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Functional expression of recombinant human luteinizing hormone / human choriogonadotropin receptor*

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The functional capacity of the recombinant human LH/hCG receptor was tested on the basis of gonadotropin stimulation of cAMP production by stable transfections of Chinese hamster ovary (CHO) cells. A CHO cell line expressed with the hLH/hCG receptor cDNA covering the entire amino acid coding region revealed the presence of LH/hCG binding site (Kd:1.45x10^{-10}M) on the plasma membrane. Treatment of transfected cells(CHO-LH/hCGR) with hCG induced dose-dependent increases in intracellular cAMP production, indicating that the expressed human LH/hCG receptor functionally couples with endogenous adenylyl cyclase. Although hCG induced dose-dependent increases in cAMP production, rat and bovine LH and human FSH did not alter cAMP levels compared to control values. Northern blot analysis with a cRNA probe derived from human LH/hCG receptor cDNA indicated the presence of three LH/hCG receptor mRNA transcripts(5.4, 3.6 and 2.4 kilobases) in RNA prepared from human ovary. Preincubation of CHO-LH/hCGR cells with hCG for 16h decreased the subsequent cAMP production caused by a 30min pulse of hCG stimulation. These results indicate that desensitization of the adenylyl cyclase response to hCG stimulation occurs in CHO-LH/hCGR cells. Therefore, this cell line provides a tool with which to pursue detailed studies on the molecular basis of LH/hCG induced desensitization.

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LH and hCG(luteinizing hormone and human chorionic gonadotropin), bind to specific receptors and the action is mediated through the G-proteins (GTP-binding protein), resulting in increased levels of cAMP. Recently cDNA clones of receptors for gonadotropins and TSH have been isolated from various animal species(1,2,3,4). We also have cloned cDNA of receptors for human gonadotropin(5,6). Human TSH receptor cDNA has also been isolated by other groups(7,8). Although the importance of LH/hCG in gonadal development and reproductive function has been established(9,10), functional analysis of human LH/hCG receptor has been hampered by the low abundance of this receptor and the limited availability of human tissue. Since the availability of a cell line that stably expressed recombinant human LH/hCG receptors has provided an unlimited source of the human receptor and made it possible for us to analyze the properties of the human LH/hCG receptor. From the clinical point of view, premature ovarian failure may be caused, in part, by the presence of antibodies to the LH/hCG receptors. Therefore, this cell line may provide a tool to detect such antibodies in patients with premature ovarian failure. Also, the measurement of cAMP induction by this cell line could offer the opportunity to analyze agonist and antagonist analogs of LH/hCG. Moreover, since the mechanism underling homologous LH/hCG desensitization has not been determined, CHO cells expressing LH/hCG receptors provide us with a specific tool for elucidating the mechanisms of LH/hCG receptor desensitization.

Materials and Methods

Hormones

Rat LH(NIDDK-rLH-I-9) was obtained from the National Hormone and Pituitary Distribution Program(Bethesda,MD); hCG(CR119)(11600IU/mg) was supplied by Dr. R.E.Canfield, College of Physicians and Surgeons of Columbia University, NY, bovine LH(USDA-bLH-I-1) was obtained from the USDA Animal Hormone Program. Purified human FSH (Fertinorm P)

was purchased from Laboratories Serono S.A., Switzerland. Purified hCG was iodinated by the chloramine T method(11), and the specific activity was about 30μCi/μg.

Expression vector constructs

The cDNA coding for the entire hLH/hCG receptor(-9 to 2987 bp) was inserted into the EcoRI site of pSD(x)(12) expression vector, under the control of SV 40 early promoter, to generate an expression plasmid(designated pSDHLHR). Re-examination of the hLH/hCG receptor cDNA leads to correction of nucleotide sequence at the following position : nucleotide 370(G to C), resulting in the substitution of amino acid at 124(Gly to Arg).

Cell transfections

To establish a cell line stably expression hLH/hCG receptor, ten micrograms of the constructed plasmid pSDHLHR was transfected into CHO cells deficient of dihydrofolate reductase(CHO-dhfr-) by the calcium phosphate method. The transformants were selected after 2 weeks at 37°C in a selection medium, which did not contain ribonucleoside and deoxyribonucleosides(αMEM2000,Gibco,Grand Island,NY). The cell populations were subjected to selection by adding 0.3 μM MTX to the medium and then subcloned by limiting dilution to obtain individual clones.

Cyclic AMP assays

MTX-resistant CHO cells($1.5x10^5$cells /culture dish) were incubated for 15min at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine(MIX, Sigma Chemical CO.,St Louis, MO). Purified hormones were added to the dish, and the incubation continued for 30min at 37°C, except for time course experiments. Intracellular cAMP levels were determined by the double antibody RIA method(13). Triplicate plates were analyzed for each data point.

Northern blot analysis of hLH/hCG receptor mRNAs

The cDNA coding for entire human LH/hCG receptor was subcloned into a Bluescript KS(+) vector(Promega, Madison,WI). The plasmid clone was

linearized with the BamHI restriction enzyme and served as a template for the production of a cRNA probe(2.9 Kb) with T7 RNA polymerase and an RNA labeling kit(Boehringer Mannheim, Mannheim, Germany). Total RNA was extracted from human ovary and placenta by the guanidinium thiocyanate method. Approximately 15μg of total RNA was loaded and fractionated by electrophoresis through a 1% agarose gel. Samples were blotted onto nitrocellulose membranes and blots were hybridized with digoxigenin-labelled cRNA probes. Under the standard protocol for the nucleic acid detection kit(Boehringer Mannheim), the membranes were then exposed to Kodak X-Omat film(Eastman Kodak, Rochester, NY).

LH/hCG receptor binding

CHO-LH/hCGR cells in each well were washed with PBS and then scraped after a rapid freeze-thaw cycle in PBS containing 20% glycerol and PMSF, and collected by centrifugation. The precipitates were then suspended in PBS and used as receptor preparations(14). LH binding was performed by incubating the receptor preparations with [^{125}I] hCG at 22°C for 18h. Non specific binding was determined by inclusion of an excess of unlabeled hormone. Equilibrium binding constants for the receptor were determined by incubating the receptor preparation at 22°C for 18h in the presence of an excess of the hormone. Data were analyzed by the methods of Scatchard, and lines were calculated by linear regression.

Results

Stable expression of the LH/hCG receptor was attempted by transfection of pSDHLHR into CHO-dhfr- cells. Following transfection and selection, MTX-resistant CHO-dhfr- cell clones were isolated. A number of colonies were screened for an increase in the level of cAMP. Consequently, several clones which responded well to hCG were selected for further characterization.

There are three LH/hCG receptor transcripts of 2.4, 3.6 and 5.4 Kb in human ovary but no detectable transcript in equivalent amount of mRNA

prepared from placenta at week 7 of pregnancy(Fig. 1). A dose-dependent increase in specifically bound ^{125}I hCG was detected in transfected cells incubated with increasing concentrations of labeled hCG, but no specific binding was observed in untransfected cells(data not shown). Scatchard plot analysis of the specific hCG binding to the CHO-LH/hCGR cells indicated a Kd value of 1.45×10^{-10}M(Fig.2).

The functional capacity of the receptor expressed in CHO-LH/hCGR cells was tested on the basis of gonadotropin stimulation of cAMP production. The time course of cAMP accumulation following hCG addition was studied. The cells responded to 30ng/ml hCG by a rapid accumulation of cAMP within the first hour, attaining steady state within this period (Fig.3). Treatment of the cells with hCG caused a dose-dependent increase in cAMP formation(ED50,50ng/ml). To assess the specificity of the hLH/hCG receptor, the CHO-LH/hCGR cells were

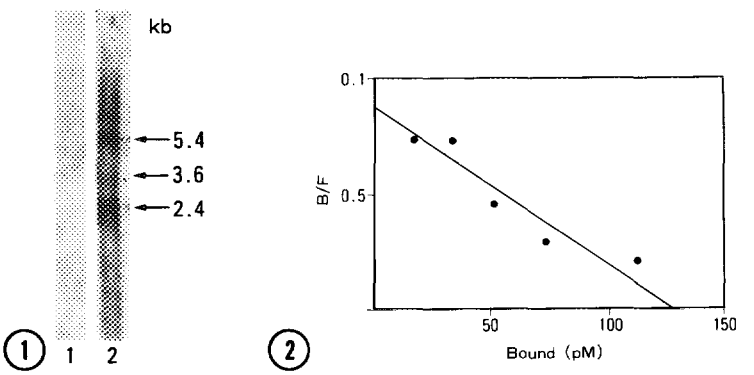


Fig. 1. Northern blot analysis of human LH/hCG receptor mRNA . Approximately 15μg of total RNA samples prepared from human placenta(lane 1) and ovary(lane 2) were loaded and fractionated by electrophoresis through a 1% agarose gel. The samples were blotted onto nitrocellulose membranes and covalently cross-linked with a UV cross-linker(Stratagene). The membranes were hybridized to a human LH/hCG receptor cRNA probe. The filter was washed and exposed to Kodak X-Omat film at room temperature.

Fig. 2. Binding of [^{125}I]hCG to hCG/LH receptors expressed in CHO-LH/hCGR cells. Cells(4×10^5) were incubated with increasing concentrations of[^{125}I]hCG in the presence or absence of an excess of unlabeled ligand. Scatchard plot analysis of the specific binding of CHO-LH/hCGR cells indicate a Kd value of 1.45×10^{-10}M.

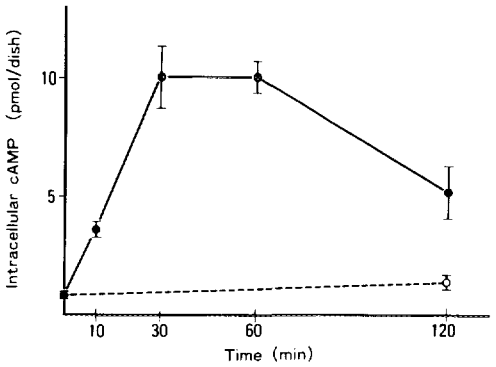
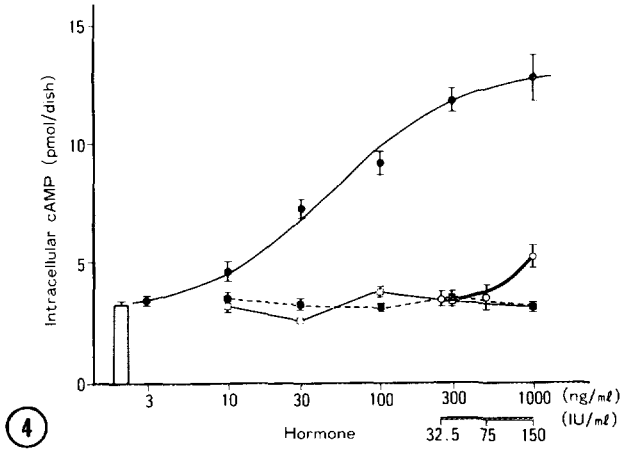


Fig. 3. Time course of cAMP production induced by hCG. Intracellular cAMP accumulation was measured after incubation of CHO-LH/hCGR cells in the presence(closed circles) or absence(open circles) of hCG(30 ng/ml) for the time indicated.

incubated with increasing doses of rat LH, bovine LH, hCG and pure human FSH(Fertinorm P). Rat and bovine LH did not increase cAMP production at doses lower than 1000 ng/ml and only a high dose of purified FSH(150 IU) increased cAMP production slightly(Fig.4). In our experiments, hCG stimulation at a dose of 100ng/ml increased intracellular cAMP levels to 2- to 3-fold and maximal stimulation was obtained after 30-60 min of exposure. However, when CHO-LH/hCGR cells were preincubated for 16h with hCG at a concentration of 100ng/ml, there was a reduction in the subsequent cAMP response to a 30 min pulse of hCG stimulation(Fig.5). These data indicate that this receptor in CHO-LH/hCGR cells undergoes desensitization.

Discussion

Previous studies have shown that in both rat and mouse Leydig cells there are several sizes of LH/hCG receptor mRNA transcripts(14,15). We also show here the existence of three LH/hCG receptor mRNAs in human ovary and no detectable transcripts in human placenta. These data suggest that either alternative or incorrect splicing or some other factor such as differences in polyadenylation is contributing to the different sizes of LH/hCG receptor mRNA transcripts in gonadal cells.



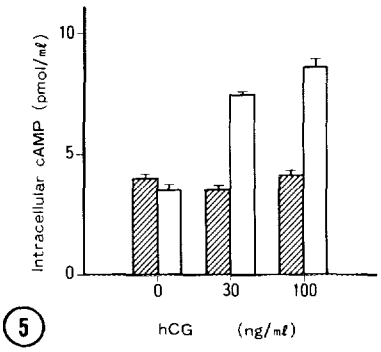


Fig.4. Gonadotropin stimulation of cAMP production. CHO-LH/hCGR cells were treated with increasing concentrations of hCG(closed circles), bovine LH(closed squares), rat LH(open squares) and human FSH(open circles) in the presence of 0.5mM methylisobutylxanthine for 30 min at 37°C. Intracellular cAMP production was measured by RIA. Data are the mean ±SE of triplicated determinations from a representative experiment.

Fig. 5. Desensitization of the cAMP response to human FSH stimulation. The cells were cultured in culture dishes in medium lacking hCG (open bars) or in the presence of hCG(100ng/ml)(hatched bars) for 16h. The medium was then replaced with fresh medium with the amounts of human FSH and 0.5mM MIX indicated. After incubation for 30 min,cellular cAMP was extracted and measured by RIA. Each bar represents the mean ±SE of triplicate values obtained in triplicate dishes. The data are representative of 3 experiments with the CHO-LH/hCGR cells.

Our data indicate that hCG stimulates cAMP production in CHO cells expressing the human LH/hCG receptor, whereas neither rat nor bovine LH is able to stimulate cAMP production in these cells. These data are in good agreement with a previous report(16) but not consistent with those in some previous reports, in which the hFSH receptor interacted with both human FSH and rat FSH(17). Scatchard analysis demonstrated that the Kd of the human LH/hCG receptors was 1.45×10^{-10}M, similar to that reported for LH/hCG receptors in the native gonads(18).

Human LH/hCG receptors expressed in CHO cells were also capable of interacting with the endogenous G-proteins of the cells to increase cAMP formation. Since it is difficult to obtain human tissues, the

availability of large amounts of the human LH/hCG receptor from this cell line will provide an important resource for clinical studies. For example, this cell line enables us to screen the circulating antibodies to LH/hCG receptor in patients with ovarian failure. Earlier reports have suggested the presence of circulating antibodies to the gonadotropin receptor in patients with premature ovarian failure (19,20).

Moreover the cloning of a cDNA for the LH/hCG receptor has now made it possible to pursue detailed studies on the molecular basis of the hCG induced uncoupling of this receptor. In a previous experiment with CHO cells expressing TSH receptors, prior TSH stimulation did not lead to functional desensitization of the TSH receptors (21). The results presented here show that the processes involved in the hCG-induced desensitization of the hCG receptor are not specific to gonadal cells. Desensitization by continued hormone stimulation is a general phenomenon involving many hormones and hormone-response tissues. The lack of appropriate experimental tools has made it difficult to search for hCG-induced post-translational modifications of LH/hCG receptor. Since the expression of LH/hCG receptors in this system is regulated by a heterologous promoter, the desensitization phenomenon detected in this experiment must be a post-transcriptional event. Therefore, this cell line may provide a good model for clarifying the post- transcriptional mechanisms of the desensitization.

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