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Formation of inositol phosphate isomers in GH_3 pituitary tumour cells stimulated with thyrotropin-releasing hormone

Acute effects of lithium ions

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With a h.p.l.c. system, the inositol mono-, bis- and tris-phosphate isomers found in [^{3}H]inositol-labelled GH_3 cells were resolved and identified. These cells possess at least ten distinct [^{3}H]inositol-containing substances when acid-soluble extracts are analysed by anion-exchange h.p.l.c. These substances were identified by their co-elution with known inositol phosphate standards and, to a limited extent, by examining their chemical structure. Two major inositol monophosphate (Ins*P*) isomers were identified, namely Ins1*P* and Ins4*P*, both of which accumulate after stimulation with the hypothalamic releasing factor (TRH) (thyrotropin-releasing hormone). Three inositol bisphosphate (InsP_2) isomers were resolved, of which two were positively identified, i.e. Ins(1,4)P_2 and Ins(3,4)P_2. TRH treatment increases both of these isomers, with Ins(1,4)P_2 being produced at a faster rate than Ins(3,4)P_2. The third InsP_2 isomer has yet to be fully identified, although it is co-eluted with an Ins(4,5)P_2 standard. This third InsP_2 is also increased after TRH stimulation. In common with other cell types, the GH_3 cell contains two inositol trisphosphate (InsP_3) isomers: Ins(1,4,5)P_3, which accumulates rapidly, and Ins(1,3,4)P_3, which is formed more slowly. The latter substance appears simultaneously with its precursor, inositol 1,3,4,5-tetrakisphosphate. We also examined the effects of acute Li^+ treatment on the rates of accumulation of these isomers, and demonstrated that Li^+ augments TRH-mediated accumulation of Ins1*P*, Ins4*P*, Ins(1,4)P_2, the presumed Ins(4,5)P_2 and Ins(1,3,4)P_3. These results suggest that the effects of Li^+ on inositol phosphate metabolism are more complex than was originally envisaged, and support work carried out by less sophisticated chromatographic analysis.

INTRODUCTION

It is now generally accepted that the phospholipase C-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) is the primary step in the signal-transduction mechanism for a class of agonists which increase intracellular Ca^{2+} (Downes & Michell, 1985). The products of PtdInsP_2 cleavage, *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], are known to act as second messengers. Diacylglycerol is an endogenous regulator of protein kinase C activity (Nishizuka, 1984), and Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores, probably within the endoplasmic reticulum (Downes & Michell, 1985).

Our understanding of the metabolic fate of Ins(1,4,5)P_3 has been substantially increased by the use of anion-exchange h.p.l.c. By using such techniques, the existence of two inositol trisphosphate isomers [Ins(1,4,5)P_3 and Ins(1,3,4)P_3], at least one inositol tetrakisphosphate isomer [Ins(1,3,4,5)P_4: Irvine *et al.* (1984, 1985); Burgess *et al.* (1985); Batty *et al.* (1985)], several inositol bisphosphate isomers [Ins(1,4)P_2, Ins(1,3)P_2 and Ins(3,4)P_2: Heslop *et al.* (1985, 1986); Hawkins *et al.* (1986); Hansen *et al.* (1986); Shears *et al.* (1987); Irvine *et al.* (1987)] and multiple inositol monophosphate isomers [primarily Ins1*P* and Ins4*P*: Siess (1985); Siess *et al.* (1986); Balla *et al.* (1986); Morgan *et al.* (1987)] has been demonstrated.

The GH_3 pituitary tumour cell line provides a convenient system for studying the role of inositol lipid metabolism in stimulus–secretion coupling. These cells contain receptors for thyrotropin-releasing hormone (TRH; thyroliberin) on their plasma membrane (Hinkle *et al.*, 1980) and release prolactin in response to stimulation with the tripeptide (Ostlund *et al.*, 1978). Furthermore, TRH stimulation is associated with a rapid breakdown of PtdInsP_2, which is mirrored in an increase in Ins(1,4,5)P_3, diacylglycerol and free intracellular Ca^{2+} (for a review, see Drummond, 1986). Treatment of GH_3 cells with Li^+, an inhibitor of Ins(1,4)P_2 4-phosphatase and Ins1*P* phosphatase (Hallcher & Sherman, 1980; Storey *et al.*, 1984), is associated with an augmentation of TRH-stimulated Ins*P*, InsP_2 and InsP_3 accumulation (Drummond *et al.*, 1984). However, the chromatographic techniques used in that study did not allow the identification of individual inositol phosphate isomers. H.p.l.c. has therefore been used to study the formation of inositol phosphate isomers in [^{3}H]inositol-labelled GH_3 pituitary tumour cells after stimulation with TRH in the absence or presence of Li^+.

EXPERIMENTAL

Culture of GH_3 pituitary cells

GH_3 pituitary tumour cells were grown on 90 mm-diam. Petri dishes as described by Macphee & Drummond (1984). At 2–3 weeks after passage, the cells were fed

Abbreviations used: PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PtdIns*P*, phosphatidylinositol 4-monophosphate; Ins*P*, Ins1*P*, Ins4*P*, *myo*-inositol monophosphate and its 1- and 4-isomers; InsP_2, Ins(1,4)P_2, Ins(1,3)P_2, Ins(3,4)P_2, Ins(4,5)P_2, *myo*-inositol bisphosphate and its 1,4-, 1,3-, 3,4- and 4,5-isomers; InsP_3, Ins(1,4,5)P_3 and Ins(1,3,4)P_3, *myo*-inositol trisphosphate and its 1,4,5- and 1,3,4-isomers; InsP_4, Ins(1,3,4,5)P_4, inositol tetrakisphosphate and its 1,3,4,5-isomer; TRH, thyrotropin-releasing hormone (thyroliberin).

with inositol-free Ham's F10 medium (Gibco, Paisley, Scotland, U.K.) containing antibiotics but supplemented with dialysed serum [donor horse (15%, v/v) and foetal calf (2.5%, v/v)]. Then 20 μCi of *myo*-[2-^{3}H]inositol (sp. radioactivity 15 Ci/mmol; A.R.C., St. Louis, MO, U.S.A.) was added to each dish. The cells were then grown for a further 3 days until the inositol-containing phospholipids had been labelled to isotopic equilibrium.

At the end of the labelling period, the cells were harvested and resuspended in a balanced salt solution (Na-BSS) of the following composition: 135 mM-NaCl, 4.5 mM-KCl, 1.5 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 5.6 mM-D-glucose, 10 mM-Hepes and 0.1% (w/v) bovine serum albumin, adjusted to pH 7.4 immediately before use with dilute NaOH. In all experiments in which the effects of Li^+ on inositol phosphate generation were examined, 10 mM-LiCl was included in the balanced salt solution (Li-BSS) and the NaCl content decreased correspondingly. A 225 μl portion of the cell suspension (approx. 3×10^6 cells) was stimulated by the addition of 25 μl of a 10 μM solution of TRH. In experiments in which the effects of Li^+ were examined, the cells were suspended in Li-BSS for 15 min before stimulation. At various times thereafter, the reactions were terminated by the addition of 250 μl of ice-cold 10% (w/v) $HClO_4$. The tubes were then left on ice for 5 min before the cell debris was sedimented by centrifugation at 12000 *g* for 2 min. A 450 μl portion of the supernatant was neutralized by the Freon/octylamine method as originally described by Sharpes & McCarl (1982), as modified by Downes *et al.* (1986). The upper phase containing the acid-soluble inositol phosphates was stored at -20 °C until assay. The recovery of radiolabelled Ins*P*, InsP_2 and InsP_3 standards after neutralization with Freon/octylamine was usually 80–95%.

Separation of inositol phosphate isomers by h.p.l.c.

[^{3}H]Inositol phosphates were separated by anion-exchange h.p.l.c. by a method similar to, but modified from, those described by Batty *et al.* (1985) and Irvine *et al.* (1985). Briefly, filtered samples were loaded, via a 2 ml injection loop, on to a 25 cm $\times$ 0.49 cm analytical column with a 5 cm $\times$ 0.49 cm guard column, both packed with Partisil-10 SAX strong anion-exchange resin (HiChrom Ltd., Reading, Berks., U.K.). Chromatographic separation of the inositol phosphate isomers was achieved with the following elution gradient: for the first 10 min, ultrapure water was pumped through the column, followed by a linear gradient rising to 2 M-ammonium formate (adjusted to pH 3.7 at 22 °C with H_3PO_4) over a period of 40 min. The eluent composition was gradually returned to ultrapure water over the next 5 min, where it remained for a further 10 min. The eluent was delivered at a rate of 1.25 ml/min. Fractions of the eluate were collected every 1 min over the first 10 min of the gradient, and every 20 s for the remainder of the gradient. Each fraction was then mixed with 3.5 ml of scintillant (Optiphase Safe; LKB-Fisons, Loughborough, Leics., U.K.), and radioactivity was determined by liquid-scintillation counting.

Preparation of radiolabelled inositol phosphate standards

L-*myo*-[U-^{14}C]Inositol 1-monophosphate (sp. radioactivity 55 mCi/mmol), D-*myo*-[2-^{3}H]inositol 1,4-bisphosphate (sp. radioactivity 1 Ci/mmol) and D-*myo*-[2-^{3}H]inositol 1,4,5-trisphosphate (sp. radioactivity 1 Ci/mmol) were purchased from Amersham International. [^{3}H]Inositol 1,3,4,5-tetrakisphosphate and inositol [4,5-^{32}P]bisphosphate were kindly given by Dr. S. Shears, of the Department of Biochemistry, University of Birmingham. Inositol 1,4,5-[4,5-^{32}P]trisphosphate and inositol 1,4-[4-^{32}P]bisphosphate were prepared from ^{32}P-labelled erythrocyte membranes as described by Downes *et al.* (1982). [^{3}H]Ins(4,5)P_2 was prepared by mild alkaline-phosphatase treatment of [^{3}H]Ins(1,4,5)P_3, by using a method similar to that described by Batty *et al.* (1985). Briefly, 10 units of calf intestine alkaline phosphatase (EC 3.1.3.1; Fluka A.G., Buchs, Switzerland) were dissolved in 500 μl of 0.1% bovine serum albumin; 50 μl of this stock solution was added to 1940 μl of 0.2 M-Tris/maleate buffer (pH 9.0) containing 2 mM-$MgCl_2$, to which 0.1 μCi of [^{3}H]Ins(1,4,5)P_3 was added. After 60 min at 37 °C, the reaction was terminated by acidification and boiling for 2 min. The sample was then neutralized, diluted and analysed by h.p.l.c. A mixture containing [^{14}C]Ins1*P* and [^{14}C]Ins2*P* was prepared by heating [^{14}C]Ins1*P* with 0.1 M-HCl as described by Tomlinson & Ballou (1961). [^{3}H]Inositol 4-phosphate was prepared by alkaline hydrolysis of [^{3}H]PtdIns*P* (Amersham) by the method of Grado & Ballou (1961). Co-elution with known inositol phosphate standards was used to identify tentatively the [^{3}H]inositol-containing peaks eluted from the h.p.l.c. column. The radioactive peaks (e and f, Fig. 1*a*) eluted with and close to the [^{3}H]- or [^{32}P]-Ins(1,4)P_2 standards were identified by the periodate-oxidation technique first described by Tomlinson & Ballou (1961), as modified by Irvine *et al.* (1987). Periodate oxidation and subsequent analysis of the non-cyclic polyols was kindly performed by Dr. R. F. Irvine of the A.F.R.C. Unit of Animal Physiology, Babraham, Cambridge, U.K.

Chemicals

All materials used in the culture of the cells were purchased from either Gibco or Flow laboratories (Irvine, Ayrshire, Scotland, U.K.). All other chemicals, unless otherwise stated, were purchased from either Sigma Chemical Co. (Poole, Dorset, U.K.) or Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and were of analytical grade.

RESULTS

Identification of the inositol phosphate isomers found in GH_3 pituitary tumour cells

The data presented in Fig. 1 show four typical chromatograms of the acid-soluble inositol phosphates present in [^{3}H]inositol-labelled GH_3 cells. Figs. 1(*a*) and 1(*b*) depict the inositol phosphates found in unstimulated GH_3 cells in the absence or presence of 10 mM-LiCl respectively. Figs. 1(*c*) and 1(*d*) depict corresponding elution profiles obtained after stimulation of the cells with TRH (1 μM) for 60 min. The h.p.l.c. separation method used in this study allows the resolution of individual inositol phosphate isomers that are not separated on Dowex columns. The retention times of these isomers were reproducible between injections, although the resolution of the inositol monophosphate isomers, in particular, declined as the column aged. The first of the [^{3}H]inositol-containing peaks was eluted

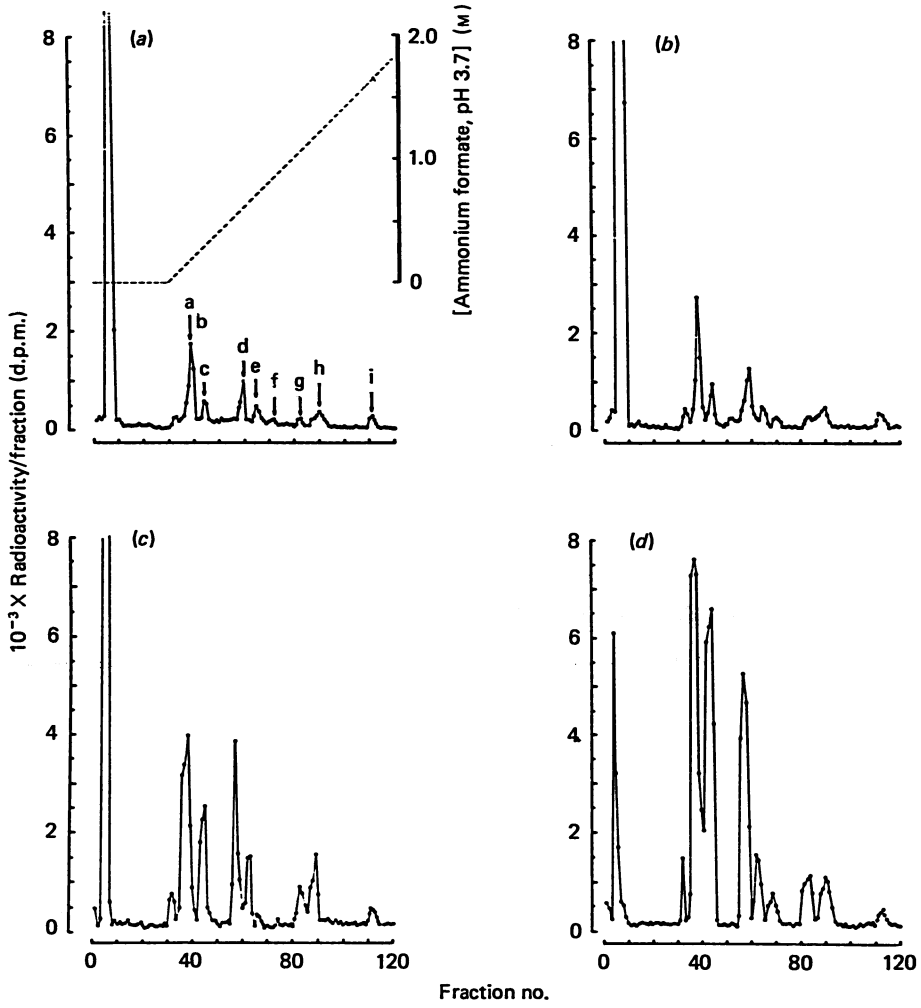

Fig. 1. Elution properties of acid-soluble [^{3}H]inositol phosphate isomers in GH_3 pituitary tumour cells

The results shown above were derived from one experiment; similar patterns were seen in six other experiments. For details, see the Experimental section. (*a*) Unstimulated GH_3 cells; (*b*) unstimulated cells after 75 min in the presence of 10 mM-LiCl; (*c*) GH_3 cells 60 min after the addition of 1 μM-TRH; (*d*) GH_3 cells stimulated for 60 min with 1 μM-TRH after 15 min treatment with 10 mM-LiCl. In (*a*), the elution gradient (----) is superimposed on the radioactivity trace. The dead space of the apparatus was approx. 4–5 min, the gradient shown in (*a*) has been drawn accordingly. The approximate elution times of individual inositol phosphate standards are indicated as follows: a, [^{14}C]Ins1*P* [retention time (T_r) = 19 min, 0.175–0.225 M-ammonium formate]; b, [^{14}C]Ins2*P* (T_r = 20.7 min, 0.250–0.325 M-ammonium formate); c, [^{3}H]Ins4*P* (T_r = 21.7 min, 0.300–0.325 M-ammonium formate); d, [^{32}P]- or [^{3}H]-Ins(1,4)P_2 (T_r = 26.8 min, 0.565–0.615 M-ammonium formate); e, the [^{3}H]inositol-containing peak eluted at the position marked 'e' (T_r = 28.8 min, 0.650–0.730 M-ammonium formate) could not be identified by its co-elution with a known inositol phosphate standard, but could be identified chemically; after periodate oxidation, reduction and dephosphorylation, most of the radioactivity found in peak e was degraded to [^{3}H]threitol, which is a characteristic of Ins(3,4)P_2 (Irvine *et al.*, 1987); f, [^{32}P]- or [^{3}H]-Ins(4,5)P_2 (T_r = 30.8 min; 0.765–0.815 M-ammonium formate); g, this peak (T_r = 34.5 min, 0.925–1.025 M-ammonium formate) co-eluted with ATP, a characteristic of Ins(1,3,4)P_3 (Irvine *et al.*, 1985); h, [^{32}P]- or [^{3}H]-Ins(1,4,5)P_3 (T_r = 36.1 min, 1.00–1.115 M-ammonium formate); i, [^{3}H]Ins(1,3,4,5)P_4 (T_r = 44.0 min, 1.400–1.500 M-ammonium formate).

4–5 min after sample injection and contained free inositol.

In GH_3 cells, at least two major Ins*P* isomers were present. The first of these (peak a in Fig. 1*a*), which had a retention time ranging from 18.5 to 19.5 min, depending on the age of the h.p.l.c. column, was co-eluted with a [^{14}C]Ins1*P* standard. The second major Ins*P* peak (peak c in Fig. 1*a*), which typically had a retention time of 21.0–22.5 min, is unlikely to be Ins2*P*, since a [^{14}C]Ins2*P* standard (peak b in Fig. 1*a*) was consistently eluted 30–60 s earlier than this peak (peak c in Fig. 1*a*). In a separate series of experiments, the inositol monophosphate isomers found in resting and stimulated GH_3 cells were isolated by anion-exchange chromatography on Dowex AG 1-X8 (formate form), freeze-dried, and the individual Ins*P* isomers were resolved by h.p.l.c. The gradient used in these experiments consisted of water for 10 min, followed by a linear ammonium formate gradient rising to 1.0 M over an 80 min period. With this separation method, three isomers were isolated: in resting cells, the initial peak co-eluted with the [^{14}C]Ins1*P* standard, and the second major Ins*P* peak co-eluted with the [^{3}H]Ins4*P*

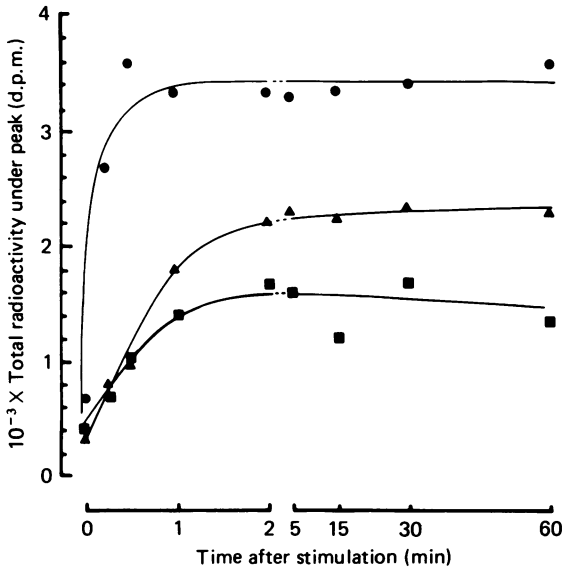

Fig. 2. Accumulation of Ins(1,3,4)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in TRH-stimulated GH_3 cells

The GH_3 cells were labelled and stimulated with TRH as described in the text. After termination of the reaction with $HClO_4$, the neutralized acid-soluble fraction was analysed by h.p.l.c. The results are expressed as the total radioactivity under a peak and represent the meaned data from six separate experiments; ●, Ins(1,4,5)P_3; ▲, Ins(1,3,4)P_3; ■, Ins(1,3,4,5)P_4. In this Figure, the standard errors are omitted in the interests of clarity, but were all approx. 8–12%.

standard produced by alkaline hydrolysis of [^{3}H]-PtdIns$P$. A small peak was present between these and co-eluted with a [^{14}C]Ins2P standard. In resting cells, this peak accounted for less than 5% of the total monophosphate isomers, and its content was unaffected by TRH stimulation (results not shown). Peak c (Fig. 1*a*) co-eluted with a [^{3}H]Ins4P standard.

Multiple isomers of InsP_2 are present in GH_3 cells. The largest of these (peak d in Fig. 1*a*) had a retention time of 26.3–27.3 min and co-eluted with an authentic [^{3}H]- or [^{32}P]-Ins(1,4)P_2 standard. Furthermore, in cell preparations that had been stimulated with TRH for 60 min in the presence of Li^+ (10 mM), more than 95% of the radioactivity contained under this peak was lost on periodate oxidation. This is a characteristic of *p*-substituted inositol bisphosphates, such as Ins(1,4)P_2 (Irvine *et al.*, 1987). The absence of any appreciable [^{3}H]ribitol associated with this peak indicates that stimulated GH_3 cells contain negligible amounts of Ins(1,3)P_2, which is known to co-elute with Ins(1,4)P_2 in this chromatography system (Irvine *et al.*, 1987). This therefore suggests that the removal of the 4-phosphate from Ins(1,3,4)P_3 is not a significant route of Ins(1,3,4)P_3 metabolism. The second [^{3}H]inositol-containing peak in this region had a retention time of 28–29.6 min (peak e in Fig. 1*a*). Since, after periodate oxidation, the radioactivity under this peak was recovered as [^{3}H]threitol, the most likely identity of this substance is Ins(3,4)P_2, a metabolite of Ins(1,3,4)P_3 (Irvine *et al.*, 1987). At present, the exact identity of the third InsP_2 peak (peak f in Fig. 1*a*) remains uncertain. It is much harder to identify, owing to the small amount present even in stimulated cells. However, it was co-eluted with authentic [^{32}P]- and [^{3}H]-Ins(4,5)P_2 standards and, since the other potential metabolites of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 are eluted elsewhere on the chromatographic profile, its most likely identity is Ins(4,5)P_2. Further work is necessary, however, to confirm this designation.

In common with other cell types, the GH_3 cell appears to contain two forms of InsP_3. In unstimulated cells, the later eluted of the [^{3}H]inositol-containing peaks (peak h in Fig. 1*a*), which had a retention time in the range 35–37.5 min, was co-eluted with an authentic [^{32}P]- or [^{3}H]-Ins(1,4,5)P_3 standard. The Ins(1,4,5)P_3 peak was preceded by a smaller [^{3}H]inositol-containing peak (peak g in Fig. 1*a*), which had a retention time in the range 33–35 min, and is most likely to represent the Ins(1,3,4)P_3 isomer. Although an authentic Ins(1,3,4)P_3 standard was not available for direct comparison with the first InsP_3 peak, it was eluted in the same position relative to Ins(1,4,5)P_3 and ATP as reported by Irvine *et al.* (1985). Under basal conditions in Na-BSS, approx. 90% of the total InsP_3 was in the form of Ins(1,4,5)P_3. The final [^{3}H]inositol-containing peak eluted from the column (peak i in Fig. 1*a*), with retention time in the range 41–45 min, co-eluted with an authentic [^{3}H]Ins(1,3,4,5)P_4 standard.

Kinetics of the accumulation of inositol phosphate isomers in GH_3 cells stimulated with TRH

Experiments were performed to clarify the kinetics of inositol phosphate accumulation in GH_3 cells after stimulation with a supramaximal concentration of TRH (1 μM). Contents of inositol phosphate isomers in unstimulated cells did not alter over a 2 h period (results not shown), although the presence of Li^+ ions did lead to an accumulation of certain isomers (see Fig. 1*b* and below).

As shown in Fig. 2, the time course of the formation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 was markedly different during the first 1 min of stimulation. In six separate experiments, an increase in the cellular content of Ins(1,4,5)P_3 was clearly evident within 15 s of stimulation, at which point its concentration was 8–10 times its baseline concentration. A steady-state value was achieved within 30 s of stimulation, and this was maintained for a further 60 min. Conversely, there was no significant increase in cellular Ins(1,3,4)P_3 until at least 30 s after receptor stimulation; subsequently its content rose steadily for a further 2 min, reaching a steady-state value approx. 7 times baseline values. Even in stimulated cell preparations at steady state, most of the total InsP_3 remained as the 1,4,5-isomer. In four experiments, there was no significant accumulation of Ins(1,3,4,5)P_4 until 30–60 s after stimulation, and its steady-state concentration (6-fold above basal) was reached within 2 min.

The effect on the cellular content of InsP_3 isomers of preincubating GH_3 cells with 10 mM-Li^+ for 15 min before TRH stimulation is shown in Fig. 3. In three experiments, acute treatment with Li^+ did not significantly affect the resting or the stimulated contents of Ins(1,4,5)P_3. However, in the same experiments, the baseline content of Ins(1,3,4)P_3 was increased by around 58% (317 ± 166 to 502 ± 106 d.p.m.; $P < 0.01$). Furthermore, the rate of accumulation of Ins(1,3,4)P_3 was approx. 3-fold faster in the presence of Li^+, with a significant increase over baseline being observed within

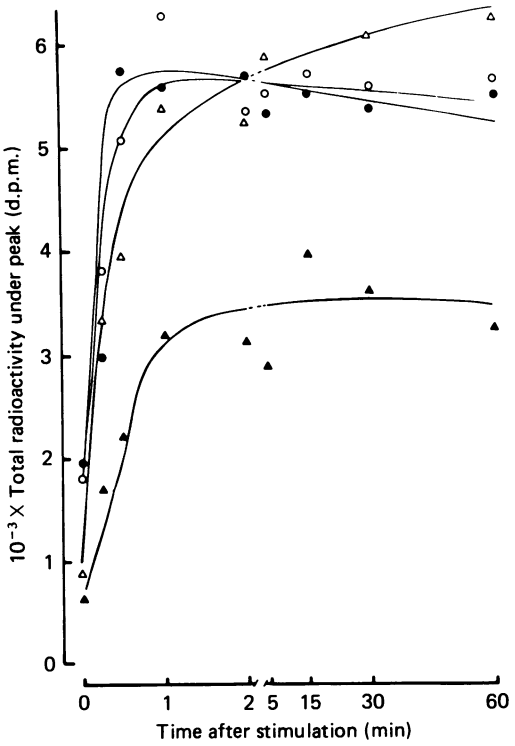

Fig. 3. Effect of 10 mM-Li^+ on the accumulation of Ins(1,3,4)P_3 and Ins(1,4,5)P_3 in TRH-stimulated GH_3 cells

GH_3 cells were treated with 10 mM-LiCl for 15 min before stimulation with 1 μM-TRH. Acid-soluble inositol phosphates were extracted and isolated as described in the Experimental section. The results are expressed as the total radioactivity under each individual peak and are shown as the mean of experiments on two separate cell preparations: ▲, △, Ins(1,3,4)P_3; ●, ○, Ins(1,4,5)P_3. △, ○, +10 mM-Li^+; ▲, ●, in Na-BSS. The standard errors are omitted for the sake of clarity, but they were typically 10–15%.

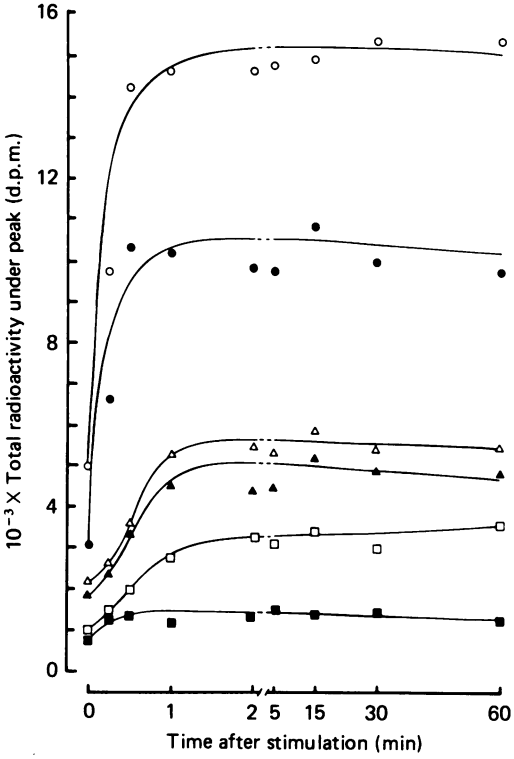

Fig. 4. Accumulation of Ins(1,4)P_2, Ins(3,4)P_2 and the third InsP_2 isomer in TRH-stimulated GH_3 cells in the absence and the presence of 10 mM-LiCl

GH_3 cells were treated with 10 mM-Li^+ for 15 min before stimulation with 1 μM-TRH. Acid-soluble inositol phosphates were extracted and isolated as described in the text. All results are expressed as the total radioactivity under a peak, and are shown as the mean value derived from three experiments performed on separate cell preparations, in which the S.E.M. was no greater than 8–15%. ○, ●, Ins(1,4)P_2; △, ▲, Ins(3,4)P_2; □, ■, the third InsP_2 isomer. ○, △, □, +10 mM-LiCl; ●, ▲, ■, in Na-BSS.

15 s of TRH addition; under these conditions and at steady state, the concentration of Ins(1,3,4)P_3 in GH_3 cells was approx. 6–8 times its new baseline concentration and 14–16 times its cellular content in the absence of Li^+. Thus, in the presence of Li^+, approximately equal amounts of the two InsP_3 isomers were produced in response to TRH addition. In one experiment, acute treatment with Li^+ did not appear to have any significant effect on either the final content or the time course of the accumulation of Ins(1,3,4,5)P_4 after TRH stimulation (results not shown).

The data presented in Fig. 4 (black symbols) illustrate the kinetics of the accumulation of the individual InsP_2 isomers. In three experiments, an increase in Ins(1,4)P_2 was observed within 15 s of stimulation, and its content was maximally elevated (2-fold) within 60 s of stimulation. Ins(3,4)P_2 accumulated at a significantly slower rate: there was no significant increase in its cellular content until 30–60 s after stimulation. Its maximum steady-state content, 3–4-fold greater than its pre-stimulation value, was achieved after 2 min of stimulation. The time course of the accumulation of the third inositol bisphosphate isomer was similar to that of Ins(1,4)P_2, with a steady state being achieved within 30 s of TRH addition, when its concentration had been increased 2-fold over baseline. At steady state, Ins(1,4)P_2 accounted for 70% of the total InsP_2 in the cell, Ins(3,4)P_2 for a further 23%, and the remaining 7% was the presumptive Ins(4,5)P_2 isomer.

The effect of acute Li^+ treatment on the time course of formation of the individual inositol bisphosphate isomers is shown in Fig. 4 (white symbols). After acute treatment with Li^+, the baseline concentration of Ins(1,4)P_2 was increased by 40% (3317±204 to 4661±437 d.p.m.; $n = 3$, $P < 0.05$) and its accumulation over the next 60 min was increased 2-fold compared with the Na^+ control samples. Acute Li^+ treatment, however, had no appreciable effect on the cellular concentration of Ins(3,4)P_2 (its baseline value being 1735±565 d.p.m. in Na-BSS and 2118±635 d.p.m. in Li-BSS; $n = 3$) or on the rate of its formation after TRH stimulation. Pretreatment with Li^+ did, however, have a marked effect on the baseline concentration and the rate of accumulation of the third bisphosphate isomer. In the presence of Li^+, the content of this isomer in untreated cells was increased by approx. 50% (796±141 to 1194±296 d.p.m.; $n = 3$, $P < 0.05$), and a significant increase in this isomer was now detectable within 1 min

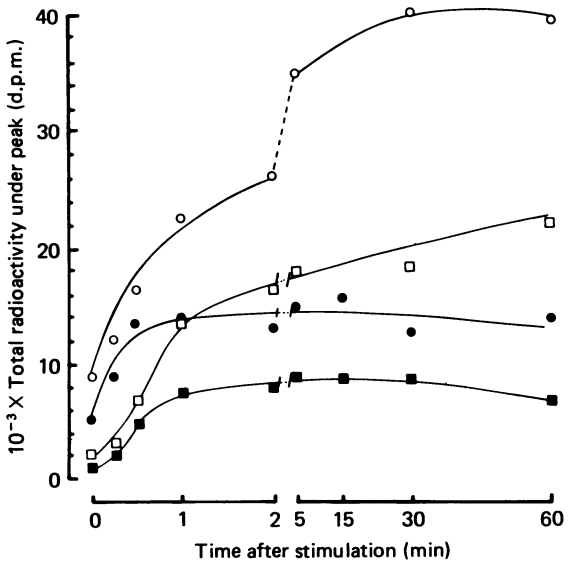

Fig. 5. Accumulation of Ins1*P* and Ins4*P* in TRH-stimulated GH_3 cells in the absence and the presence of 10 mM-LiCl

GH_3 cells were treated with 10 mM-Li^+ for 15 min before stimulation with 1 μM-TRH. Acid-soluble inositol phosphates were isolated and analysed as described in the text. All results are expressed as the total radioactivity under each peak, and are shown as the mean value obtained from three separate experiments in which the S.E.M. was typically 10–15%. ○, ●, Ins1*P*; □, ■, Ins4*P*. ○, □, +10 mM-LiCl; ●, ■, in Na-BSS.

of stimulation. It continued to accumulate until a steady state was achieved between 1 and 2 min after TRH addition, at which point its content had increased 6–7-fold in the presence of Li^+.

Fig. 5 (black symbols) shows the time course of the accumulation of Ins1*P* and Ins4*P* obtained from three separate experiments performed in the absence of Li^+. There was a rapid increase in the rate of formation of Ins1*P* that was significant within 15 s of TRH addition. The steady-state content of Ins1*P* was reached within 30 s of challenge, when its concentration was twice that found in unstimulated controls. In contrast, the rate of accumulation of Ins4*P* was significantly slower, and a steady-state value (3-fold elevated over the basal value) was reached 1–2 min after TRH addition, when its concentration was 3 times more than its baseline.

The effect of treating GH_3 cells for 15 min with 10 mM-Li^+ on the accumulation of Ins1*P* and Ins4*P* is shown in Fig. 5 (white symbols). In three experiments the basal content of Ins1*P* was increased by approx. 50% (from 5848 ± 508 to 9008 ± 1228 d.p.m.; $P < 0.02$), whereas the basal concentration of Ins4*P* was increased by approx. 75% (from 1726 ± 609 to 2922 ± 632 d.p.m.; $n = 3$, $P < 0.05$). The major effects, however, were on the time course of formation of each of the Ins*P* isomers. In the presence of Li^+, although the basal concentrations of each isomer were elevated, there was no significant enhancement of the increase in the rate of their formation during the first 30 s after stimulation. However, under these conditions, Ins1*P* continued to be produced for approx. 30 min after stimulation, and Ins4*P* continued to accumulate throughout the 60 min incubation period.

DISCUSSION

The present study confirms the observations by Rebecchi & Gershengorn (1983), Martin (1983), Drummond *et al.* (1984) and Schlegel *et al.* (1984) that, in the GH_3 cell line, Ins*P*, InsP_2 and InsP_3 accumulate within a few seconds of stimulation with TRH. In these studies, the inositol phosphates were separated by anion-exchange chromatography using Dowex columns. However, this relatively unsophisticated technique fails to resolve the closely related inositol phosphate isomers that have been identified in a variety of tissues (see the Introduction). Therefore, in the present study, we have used anion-exchange h.p.l.c. to improve the resolution of individual inositol phosphate isomers and have been able to demonstrate the existence of multiple Ins*P*, InsP_2 and InsP_3 isomers in GH_3 cells.

Stimulation of GH_3 cells was associated with the rapid accumulation of Ins(1,4,5)P_3, which was followed by a parallel increase in Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4. In Na^+-containing balanced salt solution, Ins(1,4,5)P_3 was always the predominant InsP_3 species obtained, although in the presence of Li^+ Ins(1,3,4)P_3 was produced much more rapidly, and within 2 min of challenge equal amounts of the two isomers had been formed. The relatively slow rate of appearance of Ins(1,3,4)P_3 probably represents the time taken for a proportion of the newly formed Ins(1,4,5)P_3 to be shunted through the InsP_3/InsP_4 pathway. Similar observations have been made in the closely related GH_4C_1 rat pituitary tumour cell line (Heslop *et al.*, 1985; Tashjian *et al.*, 1987), in cultured pituitary gonadotrophs (Morgan *et al.*, 1987) and in a variety of other cells or tissues (see the Introduction for references). It is noteworthy that, in the presence of Li^+, Ins(1,3,4)P_3 accumulated significantly faster; this emphasizes the enormous flux of inositol through these intermediates and the caution that is necessary in interpreting these changes in cellular content in terms of metabolic events.

While this paper was in preparation, Dean & Moyer (1987) demonstrated the existence of several InsP_2 isomers in TRH-stimulated GH_3 cells in the presence of Li^+ ions, and suggested that they included Ins(1,4)P_2, Ins(2,4)P_2 and, possibly, Ins(4,5)P_2 (in order of their increasing retention time). Additionally, they suggested that only Ins(1,4)P_2 is produced in significant amounts in response to TRH stimulation. The results in the present study are not in total agreement with the observation by Dean & Moyer (1987). In most GH_3-cell preparations, the major InsP_2 species detected was Ins(1,4)P_2, as Dean & Moyer (1987) demonstrated. However, the cellular content of all three isomers was increased in response to TRH. Dean & Moyer (1987) also showed that the second eluted of the three InsP_2 species emerged close to an Ins(2,4)P_2 standard. We suggest, however, that the true identity of this peak is Ins(3,4)P_2. If it was indeed Ins(2,4)P_2, the non-cyclic polyol that would be produced by periodate oxidation, reduction and dephosphorylation would be xylitol. Periodate oxidation of a sample of this middle InsP_2 peak yielded threitol, however, suggesting its most likely identity to be Ins(3,4)P_2. Recent work in homogenates of liver (Shears *et al.*, 1987), rat brain (Erneux *et al.*, 1987) and GH_4C_1 cells (Irvine *et al.*, 1987), suggests that Ins(1,3,4)P_3 is degraded predominantly to Ins(3,4)P_2. Our work confirms the existence of this isomer in intact cells and indicates the kinetics of its

accumulation after agonist stimulation. The demonstration of the existence of a third InsP_2 isomer was unexpected. The identity of this substance is not the 1,3-, 3,4-, 1,4- or 1,5-isomer, all of which are eluted elsewhere on the gradient (see above, and S. Shears, personal communication). Its co-elution with authentic Ins(4,5)P_2 standards suggests that this is its most likely identity. The source of this hitherto unreported isomer remains to be identified. However, recent work with sonicated GH_3-cell preparations (F. Ruiz-Larrea & A. H. Drummond, unpublished work) indicates that, in the absence of added ATP, [^{3}H]Ins(1,4,5)P_3 is metabolized to a small extent to a substance with identical elution characteristics to the third-emerging cellular InsP_2. This suggests that Ins(1,4,5)P_3 is the likely precursor for this InsP_2 isomer and would indicate the presence of a novel route of metabolism for this second messenger. However, the possibility that it may be formed by the sequential dephosphorylation of Ins(1,3,4,5)P_4 cannot yet be precluded. Therefore, further work will be necessary to confirm the identity and metabolic precursors of this radioactive peak.

The inhibition of Ins1P 1-phosphatase and Ins(1,4)P_2 4-phosphatase and the apparent insensitivity of Ins4P 4-phosphatase and Ins(1,4)P_2 1-phosphatase to inhibition by Li^+ has been well documented (Hallcher & Sherman, 1980; Berridge *et al.*, 1982; Storey *et al.*, 1984; Sherman *et al.*, 1985; Delvaux *et al.*, 1987). It might therefore have been predicted that, in cells or tissues treated with Li^+, only Ins1P would accumulate. However, other work on platelets (Siess *et al.*, 1986), adrenal glomerulosa cells (Balla *et al.*, 1986) and anterior-pituitary cells (Morgan *et al.*, 1987) has shown that the predominant Ins$P$ isomer formed after receptor stimulation is Ins4P. Moreover, in brain homogenates, Ins(1,4)P_2 is metabolized largely, if not exclusively, to Ins4P (Ackermann *et al.*, 1987; Inhorn *et al.*, 1987). Preliminary experiments with GH_3 cells suggest that Ins(1,4)P_2 is also metabolized solely to Ins4P (F. Ruiz Larrea & A. H. Drummond, unpublished work). However, in the present study with intact GH_3 cells, both Ins1P and Ins4P accumulated after TRH stimulation in the presence of Li^+. This would be consistent with the possibility that the Ins1P that accumulates after TRH stimulation of GH_3 cells is derived from the direct breakdown of PtdIns, as suggested by Imai & Gershengorn (1986). However, since the Ins1P fraction eluted from the h.p.l.c. column is likely to be contaminated with Ins3P (L-Ins1P), an Ins(3,4)P_2 metabolite, and perhaps also by Ins5P (which is present in 10-fold lower amounts than the major monophosphates in GH_3 cells; A. H. Drummond, W. R. Sherman, K. E. Ackermann & P. J. Hughes, unpublished work), and the degree to which the broken-cell studies are applicable to the intact cell is not known, it would be premature to reach any firm conclusion on this point. Further studies are needed to investigate this question.

This study confirms the observations by Drummond *et al.* (1984) that, in the presence of Li^+, a marked increase in TRH-mediated accumulation of InsP_3 is observed, and demonstrates that, in common with other cell types, the sole effect is on the Ins(1,3,4)P_3 isomer (see Burgess *et al.*, 1985; Turk *et al.*, 1986; Heslop *et al.*, 1986). Such an observation is not surprising, since Ins(1,4,5)P_3 5-phosphomonoesterase of rat brain (Delvaux *et al.*, 1987), rat liver (Storey *et al.*, 1984) or GH_3 cells (F. Ruiz Larrea & A. H. Drummond, unpublished work) is not inhibited by high concentrations of Li^+. Furthermore, it has been reported that the Ins(1,3,4,5)P_4 phosphomonoesterase is not inhibited by Li^+ (Erneux *et al.*, 1987). The effect of Li^+ on the enzymes responsible for the degradation of Ins(1,3,4)P_3 is unclear. In rat brain (Erneux *et al.*, 1987) and rat liver homogenates (Hansen *et al.*, 1986), Li^+ inhibits the Ins(1,3,4)P_3 phosphatase directly. However, Shears *et al.* (1987) have demonstrated that the Ins(1,3,4)P_3 phosphatase in rat liver is not sensitive to Li^+ alone, but is inhibited by a combination of Li^+ and Ins(1,4)P_2. This raises the possibility that, in the presence of Li^+, the concentration of Ins(1,4)P_2 can be high enough for product inhibition to occur. Further work will be necessary on GH_3 cells to ascertain which of these mechanisms applies. It is in fact conceivable that the absence of effect of Li^+ on cellular Ins(3,4)P_2 contents is due to blockade of the production of this isomer by the ion rather than the absence of any effect on its metabolism.

In summary, we have demonstrated that, in GH_3 pituitary tumour cells, TRH elicits rapid changes in the amounts of a wide range of inositol phosphates. The rates of appearance of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are consistent with their proposed roles as second messengers. Moreover, the rates of formation of two novel InsP_2 isomers have been demonstrated for the first time, and provide further insight into the complex pathways by which Ins(1,4,5)P_3 is metabolized. This study adds to the growing number of publications suggesting that the action of Li^+ on inositol phosphate accumulation is more complicated than was originally envisaged.

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