Desensitization and resensitization of lutropin receptors expressed in transfected Y-1 adrenal cells

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Abstract

Stimulation of gonadal cells by lutropins such as human chorionic gonadotropin (hCG) is often transient and followed by down-regulation and/or desensitization of lutropin receptors (LHR). Here we describe desensitization/resensitization of LHR in Y-1 adrenal cell lines (termed Y-1L) expressing a rat cDNA lacking most 5' and 3' LHR untranslated regions under the control of a metallothionein promoter. Using a simple morphological assay in which stimulated cells are round and unstimulated cells are flat, we identified clones that rounded and remained round and others that became insensitive to lutropin stimulation and reverted to their flat appearance within 2–4 h. Flattened cells were insensitive to further hormonal stimulation but rounded after treatments with cholera toxin, forskolin, or cyclic AMP, showing that loss of responsiveness was associated with an early step in signal transduction, not loss of rounding potential. Removing the lutropin stimulus for at least 90–120 min reversed hormone insensitivity, even in the presence of the protein synthesis inhibitor puromycin. The number of surface bound receptors did not change during a cycle of rounding/flattening and hCG bound to rounded or flattened cells was replaced equally by radioiodinated hCG during incubations at 4°C. Thus, desensitization/resensitization of LHR in Y-1L cells occurred in the absence of new receptor synthesis, receptor degradation, or receptor recycling. These observations suggest that LHR desensitization/resensitization in Y-1L cells was closely coupled to receptor occupancy and that this cell line may be useful for identifying factors that modulate the activities of occupied receptors.


Introduction

Follitropins and lutropins act through gonadal receptors to control reproduction and sex steroid hormone synthesis (Moyle 1980, Hsueh et al. 1984). Hormonal responsiveness can be limited by desensitization, a process in which the receptor fails to elicit a signal (Bockaert et al. 1976, Ekstrom & Hunzicker-Dunn 1989, Ekstrom et al. 1992, Sanchez-Yague et al. 1992) and/or down-regulation, a process by which receptors are lost (Hsueh et al. 1977, Metsikko & Rajaniemi 1984, Wang et al. 1991, Lakkakorpi et al. 1993). With the goal of devising a simple morphological assay for studying the abilities of human chorionic gonadotropin (hCG) and hCG analogs to elicit hormonal responses or to promote lutropin receptor (LHR) desensitization/down-regulation, we inserted the rat LHR into cells of the Y-1 adrenal cell line and monitored the ability of LHR-expressing cells to respond to hCG and other lutropins by changing their shapes. Y-1 cells were chosen for these studies because they do not express gonadotropin receptors, because their shapes have long been known to change from flat to round when they are treated with adrenocorticotropin (ACTH) (Yasamura et al. 1966), cyclic AMP (Kowal & Fiedler 1969), or agents such as cholera toxin (Kowal & Srinivasan 1975) that raise cyclic AMP levels, and because they are known to desensitize in response to hormone stimulation (Olson et al. 1991a). In addition, the propensity of Y-1 cells to mutate has led to the isolation of desensitization-resistant Y-1 cell lines that do not lose their hormone responsiveness to catecholamine stimulation (Olson et al. 1991b, Colantonio et al. 1998). These are valuable tools for understanding the desensitization process.

Since lutropin stimulation leads to cyclic AMP accumulation (Moyle 1980), we expected that treatment of LHR-transfected Y-1 adrenal cells (termed Y-1L cells) with hCG would cause them to become hormone responsive, a process readily detected by their ability to become round in appearance. As anticipated, Y-1L cells rounded in response to hCG and other lutropins. Many of these cell lines lost their ability to respond to hCG and re-acquired their flat appearance within a few hours, even though


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hCG remained present in the culture medium. This suggested that the LHR in these Y-1L clones had been down-regulated or desensitized. Here we report that loss of Y-1L responsiveness was due to desensitization of the receptor, not loss of hCG binding sites. This process was reversed in the apparent absence of de novo receptor synthesis. These cells may be useful for identifying factors that limit the signal transduction ability of the LHR.

Materials and Methods

Hormones

Purified hCG was a gift from Dr Robert Canfield, Columbia University, New York, NY, USA. Purified bovine luteinizing hormone (bLH) was a gift from Dr John Pierce, UCLA, Los Angeles, CA, USA. Iodination of hCG was performed using Iodo-Gen (Cruz Pierce, UCLA, Los Angeles, CA, USA). Iodination of bovine luteinizing hormone (bLH) was a gift from Dr John Pierce, UCLA, Los Angeles, CA, USA. Puri fi can be cloned a cDNA for the rat LHR that lacked MD, USA and transfected with a vector (pLEN) into Y-1 adrenal cells were obtained from ATCC, Rockville, MD, USA and transfected with a vector (pLEN) into which we had cloned a cDNA for the rat LHR that lacked virtually all 5’ and 3’ untranslated bases between its XbaI and BamHI endonuclease restriction sites (Bernard et al. 1990). pLEN (Kushner et al. 1990) was a gift from Dr Peter Kushner, UCSF, San Francisco, CA, USA. Y-1 cells were co-transfected with pLEN-rat LHR and pSV2-Neo (Southern & Berg 1982) and selected by their ability to grow in culture medium containing 500 µg G418/ml (Gibco-BRL, Grand Island, NY, USA). Cell lines expressing the LH receptor (i.e. Y-1L cells) were detected by their ability to bind 125I-hCG and by their ability to round after hCG stimulation.

Cell expression cell lines

Y-1 adrenal cells were obtained from ATCC, Rockville, MD, USA and transfected with a vector (pLEN) into which we had cloned a cDNA for the rat LHR that lacked virtually all 5’ and 3’ untranslated bases between its XbaI and BamHI endonuclease restriction sites (Bernard et al. 1990). pLEN (Kushner et al. 1990) was a gift from Dr Peter Kushner, UCSF, San Francisco, CA, USA. Y-1 cells were co-transfected with pLEN-rat LHR and pSV2-Neo (Southern & Berg 1982) and selected by their ability to grow in culture medium containing 500 µg G418/ml (Gibco-BRL, Grand Island, NY, USA). Cell lines expressing the LH receptor (i.e. Y-1L cells) were detected by their ability to bind 125I-hCG and by their ability to round after hCG stimulation.

Cell culture and analysis of rounding

Y-1 cells were grown in Ham’s F-12 media containing 15% horse serum and 2.5% fetal bovine serum, supplemented with 2 mM l-glutamine, 100 units penicillin/ml, and 0.1 mg streptomycin/ml in an atmosphere of 2.5% CO2/97.5% air. Culture media were changed three times weekly. Flasks of cells were transferred to a Nikon incubator (model NP-2) and their shapes monitored continuously using an inverted phase contrast microscope at 100 × magnification and a time lapse compression of 480:1. The percentage of rounded cells in most experiments was calculated from video images in which the same cells were monitored for as long as three days or from photomicrographs taken at 40 × or 100 × from random areas of the culture dishes. As noted in the figure legends, in some studies four areas were marked on the bottom of a culture dish and the rounded/flat appearance of the cells in these areas was monitored following hormone or other treatment. At least 80 and usually 100–200 cells were examined to determine the percentage of rounding. All experiments were repeated at least twice.

Receptor binding

Cells grown in 60 mm culture dishes to approximately 70% confluence were incubated in the absence of hCG (i.e. 0 min control) or with unlabeled hCG (100 ng/ml) for 40 and 240 min. The medium was removed and replaced with 2 ml ice-cold medium lacking hCG or ice-cold 100 mM NaCl–50 mM glycine buffer (pH 3), a procedure that has been shown to promote dissociation of hCG from receptors (Ascoli & Segaloff 1987). After 2 min the acid buffer was replaced with ice-cold 0.01 M phosphate buffer–0.9% NaCl solution (pH 7–4) for 2 min, and then with ice-cold media. The cells were scraped from the incubation wells and incubated for 24 h at 4 °C in tubes containing 300 000 d.p.m. 125I-hCG (approximately 4 ng) or 125I-hCG and 1 µg hCG. The cell suspension was centrifuged at 1000 × g for 10 min and the supernatant was aspirated, thereby permitting determination of the radioactivity in the cell pellet.

Cyclic AMP assays

To determine the influence of hCG on intracellular cyclic AMP accumulation we treated cells with 100 ng hCG/ml. The medium was removed and discarded, the cells were washed, and the cyclic AMP in the cell pellet was measured by radioimmunoassay (Brooker et al. 1979).

Results and Discussion

Rounding responses of Y-1L cell lines to lutropins

Treatment of all Y-1L cell clones with hCG (Figs 1 and 2) caused them to acquire a rounded appearance beginning within 10 min and reaching a maximum by approximately 40 min. Approximately 100 ng hCG/ml (2.5 nM) were required to elicit maximal rounding (Fig. 3). Some Y-1L clones retained their rounded appearance for hours; others became flat within 4 h (Figs 1 and 2). hCG treatment of one clone led to several rounds of rounding and flattening (Fig. 4). This clone was unstable, however, and the repetitive nature of the response was lost after several passages in culture.

We considered the possibility that the flattening observed following prolonged stimulation of Y-1L cells might be unique to hCG, a hormone with greater affinity for the rat LHR than most mammalian lutropins. To test this, we repeated several of the studies with bLH. Y-1L cells rounded upon treatment with bLH and then flattened in a similar manner to their response to hCG (Fig. 5). Thus, loss of responsiveness that occurred in response to occupation of the receptor by lutropins was not unique to hCG.
The observation that some Y-1L clones reverted to their flat appearance following hormone stimulation suggested that they lost their ability to respond to hCG, a process that could involve desensitization, down-regulation, or both. To distinguish these mechanisms, we further examined the loss of hormone responsiveness of one Y-1L clone.

The observation that some Y-1L clones reverted to their flat appearance following hormone stimulation suggested that they lost their ability to respond to hCG, a process that could involve desensitization, down-regulation, or both. To distinguish these mechanisms, we further examined the loss of hormone responsiveness of one Y-1L clone.

*Figure 1* Scanning electron micrographs of Y-1L cells before treatment (top) and after exposure to 100 ng hCG/ml for 40 (center) and 300 (bottom) min. Note that the retraction of the cells when they round gives them a much smaller appearance. The center panel is a composite taken to illustrate the variability in shape of the rounded cells as they retract from the surface. All photographs are at the same magnification.

*Rounding responses of Y-1L cells and hCG desensitized Y-1L cells to 8 Br-cyclic AMP and agents that raise intracellular cyclic AMP levels*

Stimulation of Y-1 cells with cyclic AMP has long been known to promote rounding (Kowal & Fiedler 1969) and

*Figure 2* Photomicrograph: time-lapse photomicrographs of Y-1L cells exposed to 100 ng hCG/ml for 0 (top), 40 (center), and 240 (bottom) min. Line drawing: summary of kinetics of rounding of Y-1L cells in response to hCG from time-lapse microscopic observation. Once cells had flattened after continuous hCG treatment, they remained flat until they divided. The flattening observed with hCG was seen in every experiment with this clone and had a similar time course (compare data with those for the first 5 h in Fig. 4).
we observed that cyclic AMP-treated Y-1L cells rounded and stayed round (not shown). Based on the known ability of hCG to stimulate cyclic AMP accumulation in gonadal cells and on the ability of cyclic AMP and the hormones to induce prolonged rounding of Y-1L cells, we reasoned that flattening observed during hCG stimulation was due either to loss of hCG-induced cyclic AMP accumulation or to loss of cyclic AMP-induced rounding. To distinguish these possibilities, we treated hCG insensitive Y-1L cells with cyclic AMP and with forskolin or cholera toxin, agents known to induce cyclic AMP formation and rounding of Y-1 cells (Kowal & Srinivasan 1975, Schimmer & Schulz 1985). While treatment of hCG-flattened cells with additional hCG did not induce them to re-round, hCG-insensitive cells rounded when treated with cyclic AMP, cholaer toxin, or forskolin (Fig. 6). This suggested that the rounding mechanism was operational but that the cells had lost their ability to make cyclic AMP in response to hCG. This was confirmed by direct measurements of intracellular cyclic AMP levels (Table 1).

*Flattened cells bind hCG, indicating that their receptors had been desensitized, not lost or sequestered in a cytosolic compartment*

The loss in responsiveness to hCG could have been caused by internalization and metabolism of hCG receptors or by reduction in the ability of the hCG–receptor complex to...
AMP treatment caused rounding of Y-1L cells that had not been cause re-rounding and (2) that cholera toxin, forskolin, and cyclic incubation of cells with additional hCG for more than 24 h did not 8-Br-cyclic AMP. In studies not shown, we found (1) that (min)

hCG treatment

<table>
<thead>
<tr>
<th>Time of Drug Addition</th>
<th>Percent Rounded Cells</th>
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<tbody>
<tr>
<td>Forskolin</td>
<td>75</td>
</tr>
<tr>
<td>Cholera Toxin</td>
<td>50</td>
</tr>
<tr>
<td>8 Br-cyclicAMP</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 6 Response of Y-1L cells to hCG and other agents known to increase cyclic AMP levels. Cells were monitored continuously by time-lapse video photomicroscopy for the periods illustrated on the abscissa. hCG (100 ng/ml) was added at the initiation of the experiment and again at 4 h. Note that the cells did not re-round after the second addition of hCG even though they were exposed to a total of 200 ng/ml. However, cells that had lost their ability to round in response to hCG rounded within 20 min following addition of 2 µg cholera toxin/ml, 10 mM forskolin, or 1 mM 8-Br-cyclic AMP. In studies not shown, we found (1) that incubation of cells with additional hCG for more than 24 h did not cause re-rounding and (2) that cholera toxin, forskolin, and cyclic AMP treatment caused rounding of Y-1L cells that had not been treated with hCG.

stimulate adenylate cyclase. Both types of responses have been seen after gonadal tissues are treated with hCG (Bockaert et al. 1976, Hsueh et al. 1977). To distinguish these possibilities, we monitored the ability of intact control and hCG-treated rounded and flattened Y-1L cells to bind radiolabeled hCG. All the cells were scraped from the dishes and washed with a neutral bu.

Table 1 Levels of intracellular cyclic AMP of Y-1L cells after hCG treatment. Cyclic AMP values are means of triplicates ± S.E.M. for approximately 60 000 cells

<table>
<thead>
<tr>
<th>hCG treatment (min)</th>
<th>Cyclic AMP (pMoles/60 000 cells)</th>
<th>Cell appearance</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0·70 ± 0·01</td>
<td>Flat</td>
</tr>
<tr>
<td>10</td>
<td>2·43 ± 0·09</td>
<td>Partially round</td>
</tr>
<tr>
<td>20</td>
<td>2·74 ± 0·10</td>
<td>Almost fully round</td>
</tr>
<tr>
<td>40</td>
<td>2·25 ± 0·11</td>
<td>Maximally round</td>
</tr>
<tr>
<td>240</td>
<td>0·72 ± 0·03</td>
<td>Flat</td>
</tr>
</tbody>
</table>

This experiment was performed four times and similar results were observed each time.

incubated with radioiodinated hCG at 4 °C for 24 h, a temperature chosen to prevent receptor internalization and/or recycling. Cells that had not been treated with hCG, rounded cells that had been treated with hCG for 40 min, and flattened cells that had been treated with hCG for 240 min bound radiolabeled hCG equally well regardless of whether they had been washed with acid or neutral buffer (Table 2). In addition to showing that flattening was not caused by hCG-induced LHR loss, these data also suggested that flattening did not involve sequestration of LHR in a cytosolic compartment that would prevent unlabeled hCG from exchanging with radioiodinated hCG at 4 °C. Thus, we concluded that flattening was the result of LHR desensitization, not LHR down-regulation. Since the receptors did not appear to be lost or internalized in desensitized cells, resensitization of the cells following hCG withdrawal may have involved dissociation of hCG from the LHR.

Kinetics of desensitization and resensitization following hCG treatment and withdrawal

Desensitization of the Y-1L cells had a slow onset and complete loss of hCG responsiveness required more than 180 min of hormone treatment (Fig. 7A). Restoration of activity also appeared to be slow and did not begin until 90–120 min after hCG had been removed from the cell surface by acid treatment (Fig. 7B). This delay was considerably longer than that needed for agents acting downstream of the LHR to promote cell rounding (Fig. 6). Thus, it may reflect the minimum time needed for the unoccupied LHR to re-acquire its responsiveness to hormonal signals. Cyclic AMP did not appear to mediate LHR desensitization. Cells that rounded in response to cyclic AMP flattened 50 min after the cyclic AMP was removed from the medium and re-rounded shortly after addition of hCG (Table 3). As seen in Fig. 7B, restoration of the rounding response to hCG-desensitized cells did not begin for 90–120 min.

LH receptor desensitization and resensitization does not require protein synthesis

Treatment of Y-1L cells with 100 µg puromycin/ml culture medium for 4 h, a concentration known to block protein synthesis completely in Y-1 cells (Kowal 1970), often caused the cells to round slowly. In most experiments puromycin treatment alone increased the number of round cells by less than 10% but in one case about 30% of the control puromycin-treated cells were rounded (not shown). Puromycin did not block or enhance the rounding response of Y-1L cells to hCG, but it impaired their ability to flatten relative to the hCG control in some, but not all studies (Fig. 8). While we do not understand the variability in the effects of puromycin on the flattening response, we cannot exclude the possibility that it is due to the toxic
effect of prolonged inhibition of protein synthesis that by itself caused the cells to round. The fact that puromycin usually did not prevent cell flattening in spite of the fact that it is toxic to the cells, suggests that protein synthesis was not required for the loss in hormone responsiveness seen following prolonged hCG treatment of Y-1L cells.

Puromycin did not prevent desensitized cells from regaining their ability to round in response to hCG (Fig. 9). Y-1L cells that had been desensitized by prolonged incubation with hCG were treated with puromycin at the start of the resensitization period, i.e. when hCG was removed from the culture medium. Treatment

Table 2  Treatment with hCG did not cause loss of LH receptors. Y-1L cells were incubated with 100 ng hCG/ml for 0, 40, or 240 min to promote rounding and flattening. After treatment with hCG, the medium was removed and replaced with 2 ml ice-cold media lacking hCG (No acid) or ice-cold 100 mM NaCl–50 mM glycine buffer, pH 3 (Acid wash). After 2 min the acid buffer was replaced with ice-cold 0.01 M phosphate buffer–0.9% NaCl solution (pH 7.4). Two min later it was replaced with ice-cold media. The cells were scraped from the incubation wells and incubated for 24 h at 4°C in tubes containing 300 000 d.p.m. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No acid</td>
<td>Acid wash</td>
</tr>
<tr>
<td>Before hCG</td>
<td>9021 ± 127</td>
<td>11 702 ± 135</td>
</tr>
<tr>
<td>40 min hCG</td>
<td>14 643 ± 121</td>
<td>12 157 ± 350</td>
</tr>
<tr>
<td>240 min hCG</td>
<td>13 147 ± 458</td>
<td>16 499 ± 271</td>
</tr>
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Figure 7  (A) Onset of desensitization. Y-1L cells were exposed to an initial treatment of 100 ng hCG/ml. Every 15 min the hCG was removed from a group of cells, the cells were acid washed (see Table 2, legend), and fresh medium lacking hCG was added. The cells were then re-exposed to 100 ng hCG/ml and the cells were photographed 40 min later. The percentage of rounded cells was calculated from the photographs. (B) Onset of resensitization. Y-1L cells were exposed to an initial treatment of 100 ng hCG/ml for 24 h. Cells were acid washed (see Fig. 6) and fresh media were added. Beginning at 90 min and every 30 min thereafter, groups of cells were re-exposed to 100 ng hCG/ml for 40 min and photographed. The percentage of rounded cells was calculated from the photographs. This experiment was performed twice and similar results were observed each time.
with hCG four hours later caused control and puromycin-treated cells to round (Fig. 9), indicating that protein synthesis was not required to re-establish responsiveness to hCG. Together with the observations that hCG treatment did not appear to alter the number of LHR on the Y-1L cells, the failure of puromycin to block resensitization suggested that receptor turnover was not required to re-establish hormone responsiveness.

**Implications of these observations**

The hormone-dependent rounding response of Y-1L cells is a rapid and simple means of characterizing LH activity. It begins within 10 min and is readily detected by observing the cells with a microscope at relatively low magnification. Since rounding of a given cell is virtually an all-or-none response, round and flat cells are easily distinguished and the response can be readily quantified. The mechanism of rounding appears to involve the cytoskeleton and while its role in steroidogenesis, if any, is not fully understood, it may be part of the process by which cholesterol, the precursor of steroid hormones, is transported to the mitochondria during hormone-stimulated steroidogenesis (Almahbobi & Hall 1990, Hall 1997).

Loss of the rounding response appears to involve modulation of an early event in LHR-mediated signaling.
Thus, hCG-insensitive flat cells retained their LH receptors and were responsive to cholera toxin, a protein known to activate the stimulatory G-protein (Gs) in Y-1 cells (Kowal & Srinivasan 1975). Loss of the hCG responsive ability to respond to hCG. Both phenomena may be related to the fact that expression of LHR mRNA levels in Y-1L cells is under the control of the metallothionein promoter, not the natural LHR gene. In addition, the construct used to express the LHR in Y-1L cells contained only a few untranslated bases in the 5′ end of the LHR cDNA and none of the untranslated bases in the 3′ end of the LHR cDNA.

During these studies we obtained several types of Y-1L cell lines that behaved differently in response to prolonged hCG treatment. Some rounded and flattened, others rounded and remained round, and one went through several cycles of rounding and flattening (Fig. 4). Originally, we anticipated that each of these cell lines would contain vastly different numbers of receptors, but all the cell lines had a similar ability to bind 125I-hCG (GA Vlamer & WR Moyle, unpublished observations). We do not understand how these differences arose or why some Y-1L clones lost their ability to respond to hCG. Both phenomena may be related to the difficulty we had in creating these lines. The Y-1 parental cell line is highly susceptible to the toxic effects of cyclic AMP and agents that promote cyclic AMP formation (Olson et al. 1991a, Mitchell et al. 1992, Parisenti et al. 1998). Therefore, transfected Y-1L cells that did not develop a mechanism for reducing their LHR activities would be at a selective disadvantage. This idea is supported by the observation that it has been much easier for us to develop stable LHR-expressing Chinese hamster ovary (CHO) cell lines, cells that are not nearly as sensitive to cyclic AMP. Comparing the differences in the responses of these cell types may reveal new insights into the mechanisms of receptor regulation.

Acknowledgements

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