Turnover of inositol pentakisphosphates, inositol hexakisphosphate and diphosphoinositol polyphosphates in primary cultured hepatocytes

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We have used a non-transformed cell model, the primary cultured hepatocyte, to explore the turnover of inositol hexakisphosphate, multiple isomers of inositol pentakisphosphate and two novel diphosphoinositol polyphosphates. All of these compounds gradually accumulated radioactivity throughout a 70 h period of labelling with [^{3}H]inositol. However, a rapid metabolic rate was revealed upon inhibition of diphosphoinositol polyphosphate biphosphatase(s) with 1 mM fluoride for 40 min: this treatment elevated levels of [^{3}H]diphosphoinositol polyphosphates up to 10-fold, indicating that their cellular pools were normally turning over at least 10 times every 40 min. This was accompanied by a turnover of about 10 % of the pool of inositol hexakisphosphate. Control experiments established that 200 nM vasopressin brought about a typical activation of phospholipase C in hepatocytes after 62 h of primary culture. This agonist treatment did not affect steady-state levels of [^{3}H]inositol pentakisphosphates, [^{3}H]inositol hexakisphosphate or [^{3}H]diphosphoinositol polyphosphates. However, prolonged treatment of hepatocytes with 2 μM thapsigargin reduced steady-state levels of [^{3}H]diphosphoinositol polyphosphates by 50–70 %. This effect of thapsigargin was also observed in the presence of fluoride, indicating that thapsigargin inhibited the rate of synthesis of diphosphoinositol polyphosphates.

INTRODUCTION

Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 have provided the primary focus for studies into the importance of inositol phosphate metabolism to mammalian cell physiology. This interest stems from Ins(1,4,5)P_3 being a Ca^{2+}-mobilizing signal, the levels of which increase several-fold upon receptor activation, due to an accelerated breakdown of PtdIns(4,5)P_2 (reviewed by Berridge and Irvine, 1989). The 3-kinase-mediated phosphorylation of Ins(1,4,5)P_3 forms Ins(1,3,4,5)P_4 (reviewed by Shears, 1992); there are several reports that Ins(1,3,4,5)P_4 augments Ca^{2+} signalling (reviewed by Irvine, 1992), although there is some controversy concerning how widespread this role of Ins(1,3,4,5)P_4 might be (Bird et al., 1991). A well characterized series of enzymes dephosphorylate both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 to inositol (see Shears, 1992), which can then be reutilized for resynthesis of the inositol lipids.

Much less attention has been given to InsP_5 and InsP_6, yet these metabolites comprise the bulk of the inositol polyphosphate content in mammalian cells. This comparative neglect in part reflects the difficulties involved in the study of these higher polyphosphates, but in addition they have often been considered to be unlikely to contribute to the short-term regulation of cell function (see Berridge and Irvine, 1989). More recently, unsuspected complexities in the metabolism of these compounds have been uncovered, and this has led to increased interest in them. Such observations include the following: (1) demonstrations that, in some transformed mammalian cells, Ins(1,3,4,5,6)P_5 is not the only InsP_5 isomer (Guse and Emmrich, 1991; McConnell et al., 1991; Stephens et al., 1991; Wong et al., 1992); (2) evidence that agonists stimulate Ins(1,3,4,5,6)P_5 dephosphorylation to Ins(3,4,5,6)P_4 (Menniti et al., 1990, 1993b); (3) the discovery that Ins(1,3,4,5,6)P_5 and InsP_6 can be converted into diphosphate derivatives, both in the AR4-2J pancreatoma cell line and in the slime mould *Dictyostelium discoideum* (Menniti et al., 1993a; Stephens et al., 1993); (4) the purification of an enzyme that dephosphorylates InsP_6 to a number of InsP_5 isomers (Nogimori et al., 1991), several of which can be phosphorylated back to InsP_6 in cell-free systems (Stephens et al., 1991; Stanley et al., 1992). There are no known functions for this baffling array of potential InsP_5/InsP_6 substrate cycles, but evidence for their association with cell signalling was recently suggested by a study with Jurkat T-lymphocytes. In the latter cells, levels of several InsP_5 isomers were regulated by occupation of phospholipase C-linked receptors, in part at least by Ca^{2+}-dependent mechanisms (Guse and Emmrich, 1991).

Many of these new findings concerning InsP_5 and InsP_6, and the novel diphosphoinositol polyphosphates, have originated from a very limited number of studies with either transformed cell lines or cell-free systems. It was therefore the goal of the current study to obtain information on these metabolites in an intact and normal, non-transformed, mammalian cell. With the further aim of correlating our data with information we have previously obtained using enzymes purified from rat liver (Nogimori et al., 1991; Abdullah et al., 1992), we have now studied the metabolism of InsP_5 and InsP_6 in the primary cultured hepatocyte.

Abbreviations used: DMSO, dimethyl sulphoxide. Inositol phosphates are abbreviated according to IUPAC nomenclature. Some inositol phosphate isomers have naturally occurring enantiomers; where these enantiomeric pairs were not individually resolved, the D/L prefix is used, i.e. D/L-Ins(1,2,4,5,6)P_5 = D-Ins(1,2,4,5,6)P_5 + L-Ins(1,2,4,5,6)P_5 [the latter may also be written as D-Ins(2,3,4,5,6)P_5]. Two diphosphate derivatives of Ins(1,3,4,5,6)P_5 and InsP_6 are denoted as *PP*-InsP_4 and *PP*-InsP_5; we have previously used the terms 'IP_5P' and 'IP_6P' respectively (Menniti et al., 1993a), but the newer abbreviations are both consistent with the format used by other workers (Stephens et al., 1993) and conform with IUPAC recommendations. We propose that there is only a single diphosphate group in each of our derivatives (the text provides data that are consistent with this assumption).

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MATERIALS AND METHODS

Materials

[^{3}H]Ins(1,3,4,5,6)P_5 was isolated from [^{3}H]inositol-labelled AR4-2J cells (Menniti et al., 1990). These cells were also the source of the ^{3}H-labelled diphosphate derivatives of Ins(1,3,4,5,6)P_5 and InsP_6, which accumulate to high levels after 1 h of treatment of the cells with 10 mM NaF (Menniti et al., 1993a). D/L-[^{3}H]Ins(1,2,4,5,6)P_6 was isolated from an extract of [^{3}H]inositol-labelled T5-1 cells, using a Partisphere SAX column (McConnell et al., 1991). All of these preparations were desalted with triethylamine bicarbonate (Menniti et al., 1993a). [^{3}H]Inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4)P_3, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]InsP_6 were purchased from New England Nuclear (Boston, MA, U.S.A.). Dexamethasone, insulin, NaF and vasopressin were obtained from Sigma (St. Louis, MO, U.S.A.) Dimethyl sulphoxide (DMSO) (J. T Baker, Phillipsburg, NJ, U.S.A.) was used to make stock concentrations of phorbol 12,13-dibutyrate and thapsigargin (LC Services Corporation, Woburn, MA, U.S.A.). Adsorbosphere h.p.l.c. columns were purchased from Alltech Associates (Deerfield, IL, U.S.A.), and Partisphere h.p.l.c. columns were supplied by Krackeler Scientific (Durham, NC, U.S.A.). Penicillin, streptomycin and fetal calf serum were obtained from Gibco (Grand Island, NY, U.S.A.).

Analysis of [^{3}H]inositol phosphates and [^{3}H]diphosphoinositol polyphosphates in hepatocytes

Primary cultured hepatocytes were maintained, at 37 °C in air/CO_2 (19:1), at a density of 10^6 cells per well (3.5 cm diameter) in 2 ml of Williams E medium supplemented with 10% (w/v) fetal calf serum, 0.004 Sigma Unit of insulin, 8 μg of dexamethasone, 20 Sigma Units of penicillin, 20 μg of streptomycin and 2 mM L-glutamine (Rooney et al., 1991). Incubations also contained 200 μCi/ml [^{3}H]inositol. After 62 h (unless otherwise indicated), this medium was removed by aspiration. For experiments on the time course of labelling with [^{3}H]inositol, cells were immediately quenched with 1.5 ml of ice-cold 6% (v/v) perchloric acid containing 1 mg/ml InsP_6, and the culture plates were placed on ice for 15 min. In other experiments, cells were washed twice with 2 ml of Williams E medium supplemented with 20 mM Hepes, pH 7.4, 0.2% (w/v) BSA and 2 mM L-glutamine. The cells were then incubated in 2 ml of this medium for 2 h at 37 °C in a shaking water bath (40 rev./min). After this time, various agents were added as detailed below. Each experimental condition was performed in triplicate wells on a single plate. To terminate these incubations, the medium was aspirated and cells were immediately quenched with perchloric acid as described above. After 15 min on ice, the acid extracts were removed from the plates and neutralized with a mixture of Freon/octylamine (Menniti et al., 1990). The plates were washed twice with 2 ml of ice-cold 6% (v/v) perchloric acid, and then the ^{3}H-labelled inositol lipids were dissolved overnight in 2 ml of 0.1 M NaOH containing 0.1% (v/v) Triton X-100; 50 μl aliquots were counted for radioactivity.

Inositol phosphates in the neutralized extracts were separated by h.p.l.c. using one of two methods. One utilized an Adsorbosphere SAX column eluted at 1 ml/min with water for 10 min, followed by a 2 h linear gradient generated by mixing water with 0–80% 1 M $NH_4H_2PO_4$, pH 3.35 (Balla et al., 1989; Menniti et al., 1990). Alternatively, inositol phosphates were separated on a Partisphere 5μ SAX column which was eluted at 1 ml/min with a gradient generated by mixing water (buffer A) with 1.15 M $(NH_4)_2HPO_4$, pH 3.8 (buffer B), by a slight modification of the method described by Stephens (1990): 0–10 min, 0% B; 10–30 min, B increased linearly from 0 to 45%; 30–130 min, B increased to 80%; 130–135 min, B = 80%.

The eluate from the Adsorbosphere column was mixed with 3 vol. of MonoFlo 4 scintillant (National Diagnostics, Manville, NJ, U.S.A.) and radioactivity was recorded on-line with a Radiomatic Flo-One (Downers Grove, IL, U.S.A.). The counting efficiency was approx. 25% throughout the gradient. The higher viscosity of the eluate from the Partisphere column led to inadequate mixing with scintillant in the flow-cell of the Radiomatic detector, progressively decreasing counting efficiency across the gradient. Therefore the eluate from the Partisphere column was quantified in individual 1 min fractions mixed with 4 vol. of MonoFlo scintillant, using a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

InsP_5, InsP_6 and diphosphoinositol polyphosphates in hepatocytes

Hepatocytes were labelled with [^{3}H]inositol during 62 h of primary culture. Cells were then quenched, neutralized and chromatographed on an Adsorbosphere SAX h.p.l.c. column; the chromatograph that is shown (Figure 1a) begins at the point where InsP_3 isomers were eluted. A number of classical inositol phosphates were identified (see legend to Figure 1): Ins(1,3,4)P_3 (peak A), Ins(1,4,5)P_3 (peak B), Ins(1,3,4,6)P_4 (peak D), Ins(1,3,4,5)P_4 (peak E), D/L-Ins(3,4,5,6)P_4 (peak F), InsP_5 (peak I) and InsP_6 (peak J). The Ins(1,4,5)P_3 peak eluted close to an unknown contaminant (peak C), which is best illustrated by the rarely achieved separation described by the inset to Figure 1(a). In that particular h.p.l.c. run, peak C was estimated to be about 40% as large as the [^{3}H]Ins(1,4,5)P_3 peak, although this was still insufficient material for its unambiguous structural characterization. The quantification of Ins(1,4,5)P_3 was further compromised by its incomplete separation from Ins(1,3,4)P_3 (Figure 1).

In most of those animal cell types that have been studied to date, almost all of the InsP_5 is the 1,3,4,5,6-isomer (Johnson and Tate, 1969; Mayr, 1988; Phillippy and Bland, 1988; Menniti et al., 1990; Mattingly et al., 1991; Stephens et al., 1991; Wong et al., 1992). Likewise, in hepatocytes nearly 98% of total [^{3}H]InsP_5 was [^{3}H]Ins(1,3,4,5,6)P_5 (peaks I and N, Figure 1). Nevertheless, additional specific InsP_5 isomers were assigned to some further peaks (G, H, O and P in Figure 1) with the aid of some standards plus the known elution properties of two h.p.l.c. systems (for details, see the legend to Figure 1): D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (1.5% of total [^{3}H]InsP_5; peaks I and P), D/L-[^{3}H]Ins(1,2,3,4,5)P_5 (0.4%; peaks H and O) and [^{3}H]Ins(1,2,3,4,6)P_5 (0.3%; peaks G and P). While these particular metabolites were present as relatively small proportions in relation to total [^{3}H]InsP_5, the absolute level of D/L-[^{3}H]Ins(1,2,4,5,6)P_6 (see above) approached that of [^{3}H]Ins(1,3,4)P_3 (which was 1.9% of total [^{3}H]InsP_5; see Figure 1 legend). One possible route of formation of the less abundant [^{3}H]InsP_5 isomers is by non-specific hydrolysis of InsP_6 by an enzyme with limited capacity; a phosphatase with just these characteristics has been purified from rat liver (Nogimori et al., 1991).

Figure 1 shows that on both the Adsorbosphere and Partisphere columns, the elution of InsP_6 (peaks J and R) was followed by the elution of a further ^{3}H-labelled compound (peaks K and S). The latter co-eluted on both h.p.l.c. systems with a diphosphate derivative of InsP_6 prepared from AR4-2J cells (see legend to Figure 1), indicating that the product formed

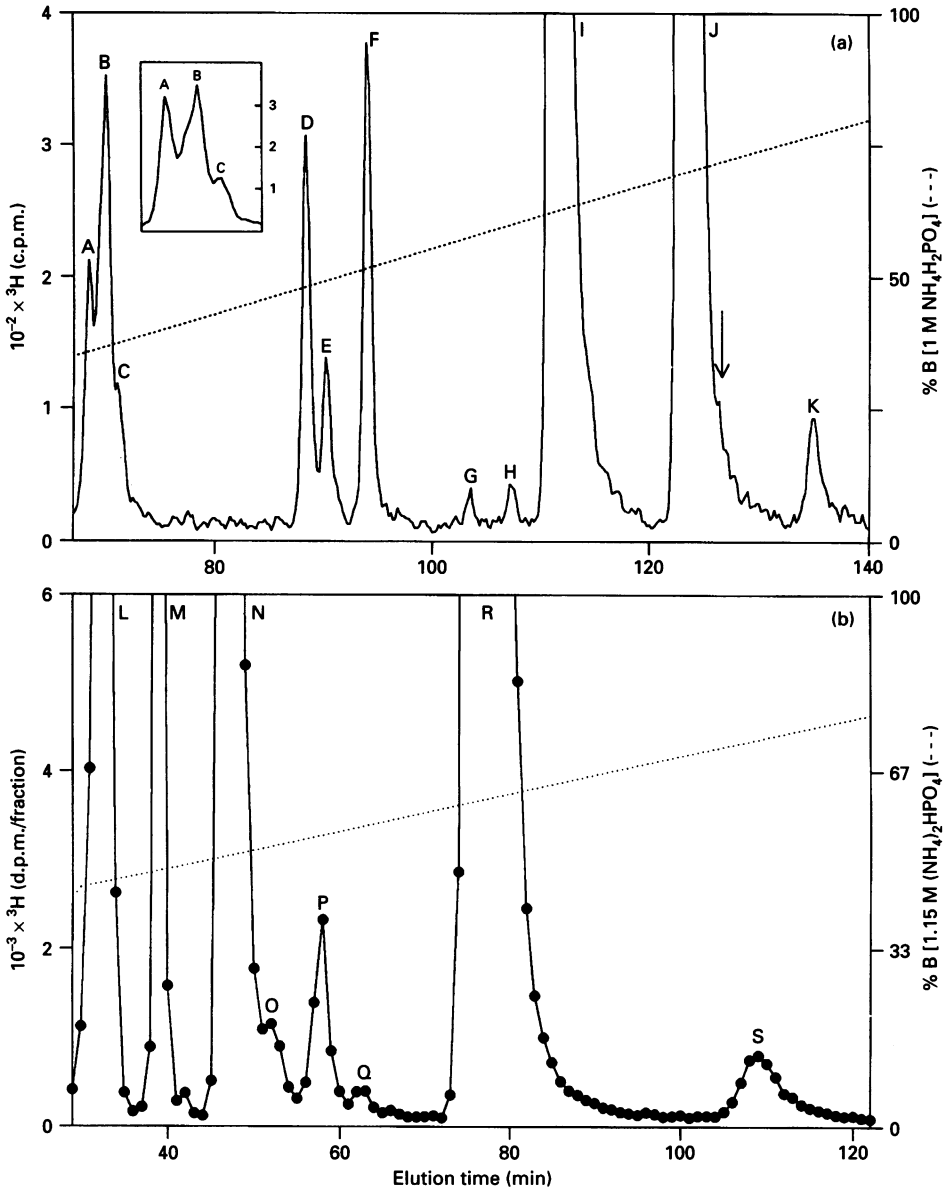

Figure 1 H.p.l.c. of extracts of [^{3}H]inositol-labelled hepatocytes

Hepatocytes were labelled for 62 h, extracted with perchloric acid and neutralized as described in the Materials and methods section. Replicate extracts were then chromatographed on either an Adsorbosphere SAX column, with radioactivity assessed on-line (**a**), or a Partisphere SAX column, with radioactivity assessed in 1 min fractions (**b**). In (**a**), isomers were identified as follows (the integrated c.p.m. for each isomer follows it in parentheses). Ins(1,3,4)P_3 (peak A; 911), Ins(1,4,5)P_3 (B; 2711), Ins(1,3,4,5)P_4 (E; 737), Ins(1,3,4,5,6)P_5 + D/L-Ins(1,2,4,5,6)P_5 (I; 46588), $InsP_6$ (J; 33711) and *PP*-InsP_5 (K; 742) were identified from their co-elution with genuine standards, the sources of which are described in the Materials and methods section. Ins(1,3,4,6)P_4 (D; 1481), D/L-Ins(3,4,5,6)P_4 (F; 1829), Ins(1,2,3,4,6)P_5 (G; 137) and D/L-Ins(1,2,3,4,5)P_5 (H; 198) were each identified from their known elution positions; for further details of the elution of isomers of $InsP_4$ on this column, see Balla et al. (1989) and Menniti et al. (1990). The elution characteristics of $InsP_5$ isomers on an Adsorbosphere SAX h.p.l.c. column were previously determined by Nogimori et al. (1991), who chromatographed a mixture of $InsP_5$s, collected the individual peaks, and rechromatographed each of them on a Dionex AS7 column (Phillippy and Bland, 1988; McConnell et al., 1991), which in turn was calibrated with genuine [^{3}H]$InsP_5$ standards (Stephens et al., 1991). An arrow marks the elution position of *PP*-InsP_4 on the Adsorbosphere column, which was determined in a sequentially performed h.p.l.c. run. The inset to (**a**) shows data from a different sample, with slightly improved resolution of Ins(1,3,4)P_3 (A; 1347), Ins(1,4,5)P_3 (B; 1914) and an unknown peak (C; 709). In (**b**) (the Partisphere SAX column), isomers were identified as follows (the d.p.m. for each isomer follows it in parentheses). *PP*-InsP_4 (Q; 527), $InsP_6$ (R; 212703) and *PP*-InsP_5 (S; 2593) were each identified from their co-elution with genuine standards (see the Materials and methods section for sources). The elution of Ins(1,3,4,5,6)P_5 (peak N) was also determined with a standard, and this peak (total = 265551 d.p.m.) includes a contribution from D/L-Ins(1,2,3,4,5)P_5 (peak O) (see Stephens, 1990). The elution of D/L-Ins(1,2,4,5,6)P_5, again confirmed with a standard, includes Ins(1,2,3,4,6)P_5 (P; total d.p.m. = 4949) (Stephens, 1990). On this column, $InsP_3$ (L; 32425) and $InsP_4$ (M; 25892) each eluted as a single unresolved peak.

by hepatocytes is very similar, if not identical. We propose that this compound contains a single diphosphate group since, during its dephosphorylation by AR4-2J homogenates, intact AR4-2J cells and a purified diphosphatase, $InsP_6$ was the only intermediate detected (Menniti et al., 1993a). A separate, later-eluting and uncharacterized compound in AR4-2J cells ('IP_6X'; Menniti et al., 1993a) might contain more than one diphosphate group. These proposals may draw some support from the work of Stephens et al. (1993). These workers have conducted a rigorous structural analysis of two diphosphate derivatives of $InsP_6$ which

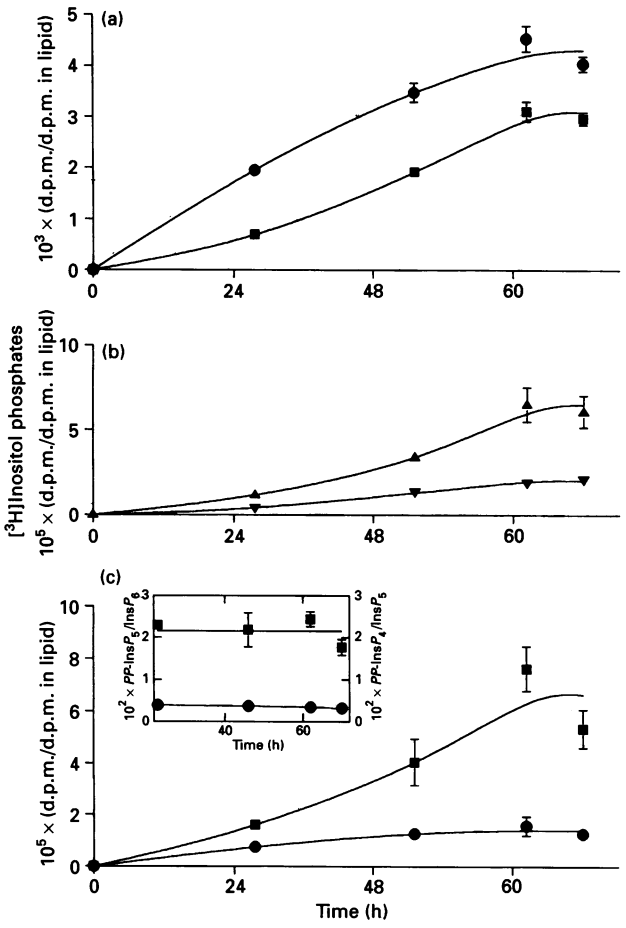

Figure 2 Time course of incorporation of [^{3}H]inositol into selected inositol polyphosphates and diphosphoinositol polyphosphates

Hepatocytes were labelled with 300 μCi/ml [^{3}H]inositol as described in the Materials and methods section. At the indicated times, cells were quenched, neutralized and chromatographed on a Partisphere SAX column as described in the Materials and methods section. Panel (**a**) shows the accumulation of [^{3}H]inositol in peaks N+O [> 98% Ins(1,3,4,5,6)P_5; see Figure 1] (●), and the [^{3}H]inositol in InsP_6 (■). Panel (**b**) shows the accumulation of [^{3}H]inositol in the combined peak P [D/L-Ins(1,2,4,5,6)P_5 + Ins(1,2,3,4,6)P_5; see Figure 1] (▲). Panel (**c**) shows the accumulation of [^{3}H]inositol in *PP*-InsP_4 (●) and [^{3}H]*PP*-InsP_5 (■). The inset to panel (**c**) describes the values of the ratios [^{3}H]*PP*-InsP_5/[^{3}H]InsP_6 (■) and [^{3}H]*PP*-InsP_4/[^{3}H]InsP_5 (●, the denominator being peaks N+O). Data are representative of four experiments. Additional experiments with a different cell preparation were analysed on an Adsorbosphere column to obtain the incorporation of [^{3}H]inositol into D/L-Ins(1,2,3,4,5)P_5 (▼ in **b**). Data are normalized to radioactivity in the inositol lipids, which attained a steady state within 24 h. Where error bars are not shown, these are smaller than the symbol.

were isolated from *Dictyostelium discoideum*. They convincingly demonstrated that the most likely structure of one of their compounds was D- and/or L-1-*PP*-InsP_5, while the other was probably D- and/or L-bis-(1,4)-*PP*-InsP_4. Stephens et al. (1993) also showed that during their h.p.l.c. procedure, which we have copied here (Figure 1b), the first of their compounds to elute after InsP_6 was *PP*-InsP_5, followed by bis-*PP*-InsP_4. It is therefore possible that the material in peaks K and S (Figure 1) is also a *PP*-InsP_5, and conceivably it is even D- and/or L-1-*PP*-InsP_5. As we have no independent information concerning the position of the diphosphate group in our compound, we shall use *PP*-InsP_5 as generic and somewhat putative terminology to describe peaks K and S. Note that in our earlier publication we referred to this compound as 'IP_6P' (Menniti et al., 1993a). The later-eluting 'IP_6X' (Menniti et al., 1993a), which we have not observed in hepatocytes (Figure 1), could be similar to bis-*PP*-InsP_4, but further work is necessary to investigate this possibility.

The level of [^{3}H]*PP*-InsP_5 in hepatocytes was similar to that of [^{3}H]Ins(1,3,4,5)P_4, but was only 3 ± 0.4 % of the level of [^{3}H]InsP_6 (n = 9). A similar ratio of [^{3}H]*PP*-InsP_5 to [^{3}H]InsP_6 has been observed in AR4-2J cells, although less routinely due to the lower levels of radioactivity previously employed (Oliver et al., 1992; Menniti et al., 1993a).

On the Partisphere SAX h.p.l.c. system, eluting between D/L-Ins(1,2,4,5,6)P_5 (peak P) and InsP_6 (peak R), we observed very small amounts of a compound that co-eluted with a standard of a diphosphate derivative of InsP_5 prepared from AR4-2J cells (peak Q in Figure 1). We have assumed that this compound is a *PP*-InsP_4, in part because the presence of more than six phosphates would probably result in it eluting after InsP_6 on this h.p.l.c. system (see Stephens et al., 1993). Furthermore, our putative *PP*-InsP_4 was metabolized by intact AR4-2J cells, AR4-2J homogenates and a purified diphosphatase directly to Ins(1,3,4,5,6)P_5 without other intermediates accumulating (Menniti et al., 1993a). Note that we previously described *PP*-InsP_4 as 'IP_5P' (Menniti et al., 1993a). Stephens et al. (1993) did not positively identify a *PP*-InsP_4 in *Dictyostelium*, although an uncharacterized peak with similar elution characteristics is apparent in their work (compare our Figure 1b with their Figure 1).

Levels of [^{3}H]*PP*-InsP_4 in hepatocytes ranged from 0.2 to 0.3 % of total [^{3}H]InsP_5 in four experiments (including that described by Figure 1). *PP*-InsP_4 was not observed on the Adsorbosphere SAX column, because of its small size and its elution close to the much larger peak of InsP_6 (Figure 1). Our use of standards to identify the diphosphoinositol polyphosphates was supplemented by demonstrating specific increases in the levels of these particular compounds in response to fluoride (see below).

Rate of incorporation of [^{3}H]inositol into InsP_5, InsP_6, *PP*-InsP_4 and *PP*-InsP_5

In these experiments, the radioactivity in the inositol lipids attained an apparent steady-state within 24 h (results not shown). In contrast, radioactivity accumulated much more slowly into a number of highly polar inositol polyphosphates (Figure 2). Despite some differences in the individual rates of labelling, there were near-constant values for the ratios of both *PP*-InsP_4/InsP_5 and *PP*-InsP_5/InsP_6 (inset to Figure 2c). These data imply that there is a close metabolic relationship between InsP_5 and *PP*-InsP_4, and between InsP_6 and *PP*-InsP_5. This is consistent with earlier evidence, obtained from AR4-2J cells, indicating that Ins(1,3,4,5,6)P_5 and InsP_6 are respectively the precursors of *PP*-InsP_4 and *PP*-InsP_5 (Menniti et al., 1993a).

Between 62 and 70 h of labelling with [^{3}H]inositol (Figure 2), there was no significant change in the levels of radioactivity in the following compounds: InsP_6, *PP*-InsP_5, *PP*-InsP_4, Ins(1,3,4,5,6)P_5, D/L-Ins(1,2,3,4,5)P_5 and the peak (denoted P in Figure 1) that contained a mixture of D/L-Ins(1,2,4,5,6)P_5 plus Ins(1,2,3,4,6)P_5. When we determined the effects of extracellular agents upon these polyphosphates (see below), we routinely labelled hepatocytes for 62 h. Within the time-frame of these experiments (e.g. 30 min in Figure 3), we can consider the polyphosphates under study to be labelled to near steady-state. Longer culture times than 70 h were possible, but then the media had to be replaced to maintain cell viability. The associated high cost of adding fresh [^{3}H]inositol precluded such an approach on a routine basis. It is possible that the relative amounts of ^{3}H in

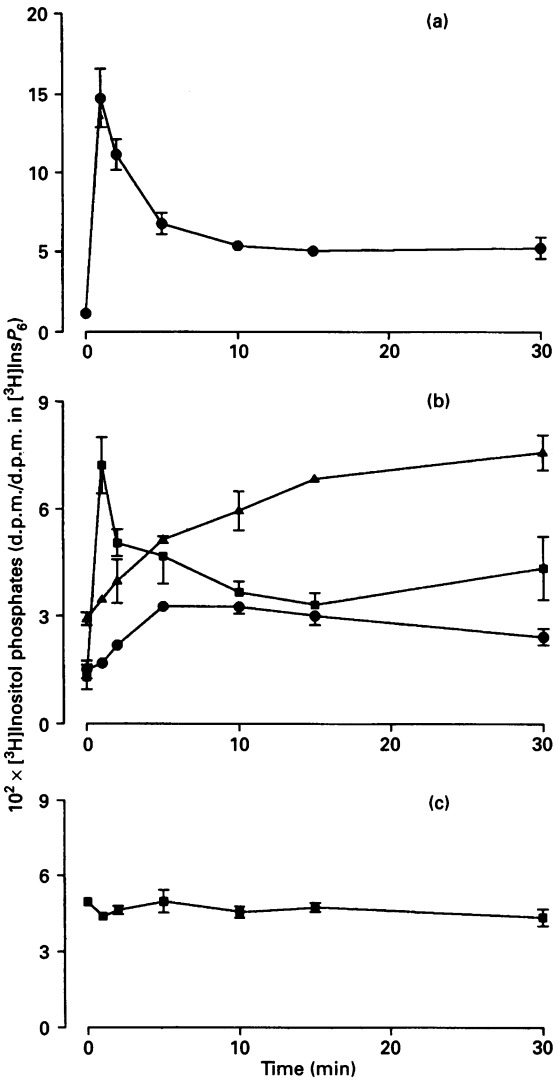

Figure 3 Effect of vasopressin upon inositol phosphates and *PP*-InsP_5 in hepatocytes

Hepatocytes were prelabelled for 62 h with [^{3}H]inositol, then incubated for 2 h in 2 ml of Williams E medium plus 20 mM Hepes, pH 7.4, 0.2% BSA and 2 mM L-glutamine (see the Materials and methods section). Then 200 nM vasopressin was added for various times to triplicate samples. Cells were quenched with perchloric acid, neutralized and chromatographed on an Adsorbosphere SAX column (see the Materials and methods section). (**a**) ●, [^{3}H]Ins(1,3,4)P_3. (**b**) ▲, [^{3}H]Ins(1,3,4,6)P_4; ■, Ins(1,3,4,5)P_4; ●, D/L-[^{3}H]Ins(3,4,5,6)P_4. (**c**) ■, [^{3}H]*PP*-InsP_5. There was no effect of the agonist upon levels of [^{3}H]InsP_6; therefore all data are presented as a ratio to [^{3}H]InsP_6. Error bars are smaller than the symbols where they are not shown. Two additional experiments gave similar results.

each polyphosphate after 62–70 h only approximately reflect their relative mass levels, due to equilibrium labelling not being fully attained.

Analysis of the relationship between phospholipase C activity and the turnover of InsP_5, InsP_6, *PP*-InsP_4 and *PP*-InsP_5

In these experiments, hepatocytes were maintained for 62 h in primary culture under the conditions that Rooney et al. (1991) have developed in order to limit the loss and desensitization of receptors that can otherwise greatly attenuate agonist-activated phospholipase C activity (Bouscarel et al., 1990). Figure 3 shows a typical time course of the effect of a maximally effective dose of vasopressin upon levels of selected inositol phosphates; these data were obtained by h.p.l.c. on an Adsorbosphere column. Since levels of [^{3}H]InsP_6 were unaffected by vasopressin (results not shown), values for individual isomers were calculated as ratios relative to [^{3}H]InsP_6, thereby correcting for any small differences in recovery of material between samples. The peak that co-eluted with standard Ins(1,4,5)P_3 was elevated 2–3-fold within 1–2 min of stimulation with vasopressin (results not shown). However, this was not considered to be an accurate assay of phospholipase C activity, due to contamination by both Ins(1,3,4)P_3 and a further, unidentified compound (Figure 1). On the other hand, Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,3,4)P_3 rose 4–6-fold and 9–12-fold respectively between 1 and 2 min of cell stimulation (Figure 3). These effects of vasopressin are close to the usual peak response of freshly isolated hepatocytes to this particular agonist [9-fold for Ins(1,3,4,5)P_4 and 15-fold for Ins(1,3,4)P_3, both achieved within 1–2 min of stimulation; see Hansen et al., 1988]. In primary cultured hepatocytes (Figure 3), levels of [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4 declined at later times of stimulation, but still remained 2–4-fold above basal for the duration of the experiment; levels of [^{3}H]Ins(1,3,4,6)P_4 rose more slowly, and less dramatically (2-fold above basal). These latter effects are again similar to the response seen in freshly isolated hepatocytes (Hansen et al., 1988).

The data in Figures 1 and 3 demonstrate that the primary cultured hepatocytes have been maintained for a sufficient time to adequately label InsP_5 and InsP_6, while also retaining the ability of vasopressin to activate phospholipase C in a manner that quantitatively and qualitatively mimics that seen in freshly isolated cells. Thus we were able to utilize the hepatocyte to investigate the relationship between phospholipase C activity and the turnover of some of the highly polar inositol phosphates. For example, we have now shown (Figure 3) that hepatocytes are similar to a number of cell types (Stephens et al., 1988; Balla et al., 1989; Menniti et al., 1990; Guse and Emmrich 1991; Barker et al., 1992) in that receptor-dependent activation of phospholipase C is accompanied by a parallel increase in levels of D/L-[^{3}H]Ins(3,4,5,6)P_4. Our data do not provide any new information on the source of D/L-Ins(3,4,5,6)P_4, which we have previously proposed is Ins(1,3,4,5,6)P_5 (Menniti et al., 1990, 1993b; Oliver et al., 1992). Although vasopressin doubled the size of the D/L-[^{3}H]Ins(3,4,5,6)P_4 peak in hepatocytes, this could have been supported by only a 4% increase in the turnover of the substantially larger peak of [^{3}H]Ins(1,3,4,5,6)P_5; such a small change could not be detected in our experiments.

Guse and Emmrich (1991) have shown that, as in hepatocytes (Figures 1 and 2), the Jurkat lymphocyte cell line contains low levels of Ins(1,2,3,4,6)P_5 in relation to total InsP_5. In the Jurkat cells, levels of this Ins(1,2,3,4,6)P_5 increased 4-fold in response to activation of phospholipase C. This was accompanied by a persistent decrease of up to 35% in levels of D/L-Ins(1,2,4,5,6)P_5. The latter response was attributed to a Ca^{2+}-dependent activation of a D/L-Ins(1,2,4,5,6)P_5 kinase (Guse and Emmrich, 1991). In hepatocytes, treatment of up to 30 min with vasopressin (or 40 min of treatment with thapsigargin, see below) did not alter the sizes of the Ins(1,2,3,4,6)P_5 peak (denoted G in Figure 1), the D/L-Ins(1,2,3,4,5)P_5 peak (denoted H in Figure 1) or the peak containing both Ins(1,2,3,4,6)P_5 and D/L-Ins(1,2,4,5,6)P_5 (denoted P in Figure 1) (results not shown). It is possible that receptor-dependent changes to InsP_5 metabolism in Jurkat cells reflect some specific response of immunological significance; it is also noteworthy that, in Jurkat cells, steady-state levels of D/L-

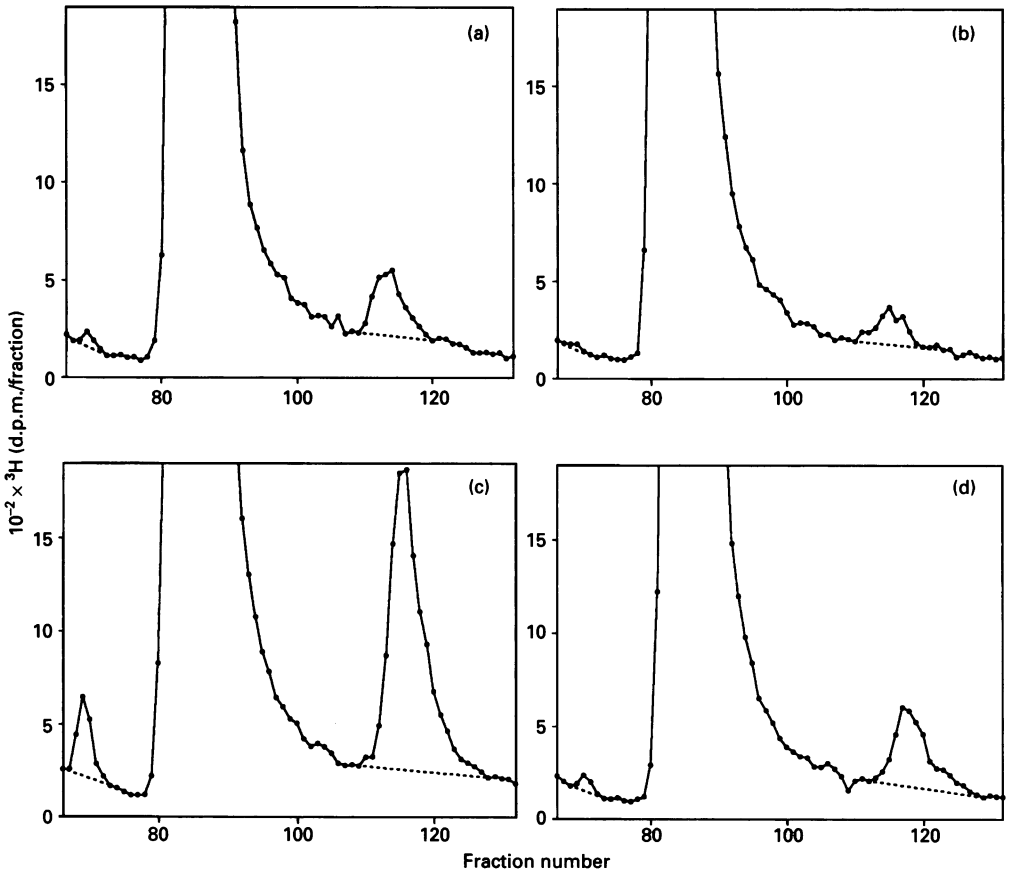

Figure 4 Effect of thapsigargin and fluoride upon levels of [^{3}H]*PP*-InsP_4 and [^{3}H]*PP*-InsP_5 in hepatocytes

Hepatocytes were prelabelled with [^{3}H]inositol for 62 h, then incubated for 2 h in 2 ml of Williams E medium plus 20 mM Hepes, pH 7.4, 0.2% BSA and 2 mM L-glutamine (see the Materials and methods section). The following additions were then made: (**a**) 5 μl of DMSO plus 30 μl of water; (**b**) 5 μl of thapsigargin (in DMSO) plus 30 μl of water; (**c**) 5 μl of DMSO plus 30 μl of NaF; (**d**) 5 μl of thapsigargin (in DMSO) plus 30 μl of NaF. The final concentrations of thapsigargin and NaF were, respectively 2 μM and 1 mM. After 40 min, incubations were quenched with perchloric acid, neutralized and chromatographed on a Partisphere SAX column. The three peaks shown in each panel are, from left to right, *PP*-InsP_4, InsP_6 and *PP*-InsP_5. The total d.p.m. in various peaks were as follows (including total amounts of [^{3}H]InsP_4 and [^{3}H]InsP_5, which are not shown in the figure). (**a**) (control): total [^{3}H]InsP_4, 18955; total [^{3}H]InsP_5, 171905; [^{3}H]*PP*-InsP_4, 265; [^{3}H]InsP_6, 153284; [^{3}H]*PP*-InsP_5, 2406. (**b**) (thapsigargin): total [^{3}H]InsP_4, 21695; total [^{3}H]InsP_5, 173378; [^{3}H]*PP*-InsP_4, 32; [^{3}H]InsP_6, 152211; [^{3}H]*PP*-InsP_5, 1283. (**c**) (NaF): total [^{3}H]InsP_4, 21668; total [^{3}H]InsP_5, 197270; [^{3}H]*PP*-InsP_4, 1488; [^{3}H]InsP_6, 139605; [^{3}H]*PP*-InsP_5, 10578. (**d**) (thapsigargin + NaF): total [^{3}H]InsP_4, 23430; total [^{3}H]InsP_5, 194123; [^{3}H]*PP*-InsP_4, 267; [^{3}H]InsP_6, 171734; [^{3}H]*PP*-InsP_5, 3516. A background of 100 d.p.m./fraction was subtracted, except for *PP*-InsP_4 and *PP*-InsP_5, where background is indicated by the baseline to each peak (---). Similar results were obtained in two additional experiments (also see the text).

Ins(1,2,4,5,6)P_5 were similar to those of Ins(1,3,4,5,6)P_5 (Guse and Emmrich, 1991).

Although vasopressin activated phospholipase C for at least 30 min, steady-state levels of [^{3}H]*PP*-InsP_5 were not affected (Figure 3). In nine experiments (including that in Figure 3) the value of the ratio of [^{3}H]*PP*-InsP_5/[^{3}H]InsP_6 in control cells was 0.031 ± 0.004. The value of this ratio after 2 and 30 min treatment with 200 nM vasopressin was, respectively, 0.035 ± 0.005 ($n = 6$, $P > 0.05$) and 0.037 ± 0.008 ($n = 6$, $P > 0.05$). *PP*-InsP_4 was not resolved from InsP_6 in these experiments, which used an Adsorbosphere h.p.l.c. column. Thus levels of [^{3}H]*PP*-InsP_4, also calculated as a ratio to InsP_6, were estimated using a Partisphere column; these were also found not to be affected by 30 min of treatment with vasopressin (control, 0.004 ± 0.001; +vasopressin, 0.006 ± 0.001; $n = 3$; $P > 0.05$).

Levels of *PP*-InsP_4 and *PP*-InsP_5 are elevated by fluoride and reduced by thapsigargin

Although vasopressin did not alter levels of either *PP*-InsP_4 or *PP*-InsP_5, we considered that the sensitivity of the search for potential control processes might be improved by a more pronounced and longer-lasting stimulus. We therefore stimulated protein kinase C by 40 min of treatment with 200 nM phorbol 12,13-dibutyrate, and elevated cytosolic [Ca^{2+}] with 40 min of treatment with 2 μM thapsigargin.

The phorbol ester treatment was without effect upon the levels of [^{3}H]*PP*-InsP_4, [^{3}H]*PP*-InsP_5 or any of the inositol phosphates described by Figure 1 (results not shown). On the other hand, thapsigargin specifically reduced the levels of both [^{3}H]*PP*-InsP_4 and [^{3}H]*PP*-InsP_5: in three separate experiments (including that described by Figure 4), thapsigargin reduced the size of the [^{3}H]*PP*-InsP_4 peak by 68 ± 14%, and the size of the [^{3}H]*PP*-InsP_5 peak decreased by 49 ± 7%. In contrast, thapsigargin did not affect the levels of either total [^{3}H]InsP_4 (see the legend to Figure 4) or individual InsP_4 and InsP_3 isomers (results now shown), in agreement with earlier reports (Jackson et al., 1988).

The decrease in levels of diphosphoinositol polyphosphates caused by thapsigargin could have resulted from an inhibition of their rate of synthesis, and/or a stimulation of their rate of breakdown. We sought to distinguish between these alternatives

by using fluoride. This anion inhibits the removal of the terminal phosphate from the diphosphate groups of *PP*-InsP_4 and *PP*-InsP_5, thereby acting as a metabolic trap that exposes their ongoing rate of synthesis (Menniti et al., 1993a). When 1 mM fluoride was added to hepatocytes for 40 min, levels of [^{3}H]*PP*-InsP_5 and [^{3}H]*PP*-InsP_4 increased 10-fold (Figure 4). In other words, flux through these compounds is normally such that these metabolic pools are turning over completely at least 10 times every 40 min; if anything, this remarkable rate of turnover is probably underestimated due to the unknown rate of fluoride uptake by hepatocytes. The absolute elevation in [^{3}H]*PP*-InsP_5 levels was of sufficient magnitude that it could be seen to be accompanied by a decrease in [^{3}H]InsP_6 levels: in five experiments (including that in Figure 4), the increase in [^{3}H]*PP*-InsP_5 accounted for 85 ± 15% of the reduction in [^{3}H]InsP_6. The absolute fluoride-dependent decrease in [^{3}H]InsP_6 was 11 ± 2.5% ($n = 5$). It therefore appears that, in hepatocytes, at least 10% of the [^{3}H]InsP_6 pool is being cycled through [^{3}H]*PP*-insP_5 every 40 min. The extent of this metabolism is not apparent from time courses of labelling with [^{3}H]inositol (e.g. Figure 2). One earlier study of the turnover of diphosphoinositol polyphosphates in intact cells (the AR4-2J pancreatoma) also indicated a quite rapid ongoing flux through these novel metabolites (Menniti et al., 1993a). Our experiments with hepatocytes now indicate that this phenomenon is not restricted to transformed cells. Note also that the fluoride-mediated increase in [^{3}H]*PP*-InsP_4 was not accompanied by a measurable decrease in levels of any [^{3}H]InsP_5 isomers (results not shown). This is not unexpected if the large pool of Ins(1,3,4,5,6)P_5 is the major precursor for the much smaller pool of *PP*-InsP_4. Fluoride at 1 mM had no effect on phospholipase C in hepatocytes (indicated by the absence of an effect on total [^{3}H]InsP_4, see the legend to Figure 4).

In three separate preparations of hepatocytes incubated with fluoride (including that described by Figure 4), thapsigargin inhibited the accumulation of [^{3}H]*PP*-InsP_4 and [^{3}H]*PP*-InsP_5 by 64 ± 20% and 55 ± 16% respectively. The degree of this effect of thapsigargin, when dephosphorylation of the diphosphoinositol polyphosphates was inhibited by fluoride, was similar to the effect of thapsigargin observed in the absence of fluoride. These data indicate that thapsigargin primarily inhibited formation of the diphosphoinositol polyphosphates, rather than their breakdown.

General conclusions

There is an enormous body of literature describing the turnover of Ins(1,4,5)P_3 and its closely related metabolites (Berridge and Irvine, 1989; Shears, 1992). A large majority of these studies have been restricted to the analysis of inositol derivatives with four phosphates or less. While a number of reports describe the presence in cells of InsP_6 and InsP_5 (often assumed to be solely the 1,3,4,5,6-isomer), there has not been any general focus upon these particular compounds, and most of the relevant knowledge we do possess has been obtained from studies with either cell-free systems or transformed cell lines (see the Introduction). It has been the intention of this study to specifically pursue these relatively neglected areas of inositol phosphate metabolism, using a non-transformed cell, the primary cultured hepatocyte. We have now shown that this cell type contains a complex array of highly polar inositol derivatives. For example, although the bulk of the InsP_5 complement in cells is composed of Ins(1,3,4,5,6)P_5, we have detected at least three additional InsP_5 isomers. The levels of these additional InsP_5 isomers, in relation to InsP_6, are consistent with their being formed by the low-capacity, non-specific InsP_6 phosphatase that has been purified from rat liver (Nogimori et al., 1991). In order to study the physiological significance of these multiple InsP_5 isomers, it might be best to concentrate future research efforts on specific cell types in which their levels respond to receptor occupation (Guse and Emmrich, 1991).

We have also found that hepatocytes contain two diphosphoinositol polyphosphates that display a rapid ongoing metabolic turnover. Steady-state levels of these compounds were unaffected by activation of phospholipase C, but were reduced by prolonged elevation of cytosolic [Ca^{2+}] (although it is possible that this effect is also due to the concomitant drop in intraluminal Ca^{2+}). Vasopressin may not imitate the effect of thapsigargin upon the turnover of *PP*-InsP_4 and *PP*-InsP_5 because the agonist elevates intracellular [Ca^{2+}] in a less sustained manner (e.g. see Rooney et al., 1991). Sustained high levels of cytosolic Ca^{2+}, such as those induced by xenobiotics for example, are ultimately toxic to the cell; hepatocytes have protective processes that act to both tolerate and reverse these potentially lethal changes (Nagelkerke et al., 1989). It is conceivable that, in response to these more dramatic increases in cytosolic [Ca^{2+}], alterations in metabolic fluxes through diphosphoinositol polyphosphates represent an event of functional significance.

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