The inositol phosphates in WRK1 rat mammary tumour cells

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1. A detailed structural survey has been made of the inositol phosphates of unstimulated and vasopressin-stimulated WRK-1 rat mammary tumour cells. Inositol phosphate peaks were separated by h.p.l.c., and structural assignments were made for more than 20 compounds by combinations of: (a) co-chromatography with labelled standards; (b) site-specific enzymic dephosphorylation; (c) complete and partial periodate oxidation, followed by h.p.l.c. of polyols and their stereospecific oxidation by dehydrogenases; and (d) ammoniacal hydrolysis. 2. The 'inositol monophosphates' fraction from unstimulated cells included an uncharacterized peak, probably containing some glycerophosphoinositol, and $\text{Ins}(1:2\text{-cyclic})P$. Stimulation provoked accumulation of both $\text{Ins}1P$ and $\text{Ins}3P$, of $\text{Ins}2P$, and of $\text{Ins}5P$ and/or the enantiomers $\text{Ins}4P$ and $\text{Ins}6P$. The proportions of $\text{Ins}1P$ and $\text{Ins}3P$ were determined by partial periodate oxidation and enantiomeric identification of the resulting glucitols. 3. Three inositol bisphosphate peaks were detected in unstimulated cells: $\text{Ins}(1,4)P_2$ [this was distinguished chemically from its enantiomer $\text{Ins}(3,6)P_2$], $\text{Ins}(3,4)P_2$ and/or $\text{Ins}(1,6)P_2$, and $\text{Ins}(4,5)P_2$ and/or $\text{Ins}(5,6)P_2$. On stimulation, $\text{Ins}(1,4)P_2$ and $\text{Ins}(3,4)P_2$ [and/or $\text{Ins}(1,6)P_2$] levels increased, and $\text{Ins}(1:2\text{-cyclic},4)P_2$ and $\text{Ins}(1,3)P_2$ were also formed. 4. Three inositol trisphosphate peaks were obtained from unstimulated cells: all increased during stimulation. These were $\text{Ins}(1,3,4)P_3$ [with some $\text{Ins}(1:2\text{-cyclic},4,5)P_3$], $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(3,4,5)P_3$ [and/or $\text{Ins}(1,5,6)P_3$]. During stimulation, another compound, probably $\text{Ins}(1,4,6)P_3$, appeared in the ' $\text{Ins}(1,4,5)P_3$ peak'. The ' $\text{Ins}(3,4,5)P_3$ peak' contained a second trisphosphate, probably $\text{Ins}(2,4,5)P_3$. 5. Three inositol tetrakisphosphates, namely $\text{Ins}(1,3,4,6)P_4$, $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(3,4,5,6)P_4$, were present in unstimulated cells, and all accumulated during stimulation. 6. $\text{Ins}(1,3,4,5,6)P_5$, which is the most abundant inositol polyphosphate in these cells, a less abundant inositol pentakisphosphate and inositol hexakisphosphate were all unresponsive to stimulation.

INTRODUCTION

The recent interest in inositol polyphosphates began with the demonstration that phosphoinositidase C (PIC)-catalysed hydrolysis of $\text{PtdIns}(4,5)P_2$ to $\text{Ins}(1,4,5)P_3$ and 1,2-diacylglycerol is a primary signalling reaction triggered by the binding of Ca^{2+} -mobilizing stimuli to their cell-surface receptors (Michell *et al.*, 1981, 1989; Creba *et al.*, 1983; Berridge, 1983, 1987; Streb *et al.*, 1983; Berridge & Irvine, 1984, 1989; Nishizuka, 1984; Downes & Michell, 1985; Putney, 1987; Berridge & Michell, 1988; Downes & MacPhee, 1990). Largely as a result of the application of analytical h.p.l.c. to cell extracts, there has been an increasing appreciation of the complexity of the inositol polyphosphate complement of mammalian cells (e.g. Irvine *et al.*, 1988). A wide variety of compounds arise through the complex metabolism of $\text{Ins}(1,4,5)P_3$, through less well characterized pathways involving novel inositol lipids bearing a 3-phosphate and through the interconversions of inositol tetrakis-, pentakis- and hexakisphosphates (reviewed in Irvine *et al.*, 1988; Berridge *et al.*, 1988; Majerus *et al.*, 1988; Berridge & Irvine, 1989; Downes, 1989; Shears, 1989a). Given the complexity of inositol phosphate metabolism, and the possibility that up to 63 different inositol phosphate isomers may exist, it is essential to identify the inositol phosphates that are present in cells. However, relatively few published studies have addressed this problem for all of the inositol phosphates present in a given cell type. A major exception is the work of Stephens and colleagues, who have characterized

the inositol phosphates of avian erythrocytes and *Dictyostelium* in great detail (Stephens *et al.*, 1988a,b, 1989a,b, 1990a,b; Stephens & Downes, 1990; Stephens & Irvine, 1990).

In following up previous studies of the inositol lipid and phosphate metabolism of the vasopressin-sensitive rat mammary tumour line WRK-1 (Monaco, 1982, 1987; Monaco & Woods, 1983; Guillon *et al.*, 1986, 1989; Kirk *et al.*, 1986; Koreh & Monaco, 1986; Maccallum *et al.*, 1989), we have rigorously identified many of the inositol phosphates of these cells. To do this, we have used h.p.l.c. techniques to resolve multiple inositol polyphosphates bearing the same number of phosphates, and we have employed the techniques of structural analysis that originated with Ballou and his colleagues (Brockerhoff & Ballou, 1962; Grado & Ballou, 1961) and have been extended by the use of stereoselective enzymes (Stephens *et al.*, 1988a, 1989a,b; reviewed by Kirk *et al.*, 1990) and partial periodate oxidation (Stephens *et al.*, 1989a, 1990b; Wong, 1989). In this paper, we present a comprehensive structural survey of the inositol phosphates of WRK1 cells. The metabolic interrelationships between these in vasopressin-stimulated cells are explored in the following paper (Barker *et al.*, 1992).

MATERIALS AND METHODS

Inositol phosphates and polyols

Amersham International supplied [$2\text{-}^3\text{H}$]inositol (10–20 Ci/mmol), [$2\text{-}^3\text{H}$]Ins(1,4,5) P_3 (1 Ci/mmol), [$2\text{-}^3\text{H}$]Ins-

Abbreviations used: PIC, phosphoinositidase C; $\text{Ins}(1:2\text{cyclic})P$, $\text{Ins}(1:2\text{cyclic},4)P_2$ and $\text{Ins}(1:2\text{cyclic},4,5)P_3$, *myo*-inositol cyclic phosphates [numbered by reference to D-*myo*-inositol 1-phosphate as $\text{Ins}1P$: see 'Numbering of atoms in *myo*-inositol', Biochem. J. (1989) 258, 1–2]; GroPIns, glycerophosphoinositol.

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(1,3,4,5) P_4 , [U- ^{14}C]Ins1 P (55 mCi/mmol) and [^{32}P]P $_1$ (carrier-free). [^3H]Ins(1,3,4) P_3 was made by dephosphorylation of the [^3H]Ins(1,3,4,5) P_4 with a human erythrocyte ghost Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5'-polyphosphatase (see below). [^{32}P]Ins4 P , [4- ^{32}P]Ins(1,4) P_2 and [4,5- ^{32}P]Ins(1,4,5) P_3 were prepared from labelled GroPIns4 P and GroPIns(4,5) P_2 , which had been obtained by methylamine deacylation (Hawkins *et al.*, 1986) of ^{32}P -labelled lipids isolated from human erythrocytes [labelled for 5 h as described by King *et al.* (1987)]. Alkaline hydrolysis of GroPIns4 P with 1 M-KOH yielded Ins4 P , and the other two compounds were obtained by mild periodate oxidation of the appropriate GroPIns ester precursors (Brown & Stewart, 1966). [^{14}C]Ins2 P was produced by acid-catalysed migration of [^{14}C]Ins3 P (Pizer & Ballou, 1959). [^3H]Ins1 P was made by ammoniacal hydrolysis of labelled Ins(1,4) P_2 and isolated by h.p.l.c. ^{14}C -labelled polyols were obtained from Amersham (glucitol) or made from the inositol lipids of HL-60 cells labelled to isotopic equilibrium with [^{14}C]inositol. [^{14}C]GroPIns4 P was partially periodate-oxidized, reduced and dephosphorylated to yield a mixture of L-altritol and D-iditol. [^{14}C]Threitol was obtained from [^{14}C]Ins(4,5) P_2 , itself derived from [^{14}C]Ins(1,4,5) P_3 in > 90% yield by incubation at 37 °C for 10 min in 5 ml of 0.1 M-glycine buffer, pH 9.0, containing 2 mM-Mg $^{2+}$, 0.5 μg of calf intestinal alkaline phosphatase/ml and 7.5 μg of bovine plasma albumin/ml, followed by h.p.l.c. [Hughes & Drummond (1987), as modified by S. B. Shears (personal communication)]. L-[^3H]Altritol was generated from [^3H]Ins(1,3,4) P_3 by periodate oxidation, reduction and dephosphorylation. Ribitol, D/L-threitol, erythritol, D-glucitol, arabinitol and xylitol were obtained from Aldrich Chemical Co. L-Altritol and D-altritol were made by borohydride reduction of L-talose and D-altrose respectively.

Other materials

Eagle's modified minimal essential medium with or without inositol (with Earle's salts, without glutamine), L-glutamine, NaHCO $_3$ (7.5%, w/v), penicillin/streptomycin (5000 units of penicillin and 5000 μg of streptomycin per ml), trypsin/EDTA solution (in modified Puck's saline A) and Dulbecco's phosphate-buffered saline (without Ca $^{2+}$, Mg $^{2+}$ and NaHCO $_3$) were from Gibco Life Technologies. Rat serum was obtained by aortic puncture from Wistar rats (> 400 g), and foetal-calf serum was from Biological Industries Ltd.

AG1X8 anion exchanger (200–400 mesh, formate form) was from Bio-Rad Laboratories, and Dowex 50W-X8 (20–50 U.S. Mesh, H $^+$ form) from BDH Chemicals. Whatman Partisil 10-SAX h.p.l.c. columns and Partisphere-WAX columns were supplied by Technicol and Laserchrom, respectively. Pellicular anion exchanger was from Whatman. The Brownlee polypore (Pb $^{2+}$) h.p.l.c. column was from Anachem.

Alkaline phosphatase (grade III, calf intestine) for the preparation of Ins(4,5) P_2 was from Boehringer. Polyol dehydrogenase, sorbitol dehydrogenase and phosphatase (product no. 5521) for dephosphorylating the products of periodate oxidation were from Sigma Chemical Co.

Growth and stimulation of [^3H]inositol-labelled WRK-1 cells, and extraction of labelled inositol phosphates

WRK-1 rat mammary tumour cells were grown and labelled to isotopic equilibrium in an inositol-depleted medium containing 10 μCi of [^3H]inositol/ml (total inositol concn. approx. 1 μM). They were stimulated with a maximally effective concentration of [arginine]vasopressin (0.4 μM) for various periods. The methods employed were as described by Wong *et al.* (1988): when LiCl was present, the cells were incubated in 10 mM-LiCl for 10 min before addition of vasopressin. Incubations were terminated either with phenol/chloroform-saturated phosphate buffer or

with HClO $_4$ (neutralization of acid extracts was with KOH), and the extracts were stored at –20 °C: the methods are specified in the legends, and are described by Wong *et al.* (1988) and Kirk *et al.* (1990).

Separation of inositol phosphate classes and of individual inositol phosphate species or h.p.l.c. peaks

Inositol phosphate classes were separated into subfractions on 2 cm $\times$ 0.6 cm columns of AG1-X8 as described by Stephens *et al.* (1988b). The separation of individual inositol phosphate peaks from these mixed fractions employed two types of anion-exchange h.p.l.c. column. Inositol mono- and bis-phosphates were separated on a 25 cm $\times$ 4.5 mm Whatman Partisil-SAX column equipped with a 5 cm $\times$ 4.5 mm guard column of pellicular resin. The isolation of a mixed Ins1 P /Ins3 P peak by sequential chromatography on AG1-X8 and Partisil-10SAX yielded samples contaminated with P $_1$. This was removed by absorption on to AG1-X8 resin, followed by elution of Ins P with 1.2 M-formic acid. Inositol tris-, tetrakis-, pentakis- and hexakis-phosphates were resolved on a 12.5 cm $\times$ 4.5 mm Whatman Partisphere-WAX column equipped with a silica guard column, by using the (NH $_4$) $_2$ HPO $_4$ gradients described in the Figure legends. Separated peaks were located by liquid-scintillation counting of samples of the collected fractions, which were pooled for detailed analysis.

Inositol 1,2-cyclic monophosphate [Ins(1:2-cyclic) P] and inositol (1,2-cyclic,4)bisphosphate [Ins(1:2-cyclic,4) P_2] are acid-labile and could only be recovered from neutral phenol extracts (Wong *et al.*, 1988). Characterization of their acid lability involved treatment with 5% (w/v) HClO $_4$ for 15 min at 37 °C, followed by neutralization with KOH.

Ammoniacal hydrolysis of inositol phosphates

Separated and freeze-dried inositol phosphates were heated with 1 ml of 22% (w/v) ammonia solution in a sealed Carius tube at 110 °C for 48 h (for bis- and tris-phosphates), 70 h (tetrakisphosphates) or 100 h (pentakisphosphates). Tubes were cooled and opened, 20 μl of an alkaline hydrolysate of phytic acid (10 mg/ml) was added, ammonia was removed under an N $_2$ stream, and the samples were freeze-dried and dissolved in water.

Complete and partial periodate oxidation of inositol phosphates, followed by h.p.l.c. analysis and stereoselective enzymic oxidation of the resulting polyols

Inositol phosphates were desalted before oxidation, usually by the method of Hawkins *et al.* (1986) or of Stephens *et al.* (1988c). Alternatively, the h.p.l.c. column eluate was neutralized with ammonia or triethylamine, diluted, loaded on to a 2 cm $\times$ 0.6 cm AG1-X8 (formate) column, and the appropriate inositol phosphate fraction(s) obtained. NH $_4^+$ was removed by Dowex-50 (H $^+$ form), and water and formic acid were removed by freeze-drying.

Periodate oxidation, with subsequent reduction and dephosphorylation, of Ins P_3 s and Ins P_4 s was as described by Stephens *et al.* (1988a,b,c), except that oxidation was sometimes extended to 8 days to maximize reaction. Oxidation of Ins P_2 samples was similar, with 0.12 M-sodium periodate at 29 °C (for 36 h or 10 days, as required). Limited partial oxidation of a mixed peak of Ins1 P and Ins3 P isolated by h.p.l.c. from the 'inositol monophosphate' fraction was with 0.05 M-sodium periodate for 5 min at 29 °C (Wong, 1989): this method is similar to that of Stephens *et al.* (1990b). The resulting dialdehydes were reduced with 0.5 M-NaBH $_4$ for 12 h. A 20 μl mixture of unlabelled polyols (normally containing 200 μg each of glucitol, inositol, arabinitol, xylitol, ribitol and erythritol) was added, and the sample was treated with Dowex-50 (H $^+$ form). NaBH $_4$ that had

not reacted was removed by two cycles of addition of 10 ml of dry methanol and evaporation of the resulting methylborane. Samples were then dephosphorylated at 30 °C for 14 h in 10 mM-ethanolamine buffer, pH 9.2, containing 5 mM-Mg²⁺ and 45 units of bovine intestinal phosphatase/ml, desalted and dried.

The resulting samples were dissolved in 15 µl of water, an internal standard (inositol or threitol, depending on the experiment) was added, and the samples were centrifuged and injected on to a Brownlee polypore column (Pb²⁺ form) at 80 °C. Elution was for 32 min with water at 0.2 ml/min. Fractions (0.1 ml) were collected and their radioactivity was determined by scintillation counting. The use of the polyol dehydrogenase from *Candida utilis* for discriminating between the enantiomers of iditol and altritol and of sorbitol dehydrogenase to distinguish glucitol enantiomers was essentially as described by Stephens *et al.* (1988a, 1989b, 1990b).

RESULTS

Some inositol phosphate peaks were observed in unstimulated cells and further accumulated in response to stimulation by vasopressin, whereas others appeared in appreciable concentrations only after stimulation or were unchanged by stimulation. For the hormone-responsive peaks that were also present in unstimulated cells, structural analyses were generally undertaken under both conditions. In these experiments our primary concern was to identify the structures present. Therefore, the numerical data presented do not allow direct comparisons between the different inositol phosphate classes (that is between mono-, bis-, tris-, pentakis- and hexakis-phosphates in Fig. 1, 3, 4 and 5 respectively). Some brief comments on the levels of individual inositol phosphates in unstimulated and stimulated WRK1 cells do appear in this paper, but detailed discussion of their relative levels and metabolic interrelationships is deferred to the accompanying paper (Barker *et al.*, 1992).

The 'inositol monophosphate' region of the chromatogram

When a shallow ammonium phosphate gradient was applied to the inositol monophosphate region of chromatograms of unstimulated and stimulated WRK1 cells, two to five labelled peaks were observed (Fig. 1). The two earliest-eluted peaks (1 and 2) emerged from the column before any monoesterified inositol monophosphate. Peaks 3–5 co-migrated respectively with standards of Ins2P, Ins3P and Ins4P, three of the six possible isomers of InsP. Co-elution with these standards only partially identifies the radioactive compounds formed in stimulated cells, since only Ins2P is eluted alone: Ins3P is accompanied by Ins1P, its enantiomer, and the peak that co-elutes with Ins4P might also include Ins6P, its enantiomer, and Ins5P.

Peak 2: Ins(1,2cyclic)P. The early elution of peaks 1 and 2 suggested that one of them might be Ins(1:2cyclic)P, whose presence was expected, since Ins(1:2cyclic,4,5)P₃ (Wong *et al.*, 1988) and Ins(1:2cyclic,4)P₂ (see below) have been seen in these cells. These peaks were isolated from control cells and those stimulated for 5 s, 30 s and 10 min, by extraction with the neutral phenol/chloroform medium: they were then acid-treated to open any acid-labile five-membered cyclic phosphate ring. Acid treatment of peak-2 samples yielded mixtures of compounds eluted as Ins1P/Ins3P and Ins2P in an approximate ratio of 2:1, as expected from Ins(1:2cyclic)P. Similar products were not obtained from peak 1. This first peak co-eluted with a [¹⁴C]-GroPIns internal standard (results not shown); however, it remains to be rigorously identified. Results have recently been obtained which suggest that some of the material present is not based on *myo*-inositol (C. J. Barker & D. J. Candy, unpublished

work). It is noteworthy that, in preliminary studies of WRK1 cells labelled with [¹⁴C]glucosamine, a precursor for the PtdIns-glycan structures, a ¹⁴C-labelled peak appeared in this region of the chromatogram.

Peak 3: Ins1P and Ins3P. Of the peaks coincident with the various InsP standards (peaks 3–5), we particularly wished to obtain separate estimates of the abundances of the enantiomers Ins1P and Ins3P in peak 3: known pathways of inositol

Fig. 1. H.p.l.c. profiles of the 'inositol monophosphates' of WRK1 cells, either unstimulated or stimulated with 400 nM-vasopressin for 5 s or 10 min

WRK1 cells were labelled with [2-³H]inositol for 5 days and were stimulated with 400 nM-vasopressin. Inositol phosphates were extracted in neutral conditions (see the Materials and methods section) followed by separation on a 25 cm Partisil-SAX column fitted with a 5 cm guard column. Samples were mixed with ¹⁴C-labelled internal standards of Ins1P/Ins3P and Ins2P: black arrows denote the positions of the internal standards, and the white arrow shows the expected elution position of Ins4P, Ins5P and Ins6P. The following gradient was used, with water in pump A and 0.1 M-(NH₄)₂HPO₄, pH 3.8 (with H₃PO₄) in pump B, at a flow rate of 1.25 ml/min: 0 min, 0% B; 5 min, 0% B; 10 min, 10% B; 40 min, 44.8% B; 44 min, 44.8% B; 46 min, 0% B. 0.3 min fractions were collected and their radioactivities determined.

Table 1. Oxidation by sorbitol dehydrogenase of Ins1P-derived D-glucitol in the mixed glucitol sample obtained from partial oxidation of a mixture of Ins1P and Ins3P

Samples of the glucitol obtained from the partial periodate oxidation of the Ins1P/Ins3P mixture isolated as peak 3 from vasopressin-stimulated WRK1 cells were mixed with a known amount of ¹⁴C-labelled D-glucitol. These samples were incubated with sorbitol dehydrogenase, and the products were analysed by h.p.l.c. using a Polypore-Pb²⁺ column (see Fig. 2). ³H radioactivity in the fructose and glucitol peaks was determined. The extents of oxidation of the internal [¹⁴C]glucitol in the 0, 10 mM- and 30 mM-Li⁺ samples were 94.6, 95.5 and 94.4 % respectively. The values quoted are means of duplicate determinations, and similar results were obtained in a second experiment.

[Li ⁺]	³ H radioactivity (d.p.m.)	
	Fructose	Residual glucitol
0	520	560
10 mM	1940	1200
30 mM	2790	780

Fig. 2. Oxidation by sorbitol dehydrogenase of the Ins1P-derived D-glucitol in the mixed glucitol sample obtained from partial oxidation of a mixture of Ins1P and Ins3P

WRK1 cells were labelled with [2-³H]inositol, stimulated for 10 min with 400 nM-vasopressin in the presence of 10 mM-LiCl and the inositol phosphates were extracted. The Ins1P/Ins3P peak (peak 3: see Fig. 1) was isolated, desalted, and oxidized with 50 mM-sodium periodate for 5 min. After reduction and dephosphorylation, the resulting polyols were separated on a Polypore-Pb²⁺ h.p.l.c. column. The product with the retention time of glucitol was incubated with sorbitol dehydrogenase in the presence of a known amount of authentic D-[¹⁴C]glucitol under conditions that achieved ~ 95 % oxidation of the standard to fructose, and the products were analysed on the Polypore column, with 0.5 min fractions being collected. The Figure shows the products of the simultaneous oxidation of authentic D-[¹⁴C]glucitol (---●---) and of the [³H]glucitol of unknown configuration that was derived from the Ins1P/Ins3P peak (—●—).

phosphate metabolism predict that both of these are likely to be produced in stimulated cells.

Until recently, the only method for resolving Ins1P and Ins3P involved derivative formation, followed by g.l.c. on a chiral column (Sherman *et al.*, 1981). In parallel, we (Wong, 1989) and Stephens *et al.* (1990b) then developed partial periodate-oxidation techniques for determining the enantiomeric composition of Ins1P/Ins3P mixtures. These techniques depend on the

Table 2. The polyols that can be formed by periodate oxidation of various myo-inositol bisphosphate isomers

Under 'complete oxidation' are listed the polyols resulting from periodate attack on all susceptible C-C bonds, followed by reduction. Under 'partial oxidation' are listed the products formed as a result of periodate oxidation of each of the two susceptible C-C bonds of each of the *para*-bisphosphates. * Denotes the InsP₂s that will produce unlabelled polyols by loss of the [2-³H]. The boxed InsP₂s are enantiomeric pairs.

Bisphosphate	Complete oxidation	Partial oxidation
1,2 and 2,3	Erythritol	
1,3	Ribitol	
1,4	Destroyed	D-Iditol + L-altritol
3,6	Destroyed	L-Iditol + D-altritol
1,5, 3,5 and *4,6	Xylitol	
1,6 and *4,5	D-Threitol	
3,5 and *5,6	L-Threitol	
2,5	Destroyed	D-Glucitol + L-glucitol
2,4	L-Arabinitol	
2,6	D-Arabinitol	

fact that if Ins1P and Ins3P are oxidized under conditions in which some of the molecules suffer cleavage of only one C-C bond, then the enantiomeric configurations of the resulting hexitols yield information on the proportions of the InsP enantiomers present in the original mixture. Theoretically, Ins1P and Ins3P can give rise to the D- and L-enantiomers, respectively, of glucitol and iditol, and either InsP will also give both enantiomers of altritol. To validate the method, authentic ¹⁴C-labelled Ins3P and [2-³H]Ins1P were oxidized for 5 min with 50 mM-periodate (see the Materials and methods section), a putative hexitol which co-chromatographed with glucitol (sorbitol) was isolated from each, and the 'glucitol' samples were subjected to oxidation by sorbitol dehydrogenase (which attacks only D-glucitol). As expected, the sample formed from Ins1P showed the same sensitivity to sorbitol dehydrogenase as authentic D-glucitol, and the putative L-glucitol derived from Ins3P was resistant to sorbitol dehydrogenase.

This procedure was then applied to peak-3 material from WRK1 cells stimulated in the absence or presence of Li⁺ (10 mM or 30 mM). When the glucitol peak derived from peak 3 of cells stimulated in the absence of Li⁺ was incubated with sorbitol dehydrogenase, approximately half was converted into fructose and the remainder was unchanged, whereas 95 % of an internal standard of D-[¹⁴C]glucitol was converted into fructose: this indicated the presence in peak 3 of a close-to-equimolar mixture of Ins1P and Ins3P (Table 1). With 10 mM-Li⁺ present during vasopressin stimulation, about 7 times more radioactivity accumulated in peak 3, with D-glucitol-yielding Ins1P comprising slightly more than 60 % of the accumulated material (Fig. 2 and Table 1). At 30 mM-Li⁺, there was only a slight further increase in the total accumulation of radioactivity in peak 3, but Ins1P was even more predominant. For a more detailed discussion of this effect of Li⁺, see Barker *et al.* (1992).

Inositol bisphosphates

The principle underlying the chemical identification of InsP₂ isomers was the same as for InsP isomers. However, the presence of two phosphates on the ring further constrained the possible sites of periodate attack and allowed intermediates in the oxidative sequence to be collected in reasonable yields more easily. Table 2 lists the polyols that can be formed by complete or partial oxidation of the various InsP₂ isomers.

Fig. 3. Resolution of five inositol bisphosphate peaks from the mixed 'inositol bisphosphate' fraction obtained from WRK-1 cells stimulated with 400 nM-vasopressin for 3 min

WRK1 cells were labelled with [2- ^3H]inositol, stimulated with vasopressin for 3 min, and the inositol phosphates were neutrally extracted. The inositol bisphosphates were separated by using a Whatman Partisil-SAX column, with the following gradient constructed from water in pump A and 0.5 M-(NH_4) $_2\text{HPO}_4$, pH 3.8 (with H_3PO_4) in pump B: 0 min, 0% B; 2 min, 0% B; 7 min, 16.0% B; 37 min, 20% B; 45 min, 20% B; 48 min, 60% B; 88 min, 100% B. 0.5 min fractions were collected and their radioactivities determined.

By using a shallow ammonium phosphate gradient to elute a Partisil-SAX column, five peaks (6–10) were well resolved in the InsP_2 region of the WRK1 chromatogram (Fig. 3). Peaks 6–9 all accumulated during stimulation with vasopressin, but peak 10 was unchanged.

Peak 6: $\text{Ins}(1,2\text{cyclic},4)\text{P}_2$. This small peak was not apparent in unstimulated cells or in cells stimulated for 3 min or less. When cell extracts were acidified, it disappeared from the chromatogram obtained from cells stimulated for 10 min, suggesting that it might be an InsP_2 isomer containing an acid-labile five-membered cyclic phosphodiester. When it was acid-treated, radioactivity was lost from peak 6 and appeared in peak 8, which is identified below as $\text{Ins}(1,4)\text{P}_2$. Because of the small quantity available, no further characterization was possible. It seems that $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_2$ was the major or sole contributor to peak 6.

Peak 7: $\text{Ins}(1,3)\text{P}_2$. This was apparent only in stimulated cells. Ammoniacal hydrolysis yielded a single peak that co-eluted with an internal [^{14}C] $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ standard. The only bisphosphate to yield this single peak would be $\text{Ins}(1,3)\text{P}_2$. The sole polyol isolated, in 70–80% yield, after periodate oxidation was the *meso*-compound ribitol; $\text{Ins}(1,3)\text{P}_2$ is the only InsP_2 isomer that can give rise to ribitol.

Peak 8: $\text{Ins}(1,4)\text{P}_2$. Peak 8 was present in unstimulated cells, but rose very rapidly to high levels after addition of vasopressin. It co-eluted on h.p.l.c. with authentic ^{32}P -labelled $\text{Ins}(1,4)\text{P}_2$. To

Fig. 4. Resolution of three inositol trisphosphates

WRK1 cells were labelled with [2- ^3H]inositol for 5 days, stimulated with vasopressin and the inositol phosphates neutrally extracted. [$4,5\text{-}^{32}\text{P}$] $\text{Ins}(1,4,5)\text{P}_3$ (---○---) was added as an internal standard, and the sample was analysed on a 12.5 cm Whatman Partisphere-WAX column, at a flow rate of 1 ml/min, yielding three separated peaks of WRK1-cell-derived material (—●—). The gradient, formed from water in pump A and 0.5 M-(NH_4) $_2\text{HPO}_4$, pH 3.2 (with H_3PO_4) in pump B, was: 0 min, 0% B; 5 min, 0% B; 15 min, 12% B; 70 min, 12% B; 90 min, 100% B; 110 min, 100% B. 0.5 min fractions were collected and their radioactivities determined. Similar results were obtained in more than 10 separate experiments.

identify this, we used a partial periodate-oxidation technique akin to that used by Stephens *et al.* (1989a) to identify the lipid $\text{PtdIns}4\text{P}$. Peak 8 was isolated from unstimulated cells and from cells stimulated for various periods. After periodate oxidation, reduction and dephosphorylation, none of these samples yielded appreciable quantities of labelled polyols, identifying peak 8 as a *para*-bisphosphate and/or a bisphosphate in which the ^3H -bearing 2-position was accessible to oxidation. $\text{Ins}(2,5)\text{P}_2$ was eliminated by the absence of $\text{Ins}2\text{P}$ from the products of ammoniacal hydrolysis. The enantiomers $\text{Ins}(4,5)\text{P}_2$ and $\text{Ins}(5,6)\text{P}_2$ were eliminated because they would be eluted with peak 10 (see below). To distinguish between $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(3,6)\text{P}_2$, peak 8 was subjected to mild periodate oxidation, and the alditol and iditol products formed after the cleavage of only one of the two susceptible C–C bonds were isolated: no other polyols were detected (results not shown). In principle, the enantiomeric configuration of the original InsP_2 could be deduced from either hexitol: we elected to analyse the iditol, because L-iditol is an excellent substrate for the enantiomer-specific polyol dehydrogenase. Iditol samples derived from peak 8 were mixed with an excess of unlabelled L-iditol, and enzymic oxidation of about 70% of the added L-iditol was followed by spectrophotometrically monitoring the reduction of NAD^+ (Stephens *et al.*, 1988a). The labelled iditol samples, whether derived from control or stimulated cells, were all resistant to the polyol dehydrogenase and were therefore of the D-configuration.

We conclude that $\text{Ins}(1,4)\text{P}_2$ was the predominant or sole InsP_2 isomer present in peak 8.

Peak 9: $\text{Ins}(3,4)\text{P}_2$ and/or $\text{Ins}(1,6)\text{P}_2$. This had the same mobility as labelled $\text{Ins}(3,4)\text{P}_2$ formed by phosphatase action on $\text{Ins}(1,3,4)\text{P}_3$ (results not shown), and there was a delay before it accumulated in stimulated cells (see Barker *et al.*, 1992): no 2-phosphate was detected after ammonia hydrolysis of this peak. After periodate oxidation, a polyol which exactly co-eluted with a [^{14}C]threitol internal standard, thus differentiating it from arabinol, was the major labelled polyol derived from the peak-9 samples from control cells and from cells stimulated for 5 s. Threitol, with a recovery of 70–80%, was the only labelled polyol detected in samples derived from cells stimulated for longer periods. This polyol can be formed from two enantiomeric InsP_2 pairs, namely $\text{Ins}(1,6)\text{P}_2/\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}(4,5)\text{P}_2/\text{Ins}(5,6)\text{P}_2$, but the latter pair of compounds is eluted in peak 10 (see below). We have not succeeded in distinguishing between the enantiomers of threitol, and we conclude that $\text{Ins}(1,6)\text{P}_2$ and/or $\text{Ins}(3,4)\text{P}_2$ are the major (no stimulus) or sole (stimulation for > 3 min) contributor(s) to peak 9.

A second polyol, tentatively identified as erythritol or ribitol, formed 25–35% of the labelled oxidation products of peak 9 derived either from unstimulated cells or from cells stimulated for 5 s. Ribitol can only come from $\text{Ins}(1,3)\text{P}_2$, which is eluted in peak 7, so the polyol must have been erythritol: the parent inositol bisphosphate(s) is thus identified as $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$. It is not clear whether this minor component of peak 9 was lost from the cells during stimulation or was simply diluted by the large amount of $\text{Ins}(3,4)\text{P}_2$ and/or $\text{Ins}(1,6)\text{P}_2$ recruited into the peak during stimulation.

Peak 10: $\text{Ins}(4,5)\text{P}_2$. This minor peak, which had the h.p.l.c. elution characteristics of $\text{Ins}(4,5)\text{P}_2$, was seen in unstimulated cells and did not change its level during 10 min of stimulation. Too little was available for fuller characterization.

Inositol trisphosphates

Maximum resolution of inositol trisphosphate peaks was achieved during a period of isocratic elution with $(\text{NH}_4)_2\text{HPO}_4$ from a Partisphere WAX column. Three inositol trisphosphate peaks (11–13) were fully resolved (Fig. 4): all increased in response to vasopressin.

Peak 11: $\text{Ins}(1,3,4)\text{P}_3$ plus $\text{Ins}(1,2\text{-cyclic},4,5)\text{P}_3$. In control cells this was the smallest InsP_3 peak, but it became the largest after 10 min or more of stimulation: it co-eluted with authentic $\text{Ins}(1,3,4)\text{P}_3$. The major polyol formed by periodate oxidation was altritol, together with small amounts of iditol and glucitol (total recovery 70–75%). The relative quantities of the different polyols varied depending on the length of stimulation: unstimulated and 5 s of stimulation yielded 70% altritol, 20% iditol, 5% glucitol and 5% of an unknown polyol (possibly inositol). After longer stimulation the proportions changed to 94%, 3%, 1% and 2% of the same compounds. Altritol can be formed from the two enantiomeric pairs $\text{Ins}(1,3,4)\text{P}_3/\text{Ins}(1,3,6)\text{P}_3$ and $\text{Ins}(1,2,4)\text{P}_3/\text{Ins}(2,3,6)\text{P}_3$. The original experiments using iditol dehydrogenase indicated that, during the long incubations required for substantial oxidation of L-altritol, the reaction did not follow first-order kinetics (Stephens *et al.*, 1989b; Shears, 1989b). This problem was circumvented by direct comparisons of the oxidation of unknown polyols with the oxidation of authentic L-[^3H]altritol (as described by Shears, 1989b). Treatment with iditol dehydrogenase of the material isolated after 30 s and 10 min of stimulation showed that at least 92% of the altritol was the L-isomer, identifying the parent InsP_3 as $\text{Ins}(1,3,4)\text{P}_3$ or $\text{Ins}(1,2,4)\text{P}_3$. $\text{Ins}(1,2,4)\text{P}_3$ cannot have been a major component because of the paucity of $\text{Ins}2\text{P}$ in the products of ammoniacal hydrolysis, so we conclude that peak 11 is largely composed of

$\text{Ins}(1,3,4)\text{P}_3$. Insufficient material was available from the unstimulated and 5 s samples for their enantiomeric configurations to be determined. The small amounts of iditol and glucitol found in the oxidation products do, however, suggest the presence in peak 11 of small amounts of $\text{Ins}(1,2\text{-cyclic},4,5)\text{P}_3$, as previously reported in these cells by Wong *et al.* (1988): opening of the cyclic phosphate ring, presumably during periodate treatment, would give rise to $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$, which would yield these two hexitols after oxidation.

Peak 12: $\text{Ins}(1,4,5)\text{P}_3$. This peak co-eluted with [$4,5\text{-}^{32}\text{P}$] $\text{Ins}(1,4,5)\text{P}_3$: it rapidly accumulated upon stimulation (within 1 s) and then declined (see also Wong *et al.*, 1988; Barker *et al.*, 1992). After periodate oxidation, the only detectable polyol was iditol (72–80% yield). Possible inositol trisphosphate precursors of iditol are the two enantiomeric pairs $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(3,5,6)\text{P}_3$ and $\text{Ins}(1,4,6)\text{P}_3/\text{Ins}(3,4,6)\text{P}_3$. Enzymic attack showed the iditol to be at least 95% in the D-form, limiting the possibilities to $\text{Ins}(1,4,5)\text{P}_3$ and/or $\text{Ins}(1,4,6)\text{P}_3$. These were distinguished by use of the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase of human erythrocytes (Downes *et al.*, 1982). Previous studies assumed that this is the only inositol polyphosphate phosphatase in human erythrocyte membranes, but these membranes also contain a highly active $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase (Doughney *et al.*, 1988; Estrada-Garcia *et al.*, 1991; A. Craxton & T. Estrada-Garcia, unpublished work). However, after solubilization a substantially purified and 3-phosphatase-free erythrocyte 5-phosphatase preparation can be isolated (A. Craxton, unpublished work). To ensure the validity of our conclusions, we incubated a mixture of ^3H -labelled peak 12 and [$4,5\text{-}^{32}\text{P}$] $\text{Ins}(1,4,5)\text{P}_3$ with this preparation. All samples showed > 95% hydrolysis of the [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$, and between 95% (unstimulated cells) and 85% (cells stimulated for 45 min) hydrolysis of the ^3H -labelled peak 12. The great majority of peak 12 is therefore $\text{Ins}(1,4,5)\text{P}_3$, together with up to 10% of a second component: this may be $\text{Ins}(1,4,6)\text{P}_3$, which also accumulates after stimulation.

Peak 13: $\text{Ins}(3,4,5)\text{P}_3$ and/or $\text{Ins}(1,5,6)\text{P}_3$. This accumulated slowly during stimulation, but was never a major InsP_3 of WRK1 cells: the small quantity made complete characterization impossible. After periodate oxidation, xylitol was usually the major polyol (90%), but together with some glucitol (up to 30%) in samples from cells stimulated for prolonged periods. Xylitol identifies a major component as $\text{Ins}(3,4,5)\text{P}_3$ and/or $\text{Ins}(1,5,6)\text{P}_3$, the InsP_3 isomers that can give rise to glucitol have been discussed above.

Inositol tetrakisphosphates

Three inositol tetrakisphosphate peaks (14–16) were apparent, particularly in samples from cells subjected to prolonged vasopressin stimulation (Barker *et al.*, 1988, 1992), but the first two were difficult to resolve fully, particularly in the large samples of cell extracts chromatographed to obtain sufficient material for detailed analysis. For this reason, many of the analyses of peaks 14 and 15 were undertaken on pooled samples containing both peaks, without prejudicing the results.

Peaks 14 and 15: $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(1,3,4,5)\text{P}_4$. Peak 14 rose slowly on stimulation, but only after 30 s. It co-eluted with $\text{Ins}(1,3,4,6)\text{P}_4$ synthesized from $\text{Ins}(1,3,4)\text{P}_3$ by rat liver 6-kinase (Shears *et al.*, 1987). Peak 15 co-eluted precisely with [$4,5\text{-}^{32}\text{P}$] $\text{Ins}(1,3,4,5)\text{P}_4$ and accumulated rapidly during stimulation. Both peaks were resistant to periodate oxidation, meaning that no constituent of either peak possessed a vicinal pair of unsubstituted hydroxyl groups. Nine InsP_4 isomers, six of which contain a 2-phosphate, share this property. Ammoniacal hydrolysis of these peaks yielded < 2% of the resulting monophosphates as $\text{Ins}2\text{P}$, leaving only $\text{Ins}(1,3,4,6)\text{P}_4$ and the $\text{Ins}(1,3,4,5)\text{P}_4/\text{Ins}(1,3,5,6)\text{P}_4$ enantiomeric pair as their possible

Fig. 5. The inositol phosphate peaks resolved by h.p.l.c. from a mixed 'inositol pentakisphosphate plus hexakisphosphate' fraction

WRK-1 cells were labelled with [2- ^3H]inositol, and the inositol phosphates were neutrally extracted from unstimulated cells. This extract was analysed on a 12.5 cm Whatman Partisphere-WAX column. Elution was at 1 ml/min, with a gradient constructed from water in pump A and 0.5 M-(NH_4) $_2\text{HPO}_4$, pH 3.2 (with H_3PO_4) in pump B: 0 min, 0% B; 5 min, 0% B; 20 min, 18% B; 70 min, 30% B; 80 min 30% B; 115 min, 80% B; 119 min, 100% B; 124 min, 100% B. 0.5 ml fractions were collected and their radioactivities determined. Only the InsP_5 and InsP_6 region of the chromatogram is shown. The Figure shows the same data plotted twice, on 70-fold different scales, to demonstrate the presence of two very minor InsP_5 peaks and two small peaks of unknown structure that were eluted after InsP_6 .

constituents. Peak 14, which was separable from authentic $\text{Ins}(1,3,4,5)\text{P}_4$ by h.p.l.c., must therefore have been $\text{Ins}(1,3,4,6)\text{P}_4$.

The likelihood that peak 15 was $\text{Ins}(1,3,4,5)\text{P}_4$ rather than its enantiomer $\text{Ins}(1,3,5,6)\text{P}_4$ was strengthened by a demonstration that it was quantitatively dephosphorylated by the partially purified $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from erythrocytes. The InsP_3 thus formed co-eluted precisely with [4- ^{32}P]- $\text{Ins}(1,3,4)\text{P}_3$. It is uncertain whether the erythrocyte 5-phosphatase can attack $\text{Ins}(1,3,5,6)\text{P}_4$, so we subjected this putative $\text{Ins}(1,3,4)\text{P}_3$ to periodate degradation and obtained alditol: this was shown using iditol dehydrogenase to be > 92% of the L-configuration. This confirmed the identity of the $\text{Ins}(1,3,4)\text{P}_3$ and thus identified peak 15 as essentially pure $\text{Ins}(1,3,4,5)\text{P}_4$.

Peak 16: $\text{Ins}(3,4,5,6)\text{P}_4$. Peak 16 was the major inositol tetrakisphosphate in unstimulated cells, and it rose 2–3-fold during 10 min of stimulation. Periodate oxidation of the peak from either unstimulated or stimulated cells yielded iditol as the major product (recovery 75–80%), which could be derived from either of the enantiomers $\text{Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$. L-Iditol dehydrogenase treatment showed that the iditol was > 95% of the L-configuration in both unstimulated and briefly stimulated cells. However, in cells stimulated for 10 min or more the iditol was only 85–90% in the L-form. Therefore, the majority of the material produced on hormone-stimulated elevation of

peak 16 is due to $\text{Ins}(3,4,5,6)\text{P}_4$; however, a small but significant proportion is attributable to its enantiomer $\text{Ins}(1,4,5,6)\text{P}_4$.

Inositol pentakisphosphates and hexakisphosphate

Peak 19: $\text{Ins}(1,3,4,5,6)\text{P}_5$. Fig. 5 shows two distinct InsP_5 peaks (17 and 19). Peak 19 was the most abundant inositol phosphate in unstimulated cells, and peak 17 was a minor and unidentified component. There were also two other, and very minor, peaks (18 and 20) that were poorly resolved from the dominant peak 19 on a Partisphere-WAX column. Peak 19 was desalted and subjected to ammoniacal hydrolysis. No $\text{Ins}2\text{P}$ was detected, despite the presence of major peaks of $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ and of $\text{Ins}4\text{P}/\text{Ins}5\text{P}/\text{Ins}6\text{P}$: $\text{Ins}(1,3,4,5,6)\text{P}_5$ is therefore the major InsP_5 of these cells.

Peak 21: InsP_6 . This was identified as InsP_6 on the basis of its h.p.l.c. mobility and the formation of material contributing to all three separable inositol monophosphate peaks during ammoniacal hydrolysis.

Peaks 22 and 23. Two small and unidentified peaks were consistently seen running after InsP_6 . They were more polar than InsP_6 , which could indicate the presence of pyrophosphate groups on the inositol ring. When peak 22 was subjected to ammonia hydrolysis it yielded the same monophosphate peaks as InsP_6 , but the proportion of the $\text{Ins}4\text{P}/\text{Ins}5\text{P}/\text{Ins}6\text{P}$ peak was lower.

DISCUSSION

As far as we are aware, the information presented here constitutes the most comprehensive structural survey to date of the diverse and metabolically interrelated inositol phosphates found in any single type of mammalian cell. The only other survey of this type was of the inositol phosphates of nucleated turkey erythrocytes by Stephens and colleagues (Stephens *et al.*, 1989b, 1990a; Stephens & Downes, 1990). There have been many studies of the metabolic interrelationships of various inositol phosphates in cell-free systems, but most previous studies of mammalian cells have focused only on the structures of one or a few of the many intracellular inositol phosphates (see Shears, 1989a, for a review). In discussing this new information, we shall concentrate particularly: (a) on the general picture that emerges from our studies of WRK1 cells; and (b) on some interesting details and differences between our findings and observations that have been made on other eukaryote cells.

Most of the inositol phosphates present in WRK1 cells are those expected as a result of the formation and metabolism of $\text{Ins}(1,4,5)\text{P}_3$

The most obvious result is the satisfying recognition that, at least in WRK1 cells labelled under the conditions that we used, most of the inositol derivatives bearing four monoesterified phosphate groups or less are precisely those expected as a result of the PIC-catalysed hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$: these will be discussed in turn. Some previous studies have rigorously characterized individual intermediates along these pathways (see below), but most workers have 'identified' many of these compounds only by their co-chromatography with authentic samples of one enantiomer of a pair. We now know that these structural assignments are correct, at least for WRK1 cells, strongly suggesting that the inferred metabolic interrelationships between these compounds in the intact cell are similarly correct (see also Barker *et al.*, 1992). As in other cells, the levels of inositol pentakisphosphates and hexakisphosphate in WRK1 cells are little affected by activation of PIC-coupled receptors.

Inositol trisphosphates. The two major InsP_3 species of WRK1 cells are $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$, both of which are expected as a consequence of receptor stimulation of PIC. Moreover, the

peaks containing these two compounds are almost homogeneous, except for a small amount of a 5-phosphatase-resistant material in the former peak (see below): we estimate that polyols derived from other isomers would have been detected if they had constituted 10% or more of either peak.

Ins(1,4,5) P_3 . Until recently, most workers have relied on quantifying the '[3H]Ins(1,4,5) P_3 peak' from h.p.l.c. traces derived from 3H -labelled cells, without further verification of its structure, despite the central role of Ins(1,4,5) P_3 in cellular regulation. This criticism has been in part addressed by the development of assays employing either a specific binding protein (e.g. Challiss *et al.*, 1990) or a purified preparation of the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase (e.g. Nogimori *et al.*, 1990), but neither of these techniques allows the identification of other compounds that co-chromatograph with Ins(1,4,5) P_3 . In this context, Stephens *et al.* (1989b) showed that in labelled avian erythrocytes this peak contained about 19% of Ins(3,4,6) P_3 and 9% of Ins(1,4,6) P_3 in addition to the expected Ins(1,4,5) P_3 : the first two compounds may have been formed by dephosphorylation of Ins(1,3,4,6) P_4 . Furthermore, Verjans *et al.* (1991) showed that 18–21% of the 'Ins(1,4,5) P_3 peak' isolated from dog thyroid cells is not degraded by a purified preparation of Ins(1,4,5)- P_3 /Ins(1,3,4,5) P_3 5-phosphatase. It is currently not clear how these other inositol phosphates influence Ins(1,4,5) P_3 -binding assays. Although it is reassuring that most of this peak in WRK1 cells proved to be genuine Ins(1,4,5) P_3 , it still remains for similar analyses to be undertaken on a variety of mammalian cells.

Ins(1,3,4) P_3 . The first unambiguous identification of this compound in any cell was that by Stephens *et al.* (1989b) in avian erythrocytes. They also showed that the Ins(1,3,4) P_3 peak of these cells contained a small amount of Ins(1,3,6) P_3 , the other enantiomer, possibly formed in the cells by dephosphorylation of the Ins(1,3,4,6) P_4 that is formed in response to stimulation (see below). Although earlier work made it fairly certain that Ins(1,3,4) P_3 was the major component of the first Ins P_3 peak in many stimulated mammalian cells, the data reported here represent the first rigorous confirmation that this is the case.

Ins(1,3,4,5) P_4 , Ins(1,3,4,6) P_4 and Ins(3,4,5,6) P_4 . Widespread acceptance of the likely coexistence of at least three Ins P_4 isomers in animal cells originated in the separate identifications: of Ins(1,3,4,5) P_4 (Batty *et al.*, 1985; Irvine *et al.*, 1986) and Ins(1,3,4,6) P_4 (Shears *et al.*, 1987) as metabolites of Ins(1,4,5) P_3 ; and of Ins(3,4,5,6) P_4 as both a relatively abundant and hormonally unresponsive compound in avian erythrocytes (Stephens *et al.*, 1988a) and as a stimulus-responsive component of macrophages (Stephens *et al.*, 1988c). In WRK1 cells, all of these compounds accumulate in response to vasopressin (Barker *et al.*, 1988, 1992; Maccallum *et al.*, 1989). Although identifications of one or more of these three compounds have since been offered in many other tissues (e.g. Balla *et al.*, 1987, 1988, 1989; Stephens *et al.*, 1988c; Barker *et al.*, 1988; Johnson *et al.*, 1989; Mayr, 1988; Pittet *et al.*, 1989; Radenberg *et al.*, 1989; Shears, 1989b; Hughes *et al.*, 1989; Mattingly *et al.*, 1991), these studies have rarely included complete structural confirmation of assigned identities, particularly of enantiomeric configurations, and only one previous study of mammalian cells has unambiguously confirmed the nature and homogeneity of all three coexisting Ins P_4 peaks (Menniti *et al.*, 1990).

Inositol bisphosphates. Three major vasopressin-responsive peaks were identified as Ins(1,4) P_2 , Ins(1,3) P_2 and Ins(3,4) P_2 [or its enantiomer Ins(1,6) P_2], all of which are expected as participants in the complex pathways of Ins(1,4,5) P_3 metabolism. Others have rigorously identified Ins(1,3) P_2 in stimulated GH3 cells (Irvine *et al.*, 1987) and rat cerebral-cortex brain slices (Batty *et al.*, 1989). Similarly, others have previously shown that the peak tentatively identified as Ins(3,4) P_2 in stimulated GH3

and GH4 cells yields threitol on oxidation (Irvine *et al.*, 1987; Hughes & Drummond, 1987). However, no study has yet achieved resolution of the threitol enantiomers, so it remains possible that the 'Ins(3,4) P_2 peak' from any cell might contain some Ins(1,6) P_2 : it is not difficult to imagine pathways to this molecule from abundant cellular inositol polyphosphates.

Ins(1,4) P_2 . The results reported here represent the first unambiguous structural identification of the major Ins P_2 of stimulated cells as Ins(1,4) P_2 . Partial identification of Ins(1,4) P_2 had been achieved previously by Hughes & Drummond (1987) and Irvine *et al.* (1987), using GH3 and GH4 cells: they showed that the putative Ins(1,4) P_2 was destroyed on periodate oxidation, thus identifying it as a *para*-isomer of Ins P_2 but not distinguishing it from its enantiomer Ins(3,6) P_2 or from Ins(2,5) P_2 .

The need structurally to discriminate between Ins(1,4) P_2 and Ins(3,6) P_2 has been emphasized by several recent observations. Gee *et al.* (1988) showed that isolated Ins(1,4) P_2 1-phosphatase attacks Ins(3,6) P_2 , albeit at a slower rate: information on susceptibility to attack by this enzyme is therefore of limited value. Moreover, Ins(1,4) P_2 was 'identified' as a constituent of stimulated cells before it was realized that mammalian cells contain a plethora of inositol polyphosphates that contain both a 3-phosphate and a 6-phosphate, in whose synthesis or degradation Ins(3,6) P_2 could easily be a participant. For example, Ins(3,6) P_2 has recently been identified as a likely intermediate in the synthesis of Ins P_6 by *Dictyostelium discoideum* (Stephens & Irvine, 1990).

Inositol monophosphates. Identification of inositol monophosphates in cells has until now relied largely on co-elution with standards on h.p.l.c.: the best available method separates the six Ins P isomers into four peaks, leaving the Ins1 P /Ins3 P and Ins4 P /Ins6 P enantiomeric pairs unresolved (Stephens *et al.*, 1988c). Derivatives of Ins1 P and Ins3 P have been separated with difficulty on chiral g.l.c. columns (Sherman *et al.*, 1981), but the limited-periodate-oxidation method described here and elsewhere (Wong, 1989; Stephens *et al.*, 1990b) has made it possible to distinguish between these enantiomers in samples from small amounts of labelled mammalian cells. Using this method, we have confirmed that both enantiomers occur in cells labelled with [3H]inositol and that their levels are independently regulated by stimulation and by the presence of Li^+ : detailed interpretation of these results is deferred to the accompanying paper (Barker *et al.*, 1992). Given that periodate attacks the *cis*-hydroxyl pairs at the 1-, 2- and 3-positions of inositol much faster than bonds adjacent to the 4- and 6-hydroxyl groups, it is unlikely that a similar strategy could be developed to discriminate the Ins4 P /Ins6 P enantiomeric pair.

A full set of inositol cyclic phosphates. We previously showed that a small quantity of Ins(1,2-cyclic,4,5) P_3 accumulates slowly in stimulated WRK1 cells (Wong *et al.*, 1988), and we have now identified small amounts of Ins(1:2-cyclic) P and Ins(1:2-cyclic,4) P_2 in these cells. Others have observed these compounds in pancreatic minilobules (Sekar *et al.*, 1987) and adrenal glomerulosa cells (Graham *et al.*, 1987) by methods similar to ours, and Ins(1:2-cyclic) P has been identified more rigorously by n.m.r. spectroscopy in rat papillary collecting-tubule cells (Shayman *et al.*, 1986) and in a rat hepatoma (Bird *et al.*, 1987). No study has yet distinguished between the enantiomers of these cyclic phosphates.

Inositol phosphates other than those expected as metabolites of Ins(1,4,5) P_3

Peaks eluted before inositol monophosphates. As a matter of routine, many studies have reported the labelling in cells, and sometimes accumulation on stimulation, of material that is eluted from Dowex-I and AG1 resins in a fraction commonly

termed 'glycerophosphoinositol' and from anion-exchange h.p.l.c. before genuine inositol monophosphates. We resolved two such peaks from neutral extracts of WRK1 cells, one of which (peak 2) appears to be inositol 1:2-cyclic phosphate. The less polar peak (peak 1) co-eluted with an internal standard of [^{14}C]GroPIns, suggesting at least some of this material may be authentic GroPIns; however, it remains to be rigorously identified. In preliminary studies of WRK1 cells labelled with [^{14}C]glucosamine, a ^{14}C -labelled peak was detected in this region of the chromatogram, suggesting that a metabolite of one of the PtdIns-glycan lipids that serve as anchors for membrane proteins, and possibly also as precursors for 'insulin mediators', might contribute to this unidentified material (Low & Saltiel, 1988; Ferguson & Williams, 1988).

Inositol bisphosphates and inositol trisphosphates. Coexisting with the three expected InsP_2 isomers in WRK1 cells were small quantities of compounds tentatively identified as $\text{Ins}(4,5)\text{P}_2$ and/or $\text{Ins}(5,6)\text{P}_2$, and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$, which did not change their levels in response to vasopressin. The final InsP_3 peak of WRK1 cells was eluted in a position sometimes assigned to $\text{Ins}(2,4,5)\text{P}_3$, on the assumption that acid extraction converts some $\text{Ins}(1,2\text{-cyclic},4,5)\text{P}_3$ into this compound. However, this peak did not co-elute with authentic $\text{Ins}(2,4,5)\text{P}_3$. The formation of xylitol on oxidation identifies its major component as $\text{Ins}(3,4,5)\text{P}_3$ and/or $\text{Ins}(1,5,6)\text{P}_3$. A similar compound was identified in avian erythrocytes by Stephens *et al.* (1989b), who suggested that the presence of an $\text{Ins}(3,4,5,6)\text{P}_4$ 3-phosphatase in those cells made $\text{Ins}(3,4,5)\text{P}_3$ more likely: we have no basis for favouring either alternative in WRK-1 cells.

Concluding comment

Inositol phosphates are central to cell regulatory processes (e.g. Berridge & Irvine, 1989) and some inositol polyphosphates are among the most abundant polyanions in many cells (e.g. French *et al.*, 1988, 1991; reviewed by Shears, 1989a). It therefore remains disturbing that only two studies, one of normal avian erythrocytes (Stephens *et al.*, 1989b; Stephens & Downes, 1990) and the other this analysis of a mammary tumour cell line, have attempted fully to catalogue the inositol phosphate complement of any cell type. Although these studies have identified many of the same compounds, inositol phosphates other than those downstream of $\text{Ins}(1,4,5)\text{P}_3$ were much more prominent components of the avian cells, emphasizing the need for similarly detailed studies of other cells. This need is further reinforced by our own similar studies of equilibrium-labelled HL60 promyelocytic cells, which have revealed both a greater variety of inositol phosphates than in WRK1 cells and the presence of some labelled and as yet unidentified 'inositol polyphosphate' peaks (French *et al.*, 1988, 1991; P. J. French, C. M. Bunce, G. Brown & R. H. Michell, unpublished work).

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