Separation of multiple isomers of inositol phosphates formed in GH₃ cells

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A high-performance-liquid-chromatography (h.p.l.c.) separation was developed, which resolves isomers of inositol monophosphate (IP), inositol biphosphate (IP₂), and inositol trisphosphate (IP₃) in a single run. In GH₃ cells labelled with [³H]inositol, treated with Li⁺ and thyrotropin-releasing hormone (TRH), radiolabelled components identified as inositol 1-phosphate (I1P), inositol 2-phosphate (I2P), inositol 4-phosphate (I4P), inositol 1,4-bisphosphate [I(1,4)P₂], inositol 1,3,4-trisphosphate [I(1,3,4)P₃] and inositol 1,4,5-trisphosphate [I(1,4,5)P₃] are present, as are multiple unidentified IP₂ peaks. After TRH stimulation, both I1P and I4P increase, the increase in I4P preceding that of I1P; I(1,4)P₂ and an unknown IP₂ increase; and both I(1,3,4)P₃ and I(1,4,5)P₃ increase, the increase in I(1,4,5)P₃ being rapid and transient, whereas the increase in I(1,3,4)P₃ is slower and more sustained. The most rapidly appearing inositol phosphates produced after TRH stimulation are I(1,4)P₂ and I(1,4,5)P₃.

INTRODUCTION

The phosphatidylinositol (PI) cycle plays a key role in signal transduction. Two components of the cycle, formed by the action of phospholipase C (PLC) on phosphatidylinositol biphosphate (PIP₂), are inositol trisphosphate (IP₃) and diacylglycerol (DAG). These compounds function as second messengers, IP₃ releasing intracellular Ca²⁺ and DAG activating protein kinase C [1,2]. The low amounts of inositol phosphates and inositol lipids in cells make chemical measurements difficult. Consequently the method of choice for investigating PI turnover has been to prelabel cells with radiolabelled inositol and to extract and separate the radiolabelled phospholipids and the radiolabelled inositol phosphates. Separation of the inositol phosphates is readily achieved on small anion-exchange columns by the method of Ellis *et al.* [3] as modified by Berridge *et al.* [4]. Many studies have been performed by this technique, which, although effective in monitoring PI turnover, gives no indication of the isomers of the inositol phosphates produced. However, multiple isomers of inositol monophosphate (IP) [5] and IP₃ [6] have been described, which appear to be widespread [7,8,9]. These observations highlight the need for the use of more sophisticated analytical techniques for the study of PI turnover.

H.p.l.c. has been used for separating isomers of IP [10], isomers of IP₃ [8] and cyclic from non-cyclic inositol phosphates [11]. However, a single procedure capable of separating all of these has not been described. We report here a separation based on anion exchange which resolves 14 different inositol-containing standards and a number of unknown compounds present in cells. The cell line used in this study is the GH₃ rat pituitary tumour

line, in which enhanced PI turnover in response to thyrotropin-releasing hormone (TRH; thyroliberin) has been well documented [12,13]. Formation of multiple isomers of the inositol phosphates by this cell line has not been previously documented. The h.p.l.c. separation revealed an unexpected heterogeneity of inositol phosphates in these cells, including the formation of two isomers of IP₃ with different kinetics.

The use of this separation will provide more detailed information about routes of phosphorylation/dephosphorylation of the inositol phosphates, and lead to a greater understanding of the relative importance of the individual inositol phosphate isomers.

EXPERIMENTAL

Materials

myo-[2-³H]Inositol (13.8 Ci/mmol), *L*-*myo*-[U-¹⁴C]-inositol 1-phosphate (55 mCi/mmol) ([¹⁴C]I1P) and *D*-*myo*-[2-³H]inositol 1,4,5-trisphosphate (1.0 Ci/mmol) {[³H]I(1,4,5)P₃} were obtained from Amersham International. *myo*-[³H]Inositol (10–20 Ci/mmol), *D*-[2-³H]-inositol 1,4-bisphosphate (2.0 Ci/mmol), {[³H]I(1,4)P₂}, *D*-[2-³H]inositol 1,4,5-trisphosphate (3.6 Ci/mmol), phosphatidyl[1,2-³H]inositol (8.4 Ci/mmol) ([³H]PI), phosphatidyl[1,2-³H]inositol 4-phosphate (1.0 Ci/mmol) ([³H]PIP) and phosphatidyl[1,2-³H]inositol 4,5-bisphosphate (2.0 Ci/mmol) ([³H]PIP₂) were obtained from New England Nuclear.

Preparation of [³H]inositol-labelled standards

[³H]Glycerophosphoinositol ([³H]GPI), [³H]glycerophosphoinositol phosphate ([³H]GPIP) and [³H]glycero-

Abbreviations used: BSS, balanced salt solution; DAG, diacylglycerol; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositol phosphate; GPIP₂, glycerophosphoinositol biphosphate; IP, inositol monophosphate; IP₂, inositol biphosphate; IP₃, inositol trisphosphate; IP₄, inositol tetrakisphosphate; IP₅, inositol pentakisphosphate; IP₆, inositol hexakisphosphate; I (1,2-cyc)P, inositol 1,2-cyclic monophosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP₂, phosphatidylinositol biphosphate; PLC, phospholipase C; TRH, thyrotropin-releasing hormone (Glp-His-Pro-NH₂).

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phosphoinositol bisphosphate ($[^3\text{H}]\text{GPIP}_2$) were prepared by the mild alkaline hydrolysis of $[^3\text{H}]\text{PI}$, $[^3\text{H}]\text{PIP}$ and $[^3\text{H}]\text{PIP}_2$ respectively [14]. $[^3\text{H}]\text{Inositol 1,2-cyclic monophosphate } \{[^3\text{H}]\text{I(1,2-cyc)P}\}$ was prepared by treating $[^3\text{H}]\text{PI}$ with a crude soluble phospholipase C preparation obtained from rat liver [15,16]. The resulting mixture of $[^3\text{H}]\text{I(1,2-cyc)P}$ and $[^3\text{H}]\text{IP}$ was separated by h.p.l.c. (see below), and the $[^3\text{H}]\text{I(1,2-cyc)P}$ was stored at -20°C . A mixture of $[^3\text{H}]\text{inositol 1-phosphate } ([^3\text{H}]\text{I1P})$ and $[^3\text{H}]\text{inositol 2-phosphate } ([^3\text{H}]\text{I2P})$ was prepared by heating $[^3\text{H}]\text{I(1,2 cyc)P}$ at 100°C for 15 min in 0.1 M-HCl [17].

Alkaline hydrolysis of ^3H -labelled inositol lipids was used to prepare different isomers of inositol phosphates. Alkaline hydrolysis of PI yields a mixture of I1P and I2P; that of phosphatidylinositol 4-phosphate (PIP) a mixture of I(1,4) P_2 , inositol 2,4-bisphosphate [I(2,4) P_2] and I4P; that of phosphatidylinositol 4,5-bisphosphate (PIP_2) a mixture of I(1,4,5) P_3 , inositol 2,4,5-trisphosphate [I(2,4,5) P_3] and inositol 4,5-bisphosphate [I(4,5) P_2] [18,19]. Briefly, 1 mg of PI, PIP or PIP_2 (Sigma) were added to 5 μCi of $[^3\text{H}]\text{PI}$, $[^3\text{H}]\text{PIP}$ or $[^3\text{H}]\text{PIP}_2$ respectively, and solvent was removed under N_2 . The samples were boiled in 2 ml of 1 M-KOH for 1 h. The resulting solutions were cooled, acidified with formic acid, washed twice with hexane and neutralized with saturated ammonia solution. This was diluted to 400 ml with distilled water and separated into IP, IP_2 and IP_3 fractions by anion-exchange chromatography [6]. These were freeze-dried, reconstituted in distilled water, and isomers were separated by h.p.l.c. $[^3\text{H}]\text{Inositol 1,3,4-trisphosphate } \{[^3\text{H}]\text{I(1,3,4)P}_3\}$ was prepared from differentiated HL-60 cells stimulated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma) for 60 s exactly as described by Burgess *et al.* [7] and separated from I(1,4,5) P_3 by h.p.l.c. $[^3\text{H}]\text{I1P}$, $[^3\text{H}]\text{I(1,4)P}_2$ and $[^3\text{H}]\text{I(1,4,5)P}_3$ were identified by co-elution with commercial standards. By deduction, I(2,4) P_2 is the IP_2 isomer formed from PIP hydrolysis that is not co-eluted with I(1,4) P_2 ; similarly I(2,4,5) P_3 is the IP_3 isomer formed from PIP_2 hydrolysis not co-eluted with I(1,4,5) P_3 . I(4,5) P_2 is the only IP_2 isomer formed by hydrolysis of PIP_2 [18,19].

Separation by h.p.l.c.

A mixture of inositol phosphates could be separated on a Whatman Partisil 10 SAX column (25 cm $\times$ 0.46 cm) with a pre-column containing Whatman pellicular anion-exchange resin, with ammonium phosphate, pH 3.8, flow rate 1 ml/min. Typically, the gradients used were 0.01–0.08 M over 30 min for $[^3\text{H}]\text{inositol}$, $[^3\text{H}]\text{GPI}$, $[^3\text{H}]\text{I(1,2-cyc)P}$, $[^3\text{H}]\text{I1P}$, $[^3\text{H}]\text{I2P}$ and $[^3\text{H}]\text{I4P}$; 0.2–0.28 M over 30 min for $[^3\text{H}]\text{GPIP}$, $[^3\text{H}]\text{I(1,4)P}_2$, $[^3\text{H}]\text{I(2,4)P}_2$ and $[^3\text{H}]\text{I(4,5)P}_2$; and 0.5–0.52 M over 30 min for $[^3\text{H}]\text{GPIP}_2$, $[^3\text{H}]\text{I(1,4,5)P}_3$ and $[^3\text{H}]\text{I(2,4,5)P}_3$. Individual isomers were recovered from the column and stored frozen. A mixture of all the above compounds was prepared which could be resolved by consecutively applying the three gradients described and collecting 0.5 ml fractions (Fig. 1). Each fraction was mixed with 6 ml of aqueous counting scintillant (Amersham) containing 5% water, and radioactivity was determined by liquid-scintillation counting. Recoveries of standards were greater than 78%, as determined by recovery of radioactivity.

GH₃ cells

To identify the inositol phosphate isomers present in GH₃ cells, a large extract of $[^3\text{H}]\text{inositol}$ -labelled cells was prepared. GH₃ cells, obtained from American Type Culture Collection, Rockville, MD, U.S.A., were grown in 150 cm² flasks in Ham's F-10 media (Advanced Biotechnologies Inc., Silver Spring, MD, U.S.A.) with 15% horse serum and 2.5% fetal-calf serum, labelled for 72 h with 100 μCi of *myo*- $[^3\text{H}]\text{inositol/flask}$, washed once with 10 ml of balanced salt solution (BSS) [12], and then suspended in 10 ml of BSS by using a cell scraper. Cells were collected by centrifugation (2 min at 2500 rev./min) and washed twice before being resuspended in 10 ml of BSS containing 10 mM-LiCl. After incubation at 37°C for 15 min, TRH (Calbiochem Behring) was added to give a final concentration of 1 μM . After 5 min the cells were centrifuged (15600 *g* for 10 s), supernatant was removed and 2 ml of cold 5% trichloroacetic acid added. Cells were removed by centrifugation (15600 *g* for 10 s), the supernatant was collected and trichloroacetic acid removed by four extractions with water-saturated diethyl ether. IP, IP_2 and IP_3 were obtained by the method of Berridge *et al.* [4]. Samples were freeze-dried, reconstituted in distilled water and stored frozen.

Kinetics of inositol phosphate production

GH₃ cells were grown, labelled with $[^3\text{H}]\text{inositol}$, washed and incubated in BSS with 10 mM-LiCl as previously described. Cell suspension (0.5 ml) was added to 1.5 ml plastic tubes and either 5 μl of 100 μM -TRH (final concn. 1 μM) or 5 μl of saline (0.9% NaCl) added. In separate experiments the reaction was terminated at the required time points by one of two ways; either by Method (i), the addition of 25 μl of 100% trichloroacetic acid (final concn. 5% trichloroacetic acid) directly to the cell suspension, or by Method (ii), addition of 0.9 ml of ice-cold 0.9% NaCl followed by centrifugation, aspiration of supernatant and the addition of 0.3 ml of 5% trichloroacetic acid. In both cases, after vortex-mixing the insoluble material was separated by centrifugation, the supernatant removed, neutralized, freeze-dried, reconstituted in distilled water and stored at -20°C until separation by h.p.l.c.

Alkaline phosphatase treatment

Neutralized trichloroacetic acid extract from GH₃ cells (25 μl), prepared as described above, was mixed with 5 μl of 10% (v/v) ethanolamine containing 390 units of bovine alkaline phosphatase (Sigma)/ml and incubated at 37°C for 3 h.

Descending paper chromatography

Isomers of inositol phosphates were separated by descending paper chromatography on Whatman no. 1 paper with propan-2-ol/ NH_3 (58%)/water (7:1:2, by vol.) for 10–30 days [19]. Chromatography paper was cut into 1 cm strips, the radioactive standards were eluted into 1 ml of distilled water and radioactivity was counted. Recoveries of standards were greater than 90%. Resolution of some standards was possible, $[^3\text{H}]\text{I4P}$ and $[^3\text{H}]\text{I2P}$ were partially co-eluted, running ahead of and distinct from $[^3\text{H}]\text{I1P}$; $[^3\text{H}]\text{I(1,4)P}_2$, $[^3\text{H}]\text{I(2,4)P}_2$ and $[^3\text{H}]\text{I(4,5)P}_2$ all separate; and $[^3\text{H}]\text{I(2,4,5)P}_3$ runs ahead of $[^3\text{H}]\text{I(1,4,5)P}_3$ and $[^3\text{H}]\text{I(1,3,4)P}_3$, which are partially co-eluted.

RESULTS

The separation described allows the resolution of isomers of IP, IP₂ and IP₃, as well as cyclic and deacetylated phosphatidylinositol compounds, by a three-step gradient (Fig. 1). Retention times are reproducible; differences in elution volumes of consecutive injections range from 0.5 to 1.5 ml ($n = 7$). Separations of extracts of [³H]inositol-labelled GH₃ cells reveal the presence of isomers not resolved by the commonly used separation of inositol phosphates on small Dowex columns (Fig. 2). To characterize these compounds more fully, IP, IP₂ and IP₃ fractions were obtained from GH₃ cells [4] which had been stimulated for 5 min with TRH in the presence of 10 mM-LiCl. These fractions could be separated into multiple peaks by h.p.l.c. revealing two major and one minor IP peaks, one major and several minor IP₂ peaks and two IP₃ peaks. The major peaks were collected and rechromatographed either by h.p.l.c. concurrently with standards or by descending paper chromatography. On h.p.l.c. the two major IP peaks co-eluted with [³H]IIP and [³H]I4P and the minor peak with [³H]I2P. The major IP₂ peak co-eluted with [³H]I(1,4)P₂ and two of the minor peaks had similar retention times to [³H]I(2,4)P₂ and [³H]I(4,5)P₂. Small IP₂ peaks with unique retention times were also observed. The earlier of the two IP₃ peaks co-eluted with [³H]I(1,3,4)P₃ and the later with [³H]I(1,4,5)P₃. Small amounts of GPI were present, which remained unchanged, and no I(1,2-cyc)P or unidentified peaks, which may represent the cyclic polyphosphate compounds described by Wilson *et al.* [11,20] were observed, even in cells extracted in neutral (chloroform/methanol) conditions.

Descending paper chromatography, which enables partial isomer separation, was used to confirm the identity of individual isomers. IIP, I4P, I(1,4)P₂ and I(1,4,5)P₃ extracted from GH₃ cells and separated by h.p.l.c. all have R_F values identical with their respective

Fig. 1. Separation of prepared [³H]inositol-labelled standards by h.p.l.c.

A mixture containing radiolabelled inositol, GPI, I(1,2-cyc)P, IIP, I2P, I4P, GPIP, I(1,4)P₂, I(2,4)P₂, I(4,5)P₂, GPIP₂, I(1,4,5)P₃, and I(2,4,5)P₃ was separated on a Whatman Partisil 10 SAX column (25 cm × 0.46 cm). Elution conditions are described in the Experimental section: the fraction size was 500 μl.

Fig. 2. Separation by h.p.l.c. of [³H]inositol-labelled compounds formed in GH₃ cells stimulated with 1 μM-TRH

Cells were extracted by Method (ii) (see the Experimental section) and the extracts chromatographed as in Fig. 1. I(1,3,4)P₃ is eluted in fractions 150–155, ahead of I(1,4,5)P₃. (a) Unstimulated cells; (b) 30 s and (c) 3 min TRH treatment.

standards when rechromatographed on paper as described.

To confirm further the identity of the radiolabelled components in GH₃ cell extracts, an extract was treated with alkaline phosphatase as described in the Experimental section. In comparison with untreated extract, alkaline phosphatase eliminated > 90% of the radiolabel associated with IP, IP₂ and IP₃ when separated by h.p.l.c.

Fig. 3. Accumulation of [^3H]inositol phosphates in [^3H]inositol-labelled GH $_3$ cells in response to 1 μM -TRH

Cells were extracted by Method (ii), except for (d), where Method (i) was used (see the Experimental section). Each point represents the mean of two separate samples in a single typical experiment. (a) IP: I1P, $\circ$ (+TRH), $\bullet$ (control); I4P, $\triangle$ (+TRH), $\blacktriangle$ (control). (b) IP $_2$: I(1,4)P $_2$, $\circ$ (+TRH), $\bullet$ (control). (c) IP $_3$: I(1,3,4)P $_3$, $\circ$ (+TRH), $\bullet$ (control); I(1,4,5)P $_3$, $\triangle$ (+TRH), $\blacktriangle$ (control). (d) IP $_3$: I(1,3,4)P $_3$, $\circ$ (+TRH), $\bullet$ (control); I(1,4,5)P $_3$, $\triangle$ (+TRH), $\blacktriangle$ (control).

Profiles of h.p.l.c. separations of inositol phosphates obtained from GH $_3$ cells stimulated with TRH for different time points are shown in Fig. 2. The kinetics of inositol phosphate formation from typical experiments are shown in Fig. 3. Experiments were normally terminated by the addition of trichloroacetic acid to cells after the removal of medium, allowing for the removal of salts which otherwise interfere with the h.p.l.c. separation (causing co-elution of I1P and I2P). This however, does not allow for reliably obtaining samples at very early times. To examine very early effects of TRH (< 30 s) on IP $_3$ 100% trichloroacetic acid was added directly to the cell suspension, to a final concentration of 5% (Fig. 3d).

Three inositol monophosphate peaks are present in unstimulated cells, with elution characteristics of I1P, I2P and I4P (Fig. 2a). Both I1P and I4P increase after TRH stimulation; however, I2P remains unchanged. I4P increases very rapidly, showing a 4-fold increase after 30 s, and continues to increase for 30 min (Fig. 3a). In contrast, I1P content is unchanged after 30 s exposure to TRH (Fig. 2b) and only begins to rise 1.5 min after TRH treatment.

In unstimulated cells IP $_2$ appears to have multiple components, the largest of which does not correspond to any prepared standard (Fig. 2a). The second largest peak is I(1,4)P $_2$, and two other peaks are present, one with a

similar retention time to I(2,4)P $_2$. The component exhibiting the largest increase after TRH treatment is I(1,4)P $_2$. It increases 3-fold after only 1 s and continues to increase linearly for 3 min before gradually falling (Fig. 3b). Another peak not seen in resting cells with a shorter retention time than I(1,4)P $_2$ appears on stimulation, which increases to become the second largest IP $_2$ component after 3 min (Fig. 2c). These multiple components not co-eluted with I(1,4)P $_2$ are present in relatively low amounts ($\sim 10\%$ of total IP $_2$ radioactivity after 3 min stimulation) and do not change as dramatically as I(1,4)P $_2$; consequently they were not further characterized. Therefore, although described as IP $_2$ isomers, owing to their elution positions relative to I(1,4)P $_2$, further characterization is necessary.

Both I(1,3,4)P $_3$ and I(1,4,5)P $_3$ are present in resting cells (Fig. 2a). I(1,4,5)P $_3$ increases immediately on stimulation and is already maximally elevated (8–10 times control) at the earliest time point obtained (1–2 s) (Fig. 3d). The concentration of I(1,4,5)P $_3$ falls to around half this by 30 s, but remains elevated above control values for at least 30 min (Fig. 3c). In contrast, I(1,3,4)P $_3$ shows no increase during the first 30 s of stimulation and gradually increases to a maximum of 3 times control after 3 min. This is followed by a gradual decrease over the next 30 min to a value twice that of control (Fig. 3c).

DISCUSSION

It is increasingly evident that the metabolism of *myo*-inositol-containing compounds by cells is more complex than had been previously described. The first indication that the dephosphorylation of IP_3 to IP_2 to IP by the sequential removal of the 5- and then the 4-phosphate groups was not all that was occurring was the observations of multiple isomers of IP [5,21] and of IP_3 [6–8]. Furthermore, more highly phosphorylated inositol phosphates, IP_4 , IP_5 and IP_6 , have also been described in animal tissue [22–24], and 1,2-cyclic forms of both $I(1,4)P_2$ and $I(1,4,5)P_3$ have been described as products of PLC hydrolysis of PIP and PIP_2 and may exist in cells [20].

Using h.p.l.c. methodology, we investigated the response of the GH_3 rat pituitary tumour-cell line to TRH. TRH increases PI turnover and the production of inositol phosphates in this cell line [25,26]. However, the isomers produced were previously unknown, and in some cases even erroneously designated [27,28]. We demonstrate here a heterogeneity of inositol phosphates, many of which are identified by co-elution with prepared radiolabelled standards.

In GH_3 cells IIP and I4P increase in response to TRH, with I2P remaining constant. I4P increases very rapidly and is elevated after only 30 s, whereas IIP only increases after 90 s (Fig. 3a). Therefore, hydrolysis of PI by PLC is not occurring at early times after stimulation, as that would lead to the formation of IIP and not I4P, which must be formed by the action of a phosphatase on $I(1,4)P_2$. The presence of multiple IP_2 peaks in cells was unexpected; however, the only component substantially increased after TRH treatment was $I(1,4)P_2$. The rapid appearance of this isomer may indicate hydrolysis of PIP or the very rapid dephosphorylation of IP_3 .

Two isomers of IP_3 are formed at very different rates after exposure to TRH. Accumulation of $I(1,4,5)P_3$ is very rapid after stimulation, whereas the concentration of $I(1,3,4)P_3$ remains stable over the first 30 s of stimulation (Fig. 3d) after which it gradually increases to around 3-fold control values after 3 min. Similarly, the increase in $I(1,4,5)P_3$ precedes that of $I(1,3,4)P_3$ in rat parotid gland [8], guinea-pig hepatocytes and HL-60 cells [7]. The rapid but transient increase in $I(1,4,5)P_3$ is consistent with its proposed role as a Ca^{2+} -releasing agent. Measurements of Ca^{2+} concentrations in GH cells have demonstrated a biphasic response to TRH [29,30]: an early spike phase ($t_{1/2}$ of 8 s), which is caused by the release of intracellular Ca^{2+} , and a later plateau of increased Ca^{2+} (30 s–20 min), which is dependent on the entrance of extracellular Ca^{2+} into the cell. In these studies measurements of total IP_3 concentrations [a mixture of $I(1,4,5)P_3$ and $I(1,3,4)P_3$] revealed a close correlation with the initial spike phase. The present study shows that at these early time points IP_3 is predominantly $I(1,4,5)P_3$, whereas at later time points amounts of $I(1,3,4)P_3$ are higher (Fig. 3d). This may be indicative of a functional dichotomy of the IP_3 isomers.

It has been proposed that $I(1,3,4)P_3$ is formed by the action of a phosphatase on $I(1,3,4,5)P_4$ [31,32]. By extending and increasing the gradient of our system, we have been able to detect more polar [3H]inositol-labelled compounds present in GH_3 cells (N.M. Dean & J.D. Moyer, unpublished work), which are probably IP_4 , IP_5 and IP_6 , previously reported in GH_4 cells [22].

GH_3 cells appear to contain inositol phosphates which are co-eluted with authentic I2P and $I(2,4)P_2$. If these are inositol isomers phosphorylated in the 2 position, then their source is unclear. It is unlikely that they are artifacts caused by the extraction procedure, either by the acid-mediated hydrolysis of (1,2)-cyclic compounds, or the acid-catalysed migration of phosphate groups, as the I2P and $I(2,4)P_2$ peaks are both present in extracts obtained under neutral conditions, under which cyclic compounds are stable. I2P and $I(2,4)P_2$ are present in relatively large amounts in resting cells; however, their amounts do not change after stimulation. It is possible that there is a gradual non-specific dephosphorylation of higher inositol phosphates (IP_5 and IP_6) in resting cells. The 2-phosphate position may be less susceptible to phosphatase action, and consequently these isomers would gradually accumulate. The inability to detect IP_3 isomers phosphorylated at the 2 position could be due to the rapid removal of other phosphates, resulting in amounts below the limit of detection.

In conclusion, h.p.l.c. can be used for routine monitoring of the concentrations of different isomers of inositol phosphates in radiolabelled GH_3 cells. Initial studies in mast cells, B lymphocytes and fibroblasts have revealed marked differences in the profiles of inositol phosphates present. It will be possible to correlate changes in the concentration of specific inositol compounds with biological changes in the cell in response to stimuli. If other inositol phosphates apart from $I(1,4,5)P_3$ have a biological role, then the routes and relative rates of phosphorylation/dephosphorylation may determine the nature and extent of the cells' response to stimulation.

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