Aggregation of luteinizing hormone receptors in granulosa cells: A possible mechanism of desensitization to the hormone

(adenylate cyclase/antibodies to gonadotropins/immunofluorescence/receptor internalization/radioautography)

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ABSTRACT The temporal relationship between redistribution of receptors to lutropin (luteinizing hormone)/human chorionic gonadotropin in cultured rat ovarian granulosa cells and the cellular response to hormonal challenge were studied. Visualization of receptor-bound human chorionic gonadotropin by indirect immunofluorescence, with hormone-specific antibodies after fixation with 2% formaldehyde, revealed the existence of small clusters around the entire cell circumference 5-20 min after exposure to the hormone at 37°C. Such small receptor aggregates were also evident if hormone incubation was at 4°C or if cells were fixed with 2% formaldehyde before incubation. Larger clusters were evident after prolonged incubation with the hormone (2-4 hr) at 37°C. The later change coincided with diminished cyclic AMP accumulation in response to challenge with fresh hormone. When the fixation step was omitted and antibodies to human chorionic gonadotropin were applied after hormonal binding, acceleration of both receptor clustering and the desensitization process was observed. This maneuver also induced capping of the hormone receptors. In contrast, monovalent Fab' fragments of the antibodies were without effect. Internalization of the bound hormone in lysosomes, and subsequent degradation, was evident 8 hr after hormonal application and was not accelerated by the antibodies. It is suggested that clustering of the luteinizing hormone receptors may play a role in cellular responsiveness to the hormone. Massive aggregation of the receptors may desensitize the cell by interfering with coupling to adenylate cyclase.

Since the observation by Frye and Edidin (1) of lateral mobility of membrane proteins, movement and clustering have been demonstrated for receptors to immunoglobulins (2) and lectins (3) as well as receptors to hormones and neurotransmitters (4-7). Moreover, it was recently suggested that receptors associated with the plasma membrane can be internalized after binding specific ligands (8-10). However, the biological significance of such a receptor redistribution is not fully understood.

Hertz and Hisaw demonstrated (11) that pituitary extracts, containing gonadotropic hormones such as folitropin (follicle-stimulating hormone; FSH), play an important role in the regulation of ovarian development. It is now known that gonadotropic hormones have receptors on the plasma membrane of ovarian target cells (12) and that they exert their effect via activation of a hormone-sensitive adenylate cyclase (13, 14).

Prolonged exposure of ovarian tissue to lutropin (luteinizing hormone; LH) or human chorionic gonadotropin (hCG), which bind to the same receptor, causes prompt stimulation of adenylate cyclase followed by desensitization of the enzyme to renewed challenge with the hormone in vitro and in vivo (13-16). A similar phenomenon was also described in other systems where hormones exert their effects via activation of adenylate cyclase (17, 18). The mechanism responsible for this refractoriness is not clear. Several explanations have been suggested, among them an agonist-induced depletion of receptor sites, referred to as "down regulation" (for review, see ref. 19).

We have previously demonstrated that internalization of the receptor-bound hCG in cultured rat granulosa cells into lysosomes occurs after hormone binding, but that this process cannot account for the loss of the cellular response to the hormone because desensitization of the hormone-stimulated adenylate cyclase preceded the massive loss of specific receptors (10). In the present study we have examined whether desensitization of granulosa cells to LH or hCG can be due to rearrangement or clustering of these receptors, reflecting their lateral mobility and the efficiency of their coupling to adenylate cyclase.

MATERIALS AND METHODS

Materials. Highly purified hCG [CR-119; 12,800 international units (IU)/mg], rat FSH, and pregnant mare serum gonadotropin were kindly provided by the National Institutes of Health (Bethesda, MD). Goat anti-rabbit IgG conjugated to fluorescein was obtained from Hyland (Costa Mesa, CA). Photographic emulsion L4 was from Ilford (Essex, England). 3-Iso-butylmethylxanthine (IBMX) was from Aldrich (Milwaukee, WI).

Granulosa Cell Culture. Granulosa cells were collected from rat preovulatory follicles on the morning of proestrus by puncture and gentle expression into McCoy's 5a (modified) medium containing 2 mM glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 20% (vol/vol) fetal calf serum. Cells (10⁶ cells per ml) were introduced into Falcon Optilux tissue culture dishes (35 × 10 mm) and cultured for 24 hr as described (20). Alternatively, granulosa cells were obtained from 26-day-old immature rats treated with pregnant mare serum gonadotropin (15 IU for 48 hr; see ref. 21) and cultured as described above. In some control experiments, cells were obtained from immature rats treated with diethylstilbestrol. These cells contain FSH but not LH receptors (22).

Preparation of Specific Antibodies to hCG. Antibodies to hCG were raised in rabbits with the highly purified hormone as the antigen. Immunoglobulin (IgG) fractions were prepared from normal and immune sera by ammonium sulfate precipitation followed by DEAE-S2 column chromatography (23). The divalent fragment F(ab')² was prepared by pepsin digestion of the IgG fraction (24) followed by removal of the Fc fragment by staphylococcal protein A absorption. The monomer, Fab', was prepared by reductive alkylation of the divalent fragment (25). The immunoglobulin fraction, the divalent fragment, and

Abbreviations: FSH, follicitropin (follicle-stimulating hormone); LH, lutropin (luteinizing hormone); hCG, human chorionic gonadotropin; [¹²⁵I]hCG, [¹²⁵I]-labeled hCG; IBMX, 3-isobutyl-1-methylxanthine; IU, international units.

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the monovalent fragment were checked for purity by Na-DodSO₄ gel electrophoresis. The titer of the specific antibodies and their fragments was checked by a double-antibody radioimmunoassay procedure (26) with ¹²⁵I-labeled hCG (¹²⁵I-hCG).

**Immunofluorescent Staining of Cells.** Cell layers were incubated with hCG (20–200 IU/ml for 5–240 min) at 37 or 4°C, followed by incubation either with antibodies to hCG [IgG, Fab', or F(ab')² fragments; 1–2 mg/ml] or with whole antiserum (diluted 1:10–1:20) for 30–90 min and finally with goat anti-antibody IgG conjugated to fluorescein (1 mg/ml for 30–45 min). Concentrations of antisera, IgG fraction, and antibody fragments were chosen to give a similar titer in radioimmunoassay (1.8000) with ¹²⁵I-hCG. In some experiments, cells were fixed with 2% formaldehyde (30 min at room temperature or 4°C) before or after incubation with the hormone and rinsed. Remaining aldehyde groups were blocked with 0.2 M Tris buffer (pH 7.4) containing 1 mg of bovine serum albumin per ml before hormones or antibodies were applied. Such a fixation procedure did not alter the specific binding of the hormone to cells, unlike glutaraldehyde which completely destroyed specific binding even at concentrations as low as 1.0%.

**Light Microscopy.** Cultures were inspected and photographed with a photomicroscope III (Zeiss, West Germany) equipped for fluorescent and phase-contrast microscopy.

**Iodination of hCG and Fragments of Antibodies.** hCG was iodinated by the lactoperoxidase method (27). The specific activity of the iodinated hormone was 50 μCi/mg (1 Ci = 3.7 × 10¹² becquerels). Iodination of Fab' and F(ab')² fragments of immunoglobulins was as described (28).

**Autoradiography.** Cells were processed for high-resolution autoradiography after binding of ¹²⁵I-hCG as described (10). Autoradiographs were examined by electron microscopy (JEOL 100-B).

**Assays.** Production of cyclic AMP in cells in the presence of a phosphodiesterase inhibitor (IBMX) was measured by the method of Gilman (29) and as described earlier (10). In some experiments the assay was performed according to Humes et al. (30). The content of trichloroacetic acid-soluble and -insoluble radioactive material in the culture incubation media, after binding of ¹²⁵I-hCG to cells, was measured as described elsewhere (10).

**RESULTS**

Specificity of hCG Antiserum to Stain Cell-Bound Hormone by Indirect Immunofluorescence Technique. When 24- to 48-hr cultured granulosa cells, obtained from preovulatory follicles, were incubated with a saturating concentration of hCG followed by anti-hCG IgG and stained with fluorescein-conjugated goat anti-rabbit IgG, bright fluorescence was evident at the circumference of the cells (Fig. 1 A–D). Because the hormone served as a specific marker for the LH/hCG receptor, it was essential to determine that the antibodies bound exclusively to the hormone molecule on the cell. Replacement of the specific antibodies with nonspecific ones abolished the fluorescent staining of the cells, whereas elimination of the hormone from the incubation resulted in only weak staining of the cells. This might be due to binding of some LH contained in the fetal calf serum of the culture medium (see Materials and Methods). However, granulosa cell cultures that had already lost their LH receptors (12 days in culture) or that contained only FSH receptors, did not show any staining. Moreover, no staining was obtained if cells from diethylstilbestrol-treated animals were incubated with a saturating concentration of rat FSH and subsequently with antibodies to hCG.

Aggregation of hCG Receptors. The effect of incubation of granulosa cells with hCG on receptor distribution was studied in detail. Fixation of the cells with 2% formaldehyde after hormone binding and before the application of the specific antibodies to the hormone allowed us to differentiate between the effect of the hormone itself and that of the antibodies. Moreover, fixation of the cells prior to hormone binding allowed us to visualize the receptor distribution before they were occupied. When the cells were incubated for 5 min with 200 IU of hCG or for 20 min with 20 IU at 37°C, followed by formaldehyde fixation and incubation with hormone-specific antibodies, small clusters of fluorescent particles were evident at the circumference of granulosa cells after the application of fluorescein-labeled goat anti-rabbit IgG (Fig. 1A). Essentially, the same pattern of fluorescence was obtained when cells were incubated with 20–200 IU of the hormone per ml at 4°C or were fixed with the aldehyde prior to incubation with hormone. This suggests that small clusters of receptors exist before application of the hormone.

Significantly, larger clusters were observed when cells were incubated with 20 IU of hCG per ml for 2 hr at 37°C and fixed with formaldehyde before the application of the antibodies (Fig. 1B). When the fixation step was omitted and antibodies were applied, bulky fluorescent agglomerates appeared (Fig. 1C), followed by cap formation after an additional 1–2 hr of incubation with antibody-free medium (Fig. 1D). Prolonged incubation with the hormone itself even up to 5 hr at 37°C did not result in formation of these bulky agglomérates or cap formation.

The integrity and ultrastructural characteristics of the cells were well preserved during incubation of the cultures with the hormones as well as with the antibodies (Figs. 1E and 2).

**Internalization and Degradation of Receptor-Bound Hormone After Application of Antibodies to hCG.** We have already shown that ¹²⁵I-hCG is internalized in lysosomes, where it is probably degraded to single amino acids (10). We found that the extent of hormone degradation after internalization is not significantly changed by external application of the specific antiserum. After a 3-hr incubation with ¹²⁵I-hCG followed by a 1-hr incubation with the antibodies and an additional 4 hr with antibody-free medium, about 20% of the radioactivity primarily associated with the cells was recovered as trichloroacetic acid-soluble material in the incubation medium. At this time, half of the radioactivity associated with the cells was found in lysosome-like structures, as revealed by high-resolution autoradiography (Fig. 2). Similar data were obtained after cells were incubated with ¹²⁵I-hCG for the same time and subsequently with hormone-free medium without antibodies (see also ref. 10). These data suggest that the formation of bulky aggregates of receptors, subsequent to hormone-induced formation of clusters of receptors, does not accelerate the rate of internalization and degradation of the receptor-bound hormone.

**Hormone-Induced Formation of Cyclic AMP.** Granulosa cell cultures not previously exposed to hCG responded to the hormone with approximately a 70-fold rise in cyclic AMP accumulation over basal levels (Tables 1 and 2). Previous studies in our laboratory indicated that incubation of such cells with hCG for 2–3 hr results in partial desensitization to the hormone, so that after rinsing and challenge with fresh hormone in the presence of a phosphodiesterase inhibitor (IBMX), cyclic AMP accumulation is reduced to about 40% of the initial response (ref. 10 and Table 1). When anti-hCG serum was added for 30 min after an initial incubation for 90 min with the hormone and excess anti-hCG was then removed (see Table 1), this desensitization was accelerated, so that only 17% of the initial cyclic
AMP response was observed at the end of a 150-min incubation. Addition of the antiserum prior to hormonal challenge did not significantly affect the cyclic AMP response. Application of second antibody (by itself, or after the first antibody) did not change the degree of desensitization (Table 1, Exp. II).

To study the effect of antibodies to the hormone in more detail, we preincubated cells with hCG for only 20 min to minimize the degree of cell desensitization due to the hormone alone. When rates of cyclic AMP accumulation in cells preincubated with the hormone and subsequently incubated in the presence or absence of antibodies were compared, 65% of inhibition in cyclic AMP formation was achieved after 1 hr of incubation with the antibodies and 90% of inhibition was seen after 90 min (Fig. 3).

Incubation of the cells with the antibodies for 30 min and subsequently with antibody-free medium for an additional 60 min gave essentially the same depression of cyclic AMP accumulation as did continuous incubation with antiserum.

When cells were preincubated with 125I-hCG (2 IU of hormone per ml), rinsed, and subsequently incubated for 90 min with the antiserum, no increase in the release of the labeled hormone was detected compared to control cultures incubated with antibody-free medium (data not shown).

Table 2 shows that hormone-induced cyclic AMP accumulation was suppressed by the whole antiserum, the IgG fraction, and the divalent F(ab')2 fragments, but not by the monovalent Fab' fragment. Introduction of excess hCG in the challenge incubation increased cyclic AMP accumulation somewhat in cells preincubated with the divalent antibody. However, cyclic AMP formation in these systems did not exceed 25–50% that produced by cultures treated with the Fab' fragments of the specific antiserum or with F(ab')2 fragments obtained from nonimmune serum. Cells preincubated with the hormone bound 125I-Fab' or 125I-F(ab')2 fragments of the specific antibodies to the same extent.

**DISCUSSION**

Loss of receptors is one explanation for the loss of cellular response to hormones. However, we have demonstrated earlier (10), as well as in this work, that refractoriness precedes massive internalization of the receptor-bound hormone and coincides with receptor aggregation. This is also consistent with the observations of Harden et al. (17) and Hoffman et al. (18), who found no change in β-adrenergic receptor number during desensitization of astrocytoma cells and turkey erythrocytes, respectively, to catecholamines.
The ovarian follicular cells respond to three different agonists, LH (hCG), FSH, and prostaglandin E₂, with increased cyclic AMP accumulation. However, the refractory state is restricted to the homologous hormone. Thus, cells that are refractory to LH are responsive to FSH and prostaglandin E₂. Therefore, it seems unlikely that the adenylate cyclase per se is affected during the desensitization period. On the other hand, the recent report (19) that fluoride-sensitive cyclase activity in luteal tissue is temporarily and partially blocked during the period of desensitization does not exclude the possibility that the enzyme might be directly affected in the refractory state.

We present evidence that failure of granulosa cells to respond to hormonal challenge after prolonged incubation with the hormone is associated with massive aggregation of the LH/hCG receptors on the cell membrane. Moreover, divalent antibodies to hCG, which enhance receptor aggregation, also accelerate the rate of desensitization in these cells. Preliminary data suggest that receptors to hCG are more aggregated in Leydig cells obtained from refractory tests than in cells obtained from the unstimulated gland (32). Therefore, clustering of receptors may play a role in the mechanism of refractoriness both in vitro and in vivo.

The process by which massive receptor aggregation may lead to inability of the cell to respond to hormonal challenge remains to be elucidated. Schlesinger et al. recently reported (33) that the clustering of receptors for insulin and epidermal growth factor that occurs after ligand binding results in a decrease of the lateral mobility of these membrane proteins. We suggest that massive aggregation of LH/hCG receptors induced by the hormone in granulosa cells may restrict the mobility of these receptors in the plane of the membrane. This may result in a decrease in efficiency of coupling between the receptor and the adenylate cyclase system.

Alteration of the lipid composition of cell membranes containing β-adrenergic receptors, which probably increase membrane fluidity, results in increased adenylate cyclase activity in response to catecholamines (34–36). This is consistent with the hypothesis that increased membrane fluidity and lateral mobility of membrane proteins increase the coupling between hormone receptors and the cyclase system, whereas restriction of receptor mobility leads to a refractory state.

Table 1. Effect of attachment of antibodies to receptor-bound hCG on adenylate cyclase activity in cultured granulosa cells

<table>
<thead>
<tr>
<th>Preincubation, min</th>
<th>Challenge incubation*</th>
<th>No hormone</th>
<th>+hCG†</th>
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<tbody>
<tr>
<td>Exp.</td>
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<td>I</td>
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<tr>
<td>0-90</td>
<td>−hCG NRS</td>
<td>&lt;5</td>
<td>197 ± 3</td>
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<tr>
<td></td>
<td>−hCG Anti-hCG NRS</td>
<td>8 ± 1</td>
<td>189 ± 14</td>
</tr>
<tr>
<td></td>
<td>+hCG NRS</td>
<td>87 ± 4</td>
<td>90 ± 11</td>
</tr>
<tr>
<td></td>
<td>+hCG Anti-hCG NRS</td>
<td>31 ± 2</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>91-120</td>
<td>−hCG NRS</td>
<td>FITC-aRIgG&lt;5</td>
<td>231 ± 13</td>
</tr>
<tr>
<td></td>
<td>+hCG NRS</td>
<td>FITC-aRIgG</td>
<td>138 ± 6</td>
</tr>
<tr>
<td>121-150</td>
<td>+hCG Anti-hCG NRS</td>
<td>53 ± 3</td>
<td>70 ± 10</td>
</tr>
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</table>

Normal rabbit serum (NRS) and anti-hCG serum were used at 1:10 dilution. Cultures were thoroughly rinsed after each incubation (at 37°C). Concentration of hCG was 20 IU/ml at the preincubation step. Fluorescein-conjugated goat anti-rabbit IgG (FITC-aRIgG) was used at 1 mg/ml. The challenge medium contained 10 μg of IBMX per ml.

* Cyclic AMP formation, given as pmol per dish per 20 min. Results are expressed as mean ± SEM (n = 3).
† 10 IU/ml.

Table 2. Effect of attachment of mono- and divalent fragments of antibodies to receptor-bound hCG on adenylate cyclase activity in cultured granulosa cells

<table>
<thead>
<tr>
<th>Incubation with antibodies (90 min)</th>
<th>Challenge incubation (20 min)*</th>
<th>No hormone</th>
<th>+hCG†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None†</td>
<td>71.0 ± 3.0</td>
<td>71.0 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>NRS</td>
<td>18.2 ± 1.7</td>
<td>20.0 ± 2.3</td>
<td></td>
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<tr>
<td>Anti-hCG serum</td>
<td>2.4 ± 0.5</td>
<td>8.4 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Anti-hCG IgG</td>
<td>3.7 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Anti-hCG P(ab)₂</td>
<td>2.5 ± 1.2</td>
<td>9.7 ± 2.9</td>
<td></td>
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<tr>
<td>Anti-hCG Fab’</td>
<td>22.4 ± 1.9</td>
<td>19.7 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Non-specific P(ab)₂</td>
<td>18.0 ± 2.4</td>
<td>18.0 ± 1.1</td>
<td></td>
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</table>

All cultures were preincubated with hCG (20 IU/ml) for 20 min and were thoroughly rinsed after each incubation (at 37°C). Normal rabbit serum (NRS) and anti-hCG serum were used at 1:10 dilution. IgG, P(ab)₂, and Fab’ fractions were used at concentrations of 2.0 mg/ml. The challenge medium contained IBMX (10 μg/ml).

* Cyclic AMP accumulation, given as relative to the basal level. Results are expressed as mean ± SEM (n = 3).
† 10 IU/ml.
‡ Immediate challenge incubation after a 20-min preincubation with hormone.
Schlegel et al. (37) reported that glucagon receptors in hepatic membranes may be present in small aggregates and that these aggregates must break down to their monomeric form in the process of combining with the catalytic unit of the cyclase. Whether this mechanism also applies to the LH/hCG-sensitive adenylate cyclase remains to be seen.

By using antibodies to insulin receptors and to receptor-bound insulin, Kahn et al. (38) indicated that aggregation of insulin receptors results in enhancement of the biological response and, thus, may be important for hormone action. However, when we used nonsaturating concentrations of hCG followed by incubation of granulosa cells with the specific antibodies to the hormone, we failed to enhance the cellular response to the hormone (unpublished observations).

The existence of small clusters of LH receptors on granulosa cells prior to hormonal incubation revealed by immunofluorescence may be of biological significance. However, the possibility that they were formed mainly after application of the antibodies to the fixed cells cannot be completely excluded because formaldehyde fixation might not completely prevent translocation of proteins within biological specimens (39). In addition, some of these aggregates might be produced after partial occupancy of the receptors by some hormone (LH) present in the serum of the culture medium (see Materials and Methods). Nonetheless, our observations clearly indicate that desensitization of granulosa cells to hormone challenge is associated with pronounced aggregation of the LH/hCG receptors. Whether such a phenomenon also occurs in other desensitized cells, in which various hormones exert their effect via activation of adenylate cyclase, remains to be seen.

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