The interrelationships of the inositol phosphates formed in vasopressin-stimulated WRK-1 rat mammary tumour cells

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1. Temporal changes in the levels of many inositol phosphates, whose structural characterization is presented in the preceding paper [Wong, Barker, Morris, Craxton, Kirk & Michell (1991) *Biochem. J.* **286**, 459–468], have been monitored in vasopressin-stimulated WRK-1 cells. 2. Upon stimulation, $\text{Ins}(1,4,5)\text{P}_3$ accumulated within 1 s, consistent with its role as a rapidly acting second messenger produced by receptor activation of phosphoinositidase C. $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4,5)\text{P}_4$, both of which are immediate products of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, also accumulated quickly. $\text{Ins}4\text{P}$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(3,4)\text{P}_2$, $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$, which are intermediates in the metabolism of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4,5)\text{P}_4$ to inositol, accumulated after seconds or within a few minutes, and in a temporal sequence consistent with their known metabolic interrelationships. 3. The stimulated accumulation of $\text{Ins}(1,3,4,6)\text{P}_4$ was delayed, as expected if it is formed by phosphorylation of $\text{Ins}(1,3,4)\text{P}_3$. 4. $\text{Ins}(3,4,5,6)\text{P}_4$ accumulated 2–3-fold in a few minutes, and mainly before $\text{Ins}(1,3,4,6)\text{P}_4$. 5. Using a $[^3\text{H}]/[^{14}\text{C}]$ -inositol double-labelling protocol, we obtained evidence that all of the compounds that accumulated upon stimulation, except $\text{Ins}(3,4,5,6)\text{P}_4$, originated from lipid-derived $\text{Ins}(1,4,5)\text{P}_3$, but that the newly formed $\text{Ins}(3,4,5,6)\text{P}_4$ came from a different source. 6. There were no consistent changes in the levels of $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 during stimulation. 7. Alongside the gradual accumulation of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ during stimulation [Wong, Barker, Shears, Kirk & Michell (1988) *Biochem. J.* **252**, 1–5], there was an accumulation of $\text{Ins}(1:2\text{-cyclic},4)\text{P}_2$ and $\text{Ins}(1:2\text{-cyclic})\text{P}$, probably as either minor side products of phosphoinositidase C action or metabolites of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$. 8. When Li^+ was present during stimulation, it redirected the dephosphorylation pathways downstream of $\text{Ins}(1,4,5)\text{P}_3$ in the manner expected from its inhibition of inositol monophosphatase and $\text{Ins}(1,4)\text{P}_2/\text{Ins}(1,3,4)\text{P}_3$ 1-phosphatase: there were marked increases in the accumulation of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$ and of monophosphates. Moreover, Li^+ shifted the $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ balance in favour of $\text{Ins}1\text{P}$, thus demonstrating redirection of the metabolism of the accumulated $\text{Ins}(1,3,4)\text{P}_3$ towards $\text{Ins}(1,3)\text{P}_2$ rather than $\text{Ins}(3,4)\text{P}_2$.

INTRODUCTION

Receptor-activated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, yielding the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol, is a universal signalling pathway in eukaryote cells, and $\text{Ins}(1,3,4,5)\text{P}_4$, the 3-phosphorylated derivative of $\text{Ins}(1,4,5)\text{P}_3$, may also be involved in the regulation of intracellular $[\text{Ca}^{2+}]$ (reviewed in Berridge & Michell, 1988; Michell *et al.*, 1989; Berridge & Irvine, 1989; Downes & MacPhee, 1990). Many studies have documented the complex metabolism of these key inositol phosphates, both in intact cells and in cell-free systems, with the intention of understanding the interrelationships between the ever-increasing number of $\text{Ins}(1,4,5)\text{P}_3$ -derived inositol phosphates found in cells. In addition, a number of laboratories have described additional intracellular inositol polyphosphates that appear metabolically to be only distantly related to the products of $\text{PtdIns}(4,5)\text{P}_2$ degradation (e.g. Heslop *et al.*, 1985; Jackson *et al.*, 1987; Stephens *et al.*, 1988a,b,c, 1989, 1990a,b; Shears, 1989b; Balla *et al.*, 1989; Pittet *et al.*, 1989; Stephens & Downes, 1990; Stephens & Irvine, 1990). In several of these publications, the time-course of the production of various inositol phosphate peaks have been monitored by h.p.l.c. analysis of compounds that accumulate when intact cells are stimulated. Some have included rigorous structural information on a limited subset of inositol phosphates (e.g. Menniti *et al.*, 1990, for

$\text{Ins}\text{P}_4\text{s}$). However, apart from the work of Stephens and colleagues cited above, few have conducted rigorous analyses to determine the structures of the complete spectrum of inositol phosphates (including the resolution of enantiomers) present during agonist stimulation. Much clearer conclusions about potential metabolic pathways have been drawn from studies of inositol phosphate metabolism in permeabilized cells or homogenates (reviewed by Shears, 1989a), but it often remains difficult to determine whether the pathways elucidated by this method reflect the situation in intact cells.

In the preceding paper (Wong *et al.*, 1992) we reported the rigorous structural characterization of most of the inositol phosphates present in WRK-1 rat mammary tumour cells. Building on this information, we now report an analysis of the metabolic interrelationships between these inositol phosphates of defined structure in vasopressin-stimulated cells. We also show how these pathways are redirected in predictable ways when the cells are stimulated to generate $\text{Ins}(1,4,5)\text{P}_3$ while loaded with Li^+ as an inhibitor of inositol monophosphatase and $\text{Ins}(1,4)\text{P}_2/\text{Ins}(1,3,4)\text{P}_3$ 1-phosphatase. Finally, we use a previously reported dual-labelling technique (Maccallum *et al.*, 1989) to examine metabolic interrelationships between the many products of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, three inositol tetrakisphosphates, InsP_5 and InsP_6 . Some of these studies have been briefly reported previously (Barker *et al.*, 1988; Kirk *et al.*, 1990).

Abbreviations used: $\text{Ins}(1:2\text{cyclic})\text{P}$, $\text{Ins}(1:2\text{cyclic},4)\text{P}_2$ and $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$, *myo*-inositol cyclic phosphates [numbered by reference to *D*-*myo*-inositol 1-phosphate as $\text{Ins}1\text{P}$: see 'Numbering of atoms in *myo*-inositol', *Biochem. J.* (1989) **258**, 1–2].

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MATERIALS AND METHODS

Materials

Most materials were from the sources given in Wong *et al.* (1992). *myo*-[2-³H]inositol (10–20 Ci/mmol and 80–100 Ci/mmol) and *myo*-[U-¹⁴C]inositol (225 mCi/mmol) were from Amersham International.

Cell culture, labelling, stimulation and extraction

Cells were cultured, labelled with [³H]inositol and stimulated with vasopressin as described in the preceding paper (Wong *et al.*, 1992). When LiCl was included, the concentration of NaCl was proportionately lowered to maintain ionic balance: cells were incubated with or without Li⁺ for 10 min before stimulation. Most incubations were terminated with a neutral phenol reagent (Wong *et al.*, 1988), and the inositol phosphates stored at –20 °C. In order also to recover the inositol lipids in the ³H/¹⁴C double-labelling experiments, these incubations were terminated with 5% HClO₄ and neutralized with KOH (Shears *et al.*, 1987).

Analysis of inositol phosphates by h.p.l.c.

Unless otherwise stated, methods were exactly as described by Wong *et al.* (1992). Inositol tris-, tetrakis-, pentakis- and hexakis-phosphates were separated either on a 12.5 cm Whatman Partisphere WAX column as described previously (Stephens *et al.*, 1988c; Barker *et al.*, 1988) or on a 25 cm column of the same type. In the latter case the gradient, made with 0.5 M-(NH₄)₂HPO₄ (pH 3.8) in pump B and water in pump A, was: 0 min, 0% B; 5 min, 0% B; 20 min, 20% B; 70 min, 25% B; 80 min, 30% B; 98 min, 90% B; 100 min, 0% B.

Dual-labelling experiments with [2-³H]inositol and [U-¹⁴C]inositol

The technique was that described by Maccallum *et al.* (1989). WRK-1 cells were grown for 3 days with 2.1 μCi of [U-¹⁴C]inositol/ml in a low-inositol medium (total concn. 0.7 μM) to achieve equilibrium labelling of all inositol-containing cell components. High-specific-radioactivity [2-³H]inositol (180 μCi/ml) was then added, and incubation was continued for a further 120 min. The cells were stimulated with vasopressin for 15 min, quenched, and inositol tetrakisphosphates were extracted and analysed.

RESULTS AND DISCUSSION

Using the turkey erythrocyte, Stephens and colleagues have achieved the most detailed description to date of the complement and metabolic interrelationships of the inositol phosphates in any cell (Stephens *et al.*, 1988a,b,c, 1989, 1990a; Stephens & Downes, 1990). In the preceding paper (Wong *et al.*, 1992), we employed the WRK1 rat mammary tumour cell line to obtain a similarly complete picture of the inositol phosphate complement of a mammalian cell type.

In the studies described here, we have monitored the changes in inositol phosphate levels that occur in these cells when, after labelling to isotopic equilibrium with [2-³H]inositol, they are intensely stimulated with vasopressin through the phosphoinositidase C-coupled V_{1a} vasopressin receptor. When the resulting structural and kinetic labelling data on the separated peaks from our highly resolving h.p.l.c. separations are combined, they yield a more complete and rigorously interpretable view of the metabolic interrelationships of the various inositol phosphates than has previously been available either in WRK1 cells (Kirk *et al.*, 1986; Mouillac *et al.*, 1989) or in most other mammalian cells (reviewed by Berridge & Irvine, 1989). Reassuringly, most of the conclusions that emerge below confirm

ideas that have been developed previously from less complete information on other cells.

Inositol phosphate profiles of unstimulated and vasopressin-stimulated WRK1 cells

Fig. 1 presents the changes in labelling observed for 18 inositol phosphates during 10 min of exposure to vasopressin, with particular emphasis on the first 1–3 min. These data are discussed below primarily in terms of two groups of compounds: (a) those on the expected pathways of metabolism of the primary second messenger Ins(1,4,5)P₃, as deduced from studies of the metabolism of these compounds in broken-cell preparations and subcellular fractions (reviewed by Shears, 1989a); and (b) those likely to be derived from other sources.

Very rapid vasopressin-stimulated production of inositol phosphates

As reported previously (Wong *et al.*, 1988), Ins(1,4,5)P₃ and Ins(1,4)P₂ increased 4-fold within approx. 1 s of the application of vasopressin (results not shown). Given our inability to detect differences in the early kinetics of the accumulation of these two compounds, we cannot distinguish in WRK1 cells between (a) direct and simultaneous stimulation of the breakdown of both PtdIns(4,5)P₂ and PtdIns4P or (b) rapid hydrolysis of PtdIns(4,5)P₂ by phosphoinositidase C, followed by very rapid conversion of the liberated Ins(1,4,5)P₃ into Ins(1,4)P₂ by 5-phosphatase action. Ins(1,4,5)P₃ levels then declined to about double the basal value within 30 s and remained at that level for the following few minutes. A small proportion (< 10%) of the 'Ins(1,4,5)P₃' present at longer time intervals can be attributed to the presence of contaminating Ins(1,4,6)P₃ and/or Ins(3,4,6)P₃ (Wong *et al.*, 1992). In the only similar study on these cells (Mouillac *et al.*, 1989), the cells were labelled for only 2 days before stimulation and the Ins(1,4,5)P₃ peak declined to the basal level much more quickly, within about 1 min. Although a possible interpretation of the difference between the two studies could be that the later phase of Ins(1,4,5)P₃ formation draws on a pool of PtdIns(4,5)P₂ which is not equilibrium-labelled during the shorter labelling period used by Mouillac *et al.* (1989), our previous studies did not reveal sufficient metabolic heterogeneity in the inositol lipids of WRK1 cells for this situation to prevail (Maccallum *et al.*, 1989).

Inositol polyphosphate products of Ins(1,4,5)P₃ metabolism and of other pathways

Ins(1,3,4,5)P₄, presumably formed by the phosphorylation of Ins(1,4,5)P₃, accumulated 7-fold within 5 s and reached a maximum of 10 times the unstimulated level within 30 s, before declining slightly over the next 10 min. Its immediate dephosphorylation product, Ins(1,3,4)P₃, increased slowly throughout the 10 min, to reach about 40 times its low starting concentration. There was a delay of about 30 s before any obvious accumulation of (1,3,4,6)P₄, presumably formed by 6-phosphorylation of Ins(1,3,4)P₃, after which its concentration rose steadily to about 6 times the initial value after 10 min. The slight fall in the level of this compound shown in Fig. 1 after 5 s was not statistically significant (means ± S.E.M. were: control, 800 ± 10 d.p.m.; 5 s, 736 ± 60 d.p.m.; *n* = 4).

The third InsP₄ peak, containing the enantiomers Ins(1,4,5,6)P₄ and Ins(3,4,5,6)P₄, was much more abundant in unstimulated cells than either Ins(1,3,4,5)P₄ or Ins(1,3,4,6)P₄, and the temporal pattern of its accumulation during stimulation was intermediate between the rates of change of the other two isomers. It rose detectably within the first 5–30 s, the accumulation becoming statistically significant at 30 s (control, 5270 ± 88 d.p.m.; 30 s, 6050 ± 330 d.p.m.; *n* = 4, *P* < 0.05), and had accumulated

Fig. 1. Changes in the levels of ^3H -labelled inositol phosphates in WRK1 cells in response to vasopressin

Cells were labelled to equilibrium with [^3H]inositol and then stimulated with 400 nM-[arginine]vasopressin for the periods indicated. Inositol phosphates were extracted and separated by h.p.l.c. on Partisil-SAX and Partisphere-WAX columns, as appropriate (see the Materials and methods section), and their radioactivities determined. The individual panels display stimulated

approx. 3-fold after 10 min: it was the most abundant InsP_4 peak at all times. Structural analyses (Wong *et al.*, 1992) indicate that this peak is mainly $\text{Ins}(3,4,5,6)\text{P}_4$, but that after stimulation 10–15% is contributed by its enantiomer $\text{Ins}(1,4,5,6)\text{P}_4$. It therefore seems likely that most of what accumulates during stimulation is $\text{Ins}(3,4,5,6)\text{P}_4$, but that the quantity of $\text{Ins}(1,4,5,6)\text{P}_4$ present probably rises many-fold from its starting level. The $\text{Ins}(1,5,6)\text{P}_3/\text{Ins}(3,4,5)\text{P}_3$ peak showed similar behaviour to $\text{Ins}(3,4,5,6)\text{P}_4$. However, the initial rise of the $\text{Ins}(1,5,6)\text{P}_3/\text{Ins}(3,4,5)\text{P}_3$ peak was statistically significant within 5 s (control, 892 ± 80 d.p.m.; 5 s, 1389 ± 75 d.p.m.; $n = 4$, $P < 0.01$).

In some experiments, there was a slight accumulation of $\text{Ins}(1,3,4,5,6)\text{P}_5$ after 30 s of stimulation (e.g. Fig. 1), but this result was inconsistent. We never saw any significant change in InsP_6 level.

Inositol bisphosphates

$\text{Ins}(1,4)\text{P}_2$ was the predominant inositol bisphosphate that accumulated during stimulation of WRK-1 cells. Taken with the rather modest increase seen in $\text{Ins}(1,3,4,5)\text{P}_4$ and its metabolite $\text{Ins}(1,3,4)\text{P}_3$, this indicates that the major pathway of inactivation of $\text{Ins}(1,4,5)\text{P}_3$ in these cells is dephosphorylation to $\text{Ins}(1,4)\text{P}_2$ rather than phosphorylation to $\text{Ins}(1,3,4,5)\text{P}_4$. However, as noted above, it remains possible that some of the accumulated $\text{Ins}(1,4)\text{P}_2$, particularly that which appears very quickly, may be directly formed by receptor-activated hydrolysis of $\text{PtdIns}4\text{P}$.

Both $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$ showed substantial accumulations that were appreciably delayed, with the latter first briefly declining and then later rising to a higher concentration. It therefore seems likely that the $\text{Ins}(1,3,4)\text{P}_3$ formed during stimulation was dephosphorylated both by 1-phosphatase and 3-phosphatase action, with the former predominating. Interpretation of the changes in labelling of the $\text{Ins}(3,4)\text{P}_2$ peak is, however, complicated by two factors: $\text{Ins}(1,6)\text{P}_2$, its enantiomer, may also be present, and about 25% of the substantial amount of material in this peak in unstimulated and 5 s-stimulated samples is probably $\text{Ins}(2,3)\text{P}_2$ and/or $\text{Ins}(1,2)\text{P}_2$ (Wong *et al.*, 1992). The tentatively identified $\text{Ins}(4,5)\text{P}_2$ peak exhibited no significant change on stimulation.

Inositol monophosphates

In unstimulated cells, the 'inositol monophosphate' region of the h.p.l.c. trace was dominated by two peaks, an unidentified compound, possibly containing some glycerophosphoinositol (results not shown; see Wong *et al.*, 1992) and $\text{Ins}(1:2\text{cyclic})\text{P}$: other inositol monophosphates were present only in trace quantities.

Stimulation with vasopressin dramatically changed this pattern. There was a rapid accumulation of the $\text{Ins}4\text{P}/\text{Ins}5\text{P}/\text{Ins}6\text{P}$ peak. Like $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$, its initial accumulation was very quick and it then maintained an elevated level, almost

changes in the levels of groups of inositol phosphates, with individual curves identified by the appended locants, in ascending order of charge (cyc, cyclic): inositol monophosphates, bisphosphates, trisphosphates, tetrakisphosphates, and pentakisphosphate and hexakisphosphate. Because we did not usually separate all of the components displayed from all cell samples, data in the various panels have been normalized to directly comparable values by using the labelling of the $\text{Ins}(1,4)\text{P}_2$ peak of unstimulated cells in each experiment as a reference value: this was chosen as the calibration reference because it is a relatively large and consistent value in all experiments. An example of a complete set of data from one experiment, at one time point, can be found in Table 1. The results presented in any panel typify those obtained from several samples of stimulated WRK1 cells. Where the changes are small, the statistical significance of the data is indicated in the relevant text.

certainly due to rapid and then sustained dephosphorylation of substantial quantities of $\text{Ins}(1,4)\text{P}_2$ to $\text{Ins}4\text{P}$. There was a slower rise in the combined $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ peak, but it achieved a similar level after 10 min. At this time, the peak comprised approximately equal quantities of $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$, and these had accumulated to about 60 times their initial levels.

Inositol cyclic phosphates

Despite a proposal several years ago that implied a central role for $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$ in receptor-activated Ca^{2+} mobilization (Wilson *et al.*, 1985; Irvine *et al.*, 1986) and subsequent detailed studies of its levels in stimulated mouse pancreatic mini-lobules (e.g. Sekar *et al.*, 1987), most of the recent evidence has indicated that inositol cyclic phosphates are minor and biologically unimportant side-products of phosphoinositidase C activity (e.g. Hawkins *et al.*, 1987; Wong *et al.*, 1988; Willcocks *et al.* 1989). We previously showed that $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$ accumulates far more slowly than $\text{Ins}(1,4,5)\text{P}_3$ in stimulated WRK1 cells (Wong *et al.*, 1988), and we have now shown that $\text{Ins}(1:2\text{cyclic},4)\text{P}_2$ also accumulates slowly and steadily when these cells are stimulated, reaching about 10 times its initial concentration within 10 min. $\text{Ins}(1:2\text{cyclic})\text{P}$ was the predominant inositol monophosphate in unstimulated WRK1 cells, and it also accumulated substantially and consistently during 10 min of stimulation. However, its behaviour over shorter periods of stimulation appeared erratic, sometimes showing a rapid rise (e.g. Fig. 1), but in other experiments showing no change or a fall. The mean results ($\pm$ S.E.M.) from four experiments were: control, 1890 ± 43 d.p.m.; 5 s, 2670 ± 344 d.p.m.; 30 s, 2340 ± 275 d.p.m. The differences between control and stimulated cells are not statistically significant. It seems likely that this apparent variability arose from technical difficulties in reproducibly assaying this weakly charged compound.

Effects of Li^+ on vasopressin-stimulated accumulation of inositol phosphates

Table 1 shows the effects of 10 mM- and 30 mM- LiCl on the stimulated accumulation of inositol phosphates after 10 min. With 10 mM- LiCl , there was a large increase in the accumulation of both $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$, presumably reflecting the inhibition of the inositol polyphosphate 1-phosphatase that hydrolyses both compounds. Further increasing the Li^+ concentration amplified these accumulations, particularly that of $\text{Ins}(1,4)\text{P}_2$.

Li^+ greatly potentiated the accumulation of $\text{Ins}(1,3)\text{P}_2$ and, at the same time, largely suppressed that of $\text{Ins}(3,4)\text{P}_2$, indicating that a second consequence of the inhibition of the 1-phosphatase by Li^+ is to divert a greater proportion of the metabolism of $\text{Ins}(1,3,4)\text{P}_3$ towards $\text{Ins}(1,3)\text{P}_2$.

A third consequence of this metabolic diversion should be to drive the metabolism of a greater proportion of these lipid-derived inositol phosphates towards $\text{Ins}1\text{P}$ [derived from the accumulated $\text{Ins}(1,3)\text{P}_2$] in preference to $\text{Ins}3\text{P}$ [from $\text{Ins}(3,4)\text{P}_2$, whose formation is suppressed]: the resulting $\text{Ins}1\text{P}$ and the $\text{Ins}3\text{P}$ should accumulate as a result of the potent Li^+ blockade of inositol monophosphatase. In the accompanying paper (Wong *et al.*, 1992), we employed a partial periodate-oxidation technique to discriminate between the enantiomers $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$ (Wong, 1989; Stephens *et al.*, 1990b). Using this technique, we have identified the compounds that contribute to the enormous accumulation of inositol monophosphates that occurs in WRK1 cells when Li^+ is present. As the Li^+ concentration was increased, there was a progressive increase in the quantity of $\text{Ins}1\text{P}$ that accumulated and a compensating decline in the fraction of the inositol monophosphates recovered as $\text{Ins}3\text{P}$, as would be

Table 1. Effects of Li^+ on the accumulation of inositol phosphates in vasopressin-stimulated WRK1 cells

Cells were labelled to equilibrium with [^3H]inositol and then stimulated with 400 nM-[arginine]vasopressin for 10 min either in the absence of Li^+ or in the presence of 10 mM- or 30 mM- LiCl . Inositol polyphosphates were neutrally extracted, separated by h.p.l.c. on Partisil-SAX and Partisphere-WAX columns, as appropriate (see the Materials and methods section and Wong *et al.*, 1992), and their radioactivities determined. The results are means of duplicate determinations from a single experiment; a further experiment gave similar results. In a separate series of experiments groups of inositol monophosphates ($\text{Ins}1\text{P}/\text{Ins}3\text{P}$, $\text{Ins}2\text{P}$ and $\text{Ins}4\text{P}/\text{Ins}5\text{P}/\text{Ins}6\text{P}$) were isolated by h.p.l.c., and the $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ peak was mildly oxidized with periodate. The resulting labelled glucitol was isolated and its enantiomeric composition was determined by oxidation with sorbitol dehydrogenase (see Wong *et al.*, 1992). The total quantities of $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$ present were then calculated by applying the resulting $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ ratio to the total radioactivity of the initially isolated mixed $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ peak.

	^3H (d.p.m.)		
	+ vasopressin	+ vasopressin + 10 mM- Li^+	+ vasopressin + 30 mM- Li^+
$\text{Ins}1\text{P}$	1460	16000	22300
$\text{Ins}3\text{P}$	1840	8600	4620
$\text{Ins}4\text{P}/\text{Ins}5\text{P}/\text{Ins}6\text{P}$	2810	32600	26700
$\text{Ins}(1,2\text{cyclic},4)\text{P}_2$	420	480	560
$\text{Ins}(1,3)\text{P}_2$	720	2650	2980
$\text{Ins}(1,4)\text{P}_2$	11000	43900	62500
$\text{Ins}(3,4)\text{P}_2$	5360	2140	1680
$\text{Ins}(1,3,4)\text{P}_3$	5550	14200	15200
$\text{Ins}(1,4,5)\text{P}_3$	3530	3610	4640
$\text{Ins}(3,4,5)\text{P}_3$	770	730	730
$\text{Ins}(1,3,4,6)\text{P}_4$	1870	2400	2320
$\text{Ins}(1,3,4,5)\text{P}_4$	570	760	770
$\text{Ins}(3,4,5,6)\text{P}_4$	4770	5280	5270
$\text{Ins}(1,3,4,5,6)\text{P}_5$	36000	34600	35400
InsP_6	16900	18900	20700

expected from the redirection of the metabolism of $\text{Ins}(1,3,4)\text{P}_3$ and the inhibition of $\text{Ins}(1,4)\text{P}_2$ degradation.

The levels of most other inositol phosphates in the cells which had been stimulated for 10 min were relatively insensitive to Li^+ . However, $\text{Ins}(1,3,4,6)\text{P}_4$ did accumulate to a slightly greater degree under these conditions, possibly because of the greater intracellular concentrations of $\text{Ins}(1,3,4)\text{P}_3$, the substrate for its formation (Table 1). Much more unexpected was the appreciable increase in InsP_6 that occurred during this relatively brief period of stimulation (Table 1): the concentration of this compound was remarkably constant under all other conditions. One other group has reported an increase in InsP_6 on agonist stimulation of adrenal chromaffin cells (Sasakawa *et al.*, 1990a,b): they also saw a rapid accumulation of InsP_6 , a phenomenon not observed in WRK-1 cells. One possible explanation of this change in InsP_6 levels might be that $\text{Ins}3\text{P}$ is the precursor of InsP_6 in WRK1 cells, as it is in *Dictyostelium* (Stephens & Irvine, 1990): in this case, the large Li^+ -induced accumulation of $\text{Ins}3\text{P}$ might lead to a secondary accumulation of InsP_6 . However, this seems unlikely, for at least two reasons. First, the accumulation of InsP_6 was greater with 30 mM- Li^+ than with 10 mM- Li^+ , even though the higher Li^+ concentration causes some redirection of metabolism towards $\text{Ins}1\text{P}$ rather than $\text{Ins}3\text{P}$. Secondly, InsP_6 that was synthesized from lipid-derived $\text{Ins}3\text{P}$ would, in the double-labelling experiment that is described next, recruit appreciable ^3H from the rapidly labelled lipid pool that is hydrolysed in stimulated cells: this did not happen (see below).

Dual-labelling experiments to compare the origins of the various inositol phosphates accumulated during stimulation

In order to discriminate between inositol phosphates that are derived from lipids, primarily or solely via $\text{Ins}(1,4,5)\text{P}_3$ formation and metabolism, and those that are formed by other routes, we used a double-labelling technique (Maccallum *et al.*, 1989). In this method, cells were cultured with [^{14}C]inositol until their components were labelled to apparent equilibrium. A very small amount of [^3H]inositol of high specific radioactivity was then added for 120 min (Fig. 2 and Table 2) before stimulation with vasopressin. The more rapid is the turnover of a particular inositol-containing constituent in cells, the greater will be the proportion of ^3H in the labelled inositol that it incorporates. Thus: (a) the $^3\text{H}:^{14}\text{C}$ ratio in any cell component can give an indication of how quickly it, or one of its immediate metabolic precursors, is synthesized from the intracellular pool of free inositol; and (b) the degree to which the $^3\text{H}:^{14}\text{C}$ ratios of any two compounds are similar offers a guide as to whether they might either be related as precursor and product or be formed from precursors that are closely related metabolically.

Table 2 shows that all of the newly formed inositol bisphosphates and trisphosphates that, on the basis of known pathways, are thought ultimately to arise from $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis showed $^3\text{H}:^{14}\text{C}$ ratios of between 4.1 and 6.8, as compared with equivalent values of 5.0–5.3 for the three inositol lipids in the same cells. Given the degree of imprecision inherent in these isotope-ratio determinations, the similarity between these two sets of ratios clearly supports, but cannot entirely confirm, this interpretation of the origin of these inositol phosphates. Similarly, the lack of any appreciable accumulation of ^3H in either $\text{Ins}(1,3,4,5,6)\text{P}_5$ or InsP_6 supports the idea that these compounds either are formed from some other precursor or are formed from $\text{Ins}(1,4,5)\text{P}_3$ at a very slow rate.

In these experiments, we focused our attention particularly on the inositol tetrakisphosphates. Known pathways predict the

relatively rapid formation of two of these, namely $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$, through the metabolism of $\text{Ins}(1,4,5)\text{P}_3$. In support of this view, preliminary studies have shown that incubation of WRK1 cell homogenates with labelled $\text{Ins}(1,4,5)\text{P}_3$ and ATP leads to formation of several products, including $\text{Ins}(1,3,4,5)\text{P}_4$ and a compound which is eluted from a Partisphere-WAX column just before $\text{Ins}(1,3,4,5)\text{P}_4$, i.e. in the position expected of $\text{Ins}(1,3,4,6)\text{P}_4$ (N. S. Wong, unpublished work). Menniti *et al.* (1990) have recently suggested that $\text{Ins}(3,4,5,6)\text{P}_4$ is formed by the action of a 1-phosphatase on $\text{Ins}(1,3,4,5,6)\text{P}_5$,

Table 2. Accumulation of labelled inositol phosphates in WRK1 cells that had been double-labelled, chronically with [^{14}C]inositol and then briefly with [^3H]inositol, before stimulation with vasopressin

WRK1 cells were cultured and labelled to equilibrium with 2 μCi of [^{14}C]inositol/ml. [^3H]inositol (40 μCi /ml) was added to the cells 120 min before hormonal stimulation, and they were then exposed to 400 nM-vasopressin for 15 min (see the Materials and methods section). Incubations were quenched with HClO_4 , inositol phosphates were separated by h.p.l.c., and their radioactivities were determined. Inositol lipids were extracted from the HClO_4 -insoluble pellets, deacylated, separated and their radioactivities determined. Methods were essentially as in Maccallum *et al.* (1989). To determine the $^3\text{H}:^{14}\text{C}$ ratio of the fraction of a particular inositol phosphate that was formed as a result of vasopressin stimulation, the quantity of each radioisotope accumulating in that component during stimulation was calculated, and the resulting values were used to calculate the relevant $^3\text{H}:^{14}\text{C}$ ratio. The results are means of duplicate determinations from a single experiment and are expressed as d.p.m. in 4×10^5 cells: a second experiment gave similar results. The $^3\text{H}:^{14}\text{C}$ ratios of the inositol lipids in this experiment were: PtdIns , 5.0; $\text{PtdIns}4\text{P}$, 5.3; and $\text{PtdIns}(4,5)\text{P}_2$, 5.2.

Fig. 2. Accumulation of labelled inositol tetrakisphosphates in WRK1 cells that had been double-labelled, chronically with [^{14}C]inositol and then briefly with [^3H]inositol, before stimulation with vasopressin

WRK1 cells were cultured and labelled to equilibrium with 2 μCi of [^{14}C]inositol/ml. [^3H]inositol (40 μCi /ml) was added to the cells 120 min before hormonal stimulation, and they were then exposed to 400 nM-vasopressin for 15 min (see Materials and methods section). Incubations were quenched with HClO_4 . Inositol tetrakisphosphates were separated by h.p.l.c. on a Partisphere-WAX column, and the radioactivities of the fractions determined. Results are from a single experiment, typical of three. Symbols: $\circ$, ^3H ; $\bullet$, ^{14}C .

	^3H (d.p.m.)	^{14}C (d.p.m.)	$^3\text{H}/^{14}\text{C}$ ratio	$^3\text{H}/^{14}\text{C}$ ratio of newly synthesized inositol phosphates
$\text{Ins}(1,3)\text{P}_2$				
Control	< 50	< 50	—	
+ Vasopressin	9 560	1 450	6.6	6.6
$\text{Ins}(1,4)\text{P}_2$				
Control	15 400	2 100	7.3	
+ Vasopressin	186 000	28 400	6.5	6.5
$\text{Ins}(3,4)\text{P}_2/\text{Ins}(1,6)\text{P}_2$				
Control	< 50	< 50	—	
+ Vasopressin	15 200	3 730	4.1	4.1
$\text{Ins}(1,3,4)\text{P}_3$				
Control	9 090	1 650	5.5	
+ Vasopressin	50 500	7 720	6.5	6.8
$\text{Ins}(1,4,5)\text{P}_3$				
Control	17 400	3 330	5.2	
+ Vasopressin	40 500	7 090	5.7	6.1
$\text{Ins}(2,4,5)\text{P}_3/\text{Ins}(3,4,5)\text{P}_3$				
Control	3 320	970	3.4	
+ Vasopressin	6 390	1 670	3.8	4.4
$\text{Ins}(1,3,4,5)\text{P}_4$				
Control	< 50	< 50	—	
+ Vasopressin	2 540	620	4.1	4.1
$\text{Ins}(1,3,4,6)\text{P}_4$				
Control	1 880	680	2.8	
+ Vasopressin	7 180	1 620	4.4	5.6
$\text{Ins}(3,4,5,6)\text{P}_4$				
Control	< 50	2 950	0	
+ Vasopressin	< 50	4 840	0	0
$\text{Ins}(1,3,4,5,6)\text{P}_5$				
Control	< 50	18 000	0	
+ Vasopressin	< 50	27 500	0	0
InsP_6				
Control	< 50	7 000	0	
+ Vasopressin	< 50	8 700	0	0

whereas studies of avian red cells have suggested that $\text{Ins}(3,4,5,6)P_4$ is formed from $\text{Ins}(1,3,4,6)P_4$ via $\text{Ins}(3,4,6)P_3$ (Stephens & Downes, 1990).

Our observation of a substantial and relatively rapid accumulation of $\text{Ins}(3,4,5,6)P_4$ in response to vasopressin was therefore unexpected, so it was important to check whether, at least in WRK1 cells, this compound might be formed quickly from one of the products of stimulated inositol lipid hydrolysis. As shown in Table 2 and Fig. 2, this is not so: whereas the newly accumulated $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4,6)P_4$ in stimulated cells had $^3\text{H}:^{14}\text{C}$ ratios (4.1 and 5.6 respectively, in the experiment of Table 2) that tallied with their expected lipid origin, we detected no ^3H transfer into $\text{Ins}(3,4,5,6)P_4$ during stimulation. The lack of incorporation of recently added inositol into $\text{Ins}(3,4,5,6)P_4$ was confirmed in a second set of experiments in which two sets of cells were separately labelled with $[2\text{-}^3\text{H}]\text{inositol}$, one set to equilibrium and the other only briefly (for 140 min). Once again, stimulation failed to elicit the increase in labelling of $\text{Ins}(3,4,5,6)P_4$ in the briefly prelabelled cells that would be expected if this material was derived from the receptor-sensitive lipid pool (results not shown). It was noticeable in the double-labelling study that the $^3\text{H}:^{14}\text{C}$ ratio of $\text{Ins}(1,3,4,6)P_4$ in unstimulated cells was markedly lower than that of other inositol phosphates that are metabolites of $\text{Ins}(1,4,5)P_3$: this might be a result of either the relatively long lifetime of the $\text{Ins}(1,3,4,6)P_4$ that was formed before the labelling of lipids with ^3H had approached its final value and/or the simultaneous formation of some $\text{Ins}(1,3,4,6)P_4$ by a second route of synthesis that employs a more slowly turned-over precursor.

From these results, we conclude that, even though the stimulated formation of $\text{Ins}(3,4,5,6)P_4$ occurs at the same time as stimulated accumulation of $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4,6)P_4$, it must arise by a pathway independent of inositol lipid hydrolysis. Instead, this compound must be formed via a pathway that draws on a relatively slowly turned-over substrate pool: neither this pathway nor the manner of its regulation is yet known. Menniti *et al.* (1990), in essentially similar experiments that compared short-term- and long-term-labelled methacholine-stimulated rat parotid acinar cells, have obtained evidence that cell stimulation can activate an $\text{Ins}(1,3,4,5,6)P_5$ 1-phosphatase which yields $\text{Ins}(3,4,5,6)P_4$. Since the response of the $\text{Ins}(1,3,4,5,6)P_5$ to agonists was inconsistent in WRK1 cells, we have been unable to use a similar approach to determine whether this is the route of stimulated $\text{Ins}(3,4,5,6)P_4$ formation in these cells.

General discussion and conclusions

In general, the observations in this and the preceding paper (Wong *et al.*, 1992) are in broad agreement with previous studies of stimulated changes in the levels of WRK-1 cell inositol phosphates that were less comprehensive and employed less highly resolving anion-exchange chromatographic methods (Kirk *et al.*, 1986; Mouillac *et al.*, 1989). The most obvious difference between our results and those of Mouillac *et al.* (1989) was our detection of an apparently more rapid and more sustained accumulation of $\text{Ins}(1,4,5)P_3$. Our results also provide new evidence that strengthens the view that $\text{Ins}(3,4,5,6)P_4$ is formed from non-lipid source(s) and turns over slowly. Perhaps most notable is the first direct confirmation that the presence of Li^+ in stimulated cells does indeed redirect the metabolism of $\text{Ins}(1,3,4)P_3$ and inhibit the breakdown of $\text{Ins}(1,4)P_2$ as would be predicted if the inositol monophosphatase and $\text{Ins}(1,4)P_2/\text{Ins}(1,3,4)P_3$ 1-phosphatases are the only significant targets of Li^+ at the concentrations used.

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