Formation of inositol phosphate isomers in GH3 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 [3H]inositol-labelled GH3 cells were resolved and identified. These cells possess at least ten distinct [3H]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 (InsP) isomers were identified, namely Ins1P and Ins4P, both of which accumulate after stimulation with the hypothalamic releasing factor (TRH) (thyrotropin-releasing hormone). Three inositol bisphosphate (InsP2) isomers were resolved, of which two were positively identified, i.e. Ins(1,4)P2 and Ins(3,4)P2. TRH treatment increases both of these isomers, with Ins(1,4)P2 being produced at a faster rate than Ins(3,4)P2. The third InsP2 isomer has yet to be fully identified, although it is co-eluted with an Ins(4,5)P2 standard. This third InsP2 is also increased after TRH stimulation. In common with other cell types, the GH3 cell contains two inositol phosphatase (InsP3) isomers: Ins(1,4,5)P3, which accumulates rapidly, and Ins(1,3,4)P3, 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 Ins1P, Ins4P, Ins(1,4)P2, the presumed Ins(4,5)P2 and Ins(1,3,4)P3. 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 (PtdInsP2) is the primary step in the signal-transduction mechanism for a class of agonists which increase intracellular Ca2+ (Downes & Michell, 1985). The products of PtdInsP2 cleavage, sn-1,2-diacylglycerol and inositol 1,4,5-trisphosphate [Ins(1,4,5)P3], are known to act as second messengers. Diacylglycerol is an endogenous regulator of protein kinase C activity (Nishizuka, 1984), and Ins(1,4,5)P3 releases Ca2+ from intracellular stores, probably within the endoplasmic reticulum (Downes & Michell, 1985).

Our understanding of the metabolic fate of Ins(1,4,5)P3 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)P3 and Ins(1,3,4)P3], at least one inositol tetrakisphosphate isomer [Ins(1,3,4,5)P4]: Irvine et al. (1984, 1985); Burgess et al. (1985); Batty et al. (1985), several inositol bisphosphate isomers [Ins(1,4)P2, Ins(1,3)P2 and Ins(3,4)P2: 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 Ins1P and Ins4P: Siess (1985); Siess et al. (1986); Balla et al. (1986); Morgan et al. (1987)] have been demonstrated.

The GH3 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 PtdInsP2, which is mirrored in an increase in Ins(1,4,5)P3, diacylglycerol and free intracellular Ca2+ (for a review, see Drummond, 1986). Treatment of GH3 cells with Li+, an inhibitor of Ins(1,4)P2 4-phosphatase and Ins1P phosphatase (Hallcher & Sherman, 1980; Storey et al., 1984), is associated with an augmentation of TRH-stimulated Ins1P, Ins4P and InsP3 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 [3H]inositol-labelled GH3 pituitary tumour cells after stimulation with TRH in the absence or presence of Li+.

EXPERIMENTAL

Culture of GH3 pituitary cells

GH3 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: PtdInsP2, phosphatidylinositol 4,5-bisphosphate; PtdInsP, phosphatidylinositol 4-monophosphate; InsP, Ins1P, Ins4P, myo-inositol monophosphate and its 1- and 4-isomers; InsP2, Ins(1,4)P2, Ins(1,3)P2, Ins(3,4)P2, Ins(4,5)P2, myo-inositol bisphosphate and its 1,4-, 1,3-, 3,4- and 4,5-isomers; InsP3, Ins(1,4,5)P3 and Ins(1,3,4)P3, myo-inositol trisphosphate and its 1,4,5- and 1,3,4-isomers; InsP4, Ins(1,3,4,5)P4, myo-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-[2-3H]inositol (sp. radioactivity 15 Ci/mmole; A.C.S., 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-CaCl₂, 0.5 mM-MgCl₂, 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 x 10⁶ 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) HClO₄. 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 Sharpe & McCollin (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 radio-labelled InsP, InsP₂ and InsP₃ standards after neutralization with Freon/octylamine was usually 80-95%.

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

[3H]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 x 0.49 cm analytical column with a 5 cm x 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 H₃PO₄) 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 radio-labelled inositol phosphate standards

L-myo-[U-14C]inositol 1-monophosphate (sp. radioactivity 55 mCi/mmole), D-myo-[2-3H]inositol 1,4-bisphosphate (sp. radioactivity 1 Ci/mmole) and D-myophosphatidyl ethanolamine, [2-3H]inositol 1,4,5-trisphosphate (sp. radioactivity 1 Ci/mmole) were purchased from Amersham International. [3H]inositol 1,3,4,5-tetrakisphosphate and inositol 1,4,5-[32P]trisphosphate were kindly given by Dr. S. Shears, of the Department of Biochemistry, University of Birmingham. Inositol 1,4,5-[4-3H]trisphosphate and inositol 1,4-[4-3H]bisphosphate were prepared from [3P]-labelled erythrocyte membranes as described by Downes et al. (1982). [3H]Ins(4,5)P₃ was prepared by mild alkaline-phosphatase treatment of [3H]Ins(1,4,5)P₃, 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-MgCl₂, to which 0.1 μCi of [3H]Ins(1,4,5)P₃ 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 [4C]Ins1P and [4C]Ins2P was prepared by heating [4C]Ins1P with 0.1 M-HCl as described by Tomlinson & Ballou (1961). [3H]inositol 4-phosphate was prepared by alkaline hydrolysis of [3H]PtdInsP (Amersham) by the method of Grado & Ballou (1961). Co-elution with known inositol phosphate standards was used to identify tentatively the [3H]inositol-containing peaks eluted from the h.p.l.c. column. The radioactive peaks (e and f, Fig. 1a) eluted with and close to the [3H]- or [3P]-Ins(1,4)P₂ standards 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 GH₄ pituitary tumour cells

The data presented in Fig. 1 show four typical chromatograms of the acid-soluble inositol phosphates present in [3H]inositol-labelled GH₄ cells. Figs. 1(a) and 1(b) depict the inositol phosphates found in unstimulated GH₄ cells in the absence or presence of 10 mM-LI, 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 [3H]inositol-containing peaks was eluted.
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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 GH₃ cells; (b) unstimulated cells after 75 min in the presence of 10 mM-LiCl; (c) GH₃ cells 60 min after the addition of 19 μM-TRH; (d) GH₃ 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, [14C]Ins1P [retention time (Tₑ) = 19 min, 0.175–0.225 M-ammonium formate]; b, [14C]Ins2P (Tₑ = 20.7 min, 0.250–0.325 M-ammonium formate); c, [3H]Ins3P (Tₑ = 21.7 min, 0.300–0.325 M-ammonium formate); d, [3H] or [3H]Ins(1,4)P₃ (Tₑ = 26.8 min, 0.565–0.615 M-ammonium formate); e, the [3H]inositol-containing peak eluted at the position marked 'c' (Tₑ = 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 c was degraded to [3H]threitol, which is a characteristic of Ins(3,4)P₂ (Irvine et al, 1987); f, [3H] or [3H]Ins(4,5)P₃ (Tₑ = 30.8 min; 0.765–0.815 M-ammonium formate); g, this peak (Tₑ = 34.5 min, 0.925–1.025 M-ammonium formate) co-eluted with ATP, a characteristic of Ins(1,3,4)P₃ (Irvine et al., 1985); h, [3H] or [3H]Ins(1,4,5)P₃ (Tₑ = 36.1 min, 1.00–1.115 M-ammonium formate); i, [3H]Ins(1,3,4,5)P₄ (Tₑ = 44.0 min, 1.400–1.500 M-ammonium formate).

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

In GH₃ cells, at least two major InsP isomers were present. The first of these (peak a in Fig. 1a), 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 [14C]Ins1P standard. The second major InsP peak (peak c in Fig. 1a), which typically had a retention time of 21.0–22.5 min, is unlikely to be Ins2P, since a [14C]Ins2P standard (peak b in Fig. 1a) was consistently eluted 30–60 s earlier than this peak (peak c in Fig. 1a). In a separate series of experiments, the inositol monophosphate isomers found in resting and stimulated GH₃ cells were isolated by anion-exchange chromatography on Dowex AG 1-X8 (formate form), freeze-dried, and the individual InsP 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 [14C]Ins1P standard, and the second major InsP peak co-eluted with the [3H]Ins4P.
The GH₃ cells were labelled and stimulated with TRH as described in the text. After termination of the reaction with HClO₄, 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 mean data from six separate experiments: ○, Ins(1,4,5)P₃; ▲, Ins(1,3,4)P₃; ■, Ins(1,3,4,5)P₄. 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 [³²P]-PtdlnsP. A small peak was present between these and co-eluted with a [¹⁴C]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. 1a) co-eluted with a [³²P]Ins4P standard.

Multiple isomers of InsP₃ are present in GH₃ cells. The largest of these (peak d in Fig. 1a) had a retention time of 26.3–27.3 min and co-eluted with an authentic [²³]H- or [³²P]-Ins(1,4)P₃ 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)P₃ (Irvine et al., 1987). The absence of any appreciable [³²P]riboitol associated with this peak indicates that stimulated GH₃ cells contain negligible amounts of Ins(1,3)P₂, which is known to co-elute with Ins(1,4)P₃ in this chromatography system (Irvine et al., 1987). This therefore suggests that the removal of the 4-phosphate from Ins(1,3,4)P₃ is not a significant route of Ins(1,3,4)P₃ metabolism. The second [³²P]inositol-containing peak in this region had a retention time of 28–29.6 min (peak e in Fig. 1a). Since, after periodate oxidation, the radioactivity under this peak was recovered as [³²P]threitol, the most likely identity of this substance is Ins(3,4)P₂, a metabolite of Ins(1,3,4)P₃ (Irvine et al., 1987). At present, the exact identity of the third InsP₃ peak (peak f in Fig. 1a) 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 [³²P]- and [³²H]-Ins(4,5)P₃ standards and, since the other potential metabolites of Ins(1,4,5)P₃ and Ins(1,3,4)P₃ are eluted elsewhere on the chromatographic profile, its most likely identity is Ins(4,5)P₃. Further work is necessary, however, to confirm this designation.

In common with other cell types, the GH₃ cell appears to contain two forms of InsP₃. In unstimulated cells, the later eluted of the [³²H]inositol-containing peaks (peak h in Fig. 1a), which had a retention time in the range 35–37.5 min, was co-eluted with an authentic [³²P]- or [³²H]-Ins(1,4,5)P₃ standard. The Ins(1,4,5)P₃ peak was preceded by a smaller [³²H]inositol-containing peak (peak g in Fig. 1a), which had a retention time in the range 33–35 min, and is most likely to represent the Ins(1,3,4)P₃ isomer. Although an authentic Ins(1,3,4)P₃ standard was not available for direct comparison with the first InsP₃ peak, it was eluted in the same position relative to Ins(1,3,4)P₃ and ATP as reported by Irvine et al. (1985). Under basal conditions in Na-BSS, approx. 90% of the total InsP₃ was in the form of Ins(1,4,5)P₃. The final [³²H]inositol-containing peak eluted from the column (peak i in Fig. 1a), with retention time in the range 41–45 min, co-eluted with an authentic [³²H]Ins(1,3,4,5)P₄ standard.

Kinetics of the accumulation of inositol phosphate isomers in GH₃ cells stimulated with TRH

Experiments were performed to clarify the kinetics of inositol phosphate accumulation in GH₃ 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. 1b and below).

As shown in Fig. 2, the time course of the formation of Ins(1,4,5)P₃ and Ins(1,3,4)P₃ 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)P₃ 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)P₃ 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 InsP₃ remained as the 1,4,5-isomer. In four experiments, there was no significant accumulation of Ins(1,3,4,5)P₄ 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 InsP₃ isomers of preincubating GH₃ 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)P₃. However, in the same experiments, the baseline content of Ins(1,3,4)P₃ 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)P₃ was approx. 3-fold faster in the presence of Li⁺, with a significant increase over baseline being observed within
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Fig. 3. Effect of 10 mM-Li⁺ on the accumulation of Ins(1,3,4)P₃ and Ins(1,4,5)P₃ in TRH-stimulated GH₃ cells

GH₃ 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)P₃; ○, Ins(1,4,5)P₃; △, ○, +10 mM-Li⁺; △, ○, in Na-BSS. The standard errors are omitted for the sake of clarity, but they were typically 10–15%.

15 s of TRH addition; under these conditions and at steady state, the concentration of Ins(1,3,4)P₃ in GH₃ 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 InsP₃ 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)P₄ after TRH stimulation (results not shown).

The data presented in Fig. 4 (black symbols) illustrate the kinetics of the accumulation of the individual InsP₃ isomers. In three experiments, an increase in Ins(1,4)P₃ was observed within 15 s of stimulation, and its content was maximally elevated (2-fold) within 60 s of stimulation. Ins(3,4)P₃ 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)P₃, 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)P₃ accounted for 70% of the total InsP₃ in the cell, Ins(3,4)P₃ for a further 23%, and the remaining 7% was the presumptive Ins(4,5)P₃ 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)P₃ 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)P₃ (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.
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 Ins1P and Ins4P obtained from three separate experiments performed in the absence of Li⁺. There was a rapid increase in the rate of formation of Ins1P that was significant within 15 s of TRH addition. The steady-state content of Ins1P was reached within 30 s of challenge, when its concentration was twice that found in unstimulated controls. In contrast, the rate of accumulation of Ins4P 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 GH₃ cells for 15 min with 10 mM-Li⁺ on the accumulation of Ins1P and Ins4P is shown in Fig. 5 (white symbols). In three experiments the basal content of Ins1P was increased by approx. 50 % (from 5848 ± 508 to 9008 ± 1228 d.p.m.; P < 0.02), whereas the basal concentration of Ins4P 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 InsP 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, Ins1P continued to be produced for approx. 30 min after stimulation, and Ins4P 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 GH₃ cell line, InsP, Ins4P and Ins3P 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 this 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 InsP, Ins3P and Ins4P isomers in GH₃ cells.

Stimulation of GH₃ cells was associated with the rapid accumulation of Ins(1,4,5)P₃, which was followed by a parallel increase in Ins(1,3,4,5)P₄ and Ins(1,3,4,5,6)P₅. In Na⁺-containing balanced salt solution, Ins(1,4,5)P₃ was always the predominant InsP, species obtained, although in the presence of Li⁺ Ins(1,3,4)P₃ 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)P₃ probably represents the time taken for a proportion of the newly formed Ins(1,4,5)P₃ to be shunted through the InsP₃/InsP₄ pathway. Similar observations have been made in the closely related GH₄C₁ 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)P₃ 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 InsP₄ isomers in TRH-stimulated GH₃ cells in the presence of Li⁺ ions, and suggested that they included Ins(1,4)P₄, Ins(2,4)P₄ and, possibly, Ins(4,5)P₅ in (order of their increasing time retention). Additionally, they suggested that only Ins(1,4)P₄ 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 GH₃-cell preparations, the major InsP₄ species detected was Ins(1,4)P₄, 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 InsP₄ species emerged close to an Ins(2,4)P₄ standard. We suggest, however, that the true identity of this peak is Ins(3,4)P₄. If it was indeed Ins(2,4)P₄, 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 InsP₄ peak yielded threitol, however, suggesting its most likely identity to be Ins(3,4)P₄. Recent work in homogenates of liver (Shears et al., 1987), rat brain (Erneux et al., 1987) and GH₄C₁ cells (Irvine et al., 1987), suggests that Ins(1,3,4)P₃ is degraded predominantly to Ins(3,4)P₄. Our work confirms the existence of this isomer in intact cells and indicates the kinetics of its
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...accumulation after agonist stimulation. The demonstration of the existence of a third InsP₅ 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)P₂ 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 GH₃-cell preparations (F. Ruiz-Larrea & A. H. Drummond, unpublished work) indicates that, in the absence of added ATP, [3H]Ins(1,4,5)P₃ is metabolized to a small extent to a substance with identical elution characteristics to the third-emerging cellular InsP₅. This suggests that Ins(1,4,5)P₃ is the likely precursor for this InsP₅ 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)P₄ 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)P₂ 4-phosphatase and the apparent insensitivity of Ins4P 4-phosphatase and Ins(1,4)P₂ 1-phosphatase to inhibition by Li⁺ has been well documented (Hallicher & 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 InsP isomer formed after receptor stimulation is Ins4P. Moreover, in brain homogenates, Ins(1,4)P₂ is metabolized largely, if not exclusively, to Ins4P (Ackermann et al., 1987; Inhorn et al., 1987). Preliminary experiments with GH₃ cells suggest that Ins(1,4)P₂ is also metabolized solely to Ins4P (F. Ruiz Larrea & A. H. Drummond, unpublished work). However, in the present study with intact GH₃ 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 GH₃ 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 (i.e. Ins1P), an Ins(3,4)P₂ metabolite, and perhaps also by Ins5P (which is present in 10-fold lower amounts than the major monophosphates in GH₃ 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 InsP₅ is observed, and demonstrates that, in common with other cell types, the sole effect is on the Ins(1,3,4,5)P₄ 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)P₂ 5-phosphomonoesterase of rat brain, delvaux et al., (1987), rat liver (Storey et al., 1984) or GH₃ 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)P₄ 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)P₂ is unclear. In rat brain (Erneux et al., 1987) and rat liver homogenates (Hansen et al., 1986), Li⁺ inhibits the Ins(1,3,4)P₂ phosphatase directly. However, Shears et al. (1987) have demonstrated that the Ins(1,3,4)P₂ phosphatase in rat liver is not sensitive to Li⁺ alone, but is inhibited by a combination of Li⁺ and Ins(1,4)P₂. This raises the possibility that, in the presence of Li⁺, the concentration of Ins(1,4)P₂ can be high enough for product inhibition to occur. Further work will be necessary on GH₃ cells to ascertain which of these mechanisms applies. It is in fact conceivable that the absence of effect of Li⁺ on cellular Ins(3,4,5)P₄ 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 GH₃ 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)P₃ and Ins(1,3,4,5)P₄ are consistent with their proposed roles as second messengers. Moreover, the rates of formation of two novel InsP₅ isomers have been demonstrated for the first time, and provide further insight into the complex pathways by which Ins(1,4,5)P₃ 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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