Inositol 1,2,3-trisphosphate and inositol 1,2- and/or 2,3-bisphosphate are normal constituents of mammalian cells

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1. An inositol trisphosphate (InsP_3) distinct from $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$, which we previously observed in myeloid and lymphoid cells [French, Bunce, Stephens, Lord, McConnell, Brown, Creba and Michell (1991) *Proc. R. Soc. London B* **245**, 193–201; Bunce, French, Allen, Mountford, Moore, Greaves, Michell and Brown (1993) *Biochem. J.* **289**, 667–673], is present in WRK1 rat mammary tumour cells and pancreatic endocrine β -cells. 2. It has been identified as $\text{Ins}(1,2,3)\text{P}_3$ by a combination of oxidation to ribitol, a structurally diagnostic polyol, and ammoniacal hydrolysis to identified inositol monophosphates. 3. $\text{Ins}(1,2,3)\text{P}_3$ concentration in HL60 cells changed little during stimulation by ATP or fMetLeuPhe or during neutrophilic or monocytic differentiation, and $\text{Ins}(1,2,3)\text{P}_3$ was unresponsive to vasopressin in WRK1 cells. 4. $\text{Ins}(1,2,3)\text{P}_3$ was usually more

abundant than $\text{Ins}(1,4,5)\text{P}_3$, often being present at concentrations between $\sim 1 \mu\text{M}$ and $\sim 10 \mu\text{M}$. 5. HL60, WRK-1 and lymphoid cells also contain $\text{Ins}(1,2)\text{P}_2$ or $\text{Ins}(2,3)\text{P}_2$, or a mixture of these two enantiomers, as a major InsP_2 species. 6. $\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$ are readily detected in cells labelled for long periods, but not in acutely labelled cells. This behaviour resembles that of InsP_6 , the most abundant cellular inositol polyphosphate that includes the 1,2,3-trisphosphate motif, which also achieves isotopic equilibrium with inositol only slowly. 7. $\text{Ins}(1,2,3)\text{P}_3$ is the major InsP_3 that accumulates during metabolism of InsP_6 by WRK-1 cell homogenates. 8. Possible metabolic relationships between $\text{Ins}(1,2,3)\text{P}_3$, $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$ and other inositol polyphosphates in cells, and a possible role for $\text{Ins}(1,2,3)\text{P}_3$ in cellular iron handling, are considered.

INTRODUCTION

Since the recognition that $\text{Ins}(1,4,5)\text{P}_3$ is a widespread intracellular messenger molecule, there has been growing recognition that mammalian cells contain many of the 62 other *myo*-inositol derivatives that bear only monoesterified phosphate groups. Some are known metabolites of $\text{Ins}(1,4,5)\text{P}_3$ [including $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,3,4,6)\text{P}_4$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(3,4)\text{P}_2$, $\text{Ins}1\text{P}$, $\text{Ins}3\text{P}$, $\text{Ins}4\text{P}$], and others are members of a more abundant and slowly metabolized family of inositol polyphosphates that includes InsP_6 and several isomers of InsP_4 and InsP_5 [for reviews, see Menniti et al., 1993; Hughes and Michell, 1993].

Relatively few of the above compounds include a 2-phosphate group. Of the many possible 2-phosphate-containing inositol polyphosphates, $\text{Ins}(1,2,3,4,6)\text{P}_5$ and $\text{Ins}(1,2,4,5,6)\text{P}_5$ are abundant in *Dictyostelium* (Stephens et al., 1991), and $\text{Ins}(1,2,3,5,6)\text{P}_5$, $\text{Ins}(1,2,3,4)\text{P}_4$ and $\text{Ins}(1,2,4,5)\text{P}_4$ are prominent InsP_4 metabolites formed by plant and fungal phytases (Cosgrove, 1969; and see Stephens, 1990). Evidence for 2-phosphate-containing inositol polyphosphates other than InsP_6 in most mammalian cells is more scant. The pentakisphosphates $\text{Ins}(1,2,3,4,6)\text{P}_5$ and $\text{Ins}(1,2,4,5,6)\text{P}_5$ [or $\text{Ins}(2,3,4,5,6)\text{P}_5$] are present in HL60 promyeloid cells and an NG-115-derived neuroblastoma cell line in trace quantities, and these neuroblastoma cells also contain traces of $\text{Ins}(1,2,3,4,5)\text{P}_5$ [or $\text{Ins}(1,2,3,5,6)\text{P}_5$] (Stephens et al., 1991). Other transformed cell lines contain detectable amounts of $\text{Ins}(1,2,3,4)\text{P}_4$, $\text{Ins}(1,2,3,6)\text{P}_4$, $\text{Ins}(1,2,4,5,6)\text{P}_5$, $\text{Ins}(2,3,4,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,5)\text{P}_5$ [or $\text{Ins}(1,2,3,5,6)\text{P}_5$] (Menniti et al., 1990; Mattingley et al., 1991; McConnell et al., 1991; Guse and Emmrich, 1991), and small amounts of $\text{Ins}(1,2,3)\text{P}_3$ and of

either $\text{Ins}(1,2,6)\text{P}_3$ or $\text{Ins}(2,3,4)\text{P}_3$ have been briefly reported in human B lymphocytes (McConnell et al., 1992).

Despite the apparent scarcity of most 2-phosphate-containing inositol polyphosphates in non-transformed animal cells, a recent study suggests that inositol polyphosphates that possess a 1,2,3-trisphosphate motif are especially potent in sequestering Fe^{3+} into complexes and thus preventing Fe^{3+} from initiating free-radical cascades, an observation that makes 1,2,3-trisphosphate-containing inositol polyphosphates into interesting potential candidates as intracellular iron-transport agents and/or cellular antioxidants (Hawkins et al., 1993).

We previously reported that HL60 promyeloid cells, B-lymphoid cells and T-helper lymphoid cells that are equilibrium-labelled with [^3H]inositol all contain as their most abundant ^3H -labelled inositol trisphosphate an unidentified compound that is neither $\text{Ins}(1,4,5)\text{P}_3$ nor its metabolite $\text{Ins}(1,3,4)\text{P}_3$ (French et al., 1991; Bunce et al., 1992, 1993). More recently, we have found a similar compound in equilibrium-labelled WRK-1 mammary tumour cells and in endocrine pancreatic β -cells. We now report that this novel inositol trisphosphate is $\text{Ins}(1,2,3)\text{P}_3$, which is present in some cells at concentrations of $\geq 10 \mu\text{M}$. At least in some cells, $\text{Ins}(1,2,3)\text{P}_3$ is accompanied by a potentially related inositol bisphosphate [$\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$].

MATERIALS AND METHODS

Most experimental details have been reported in previous papers (French et al., 1991; Bunce et al., 1992, 1993; Wong et al., 1992; Barker et al., 1991, 1992, 1994). Unlabelled polyols were from

Abbreviations used: Ins , InsP , InsP_2 , InsP_3 etc. [and equivalent abbreviations with added locants, e.g. $\text{Ins}(1,2,3)\text{P}_3$], *myo*-inositol and *myo*-inositol phosphates [numbered by reference to *D*-*myo*-inositol 1-phosphate as $\text{Ins}1\text{P}$: see Biochem. J. (1989) **258**, 1–2].

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Table 1 Estimated concentrations of $\text{Ins}(1,2,3)\text{P}_3$ and of $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ in cultured mammalian cells labelled to or close to isotopic equilibrium with $[^3\text{H}]\text{inositol}$

The estimated concentrations presented were, in general, calculated from studies of equilibrium (or near-equilibrium) labelled cells, by using the known specific radioactivities of the $[^3\text{H}]\text{inositol}$ in the culture media, experimentally measured cell numbers and volumes, and the assumption [proven only for HL60 cells (French et al., 1991) and for WRK-1 cells (R. Fox and C. J. Barker, unpublished work)] that the cells do not synthesize a significant proportion of their total inositol requirement. For details, see French et al. (1991), Bunce et al. (1993) and Mountford et al. (1994). The effectiveness of our chromatographic separations means that the $\text{Ins}(1,2,3)\text{P}_3$ concentrations are likely to be relatively accurate. The $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$ concentrations in lymphoid cells are recorded as maximum possible values, because our h.p.l.c. methods would not have resolved these InsP_2 isomers from $\text{Ins}(3,4)\text{P}_2$, and structural analyses were not undertaken on peak H from these cells. Despite this qualification, $\text{Ins}(3,4)\text{P}_2$ is likely to have been of relatively low abundance, since the cells were not overtly treated with any potent $\text{Ins}(1,4,5)\text{P}_3$ -generating stimulus. Abbreviations: n.d., not determined; PMA, phorbol 12-myristate 13-acetate.

Cell type	$[\text{Ins}(1,2,3)\text{P}_3]$ (μM)	$[\text{Ins}(1,2)\text{P}_2/$ $\text{Ins}(2,3)\text{P}_2]$ (μM)	Reference
Human myeloid blasts (primary)			
Undifferentiated	1.0 ± 0.3	≤ 12	Mountford et al. (1994)
Neutrophil-differentiated	2.9 ± 0.8	≤ 13	Mountford et al. (1994)
HL60 (human promyeloid leukaemia-derived)			
In $200 \mu\text{M}$ Ins (growing)	13.1 ± 2.1	$\sim 11^*$	Bunce et al. (1993)
In $5 \mu\text{M}$ Ins (growing)	1.6 ± 0.4	~ 11	French et al. (1991)
In $5 \mu\text{M}$ Ins (neutrophil-differentiated: dimethyl sulphoxide for 5 days)	1.0 ± 0.2	~ 8	French et al. (1991)
In $5 \mu\text{M}$ Ins (monocyte-differentiated: PMA for 2 days)	~ 0.8	~ 4	French (1990)
WRK-1 rat mammary tumour cells	$\sim 0.6\text{--}1.2$	$\sim 2\text{--}6$	The present paper
Murine interleukin-3-dependent B-cell progenitor cells (BAF3)	3.4 ± 2.7	≤ 14	Bunce et al. (1993)
Bcr-abl-transformed BAF3 cells (p210)	5.6 ± 2.9	≤ 13	Bunce et al. (1993); Mountford et al. (1994)
Mature B lymphocyte line (NSI)	2.8 ± 0.8	≤ 30	Mountford et al. (1994)
Immature T-lymphoid cells (S49)	n.d.	≤ 17	Mountford et al. (1994)
Human cord blood T-helper cells (primary culture)	4.8 ± 1.5	≤ 42	Bunce et al. (1993)
HTLV1-transformed human T-helper cell line (C8166)	2.2 ± 0.3	≤ 12	Bunce et al. (1993)

* In a second study (Mountford et al., 1994) the total InsP_2 concentrations in this peak were $22.2 \pm 1.6 \mu\text{M}$ (undifferentiated HL60 cells), $27 \pm 0.4 \mu\text{M}$ (neutrophil-differentiated in 10 nM all-*trans*-retinoic acid plus 30 ng/ml granulocyte-colony stimulating factor) and $34 \pm 0.9 \mu\text{M}$ (monocyte-differentiated in $0.1 \mu\text{M}$ vitamin D_3).

Aldrich Chemical Co., and $[^{14}\text{C}]\text{ribitol}$ was a gift from Dr. L. R. Stephens.

The isolation and structural studies of inositol polyphosphates involved extraction, h.p.l.c., ammoniacal hydrolysis and periodate oxidation by methods of the general types described by Stephens (1990) and Kirk et al. (1990): detailed methods are reported by French et al. (1991) and Wong et al. (1992), except for polyol separations and some InsP_3 separations (see below).

H.p.l.c. separation of polyols and of InsP_3 isomers

Polyols were separated by h.p.l.c. on a Bio-Rad Aminex HPX-87C column eluted with water at 65°C and 0.6 ml/min . Unlabelled standard polyols were detected by a refractometric detector (RefractoMonitor IV; LDC Analytical, Stone, Staffs., U.K.), and radioactive polyols by scintillation counting (Wong et al., 1992).

The h.p.l.c. method used to achieve optimum separation of multiple InsP_3 species from WRK-1 cells (see Figure 3) used a 25 cm Whatman Partisphere-SAX column (Laserchrom U.K. Ltd.). The column was pre-equilibrated with water, and the sample was loaded and eluted by using the following gradient, generated from deionized water (Pump A) and two successive concentrations of $(\text{NH}_4)_2\text{HPO}_4$ (0.25 M to 30 min and 1.25 M thereafter) adjusted to $\text{pH } 4.4$ with H_3PO_4 (Pump B). The gradient was: $0\text{--}5 \text{ min}$, 0% B; 6.5 min , 6% B; 30 min , 50% B; switch solvents; 30.1 min , 10% B; 32 min , 10% B; 45 min , 45% B; $65\text{--}89 \text{ min}$, 60% B; 95 min , 70% B; 105 min , 90% B; 120 min , 100% B. Samples were collected every 0.25 min in the InsP_3 region of the chromatogram, and a small sample was removed for determination of radioactivity (Canberra Packard). The

labelled peaks were neutralized, pooled, desalted, and the structures were determined (see above). This gradient, using a relatively new h.p.l.c. column, was particularly suitable for the separation of inositol phosphates from the larger amounts of labelled cell extracts required to yield sufficient material for structural determination (see Figure 3).

For routine separations of inositol phosphates from mouse pancreatic β -cells and InsP_6 dephosphorylation assays (as in Figures 4 and 5), an older Partisphere SAX column ($50\text{--}150$ runs before the experiment) was used, with the following modified gradient (using the same elution buffers as above, and a solvent switch at 23.2 min): $0\text{--}5 \text{ min}$, 0% B; 6.5 min , 6% B; 23 min , 9% B; 23.2 min , switch solvents, then 2% B; 25 min , 10% B; 44 min , 15% B; 49 min , 20% B; 50 min , 46% B; 59 min , 50% B; 68 min , 50% B; 83 min , 97% B; 93 min , 100% B.

Equilibrium labelling of WRK-1 cells and of endocrine pancreatic β -cells with $[^3\text{H}]\text{inositol}$

WRK-1 cells were labelled as described by Wong et al. (1992), except that the concentration of inositol in the cultures was $\sim 10 \mu\text{M}$ rather than $\sim 1 \mu\text{M}$. Mouse β -cells were labelled in primary culture as described by Barker et al. (1994), but for 48 h rather than 24 h .

Assay of InsP_6 metabolism in WRK-1 cell homogenates

WRK-1 cells were washed twice with PBS and once with homogenization medium consisting of 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethanesulphonyl fluoride, 2 mM mer-

captoethanol, 25 mM Hepes, pH 7.2, and 5 μ g/ml each of antipain, aprotinin, leupeptin and E64. Cells from two plates were scraped into 0.5 ml of homogenization buffer and disrupted with three 5 s pulses from a probe sonicator, with cooling to 4 °C between pulses. This homogenate was incubated for 2 h at 37 °C with 2.5 ml of an 'intracellular buffer' [final composition: 100 mM KCl, 6 mM $MgSO_4$, 10 mM NaCl, containing 2 mg/ml saponin] supplemented with 1 μ M $InsP_6$ containing ~ 5000 d.p.m. of [3H] $InsP_6$ (NEN-DuPont)]. The resulting inositol phosphates were mixed with a mixture of ^{14}C -labelled $Ins(1,2,3)P_3$, $Ins(1,3,4)P_3$ and $Ins(1,4,5)P_3$ from HL-60 cells and analysed by h.p.l.c. (see above).

RESULTS

Identification of a novel $InsP_3$ in HL60 cells as $Ins(1,2,3)P_3$

When we first described the presence in HL60 cells of an

unidentified $InsP_3$ (peak K in French et al., 1991), its intracellular concentration was calculated to be ~ 1 μ M, i.e. several-fold higher than that of $Ins(1,4,5)P_3$ (see Table 1). These results came from cells grown at a low inositol concentration (~ 5 μ M) so as to produce maximum labelling even of minor intracellular metabolites. However, this is only a small fraction of the concentration of inositol to which cells are exposed *in vivo*. When the same cells were equilibrium-labelled with ~ 200 μ M [3H]inositol, much closer to the concentrations found in extracellular fluid, the intracellular concentration of the unidentified $InsP_3$ was > 10 μ M, i.e. ~ 10-fold higher. Under these conditions, it became one of the small number of relatively abundant intracellular inositol phosphate species, even though the concentrations of most other inositol phosphates were little changed (see Table 1, and Bunce et al., 1993). In our original reports, we were unable to identify the novel $InsP_3$ of HL60 cells, but its structure has now been established by two experimental

Figure 1 Identification of the polyols formed by oxidation of the unknown $InsP_3$ from HL60 cells and WRK-1 cells as ribitol

(a) Elution of polyol standards from the Aminex h.p.l.c. column. (b) Separation of 3H -labelled peak K-derived polyols (■) from HL60 cells together with ^{14}C -labelled standard polyols (□). (c) Separation of 3H -labelled polyols derived from the unknown $InsP_3$ peak (see Figure 3) of WRK-1 cells (■), chromatographed together with ^{14}C -labelled standard polyols (□). Polyols were isolated and subjected to h.p.l.c. as described in the Materials and methods section.

Figure 2 Anion-exchange h.p.l.c. separation of the inositol monophosphates formed during ammoniacal hydrolysis of (a) peak K and (b) peak I of HL60 cells

See the text for technical details.

approaches, periodate oxidation to a diagnostic polyol and ammoniacal hydrolysis.

First, the isolated InsP_3 was subjected to periodate oxidation, followed by reduction and dephosphorylation to yield a polyol derived from the segment of its carbon backbone that the phosphate groups shield. The major ^3H -labelled polyol ($\sim 85\%$ of oxidation products) was eluted from an Aminex column in the position characteristic of the *meso*-pentitol ribitol ($\equiv$ adonitol), which can only be formed from one InsP_3 , the *meso*-compound $\text{Ins}(1,2,3)\text{P}_3$ (see Stephens, 1990; Kirk et al., 1990; McConnell et al., 1992). The second, smaller, peak ($\sim 10\%$) was unchanged inositol (Figure 1b), which could either be derived from a periodate-resistant InsP_3 or be the remnant of an incompletely oxidized InsP_3 isomer: the former explanation seems more likely, since $\text{Ins}(1,2,3)\text{P}_3$ should be readily oxidized by periodate.

Secondly, ammoniacal hydrolysis, which dephosphorylates inositol polyphosphates without phosphate-group migration and thus yields samples of inositol monophosphates that correspond to the positions of the phosphate groups in the parent compound, yielded two major inositol monophosphate peaks corresponding to Ins2P and to the enantiomeric mixture $\text{Ins1P}/\text{Ins3P}$, which are expected as products of $\text{Ins}(1,2,3)\text{P}_3$ dephosphorylation, plus a small amount of Ins4P (or Ins6P) (Figure 2a).

The six InsP_3 isomers (three enantiomeric pairs) other than $\text{Ins}(1,2,3)\text{P}_3$ that include both a 2-phosphate and a 1- or 3-phosphate are: $\text{Ins}(1,2,4)\text{P}_3$ and $\text{Ins}(2,3,6)\text{P}_3$; $\text{Ins}(1,2,5)\text{P}_3$ and $\text{Ins}(2,3,5)\text{P}_3$; and $\text{Ins}(1,2,6)\text{P}_3$ and $\text{Ins}(2,3,4)\text{P}_3$. Ribitol, derived from $\text{Ins}(1,2,3)\text{P}_3$, was readily distinguishable on the Aminex

Figure 3 High-resolution h.p.l.c. separation of four InsP_3 peaks from WRK-1 cells

The unknown peak (X), whose analysis is described in the text, was the first to emerge from the column. The subsequent peaks were identified by degradation methods as: peak A, $\text{Ins}(1,5,6)\text{P}_3$ and/or $\text{Ins}(3,4,5)\text{P}_3$; peak B, $\text{Ins}(1,3,4)\text{P}_3$ and/or $\text{Ins}(1,3,6)\text{P}_3$; peak C, $\text{Ins}(1,4,5)\text{P}_3$ and/or $\text{Ins}(3,5,6)\text{P}_3$ (C. J. Barker, unpublished work).

column from altritol, glucitol and arabitol, the polyols that would be formed by oxidation of these other isomers (compare Figures 1a and 1b), and the latter polyols were not found in the polyol sample derived from the unknown InsP_3 .

These results indicate that most of the previously uncharacterized $[\text{H}]\text{InsP}_3$ peak in labelled HL60 cells consists of $\text{Ins}(1,2,3)\text{P}_3$, a compound that has only once been briefly described in mammalian cells (McConnell et al., 1992), and it seems likely that this is also the compound with similar elution characteristics that is seen in other equilibrium-labelled haemopoietic cells. The small amount of Ins4P formed by hydrolysis of the InsP_3 peak indicates that another InsP_3 co-eluted with $\text{Ins}(1,2,3)\text{P}_3$. This is most likely to be an $\sim 10\%$ contribution of $\text{Ins}(2,4,6)\text{P}_3$, an InsP_3 that: (a) lacks a periodate-sensitive hydroxyl pair and would therefore yield unchanged inositol after periodate treatment; and (b) will yield only Ins2P and $\text{Ins4P}/\text{Ins6P}$ during ammoniacal hydrolysis.

The concentration of $\text{Ins}(1,2,3)\text{P}_3$ in HL60 cells did not change strikingly during either neutrophilic or monocytic differentiation (Table 1), during which there were a number of changes in the concentrations of other inositol polyphosphates. Its concentration remained stable when HL60 cells were stimulated for 30 s with $10\ \mu\text{M}$ ATP (undifferentiated or neutrophil-differentiated cells) or $1\ \mu\text{M}$ fMetLeuPhe (neutrophil-differentiated cells), even though both of these treatments provoke rapid rises in the concentrations, among others, of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ (Dubyak et al., 1988; Cowen et al., 1989; Pittet et al., 1989a,b; French, 1990).

$\text{Ins}(1,2,3)\text{P}_3$ in WRK-1 rat mammary tumour cells

The inositol phosphates present in WRK-1 rat mammary tumour cells, and the manner in which their concentrations change in response to stimulation, have been analysed in greater detail than for most other mammalian cells (Wong et al., 1992; Barker et al., 1992). The first-eluted InsP_3 peak resolved in those studies (peak 11) was mainly $\text{Ins}(1,3,4)\text{P}_3$ in vasopressin-stimulated cells, but it

Figure 4 High-resolution h.p.l.c. separation of multiple ^3H -labelled InsP_2 and InsP_3 peaks from extracts of mouse pancreatic β -cells

A [^3H]inositol-labelled β -cell extract (continuous line) was co-chromatographed under the conditions described in the Materials and methods section, together with a ^{14}C -labelled extract isolated from HL60 cells that had been equilibrium-labelled with [^{14}C]inositol (dashed line). Only the InsP_2 and InsP_3 regions of the h.p.l.c. chromatogram are shown. The second InsP_2 peak eluted (B) and the first InsP_3 peak eluted (D) from the β -cells co-chromatographed precisely with the peaks from HL60 cells that are identified above as $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ and as $\text{Ins}(1,2,3)\text{P}_3$.

also yielded small amounts of $\text{Ins}2\text{P}$ and of unidentified polyols (especially from unstimulated cells), the origins of which were not clear (Wong et al., 1992). The present studies used WRK-1 cells labelled with a higher concentration of inositol than previously (see the Materials and methods section). Using cells labelled under these conditions, we have found that an InsP_3 with elution characteristics similar to those of the novel InsP_3 from HL60 cells becomes more abundant and is resolvable from $\text{Ins}(1,3,4)\text{P}_3$ by h.p.l.c. of cell extracts (Peak X, Figure 3). As in the HL60-cell extracts, periodate oxidation of this peak yielded ribitol and a small proportion of unchanged inositol (Figure 1c), showing that it is predominantly $\text{Ins}(1,2,3)\text{P}_3$, with $\leq 15\%$ of a second InsP_3 present [possibly $\text{Ins}(2,4,6)\text{P}_3$]. The concentration of $\text{Ins}(1,2,3)\text{P}_3$ in WRK-1 cells, estimated from its ^3H content in cells labelled to equilibrium with $\sim 10\ \mu\text{M}$ extracellular [^3H]inositol, was of the order of $1\ \mu\text{M}$ (Table 1).

$\text{Ins}(1,2,3)\text{P}_3$ in pancreatic β -cells

We recently reported that murine pancreatic β -cells contain an unidentified InsP_3 that, unlike $\text{Ins}(1,4,5)\text{P}_3$, does not change its concentration in synchrony with intracellular $[\text{Ca}^{2+}]$ oscillations (Barker et al., 1994). When a ^3H -labelled InsP_3 fraction from cells which had been exposed to $20\ \text{mM}$ glucose for 2 min as described in Barker et al. (1994) was mixed with a ^{14}C -labelled InsP_3 fraction from HL60 cells, the unidentified InsP_3 co-eluted precisely with the compound identified above as $\text{Ins}(1,2,3)\text{P}_3$ (Figure 4). Similar levels of this compound were also detected in

β -cells which had not been treated with glucose (results not shown).

Probable occurrence of $\text{Ins}(1,2,3)\text{P}_3$ in other myeloid cells and in lymphoid cells

During studies of the levels of inositol polyphosphates during cell differentiation and transformation, undertaken in parallel with the HL60-cell studies, an InsP_3 peak with elution characteristics of $\text{Ins}(1,2,3)\text{P}_3$ was seen in several other cell populations that had been equilibrium-labelled with [^3H]inositol (Bunce et al., 1993; Mountford et al., 1994; see Table 1). This material, which has not been structurally analysed, is likely to have been $\text{Ins}(1,2,3)\text{P}_3$.

$\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ are present in WRK-1 cells, HL60 cells, pancreatic β -cells and probably also lymphoid cells

We previously reported that the ' $\text{Ins}(3,4)\text{P}_2$ ' peak isolated from WRK-1 cells, especially from cells that had not been stimulated through receptors that provoke the generation of $\text{Ins}(1,4,5)\text{P}_3$, includes 25–35% of a compound with the properties of $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$: the diagnostic feature was formation of erythritol after periodate oxidation (Wong et al., 1992).

A major InsP_2 peak from HL60 cells that has similar characteristics [peak I of French et al. (1991), equivalent to peak H of Bunce et al. (1993): see the h.p.l.c. chromatograms in the two papers] has now been analysed in detail. After periodate oxidation, $\sim 90\%$ of this peak was converted into erythritol (results not shown). Ammoniacal hydrolysis yielded $\text{Ins}2\text{P}$, $\text{Ins}1\text{P}/\text{Ins}3\text{P}$, and a trace of $\text{Ins}4\text{P}$ (Figure 2b). After hydrolysis of peak I [putative $\text{Ins}(1,2)\text{P}_2$], the ratio of labelling in [^3H] $\text{Ins}2\text{P}$ divided by labelling in [^3H] $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ was about double that seen in the hydrolysate of peak K [$\text{Ins}(1,2,3)\text{P}_3$]. This suggests, as expected, that the single 2-phosphate group makes a 2-fold greater contribution to the phosphate complement of this bisphosphate molecule, relative to the 1- and/or 3-phosphate group(s), than in $\text{Ins}(1,2,3)\text{P}_3$ (compare Figure 2a with Figure 2b). Both HL60 cells and WRK-1 cells therefore contain appreciable concentrations of $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ (Table 1), but we have not been able to determine the relative contributions of these two enantiomers.

A ^3H -labelled InsP_2 that co-elutes with the ^{14}C -labelled $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$ -dominated InsP_2 peak from HL60 cells is also present in extracts of pancreatic β -cells (Figure 4, peak B), and a relatively abundant InsP_2 peak (peak H) of similar elution characteristics was also noted in the lymphoid cells analysed by Bunce et al. (1993) (Table 1). However, since $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$ approximately co-eluted in the h.p.l.c. system used by Bunce et al. (1993), we did not determine their relative contributions to peak H in these cells.

$\text{Ins}(1,2,3)\text{P}_3$ is a product of InsP_6 metabolism by WRK-1 cell homogenates

Since $\text{Ins}(1,2,3)\text{P}_3$ is a known product of InsP_6 degradation by phytase in some organisms, we investigated whether it would be produced during InsP_6 metabolism by homogenates of WRK-1 cells. When such homogenates were incubated for 2 h with InsP_6 , $\sim 70\%$ of the InsP_6 was degraded and the main products had the elution characteristics of two InsP_3 isomers, one InsP_4 and two types of InsP_3 (Figure 5); there was very little de-

Figure 5 Formation of $\text{Ins}(1,2,3)\text{P}_3$ during dephosphorylation of InsP_6 by WRK-1 cell homogenates

WRK-1 cell homogenates were incubated with $[^3\text{H}]\text{InsP}_6$ for 2 h, and the products were then spiked with an HL60-cell-derived mixture of ^{14}C -labelled inositol polyphosphates [$\text{Ins}(1,2,3)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(3,4,5,6)\text{P}_4$, $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6] and analysed by h.p.l.c. (see the Materials and methods section). For clear presentation of the InsP_3 region of the resulting chromatogram, the time scale of the elution is expanded 5-fold in the left-hand panel of the Figure. The elution profile shown is representative of those obtained in three experiments that gave similar results.

phosphorylation to Ins , InsP or InsP_2 . Of these products, the main InsP_3 was $\text{Ins}(1,2,3)\text{P}_3$, which constituted about 20% of the accumulated metabolites: detailed analyses to identify the other products, some of which are assigned possible identities in Figure 5, have yet to be undertaken.

DISCUSSION

Most of the inositol phosphates that have been fully characterized in mammalian cells are either (a) mono-, bis-, tris-, or tetrakis-phosphates that lie on metabolic pathways downstream of the intracellular messenger $\text{Ins}(1,4,5)\text{P}_3$ (see the Introduction), or (b) precursors or metabolites of InsP_6 that lack a 2-phosphate group, including $\text{Ins}(1,3,4,5,6)\text{P}_5$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$. Relatively few inositol polyphosphates that include a 2-phosphate group have been reported in mammalian cells (see the Introduction).

$\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ are present in mammalian cells

Combination of the new information reported here with our previous observations (French et al., 1991; Bunce et al., 1992, 1993) and the work of McConnell et al. (1992) establishes the presence of $\text{Ins}(1,2,3)\text{P}_3$ in a variety of mammalian cells, and of $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ in at least some of these cells. Although limited, the selection of cells studied is functionally diverse, encompassing normal and transformed myeloid and lymphoid cells, a mammary tumour line and primary cultured endocrine secretory cells. Since $\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ were present in all of the cells that we examined carefully, they seem likely to be ubiquitous in mammalian cells, and maybe other eukaryote cells, but previously have remained largely unrecognized. It has been known for some time that mammalian cells contain appreciable amounts of $\text{Ins}2\text{P}$, of unknown origin (e.g. French et al., 1991; Bunce et al., 1993), which may be a metabolite of these newly recognized polyphosphates.

Three factors may account for previous failures to recognize the presence in cells of $\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2)\text{P}_2/\text{Ins}(2,3)\text{P}_2$. The first is that $\text{Ins}(1,2,3)\text{P}_3$ is eluted from anion-exchange h.p.l.c. columns in the same general region as $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ [its exact elution position varies with the type of anion-exchange column and the nature and pH of the elution gradient] and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ are eluted close to $\text{Ins}(3,4)\text{P}_2$, a known $\text{Ins}(1,4,5)\text{P}_3$ metabolite. As a result, $\text{Ins}(1,2,3)\text{P}_3$ has probably been overlooked whenever the elution positions of InsP_3 peaks from labelled cells have simply been compared with those of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ standards, and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$ will similarly have been misidentified as $\text{Ins}(3,4)\text{P}_2$. When, as is often noted in published studies, intracellular $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4)\text{P}_3$ levels in unstimulated cells seem unexpectedly high, this may sometimes reflect the presence of receptor-unresponsive $\text{Ins}(1,2,3)\text{P}_3$ as a 'contaminant' in the InsP_3 peaks. If such errors are to be avoided in future studies, then attempts to identify multiple labelled InsP_3 isomers primarily based on the basis of h.p.l.c. separations should employ a separation method, such as that used for the β -cell extracts in this paper, that fully resolves $\text{Ins}(1,2,3)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$. Alternatively, estimation of how much $[^3\text{H}]\text{ribitol}$ is formed when a ^3H -labelled InsP_3 peak is oxidized by periodate allows calculation of the $[^3\text{H}]\text{Ins}(1,2,3)\text{P}_3$ content of such mixed peaks.

A second reason for previous failures to recognize the presence of these relatively abundant 2-phosphate-containing inositol phosphates in labelled cells is that $\text{Ins}(1,2,3)\text{P}_3$, $\text{Ins}(1,2)\text{P}_2$ and $\text{Ins}(2,3)\text{P}_2$ are likely to be metabolically linked most closely to InsP_6 and the various 2-phosphate-containing inositol pentakis- and tetrakis-phosphates mentioned in the Introduction. These are metabolically inter-linked compounds that have relatively slow rates of synthesis, and thus of $[^3\text{H}]\text{inositol}$ incorporation, and which therefore recruit label much more slowly than $\text{Ins}(1,4,5)\text{P}_3$ and its immediate metabolites (see Menniti et al., 1993; Hughes and Michell, 1993). The likelihood that $\text{Ins}(1,2,3)\text{P}_3$ is metabolically related to these 'higher' inositol polyphosphates in intact cells is greatly strengthened by our

observation that WRK-1 cell homogenates form Ins(1,2,3) P_3 in relatively high yield during the dephosphorylation of Ins P_6 (Figure 5). The true magnitude of the contributions of Ins(1,2,3) P_3 , Ins(1,2) P_2 and Ins(2,3) P_2 to the cellular inositol polyphosphate complement may therefore only be recognized when cells are labelled for long enough to bring all inositol phosphates to similar, near-equilibrium, specific radioactivities: the cells used in this study were all labelled for ≥ 48 h.

Thirdly, we have seen that Ins(1,2,3) P_3 is more readily observed both in HL60 cells and in WRK-1 cells when they are cultured with ≥ 10 μ M inositol than at lower extracellular inositol concentrations (see Table 1 and the text). Most workers label cells with the minimum [3 H]inositol concentrations needed to sustain the health of cultures, both to minimize the cost of labelled inositol and to maximize the labelling of inositol polyphosphates and lipids. At least in HL60 and WRK-1 cells, this type of experimental approach may give the misleading impression that Ins(1,2,3) P_3 is a quantitatively minor cellular inositol polyphosphate.

Why do cells contain Ins(1,2,3) P_3 , and does Ins(1,2,3) P_3 have a specific cellular function?

Firstly, are Ins(1,2,3) P_3 and Ins(1,2) P_2 /Ins(2,3) P_2 simply intermediates in the metabolism of Ins P_6 or other inositol polyphosphates? In green plants and fungi, Ins P_6 metabolism by 'phytases' seems to go by one of two alternative routes, via Ins(1,2,3) P_3 or via Ins(1,2,6) P_3 (Cosgrove, 1969; and see Stephens, 1990), and the present work and that of McConnell et al. (1992) together demonstrate that both of these inositol trisphosphates occur as normal metabolites in mammalian cells, thus making either pathway plausible. Moreover, the results presented here show that WRK-1 cells possess the necessary enzymic apparatus to catalyse Ins P_6 dephosphorylation via Ins(1,2,3) P_3 .

However, other possible cellular functions of inositol polyphosphates that have an equatorial/axial/equatorial trisphosphate array on the 1,2,3-face of the inositol ring have been highlighted by the work of Graf and colleagues (Graf et al., 1984, 1987; Graf and Eaton, 1990) and of Hawkins et al. (1993). The former authors showed that phytic acid [Ins(1,2,3,4,5,6) P_6] has appropriate properties for it to serve as an endogenous antioxidant in plants, where it is present in high concentrations, and possibly also in mammalian cells, wherein it is less abundant but is still one of the major polyanions of the cytosol compartment (e.g. in HL60 promyeloid cells: see French et al., 1991; Bunce et al., 1993; Stuart et al., 1994). Apparently uniquely among known components of animal cells, Ins P_6 abolished potentially lethal iron-catalysed hydroxyl radical (HO $\cdot$) generation in model assays, rather than merely decreasing its rate (Graf et al., 1987).

The work of Hawkins et al. (1993) then showed that, for inositol polyphosphates to have this ability to prevent HO $\cdot$ generation, they should possess a 1,2,3-trisphosphate array: Ins P_6 , Ins(1,2,3,4,6) P_5 and a racemic Ins(1,2,3,4,5) P_5 /Ins(1,2,3,5,6) P_5 mixture were effective, but Ins(1,3,4,5,6) P_5 , the predominant Ins P_5 of mammalian cells, was only partially inhibitory. Thus they suggested that cytosolic Ins P_6 , then thought to be the only relatively abundant 1,2,3-trisphosphate-containing inositol polyphosphate in mammalian cells, might be an intracellular suppressor of free-radical generation, and also that it might shuttle the toxic Fe $^{3+}$ ion from the transferrin receptor to sites in the cell where it is used, e.g. for haem synthesis. The information reported here makes Ins(1,2,3) P_3 an additional candidate for these functions. Presumably Fe $^{3+}$ will bind to its protective equatorial/axial/equatorial 1,2,3-trisphosphate array

without intramolecular competition by other phosphate groups. In the cells we examined, its concentration was usually in the range ~ 2 –12 μ M, near the middle of the inhibitory concentration range for Ins P_6 and between one-third and one-twentieth of the concentration of Ins P_6 in the same cells. However, Ins(1,2,3) P_3 concentration seems likely to be more sensitive to changes in inositol supply than are the levels of other inositol polyphosphates, so our assays on HL60 cells grown in 200 μ M inositol, in which we saw the highest Ins(1,2,3) P_3 concentration, may give a truer reflection of its normal concentration in cells *in vivo*.

At least in HL60 cells and WRK-1 cells, the concentration of Ins(1,2,3) P_3 changed little either during stimulation through G-protein-coupled receptors that activate phosphoinositidase C or during cell differentiation. However, Ins(1,2,3) P_3 levels do appear to fluctuate about 2-fold during the WRK-1 cell cycle (Barker et al., 1995), a phenomenon that we are currently investigating in detail.

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