Induction of lutropin receptors by lutropin and cyclic AMP in cultured mouse tumour (MA10) Leydig cells

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The role of cyclic AMP in the regulation of lutropin (luteinizing hormone, LH) receptors has been investigated in cultured mouse tumour (MA10) Leydig cells. The LH receptors were quantified by measuring the binding of ^{125}I -labelled human chorionic gonadotropin (hCG). LH (0.03 nM) in the presence of 1 mM-dibutyl- γ -cyclic AMP [(Bu) $_2$ cAMP] caused a 3–8-fold increase in subsequent ^{125}I -hCG binding. (Bu) $_2$ cAMP (1 mM), cholera toxin (11.9 nM) and forskolin (1 μM) each caused a 2–4-fold increase in binding. In the presence of translation (cycloheximide) and transcription (actinomycin D) inhibitors, there was a loss of detectable binding sites. (Bu) $_2$ cAMP increased the rate of recovery of binding sites after trypsin treatment of MA10 cells, with a concomitant 2-fold increase in the level of binding sites. Under conditions where receptor levels were increased by 3–8-fold there was also a significant increase in pregnenolone production. It is concluded that LH and cyclic AMP have positive regulatory effects on LH receptors in MA10 cells by inducing the synthesis of new receptors. These induced receptors are functionally coupled to steroidogenesis.

INTRODUCTION

Lutropin (luteinizing hormone, LH) and human chorionic gonadotropin (hCG) are polypeptide hormones that interact with the same cell surface receptor to stimulate steroidogenesis in Leydig cells in the testis via the second messenger cyclic AMP, and also by other mechanisms involving chloride channels (Choi & Cooke, 1990), Ca^{2+} and arachidonic acid and/or its metabolites (Abayasekara *et al.*, 1990). The LH receptor also exhibits ligand-induced loss, i.e. down-regulation of cell surface LH binding capacity (Freeman & Ascoli, 1981).

The mechanism(s) involved in the synthesis of LH receptors in Leydig cells is not well understood. Increases in LH receptor concentrations have been demonstrated *in vivo* (Huhtaniemi *et al.*, 1981) and *in vitro* (Baranao & Dufau, 1983), but the increases were transient and the receptors were shown not to be functionally coupled.

Cyclic AMP has been demonstrated in other cell systems to have a role in the regulation of plasma membrane receptor synthesis and growth of cultured cells. However, both positive and negative effects have been found. For example, the growth hormone receptor is down-regulated in the presence of cyclic AMP (Gorin *et al.*, 1988), whereas the expression of thyroid-stimulating hormone receptors (Lissitzky *et al.*, 1975) and insulin receptors (Thomopoulos *et al.*, 1977) is increased by cyclic AMP. Cyclic AMP also has effects on the growth of cells, either stimulating (Dumont *et al.*, 1989) or inhibiting (Pastan *et al.*, 1975) growth.

In granulosa cells, up-regulation of LH receptors occurs *in vitro* when the cells are stimulated with follicle-stimulating hormone, acting via cyclic AMP (Knecht & Catt, 1982) and oestrogens (Knecht *et al.*, 1984). The increase in receptor numbers is derived from the synthesis of new receptors and not from a pool of receptors (Segaloff & Limbird, 1983).

In this study, we present evidence to show that there is a cyclic AMP-dependent mechanism for the up-regulation of LH

receptors in cultured mouse tumour (MA10) Leydig cells and furthermore that these new receptors are functionally coupled to steroidogenesis.

MATERIALS AND METHODS

Materials

Ovine LH (batch oLH-26, potency 2.3 units/mg) and hCG (batch CR-127, potency 14900 units/mg) were obtained from the National Institute of Child Health and Human Development (Bethesda, MD, U.S.A.). MA10 cells were a donation from Dr. M. Ascoli, The Population Council (New York, NY, U.S.A.). Crude hCG (5000 units) was obtained from Serono Laboratories (Welwyn Garden City, Herts., U.K.). Forskolin, cholera toxin and dibutyl- γ -cyclic AMP [(Bu) $_2$ cAMP] were purchased from Sigma (Poole, Dorset, U.K.). Powered media and sera were obtained from Gibco (Uxbridge, Middlesex, U.K.). Glass microfibre filters (GF/C) were purchased from Whatman (Maidstone, Kent, U.K.). Carrier-free Na^{125}I was obtained from Amersham International (Amersham, Bucks., U.K.). [^3H]Methionine was purchased from NEN-Dupont (Stevenage, Herts, U.K.). Cyanoketone and SU10603 (inhibitors of pregnenolone metabolism) were obtained from Sterling Research Group Europe (Guildford, Surrey, U.K.). All other reagents were purchased from Sigma or BDH (Poole, Dorset, U.K.).

MA10 cell propagation

Stock cultures of MA10 cells (Ascoli, 1981) were maintained in Waymouth MB752/1 medium, containing 20 mM-Hepes (pH 7.7), 1.12 g of NaHCO_3 /l, 40 μg of gentamycin/ml and 15% horse serum (growth medium), in a humid atmosphere of CO_2/O_2 (1:39) at 34 °C. The cells were subcultured by trypsin treatment [0.05% (w/v) trypsin] and experimental cultures were plated in growth medium or serum-free media [containing 0.1% (w/v) BSA, 4 mg of insulin/l and 10 mg of transferrin/l] and

Abbreviations used: LH, lutropin (luteinizing hormone); hCG, human chorionic gonadotropin; (Bu) $_2$ cAMP, dibutyl- γ -cyclic AMP; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

used 2–3 days after subculture. The growth medium was replaced on alternate days in culture. Cell numbers were determined by using a haemocytometer and viability of the cells by the Trypan Blue exclusion method (Jeejeebhoy *et al.*, 1975) and diaphorase histochemistry (Aldred & Cooke, 1983).

Iodination of hCG

Highly purified hCG (CR-127) was iodinated with ^{125}I using lactoperoxidase, to give a specific radioactivity of about 100 Ci/g, and purified by Sephadex G-25M chromatography (Thorell & Johansson, 1971). ^{125}I -hCG was stored at -20°C and used within 4 weeks of preparation. Characterization of ^{125}I -hCG was by a competitive binding assay with known concentrations of purified hCG.

Measurement of LH/hCG receptors

Cultured cells were placed on ice, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS; 0.01 M-phosphate/0.9% NaCl, pH 7.4) containing 0.1% BSA (PBS/BSA). If the cells had been incubated in the presence of LH/hCG, they were further washed with 50 mM-glycine-buffered 0.9% NaCl (pH 3) for 2 min on ice to remove surface-bound LH/hCG (Ascoli, 1982). After washing again with 0.01 M-PBS/BSA, the cells were reincubated overnight in 0.1% BSA-containing Waymouth MB752/1 medium at 4°C with ^{125}I -hCG (0.56 nM) and with 0, 0.14 or 0.28 nM highly purified hCG. The level of binding was determined by aspirating the medium, washing twice with 0.01 M-PBS/BSA to remove unbound hormone and dissolving the cells in 0.5 M-NaOH before determination of radioactivity by γ -counting. Non-specific binding, which was approx. 10% of the total binding, was determined in the presence of 400 units of unlabelled crude hCG.

Measurement of pregnenolone and cyclic AMP production

Cells were preincubated with inhibitors of pregnenolone metabolism [cyanoketone (5 μM) or SU10603 (20 μM) dissolved in ethanol] for 30 min and then incubated with LH, hCG, cholera toxin or forskolin for 2 h at 34°C . Pregnenolone and cyclic AMP were extracted from the cells by the addition of HClO_4 (final concentration 0.5 mol/l), which was neutralized by adding K_3PO_4 (final concentration 0.23 mol/l). The levels of cyclic AMP were determined by radioimmunoassay by the method of Steiner *et al.* (1972), as modified by the acetylation procedure described by Harper & Brooker (1975). Pregnenolone production was determined by radioimmunoassay by the method of van der Vusse *et al.* (1975). Each determination was carried out in triplicate.

Measurement of protein synthesis using the incorporation of [^3H]methionine

Cells were preincubated for 14 h in the presence or absence of LH (0.033 nM) and $(\text{Bu})_2\text{cAMP}$ (1 mM) in 3 ml of growth medium at 34°C . Following this incubation the cells were washed twice with 1 ml of methionine-free medium [Dulbecco's modified Eagle's medium (DMEM)/F12 plus 15% horse serum] and recultured in 1 ml of the same medium in the presence or absence of LH/ $(\text{Bu})_2\text{cAMP}$ or $(\text{Bu})_2\text{cAMP}$ plus 5 μCi of [^3H]methionine (200 mCi/mmol). After 6 h the cells were washed twice with DMEM/F12 containing 10 mM-methionine, scraped from the dish in 1 ml of PBS containing 0.1% BSA and 10 mM-methionine and homogenized. Portions of 100 μl were pipetted on to Whatman GF/C filters (prewashed in PBS/BSA/methionine) and the protein was precipitated and washed with ice-cold 30% (w/v) trichloroacetic acid/10 mM-methionine by a vacuum filtration method.

RESULTS

In this study the regulation of LH receptors was carried out using LH and the binding sites were assessed using ^{125}I -hCG. LH was found to have similar effects to those of hCG (results not shown). Ascoli & Segaloff (1987) have also demonstrated that, in the presence of excess ^{125}I -LH or ^{125}I -hCG, the hormones undergo similar fates after binding to the LH receptor in MA10 cells.

In order to determine the effects of LH and cyclic AMP on the rate of growth of the MA10 cells, the latter were incubated with cholera toxin (11.9 nM), forskolin (1 μM) or $(\text{Bu})_2\text{cAMP}$ (1 mM) for 72 h (Fig. 1). All of these compounds decreased the growth rate of the cells. Incubating the cells with LH (0.033 nM) over the same time period resulted in no change in the growth rate of the MA10 cells (results not shown).

Figs. 2 and 3 show the effects on LH receptor levels when MA10 cells are incubated in the presence of LH, $(\text{Bu})_2\text{cAMP}$, cholera toxin or forskolin. LH at all concentrations (0.033–3.3 nM) caused a significant loss of receptors (Fig. 2a). The degree of loss was both concentration- and time-dependent. There was also a significant recovery of binding sites, back to control levels, in the incubations containing 0.033 or 0.33 nM-LH. Cholera toxin (11.9 nM) caused a 4-fold increase of binding sites above basal levels by 24 h. Forskolin (1 μM) and $(\text{Bu})_2\text{cAMP}$ (1 mM) produced a similar profile, inducing a 3-fold increase above basal levels by 48 h (Fig. 2b). Higher concentrations of $(\text{Bu})_2\text{cAMP}$ (2.5–5 mM) had no effect on the level of detectable binding sites (cf. controls) in MA10 cells over 48 h (results not shown). During the incubation period, control cells demonstrated fluctuations in the numbers of ^{125}I -hCG binding sites, ranging from 13.8 ± 5.5 to 97.0 ± 13.8 fmol/ 10^6 cells; therefore results refer to the appropriate control at the time of determination.

Fig. 3 shows the effects of adding 1 mM- $(\text{Bu})_2\text{cAMP}$ with LH, cholera toxin or forskolin. $(\text{Bu})_2\text{cAMP}$ in the presence of LH (0.033 nM) induced an 8-fold increase in receptor number. The average increase in the numbers of binding sites, from different experiments, of cells treated with LH (0.033 nM) and $(\text{Bu})_2\text{cAMP}$ (1 mM) was 5 ± 2 -fold (mean $\pm$ S.D., $n = 5$). $(\text{Bu})_2\text{cAMP}$ had no effect on the loss of binding sites induced by 3.3 nM-LH, but increased the rate of loss of receptors with 0.33 nM-LH (Fig. 3a), i.e. by 48 h there was a total loss of binding sites compared with

Fig. 1. The effects of $(\text{Bu})_2\text{cAMP}$, cholera toxin and forskolin on the growth of MA10 cells

Cells were plated at 200×10^3 /well in 12-well plates, incubated at 34°C and used 48 h later. At 48 h, 1 mM- $(\text{Bu})_2\text{cAMP}$ (△), 11.9 nM-cholera toxin (■) or 1 μM -forskolin (□) was added, and cell numbers were determined at the times indicated. ○, Control cells. The results are means $\pm$ S.D. Statistical analysis was by Student's paired *t* test, $n = 6$ (* $P < 0.01$, ** $P < 0.001$). The data are representative of six experiments.

Fig. 2. Time courses of the effects of LH, cholera toxin, forskolin and cyclic AMP on the levels of binding sites in MA10 cells

Cells were plated at a density of 150×10^3 /well in 24-well plates and used 2 days later. (a) Cells were incubated at 34°C , with LH [0.033 ($\square$), 0.33 ($\circ$) and 3.3 ($\triangle$) nM]. At the indicated times the cells were placed on ice and washed and ^{125}I -hCG binding was determined. (b) Cells were treated as above with 1 mM-(Bu)₂cAMP ($\square$), 11.9 nM-cholera toxin ($\circ$) or 1 μM -forskolin ($\triangle$). Results are the means $\pm$ S.D. Statistical analysis was by Student's paired *t* test; $n = 6$ (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$). The results are representative of two similar experiments.

only a 30% loss with 0.33 nM-LH (Fig. 2a) on its own at the same time point. Incubating cells with 1 mM-(Bu)₂cAMP and cholera toxin (Fig. 3b) enhanced the induced levels of binding sites from 4–6-fold. (Bu)₂cAMP (1 mM) had no effect on forskolin (1 μM)-stimulated induction of binding sites (Fig. 3b) and inhibited the increase observed with 10 μM -forskolin (results not shown).

In order to determine the mechanism involved in the observed increase in receptors, the effects of transcription and translation inhibitors on binding sites were investigated (Fig. 4). Parallel protein synthesis assays were also undertaken on cells derived from the same population. Cells were preincubated for 14 h in the absence or the presence of LH (0.033 nM) and (Bu)₂cAMP (1 mM) and then further incubated for 6 h after the addition of cycloheximide (3.5 and 35 mM) or actinomycin D (3.98 mM). It was found that incorporation of the labelled amino acid into proteins during the final 6 h of incubation was increased when LH and (Bu)₂cAMP were present together [control $(3.2 \pm 0.6) \times 10^4$ c.p.m./ 10^6 cells; stimulated $(5.2 \pm 0.3) \times 10^4$ c.p.m./ 10^6 cells; $P < 0.05$]. When LH was removed for the last 6 h [i.e. cells incubated in the presence of (Bu)₂cAMP only] this increase was not detectable. The effects of cycloheximide and

Fig. 3. Time courses of the effects of 1 mM-(Bu)₂cAMP plus LH, cholera toxin or forskolin on the levels of binding sites in MA10 cells

Cells were treated as in Fig. 2 in the presence of 1 mM-(Bu)₂cAMP plus (a) various LH concentrations [0.033 ($\square$), 0.33 ($\circ$) and 3.3 ($\triangle$) nM]; (b) 11.9 nM-cholera toxin ($\circ$) or 1 μM -forskolin ($\triangle$). Results are means $\pm$ S.D. Statistical analysis was by Student's paired *t* test; $n = 6$ (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$). The results are representative of one of two similar experiments.

actinomycin D on receptor levels in the MA10 cells during the 6 h incubation period are shown in Fig. 4. Both inhibitors caused a loss of binding sites in control cells. In cells that had been pretreated for 14 h with LH and (Bu)₂cAMP, and then for 6 h in the presence of the inhibitors, a 50% decrease in the levels of induced binding sites was observed. At the concentrations of inhibitors used there was only a 5% decrease in the viability of the cells, which would not account for the observed loss of binding sites.

The effect of (Bu)₂cAMP on the rate of recovery after trypsin treatment of MA10 cells was also studied. Cells were treated for 4 min with 0.05% trypsin which resulted in a complete loss of detectable binding sites. Trypsin-treated cells were incubated in the absence or the presence of (Bu)₂cAMP (1 mM) and the rate of recovery of the receptors was observed by measuring binding sites at various time points (Fig. 5). In control cells, binding returned to pre-trypsin-treated levels by 48 h. In the presence of (Bu)₂cAMP, receptor levels reached a maximum by 24 h, which was maintained for 72 h and was 2-fold higher than control levels. (Bu)₂cAMP also prevented the loss of binding sites that is observed when MA10 cells are incubated in serum-free conditions for longer than 24 h (results not shown).

Fig. 4. The effects of translation and transcription inhibitors on the level of hCG binding sites in MA10 cells

Cells were plated at a density of 150×10^3 /well in 24-well plates in a volume of 1 ml at 34°C and used 2 days later. Cells were preincubated as for Fig. 3. At the end of 14 h the inhibitors were added ($\square$, basal; $\boxtimes$, 3.98 mM-actinomycin D; $\blacksquare$, 3.5 mM-cycloheximide; and $\boxdot$, 35 mM-cycloheximide), and incubated for a further 6 h. At the end of the total incubation time cells were placed on ice and washed, and ^{125}I -hCG binding was determined. Results are means $\pm$ S.D. Statistical analysis was by Student's paired t test; $n = 6$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The data represent one of two similar experiments.

The effect of preincubating MA10 cells for 24 h with LH (0.033 nM) and/or $(\text{Bu})_2\text{cAMP}$ (1 mM) on the subsequent LH-stimulated pregnenolone production of these cells when incubated with 3.3 nM-LH is shown in Fig. 6. The results show that in the cells treated with $(\text{Bu})_2\text{cAMP}$ alone or with LH there was a marked increase in pregnenolone production both in the presence and the absence of LH in the second incubation (Fig. 6). These increases correlated with the increases in receptor numbers (Figs. 2b and 3a). In order to remove residual bound LH the cells were acid-washed before the second incubation; this was found to decrease pregnenolone production in the control cells by 50% (Fig. 6). Table 1 shows the production of cyclic AMP in pretreated cells on stimulation with LH (3.3 nM). The washing procedure used appears to have inhibited the production of cyclic AMP,

Fig. 5. Time course of the recovery of LH receptors after trypsin treatment

Cells were grown to a density of 200×10^3 /well (24-well plates) and treated with 0.05% (w/v) trypsin for 4 min. The trypsin-treated cells were recultured in 1 ml of growth medium at 34°C in the absence ($\circ$) or presence ($\triangle$) of 1 mM- $(\text{Bu})_2\text{cAMP}$. At the times indicated, the degree of ^{125}I -hCG binding was determined as described in the Materials and methods section. Results are means $\pm$ S.D. Statistical analysis was by Student's paired t test; $n = 12$ (* $P < 0.001$).

Fig. 6. Pregnenolone production by cells pretreated for 24 h to induce LH receptors

Cells were plated at a density of 150×10^3 /well and used 2 days later. The cells were preincubated with LH (0.033 nM, $\boxtimes$), $(\text{Bu})_2\text{cAMP}$ (1 mM, $\boxdot$) or LH + $(\text{Bu})_2\text{cAMP}$ ($\blacksquare$) for 24 h. At the end of this incubation period the cells were washed twice with 50 mM-glycine-buffered saline (pH 3) and four times with medium, and recultured with 1 ml of fresh growth medium. Non-pretreated cells were also given an acid wash ($\boxtimes$) or not treated ($\square$) to act as controls. Cells were then further incubated for 30 min with inhibitors of pregnenolone metabolism before being incubated for 2 h in the absence or presence of LH (3.3 nM). The reaction was stopped using HClO_4 . Pregnenolone was determined as described in the Materials and methods section. Results are means $\pm$ S.D. Statistical analysis was by Student's paired t test; $n = 6$ (* $P < 0.01$, ** $P < 0.001$).

Table 1. Cyclic AMP production by cells pretreated for 24 h to induce LH receptors

Cells were prepared and treated as for Fig. 6, and cyclic AMP production was determined as described in the Materials and methods section. Results are means $\pm$ S.D.

Pretreatment	Cyclic AMP (pmol/h per 10^6 cells)	
	Basal	+ LH (3.3 nM)
Control (not acid-washed)*	5.0 ± 2.0	125.0 ± 25.0
Control	0.5 ± 1.0	2.0 ± 1.0
LH (0.033 nM)	0.3 ± 0.3	0.5 ± 0.4

* These cells were not washed with 50 mM-glycine-buffered saline before ^{125}I -hCG binding was determined.

though there is still a 50% maximal stimulated production of pregnenolone observed in these cells.

DISCUSSION

In this study we present evidence for the positive modulation of the synthesis of LH receptors by LH and cyclic AMP in cultured mouse tumour (MA10) Leydig cells. Previously the up-regulation of LH receptors in rat testis Leydig cells was shown to occur over a short period of time and to not be a direct effect of LH on its receptor, but an effect on the plasma membrane (Huhtaniemi *et al.*, 1981). LH in our studies did not exhibit this short-term effect, and all concentrations of LH used eventually resulted in receptor down-regulation. This is similar to the situation in rat testis Leydig cells where it is established that LH-induced desensitization and eventual down-regulation regulate the LH receptor (Cooke & Rommerts, 1988; Rommerts & Cooke, 1988). These events are not thought to be mediated by

cyclic AMP but probably involve LH-regulated protein kinase C (Dix *et al.*, 1987). Cyclic AMP in the present experiments prevented the loss of LH receptors at low doses of LH and increased levels of binding. This may explain the recovery of binding sites observed after down-regulation with 0.033 nM-LH (48 h) and 0.33 nM-LH (24 h). Generation of cyclic AMP by activation of the GTP-binding protein G_s by cholera toxin and direct stimulation of adenylate cyclase by forskolin also resulted in an increase in binding sites.

LH and cyclic AMP have previously been shown to induce the synthesis of mitochondrial proteins involved in steroid synthesis in MA10 cells, but it was found that the overall protein synthesis capacity was not affected during a 6 h incubation period (Stocco & Kilgore, 1988). In our study, preincubation with LH and cyclic AMP for 14 h resulted in a detectable increase in total protein synthesis during a further 6 h incubation with a labelled amino acid. The constant presence of LH was required for this increase to occur. The effect of the translation and transcription inhibitors on the levels of LH receptors indicated that, for the maintenance of receptors, continual protein synthesis is required. The initiation of this process may occur at the gene level, as was demonstrated in porcine granulosa cells (Segaloff & Limbird, 1983), and not from a pool of mRNA. The stimulatory effect of cyclic AMP on the induction of binding sites was also shown in cells which had been trypsin-treated; cyclic AMP increased the rate of recovery of binding sites in these cells, as well as raising the number of detectable binding sites.

The results obtained by pretreating the cells for 24 h with LH and (Bu)₂cAMP and then adding LH (3.3 nM) show that there is an increase in functionally coupled LH receptors. The results also indicated that there was no detectable production of cyclic AMP in cells that have been acid-washed. The maximal pregnenolone production in control cells could therefore be the result of two pathways acting together; one of these pathways may not be cyclic AMP-dependent. Previously it was shown that intracellular levels of cyclic AMP could not be restimulated after exposure to 1.12 nM-hCG for 15, 30 or 120 min using an acid wash to remove the hCG (Segaloff & Ascoli, 1981).

To conclude, the results of the present study support the concept of a role for cyclic AMP in the maintenance and regulation of LH receptors in MA10 cells. These cells are similar to granulosa cells in that factors acting via cyclic AMP maintain the LH receptor. In the MA10 cells, LH also appears to have two effects on its receptor. In the presence of LH, down-regulation occurs; this is probably due to an increased rate of degradation of the receptor upon binding of LH. When cyclic AMP is added in the presence of low doses of LH (< 0.033 nM) up-regulation occurs, which is probably due to an increased rate of synthesis of the LH receptor.

We are grateful to the Medical Research Council, the Science and Engineering Research Council and the Nuffield Foundation for financial support, to Professor E. Barnard for helpful discussions and to Dr. M. Ascoli for generously donating the MA10 cells.

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