described by Meeker & Harden (1982) and labelled with [³H]*Ins* (50–100 μCi/ml) for 2 days in 3 cm-diameter Petri dishes

Avian erythrocytes were prepared and labelled with [³H]*Ins* as described previously (Stephens *et al.*, 1988a).

Rat brain homogenates were prepared in a buffer containing 0.25 M-sucrose, 50 mM-Hepes, 2 mM-EGTA, 15 mM-2-mercaptoethanol, 50 μM-PMSF, and 1 μg each of antipain, pepstatin A and leupeptin/ml (pH 7.0 at 4 °C) at a tissue density of approx. one brain/5 ml as described previously (Stephens *et al.*, 1988b). Homogenates of primary-cultured macrophages were prepared by lysing the adherent cells as described by Stephens *et al.* (1988b), and typically possessed protein concentrations of 4–5 mg/ml

Preparation of human erythrocyte ghosts

Human erythrocyte ghosts were prepared as described previously (Hawkins *et al.*, 1984).

Extraction of inositol phosphates

Acid cell extracts were prepared by quenching with ice-cold HClO₄ (5%, v/v) and were processed as described by Stephens *et al.* (1988a).

Neutral-pH aqueous extracts of cells were prepared as described by Hawkins *et al.* (1987).

H.p.l.c. separations

Separation of polyols was achieved on a Brownlee Polypore cation-exchange column (in the Pb²⁺ mode) as described by Stephens *et al.* (1988a).

Abbreviations used: *Ins*, *myo*-inositol; *InsP*, *myo*-inositol phosphate; *InsP*₂, *myo*-inositol bisphosphate; *InsP*₃, *myo*-inositol trisphosphate; *InsP*₄, *myo*-inositol tetrakisphosphate; *InsP*₅, *myo*-inositol pentakisphosphate (with isomeric numbering of phosphates as appropriate; note that, in line with the forthcoming NC-IUB recommendation which allows the relaxation of the lowest-number rule of the JCBN if it 'clarifies the relationships in biochemical pathways', all numbering is D unless otherwise stated); PMSF, phenylmethanesulphonyl fluoride; FPLC, fast protein liquid chromatography; SAX, strong-anion-exchange; WAX, weak-anion-exchange; BSA, bovine serum albumin; PAF, platelet-activating factor.

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Partisil 10-SAX anion-exchange h.p.l.c. columns (25 cm $\times$ 0.46 cm) were used as described previously (Stephens *et al.*, 1988a).

Ins P_4 s were resolved on a weak anion-exchange h.p.l.c. column (Partisphere WAX 5 μ m particle size, in a 12.5 cm cartridge; Whatman). The column was pre-conditioned with water (50 ml, at 1.0 ml/min), and then used with a gradient delivered from two independent pumps drawing on reservoirs containing water (pump A) and 0.25 M-(NH $_4$) $_2$ HPO $_4$ (pH 3.2 with H $_3$ PO $_4$ at 25 °C; pump B), at a flow rate of 1.0 ml/min, as follows: 0 min, 0% B; 5 min, 0% B; 6 min, 60% B; 80 min, 60% B; 81 min, 100% B; 90 min, 100% B; 91 min, 0% B. Under these conditions a new column would retain inositol tetrakisphosphates for 50–60 min and achieve a baseline separation of Ins(1,3,4,6) P_4 , Ins(1,3,4,5) P_4 and Ins(3,4,5,6) P_4 (elution in that order; see below). As the column was used, the %B required to maintain this retention time [if the retention time falls below approx. 20 min with this gradient, Ins(1,3,4,6) P_4 and Ins(1,3,4,5) P_4 are not resolved] had to be decreased. The %B required to maintain good separation often fell to 30% of its original value.

The SAX version of the same column, when eluted with similar buffers [A = water, B = 1.25 M-(NH $_4$) $_2$ HPO $_4$ /H $_3$ PO $_4$, pH 3.8], failed to resolve Ins(1,3,4,5) P_4 and Ins(3,4,5,6) P_4 . However, Ins(1,3,4,6) P_4 was eluted well after these poorly resolved isomers, in contrast with the order of elution noted above for the corresponding WAX column. The SAX column, eluted with the same buffers, also was used to separate inositol bisphosphates, by using the following gradient: 0 min, 0% B; 5 min, 0% B; 6 min, 10% B; 60 min, 10% B; 61 min, 0% B. The flow rate was 1 ml/min.

A Partisphere WAX column with a triethylamine/formic acid gradient was employed in the purification of some standards. The following gradient was used (A = water, B = 2.0 M-triethylamine, pH 4.0 with formic acid): 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, although only compounds with a relatively low affinity for the resin (less than Ins P_3 at pH 4.0) could be quantitatively eluted.

D/L-Ins1P, Ins2P, Ins5P, D/L-Ins4P and P_i were separated on a strong anion-exchange h.p.l.c. system. This utilized two 12.5 cm Partisphere SAX (5 μ m particle size) cartridges connected in series by a low dead-volume stainless-steel adaptor (generously given by Whatman Research Laboratories) eluted at 1.0 ml/min with a gradient based on (A) water and (B) 0.2 M-sodium acetate (pH 3.75 at 25 °C, with acetic acid): 0 min, 0% B; 10 min, 0% B; 11 min, 48% B; 80 min, 48% B; 81 min, 100% B; 85 min, 100% B; 86 min, 0% B.

Desalting h.p.l.c. eluates

Fractions of h.p.l.c. eluates were desalted by adjusting their pH to 6–7 with triethylamine, diluting them 10-fold with water and applying them to an anion-exchange resin column (300 μ l packed volume; Bio-Rad AG 1X8, 200–400 mesh formate form).

P_i was eluted with 10 ml of 0.2 M triethylamine (pH 4.0 with formic acid). Ins P_2 s, Ins P_3 s and Ins P_4 s were eluted by washing the resin (which had stood for 5 min after resuspension in the solvent) with 2 $\times$ 2 ml of 2 M-triethylamine (pH 3.8 with formic acid). The resulting

solution was freeze-dried directly. Ins P_5 s were eluted by washing the resin with 2 $\times$ 2 ml of 2 M-ammonium formate/0.1 M-formic acid, and this solution was freeze-dried directly. Trace quantities of Ins P_6 s, particularly if concentrated by freeze-drying from solutions containing triethylamine, cannot be quantitatively removed from glass vessels with water. Ins P_5 s were therefore dried down in polypropylene vials during desalting; the material remaining could then be quantitatively removed with water.

Periodate oxidation of inositol phosphates

Inositol phosphates were oxidized with either periodic acid (0.1 M; pH 2.0 with NaOH at 25 °C) or sodium periodate (0.1 M; pH 4.5 at 25 °C) for 36 h at 25 °C in the dark. Samples were reduced and dephosphorylated as described previously (Stephens *et al.*, 1988a).

Glycerol was cleaved from glycerophosphoinositol phosphates with 10 mM-sodium periodate and 1,1-dimethylhydrazine as described previously (Brown & Stewart, 1966; Stephens *et al.*, 1988a).

Partial dephosphorylation of inositol phosphates

This was achieved for Ins P_5 samples by incubation with human erythrocyte ghosts, and for all other inositol phosphates either by ammonia hydrolysis or by incubation with alkaline phosphatase.

[3 H]Ins P_5 or Ins[32 P] P_5 was incubated with human erythrocyte ghosts (prepared as described above) for 90 min (5 ml assay volume, containing 2 ml of packed ghosts, 2 mM-MgCl $_2$, 2 mM-EGTA, 50 mM-Hepes and 1 mg of BSA/ml, pH 7.0, 37 °C). The reaction was terminated by the addition of 50 μ l of ice-cold 72% HClO $_4$.

The precipitate was sedimented by centrifugation and the supernatant neutralized with 2 M-KOH/0.2 M-sodium Hepes; the KClO $_4$ precipitate formed was pelleted, 250 μ l of 0.1 M-EDTA added and, after filtration (pore size 0.22 μ m; Millipore GS filter), the sample could be applied directly to an h.p.l.c. column.

For dephosphorylation with NH $_3$, a sample of the relevant inositol phosphate was mixed with 1 mg of sodium phytate and passed through a 200 μ l column of Bio-Rad AG 50 WX4 anion-exchange resin (200–400 mesh; NH $_4^+$ form), freeze-dried and then reconstituted in 10 M-NH $_3$. Dephosphorylation was achieved in a sealed Carius tube at 110 °C (optimum yields of InsPs were obtained after 30 h for Ins P_3 s, 50 h for Ins P_4 s and 100 h for Ins P_5 s).

Partial dephosphorylation of inositol phosphates with alkaline phosphatase was achieved by incubating a sample of the inositol phosphate in a buffer containing 0.1 mM-ZnCl $_2$, 0.1% BSA, 10 mM-ethanolamine and 5 units of alkaline phosphatase (units as defined by Sigma; type P-5521)/ml at pH 9.5 at 25 °C for various times. Reactions were quenched with ice-cold 5% HClO $_4$, and the samples neutralized with tri-*N*-octylamine/Freon as described by Stephens *et al.* (1988a).

Preparation of standards

Ins[32 P](1,4,5) P_3 was prepared from [32 P] P_i -labelled human erythrocytes (Hawkins *et al.*, 1986), purified on a Partisil 10 SAX anion-exchange h.p.l.c. column and desalted as described above.

Ins[32 P](1,3,4,5) P_4 and [3 H]Ins(1,3,4,5) P_4 were prepared from Ins[32 P](1,4,5) P_3 and [3 H]Ins(1,4,5) P_3 (Amer-

sham International, Amersham, Bucks., U.K.) respectively, by incubating the substrates with a partially purified preparation of rat brain cytosol Ins(1,4,5) P_3 3-OH kinase (Stephens *et al.*, 1988a).

The purity of the [^3H]Ins(1,3,4,5) P_4 preparation was confirmed as follows. (a) It was insensitive to periodate (0.1 M, pH 2.0), yielding only [^3H]inositol after reduction and dephosphorylation. (b) When the [^3H]Ins P_4 was incubated with human erythrocyte ghosts (as described above), 70 % of the starting material was recovered in a [^3H]Ins P_3 that gave [^3H]altritol as the sole polyol upon periodate (0.1 M, pH 4.5) oxidation, reduction and dephosphorylation. (c) Incubation of the [^3H]Ins P_4 preparation with alkaline phosphatase (for 2.5 h under the conditions described above) resulted in 26 % of the radioactivity being recovered in [^3H]Ins P_3 (s) that were purified by h.p.l.c. (Partisil 10-SAX) and desalted (see above). Oxidation with periodate (0.1 M, pH 4.5), followed by reduction and dephosphorylation, gave only [^3H]xylitol with 82 % recovery from the starting Ins P_3 , consistent with it being Ins(3,4,5) P_3 (Batty *et al.*, 1985).

The purity of the Ins[^{32}P](1,3,4,5) P_4 preparation was confirmed by measuring the $^3\text{H}/^{32}\text{P}$ ratio of the Ins P_4 produced in a parallel incubation with an identical concentration of Ins[^{32}P](1,4,5) P_3 spiked with a tracer quantity of [^3H]Ins(1,4,5) P_3 . The product possessed the same $^3\text{H}/^{32}\text{P}$ ratio as the substrate. When subjected to anion-exchange h.p.l.c. with a Partisphere WAX column (see above), only a single peak of ^{32}P could be detected, which was separate from [^3H]Ins(1,3,4,6) P_4 and [^3H]Ins(3,4,5,6) P_4 (see below).

Ins[^{32}P](1,3,4) P_3 and [^3H]Ins(1,3,4) P_3 were prepared from Ins[^{32}P](1,3,4,5) P_4 and [^3H]Ins(1,3,4,5) P_4 respectively, by incubating the substrates with human erythrocyte ghosts (1 h at 37 °C) in a medium containing 2 ml of packed ghosts and 5 ml of a buffer containing 2 mM-MgCl $_2$, 50 mM-Hepes, 2 mM-EGTA and 1 mg of BSA/ml, pH 7.0. [^3H]Ins P_3 and Ins[^{32}P] P_3 were purified from the assay mixtures by chromatography on a Partisil 10-SAX anion-exchange h.p.l.c. column and desalted (as described above). After periodate (0.1 M, pH 4.5) oxidation, reduction and dephosphorylation, 88 % of the [^3H]Ins P_3 was recovered in [^3H]altritol; no other [^3H]polyol could be detected.

[^3H]Ins(1,3,4,6) P_4 and Ins[^{32}P](1,3,4,6) P_4 were prepared by incubating [^3H]Ins(1,3,4) P_3 and Ins[^{32}P](1,3,4) P_3 respectively with a partially purified preparation of Ins(1,3,4) P_3 kinase (see the Results section) in a buffer containing 5 mM-ATP, 80 mM-KCl, 50 mM-Hepes, 2 mM-EGTA, 0.2 mM-CaCl $_2$, 5.9 mM-MgCl $_2$, 15 mM-2-mercaptoethanol and 100 μl of kinase preparation [added in a mixture of glycerol and 50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol (1:1, v/v)], pH 7.0 at 37 °C (this buffer contained a free Ca $^{2+}$ concentration of 0.1 μM and a theoretical free Mg $^{2+}$ concentration of 1 mM; see Stephens *et al.*, 1988b) for 1 h. The assays were quenched with HClO $_4$ and processed for application to a Partisphere WAX h.p.l.c. column as described above. Some 60 % of the ^3H and 70 % of the ^{32}P preparations were typically recovered as Ins P_4 (see the Results section).

[^3H]Ins(3,4,5,6) P_4 was prepared from [^3H]Ins-prelabelled 5-day-old chick erythrocytes (as described by Stephens *et al.*, 1988a), purified on a Partisphere WAX h.p.l.c. column and desalted (as described above). The [^3H]Ins P_4 so produced yielded [^3H]iditol (the final

recovery of ^3H was 74 %) upon periodate (0.1 M, pH 2.0) oxidation, reduction and dephosphorylation (Johnson & Tate, 1969; Stephens *et al.*, 1988a), which was oxidized by L-iditol dehydrogenase (EC 1.1.1.14) to [^3H]sorbose at the same rate as internal L-iditol (assayed as described by Stephens *et al.*, 1988a).

Ins[^{32}P](3,4,5,6) P_4 was prepared from [^{32}P]P $_i$ -labelled 5-day-old chick erythrocytes as described by Stephens *et al.* (1988a), except that the HClO $_4$ extract was mixed with 5 mg of acid-washed Darco-G60 charcoal. After 10 min on ice, 1 mg of BSA was added to the mixture, and the charcoal was pelleted in a Micro-Centaur bench-top centrifuge. The supernatant was neutralized, applied to a Partisil 10-SAX h.p.l.c. column, and the fractions containing the Ins[^{32}P] P_4 were eluted and desalted as described above. The desalted Ins[^{32}P] P_4 preparation was applied to a Partisphere WAX h.p.l.c. column and eluted as described above. Three major ^{32}P -labelled compounds were resolved that possessed the mobilities expected for an Ins P_4 . The compound with the longest retention time accounted for 70–80 % of the total ^{32}P recovered (when the erythrocytes had been labelled with [^{32}P]P $_i$ for 24 h). The fractions containing this compound were pooled and desalted, and a sample of the preparation was mixed with [^3H]Ins(3,4,5,6) P_4 (prepared as described above) and applied to a Partisphere WAX h.p.l.c. column, which was eluted as described above. The ^{32}P -labelled compound co-chromatographed precisely with [^3H]Ins(3,4,5,6) P_4 .

[^3H]Ins(1,3,4,5,6) P_5 and Ins[^{32}P](1,3,4,5,6) P_5 were prepared from 5-day-old chick erythrocytes labelled with either [^{32}P]P $_i$ or [^3H]Ins as described above for Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins(3,4,5,6) P_4 except that the only purification required for both ^3H - and ^{32}P -labelled preparations was achieved on a Partisil 10-SAX h.p.l.c. column as described above.

[^{14}C]Ins2P and D/L-[^{14}C]Ins1P were prepared from Ptd[^{14}C]Ins (Amersham International) as described previously (Stephens *et al.*, 1988b).

[^{14}C]Ins4P was prepared from Ptd[^{14}C]Ins4P (in turn prepared by incubation of Ptd[^{14}C]Ins with human erythrocyte membranes) by deacylation (Clarke & Dawson, 1981) and alkaline hydrolysis (Grado & Ballou, 1961).

Ins[^{32}P]5P was prepared by dephosphorylation of the ammonium salt of Ins[^{32}P](1,4,5) P_3 as described above.

Protein separation techniques

Soluble proteins were separated by using an anion-exchange FPLC column (Mono-Q H/R 10/10 analytical FPLC column; Pharmacia) and a gel-filtration FPLC column (Superose-12; Pharmacia) as described previously (Stephens *et al.*, 1988b).

(NH $_4$) $_2$ SO $_4$ precipitations were carried out on ice as described by Stephens *et al.* (1988b).

Assay of inositol phosphate kinase and phosphatase activities

All assays were performed in an incubation buffer of the composition defined by Stephens *et al.* (1988b) and above in the section describing the preparation of [^3H]Ins(1,3,4,6) P_4 , either with or without MgNa $_2$ ATP.

Reagents and radiochemicals were obtained from sources previously defined (Stephens *et al.*, 1988a,b).

Table 1. Metabolism of [^3H]Ins[^{32}P](1,4,5) P_3 by rat brain cytosol in the presence of ATP

A 1 nmol portion of [^3H]Ins[^{32}P](1,4,5) P_3 (prepared as described in the Materials and methods section, containing 71 % of the ^{32}P in the 5-phosphate and 29 % in the 4-phosphate; the distribution of radioactivity was determined as described by Hawkins *et al.*, 1984) was incubated with a rat brain cytosol fraction for various times [1 ml total assay volume, containing 20 μl of cytosol preparation and an incubation buffer of final composition 80 mM-KCl; 50 mM-Hepes; 2 mM-EGTA; 1 mM-MgCl $_2$; 0.2 mM-CaCl $_2$; 15 mM-2-mercaptoethanol; 1 mg of BSA/ml; 5 mM-MgNa $_2$ -ATP, which was omitted from phosphatase assays (see below); pH 7.0, 37 °C]. If the assays contained a portion of a homogenate or soluble or particulate fraction, then 10 mM-phosphocreatine and 5 units (as defined by Sigma) of creatine kinase/ml were included in the buffer. The samples were quenched and processed for chromatography, and the data were collected and presented as described in the legend to Table 2. The mean range of the duplicates was 5.2 %.

Incubation time (min)	Radio-isotope	$^3\text{H}/^{32}\text{P}$ -labelled metabolites (d.p.m.)							
		Ins	InsP	P $_i$	InsP $_2$ s	Ins(1,3,4) P_3	Ins(1,4,5) P_3	InsP $_4$	Totals
0	^3H	133	283		474	—	49 140 (22)	—	50030 (23)
	^{32}P		—	—	—	—	2195	—	2195
1	^3H	128	158		2163	733 (91)	11 549 (21)	37 775 (23)	52 378 (24)
	^{32}P		—	40	17	8	555	1608	2228
15	^3H	840	3095		9211	6248 (77)	657 (16)	32 888 (32)	52 972 (23)
	^{32}P		—	1022	93	81	42	1035	2287
45	^3H	3785	8925		10 899	1911 (64)	270 (15)	23 166 (51)	53 722 (23)
	^{32}P		—	1654	112	30	18	455	2332

RESULTS

Metabolism of [^3H]Ins[^{32}P](1,4,5) P_3 by rat brain and murine macrophage homogenates

Incubation of [^3H]Ins[^{32}P](1,4,5) P_3 (prepared as described in the Materials and methods section, with the ^{32}P located in the 4 and 5 positions only) with a rat brain soluble fraction, in a buffer of intracellular ionic composition, results in the rapid metabolism of the substrate. Dephosphorylation is catalysed by a soluble Ins(1,4,5) P_3 5-phosphate phosphomonoesterase, yielding [^3H]Ins[^{32}P](1,4) P_2 (results not shown; Storey *et al.*, 1984) and, in the presence of an ATP-regenerating system, phosphorylation is catalysed by Ins(1,4,5) P_3 3-OH kinase (Irvine *et al.*, 1986), giving [^3H]Ins[^{32}P](1,3,4,5) P_4 (Batty *et al.*, 1985; see the Materials and methods section).

The dephosphorylation of [^3H]Ins[^{32}P](1,3,4,5) P_4 in brain soluble fractions (in the absence of ATP) proceeds via a single detectable [^3H]Ins[^{32}P] P_3 , which yields [^3H]altritol upon periodate oxidation (0.1 M, pH 4.5), reduction and dephosphorylation [consistent only with the structures D/L-Ins(1,3,4) P_3 and/or D/L-Ins(1,2,4) P_3]. This Ins P_3 is in turn dephosphorylated by two routes in rat brain cytosol. An EDTA-sensitive phosphatase yields a [^3H]Ins[^{32}P] P_2 that gives [^3H]threitol upon periodate oxidation (0.1 M, pH 4.5), reduction and dephosphorylation, consistent only with the structure D/L-Ins[^{32}P](3,4) P_2 , and an EDTA-resistant route yields a [^3H]Ins P_2 that gives [^3H]adonitol upon periodate oxidation (0.1 M, pH 4.5), reduction and dephosphorylation, consistent only with the structure [^3H]Ins(1,3) P_2 (results not shown). These results [and those in the Materials and methods section, which are identical in form with those of Irvine *et al.* (1984), Batty *et al.* (1985), Hansen *et al.* (1986), Bansal *et al.* (1987) and Shears *et al.* (1987a,b)] are consistent only with the original [^3H]Ins[^{32}P] P_4 possessing the structure D- or L-[^3H]Ins[^{32}P](1,3,4,5) P_4 .

If the starting material is assumed to be D-[^3H]Ins[^{32}P](1,4,5) P_3 , then it is possible to assign to the Ins P_4 the structure D-Ins(1,3,4,5) P_4 . This assumption is based on two forms of stereochemical observation; initially by a comparison of the specific optical rotation of biologically derived inositol phosphates with those of chemically synthesized standards (Ballou & Pizer, 1960; Grado & Ballou, 1961), and more recently by use of a stereochemically selective enzyme (Stephens *et al.*, 1988a).

Prolonged incubation of radiochemically pure [^3H]Ins[^{32}P](1,4,5) P_3 with rat brain cytosol in the presence of an ATP-regenerating system resulted in the appearance of a highly polar compound with the chromatographic properties of an Ins P_5 (see Tables 1, 2 and 4 and Fig. 1). Similarly, incubation of [^3H]Ins(1,4,5) P_3 and an ATP-regenerating system with murine macrophages (over 98 % pure) homogenates results in the formation of a ^3H -labelled compound that ran precisely with an internal Ins[^{32}P](1,3,4,5,6) P_5 standard during anion-exchange h.p.l.c. (results not shown).

The [^3H]Ins[^{32}P] P_4 (s) produced after 1, 5 and 20 min progress of such an assay with rat brain cytosol were purified by anion-exchange h.p.l.c., desalted, oxidized with periodate (0.1 M, pH 2.0), reduced and dephosphorylated. At all three times the only product was [^3H]Ins (obtained in 58, 65 and 72 % final recoveries respectively).

The $^3\text{H}/^{32}\text{P}$ ratio of this Ins P_4 (s) peak, resolved on a

Table 2. Metabolism of [^3H]Ins[^{32}P](1,3,4,5) P_4 by rat brain cytosol in the presence of ATP

A 1.5 nmol portion of [^3H]Ins[^{32}P](1,3,4,5) P_4 which was prepared from the Ins[^{32}P](1,4,5) P_3 used as a substrate in the experiments described in Table 1, and hence had the same ^{32}P distribution) was incubated with 40 μl of cytosol preparation (in a final volume of 200 μl with the assay buffer described in the legend to Table 1), for various times, then quenched with ice-cold HClO_4 , processed for application to a Partisil 10-SAX h.p.l.c. column and eluted as described in the Materials and methods section. The data shown are means ($n = 2$) for the ^3H and ^{32}P contents of the metabolites indicated (the $^3\text{H}/^{32}\text{P}$ d.p.m. ratio is shown in parentheses), measured in a standard liquid-scintillation counter with a $^3\text{H}/^{32}\text{P}$ dual-label quench-corrected program. The mean range of the duplicates was 4.3%.

Incubation time (min)	Radioisotope	$^3\text{H}/^{32}\text{P}$ labelled metabolites (d.p.m.)						
		Ins	InsP	P_i	Ins(1,3) P_2	Ins(3,4) P_2	Ins(1,3,4) P_3	Totals
0	^3H	60	—	—	—	—	72	5296 (1.46)
	^{32}P	—	—	—	—	—	—	3617
15	^3H	73	440	—	346	941 (4.3)	478 (4.5)	4925 (1.46)
	^{32}P	—	—	1887	13	217	106	3383
45	^3H	858	978	—	325	513 (4.3)	75 (3.4)	5154 (1.5)
	^{32}P	—	—	2719	4	119	22	3424
								Ins P_5
								112 (4.3)
								26
								576 (4.0)
								144

Partisil 10-SAX column, rose with time of incubation (see Table 1), from an initial value identical with that of the starting material eventually to a value similar to that of the [^3H]Ins[^{32}P](1,3,4) P_3 produced in parallel. Similarly, if [^3H]Ins[^{32}P](1,3,4) P_4 was incubated with a brain soluble fraction, then the $^3\text{H}/^{32}\text{P}$ ratio of the Ins P_4 peak rose with time, and a compound with the chromatographic properties of an Ins P_5 appeared which possessed a $^3\text{H}/^{32}\text{P}$ ratio significantly higher than that of the initial [^3H]Ins[^{32}P](1,3,4,5) P_4 and similar to that of the [^3H]Ins[^{32}P](1,3,4) P_3 produced in the same incubation (see Table 2). When a sample from an experiment involving incubation of [^3H]Ins[^{32}P](1,4,5) P_3 with a rat brain soluble fraction was applied to a more chromatographically efficient anion-exchange column (Partisphere SAX) and eluted as described above, then, after 30 min two Ins P_4 compounds were resolved (see Fig. 1). The earlier-eluted peak had precisely the same $^3\text{H}/^{32}\text{P}$ ratio as the substrate and the single [^3H]Ins[^{32}P]- P_4 peak that was present after only 1 min of incubation (see Fig. 1b). The later-eluted peak had a substantially higher $^3\text{H}/^{32}\text{P}$ ratio that was the same as for the [^3H]Ins[^{32}P](1,3,4) P_3 formed in the same assay. The [^3H]Ins[^{32}P] P_5 formed in these assays (see Fig. 1 and Tables 1 and 2) showed a mean $^3\text{H}/^{32}\text{P}$ ratio that was $89 \pm 7.4\%$ (mean $\pm$ S.E.M., $n = 9$) of that of the internally produced [^3H]Ins[^{32}P](1,3,4) P_3 .

Incubation of [^3H]Ins(1,3,4) P_3 with rat brain homogenates

[^3H]Ins(1,3,4) P_3 (prepared as described in the Materials and methods section) was incubated with rat brain homogenates, and the soluble and particulate fractions derived from them, in buffers with an ionic composition like that of the cytosol (see the Materials and methods section).

The [^3H]Ins(1,3,4) P_3 was phosphorylated in homogenates and soluble fractions to yield a [^3H]Ins P_4 (see Tables 3 and 4) by an activity that was dependent on the presence of ATP and, in soluble fractions, unaffected by either 2 μM free Ca^{2+} or 10 μM cyclic AMP (results not shown). At later times a compound with the chromatographic properties of a [^3H]Ins P_5 appeared in the incubations (see Table 4). The [^3H]Ins P_4 did not co-chromatograph with Ins[^{32}P](1,3,4,5) P_4 (on Partisphere SAX or WAX anion-exchange h.p.l.c. columns; results not shown) or Ins[^{32}P](3,4,5,6) P_4 (on a Partisphere WAX h.p.l.c. column; results not shown) and, when treated with periodic acid (0.1 M, pH 2.0), reduced and dephosphorylated, yielded [^3H]Ins (75% overall recovery of ^3H through the processing).

To enable substantial quantities of the [^3H]Ins P_4 product of [^3H]Ins(1,3,4) P_3 phosphorylation to be prepared, a partial purification of the rat brain cytosol activity was undertaken (see Table 3 and Fig. 2). $(\text{NH}_4)_2\text{SO}_4$ fractionation of a rat brain cytosol fraction combined with anion-exchange chromatography gave a 230-fold purification of the activity over that present in a rat brain homogenate (see Table 3). The kinase activity was eluted from a calibrated gel-filtration column as a single peak, at a volume suggesting a native M_r of 30 000 (see Fig. 3). When incubated with the partially purified kinase preparation, Ins[^{32}P](1,3,4) P_3 was converted into an Ins[^{32}P] P_4 that did not chromatograph with [^3H]Ins(1,3,4,5) P_4 or [^3H]Ins(3,4,5,6) P_4 (see below) when eluted from a Partisphere WAX h.p.l.c. column. The

Fig. 1. H.p.l.c. separation of the products of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4,5)P_3$ metabolism in rat brain cytosol

Assays contained 40 000 d.p.m. of D- $[^3\text{H}]\text{Ins}(1,4,5)P_3$ and 14 000 d.p.m. of $\text{Ins}[^{32}\text{P}](1,4,5)P_3$ [the final concn. of $\text{Ins}(1,4,5)P_3$ was $0.75\ \mu\text{M}$] in a buffer of final composition identical with that defined in the legend to Table 1, and $40\ \mu\text{l}$ of rat brain cytosol preparation (in a total volume of $200\ \mu\text{l}$). The incubations were at $37\ ^\circ\text{C}$ for (a) 0, (b) 1 or (c) 30 min. The reactions were quenched with ice-cold 5% HClO_4 processed for application to a Partisphere SAX h.p.l.c. column and eluted from the column as described in the Materials and methods section. Fractions were collected every 0.25 min, mixed with scintillation fluid and counted for

Table 3. Partial purification of rat brain cytosol Ins(1,3,4) P_3 kinase

The fractions of rat brain protein were prepared as described in the Materials and methods section (and Fig. 3). Assays contained 3500 d.p.m. of [^3H]Ins(1,3,4) P_3 , in a mixture with a total volume of 200 μl and a final composition identical with that described in the legend to Table 1. The final concentration of [^3H]Ins(1,3,4) P_3 was 8.0 nM. The assays were terminated before 50% of the initial substrate had been metabolized. Data are presented as means ($n = 2-8$, from four different experiments) converted (into [^3H]Ins, [^3H]Ins P and [^3H]Ins P_2 for the phosphatase activity and into [^3H]Ins P_4 in the kinase assays) in 5 min per mg of protein. The products were separated on small anion-exchange resin columns as described previously (Stephens *et al.*, 1988b). MgNa_2ATP (5 mM) caused a 60–80% decrease in the total measured phosphatase activity.

Fraction	Activity against [^3H]Ins(1,3,4) P_3 (d.p.m. converted/5 min per mg of protein)	
	Total phosphatase	Kinase
Homogenate	21 589	3751
Particulate fraction	12 327	240
Soluble fraction	54 472	15 666
30–50% satd.-(NH_4) $_2\text{SO}_4$	17 594	19 343
Anion-exchange peak	6651 ($\times \frac{1}{3.2}$)	878 000 ($\times 234$)

[^3H]Ins P_4 produced by the partially purified kinase yielded only [^3H]Ins upon periodate oxidation (0.1 M, pH 2.0), reduction and dephosphorylation (the final recovery of ^3H through this process was 82%). When incubated with alkaline phosphatase (as described in the Materials and methods section, or when MgCl_2 was substituted for ZnCl_2 in the buffer), the [^3H]Ins P_4 did not yield a significant quantity of [^3H]Ins P_3 . The only dephosphorylation products which accumulated significantly were [^3H]Ins P_2 (s) and [^3H]Ins. These results are only consistent with the product of Ins(1,3,4) P_3 phosphorylation, catalysed by both rat brain cytosol and the partially purified kinase preparation, being Ins(1,3,4,6) P_4 (see below for discussion).

Metabolism of [^3H]Ins P_4 product of D-[^3H]Ins(1,3,4) P_3 phosphorylation by rat brain homogenates

The [^3H]Ins P_4 product of [^3H]Ins(1,3,4) P_3 phosphorylation by the partially purified kinase of rat brain cytosol {i.e. [^3H]Ins(1,3,4,6) P_4 } was incubated with rat brain homogenates, or the cytosolic and/or particulate fractions derived from them, in the presence of ATP (see Table 5). A highly polar product was formed that was chromatographically indistinguishable from Ins[^{32}P](1,3,4,5,6) P_5 .

The [^3H]Ins P_5 produced in an assay containing rat brain cytosol and [^3H]Ins(1,3,4,6) P_4 was purified by anion-exchange h.p.l.c., desalted (see above), and incubated with human erythrocyte ghosts (see the Materials and methods section; this dephosphorylation, which occurs at a low rate under 'extreme' conditions, was merely employed as a convenient mechanism by which to

Table 4. Metabolism of [^3H]Ins(1,3,4) P_3 and [^3H]Ins(1,4,5) P_3 by rat brain cytosol fractions

[^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4) P_3 were incubated with 40 μl of rat brain cytosol fraction (143 μg of protein, in a volume of 200 μl ; the assay buffer had a final composition identical with that described in the legend to Table 1) for various times and quenched with ice-cold 5% HClO_4 . The samples were processed as described in the Materials and methods section, and the [^3H]inositol phosphates were separated on small anion-exchange columns as described previously (Stephens *et al.*, 1988b). Each value is a mean ($n = 3$), the standard errors of which were all within 9%.

Time (min)	Substrate	Radioactivity (d.p.m.)						Total
		Ins	Ins P	Ins P_2	Ins P_3	Ins P_4	Ins P_5	
0	[^3H]Ins(1,4,5) P_3	0	49	116	5835	191	0	6191
	[^3H]Ins(1,3,4) P_3	0	27	8	3155	10	9	3209
0.25	[^3H]Ins(1,4,5) P_3	0	42	136	554	5562	0	6294
	[^3H]Ins(1,3,4) P_3	0	27	23	3049	26	1	3126
1	[^3H]Ins(1,4,5) P_3	0	43	154	347	5448	18	6010
	[^3H]Ins(1,3,4) P_3	0	18	190	2759	252	0	3219
5	[^3H]Ins(1,4,5) P_3	0	107	609	1049	4190	42	5997
	[^3H]Ins(1,3,4) P_3	0	73	802	869	1355	47	3146
10	[^3H]Ins(1,4,5) P_3	56	303	1107	783	3586	126	5958
	[^3H]Ins(1,3,4) P_3	0	190	934	265	1667	119	3175
20	[^3H]Ins(1,4,5) P_3	400	920	1204	393	3091	372	6380
	[^3H]Ins(1,3,4) P_3	0	383	774	72	1618	300	3147

^3H and ^{32}P radioactivity by standard dual-label liquid-scintillation-counting techniques. Neighbouring pairs of fractions containing background radioactivity were pooled and meaned for the purposes of presentation. The value ^3H d.p.m./ ^{32}P d.p.m. ratio for each peak is shown on the Figure. The overall $^3\text{H}/^{32}\text{P}$ d.p.m. ratios for all recovered radioactivities in the 0, 1 and 30 min samples were 2.80, 2.78 and 2.70 respectively. The abscissa of Fig. 1(c) is divided by an additional ordinate. Points to the left of this line are plotted at co-ordinates defined by the scale on the ordinate to the left, and points to the right are plotted at co-ordinates defined by the ordinate at the right-hand side of the Figure.

Fig. 2. Separation of rat brain cytosol $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,6)\text{P}_4$ kinase and phosphatase activities on an anion-exchange column

A portion (0.38 ml, containing 10 mg of protein) of a solubilized [in 50 mM-Tris/1 mM-EGTA/15 mM-2-mercaptoethanol/50 μM -PMSF; containing antipain, leupeptin and pepstatin A (each 1 $\mu\text{g}/\text{ml}$); pH 7.2 at 4 $^{\circ}\text{C}$] 20–50 % satd. $(\text{NH}_4)_2\text{SO}_4$ fraction from rat brain cytosol was applied to a Mono-Q FPLC column and eluted with a gradient based on buffer A (identical with the buffer in which the sample was loaded; see above) and B (as above but also containing 1 M-NaCl), delivered as follows: 0–10 min, 0% B; 10–50 min, 0–30% B; 50–60 min, 30–100% B (at 0.5 ml/min; fractions were collected at 1 min intervals). (a) Samples (5 μl) of each fraction were assayed for $\text{Ins}(1,3,4)\text{P}_3$ kinase as described in the legend to Table 1(b). Samples (15 μl) of each fraction were assayed for $\text{Ins}(1,3,4,6)\text{P}_4$ kinase activity (the assays were of a final composition and volume identical with those for $\text{Ins}(1,3,4)\text{P}_3$ kinase described above, except that they contained 2500 d.p.m. of $[^3\text{H}]\text{Ins}(1,3,4,6)\text{P}_4$). The $[^3\text{H}]\text{Ins}$ -labelled products formed in each assay were separated on small anion-exchange columns as described previously (Stephens *et al.*, 1988b). The data shown are representative of three identical independent experiments. The fractions containing $\text{D-Ins}(1,3,4)\text{P}_3$ kinase activity, without significant $\text{Ins}(1,3,4)\text{P}_3$ phosphatase activity, were pooled and dialysed against 1:1 (v/v) mixture of 50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol (pH 7.0, 4 $^{\circ}\text{C}$) and glycerol, and stored at -20°C .

obtain a reasonable quantity of a structurally defined breakdown product that would allow the positions of the phosphate groups in the parent InsP_5 to be deduced) in parallel with a sample of $[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ (prepared as described above). Both $[^3\text{H}]\text{InsP}_5$ samples were significantly dephosphorylated, to yield families of ^3H -labelled InsPs , InsP_2s , InsP_3s and InsP_4s (results not shown). The $[^3\text{H}]\text{InsP}_4$ formed from both $[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ and the unassigned $[^3\text{H}]\text{InsP}_5$ co-chromatographed precisely with $\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ on a Partisphere WAX anion-exchange h.p.l.c. column (results not shown; see the Materials and methods section). If the $[^3\text{H}]\text{InsP}_4\text{(s)}$ produced in these incubations were purified by anion-exchange h.p.l.c. and desalted, then upon periodate oxidation (0.1 M, pH 2.0), reduction and dephosphorylation, they both yielded $[^3\text{H}]\text{iditol}$, which was resistant to oxidation by L-iditol dehydrogenase (results not shown). The $[^3\text{H}]\text{InsP}_3\text{(s)}$, produced from both $[^3\text{H}]\text{InsP}_5$ samples during incubations with human erythro-

cyte ghosts, yielded $[^3\text{H}]\text{iditol}$ (79 % of recovered radioactivity) and $[^3\text{H}]\text{xylitol}$ (19 % of recovered radioactivity), and the $[^3\text{H}]\text{InsP}_2\text{(s)}$ gave $[^3\text{H}]\text{adonitol}$ (4 % of recovered radioactivity), $[^3\text{H}]\text{threitol}$ (7 % of recovered radioactivity) $[^3\text{H}]\text{altritol}$ (14 % of recovered radioactivity) and $[^3\text{H}]\text{iditol}$ (75 % of recovered radioactivity) upon periodate oxidation (0.1 M, pH 4.5), reduction and dephosphorylation.

Occurrence in cells of $[^3\text{H}]\text{InsP}_4\text{(s)}$ derived from the phosphorylation of $\text{D-Ins}(1,3,4)\text{P}_3$

When acid extracts of $[^3\text{H}]\text{Ins}$ -prelabelled, primary-cultured, murine bone-marrow-derived macrophages, 1321 N1 astrocytoma cells or avian erythrocytes were mixed with $\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$, $\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ and $\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ standards and applied to a high-efficiency anion-exchange h.p.l.c. column (Partisphere

Fig. 3. Separation of Ins(1,3,4)P₃ and Ins(1,3,4,6)P₄ kinase and phosphatase activities on a gel-filtration column

A 50 μ l portion of a reconstituted 20–50 % -satd.-(NH₄)₂SO₄ fraction [containing 1.5 mg of protein, in 0.5 M-KCl/50 mM-Hepes (pH 7.0, 4 °C)/2 mM-EGTA/15 mM-2-mercaptoethanol/50 μ M-PMSF and antipain, pepstatin A and leupeptin (each 1 μ g/ml)] from a rat brain cytosol was applied to a Superose-12 FPLC column equilibrated with the sample buffer at 4 °C. Fractions were collected every 1.66 min with a flow rate of 0.3 ml/min, between the V₀ (7 ml) and the V_i (19 ml), and the A₂₈₀ of the eluate was monitored continuously. A 340 μ l portion of each fraction was assayed for Ins(1,3,4,6)P₄ kinase (the final assay volume was 1.1 ml, with an ionic composition identical with that defined in the legend to Table 1; it contained 2000 d.p.m. of [³H]Ins-(1,3,4,6)P₄). (b) A 75 μ l portion from each fraction was assayed for Ins(1,3,4)P₃ kinase activity exactly as described in the legend to Table 3. The [³H]inositol phosphates were separated and quantified as described by Stephens *et al.* (1988b). The elution position of M_r ($\times 10^{-3}$) markers are indicated in part (a); they were: cytochrome *a*, 12400; carbonic anhydrase, 29000; ovalbumin, 45000; BSA, 66000; alcohol dehydrogenase, 150000; β -amylase, 200000; apo-ferritin, 440000. The data shown are typical of those from three independent experiments.

WAX; see the Materials and methods section), three peaks of ³H that were eluted in parallel with the Ins-[³²P]P₄ standards (see Fig. 4 and Table 6) were detected. These three peaks of ³H could still be detected in neutral, phenol-based, extracts of the tissues (see the Materials and methods section).

The [³H]InsP₄ from avian erythrocytes, which co-chromatographed with Ins[³²P](1,3,4,6)P₄, was analysed in more detail. A 0.13 μ Ci portion was purified on a Partisphere WAX h.p.l.c. column and then desalted. A 0.12 μ Ci portion was dephosphorylated in 10 M-NH₃ (a process which occurs without phosphate migration; see the Materials and methods section). The resulting family of [³H]inositol phosphates was mixed with D/L-[¹⁴C]Ins1P, [¹⁴C]Ins2P, [¹⁴C]Ins4P and Ins[³²P]5P and separated on an anion-exchange h.p.l.c. column (see the Materials and methods section). The only [³H]Ins-labelled InsPs that could be detected co-chromatographed with D/L-[¹⁴C]Ins1P and [¹⁴C]Ins4P. A sample of the remaining [³H]InsP₄ was subjected to periodate (0.1 M, pH 2.0) oxidation, reduction and dephosphorylation. The only [³H]polyol that could be detected was Ins (the overall recovery of ³H was 65 %).

Table 5. [³H]Ins(1,3,4,6)P₄ metabolism by rat brain homogenates

[³H]Ins(1,3,4,6)P₄ was incubated with 40 μ l portions of homogenate, soluble or particulate fractions (with an assay buffer of a final composition as described in the legend to Table 1, in a total volume of 200 μ l). The reactions were quenched with ice-cold 5 % HClO₄ and, after extraction of the acid, the products were separated on anion-exchange resin columns as described previously (Stephens *et al.*, 1988b). Less than 40 % of the starting substrate was metabolized in each of the assays. The data presented are means of triplicate determinations, and four separate preparations gave similar results.

	Conversion into InsP ₅ (d.p.m./5 min per mg of protein)	Total dephos- phorylated (d.p.m./5 min per mg of protein)
Homogenate	76	68
Soluble	458	83
Particulate	40	26

Fig. 4. H.p.l.c. separation of [^3H]inositol phosphates extracted from control and PAF-stimulated macrophages

Petri dishes (10 cm diam.) of [^3H]Ins-prelabelled ($130 \mu\text{Ci/ml}$ for 21 h) primary-cultured macrophages (see the text) were quenched with ice-cold 5% HClO_4 (after aspiration of the medium) in either their basal state or after various periods of stimulation with PAF ($2 \mu\text{g/ml}$ final concn.). After neutralization, half of the acid extract was mixed with Ins[^{32}P](1,4,5) P_3 , Ins[^{32}P](1,3,4,5) P_4 , Ins[^{32}P](1,3,4,6) P_4 , and Ins[^{32}P](3,4,5,6) P_4 and Ins[^{32}P](1,3,4,5,6) P_5 , applied to a Partisphere WAX h.p.l.c. column and eluted as described in the Materials and methods section, yielding the 'inositol phosphate' data given in Table 6 and the two representative traces shown in the Figure. The abscissae of both graphs are divided into three sections, the ordinate values of the ^3H points (●) being defined by the scale on the left-hand side of that section.

Table 6. H.p.l.c. separation of [^3H]inositol phosphates extracted from control and PAF-stimulated macrophages

Acid extracts of [^3H]Ins-prelabelled control or PAF-stimulated macrophages were prepared as described in the legend to Fig. 4. The total ^3H (d.p.m.) collected in each identified peak (see Fig. 4) is given in the column headed 'Inositol phosphates' (this constituted the radioactivity found in that compound in half of the total acid extract; see Fig. 4). The remaining half of the acid extract was applied to a Partisil 10-SAX h.p.l.c. column, and the [^3H]Ins P_4 peak was collected, desalted, oxidized with periodic acid (0.1 M, pH 2.0), reduced and dephosphorylated. The [^3H]polyols produced were separated and quantified. The data were corrected for total losses in processing (recoveries were in the range 78–90%) to give the total radioactivity (d.p.m.) in [^3H]Ins P_4 (s) that yielded [^3H]Ins or [^3H]iditol (in half of the acid extract). All the PAF-stimulated data are presented as percentage differences from the control value of that metabolite. The mean range of the duplicates was 3.9%.

^3H -labelled metabolite	Control (d.p.m.)		PAF-stimulated (% above control)			
			40 s		5 min	
	Inositol phos- phates	Polyols	Inositol phos- phates	Polyols	Inositol phos- phates	Polyols
[^3H]Ins P + [^3H]Ins P_2	215 791		1079		234	286
[^3H]Ins(1,3,4) P_3	3148		6181		1710	229
[^3H]Ins(1,4,5) P_3	11372		778		172	218
[^3H]Ins(1,3,4,6) P_4	1927	[^3H]Ins3860	250	Ins 1254	430	540
[^3H]Ins(1,3,4,5) P_4	751		6375		1365	1550
[^3H]Ins(3,4,5,6) P_4	3885	[^3H]Iditol 2703	180	Iditol 154	249	370
[^3H]Ins P_5	12 348		95		185	96

Upon stimulation of primary-cultured murine bone-marrow-derived macrophages with a maximal dose of PAF (final concn. 2 $\mu\text{g}/\text{ml}$), the amounts of all three [^3H]Ins P_4 (s) rose. The ^3H compound that co-migrated with Ins[^{32}P](1,3,4,5) P_4 was at its maximum measured value (63-fold above control) at the first observation point (40 s). By contrast, the other [^3H]Ins P_4 s reached their highest measured values after 10 min stimulation (see Fig. 4 and Table 6).

Parallel quantitative periodate oxidation, reduction and dephosphorylation of the [^3H]Ins P_4 (s) present in control and stimulated macrophages revealed a pattern of change in the proportions of the [^3H]polyols recovered that was entirely consistent with the anion-exchange h.p.l.c. data (see Table 6).

DISCUSSION

We have previously reported that Ins(3,4,5,6) P_4 , the major Ins P_4 in many cell types, can act as substrate for a widely distributed soluble D-1-hydroxyl kinase synthesizing Ins(1,3,4,5,6) P_5 (Stephens *et al.*, 1988a,b). The metabolic origin of Ins(3,4,5,6) P_4 remains unknown. In experiments designed to test whether Ins(1,4,5) P_3 might act as an indirect precursor of Ins(3,4,5,6) P_4 , [^3H]Ins(1,4,5) P_3 was added to homogenates of rat brain and primary-cultured macrophages, and the eventual appearance of [^3H]Ins P_5 was detected (see Fig. 1 and Table 4). This catalytic ability of a homogenate derived from a homogeneous population of macrophages (Stephens *et al.*, 1988a) suggests that all of the relevant enzyme activities are found within a single cell type and that the pathway responsible is not the sole result of the pooling of enzymes from some of the different cell types that would be present in brain homogenates (although whether the enzymes within a single cell type would normally have similar access to the relevant substrate/product pools *in vivo* remains unanswered). The [^3H]Ins P_4 (s) present in these incubations (after 1, 15 and

30 min) were insensitive to oxidation by periodate under conditions in which Ins(3,4,5,6) P_4 was cleaved. Presumably a periodate-resistant Ins P_4 was able to act as a precursor for the [^3H]Ins P_5 . Previous work (Stephens *et al.*, 1988b) demonstrated that, when [^3H]Ins(3,4,5,6) P_4 and Ins[^{32}P](1,3,4,5) P_4 were incubated together in a primary-cultured macrophages homogenate, an Ins P_5 that was significantly labelled only with ^3H was produced. Consequently it was concluded that Ins(1,3,4,5) P_4 could not act as a direct precursor of Ins P_5 .

The lag in the conversion of [^3H]Ins(1,4,5) P_3 into [^3H]Ins P_5 by brain soluble fractions, even once all of the initial substrate had been converted into [^3H]Ins(1,3,4,5) P_4 (see the Materials and methods section and Table 4) is also in agreement with this conclusion. When the substrate [either Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4] in one of these experiments was dual-labelled with [^3H]Ins and [^{32}P]P $_i$, with the ^{32}P located in the 5 (70%) and the 4 (30%) phosphates only, then the Ins P_5 formed possessed a $^3\text{H}/^{32}\text{P}$ ratio that was approx. 90% of that of the [^3H]Ins-[^{32}P](1,3,4) P_3 , [^3H]Ins-[^{32}P](1,4) P_2 , [^3H]Ins[^{32}P](3,4) P_2 and [^3H]Ins[^{32}P]4P that are (or would be) produced in the same incubations. This discrepancy in $^3\text{H}/^{32}\text{P}$ ratio was statistically significant ($P < 0.05$), and could mean that a proportion of the Ins P_5 was produced by direct phosphorylation of Ins(1,3,4,5) P_4 . By contrast, the [^3H]Ins-[^{32}P]P $_4$ formed initially had a $^3\text{H}/^{32}\text{P}$ ratio identical with that of the substrate. Moreover, when the [^3H]Ins[^{32}P]P $_4$ (formed after 1 min) was incubated with human erythrocyte ghosts or alkaline phosphatase [^3H]Ins P_3 s were obtained that yielded [^3H]altritol and [^3H]xytilol respectively upon periodate oxidation (0.1 M, pH 4.5), reduction and dephosphorylation. These results are consistent only with the Ins P_4 having the structure Ins(1,3,4,5) P_4 if it is derived by direct phosphorylation of Ins(1,4,5) P_3 . The $^3\text{H}/^{32}\text{P}$ ratio of the Ins P_4 fraction rose with time (see Tables 1 and 2), eventually approaching the ratio of the [^3H]Ins[^{32}P](1,3,4) P_3 formed in parallel (see above). Similarly, with [^3H]Ins[^{32}P](1,3,4,5) P_4 as the

starting material, the $^3\text{H}/^{32}\text{P}$ ratio of the total $\text{Ins}P_4$ rose with time, eventually reaching the same value as for the $\text{Ins}(1,3,4)P_3$ being formed in the same incubations. These observations suggest an $\text{Ins}P_4(\text{s})$ is/are being produced that lacks a 5-phosphate, but retains the ^{32}P -labelled 4-phosphate and which presumably acts as a precursor for the $\text{Ins}P_5$ formed.

When resolved on a high-efficiency anion-exchange h.p.l.c. column (see the Materials and methods section and Fig. 1), the appearance of a second $\text{Ins}P_4$ that possessed a $^3\text{H}/^{32}\text{P}$ ratio consistent with the lack of a 5-phosphate was detected. This compound yielded $[^3\text{H}]\text{Ins}$ upon periodate oxidation, reduction and dephosphorylation, and hence is indistinguishable from the $\text{Ins}P_4$ described by Shears *et al.* (1987b) and Balla *et al.* (1987) as the product of $\text{Ins}(1,3,4)P_3$ phosphorylation in liver homogenates and permeabilized adrenal glomerulosa cells respectively.

Possible precursors, on the basis of the $^3\text{H}/^{32}\text{P}$ ratio, in the mixture of inositol phosphates formed in these soluble fractions would include $\text{Ins}(1,3,4)P_3$, $\text{Ins}(3,4)P_2$, $\text{Ins}(1,4)P_2$ and D- $\text{Ins}4P$. However, h.p.l.c.-purified and desalted $[^3\text{H}]\text{Ins}(1,4)P_2$, $[^3\text{H}]\text{Ins}(3,4)P_2$, $[^{14}\text{C}]\text{Ins}4P$ and $[^3\text{H}]\text{Ins}(1,3,4)P_3$ added back to rat brain homogenates and soluble fractions, only $[^3\text{H}]\text{Ins}(1,3,4)P_3$ was phosphorylated. Moreover, the ability of partially purified fractions of rat brain cytosol to convert $[^3\text{H}]\text{Ins}(1,3,4)P_3$ into $[^3\text{H}]\text{Ins}P_4$ in the absence of any detectable $[^3\text{H}]\text{Ins}(1,3,4)P_3$ phosphatase activity similarly suggests that $\text{Ins}(1,3,4)P_3$ itself, and not one of its dephosphorylation products, can act as a substrate for a kinase.

Although phosphorylation of $\text{Ins}(1,3,4)P_3$ by rat brain cytosol was readily detected, it should be pointed out that the first-order rate constant for this process is approx. 1% of that for $\text{Ins}(1,4,5)P_3$ phosphorylation.

The product of $[^3\text{H}]\text{Ins}(1,3,4)P_3$ phosphorylation did not co-chromatograph with $\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ or $\text{Ins}[^{32}\text{P}](1,3,4,5)P_4$ (eliminating four of the 15 possible $\text{Ins}P_4$ structures) and yielded $[^3\text{H}]\text{Ins}$ upon periodate oxidation, reduction and dephosphorylation (eliminating another four possible structures). When dephosphorylated with alkaline phosphatase, the major intermediates were $[^3\text{H}]\text{Ins}P_2(\text{s})$; $[^3\text{H}]\text{Ins}P_3(\text{s})$ and $[^3\text{H}]\text{Ins}P(\text{s})$ did not accumulate significantly (results not shown). In the light of the well-described selectivity of alkaline phosphatase for monoester phosphate groups flanked by free hydroxyl groups (Tomlinson & Ballou, 1961), which results in $[^3\text{H}]\text{Ins}(3,4,5)P_3$ constituting 82% of the $[^3\text{H}]\text{Ins}P_3(\text{s})$ produced from $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ by alkaline phosphatase, this suggests that the parent compound (as it is periodate-insensitive and hence does not contain four phosphate groups, in a row) contains two pairs of vicinal phosphate groups and not a 3 and 1 grouping. The only likely structures are thus D or L- $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4,6)P_4$.

As the starting material was $\text{Ins}(1,3,4)P_3$, and no other $[^3\text{H}]\text{Ins}P_3$ could be detected in the reaction mixtures, these data are consistent only with the structure of the product of $[^3\text{H}]\text{Ins}(1,3,4)P_3$ phosphorylation in rat brain cytosol being $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$. An activity probably catalysing this reaction has been described previously in both rat liver and primary-cultured adrenal glomerulosa cells (Shears *et al.*, 1987b; Balla *et al.*, 1987) and would hence appear to be a widely distributed enzyme. The behaviour of the rat brain cytosol enzyme during its

partial purification demonstrated that it is distinct from $\text{Ins}(1,4,5)P_3$ 3-OH kinase (on elution from an anion-exchange column; results not shown), though it is very similar to $\text{Ins}(3,4,5,6)P_4$ 1-OH kinase on both anion-exchange and gel-filtration columns (results not shown). Its native M_r is approx. 30000.

The total $\text{Ins}(1,3,4)P_3$ phosphatase activity, which was separated from the $\text{Ins}(1,3,4)P_3$ kinase activity, could be resolved into two peaks of activity in both chromatography systems employed; these presumably correspond to the Mg^{2+} -dependent 'inositol polyphosphate 1-phosphatase' forming $\text{Ins}(3,4)P_2$, and the Mg^{2+} -independent 'inositol polyphosphate 4-phosphatase' that yields $\text{Ins}(1,3)P_2$ (Bansal *et al.*, 1987; Inhorn *et al.*, 1987).

When $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ was added to rat brain homogenate or cytosol fractions (Table 5), in the presence of ATP, the major product of its metabolism was a $[^3\text{H}]\text{Ins}P_5$ [on the basis of its co-chromatography with $\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ on an anion-exchange h.p.l.c. column]. The product of this reaction was shown by indirect means to be $\text{Ins}(1,3,4,5,6)P_5$, since, when the $[^3\text{H}]\text{Ins}P_5$ product was dephosphorylated, by incubation with human erythrocyte ghosts, it yielded $[^3\text{H}]\text{Ins}P_4(\text{s})$ which gave D- $[^3\text{H}]\text{iditol}$ upon periodate oxidation, reduction and dephosphorylation. There is only one $\text{Ins}P_4$ consistent with this result, $\text{Ins}(1,4,5,6)P_4$; hence the parent $[^3\text{H}]\text{Ins}P_5$, if derived from $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$, has the structure $[^3\text{H}]\text{Ins}(1,3,4,5,6)P_5$.

The enzyme responsible for catalysing the above reaction was soluble and, although very similar in its chromatographic properties to $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(3,4,5,6)P_4$ kinases, could be reproducibly differentiated from each of these activities by both anion-exchange and gel-filtration chromatography [results for $\text{Ins}(3,4,5,6)P_4$ kinase not shown]. Two peaks of $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ phosphatase activity could also be resolved from the $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ kinase activity [in both the presence (see Figs. 2 and 3) and the absence of ATP (results not shown)]. Again it was possible to identify fractions of partially purified protein eluted from the FPLC columns that contained $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ kinase activity, but no detectable phosphatase activity, strongly suggesting that $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ is a direct substrate for the $\text{Ins}P_4$ kinase.

The obvious question remains; is $\text{Ins}(1,3,4,6)P_4$ present in cells? $[^3\text{H}]\text{Ins}$ -prelabelled avian erythrocytes, primary-cultured murine bone-marrow-derived macrophages and human astrocytoma (1321 N1) cells contain $[^3\text{H}]\text{Ins}$ -labelled compounds that co-chromatograph with ^{32}P -labelled $\text{Ins}(1,3,4,6)P_4$, $\text{Ins}(3,4,5,6)P_4$ and $\text{Ins}(1,3,4,5)P_4$ {a neutral extract of $[^3\text{H}]\text{Ins}$ -prelabelled macrophages similarly contained three $[^3\text{H}]\text{Ins}$ -labelled compounds and co-chromatographed with the three $\text{Ins}[^{32}\text{P}]P_4(\text{s})$ used above}. The $[^3\text{H}]\text{Ins}$ -labelled compound that ran with $\text{Ins}[^{32}\text{P}](1,3,4,6)P_4$ was identified as $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$, essentially on the basis of three observations: (1) lack of coincidence with $\text{Ins}[^{32}\text{P}](1,3,4,5)P_4$ or $\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ on elution from h.p.l.c. columns; (2) its resistance to oxidation by periodic acid; (3) when randomly dephosphorylated with 10 M-NH₃, the only detectable inositol monophosphates formed were D/L- $[^3\text{H}]\text{Ins}1P$ and D/L- $[^3\text{H}]\text{Ins}4P$; if the parent compound is an $\text{Ins}P_4$, then this result is consistent only with the structure $\text{Ins}(1,3,4,6)P_4$.

A quantitative analysis of the changes in the amounts

of the [^3H]Ins P_4 present in the primary-cultured macrophages during stimulation with a maximal dose of PAF revealed that the concentration of [^3H]Ins(1,3,4,6) P_4 rose steadily during prolonged periods of stimulation (see Table 6). In comparison with the changes in concentrations of [^3H]Ins(1,3,4,5) P_4 (47-fold rise after 40 s, falling to 13-fold at 5 min) and [^3H]Ins(1,3,4) P_3 (61-fold rise at 40 s, falling to 17-fold at 5 min), these are relatively small changes that occur after a considerable time lag, but consistent with an indirect route of synthesis from an inositol phosphate pool whose concentrations are acutely hormone-sensitive. Precise interpretations, in terms of precursor/product relationships, from the patterns of changes in the concentrations of Ins P_4 isomers, must be guarded, as many other cellular processes are altered by PAF, some of which will undoubtedly impinge on inositol phosphate metabolism, and because the turnover times of these pools of inositol phosphates are unknown.

The concentration of [^3H]Ins(3,4,5,6) P_4 also rose during prolonged stimulation, although only by a small degree. The metabolic origin of this Ins P_4 is currently undefined, although the absence of a D-1 phosphate group would suggest an indirect relationship with the hormone-sensitive lineage of Ins(1,4,5) P_3 -derived inositol phosphates (Stephens *et al.*, 1988a).

In the three cell types investigated, avian erythrocytes (labelled with [^3H]Ins for 24 h), primary-cultured macrophages (labelled with [^3H]Ins for 22 h) and 1321 N1 cells (labelled with [^3H]Ins for 60 h), [^3H]Ins(1,3,4,5) P_4 represented only 4%, 11% and 23%, respectively, of the total [^3H]Ins P_4 found under non-stimulated conditions. Clearly the true scale of the rise in the concentration of [^3H]Ins(1,3,4,5) P_4 reported upon hormone stimulation in many cells is substantially greater than the simple increase in total [^3H]Ins P_4 measured on either batch-eluted anion-exchange resin columns or Partisil 10-SAX h.p.l.c. columns.

The changes during stimulation in the concentrations of [^3H]Ins P_5 follow no pattern that could be clearly stated as evidence for a significant rise in the rate of Ins P_5 synthesis from either Ins(3,4,5,6) P_4 or Ins(1,3,4,6)- P_4 , but the problems in interpreting this form of data have been mentioned above.

The cellular significance of the series of reactions responsible for synthesis of Ins(1,3,4,5,6) P_5 from Ins(1,4,5) P_3 via Ins(1,3,4,5) P_4 , Ins(1,3,4) P_3 and Ins(1,3,4,6)- P_4 , and the possible functions, if any, of the individual intermediates, are unknown. It remains possible that compartmentation within the cell blocks the full expression of this series of reactions, or limits its operation to particular locations. This notion of compartmentation is further emphasized by recent work describing possible extracellular functions of inositol polyphosphates, such

as Ins P_5 , as putative neurotransmitters in localized regions of the brain (Vallejo *et al.*, 1987). Moreover, these studies underscore the need to define precisely the metabolic pathways of Ins P_5 synthesis and degradation.

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