ABSTRACT

Previous studies have demonstrated that TRH is a potent stimulator of α-MSH secretion from frog pituitary melanotrophs. In order to determine the intracellular events responsible for TRH-evoked α-MSH release, we have investigated the effect of TRH on polyphosphoinositide breakdown in frog pars intermedia. Neurointermediate lobes were labelled to isotopic equilibrium with myo-[3H]inositol.

TRH stimulated the rate of incorporation of [3H]inositol into the phospholipid fraction. The effect of TRH was concentration-dependent; half-maximal stimulation of α-MSH release and inositol incorporation occurred at 12 and 28 nmol TRH/l respectively. In prelabelled neurointermediate lobes, lithium (10 mmol/l) enhanced the radioactivity in inositol monophosphate, bisphosphate (IP2) and trisphosphate (IP3). LiCl (10 mmol/l) induced a 38% inhibition of α-MSH release from perfused neurointermediate lobes but did not impair TRH-induced α-MSH secretion. In the presence of LiCl, TRH (1 μmol/l) induced a transient increase of the radioactivity in IP3, which was evident by 30 s and maximal by 1 min (+100%). TRH treatment also increased the radioactivity in IP2, which reached a plateau after 5 min (+100%). The increase in radioactivity in IP3 induced by TRH was closely paralleled by a rapid loss of [3H]phosphatidylinositol bisphosphate (PIP2), which was maximal by 1 min (−70%).

These results indicate that, in frog pars intermedia, TRH-evoked α-MSH secretion is coupled to breakdown of PIP2. The data suggest that, in amphibian melanotrophs, as previously shown in GH3 tumour cells and in rat pituitary mammotrophs, TRH causes rapid stimulation of polyphosphoinositide-hydrolysing phospholipase C. *Journal of Molecular Endocrinology* (1990) 5, 129–136

INTRODUCTION

The melanotropic cells of the intermediate lobe of the pituitary synthesize the precursor protein pro-opiomelanocortin which is processed to a number of small biologically active peptides (Martens, Jenks & van Overbeeke, 1982; Vaudry, Jenks & van Overbeeke, 1984). In amphibia, the pars intermedia is richly innervated by both aminergic and peptidergic fibres (Tonon, Danger, Lamacz et al. 1988). Many of the factors contained in these nerve endings act directly on melanotrophs. In particular, thyrotrophin-releasing hormone (TRH) exerts direct stimulatory control on the secretion of α-melanocyte-stimulating hormone (α-MSH) (Tonon, Leroux, Leboulenger et al. 1980b; Louiset, Cazin, Lamacz et al. 1989). The effect of TRH on α-MSH release is dependent upon the concentration of extracellular calcium (Lamacz, Tonon, Guy et al. 1988). In general, receptors which elicit a Ca2+-dependent response exhibit the common ability to enhance the breakdown of polyphosphoinositides (Abdel-Latif, 1986). GH3 cells, a clone of rat pituitary tumour cells, have been widely used as a model to study the mechanism of action of TRH, and it is now well established that TRH accelerates the turnover of polyphosphoinositides in these cells (for review see Gershengorn, 1986). First, TRH induces hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2), resulting in an increase in the level of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DG). IP3 mobilizes calcium from intracellular stores, whereas DG enhances the activity of protein kinase C (Ronning, Heatley &...
The intermediate lobe of the frog pituitary, which is composed of a homogeneous population of cells, is a valuable model to investigate the mechanism of action of TRH in non-tumoral endocrine cells. In the present study, we have examined the role of phospholipid metabolism in the mechanism of action of TRH in the amphibian pars intermedia.

**MATERIALS AND METHODS**

**Materials**

Myo-[³H]inositol (100 Ci/mmol) and [³H]PIP₂ (1 Ci/mmol) were obtained from Amersham International plc (Versailles, France). Synthetic pyro-Glu-His-Pro-NH₂ (TRH; purity 98.5%) was kindly supplied by Dr. K. Kupryszeewski (University of Gdansk, Poland) and synthetic α-MSH was a gift from Drs. A. Andreatta and K. Scheibl (Ciba-Geigi, Basle, Switzerland). Leibovitz culture medium, Hepes, Dowex-1 resin (chloride form, 100–200 mesh), L-α-phosphatidylinositol (PI), L-α-phosphatidylserine (PS) and a mixture of PI, PIP₂, PS and phosphatidylinositol monophosphate (PIP) were from Sigma Chemical Company (St Louis, MO, U.S.A.). Kanamycin, antibiotic–antimycotic solution and bovine serum albumin (BSA) were from Boehringer Mannheim (Meylan, France). Silica gel 60 F₂₅₄ HPTLC plates were from Merck (Paris, France). The scintillation fluid (Hydroluma) was purchased from Lumac (Langraaf, The Netherlands).

**Animals**

Adult male frogs (*Rana ridibunda*) of about 30 g body weight, originating from Yugoslavia, were purchased from a commercial supplier (Couétard, Saint-Hilaire de Riez, France) and maintained in a temperature-controlled room (8 ± 0.5 °C) with an established photoperiod of 12 h light/day (lights on from 06.00 to 18.00 h). The animals were kept unfed under running water for at least 1 week before being killed. They were killed by decapitation and the neurointermediate lobes carefully dissected out under a microscope.

**Labelling of frog pituitaries with [³H]inositol**

Phosphoinositides and inositol phosphates were labelled by incubating neurointermediate lobes in Leibovitz culture medium (2:3 dilution), supplemented with 0.2 g glucose/l, 80 mg CaCl₂/l, 1% (v/v) kanamycin and 1% (v/v) antibiotic–antimycotic solution, with myo-[³H]inositol (100 μCi/ml) at 21 °C for 0–18 h.

For the incorporation studies, TRH (1 μmol/l) was added to the incubation medium for 0–7 h. Incubation was stopped by transferring neurointermediate lobes into Krebs–Ringer solution (112 mmol NaCl/l, 2 mmol KCl/l, 2 mmol CaCl₂/l and 15 mmol Hepes/l) supplemented with 0.8 g glucose/l and 0.3 g BSA/l. The solution was gassed for 15 min with moistened O₂:CO₂ (95:5) before use and the pH was adjusted to 7.4. After six washes in the same medium, 500 μl ice-cold 10% (v/v), trichloroacetic acid (TCA) were added. Neurointermediate lobes were then homogenized, and precipitated proteins removed by centrifugation.

For the equilibrium studies, neurointermediate lobes were prelabelled in Leibovitz medium with myo-[³H]inositol (100 μCi/ml) for 18 h at 21 °C. After withdrawal of the labelling medium, neurointermediate lobes were washed six times with Krebs–Ringer and then incubated for short times (0.5–20 min) with TRH diluted in Krebs–Ringer. Most of the experiments were carried out in the presence of 10 mmol LiCl/l. In those cases the NaCl content of the medium was proportionally lowered to keep a constant osmolarity, and neurointermediate lobes were preincubated for 10 min with Krebs–Ringer–LiCl solution before stimulation with TRH. Incubations were stopped by adding 250 μl ice-cold 20% TCA. Neurointermediate lobes were then homogenized, and precipitated proteins removed by centrifugation.

The acid-soluble fractions were stored at −20 °C until analysis of [³H]inositol phosphates. Phospholipids were extracted from protein pellets with 200 μl chloroform/methanol (2:1, v/v).

**Analysis of inositol phosphates**

Inositol phosphates were separated by anion-exchange chromatography as described previously (Drummond, Bushfield & Macphee, 1984) on columns (1 x 2 cm) of Dowex. Free [³H]inositol and inositol monophosphate (IP₁), inositol bisphosphate (IP₂) and IP₃ were sequentially eluted with 30 ml distilled water and with 30 ml each of solutions of 30, 90 and 500 mmol HCl/l respectively. The recoveries of IP₁ and IP₂ from the column were 98.6 ± 0.8% and 99.9 ± 0.1% respectively. Frozen aliquots of acid-soluble inositol polyphosphates were thawed, neutralized with NaOH and then applied onto columns. For each column, 60 fractions (2 ml each) were collected. Scintillation fluid (10 ml)
was added to each fraction and the radioactivity determined by counting in a 1217 Rackbeta spectrometer (63% efficiency). The radioactivity in IP₁, IP₂ and IP₃ was calculated as the net area under the peak.

**Analysis of phospholipids**

For determination of polyphosphoinositide breakdown, [³H]inositol-containing phospholipids were separated by thin-layer chromatography (TLC) on precoated silica gel 60 F₂₅₄ plates in chloroform/methanol/acetic acid/water (40:13:15:12:8, by vol.) as described previously (Martin, 1983). The dried samples were reconstituted with 20 μl chloroform/methanol (2:1, v/v) and applied to TLC plates. After 2 h, corresponding to 17 cm development length, [³H]inositol-containing phospholipids were identified by comparison with reference standards (PS, PI, [³H]PIP₂ and a mixture of PS, PI, PIP and PIP₂) visualized on the TLC plates by exposure to iodine vapor and by counting the radioactivity. Using the solvent system described above, phospholipids migrated with approximate relative band speeds of 0·1 (PIP₂), 0·42 (PIP) and 0·8 (PI). For each sample, the phospholipid-containing areas were scraped into vials, and the radioactivity corresponding to PI, PIP and PIP₂ was measured by scintillation counting.

**Perfusion experiments**

The perfusion system employed to study α-MSH release from frog whole neurointermediate lobe has been described previously (Tonon, Leboulenger, Delarue et al. 1980a). After dissection, three neurointermediate lobes were preincubated in Krebs–Ringer solution. For each experiment, neurointermediate lobes were suspended in a Bio-Gel P2 matrix and transferred into a siliconized glass tube (0·9 cm internal diameter). The glands were perfused with Krebs–Ringer solution at a constant flow rate (0·6 ml/min) and temperature (21 °C) throughout the experiment. After a 90-min stabilization period, test substances (secretagogues) freshly dissolved in the medium were infused for various periods. The effluent perifusate was collected as 7·5-min fractions during stabilization periods, or as shorter fractions (0·5, 10 and 2·5 min) during infusion of secretagogues, and immediately chilled on ice. The α-MSH concentration of each fraction was measured on the same day as the perfusion experiment.

**Radioimmunoassay of α-MSH**

Concentrations of α-MSH were measured using a double-antibody radioimmunoassay method described previously (Vaudry, Tonon, Delarue et al. 1978). Briefly, synthetic α-MSH (1 μg) was labelled with 0·5 mCi Na¹²⁵I and 20 μg chloramine T in 20 μl phosphate buffer (pH 7·4). Labelling of the molecule was completed within 15 s and the reaction stopped by adding 60 μg sodium metabisulphite. Labelled α-MSH was purified by adsorption on QUSO G32 glass powder and then kept frozen for 4 weeks without noticeable loss of immunoreactivity. The radioimmunoassay was performed in 500 μl veronal–mercaptoethanol–BSA buffer (VMA; 0·02 mol/l–0·1%, v/v, -0·4%, w/v; pH 8·6). The final dilution of the antiserum was 1:40 000, and the total amount of labelled α-MSH was 5000 c.p.m./tube. After a first incubation for 2 days at 4 °C, the antibody-bound α-MSH was immunoprecipitated by 200 μl each of anti-rabbit goat γ-globulin and normal rabbit serum diluted 1:30 and 1:200 respectively in VMA. The incubation was carried on for another 2 days at 4 °C. After centrifugation, the supernatant was removed and the pellet containing the bound fraction was counted on a gamma counter (LKB Wallak, model 1274). The standard curve was set up with synthetic α-MSH at concentrations ranging from 10 to 2·5 x 10³ pg/tube. Peptide concentrations in perfusion fractions were calculated with a Hewlett-Packard model C (HP 85) from the parameters of the standard curve linearized by means of logit–log transformation.

The effects of secretagogues on α-MSH release were calculated as the net area under the peak (stimulatory effect) or above the basal level (inhibitory effect) and expressed as percentages of the control level. Controls were calculated as the mean of four consecutive fractions (7·5 min each) collected under basal conditions.

Student’s t-test for variation analysis was used to compare the means of different values within the same set of experiments.

**RESULTS**

**Time-course of incorporation of [³H]inositol into phospholipids**

To determine the time needed to label polyphosphoinositides to a constant specific activity, neurointermediate lobes were incubated with Leibovitz medium containing [³H]inositol for various periods of time (Fig. 1). The radioactivity of the acid–water phase, corresponding to free [³H]inositol and inositol polyphosphates, rapidly increased and reached a maximum by 12 h of incubation. As shown in Fig. 1, significant incorporation of [³H]inositol in the phospholipid fraction was observed as early as 2 h after the beginning of labelling. Polyphosphoinosi-
Effect of lithium chloride on inositol phosphate levels

In tissues prelabelled with \( ^{3}\text{H} \text{inositol} \) (18 h), analysis of inositol polyphosphates by Dowex ion-exchange chromatography indicated that the rate of formation of IP\(_{1}\) was 7.5 times higher than that of IP\(_{2}\) (15 400 \( \pm \) 1000 (s.e.m.) and 2040 \( \pm \) 80 c.p.m./sample respectively). In these conditions the radioactivity in IP\(_{3}\) was very low or undetectable. LiCl (10 mmol/l for 10 min) caused an increase in the radioactivity in all cellular inositol polyphosphates, and IP\(_{4}\) became detectable (56 \( \pm \) 8 c.p.m./sample (Fig. 2)). LiCl also enhanced the radioactivity in IP\(_{1}\) and IP\(_{2}\) by 160 and 50% of the control values respectively.

Effect of TRH on \( \alpha \)-MSH secretion

In order to determine whether neurointermediate lobes could still respond properly after an 18-h incubation period, freshly dissected or preincubated lobes (Leibovitz medium for 18 h at 21 °C) were transferred into perfusion columns. After a 90-min stabilization period, TRH (1 \( \mu \text{mol/l} \) ) was added for 10 min. As shown in Fig. 3, in both freshly removed and preincubated tissues, TRH induced a twofold increase in \( \alpha \)-MSH release. Infusion of LiCl (10 mmol/l) to perfused frog neurointermediate lobes caused a 38% inhibition of \( \alpha \)-MSH secretion but did not impair TRH-induced \( \alpha \)-MSH release (Fig. 4).

\textit{Journal of Molecular Endocrinology} (1990) 5, 129–136

**FIGURE 1.** Time-course of \( ^{3}\text{H} \text{inositol} \) labelling of phosphoinositides in frog neurointermediate lobes. Phospholipids were extracted at the times indicated. Values are means \( \pm \) S.E.M. for incorporation of \( ^{3}\text{H} \text{inositol} \) into phosphoinositides (\( \bullet \)) and for radioactivity in the acid–water phase (inositol phosphates; \( \triangle \)).

**FIGURE 2.** Effect of lithium on inositol polyphosphate levels in frog neurointermediate lobes. Neurointermediate lobes were prelabelled with \( ^{3}\text{H} \text{inositol} \) for 18 h at 21 °C. The tissues were then incubated for 10 min in the absence (open bars) or presence (hatched bars) of lithium (10 mmol/l). IP\(_{1}\), inositol monophosphate; IP\(_{2}\), inositol bisphosphate; IP\(_{3}\), inositol trisphosphate. Values are means \( \pm \) S.E.M.

**FIGURE 3.** Effect of TRH on \( \alpha \)-MSH release from perfused frog neurointermediate lobes. Freshly dissected neurointermediate lobes (solid bar) or lobes which had been preincubated in Leibovitz medium for 18 h at 21 °C (hatched bar) were stimulated for 10 min with TRH (1 \( \mu \text{mol/l} \) ). The effect of TRH on \( \alpha \)-MSH secretion was calculated as the net area under the peaks, corresponding to a 15-min period starting after the onset of TRH infusion. ***\( P<0.001 \) compared with control (open bar; Student's \( t \)-test). Values are means \( \pm \) S.E.M. NS, not significant (\( P>0.05 \)).

**Effect of TRH on incorporation of \( ^{3}\text{H} \text{inositol} \) into phospholipids**

TRH (1 \( \mu \text{mol/l} \) ) increased \( ^{3}\text{H} \text{inositol} \) incorporation into the phospholipid fraction (Fig. 5). After a
3-h period, TRH significantly \((P < 0.05)\) enhanced the radioactivity in phospholipids. The stimulatory effect of TRH on the incorporation of \([3H]\)inositol reached 71\% above the control level at 7 h. In addition, TRH induced a dose-dependent increase in radioactivity in phosphoinositides (median effective dose = 28 nmol/l), with a maximal effect for 1 \(\mu\)mol TRH/l (Fig. 6).

**Effect of TRH on the rate of accumulation of phosphatidylinositol metabolites**

The time-course of the effect of TRH on the accumulation of IP\(_2\) and IP\(_3\) and on the disappearance of PIP\(_2\) in neurointermediate lobes prelabelled with \([3H]\)inositol (18 h) is shown in Fig. 7. In these experiments, LiCl (10 mmol/l) was added 10 min before TRH (1 \(\mu\)mol/l). An increase of the radioactivity in IP\(_3\) was observed as early as 30 s after the addition of TRH and reached a maximum by 1 min (100\% above control). The radioactivity in IP\(_3\) then declined to the basal value within 5 min. The radioactivity in IP\(_2\) reached a plateau within 5 min and remained at this high level for at least 20 min (Fig. 7a). Concurrently, TRH induced a rapid loss of \([3H]\)PIP\(_2\) which reached a minimum (-70\%) by 1 min (Fig. 7b). PIP\(_2\) returned to the basal value within 20 min.

**DISCUSSION**

Previous studies have demonstrated that, in frogs (Tonon et al. 1980a,b) and toads (Verburg-van Kemenade, Jenks, Visser et al. 1987), TRH is a potent stimulator of \(\alpha\)-MSH secretion from pars intermedia cells. However, the intracellular events involved in the response of melanotropic cells to TRH have never been investigated. The mechanism of action of TRH on pituitary cells has been extensively studied in GH\(_3\) cells. In this pituitary tumour cell line it has been demonstrated that TRH stimulates hydrolysis of polyphosphoinositides. Leung, Raymond & Labrie (1982) have also shown that, in rat anterior pituitary cells, TRH activates the incorporation of \(^{32}\)P into phosphatidylinositol. However, studies on the mechanism of action of TRH in normal pituitary cells are complicated by the heterogeneity of the cell types in the adenohypophysis. Therefore, the pars intermedia of amphibians, which is composed of a homogeneous
population of melanotropic cells, appears to be an interesting model in which to investigate the mechanism of action of TRH on non-tumoral endocrine cells. The results of the present study indicate that, in frog melanotrophs, TRH stimulates polyphosphoinositide breakdown.

We first showed that TRH stimulates the incorporation of 

\[ ^3H \text{inositol} \]

into phospholipids in a dose-dependent manner. The concentration of TRH which induced a half-maximal increase in 

\[ ^3H \text{inositol} \]

incorporation into phospholipids (28 nmol/l) was in the same range as that required to obtain half-

maximal stimulation of a-MSH secretion from perifused frog neurointermediate lobes (12 nmol/l). Similarly, in the rat anterior pituitary, the concentration of TRH required for half-maximal stimulation of inositol phosphate turnover compares closely with the corresponding value for TRH-

stimulated prolactin (or thyrotrophin) release (Leung et al. 1982; Lo & Hughes, 1987). It is well documented that activation of phospholipase C is

the initial process involved in signal-induced enhancement of phosphatidylinositol turnover (Martin, 1983). In turn, phospholipase C hydrolyses

\[ \text{PIP}_2 \]

to generate two compounds: IP3 and DG (for reviews see Berridge, 1984; Nishizuka, Takai, Kishimoto et al. 1984). In order to show that the action of TRH is mediated through phospholipid hydrolysis, we have measured the levels of IP3 and PIP2 in frog neurointermediate lobes exposed to TRH. Under basal conditions, IP1 and IP2 represented approximately 98% of the inositol polyphosphates, and the non-stimulated level of IP3 was very low. Several studies have demonstrated that lithium blocks inositol 1-phosphomonoesterase (for review see Berridge, Downes & Hanley, 1989) and induces

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*Journal of Molecular Endocrinology (1990) 5, 129-136*
accumulation of inositol polyphosphates in GH₃ cells (Drummond et al. 1984; Schlegel, Roduit & Zahnd, 1984). In our model, addition of 10 mmol LiCl/l enhanced the concentration of all inositol phosphates. Although lithium at this high concentration caused an inhibition of α-MSH release from perfused frog neurointermediate lobes, it did not impair the secretory response of pituitary melanotrophs to TRH. Since Na⁺ channels are made permeable by Li⁺ (Hille, 1984), the inhibitory effect of Li⁺ on α-MSH release most likely reflects a loss of Na⁺ from the cells, which in turn is responsible for inhibition of the intracellular processes involved in hormonal release. In lithium-pretreated lobes, TRH induced a rapid (30 s) and transient (<5 min) increase of radioactivity in IP₃, whereas the radioactivity in IP₂ remained increased for at least 20 min.

Recently, electrophysiological studies on frog melanotrophs have shown that TRH induces a slight and transient polarization, followed by a rise in spike discharges (Louiset et al. 1989). In the excised patch configuration, TRH increases the open probability of Ca²⁺-activated K⁺ channels via intracellular messengers. Since IP₃ mobilizes calcium from intracellular stores (Ronning et al. 1982; Gershengorn et al. 1984), the TRH-induced increase in the radioactivity in IP₃ can be regarded as the potential intracellular messenger involved in the outward K⁺ current in the frog. In addition, TRH induced a rapid (30 s) reduction of the radioactivity in IP₂. This decrease was concomitant with a rise in the radioactivity in cellular IP₃. It was noted that PIP₂ recovered its basal radioactivity later than IP₃. This difference can be accounted for by rapid dephosphorylation of IP₃ (Drummond et al. 1984; Ramsdell & Tashjian, 1986).

Enhanced hydrolysis of polyphosphoinositides raises the levels of another potential intracellular mediator, DG, which activates protein kinase C (Wolf, Sahyoun, Levine & Cuatrecasas, 1984). It has been proposed that phospholipase C may hydrolyse both PIP and PIP₂, and to a lesser extent, PI (Macphee & Drummond, 1984; Osborne, Tobin & Ghazi, 1988). Since TRH did not induce a rise in IP₁, our results indicate that breakdown of PI is not the initial event involved in the mechanism of action of TRH. Nevertheless, a weak decline in the radioactivity in PI occurred at 20 min, at a time when PIP₂ is resynthesized. The fact that no modification in the radioactivity in IP₁ was observed suggests that the decrease in PI probably results from its conversion into PIP₂.

In summary, our results demonstrate that TRH causes a dose-dependent increase in polyphosphoinositol turnover in frog melanotrophs. These data suggest that TRH-induced PIP₂ hydrolysis is the first intracellular event involved in the mechanism of action of TRH on α-MSH secretion by pars intermedia cells.

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