Thyroid-stimulating hormone stimulates increases in inositol phosphates as well as cyclic AMP in the FRTL-5 rat thyroid cell line

James B. FIELD,* Patricia A. EALEY,† Nicholas J. MARSHALL† and Shamshad COCKCROFT*

*Department of Experimental Pathology and †Department of Chemical Pathology, School of Medicine, University College London, University Street, London WC1E 6JJ, U.K.

Studies were conducted to determine whether thyroid-stimulating hormone (TSH; thyrotropin), a hormone known to increase cytosol concentrations of cyclic AMP, also stimulates the formation of inositol phosphates in thyroid cells. TSH and noradrenaline both stimulated [^3H]inositol phosphate formation in a concentration-dependent manner in the rat thyroid cell line, FRTL-5 cells, which had been prelabelled with [^3H]inositol. The threshold concentration of TSH required to stimulate inositol phosphate formation was more than 20 munits/ml, which is approx. 10^3 -fold greater than that required for cyclic AMP accumulation and growth in these cells. We also demonstrate that membranes prepared from FRTL-5 cells possess a guanine nucleotide-activatable polyphosphoinositide phosphodiesterase, which suggests that activation of inositide metabolism in these cells may be coupled to receptors by the G-protein, G_p . Our findings suggest that two second-messenger systems exist to mediate the action of TSH in the thyroid.

INTRODUCTION

The thyroid produces thyroid hormones in response to thyroid-stimulating hormone (TSH) released from the pituitary. TSH activates glucose oxidation, enhanced turnover of phosphatidylinositol (PI), iodine organification, endocytosis of colloid, fusion of phagosomes with lysosomes and finally release of thyroid hormones (Field, 1978). Although most of the effects of TSH on the thyroid are mediated via activation of the adenylate cyclase–cyclic AMP system, some responses cannot be explained on this basis (Field, 1975). These include such parameters as ^{32}P incorporation into phosphatidic acid and PI (Morton & Schwartz, 1953; Freinkel, 1957; Zor *et al.*, 1968; Scott *et al.*, 1970), induction of desensitization of adenylate cyclase (Shuman *et al.*, 1976; Hirayu *et al.*, 1985) and formation of diacylglycerol (Igarashi & Kondo, 1980).

The existence of another signalling system, involving the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) with the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, has been demonstrated in a wide variety of tissues (Downes & Michell, 1985; Berridge, 1986), and this could mediate the effects of TSH on the thyroid which are not due to cyclic AMP. The IP_3 generated facilitates an increase in intracellular Ca^{2+} (Berridge & Irvine, 1984), whereas diacylglycerol activates the Ca^{2+} –phospholipid-dependent protein kinase (protein kinase C) (Nishizuka, 1984).

The present studies were undertaken to examine the effect of TSH and other agonists on inositol phosphate production as a result of PPI hydrolysis in a clonal strain of rat thyroid cells (FRTL-5). While they were in progress, Philp & Grollman (1986) reported that TSH

and noradrenaline stimulated PIP_2 hydrolysis in FRTL-5 cells, a line of rat thyroid cells.

METHODS AND MATERIALS

Culture of FRTL-5 cells

FRTL-5 cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% (v/v) newborn-calf serum containing TSH (1 munit/ml), transferrin (5 $\mu\text{g}/\text{ml}$), insulin (10 $\mu\text{g}/\text{ml}$), somatostatin (10 ng/ml), cortisone (10 nM) and the tripeptide glycyl-L-histidyl-L-lysine acetate (10 ng/ml) in plates with 24 wells as previously described (Ambesi-Impimbato *et al.*, 1980). Cells were plated out at a density of 2×10^5 cells/ml, and each well contained 0.5 ml. At 7 days before labelling of the cells with [^3H]inositol, they were placed in the same tissue-culture medium containing all of the above supplements except TSH. At 1–2 days before the cells were used, they were transferred to Medium 199 containing [^3H]inositol (10 $\mu\text{Ci}/\text{ml}$), insulin (10 $\mu\text{g}/\text{ml}$) and transferrin (5 $\mu\text{g}/\text{ml}$).

Stimulation of FRTL-5 cells

Following the labelling of the cells with [^3H]inositol, the cells were washed twice with a buffered salt solution (pH 7.4) which consisted of: 137 mM-NaCl; 2.7 mM-KCl; 1 mM- MgCl_2 ; 1 mM- CaCl_2 ; 20 mM-Hepes; 5.6 mM-glucose; 1 mg of bovine serum albumin/ml; 1 mM-*myo*-inositol; and 10 mM-LiCl to prevent the hydrolysis of IP_1 to inositol (Berridge *et al.*, 1982). LiCl and unlabelled inositol were also present in the subsequent incubation with buffer or agonist. FRTL-5 cells were incubated in each well with 0.5 ml of buffer or agonist for 1 h, except

Abbreviations used: TSH, thyroid-stimulating hormone (thyrotropin); PIP_2 , phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; IP_3 , total inositol phosphates; IP_1 , inositol monophosphate; IP_2 , inositol bisphosphate; IP_3 , inositol trisphosphate; GPI, glycerophosphoinositol; PPI-pde, polyphosphoinositide phosphodiesterase; GTP[S], guanosine 5'-[γ -thio]triphosphate; PPI, polyphosphoinositides.

for the time-course experiment in Fig. 2, where the cells were quenched at the indicated times.

Extraction and analysis of inositol phosphates

Incubations were terminated by addition of 0.025 ml of conc. HClO_4 . The contents of the tissue-culture wells were transferred to centrifuge tubes, and the wells were rinsed with 0.5 ml of buffer, which was combined with the homogenate before centrifugation at 3000 rev./min for 5 min. After removal of the supernatant, the pellet was resuspended in 0.5 ml of 1 M- HClO_4 and re-centrifuged. The supernatant was combined with the initial supernatant and then neutralized with 1 M-KOH. After centrifugation the supernatant was diluted to 10 ml with water, and the glycerophosphoinositol (GPI) and the inositol phosphates (IPs) were separated on Dowex resin (formate form) (Berridge *et al.*, 1983). Free [^3H]inositol was washed through with water. GPI was eluted with 5 mM-sodium borate/60 mM-sodium formate. IPs were eluted with increasing concentrations of ammonium formate in 100 mM-formic acid: IP_1 , 200 mM-ammonium formate; IP_2 , 400 mM-ammonium formate; IP_3 , 1 M-ammonium formate. Fractions (3 ml) were collected and counted for radioactivity in PCS scintillator (Amersham) in a liquid-scintillation spectrometer. Previous studies have indicated that isomers of IPs are formed, but are most likely derived from inositol 1,4,5-trisphosphate (Hawkins *et al.*, 1986; Downes *et al.*, 1986). Since the purpose of the present study was to study whether TSH could couple to inositolide metabolism, no attempts were made to characterize the different isomers.

Extraction and analysis of phospholipids

The pellet from the centrifugation was dissolved in 1.9 ml of acidified chloroform/methanol/water (5:10:4, by vol.), and, after 10 min, 0.5 ml of chloroform and 0.5 ml of 1 M-HCl were added. The tubes were mixed and centrifuged at 3000 rev./min for 5 min to separate the chloroform and aqueous phases. The chloroform layer was removed, dried down and resuspended in 50 μl of chloroform. The phospholipids were separated by t.l.c. (Cockcroft *et al.*, 1985). PPI were included as a carrier with each sample, to aid localization with iodine vapour. The individual spots containing PI, PIP and PIP_2 were scraped into scintillation vials, suspended in 0.5 ml of methanol and counted for radioactivity in PCS scintillator.

Measurement of cyclic AMP

Cyclic AMP was measured as previously reported (Bidey *et al.*, 1984).

Activation of polyphosphoinositide phosphodiesterase in FRTL-5 membranes by GTP[S] and GTP

FRTL-5 cells were prelabelled with [^3H]inositol in the same way as described above, except that they were grown in Petri dishes seeded with 5 ml containing 2×10^5 cells/ml. The labelled cells were washed with ice-cold buffered salt solution (pH 7.2) containing 137 mM-NaCl, 2.7 mM-KCl and 20 mM-Hepes. All operations were carried out at 4 °C. After washing, the cells were scraped with a rubber policeman into 12 ml of 250 mM-sucrose buffer (pH 7.2) containing 20 mM-Hepes and 0.1 mM-EGTA. The cells were sonicated with an MSE probe at amplitude 3 at a medium setting for 5 s. Any

remaining intact cells were removed by centrifugation at 600 *g* for 5 min. The resulting homogenate was further centrifuged at 4 °C for 20 min at 86 500 *g*_{av.} (35 000 rev./min) to pellet the membranes. They were resuspended in buffer (pH 6.8) containing Pipes (20 mM), KCl (150 mM), 2,3-bisphosphoglycerate (2 mM), LiCl (10 mM), ATP (5 mM), MgCl_2 (10 mM) and free Ca^{2+} at 0.1 μM buffered with EGTA (3 mM) as described previously (Barrowman *et al.*, 1987). In the experiments with GTP, a triphosphate-regenerating system was also present: phosphoenolpyruvate (1 mM) and pyruvate kinase (40 $\mu\text{g}/\text{ml}$) (Bennett & Dupont, 1985). The reaction was initiated by adding 100 μl of membranes to an equal volume of buffer containing the indicated additions at 37 °C. Incubations were terminated by addition of 1.5 ml of chloroform/methanol (1:2, v/v). Phase separation of the aqueous from the organic phase was achieved by addition of 0.7 ml of water and 0.5 ml of chloroform. Then 1.4 ml of the top phase was analysed for IPs as described above.

Expression of results

In each experiment, each condition was done in triplicate or quadruplicate. The results are presented as means $\pm$ S.E.M. The results shown are representative of at least two other experiments.

Fig. 1. Concentration-dependence of TSH on (a) cyclic AMP formation and (b) IP_1 formation in FRTL-5 cells

(a) For the determination of cyclic AMP, the cells were incubated with increasing doses of TSH and the cyclic AMP was determined after 20 min. Average of duplicate determinations, which did not vary by more than 5%, are shown. (b) [^3H]inositol-labelled FRTL-5 cells were incubated with different concentrations of TSH for 1 h and the formation of IP_1 was determined. The values are means $\pm$ S.E.M. for four replicate determinations. In this experiment there were 70 500 d.p.m./10⁵ cells in the total inositol lipids.

Table 1. Distribution of [³H]inositol radioactivity in the water-soluble IPs in FRTL-5 cells stimulated with TSH

[³H]Inositol-prelabelled FRTL-5 cells were stimulated with TSH (500 munits/ml) for 1 h, and the distribution of [³H]inositol radioactivity in the phosphate esters was determined. The results are means $\pm$ S.E.M. of quadruplicate incubations for each compound. *P* values for the individual TSH-stimulated inositol phosphates compared with control incubations are < 0.001 .

	³ H (d.p.m./10 ⁵ FRTL-5 cells)	
	Control	TSH
GPI	13874 $\pm$ 632	28518 $\pm$ 758
IP ₁	2864 $\pm$ 301	7187 $\pm$ 547
IP ₂	490 $\pm$ 53	1021 $\pm$ 86
IP ₃	333 $\pm$ 46	826 $\pm$ 97

Materials

TSH (1 or 30 units/mg) was obtained from Sigma or the National Institute of Diabetes, Digestive and Kidney Diseases, N.I.H., Bethesda, MD, U.S.A., respectively. GTP[S] and GTP were purchased from Boehringer and were used without further purification (Cockcroft & Gomperts, 1985). Coon's modified Ham's F-12 medium was obtained from Gibco.

RESULTS

Studies with intact FRTL-5 cells

Analysis of the incorporation of [³H]inositol into inositol-containing lipids by t.l.c. indicated that the percentage distribution of radioactivity was as follows: PI, 78%, PIP, 16.8%, PIP₂, 5.2%. This percentage distribution of [³H]inositol was not changed in the cells which were incubated with agonists. Although TSH stimulated an increase in all of the IPs in FRTL-5 cells, the radioactivity was greatest in IP₁ (Table 1). Although hydrolysis of PIP₂ would yield IP₃, during the incubation, IP₃ is sequentially dephosphorylated to IP₂ and IP₁, and LiCl inhibits IP₁ breakdown to inositol. TSH also increased accumulation of GPI.

Fig. 1 shows the concentration requirement for IP₁ formation and cyclic AMP formation. The data in Fig. 1(b) indicate that 2 and 20 munits of TSH/ml did not induce the stimulation of IP₁, and the effect of 500 munits/ml was greater than that of 200 munits/ml. In other experiments, 100 munits/ml also produced significant stimulation (results not shown). In contrast, doses of TSH between 0.1 and 0.5 munits/ml had a marked effect on cyclic AMP accumulation (Fig. 1a). Cyclic AMP accumulation reached a peak at 2 munits of TSH/ml (results not shown).

No effect of TSH on IP₁, IP₂ or IP₃ production was apparent during a 5 min incubation, but stimulation was obtained by 15 min and continued to increase for at least 60 min, the longest incubation time that was used (Fig. 2). Noradrenaline also stimulated IP₁ accumulation in FRTL-5 cells (Fig. 3). The maximum effect was obtained with 10 μ M, but that of 0.1 μ M was not significantly greater than the control. In separate experiments, noradrenaline tested over this dose range has been shown

to stimulate neither cyclic AMP accumulation nor cell division in the FRTL-5 cells (results not shown). The latter was assessed by using the metaphase-index assay (Ealey *et al.*, 1985). In contrast, carbachol at a concentration which consistently increased IP₁ accumulation in dog and sheep thyroid slices (results not shown) had no effect on FRTL-5 cells (results not shown).

Studies with membranes prepared from FRTL-5 cells

The membrane-bound enzyme responsible for hydrolysing PPI in many cells is activated by guanine nucleotides (Cockcroft & Gomperts, 1985; Martin *et al.*, 1986; Uhing *et al.*, 1986), and we conducted a number of experiments to demonstrate the presence of such an enzyme in FRTL-5 cells. The conditions used to assay for the activity of PPI-pde were similar to those used for hepatocytes (Cockcroft & Taylor, 1987). MgATP was included in the incubation medium to maintain the amounts of PPI. PPI-pde activation was monitored by measuring the increase in IPs generated from the hydrolysis of [³H]inositol-labelled lipids. 2,3-Bisphosphoglycerate can prevent the conversion of IP₃ into IP₂ (Downes *et al.*, 1982; Cockcroft & Taylor, 1987), and LiCl can inhibit the hydrolysis of IP₁ back to inositol (Berridge *et al.*, 1982). Both were included in the incubation. We have previously shown that the PPI-pde requires 100 nM-Ca²⁺ for activation (Cockcroft, 1986),

Fig. 2. Time course of IP₁ formation with TSH in FRTL-5 cells

[³H]Inositol-labelled FRTL-5 cells were incubated with and without TSH (500 munits/ml) for the indicated periods of time. Radioactivity in all three inositol phosphates was analysed, and no change in IP₂ or IP₃ was evident at 5 min, but these increased at later times similarly to IP₁. Only results for IP₁ are shown, for reasons of clarity. The results are means $\pm$ S.E.M. of quadruplicate determinations. $\circ$, Control; $\bullet$, incubations with TSH. At 15 min $P < 0.005$ and at 30 and 60 min $P < 0.001$ compared with the control incubations. In this experiment there were 109600 ± 13000 d.p.m./10⁵ cells in the total inositol lipids.

Fig. 3. Concentration-dependence of noradrenaline on IP_1 formation in FRTL-5 cells

[3H]Inositol-labelled FRTL-5 cells were incubated with different concentrations of noradrenaline for 1 h and the formation of IP_1 was determined. Results presented are means $\pm$ S.E.M. of quadruplicate determinations. In this experiment there were $83\,000 \pm 10\,000$ d.p.m./ 10^5 cells in the total inositol lipids.

and therefore all the experiments were performed in the presence of this concentration of Ca^{2+} .

Both GTP and GTP[S], in a concentration-dependent manner, stimulate the formation of IP_2 and IP_3 , but not IP_1 , in membranes from FRTL-5 cells (Fig. 4). The absolute increase in IP_3 was much larger than the increase in IP_2 , suggesting that PIP_2 is the major substrate for the enzyme.

DISCUSSION

The present study has examined the effects of TSH and noradrenaline on the hydrolysis of inositol lipids in a cultured rat thyroid-cell line (FRTL-5), by measuring the accumulation of IPs. TSH and noradrenaline stimulated the formation of IP_1 , IP_2 and IP_3 in FRTL-5 cells. These results are consistent with the report that TSH and noradrenaline rapidly stimulated the hydrolysis of PIP and PIP_2 (Philp & Grollman, 1986). The amount of TSH required for stimulation was approximately 3 orders of magnitude greater than that necessary for either growth of the cells (Ealey *et al.*, 1985) or augmentation of cyclic AMP (Bidey *et al.*, 1984; Corda *et al.*, 1985; the present paper). This result is consistent with the observation that concentrations of TSH 3 orders of magnitude higher are needed to increase the intracellular Ca^{2+} concentration of FRTL-5 cells (Corda *et al.*, 1985). Thus it is very likely that the increase in cytosol Ca^{2+} observed in these cells is a function of TSH-stimulated IP_3 production.

Both cyclic AMP formation and IPs formation are events that are a function of receptor occupancy. Thus the observation that markedly different concentrations

Fig. 4. GTP and GTP[S] stimulation of IP_2 and IP_3 accumulation in membranes prepared from [3H]inositol-labelled FRTL-5 cells

Membrane preparations of FRTL-5 cells which had been prelabelled with [3H]inositol were incubated with various concentrations of GTP or GTP[S] for 10 min. The samples were quenched directly with chloroform/methanol and, after phase separation (as described in the Methods and materials section), the individual IPs were analysed. The results for GTP and GTP[S] were obtained from two independent experiments. The radioactivity in IP_1 was 286 ± 24 and 168 ± 25 d.p.m. in the control incubations, and did not change with the addition of GTP[S] or GTP respectively (results not shown). The results are the means $\pm$ S.E.M. of triplicate determinations. For the experiment with GTP[S], there were 2×10^5 d.p.m. in the inositol phospholipids used per incubation, and for the experiment with GTP there were 1.3×10^5 d.p.m. The increase in IP_2 and IP_3 with GTP[S] represents hydrolysis of 6% of the total inositol lipids. Δ , $\blacktriangle$, IP_3 ; $\circ$, $\bullet$, IP_2 ; Δ , $\circ$, experiment with GTP[S]; $\blacktriangle$, $\bullet$, experiment with GTP.

of TSH are necessary for IPs formation compared with cyclic AMP formation raises the possibility that there are two different TSH receptors. Although several reports have indicated curvilinear Scatchard plots of TSH binding (Manley *et al.*, 1972; Kotani *et al.*, 1975; Tramontano & Ingbar, 1986), these have been interpreted on the basis of negative co-operativity rather than two different classes of TSH receptors. However, some investigators have reported only a single class of TSH receptors (Smith & Hall, 1974; Verrier *et al.*, 1974).

Piot & Jacquemin (1980) reported a dissociation of the effects of TSH on cyclic AMP concentrations and PI labelling in a mouse thyroid tumour. The tissue res-

ponded to TSH with regard to PI labelling but not accumulation of cyclic AMP. It is not uncommon for a cell to have two separate receptors systems coupled to different signalling systems responding to a single agonist. Thus 5-hydroxytryptamine in blowfly salivary glands (Berridge & Heslop, 1981), glucagon in hepatocytes (Wakelam *et al.*, 1986), luteinizing hormone in rat granulosa cells (Davis *et al.*, 1986), corticotropin in rat adrenal cells (Farese *et al.*, 1986) and secretin in rat pancreas (Trimble *et al.*, 1986) all stimulate inositide metabolism and cyclic AMP formation, showing different concentration-dependencies for the two signalling systems. In some of the studies (Berridge & Heslop, 1981; Wakelam *et al.*, 1986) it was clearly established that this was due to the presence of two different receptor classes rather than a single receptor class coupling to two signalling systems.

Cyclic AMP can mimic many of the TSH-stimulated responses, such as growth, iodine organification, glucose oxidation, endocytosis of colloid, fusion of phagosomes with lysosomes or release of thyroid hormones (Field, 1975), but not desensitization of adenylate cyclase or inositol lipid metabolism (Zor *et al.*, 1968; Shuman *et al.*, 1976; Igarashi & Kondo, 1980; Hirayu *et al.*, 1985). Since the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate is able to inhibit the TSH stimulation of adenylate cyclase (Yoshimura *et al.*, 1986), it could be speculated that diacylglycerol may be responsible for desensitization by stimulating protein kinase C. Evidence has been presented that phosphorylation by protein kinase C can desensitize receptors that are coupled to adenylate cyclase (Rebois & Patel, 1985; Nambi *et al.*, 1985). In this way not only would TSH stimulate the functional responses of the thyroid through one transmembrane signalling system, i.e. cyclic AMP, but also it could, by interacting with another system, operating through activation of inositide metabolism, provide a negative-feedback control in the regulation of thyroid function.

Our results indicate other effects of TSH on inositide metabolism beside formation of IPs, since GPI, most probably derived from PI deacylation, was also consistently increased. This could reflect the action of phospholipase A₂ for the generation of arachidonic acid (Irvine, 1982). TSH stimulation of phospholipase A₂ has been previously reported in thyroid cells (Haye & Jaquemin, 1974; Burch *et al.*, 1986).

We have also studied the presence of the enzyme (PPI-pde) that is responsible for hydrolysing PPI in membranes from FRTL-5 cells. It has been shown previously that PPI-pde is able to be directly activated by guanine nucleotides (Cockcroft & Gomperts, 1985; Martin *et al.*, 1986; Uhing *et al.*, 1986), supporting the notion that PPI-pde is regulated by a guanine nucleotide regulatory protein, G_v, in a manner analogous to adenylate cyclase regulation by G_s (Gilman, 1984). Like a number of other cell types, FRTL-5 cells also have a guanine-nucleotide-regulated PPI-pde. From the composition of the water-soluble products in FRTL-5 membranes stimulated with GTP[S], it is evident that PIP₂ is the major substrate to be hydrolysed, since that is the only source of IP₃. We cannot rule out that some PIP may also be directly hydrolysed, since IP₂ also accumulated. However, it may be derived from either IP₃ or PIP.

In conclusion, we have demonstrated that TSH can couple to two separate transmembrane signalling systems, i.e. the cyclic AMP/adenylate cyclase system

and IP₃/diacylglycerol/PPI-pde. Both systems are regulated by guanine nucleotide regulatory proteins, G_s and G_v, respectively.

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