Subsecond and second changes in inositol polyphosphates in GH₄C₁ cells induced by thyrotropin-releasing hormone

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It has been demonstrated previously that thyrotropin-releasing hormone (TRH) induces changes in inositol polyphosphates in the GH₃ and GH₄C₁ strains of rat pituitary cells within 2.5–5.0 s. TRH also causes a rapid rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in these cells which is due largely to redistribution of cellular calcium stores. Therefore, it has been concluded that TRH acts to release sequestered calcium in these cells via enhanced generation of inositol 1,4,5-trisphosphate $[Ins(1,4,5)P_3]$. If this conclusion were correct, TRH-enhanced accumulation of $Ins(1,4,5)P_3$ should occur at least as rapidly as the increase in $[Ca^{2+}]_i$. We have shown previously that the rise in $[Ca^{2+}]_i$ induced by TRH occurs within about 400 ms; thus, it was important to investigate the subsecond time-course of changes in inositol phosphates caused by TRH. Using a rapid mixing device, we have measured changes in inositol polyphosphates on a subsecond time scale in GH₄C₁ cells prelabelled with *myo*-[2-³H]inositol. Although TRH did alter inositol polyphosphate metabolism within 500 ms, the changes observed did not reveal a statistically significant increase in $Ins(1,4,5)P_3$ within time intervals of less than 1000 ms. Thus, we have been unable to demonstrate that a TRH-induced rise in $Ins(1,4,5)P_3$ precedes or occurs concomitantly with the rise in $[Ca^{2+}]_i$ in GH₄C₁ cells. Although these results do not disprove the current view that $Ins(1,4,5)P_3$ mediates the action of TRH on intracellular calcium redistribution, we conclude that caution should be exercised in this, and possibly other cell systems, in accepting the dogma that all of the rapid, agonist-induced redistributions of intracellular calcium are mediated by $Ins(1,4,5)P_3$.

INTRODUCTION

It is now widely held that agonist-induced redistribution of intracellular calcium is mediated by the rapid generation of $Ins(1,4,5)P_3$ which then acts to release sequestered calcium into the cytosol (Berridge & Irvine, 1984; Fain, 1984; Downes & Michell, 1985; Hokin, 1985). Some of the evidence consistent with this view has been derived from studies on the GH strains of hormone-responsive rat pituitary cells (Tashjian, 1979). These cells respond to the tripeptide TRH by a receptor-mediated activation of phospholipase C which causes hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield $Ins(1,4,5)P_3$ (Martin, 1983; Macphée & Drummond, 1984; Gershengorn, 1985). The most rapid increases in accumulation of $Ins(1,4,5)P_3$ reported in intact GH₃ or GH₄C₁ cells induced by TRH have been 1.5 s (Martin, 1983; Gershengorn, 1985; Heslop *et al.*, 1985). Exogenous $Ins(1,4,5)P_3$ has also been shown to mobilize cellular calcium stores in permeabilized GH₃ cells (Gershengorn *et al.*, 1984; Biden *et al.*, 1986). Using quin2-loaded GH₄C₁ cells, Albert & Tashjian (1984a) have shown biphasic changes in $[Ca^{2+}]_i$ induced by TRH. The rapid upstroke of the rising phase of the TRH-induced burst in $[Ca^{2+}]_i$ is observed within 400 ms of addition of TRH to the cuvette containing quin2-

loaded GH₄C₁ cells (Albert & Tashjian, 1984b). Thus, on the basis of available data, a rise in $[Ca^{2+}]_i$ induced by TRH might precede the rise in $Ins(1,4,5)P_3$. If an increase in cellular $Ins(1,4,5)P_3$ is the mediator of the TRH-induced rise in $[Ca^{2+}]_i$ due to release of sequestered intracellular calcium, it would be expected that $Ins(1,4,5)P_3$ should be generated in response to TRH receptor occupancy at least as rapidly as the increase in $[Ca^{2+}]_i$.

The present experiments, therefore, were undertaken to examine the subsecond kinetics of TRH-induced changes in inositol polyphosphates in intact GH₄C₁ cells. Under conditions of rapid mixing of TRH and the cells, we find no increase in $Ins(1,4,5)P_3$ before at least 1000 ms, a time interval well after TRH has caused a rise in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Cell culture

The general methods of cell culture have been described in detail previously (Albert & Tashjian, 1984a,b). In brief, the GH₄C₁ strain of rat pituitary cells were grown in 100 mm plastic dishes with 8 ml of Eagle's minimum essential medium supplemented with 11% foetal bovine serum. For the last 72–96 h before

Abbreviations used: TRH, thyrotropin-releasing hormone; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$, inositol mono-, bis-, tris- and tetrakis-phosphates, respectively; locants where appropriate are in parentheses, e.g. $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate.

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harvest, fresh medium was added which contained 72 μCi of *myo*-[2- ^3H]inositol per dish. Cells washed with nonradioactive culture medium were harvested in Ca^{2+} -free assay medium containing 0.02% EDTA, washed twice in assay medium (118 mM-NaCl, 4.6 mM-KCl, 1.0 mM- CaCl_2 , 10 mM-D-glucose and 20 mM-Hepes, pH 7.2), and resuspended in assay medium. The cell suspension $[(5-10) \times 10^6 \text{ cells/ml}]$ was placed in a plastic tube in crushed ice and gently agitated before sampling.

Stimulation of TRH

Aliquots (100 μl) of the labelled cell suspension were warmed to 37 °C for 2 min and mixed rapidly with 200 μl of TRH (final concentration $1 \times 10^{-6} \text{ M}$). The reaction was stopped by rapid addition of 300 μl of 10% (w/v) trichloroacetic acid, and the mixture was allowed to extract for 5–10 min on ice. Rapid application and mixing of TRH and cells followed by quenching of the reaction with trichloroacetic acid at short time intervals were made by using an apparatus described previously (Berridge *et al.*, 1984). In brief, electronically controlled solenoids allowed a high-pressure stream of air to drive the small volume of TRH solution into the cell suspension with nearly instantaneous mixing followed by a second jet stream which drove and mixed the trichloroacetic acid solution. The original instrument was modified slightly to permit intervals between agonist and quench solutions to be as short as 100 ms. In all experiments described in this report, the zero time control value was obtained by injecting into the cell suspension a mixture of TRH and trichloroacetic acid to give the same final concentrations of each reagent as occurred when these materials were injected sequentially. The zero time control values obtained by this technique did not differ significantly ($P > 0.5$) from those obtained by application of trichloroacetic acid alone or sequential application of the TRH vehicle (deionized water) and trichloroacetic acid.

Most experiments were performed in assay medium as described above. In experiments using Li^+ , the harvested cells were preincubated with 10 mM-LiCl for 10 min at 37 °C immediately prior to stimulation with TRH. Li^+ was present during stimulation as well as in the preincubation period.

Analytical methods

Separation of inositol phosphates was achieved by the h.p.l.c. technique described by Irvine *et al.* (1985). All samples were filtered through a 0.45 μm filter (ACRO LC13, Gelman Sciences). Separation of inositol phosphates was achieved on a Partisil 10 SAX column (Technicol, Higher Hillgate, Stockport, Cheshire, U.K.) using a linear gradient from water to 0.86 M-ammonium formate (buffered to pH 3.7 with phosphoric acid). The flow rate was 2.5 ml/min and fractions were collected for liquid scintillation counting at 30 s intervals. The [^3H]Ins(1,4,5) P_3 peak co-chromatographed with [^{32}P]Ins(1,4,5) P_3 (Downes *et al.*, 1982) and the preceding [^3H]Ins(1,3,4) P_3 peak eluted with the ATP marker. The Ins P_2 region of the chromatogram showed two incompletely separated peaks (Heslop *et al.*, 1985). The earlier of these is Ins(1,4) P_2 ; the later is unidentified and is possibly Ins(3,4) P_2 (Heslop *et al.*, 1986).

Some preliminary experiments were performed in

which the inositol phosphates were separated by Dowex-1 anion-exchange chromatography as described by Berridge *et al.* (1983). This method does not separate the various isomers of Ins P_2 and Ins P_3 from each other.

Statistical analysis

Values given for each data point are means of four to six separate samples taken from a common pool of labelled cells. The S.E.M. values were calculated from an analysis of variance and the appropriate P value determined as indicated in the text or Figure legends.

Materials

Plastic cultureware was from Nunc (Denmark) and foetal bovine serum was purchased from Gibco Life Technologies (Paisley, Scotland, U.K.). TRH was obtained from Peninsula Laboratories (Belmont, CA, U.S.A.) *myo*-[2- ^3H]inositol (sp. radioactivity 16.3 Ci/mmol) was purchased from Amersham International. All other chemicals were reagent grade or better.

RESULTS

Early changes in inositol phosphates assessed by h.p.l.c.

When GH_4C_1 cells were stimulated by rapid addition of TRH, there was no significant increase in Ins(1,4,5) P_3 at 490 or 750 ms; however, Ins(1,4,5) P_3 was elevated ($P < 0.01$) at 1.0 s and beyond (Fig. 1). This result was

Fig. 1. Time course of changes in inositol polyphosphates induced by TRH as assessed by h.p.l.c.

GH_4C_1 cells labelled with *myo*-[2- ^3H]inositol were stimulated by rapid addition of TRH ($1 \times 10^{-6} \text{ M}$) at time zero as described in the Materials and methods section. The inositol phosphates were separated by h.p.l.c. Each data point gives the mean value of three or four separate samples. The S.E.M. values were within the symbol size. All values at 1.00 and 1.50 s were greater than the zero-time controls ($P < 0.01$); values at 490 and 750 ms were not elevated.

Fig. 2. Early time course of changes in inositol phosphates in GH₄C₁ cells induced by TRH and assessed by h.p.l.c. analysis

This independent experiment was performed as described in the legend to Fig. 1. Each data point gives the mean value of four separate samples, except for the zero-time control which was composed of six samples. The S.E.M. values were all within symbol size. The time points shown are 0.49, 0.75, 1.00, 1.50 and 3.0 s. No value at 1.00 s or less was elevated above control. All values for Ins(1,4)P₂ and Ins(1,4,5)P₃ at 1.50 and 3.0 s were greater than control ($P < 0.01$).

confirmed in several additional experiments of the type illustrated in Fig. 2. No significant increase in Ins(1,4,5)P₃ was observed at times after TRH of less than 1.0 s; however, at 1.5 and 3.0 s after TRH, Ins(1,4,5)P₃ was significantly ($P < 0.01$) elevated. The results in Fig. 2 also show that GH₄C₁ cells synthesize Ins(1,3,4)P₃, but that rapid changes in this isomer of InsP₃ do not occur in response to TRH.

Time course of changes in Ins(1,3,4)P₃

The data presented in Fig. 2 and our previous results (Heslop *et al.*, 1985) demonstrate that GH₄C₁ cells produce Ins(1,3,4)P₃ but that an increase in the accumulation of this isomer of InsP₃ does not occur very rapidly over exposure of the cells to TRH. In preliminary experiments, we did observe a significant ($P < 0.01$) increase in Ins(1,3,4)P₃ in GH₄C₁ cells treated with TRH for 1.0 min. In order to examine the time course of action of TRH of Ins(1,3,4)P₃ accumulation in greater detail we used cells preincubated with Li⁺, which augments the accumulation of Ins(1,3,4)P₃.

In Li⁺-pretreated GH₄C₁ cells the usual large increase in Ins(1,4,5)P₃ was observed (Fig. 3). A smaller and more gradual increase in Ins(1,3,4)P₃ was also seen, reaching about three times the basal level of Ins(1,3,4)P₃ after 5 min of exposure to TRH in Li⁺-treated cells. The time-course of change in Ins(1,3,4)P₃ in GH₄C₁ cells is

Fig. 3. Time course of changes in Ins(1,3,4)P₃ and Ins(1,4,5)P₃ induced by TRH in Li⁺-pretreated GH₄C₁ cells

Cells labelled with *myo*-[2-³H]inositol were incubated with 10 mM-Li⁺ for 10 min at 37 °C before rapid addition of TRH (1×10^{-6} M) at time zero as described in the Materials and methods section. Inositol phosphates were separated by h.p.l.c. Each data point gives the mean value of five separate samples. The S.E.M. values were within symbol size. The time points shown are 3 and 15 s and 1.0, 2.0 and 5.0 min. All values for both Ins(1,3,4)P₃ and Ins(1,4,5)P₃ after zero time were statistically greater than control ($P < 0.01$).

similar to that recently described in insulin-secreting RINm5F cells stimulated by carbamoylcholine (Wollheim & Biden, 1986).

DISCUSSION

It has been generally assumed in the GH cell system, as in many other cell types, that relatively rapid (within 2–5 s) agonist-induced generation of Ins(1,4,5)P₃ is the mediator of the rapid phase in intracellular calcium redistribution leading to a spike in [Ca²⁺]_i. Results of the experiments described in this report are not entirely consistent with present dogma and raise a question as to whether Ins(1,4,5)P₃ is the sole mediator of the spike phase of change in [Ca²⁺]_i in GH₄C₁ cells. If Ins(1,4,5)P₃ causes the TRH-induced spike in [Ca²⁺]_i by releasing calcium from non-mitochondrial stores, it would be anticipated that a measurable rise in Ins(1,4,5)P₃ should occur before or at least simultaneous with the rising phase of [Ca²⁺]_i. Our results show that the rise in Ins(1,4,5)P₃ is considerably slower than, and in fact lags behind, the rise in [Ca²⁺]_i.

Using the fluorescent indicator quin2, we have shown a significant rise in [Ca²⁺]_i in GH₄C₁ within 400 ms (Albert & Tashjian, 1984b). By h.p.l.c. analysis, there was no significant increase in Ins(1,4,5)P₃ until at least 1000 ms after exposure to TRH (Figs. 1 and 2). Thus, we have been unable to demonstrate a rise in Ins(1,4,5)P₃ which precedes or even occurs with the rise in [Ca²⁺]_i.

There are several possible interpretations of our findings. First, it is possible that Ins(1,4,5)P₃ is not the mediator of the initial rise of the spike phase of [Ca²⁺]_i.

in GH_4C_1 cells. If this interpretation is correct, then it will be necessary to determine whether $\text{Ins}(1,4,5)\text{P}_3$ acts in concert with another intracellular mediator to cause the initial rising phase of the spike, or whether $\text{Ins}(1,4,5)\text{P}_3$ acts only after about 1000 ms to augment the magnitude of the spike in $[\text{Ca}^{2+}]_i$. Other potential mediators which might play a direct role in calcium mobilization include GTP (Gill *et al.*, 1986) and arachidonic acid (Wolf *et al.*, 1986). The second interpretation is that $\text{Ins}(1,4,5)\text{P}_3$ is the sole mediator of the spike in $[\text{Ca}^{2+}]_i$ but that our experimental approaches failed to detect a rise in this polyphosphoinositol before the increase in $[\text{Ca}^{2+}]_i$. The following points need to be considered. 1. A small rise in $\text{Ins}(1,4,5)\text{P}_3$ was induced within < 400 ms by TRH but we failed to detect it. Using our methods, a 20–25% change in $\text{Ins}(1,4,5)\text{P}_3$ can be measured as significantly ($P < 0.05$) different from control. Changes of less than 20–25% are obscured by the sum of sample-to-sample variation, sampling errors, and analytical errors. Thus, a rise of intracellular $\text{Ins}(1,4,5)\text{P}_3$ of $< 20\%$ would not have been detected. The concentration of TRH used in our experiments (1×10^{-6} M) is supramaximal with respect to receptor occupancy and stimulated hormone release. It is also supramaximal with respect to rapid enhancement of $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells (Albert & Tashjian, 1984b). It is possible that the increases in inositol phosphates which we measured at times after 1.0 s are in excess of those required to release intracellular calcium stores; however, the functional significance of a 20% rise in $\text{Ins}(1,4,5)\text{P}_3$ in GH_4C_1 cells is not clearly known. 2. A rise in $\text{Ins}(1,4,5)\text{P}_3$ of substantially more than 20% could have occurred in some localized cellular compartment that was functionally important but not detected by measurement of bulk cellular $\text{Ins}(1,4,5)\text{P}_3$. This interpretation cannot be ruled out, especially if it is argued that the non-mitochondrial (possibly endoplasmic reticulum) reservoir of intracellular calcium is closely associated with the plasma membrane of GH_4C_1 cells (Tan & Tashjian, 1981), which is also the likely site of generation of $\text{Ins}(1,4,5)\text{P}_3$. 3. A rapid rise in $\text{Ins}(1,4,5)\text{P}_3$ was not detected because of immediate conversion to $\text{Ins}(1,4)\text{P}_2$ or $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1986). As shown in Figs. 1 and 2, $\text{Ins}(1,4)\text{P}_2$ did not rise more rapidly than did $\text{Ins}(1,4,5)\text{P}_3$, suggesting that the lack of more rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ was not due to its immediate conversion to $\text{Ins}(1,4)\text{P}_2$. On the other hand, we have reported that InsP_4 rises more rapidly in GH_4C_1 cells in response to TRH than does $\text{Ins}(1,4,5)\text{P}_3$ (Heslop *et al.*, 1985); however, we have not shown that, under these conditions, the tetrakisphosphate is derived from $\text{Ins}(1,4,5)\text{P}_3$. 4. Because the measurements of $[\text{Ca}^{2+}]_i$ (Albert & Tashjian, 1984b) and inositol polyphosphates (the present paper) were not performed under identical conditions, it could be argued that the action of TRH was expressed more rapidly in the experiments of Albert & Tashjian (1984b) than in the present case. However, using the same methods of mixing TRH and cells, we have demonstrated TRH-induced subsecond increases in InsP_4 in these cells (Heslop *et al.*, 1985). Taken together, these findings demonstrate rapid (< 500 ms) mixing of TRH with the target cell suspension by using our experimental protocol.

At the present time our data do not allow us to reject

the widely held view (see Gershengorn, 1985, for a recent summary) that $\text{Ins}(1,4,5)\text{P}_3$ is the mediator of the TRH-induced spike phase of change in $[\text{Ca}^{2+}]_i$ in GH cells. On the other hand, we believe, until additional data are in hand on rapid kinetics of both $[\text{Ca}^{2+}]_i$ and inositol lipid and inositol phosphates, that caution should be exercised in this as well as other cell systems in accepting the conclusion that all rapid agonist-induced redistributions of intracellular calcium are mediated entirely by $\text{Ins}(1,4,5)\text{P}_3$.

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