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Inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate formation in Ca^{2+}-mobilizing-hormone-activated cells

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The inositol trisphosphate liberated on stimulation of guinea-pig hepatocytes, pancreatic acinar cells and dimethyl sulphoxide-differentiated human myelomonocytic HL-60 leukaemia cells is composed of two isomers, the 1,4,5-trisphosphate and the 1,3,4-trisphosphate. Inositol 1,4,5-trisphosphate was released rapidly, with no measurable latency on hormone stimulation, and, consistent with its proposed role as an intracellular messenger for Ca^{2+} mobilization, there was good temporal correlation between its formation and Ca^{2+}-mediated events in these tissues. There was a definite latency before an increase in the formation of inositol 1,3,4-trisphosphate could be detected. In all of these tissues, however, it formed a substantial proportion of the total inositol trisphosphate by 1 min of stimulation. In guinea-pig hepatocytes, where inositol trisphosphate increases for at least 30 min after hormone application, inositol 1,3,4-trisphosphate made up about 90% of the total inositol trisphosphate by 5–10 min. In pancreatic acinar cells, pretreatment with 20 mM-Li^+ caused an increase in hormone-induced inositol trisphosphate accumulation. This increase was accounted for by a rise in inositol 1,3,4-trisphosphate; inositol 1,4,5-trisphosphate was unaffected. This finding is consistent with the observation that Li^+ has no effect on Ca^{2+}-mediated responses in these cells. The role, if any, of inositol 1,3,4-trisphosphate in cellular function is unknown.

INTRODUCTION

When many tissues are stimulated with Ca^{2+}-mobilizing hormones, one of the earliest effects is the phosphodiesteratic breakdown of the polyphosphoinositide $PI(4,5)P_2$ to form diacylglycerol and the water-soluble inositol phosphate $(1,4,5)IP_3$ (Kirk *et al.*, 1981; Berridge *et al.*, 1983; Creba *et al.*, 1983; Thomas *et al.*, 1984; Aub & Putney, 1984; Dougherty *et al.*, 1984; Rubin, 1984). For many of these same tissues $(1,4,5)IP_3$, which is the expected breakdown product of $PI(4,5)P_2$, has been shown to release Ca^{2+} from an intracellular store believed to be a component of the endoplasmic reticulum (Streb *et al.*, 1983; Berridge *et al.*, 1984; Burgess *et al.*, 1984*a*, *b*, *c*; Dawson & Irvine, 1984; Joseph *et al.*, 1984; Prentki *et al.*, 1984; Suematsu *et al.*, 1984; for a review see Berridge & Irvine, 1984). Because of these findings, it has been proposed that $(1,4,5)IP_3$ may be the messenger which signals Ca^{2+} release from the hormone-sensitive pool after Ca^{2+}-mobilizing-hormone-receptor activation.

Recently Irvine *et al.* (1984, 1985) demonstrated that a large proportion of the IP_3 that is formed on stimulation of the parotid gland with the Ca^{2+}-mobilizing agonist carbachol is not the 1,4,5 isomer but is in the form D- or L-*myo*-$(1,3,4)IP_3$. As yet neither the route of synthesis nor the role of this molecule, if any, in cell function is known. It was important, therefore, to determine whether $(1,3,4)IP_3$ was also produced in other cell types.

In this study we have examined the isomeric forms of IP_3 in two tissues for which we have previously demonstrated the Ca^{2+}-releasing activity of $(1,4,5)IP_3$, specifically the guinea-pig hepatocyte (Burgess *et al.*, 1984*a*, *b*) and the DMSO-differentiated HL-60 cell (Burgess *et al.*, 1984*c*). In addition, we have examined the effects of Li^+ on the accumulation of isomers of IP_3 in pancreatic acinar cells. This latter preparation was chosen because Li^+ pre-treatment has previously been shown to increase substantially the stimulated content of total IP_3 in this tissue (Rubin, 1984).

MATERIALS AND METHODS

Preparation of isolated guinea-pig hepatocytes

Isolated hepatocytes were prepared by collagenase digestion of the livers of male Hartley guinea pigs (Burgess *et al.*, 1981). The cells were then incubated in Eagle's medium containing 2% (w/v) bovine serum albumin. The pH was maintained at 7.2 by equilibration with CO_2/O_2 (1:19). Cell viability was assessed by Trypan Blue exclusion and was 85–95%.

Preparation of DMSO-differentiated HL-60 cells

HL-60 myelomonocytic leukaemia cells were cultured in roller bottles as described previously by Dougherty *et al.* (1984). The cells were differentiated into granulocytes by culture for 7 days in the presence of DMSO (1.3%, v/v) and dexamethasone (1 μM). After this treatment they respond to fMet-Leu-Phe and will perform many of the functions of human neutrophils (Dougherty *et al.*, 1984).

Preparation of isolated rat pancreatic acinar cells

Pancreatic acinar cells were isolated from male rats as described by Putney *et al.* (1980), and were more than

Abbreviations used: PI, phosphatidylinositol; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; $(1,4,5)IP_3$, inositol 1,4,5-trisphosphate; $(1,3,4)IP_3$, inositol 1,3,4-trisphosphate; PIP_2, phosphatidylinositol bisphosphate; IP, inositol monophosphate; IP_2, inositol bisphosphate; DMSO, dimethyl sulphoxide; fMet-Leu-Phe, formylmethionyl-leucylphenylalanine.

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90% viable as assessed by Trypan Blue. The acinar cells were incubated in a Tris-buffered Krebs–Ringer medium and gassed with O_2.

Generation of [^{3}H]inositol trisphosphate samples for isomer determination

Hepatocytes and pancreatic acinar cells (3–5 mg of cell protein/ml) were incubated in their respective media containing 10 μCi of [^{3}H]inositol/ml for 90 min at 37 °C. At the end of this period the labelled medium was removed by centrifugation (50 *g* for 2 min), and the cells were then placed in fresh non-radioactive medium and incubated for 20 min.

On day 6 of differentiation, the HL-60 cells were concentrated from 10^6/ml to 2×10^6/ml and transferred from RPMI 60 to Eagle's medium (which has a lower inositol concentration). They were then placed in flat-bottomed culture flasks and loaded with 10 μCi of [^{3}H]inositol/ml for approx. 18 h. The cells were then washed by centrifugation (50 *g* for 2 min) and placed in non-radioactive Eagle's medium, which was maintained at pH 7.2 with CO_2/O_2 (1:19), at a final concentration of 10^7 cells/ml. As with the other cell types, the HL-60 cells were then preincubated for 20 min before the start of the experiment. If the effect of Li^+ was to be tested, the cells were preincubated with either LiCl (20 mM) or NaCl (20 mM) for 20 min before the addition of agonist.

At the end of the preincubation period, a Ca^{2+}-mobilizing agonist was added to the cell suspension; 1 ml samples were taken at various time intervals, rapidly added to 2 ml of ice-cold 4.5% (w/v) $HClO_4$ and centrifuged (1000 *g* for 5 min). The pH of the supernatant was adjusted to 8–9 with a buffer containing 9 mM-sodium tetraborate and 0.5 M-KOH. The samples were then centrifuged (1000 *g* for 5 min) to pellet the $KClO_4$ precipitate, and the supernatants were processed for analysis of the radioactive inositol phosphates as described by Berridge *et al.* (1983), except that the IP and IP_2 were generally eluted from the Dowex formate columns together, and the columns were washed with 16 ml of water before the IP_3 was eluted.

The IP_3 fractions from the Dowex columns were acidified by treatment with Dowex 50 (H^+ form), frozen, and then freeze-dried to remove the formic acid. The samples were then stored at −70 °C until further analysis.

Separation of IP_3 isomers by h.p.l.c.

[^{32}P](1,4,5)IP_3 prepared from erythrocyte ghosts as described by Downes *et al.* (1982) and [^{3}H]IP_3 from the experimental samples were mixed together in a small volume of water (< 1 ml), to which was added ATP (30 μg/ml), which co-chromatographed with the 1,3,4 isomer (Irvine *et al.*, 1985). This mixture was filtered and the isomers of inositol trisphosphate were separated by a slight modification of the method of Irvine *et al.* (1985). The sample was eluted at 1.2 ml/min by a non-linear gradient (convex No. 4 on a Waters gradient former) increasing from water to 100% 1.0 M-ammonium formate (adjusted to pH 3.7 with phosphoric acid) in 25 min from a Whatman Partisil 10 SAX anion-exchange column ($H_2PO_4^-$ form). The ammonium formate buffer (100%) was run for an additional 5 min and then the eluent was returned linearly to water over 10 min. Water was run through the column for a further 10 min before the next sample was injected. U.v. absorbance was monitored at 259 mm in order to detect ATP, and 1 min fractions were collected for about the first 18 min. From 18 to 25 min, 0.3 min fractions were collected, and 1 min fractions were collected from 25 to 30 min. The volumes of the smaller fractions were adjusted to 1.2 ml with water, and after addition of 20 ml of Beckman Ready-Solve E.P. scintillant the radioactivity in both ^{32}P and ^{3}H was determined in a liquid-scintillation counter (samples were counted twice for 10 min). Approx. 90% of the radioactivity loaded on to the columns was recovered, and no peaks were detected in random blank runs done between sample runs. For each experiment total lipid radioactivity was determined in two chloroform/methanol extracts, and, as it has been found that the radioactivity in these samples does not change during the course of these experiments (Burgess *et al.*, 1984*a*),

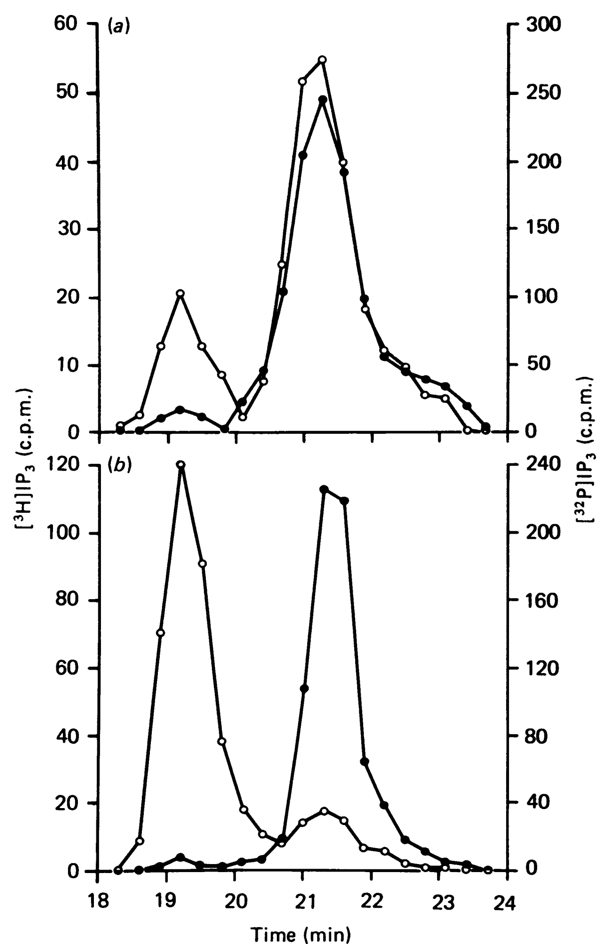

Fig. 1. Separation of inositol trisphosphate isomers by h.p.l.c.

Guinea-pig hepatocytes labelled with [^{3}H]inositol were stimulated with angiotensin (1 μM). The [^{3}H]IP_3 fractions purified from Dowex (formate form) columns were mixed together with [^{32}P]IP_3 from erythrocyte ghosts as a standard, and with ATP as a marker. This mixture was injected on to the h.p.l.c. anion-exchange column and eluted as described in the Materials and methods section. Panels (*a*) and (*b*) illustrate experiments where the hepatocytes were stimulated with angiotensin for 5 s or 60 s respectively. Fig. 1 shows one of four experiments which gave similar results and which were used to complete the time course in Fig. 3. ○, ^{3}H; ●, ^{32}P.

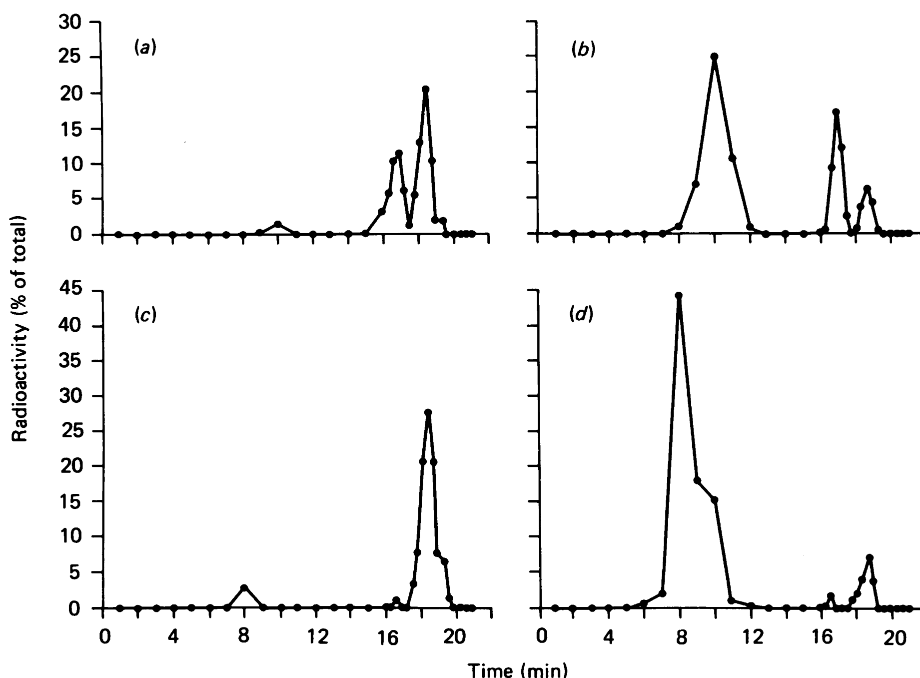

Fig. 2. Effect of erythrocyte ghost IP_3 5′-phosphomonoesterase on the two isomers of [^{3}H]IP_3 from guinea-pig hepatocytes

A [^{3}H]IP_3 sample from angiotensin (1 μM for 15 s)-stimulated hepatocytes was mixed together with [^{32}P]IP_3 from erythrocyte ghosts and incubated with erythrocyte ghost IP_3 5′-phosphomonoesterase in the presence of either EDTA (2 mM, control) or 2 mM-$MgCl_2$. The samples were then injected on to the h.p.l.c. column and eluted as described in the Materials and methods section. Panels (*a*) and (*b*), ^{3}H c.p.m. of sample incubated with enzyme and EDTA or with enzyme and Mg^{2+}, respectively; panels (*c*) and (*d*), ^{32}P c.p.m. of sample incubated with enzyme and EDTA, or with enzyme and Mg^{2+}, respectively. Results are shown of one of two experiments giving similar results.

the radioactivity associated with [^{3}H]IP_3 was expressed as a percentage of the lipid radioactivity, assumed to be predominantly [^{3}H]phosphatidylinositol.

Determination of the nature of the IP_3 isomers

(***a***) **IP_3 5′-phosphomonoesterase assay.** IP_3 5′-phosphomonoesterase, which specifically removes the 5′-phosphate from (1,4,5)IP_3, was prepared from erythrocytes by the method of Downes *et al.* (1982). [^{32}P](1,4,5)IP_3 from erythrocyte ghosts and [^{3}H]IP_3 from liver were mixed together in 900 μl of 30 mM-Hepes buffer (pH 7.0) to which was added human erythrocyte membrane (approx. 0.2 mg of protein) containing the 5′-phosphomonoesterase enzyme, and either 2 mM-$MgCl_2$ or 2 mM-EDTA (control), to a total volume of 1 ml. This mixture was incubated at 37 °C for 30 min, at which time an extra 0.2 mg of membrane protein was added to increase the amount of the (1,4,5)IP_3 hydrolysed. The incubation was continued for a further 30 min and the reaction was stopped by dilution of 10 ml of ice-cold water. The supernatant (15000 *g* for 20 min) was then frozen and freeze-dried and applied to the h.p.l.c. system described above.

(***b***) **Chemical identification.** The distribution of the phosphate groups of the [^{3}H]IP_3 isomer that were not eluted with the [^{32}P](1,4,5)IP_3 standard in the h.p.l.c. system was determined by periodate oxidation as described by Irvine *et al.* (1984).

Materials

Collagenase was obtained from Boehringer Mannheim. *myo*-[^{3}H]Inositol was obtained from American Radiolabelled Chemicals, and [^{32}P]phosphate from I.C.N. Caerulein was from Peninsula Laboratories, and all other chemicals were obtained from Sigma.

RESULTS

Presence of more than one isomer of IP_3 in liver cells

To determine whether the IP_3 formed on stimulation of guinea-pig hepatocytes was only partly in the 1,4,5 isomeric form, as has been reported for parotid cells (Irvine *et al.*, 1984, 1985), suspensions of hepatocytes were stimulated for periods of either 5 s or 1 min with angiotensin (1 μM, a maximal concentration for IP_3 formation). [^{3}H]IP_3 fractions were purified from these stimulated cells on Dowex formate columns, and then applied, along with [^{32}P]IP_3 from erythrocyte ghosts, and ATP to an h.p.l.c. Partisil SAX 10 anion-exchange column, and eluted as described in the Materials and methods section. Fig. 1 shows that the [^{32}P]IP_3 standard was eluted from the column as two peaks, one major peak, which was assumed to be the 1,4,5 isomer, at 21.3 min, and a much smaller peak, amounting to 1–5% of the total radioactivity, at 19.2 min. The minor peak is not predominantly an inositol trisphosphate, and its identity is at present unknown (Irvine *et al.*, 1985).

The elution profile of the [^{3}H]IP_3 from liver cells shows two peaks, and, as shown by contrasting Fig. 1(*a*), which

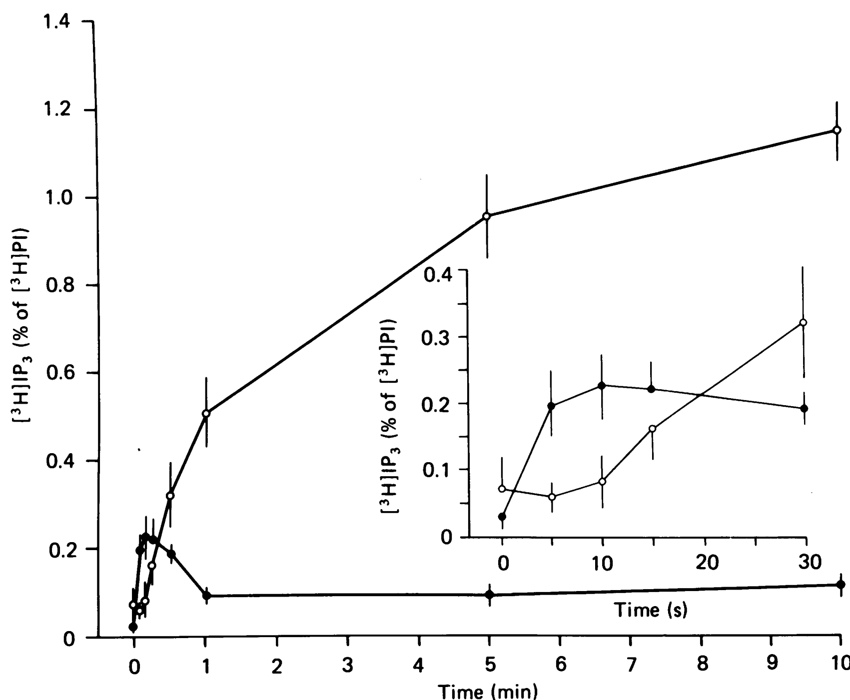

Fig. 3. Time course of formation of $[^3H](1,4,5)IP_3$ and $[^3H](1,3,4)IP_3$ in guinea-pig hepatocytes

Guinea-pig hepatocytes labelled with $[^3H]$inositol were incubated with 1 μM-angiotensin for the times indicated. The $[^3H]IP_3$ fractions, which had been pre-purified on Dowex (formate form) columns, were then mixed with $[^{32}P]IP_3$ from erythrocyte ghosts and ATP (as a marker). They were then injected on to the h.p.l.c. anion-exchange column and eluted as described in the Materials and methods section to separate the two isomers of IP_3. The ^3H radioactivity in the two fractions identified as $(1,4,5)IP_3$ (●) and $(1,3,4)IP_3$ (○) was then expressed as a percentage of $[^3H]PI$. The bars indicate S.E.M. values for four experiments.

represents 5 s of stimulation with angiotensin, with Fig. 1(*b*), which represents 1 min of stimulation, the ratio of the two peaks changed with length of stimulation. The ^3H peak with the longer retention time co-chromatographed with $[^{32}P](1,4,5)IP_3$, indicating that it was probably also the 1,4,5 isomer. The first ^3H peak (19.2 min) appeared to correspond to the isomer of IP_3 identified as 1,3,4 by Irvine *et al.* (1984, 1985), because of its relative retention time compared with $(1,4,5)IP_3$, and because it co-eluted from the column with ATP (see Irvine *et al.*, 1985). The ^3H peak presumed to be $(1,4,5)IP_3$ (21.3 min) predominated at the shorter time point, and the other isomer (probably 1,3,4) was predominant at 60 s.

Determination of the nature of the two isomers of IP_3

To verify that the $[^3H]$- and $[^{32}P]$-IP_3 peaks with the longer retention time were in fact $(1,4,5)IP_3$, partially purified $[^3H]IP_3$ from liver (15 s of stimulation with 1 μM-angiotensin) and $[^{32}P]IP_3$ from erythrocyte ghosts were mixed and incubated with erythrocyte ghost 5′-phosphomonoesterase in the presence of either EDTA (2 mM) to inhibit the enzyme (control) or Mg^{2+} (2 mM) to activate the enzyme. Fig. 2(*c*) shows the h.p.l.c. elution pattern of the $[^{32}P]IP_3$ incubated with non-activated enzyme. As in Fig. 1, there is one major peak at 18.4 min, which presumably corresponds to $(1,4,5)IP_3$ plus the very small peak at 16.6 min (see above). (This was an older column, and retention times tended to shorten with use of the column; however, ATP always co-eluted with the first IP_3 peak, and the elution pattern in general did not change.) Fig. 2(*d*) shows the h.p.l.c. elution pattern of the $[^{32}P]IP_3$ if it was first incubated with the activated enzyme. Here the large peak at 18.4 min has been greatly decreased (by about 80%), which would be expected if it corresponded to $(1,4,5)IP_3$. The smaller peak at 16.6 min remained unchanged, and a large broad peak appeared at 8–10 min, corresponding to P_i and IP_2 (see Downes *et al.*, 1982), which are eluted similarly in this system. The $[^3H]IP_3$ from liver shows two clear peaks after incubation with the non-activated (in the presence of EDTA) 5′-phosphomonoesterase (Fig. 2*a*), one at 16.9 min, which had the same retention time as ATP, and one at 18.4 min, corresponding to $[^{32}P](1,4,5)IP_3$ from the erythrocyte ghosts. Fig. 2(*b*) shows that after incubation with the activated enzyme the later peak (18.4 min) was again much decreased (in this case by 78%), whereas the other peak, at 16.9 min, was unaffected. The radioactivity lost from the peak at 18.4 min could all be accounted for by the appearance of a peak at 10 min which corresponds to $[^3H]IP_2$, the expected breakdown product of the $[^3H]IP_3$.

It would appear that the ^3H radioactivity in the later peak which was broken down by the 5′-phosphomonoesterase was indeed $(1,4,5)IP_3$, and therefore a valid comparison can be made between this h.p.l.c. system and that of Irvine *et al.* (1985). [It is possible that the small amount of ^3H and ^{32}P radioactivity remaining in the later peak after enzyme treatment is also $(1,4,5)IP_3$, and would have been broken down by more prolonged incubation or addition of more enzyme; see the Materials and methods section.] In all likelihood the early peak which was unaffected by the 5′-phosphomonoesterase, and

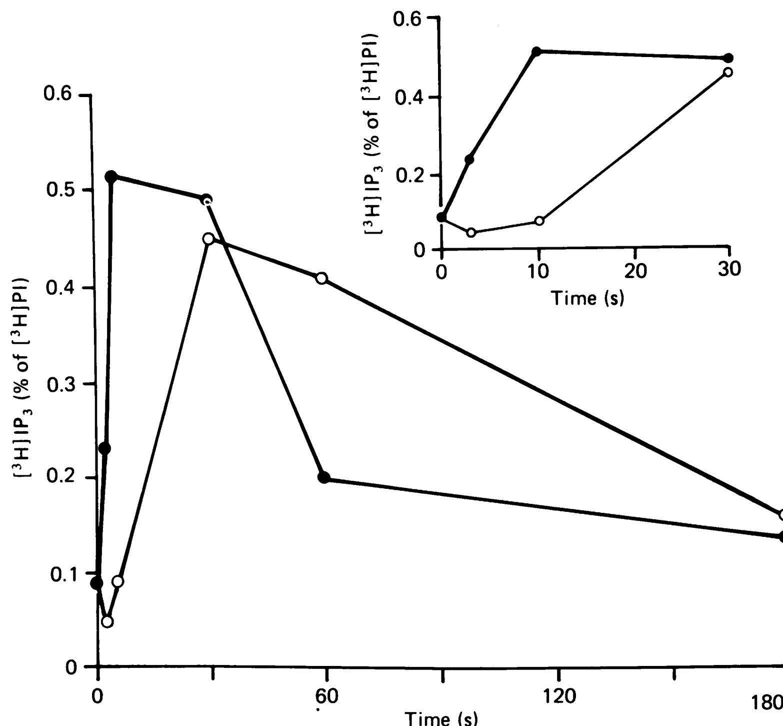

Fig. 4. Time course of formation of $[^3H](1,4,5)IP_3$ and $[^3H](1,3,4)IP_3$ in DMSO-differentiated HL-60 cells

DMSO-differentiated HL-60 cells which had been labelled with [³H]inositol were incubated with fMet-Leu-Phe (1 μM) for the times indicated. The $[^3H]IP_3$ fractions from these cells were then injected on to the h.p.l.c. column as described for Fig. 3, so that the rate of appearance of the two isomers could be followed. Fig. 4 shows results of one of three experiments which gave similar results. ●, $[^3H](1,4,5)IP_3$; ○, $[^3H](1,3,4)IP_3$.

which, as in the above reference, co-eluted with ATP, was the isomer that has been identified as $(1,3,4)IP_3$. Chemical analysis by periodate oxidation as described by Irvine *et al.* (1984) of the liver IP_3 in this early peak yielded altritol, confirming that it was indeed likely to be $(1,3,4)IP_3$.

Kinetics of formation of $[^3H](1,4,5)IP_3$ and $[^3H](1,3,4)IP_3$ in guinea-pig hepatocytes

As it seemed from experiments like that illustrated in Fig. 1 that the proportion of the two isomers of IP_3 changed, depending on the duration of stimulation, the time course of formation of the isomers was investigated by determining the h.p.l.c. elution pattern of $[^3H]IP_3$ samples from hepatocytes stimulated for various times with angiotensin (1 μM). It has previously been shown that total $[^3H]IP_3$ production in α-adrenoceptor-activated hepatocytes continues to rise until at least 30 min (Burgess *et al.*, 1984*a*). The same was found to be true for angiotensin (results not shown). Fig. 3 shows the time courses of formation of the two isomers of $[^3H]IP_3$ on stimulation with 1 μM-angiotensin. $[^3H](1,4,5)IP_3$ increased very steeply initially and was almost maximal by 5 s. It reached a plateau between 5 and 15 s, at which point its concentration was 7.7 times the basal value, and then began to decrease until it reached a steady value between 1 and 10 min which was around 4 times the basal value. In several experiments where the hepatocytes were stimulated with angiotensin (1 μM) for up to 30 min (results not shown), $(1,4,5)IP_3$ concentrations remained several times higher than the control value. The time course of formation of $[^3H](1,3,4)IP_3$ was quite different from that of the 1,4,5 isomer. The former did not show any significant increase until 15 s, and then began to increase quite steadily until 10 min, when it reached a value of about 1.15% of [³H]PI, which is 15.5 times its basal value (Fig. 3). After 60 s of stimulation with angiotensin, up to 90% of the IP_3 in the cell was in the 1,3,4 form.

Kinetics of formation of $[^3H](1,4,5)IP_3$ and $[^3H](1,3,4)IP_3$ in DMSO-differentiated HL-60 cells

The formation of total $[^3H]IP_3$ in fMet-Leu-Phe-stimulated DMSO-differentiated HL-60 cells does not follow the same time course as in hormone-stimulated hepatocytes. Dougherty *et al.* (1984) showed that there was a very rapid increase in $[^3H]IP_3$, which decreased back to initial values in about 30 min. Therefore, unlike the hepatocyte, there is a reasonable temporal correlation between hormone-induced $[^3H]IP_3$ formation and other physiological responses of these neutrophil-like cells which are not sustained for long periods. Fig. 4 shows one of several similar experiments where DMSO-differentiated HL-60 cells, loaded with [³H]inositol, were stimulated with fMet-Leu-Phe (1 μM) for the times indicated, and the isomeric content of the $[^3H]IP_3$ formed was analysed by h.p.l.c. Although the time course of its formation is different from that in liver cells, the HL-60 cells do produce quite a large proportion of the 1,3,4 isomer. Again, the $[^3H](1,4,5)IP_3$ content began to rise first and was maximal by about 10 s. The amount of $[^3H](1,4,5)IP_3$ remained at about 0.5% of [³H]PI (about 5.5 times the control value) until 30 s and then began to decrease, until at 2 min it was only just above the control value. There was a definite lag period of at least 10 s before the

Table 1. Differential effects of Li^+ on caerulein-induced formation of (1,4,5)IP_3 and (1,3,4)IP_3 in pancreatic acinar cells

Pancreatic acinar cells, labelled with [^{3}H]inositol, were incubated with either 20 mM-NaCl as a control or 20 mM-LiCl. They were then incubated for a further 2.5 min with caerulein (0.1 μM). The [^{3}H]IP_3 formed was purified on Dowex (formate form) columns and then injected on to the h.p.l.c. column as described in the Materials and methods section. Results are means ± S.E.M. for three observations. The difference between Na^+ and Li^+ is significant (*$P < 0.05$), based on two-way analysis of variance.

Incubation conditions	[^{3}H]IP_3 (% of total PI)	
	1,4,5	1,3,4
Na^+	0.140 ± 0.006	0.493 ± 0.084
Li^+	0.155 ± 0.020	0.897 ± 0.121*

[^{3}H](1,3,4)IP_3 began to increase, at which time the [^{3}H](1,4,5)IP_3 amount was maximal. The amount of the 1,3,4 isomer was maximal at 30 s and declined subsequently, again reaching almost control values by 2 min.

Effect of Li^+ on the formation of [^{3}H](1,4,5)IP_3 and [^{3}H](1,3,4)IP_3

In most tissues, preincubation with Li^+ causes an inhibition of inositol-1-phosphatase, and on stimulation of the cells with an agonist leads to accumulation specifically of inositol 1-phosphate (Sherman *et al.*, 1981; Berridge *et al.*, 1982; Godfrey & Putney, 1984). However, in a few tissues, including pancreatic acini (Rubin, 1984), and rat hepatocytes (Thomas *et al.*, 1984), Li^+ also increases agonist-induced IP_2 and IP_3 accumulation, either by inhibiting the specific phosphatases which break down IP_3 and IP_2, or possibly because of indirect effects of the build-up of IP. However, in those tissues where agonist-induced [^{3}H]IP_3 accumulation is increased by Li^+, there is no affect of the Li^+ on the final physiological responses of the cell, e.g. amylase secretion in pancreatic acini (Rubin, 1984), or phosphorylase activation in rat hepatocytes (Thomas *et al.*, 1984). If IP_3 is the second messenger responsible for mobilizing Ca^{2+}, this Li^+-induced increase in IP_3 might be expected to result in a greater biological response. It seemed possible therefore that in those tissues where Li^+ did cause an increase in IP_3 accumulation, the 1,3,4 isomer, for which no role has yet been ascribed, might be increased rather than (1,4,5)IP_3, which is likely to be the intracellular signal responsible for the hormone-induced rise in cytosolic Ca^{2+} concentration. In order to investigate this possibility, the effect of LiCl (20 mM) on the isomeric content of the [^{3}H]IP_3 formed in response to caerulein (0.1 μM), in rat pancreatic acinar cells was studied. Pancreatic acinar cells were chosen because Li^+ causes a large increase in agonist-induced [^{3}H]IP_3 accumulation in this tissue, e.g. a 24-fold amplification of the response to caerulein at 30 min has been reported (Rubin, 1984). {In guinea-pig hepatocytes, the [^{3}H]IP_3 response to angiotensin was increased at most by 20% by Li^+; Thomas *et al.* (1984) obtained somewhat greater effects in rat hepatocytes.} The h.p.l.c. elution pattern of [^{3}H]IP_3 purified from pancreatic acinar cells that had been stimulated for 2.5 min with caerulein was similar to that for hepatocytes and HL-60 cells. A large peak at 19.2 min was eluted with ATP and corresponded to [^{3}H](1,3,4)IP_3, and a small peak at 21 min corresponded to the 1,4,5 isomer of [^{3}H]IP_3. If the pancreatic acinar cells were pretreated with LiCl (20 mM) for 20 min, caerulein-induced total [^{3}H]IP_3 accumulation was increased by 60% at 2.5 min. This increase in the radioactivity of the [^{3}H]IP_3 fraction [which is comparable with the increase reported by Rubin (1984) at 2.5 min] could be completely accounted for by an increase in [^{3}H](1,3,4)IP_3. As shown in Table 1, the [^{3}H](1,3,4)IP_3 increased from about 0.5% to about 0.9% of [^{3}H]PI. Table 1 also quite clearly illustrates that Li^+ had no effect whatsoever on the amount of [^{3}H](1,4,5)IP_3.

DISCUSSION

Michell (1975) has suggested that inositol lipid turnover may play a role in receptor-regulated Ca^{2+} mobilization, and Berridge (1983; see also Berridge & Irvine, 1984) has suggested that this might occur through the breakdown product of PI(4,5)P_2, (1,4,5)IP_3, acting as a second messenger to signal intracellular Ca^{2+} release. Previous work from our laboratory provided evidence supporting this hypothesis by using two distinct cell types, the guinea-pig hepatocyte (Burgess *et al.*, 1984*a*, *b*) and the DMSO-differentiated HL-60 cell (Dougherty *et al.*, 1984; Burgess *et al.*, 1984*c*). The main points of evidence were: (1) purified (1,4,5)IP_3 applied to permeable hepatocytes or HL-60 cells induced ^{45}Ca release from an ATP-dependent non-mitochondrial pool; and (2) receptor activation by appropriate agonists resulted in an increased cellular content of [^{3}H]IP_3 formed from lipids prelabelled with [^{3}H]inositol.

The relevance of these latter measurements, however, could be questioned in the light of the findings by Irvine *et al.* (1984, 1985) showing that parotid cells respond to a Ca^{2+}-mobilizing agonist with the formation of two isomers of IP_3, the expected 1,4,5 isomer, and also a 1,3,4 isomer. We have therefore examined the [^{3}H]IP_3 formed in guinea-pig hepatocytes and HL-60 cells to determine which isomers are formed, and in what relative quantities.

The previously reported kinetics of total [^{3}H]IP_3 production in these two cell types differed markedly; total IP_3 in hepatocytes increased slowly over 30 min, whereas IP_3 concentrations in HL-60 cells were maximal in 5 s. However, when the IP_3 formed in these cells was analysed for isomers, a number of similarities (to one another, and to previous data with the parotid gland) were noted. Specifically, in both cases (1) both (1,4,5)IP_3 and (1,3,4)IP_3 were present in non-stimulated cells, and both were increased on stimulation, (2) (1,4,5)IP_3 increased rapidly, whereas there was a definite latency before (1,3,4)IP_3 increased, such that (3) at early times after stimulation (1,4,5)IP_3 predominated, whereas later most of the IP_3 was the 1,3,4 isomer. Furthermore, since the resting IP_3 is a mixture of the two isomers, the relative increase in (1,4,5)IP_3 in the first few seconds is much greater than originally believed on the basis of total IP_3 measurements. The [^{3}H](1,4,5)IP_3 was increased 7.7-fold in the hepatocytes and 5.5-fold in the HL-60 cells; previous findings based on total [^{3}H]IP_3 indicated only about a doubling (Burgess *et al.*, 1984*a*; Dougherty *et al.*, 1984). Thus, in terms of the suggested function of

$(1,4,5)IP_3$, these data would seem to add further support to this hypothesis. In cell types in which $(1,4,5)IP_3$ has been shown to release internal Ca^{2+}, $(1,4,5)IP_3$ is indeed formed and the kinetics of the increase of this specific isomer are more consistent with its proposed second-messenger status than were the previously published kinetics of total IP_3.

The findings on the action of Li^+ on pancreatic inositol phosphates serve to resolve a standing paradox, and may also be relevant to the question of whether the $(1,3,4)IP_3$ has any role in stimulus–response coupling. An apparent paradox was created by the data of Thomas *et al.* (1984) and Rubin (1984), which indicated that Li^+ was able to potentiate agonist-induced IP_3 formation, but not the appropriate Ca^{2+}-mediated response. The data in Table 1 show, however, that in pancreatic acinar cells, the 1,4,5 isomer, which has been shown to be biologically active, is not affected by Li^+ at a time when both agonist-induced Ca^{2+} mobilization (Putney *et al.*, 1983) and agonist-induced secretion (Rubin, 1984) are near maximal. Only the 1,3,4 isomer was increased in the presence of Li^+. On the basis of this finding (by using logic only slightly circular) it is also suggested that: (1) $(1,3,4)IP_3$ neither releases Ca^{2+} nor inhibits the action of $(1,4,5)IP_3$, (2) the enzyme or enzymes which degrade $(1,3,4)IP_3$ are different from that for $(1,4,5)IP_3$, and (3) $(1,3,4)IP_3$ does not regulate the rate of formation or degradation of $(1,4,5)IP_3$.

It may be that the 1,3,4 isomer is not active as an agonist or antagonist, but is formed as a means of desensitizing the Ca^{2+} response while maintaining a high rate of production of diacyglycerol. The apparent widespread occurrence of $(1,3,4)IP_3$ makes it seem likely that it may have some such role in cell metabolism.

The route of synthesis of $(1,3,4)IP_3$ is unknown. It has been suggested (Irvine *et al.*, 1985) that it might be synthesized either by isomerization from $(1,4,5)IP_3$ or that there might be some $PI(3,4)P_2$ lipid which can also be broken down by phospholipase C. As $(1,3,4)IP_3$ seems to be insensitive to the phosphatase responsible for breaking down $(1,4,5)IP_3$, it would tend to accumulate. However, when erythrocyte $[^{32}P](1,4,5)IP_3$ was added to permeable hepatocytes (saponin-treated; see Burgess *et al.*, 1984*a*), the material was degraded to $[^{32}P]IP_2$ and $[^{32}P]P_i$ without the formation of any detectable $^{32}P(1,3,4)IP_3$ (results not shown). Preliminary analysis of PIP_2 from HL-60 cells and hepatocytes (see Irvine *et al.*, 1985, for methods) have revealed that more than 98% of the PIP_2 is $PI(4,5)P_2$; the specific radioactivity of the samples was not high enough to say conclusively whether the remainder was $PI(3,4)P_2$. It is possible, however, that $PI(3,4)P_2$ must first be increased by Ca^{2+}-mobilizing-hormone stimulation, for example by the increase in diacylglycerol (Taylor *et al.*, 1984; Irvine *et al.*, 1985), before it can be broken down by the phosphodiesterase. This might account for the latency in the increase in $[^3H](1,3,4)IP_3$ (control IP_3 samples from the cells in this study did, however, contain approximately equivalent amounts of each isomer).

In summary, stimulation by appropriate agonists of guinea-pig hepatocytes, DMSO-differentiated HL-60 cells and rat pancreatic acinar cells leads to the production of $(1,3,4)IP_3$ and $(1,4,5)IP_3$. The kinetics of the formation of $(1,4,5)IP_3$ are rapid and are consistent with its suggested second-messenger role. The amount of $(1,3,4)IP_3$ increases more slowly, but to a greater extent. In the pancreas Li^+ potentiates the agonist-induced increase in $(1,3,4)IP_3$ [but not in $(1,4,5)IP_3$], presumably by inhibiting its degradation. The origin and biological significance of $(1,3,4)IP_3$ are unknown.

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