Mutations That Induce Constitutive Activation and Mutations That Impair Signal Transduction Modulate the Basal and/or Agonist-stimulated Internalization of the Lutropin/Choriogonadotropin Receptor*

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The lutropin/choriogonadotropin receptor (LHR) is a member of the rhodopsin-like subfamily of G protein-coupled receptors (GPCRs) (reviewed in Ref. 1) that has been shown to occur in the stimulation of G proteins and in triggering the internalization of the bound agonist.

We have now analyzed two naturally occurring, constitutively active mutants of the human LHR. These mutations were introduced into the rat LHR (rLHR) and are designated L435R and D556Y. Cells expressing rLHR-D556Y bind human choriogonadotropin (hCG) with normal affinity, exhibit a 25-fold increase in basal cAMP and respond to hCG with a normal increase in cAMP accumulation. This mutation does not affect the internalization of the free receptor, but it enhances the internalization of the agonist-occupied receptors 3-fold. Cells expressing rLHR-L435R also bind hCG with normal affinity, exhibit a 47-fold increase in basal cAMP, and do not respond to hCG with a further increase in cAMP accumulation. This mutation enhances the internalization of the free and agonist-occupied receptors 2- and 17-fold, respectively. We conclude that the state of activation of the rLHR can modulate its basal and/or agonist-stimulated internalization.

Since the internalization of hCG is involved in the termination of hCG actions, we suggest that the lack of responsiveness detected in cells expressing rLHR-L435R is due to the fast rate of internalization of the bound hCG. The finding that membranes expressing rLHR-L435R (a system where internalization does not occur) respond to hCG with an increase in adenyl cyclase activity supports this suggestion.

The lutropin/choriogonadotropin receptor (LHR) is a member of the rhodopsin-like subfamily of G protein-coupled receptors (GPCRs) (reviewed in Ref. 1) that has been shown to mediate the internalization of its two naturally occurring agonists, lutropin and choriogonadotropin (CG). These studies, which have been conducted in target or transfected cells, have shown that the free LHR is randomly distributed in the plasma membrane, but that it clusters in clathrin-coated pits upon agonist activation (2). The clustered agonist-receptor complex is internalized by a dynamin-dependent pathway and traverses the endosomal compartment without agonist dissociation (3–5). Dissociation of the agonist-receptor complex occurs in the lysosomes, where both the agonist and the receptor are degraded (2, 4–6). The receptor-mediated endocytosis of lutropin and CG serves two important functions in regulating cellular responsiveness. First, the internalization of the receptor-bound agonist effectively stops activation of downstream effectors such as adenyl cyclase (7). Second, the accumulation of the agonist-receptor complex in the lysosomes promotes receptor degradation and is ultimately responsible for the agonist-induced down-regulation of the cell surface LHR, an outcome that prevents further activation of the cells (4, 8, 9).

The lysosomal accumulation of the rLHR-agonist complex and the involvement of rLHR internalization in the termination of hormone action (i.e. desensitization) place the LHR in an unusual subgroup of GPCRs. Thus, the majority of internalized GPCRs recycle back to the plasma membrane instead of being routed to the lysosomes, and internalization is thought to play an important role in receptor resensitization rather than desensitization (10–12). Another notable difference between the LHR and other GPCRs is on the rate of internalization. The t½ of internalization of most GPCRs that are internalized by a nonvisual arrestin/clathrin/dynamin-dependent pathway is 10–15 min (11, 12), while the t½ of internalization of the LHR, which is also internalized by a nonvisual arrestin/clathrin/dynamin-dependent pathway (2, 3) is 20–60 min in target cells (5, 8, 13) and 100–140 min in 293 cells expressing the recombinant rLHR (3, 14, 15). It is not known if this slower rate of internalization of the LHR agonist complex is a reflection of a weak interaction of the LHR with the nonvisual arrestins or if it is due to the involvement of other adapter proteins (see Ref. 3).

Recent studies conducted with several members of the rhodopsin-like subfamily of GPCRs, including the LHR, have led to the suggestion that the same active conformation of GPCRs is involved in the stimulation of G proteins and the endocytosis of the receptor. For example, when occupied by weak partial agonists or antagonists, the mouse LHR (13), and the β2-adrenergic receptor (16) are internalized at much slower rates than when occupied by agonists. It is also known that the slower internalization of the LHR induced by a weak partial agonist is not due to the reduced levels of cAMP, because the addition of cAMP analogs does not increase the rate of internalization of the weak partial agonist (13). Moreover, the LHR-mediated

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§ The abbreviations used are: LHR, lutropin/choriogonadotropin receptor; rLHR, rat LHR; hLHR, human LHR; GPCR, G protein-coupled receptor; CG, choriogonadotropin; hCG, human CG; PCR, polymerase chain reaction.
internalization of hCG is normal in a cell line that expresses a cAMP-resistant phenotype (13). More recent experiments conducted with several agonists of the m3 muscarinic receptor have shown that there is a linear correlation between their intrinsic agonistic activity and their rate of endocytosis (17). Likewise, point mutations of the rat LHR (rLHR) (14) or the β2-adrenergic receptor (18) that impair signal transduction also impair endocytosis of their respective agonists. Universal statements about agonist-induced activation and endocytosis of GPCRs cannot be made, however, because there are point mutations of some GPCRs such as the β2-adrenergic receptor (19) and the α1A receptor (20) that impair signal transduction without affecting endocytosis. Last, three potent agonists of the µ-opioid receptor (enkephalin, etorphine, and morphine) capable of stimulates the cell surface transduction, but only two of them (enkephalin and etorphine) stimulate receptor internalization (21).

Since the use of a weak partial agonist and signaling-impairing mutations of the LHR have established that impairments in signal transduction slow down the endocytosis of this receptor (13, 14), we hypothesized that mutations of the LHR that make it constitutively active should enhance endocytosis. The experiments described herein take advantage of two activating mutations of the LHR associated with human disease (22, 23) to examine this hypothesis.

Materials and Methods

Plasmids and Cells—The cloning of the rat lutropin/CG receptor cDNA and the template plasmid containing the full-length coding region plus portions of the 5′- and 3′-untranslated regions of the wild-type rLHR cDNA have been previously described (24). The preparation and characterization of myc-rLHR-wt, a modified form of the rLHR containing the myc epitope at the N terminus has also been described (25). Point mutations were introduced using PCR strategies, and the sequence of the entire region of each mutant cDNA generated by PCR was verified by automated DNA sequencing. The mutant and wild-type rLHR cDNAs were subcloned into the eukaryotic expression vector pcDNA1/Neo (Invitrogen) for transfection.

Transfections of human embryonic kidney (293) cells were done using calcium phosphate as described by Chen and Okayama (26). Cells plated in 100-mm dishes were transfected when 70–80% confluent and using 10 µg of each plasmid. After an overnight incubation, the cells were washed and they were placed back in culture medium for 24 h. The cells were then trypsinized, plated at low densities in 100-mm dishes, and selected using 700 µg/ml G418. Stably transfected cell lines were obtained following G418 selection and cloning as described elsewhere (14, 27).

The establishment and properties of a clonal cell lines expressing rLHR-wt (designated 293L/wt-12) and a clonal cell line expressing myc-rLHR-wt (designated 293Lmycwt-11) have been described (15, 25, 28).

Hormone Binding and Signal Transduction Assays—Equilibrium binding parameters for hCG were measured during an overnight incubation (4 °C) of intact cells with seven different concentrations of 125I-hCG (27, 28). Concentration-response curves for the hCG-induced increases in cAMP accumulation were obtained by measuring total cAMP levels in cells that had been incubated with at least five different concentrations of hCG for 30 min at 37 °C in the presence of a phosphodiesterase inhibitor. The different parameters that describe the binding or concentration response curves were calculated as described elsewhere (27, 29).

The methods used for the isolation of membranes and adenyl cyclase assays have also been described (29).

Internalization Assays—The endocytosis of 125I-hCG was measured as follows (5, 8). Cells, plated in 35-mm dishes, were preincubated in 1 ml of Waymouth’s MB752/1 medium supplemented with 20 mM Hepes, 15% horse serum, 20 mM Hepes, 50 mM glucose, 100–200 µCi/ml Tran35S-label. Cell lysates were prepared at different times and, depending on the cell type used, the 35S-labeled receptor was immunoprecipitated using antibodies raised against synthetic peptides derived from the rLHR-wt or an antibody (designated 9E10) directed against the myc epitope (25, 27, 28, 32–34). Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and fluorograms of the dried gels were obtained using a Bio-Rad Molecular Imaging System, and the intensity of the band corresponding to the intracellular rLHR precursor (~68 kDa, see Refs. 25 and 32) was quantitated and captured in a digital format. The rate constants for degradation (kdeg) were calculated by a nonlinear fit of plots of the amount of radioactivity incorporated into the rLHR precursor versus time (cf. Equation 4). Nonlinear regressions were performed using the Origin software package.

Hormones and Supplies—Purified hCG (CR-127) was obtained from the Human Hormone and Pituitary Agency of the NIDDK, National Institutes of Health. 125I-hCG was prepared as described previously (35) to give a specific radioactivity of 25,000–30,000 cpm/ng. Nonidet P-40, sodium deoxycholate, protease inhibitors, N,N′-N′-triacetylimido- triose, protein A-agarose, bovine serum albumin, and protease type XIV were from Sigma. Wheat germ agglutinin agarose was from Vector Laboratories. Cell culture supplies and reagents were obtained from Corning and Life Technologies, Inc., respectively. Tran35S-label was from ICN. Human kidney 293 cells and the 9E10 hybridoma cell line were purchased from the American Type Culture Collection. All other materials were obtained from commonly used suppliers.

Results

Establishment and Characterization of Clonal Cell Lines Expressing Constitutively Active and Signaling-impaired Mutants of the LHR—There are now a large number of activating and signaling-impaired mutants of the human LHR (hLHR) that have been found to be associated with gonadotropin-independent, male-limited precocious puberty (reviewed in Ref. 36). We chose two of these mutations, an Asp to Tyr mutation in TM6 (22) and a Leu to Arg mutation in TM3 (23) to pursue the experiments outlined below. These two residues are fully conserved in the rLHR, and the corresponding mutants are designated rLHR-D556Y and

Buffer washes were combined and counted, and the cells were solubilized with 100 µl of 0.5% NaOH, collected with a cotton swab, and counted to determine the amount of internalized hormone. At least five different data points collected at 5–10 min intervals were used in each experiment, and the endocytotic rate constant (kdeg) was calculated from the slope of the line obtained plotting the internalized radioactivity against the integral of the surface-bound radioactivity (30). The half-life of internalization (t1/2) is defined as 0.693/kdeg.

Measurement of the Rate Constant for Internalization of the Free Cell Surface rLHR—Since proteolysis of intact cells under mild conditions has been shown to destroy the cell surface rLHR (25), the rate constant for internalization of the free rLHR can be measured by following the time course of recovery of 125I-hCG binding to the cell surface as a function of time after removal of the protease (31). Cells (plated in 100-mm dishes) were placed on ice and washed twice with 4 ml portions of cold Hanks’ balanced salt solution. The cell surface rLHR was then proteolyzed by incubating the cells on ice for 30–45 min in cold Hanks’ balanced salt solution supplemented with 250 µg/ml Protease type XIV (25). Protease activity was quenched by the addition of 4 ml of Waymouth’s MB752/1 medium supplemented with 20 µg Hepes, 15% horse serum, 1 mM phenylmethyl sulfonyl fluoride, 2 mM EDTA, and 5 µg/ml N-ethylmaleimide. The cells were then scraped from the plate and collected by centrifugation. The pellet was resuspended in the same medium, and the cells were collected by centrifugation again and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum, 200 µg/ml gentamicin, and 100–200 µCi/ml Tran35S-label. Cell lysates were prepared at different times and, depending on the cell type used, the 35S-labeled receptor was immunoprecipitated using antibodies raised against synthetic peptides derived from the rLHR-wt or an antibody (designated 9E10) directed against the myc epitope (25, 27, 28, 32–34). Immunoprecipitates were resolved on SDS gels, and fluorograms of the dried gels were obtained using Kodak BioMax MS film. All fluorograms were scanned using a Bio-Rad Molecular Imaging System, and the intensity of the band corresponding to the intracellular rLHR precursor (~68 kDa, see Refs. 25 and 32) was quantitated and captured in a digital format. The rate constants for degradation (kdeg) were calculated by a nonlinear fit of plots of the amount of radioactivity incorporated into the rLHR precursor versus time (cf. Equation 4). Nonlinear regressions were performed using the Origin software package.

Hormones and Supplies—Purified hCG (CR-127) was obtained from the Human Hormone and Pituitary Agency of the NIDDK, National Institutes of Health. 125I-hCG was prepared as described previously (35) to give a specific radioactivity of 25,000–30,000 cpm/ng. Nonidet P-40, sodium deoxycholate, protease inhibitors, N,N′-N′-triacetylimido-triose, protein A-agarose, bovine serum albumin, and protease type XIV were from Sigma. Wheat germ agglutinin agarose was from Vector Laboratories. Cell culture supplies and reagents were obtained from Corning and Life Technologies, Inc., respectively. Tran35S-label was from ICN. Human kidney 293 cells and the 9E10 hybridoma cell line were purchased from the American Type Culture Collection. All other materials were obtained from commonly used suppliers.
Activation and Endocytosis of the LHR

10–20% of the maximally attainable levels, however, and the cells expressing rLHR-D556Y still responded to hCG with a robust increase in cAMP accumulation. The maximal cAMP response to hCG detected in 293L(D556Y-6) cells corresponded to about 75% of the maximal response of 293L(wt-12) cells (as judged by the response ratio), and the EC50 for this response was comparable between these two cell lines. The hCG responsiveness of cells expressing rLHR-D556Y (Fig. 1 and Table I) seems higher than that of cells expressing the equivalent mutation of the hLHR (22, 39). It is not known if this is due to intrinsic differences between the rat and human receptor, to differences in the cells used for transfections (COS-7 cells were used in Ref. 39), or to the use of transient (22, 39) versus stable transfections (Fig. 1 and Table I).

When compared with 293Lmyc(wt-11) cells, 293Lmyc(L435R-2) cells displayed a 42-fold increase in the levels of cAMP detected under basal conditions. This elevated basal level of cAMP corresponds to 40–50% of the maximal response detected in 293Lmyc(wt-11) cells. 293Lmyc(L435R-2) cells did not respond to further stimulation by hCG, however, despite the fact that they bind hCG with normal affinity (Table I). These results are in agreement with the data reported for the equivalent mutation of the hLHR (23).

Endocytosis of [125I]hCG Mediated by Constitutively Active and Signaling-impaired Mutants of the rLHR—The binding of hCG to the LHR occurs with very high affinity (~200 pM, cf. Table I), and once formed, the hCG-LHR complex dissociates with a half-life of several hours at neutral pH (5, 40). In fact, the nature of this association is such that the hCG-LHR complex does not dissociate readily unless the pH is lowered to ~3 (4). Thus, previous biochemical (4) and morphological studies (2) have shown that the hCG-LHR complex remains associated as it traffics from the cell surface to coated vesicles and endosomes, and it does not dissociate until it reaches the lysosomes. In this respect, the LHR is different from many other GPCRs in that endocytosis (or internalization) leads to the lysosomal accumulation and degradation of the cell surface LHR, thus resulting in a net loss of the cell surface LHR (down-regulation). For many other GPCRs, the initial internalization of the receptor, a process that is often referred to as sequestration, is quickly followed by the return of the internalized receptor to the cell surface, a process that is also known as recycling (reviewed in Refs. 11 and 12). As such, the methods used to measure internalization of the agonist-occupied and free (see below) LHR take advantage of these unique properties of the LHR and are often different from the methods commonly used to measure internalization of many other GPCRs.

### Table I

<table>
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<tr>
<th>Cell line</th>
<th>hCG bindinga</th>
<th>cAMP responsivenessb</th>
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<tbody>
<tr>
<td></td>
<td>Kd, pmol/10^6 cells</td>
<td>R_max, pmol/10^6 cells</td>
</tr>
<tr>
<td>293L(wt-12)</td>
<td>196 ± 24</td>
<td>99,000 ± 11,000</td>
</tr>
<tr>
<td>293L(Y524F-22)</td>
<td>241 ± 27</td>
<td>120,000 ± 12,000</td>
</tr>
<tr>
<td>293L(D556Y-6)</td>
<td>166 ± 22</td>
<td>96,000 ± 28,000</td>
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<tr>
<td>293Lmyc(wt-11)</td>
<td>199 ± 14</td>
<td>86,000 ± 10,000</td>
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<tr>
<td>293Lmyc(L435R-2)</td>
<td>249 ± 27</td>
<td>70,000 ± 20,000</td>
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**a** Equilibrium binding parameters were determined during an overnight incubation (4°C) of intact cells with increasing concentrations of [125I]hCG as described under “Materials and Methods.” Each value represents the average ± S.E. of 4–7 independent experiments.

**b** Total levels of cAMP were measured in cells incubated at 37 °C in the presence of 1 μM isobutylmethylxanthine and increasing concentrations of hCG for 15 min as described under “Materials and Methods.” The EC50 and maximal cAMP responses were calculated by fitting the dose-response curves to a sigmoidal equation as described under “Materials and Methods.” The maximal response to cholera toxin was calculated by measuring total cAMP levels in cells that had been incubated with a single, saturating concentration of cholera toxin (500 ng/ml) for 3 h at 37 °C. The response ratio shown in the last column was calculated by dividing the maximal hCG response by the maximal cholera toxin response. Each value represents the average ± S.E. of 4–7 independent experiments.

An EC50 could not be calculated because there is little or no increase in cAMP stimulation by hCG (i.e. the basal and R_max are very similar). Also see lower panel of Fig. 1.

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rLHR-L435R. For comparative purposes, we also constructed and analyzed a Tyr to Phe mutation in TM5 (designated rLHR-Y524F), which is predicted to impair signaling of the rLHR. This prediction is based on the high degree of conservation of this residue among the rhodopsin-like subfamily of GPCRs (37) and on mutagenesis studies conducted on the AT1A receptor showing that the equivalent Tyr to Phe mutation prevents G protein activation (38). Rat LHR-Y524F and D556Y were constructed in the context of the native rLHR sequence, while rLHR-L435R was constructed in the context of the myc-tagged rLHR.

Clonal lines of human kidney 293 cells stably expressing each of these constructs were established and tested for [125I]hCG binding. All mutant receptors bind hCG with an affinity comparable with that detected in cells expressing rLHR-wt (Table I). Cell lines expressing a density of about 100,000 cell surface receptors/cell were matched with previously established cell lines designated 293L(wt-12) and 293Lmyc(wt-11) expressing a comparable density of the native or myc-tagged rLHR-wt (15, 25) as shown in Table I. The data presented in Table I and Fig. 1 also show a comparison of the basal and hCG-stimulated cAMP levels in the clonal lines expressing the wild type and mutant receptors. The responsiveness of these cell lines to a maximally effective concentration of cholera toxin and a response ratio, calculated by dividing the maximal hCG response over the maximal cholera toxin response, is also shown in Table I. These data are included in an attempt to correct for clonal variation and for an inherent variability in the basal and hCG-stimulated levels of cAMP.

As predicted (see above) the Y524F mutation is a signaling-imparing mutation. The EC50 for the hCG-induced increase in cAMP levels is 27-fold higher in 293L(Y524F-22) than in 293L(wt-12). 293L(Y524F-22) cells also display a low to normal level of cAMP under basal conditions, but their maximal response to hCG is only 14% lower (as judged by the response ratio) than the response of 293L(wt-12) cells.

In agreement with previous studies on the equivalent mutation of the hLHR (22, 39), the D556Y mutation induced constitutive activation of the rLHR as illustrated by a 25-fold increase in the levels of cAMP detected in cells incubated without hormone. This elevated basal level of cAMP corresponds to only

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The equivalent mutations described in the hLHR are D578Y and L559Y (22, 23), respectively, simply because codons in the hLHR have always been numbered including a 22-amino acid signal peptide, while codons in the rLHR have always been numbered starting from the known N terminus of the mature protein.
In agreement with previous results obtained using target cells (4), we found that 90% of the 125I-hCG bound to the surface of 293L(wt-12) cells was receptor-bound (as determined by precipitation with polyethylene glycol; see Ref. 4), while 70% of the internalized radioactivity recovered at the end of a 60-min incubation (the longest time point used below) remained receptor-bound. In parallel experiments, we found that the percentage of internalized radioactivity that remained receptor-bound in target cells was ~45%. In either case, the percentage of internalized radioactivity that remained receptor-bound after 60 min of internalization could be increased to 90% by the inclusion of 10 mM NH4Cl (also see Ref. 4). NH4Cl was not routinely used in the internalization assays, however, because these assays simply rely on the measurement of the total (i.e., free and receptor-bound) radioactivity, and NH4Cl did not affect this parameter at time points of up to 60 min (the longest time point used in the experiments described below). Thus, when occupied by hCG, the fate of the rLHR can be conveniently tracked by following the fate of 125I-hCG.

When cells expressing rLHR-wt are exposed to 125I-hCG, the amount of internalized hormone is only about 20% during a 60-min incubation (Fig. 2). $k_c$ can be calculated from these time courses by plotting the internalized radioactivity against the integral of the surface radioactivity (Fig. 3). The slope of this line gives $k_c$ (30), and the half-life of internalization ($t_{1/2}$) is

**Fig. 1.** Concentration-response curves for hCG-induced cAMP accumulation in 293 cells stably transfected with the rLHR-wt or mutants thereof. Cells were plated in 35-mm wells and preincubated at 37 °C in the presence of 1 mM isobutylmethylxanthine for 15 min. The indicated concentrations of hCG were then added, and the incubation was continued for another 15 min at 37 °C. Total (i.e., intracellular plus extracellular) cAMP was then measured in duplicate wells as described under "Materials and Methods." The results of a representative experiment performed with the indicated cell lines are shown. The lines are nonlinear least square fits of the experimental data (see "Materials and Methods" for details).

**Fig. 2.** Time course of internalization of 125I-hCG in 293 cells stably transfected with the rLHR-wt or mutants thereof. Cells were preincubated for 60 min at 37 °C. 125I-hCG was then added (at $t = 0$ in the figure) to give a final concentration of 40 ng/ml, and the incubation was continued. At the times indicated, triplicate wells were used for the determination of the surface-bound and internalized radioactivity as described under "Materials and Methods." The internalized hormone is expressed as a percentage of the total cell-associated radioactivity (i.e., surface plus internalized). The results of a representative experiment performed with the indicated cell lines are shown. Top, ●, 293L9wt-12; △, 293LD556Y-6; ○, 293LY524F-22. Bottom, ■, 293Lmyc(wt-11); ◊, Lmyc(L435R-2).
Internalization of Constitutively Active and Signaling-impaired Mutants of the rLHR

The half-life of internalization of the agonist-receptor complex formed by the constitutively active and signaling-impaired mutants of the rLHR described above is presented in Table II. For comparison, we also included a previously described cell line (designated 293L(D383N-9)) that expresses another signaling-impaired mutant that has been previously shown to activate and endocytose the cell surface receptor following mild proteolysis of intact cells (25). This analysis is based entirely on the appearance of the cell surface receptor following mild proteolysis (25). This analysis is based entirely on the appearance of the cell surface receptor following mild proteolysis (25). Unfortunately, the gels used to resolve the immunoprecipitates could also be directly measured by surface biotinylation of intact cells (25). This method can be readily labeled with [35S]cysteine/methionine (32) pulse-chase experiments (not shown) revealed that not all the precursor is converted to the mature cell surface rLHR even during a 12-h chase. As such, we were unable to find conditions where all of the radioactivity initially incorporated into the intracellular precursor was transferred to the cell surface rLHR. Since this is a prerequisite for measuring the rate of internalization of the free rLHR, we were unable to use this experimental paradigm. Last, the internalization of receptors can also be directly measured by surface biotinylation (using a disulfide-bonded form of biotin), stripping the biotin off of the cell surface and following the rate of disappearance of the antibody from the cell surface. While anti-rLHR or anti-myc antibodies are available, we opted against this procedure, however, because we do not have a way to independently determine if antibody binding affects the internalization of the rLHR. Quantitative measurements of the steady state distribution of the rLHR between the cell surface and intracellular compartments (which could also be accomplished using anti-rLHR or anti-myc antibodies) were also not possible, because transfected cells express a large amount of an intracellular rLHR precursor (25), which is difficult to distinguish (by simple microscopy) from internalized receptor. We also attempted to measure the rate of internalization of the free rLHR by following the rate of disappearance of the cell surface rLHR following metabolic labeling with [35S]cysteine/methionine. This method also proved difficult because of the large amount of intracellular 68-kDa rLHR precursor and the kinetics of conversion of the precursor to the cell surface 85-kDa rLHR (see below and Refs. 25 and 32). Although the intracellular rLHR precursor can be readily labeled with [35S]cysteine/methionine (32) pulse-chase experiments (not shown) revealed that not all the precursor is converted to the mature cell surface rLHR even during a 12-h chase. As such, we were unable to find conditions where all of the radioactivity initially incorporated into the intracellular precursor was transferred to the cell surface rLHR. Since this is a prerequisite for measuring the rate of internalization of the rLHR described above is presented in Table II. For comparison, we also included a previously described cell line (designated 293L(D383N-9)) that expresses another signaling-impaired mutant that has been previously shown to activate and endocytose the bound agonist at a slower rate than rLHR-wt (14). These data clearly show that the two signaling-imparing mutations, rLHR-D383N and rLHR-Y524F, decrease the rate of internalization of hCG, while the two constitutively active mutants, rLHR-D556Y and myc-rLHR-L435R, enhance the rate of internalization of hCG.

Internalization of Constitutively Active and Signaling-impaired Mutants of the rLHR in the Absence of Agonist—While the data presented above show a clear correlation between signaling and internalization of 125I-hCG, it was particularly important to determine if constitutive activation affected the turnover of the rLHR when not stimulated by agonist.

The endocytotic rate constants ($k_e$) were calculated from the slopes of plots such as those shown in Fig. 3. The half-life of internalization was calculated by the equation $t_{1/2} = \ln(2)/k_e$. Each value represents the mean ± S.E. of the indicated number of experiments.

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<tr>
<th>Cell line</th>
<th>$t_{1/2}$ of internalization of hCG</th>
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<td>rLHR-wt</td>
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</tbody>
</table>

Although the rate of internalization of the hCG-receptor complex could be conveniently measured by tracking the radio-labeled ligand (see above), measuring the rate of internalization of the free rLHR necessitated the establishment of a quantitative procedure to directly track the receptor. Some investigators (see Ref. 41 for an example) have quantitatively tracked free GPCRs by forming a receptor-antibody complex at the cell surface and following the rate of disappearance of the antibody from the cell surface. While anti-rLHR or anti-myc antibodies are available, we opted against this procedure, however, because we do not have a way to independently determine if antibody binding affects the internalization of the rLHR. Quantitative measurements of the steady state distribution of the rLHR between the cell surface and intracellular compartments (which could also be accomplished using anti-rLHR or anti-myc antibodies) were also not possible, because transfected cells express a large amount of an intracellular rLHR precursor (25), which is difficult to distinguish (by simple microscopy) from internalized receptor. We also attempted to measure the rate of internalization of the free rLHR by following the rate of disappearance of the cell surface rLHR following metabolic labeling with [35S]cysteine/methionine. This method also proved difficult because of the large amount of intracellular 68-kDa rLHR precursor and the kinetics of conversion of the precursor to the cell surface 85-kDa rLHR (see below and Refs. 25 and 32). Although the intracellular rLHR precursor can be readily labeled with [35S]cysteine/methionine (32) pulse-chase experiments (not shown) revealed that not all the precursor is converted to the mature cell surface rLHR even during a 12-h chase. As such, we were unable to find conditions where all of the radioactivity initially incorporated into the intracellular precursor was transferred to the cell surface rLHR. Since this is a prerequisite for measuring the rate of internalization of the rLHR described above is presented in Table II. For comparison, we also included a previously described cell line (designated 293L(D383N-9)) that expresses another signaling-impaired mutant that has been previously shown to activate and endocytose the bound agonist at a slower rate than rLHR-wt (14). These data clearly show that the two signaling-imparing mutations, rLHR-D383N and rLHR-Y524F, decrease the rate of internalization of hCG, while the two constitutively active mutants, rLHR-D556Y and myc-rLHR-L435R, enhance the rate of internalization of hCG.

Internalization of Constitutively Active and Signaling-impaired Mutants of the rLHR in the Absence of Agonist—While the data presented above show a clear correlation between signaling and internalization of 125I-hCG, it was particularly important to determine if constitutive activation affected the turnover of the rLHR when not stimulated by agonist.

The endocytotic rate constants ($k_e$) were calculated from the slopes of plots such as those shown in Fig. 3. The half-life of internalization was calculated by the equation $t_{1/2} = \ln(2)/k_e$. Each value represents the mean ± S.E. of the indicated number of experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$t_{1/2}$ of internalization of hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLHR-wt</td>
<td>99 ± 6 (n = 14)</td>
</tr>
<tr>
<td>293L(Y524F-22)</td>
<td>188 ± 14 (n = 5)</td>
</tr>
<tr>
<td>293L(D383N-9)</td>
<td>288 ± 12 (n = 6)</td>
</tr>
<tr>
<td>293L(D556Y-6)</td>
<td>37 ± 2 (n = 5)</td>
</tr>
<tr>
<td>293Lmyc(wt-11)</td>
<td>119 ± 11 (n = 7)</td>
</tr>
<tr>
<td>293Lmyc(L435R-2)</td>
<td>7 ± 2 (n = 4)</td>
</tr>
</tbody>
</table>
the rate of externalization of the receptor (E) and the rate constant for internalization of the receptor (k_int) as defined by Equation 1 (8, 31, 43).

\[
[rLHR_85] = \frac{E}{k_{\text{int}}} \tag{Eq. 1}
\]

As initially described by Berlin and Schimke (31), when the steady state level of a protein is perturbed, the time course required to attain a new steady state is based entirely on the rate constant for degradation of the protein. This analysis can be readily modified to describe the dynamics of a cell surface receptor as shown by Equation 2 (8, 31).

\[
[rLHR_85] = \left(\frac{E}{k_{\text{int}}} - \frac{[rLHR_85]}{0}\right)e^{-k_{\text{int}}t} \tag{Eq. 2}
\]

where \([rLHR_85]_t\) represents the density of the cell surface 85-kDa rLHR at time \(t\); \([rLHR_85]\)_0 represents the density of the cell surface 85-kDa rLHR at time 0; \(E\) is the rate of externalization of the rLHR; and \(k_{\text{int}}\) is the rate constant for internalization of the rLHR. The half-life of internalization of the free receptor can then be calculated from Equation 3.

\[
t_{1/2} \text{of internalization} = \frac{0.693}{k_{\text{int}}} \tag{Eq. 3}
\]

Thus, in order to measure the rate of internalization of the mature, cell surface rLHR in the absence of agonist activation, we first perturbed the steady state levels of the cell surface rLHR by exposing intact cells to protease XIV (a condition that was previously shown to result in a quantitative loss of the cell surface rLHR; see Ref. 25) and then followed the time course of recovery of the cell surface rLHR (which can be readily assessed by measuring \(^{125}\text{I}-\text{hCG} binding to the cell surface} after removal of the protease (25)) as shown in Fig. 4. Since, upon removal of the protease, the density of cell surface rLHR returns to preproteolysis levels (25), one can readily measure \(E\) and \(k_{\text{int}}\) by fitting the results shown in Fig. 4 to Equation 2. The \(t_{1/2}\) of internalization of the free rLHR can then be readily calculated by substituting the calculated value of \(k_{\text{int}}\) into Equation 3.

The results of these experiments are summarized in Table III and show that the \(t_{1/2}\) of internalization of the free rLHR-wt is \(~450\) min. This \(t_{1/2}\) of internalization of the unoccupied receptor is slower than the \(t_{1/2}\) of internalization measured when the rLHR-wt is occupied by agonist (\(~100\) min; see Table II), and it is remarkably similar to the \(t_{1/2}\) of internalization of the free mouse LHR expressed in target cells (\(~400\) min; see Ref. 8).

The data presented in Table III also show that one mutation that impairs signal transduction (Y524F) and one mutation that induces constitutive activation (D556Y) had little or no effect on the \(t_{1/2}\) of internalization of the mature free rLHR. The \(t_{1/2}\) of internalization of the mature free myc-rLHR-wt is somewhat slower than that of the free mature untagged receptor. More importantly, however, the data presented in Table III show that the \(t_{1/2}\) of internalization of the free mature myc-rLHR-L435R is about twice as fast as that of the mature myc-rLHR-wt.

Taken together, these results show that although both activating mutations of the LHR (i.e. D556Y and L435R) increase the rate of internalization of the agonist-occupied receptor, only the stronger activating mutation (i.e. the L435R mutation) increases the internalization of the free receptor.

Last, it should be stressed that the conditions used here to proteolyze the cell surface rLHR (see “Materials and Methods” for details) resulted in a quantitative loss (i.e. 96–98%) of \(^{125}\text{I}-\text{hCG} binding to cells expressing rLHR-wt, rLHR-Y524F, rLHR-D556Y, and myc-rLHR-wt. The loss of binding activity detected in cells expressing myc-rLHR-L435R was at best 85%, however (Fig. 4).
The rate constants for internalization (k_{int}) of the cell surface rLHR were calculated by fitting the individual experiments shown in Fig. 4 to Equation 2, and the t_{1/2} values of internalization of the free rLHR were calculated from Equation 3. The rate constants for degradation (k_{deg}) of the intracellular rLHR precursor were calculated as described in Equations 4 and 5 and under "Materials and Methods."

The half-life of internalization of the free receptor can then be calculated from Equation 5. The rate constants for degradation of the intracellular 68-kDa rLHR were calculated using Equation 5. The half-life of internalization of the free rLHR was calculated using non-linear fits of these data to Equation 4, and the t_{1/2} values of internalization of the free rLHR were calculated from Equation 3. The rate constants for degradation of the intracellular 68-kDa rLHR precursor were calculated as described in Equations 4 and 5 and under "Materials and Methods."

In this case, the steady state was not perturbed, but the rate constant for degradation of the intracellular 68-kDa rLHR precursor can be simply measured by following the time course of incorporation of Tran^35S-label into this precursor. The rate constants for degradation (k_{deg}) were calculated using non-linear fits of these data to Equation 4, and the t_{1/2} values for degradation were calculated using Equation 5.

The results of these calculations are summarized in Table III and show that the intracellular rLHR precursor is degraded rather than converted to the mature 85-kDa rLHR (32, 44, 45), the differences in their turnover rate imply that most of the 68-kDa rLHR precursor is degraded rather than converted to the mature 85-kDa rLHR. This finding also provides an explanation for the finding that pulse-chase experiments performed with Tran^35S-label fail to show a complete translational of the radioactivity initially associated with the 68-kDa rLHR into the 85-kDa rLHR (see above).

Effect of hCG on Adenylyl Cyclase Activity of Membranes Prepared from Cells Expressing rLHR-L435R—Based on the findings that rLHR-L435R binds hCG with high affinity (cf. Table I) and that the internalized hCG is biologically inactive (7), we reasoned that the extremely fast rate of internalization of hCG mediated by rLHR-L435R may be responsible for the lack of responsiveness displayed by cells expressing this mutant (see Fig. 1 and Table I). This possibility was addressed by measuring the effects of hCG in isolated membranes, a subcellular fraction where signal transduction can be measured in the absence of internalization.

Table III

<table>
<thead>
<tr>
<th>Cell line</th>
<th>~85-kDa cell surface rLHR (t_{1/2} of internalization)</th>
<th>~68-kDa intracellular rLHR (t_{1/2} of degradation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>293L(wt-12)</td>
<td>458 ± 97 (n = 3)</td>
<td>107 ± 22 (n = 5)</td>
</tr>
<tr>
<td>293L(Y524F-22)</td>
<td>534 ± 48 (n = 3)</td>
<td>103 ± 4 (n = 3)</td>
</tr>
<tr>
<td>293L(D556Y-6)</td>
<td>522 ± 90 (n = 3)</td>
<td>ND*</td>
</tr>
<tr>
<td>293Lmyc(wt-11)</td>
<td>625 ± 102 (n = 5)</td>
<td>97 ± 13 (n = 5)</td>
</tr>
<tr>
<td>293Lmyc(L435R-2)</td>
<td>334 ± 49 (n = 3)</td>
<td>108 ± 13 (n = 4)</td>
</tr>
</tbody>
</table>

* Not determined.

As shown in Table I, membranes prepared from 293Lmyc(wt-11) cells respond to hCG with a 1.7-fold increase in adenylyl cyclase activity. This is a rather weak signal compared with that detected in intact cells (cf. Fig. 1 and Table I), but it is consistent with previous experiments using membranes prepared from 293 cells transfected with the rLHR (29, 46). Under the same assay conditions used here, a maximally effective concentration of hCG stimulates adenylyl cyclase activity ~3-fold in membranes from 293L(wt-12) cells (29). The 1.7-fold increase reported in Table IV for membranes prepared from 293Lmyc(wt-11) cells is consistent with the reduced responsiveness detected in cells expressing the myc-tagged rLHR (Table I and Ref. 25). As expected from the data obtained using intact cells expressing rLHR-L435R (cf. Fig. 1 and Table I), we found that membranes prepared from these cells exhibit a higher level of basal adenylyl cyclase activity than membranes expressing rLHR-wt (Table IV). In contrast to the data obtained with intact cells expressing rLHR-L435R, which do not respond to hCG with further cAMP accumulation (cf. Fig. 1 and Table I), we found that membranes prepared from these cells do respond to hCG with an increase in adenylyl cyclase activity (Table IV). The magnitude of this increase (~2-fold) is comparable with the 1.7-fold increase in adenylyl cyclase detected upon the addition of hCG to membranes prepared from cells expressing rLHR-wt.

These data show that whatever constraints are imposed by intact cells on the signaling properties of rLHR-L435R are relieved when the cells are lysed and isolated membranes are used to measure signaling.

**DISCUSSION**

The simplest version of the current model for GPCR activation states that GPCRs exist in an equilibrium between an inactive (R) and an active conformation (R*) which is responsible for G protein activation. In this model, agonists are said to have a higher binding affinity for R* than for R. Thus, the preferential binding of an agonist to R* activates GPCRs by shifting the equilibrium toward R* (47). It is also clear that R* can exist in the absence of agonist activation, because many mutations of GPCRs in general (47) and the LHR in particular (36) have now been found to cause constitutive (i.e. agonist-independent) activation.

Three previous observations from this laboratory have suggested that the same active conformation of LHR is involved in the stimulation of G proteins and the endocytosis of the hCG-LHR complex. First, when occupied by agonist, the t_{1/2} of internalization of the mouse LHR expressed in target cells is faster than that of the free receptor (30–60 versus 400 min; see Ref. 8). This is also the case in transfected cells, since the t_{1/2} of internalization of the agonist-occupied rLHR-wt expressed in 293 cells is several times faster (99–119 min; see Table II) than...
that of the free LHR-wt (458–625 min; see Table III). Second, the rate of internalization of the mouse LHR occupied by a weak partial agonist is much slower than that of the mouse LHR occupied by the full agonist (30–60 versus 250 min; see Ref. 13). Third, two distinct point mutations of the rLHR that impair signal transduction (an Arg to His mutation in the second intracellular loop and an Asp to Asn mutation in TM2) also slow down the internalization of hCG (14). The results presented here show that a third mutation of the rLHR that impairs signal transduction (a Tyr to Phe mutation in TM5) also retards the internalization of the bound hCG (Tables I and II). It is important to note, however, that the rate of internalization of the endogenous mouse LHR occupied by a weak partial agonist is still faster than the rate of internalization of the unoccupied receptor (8, 13). Likewise, the $t_{1/2}$ values of internalization of hCG in cells expressing signaling-imparing mutations (i.e. 293L(Y524F-22) and 293L(D383N-9)) cells are still faster (200–300 min; see Table II) than the $t_{1/2}$ of internalization of the free rLHR-wt (~450 min; see Table III).

Based on the activation model and the observations summarized above, we reasoned that mutations of the rLHR that induce constitutive activation should be internalized at a faster rate than rLHR-wt. The experiments presented herein were thus conducted to test this prediction by analyzing two naturally occurring mutations (an Asp to Tyr mutation in TM6 and a Leu to Arg mutation in TM3) that have been previously shown to induce constitutive activation of the hLHR (22, 23, 39). As shown here, the equivalent mutations also induced activation of the rLHR (Table I and Fig. 1). Several properties of these two mutants suggest that their active configurations are different, however. First, cells expressing the D556Y mutant have an elevated basal level of cAMP corresponding to 10–20% of the maximal cAMP response detected in cells expressing rLHR-wt, while cells expressing the L435R mutant have an elevated basal level of cAMP corresponding to 40–50% of the maximal cAMP response detected in cells expressing rLHR-wt (Table I and Fig. 1). Second, whereas cells expressing either of these two mutants bind hCG with high affinity (Table I), cells expressing the L435R mutant do not respond to further hCG stimulation, while cells expressing the D556Y mutant show a normal response to hCG stimulation (Table I and Fig. 1). Third, proteolysis of intact cells at 4 °C degrades 98% of the $^{125}$I-hCG binding activity detected in cells expressing the D556Y mutant but only about 85% of the $^{125}$I-hCG binding activity detected in cells expressing the L435R mutant (Fig. 4).

If the conformation of LHR-D556Y and rLHR-L435R is the same as the conformation induced by agonist binding to rLHR-wt, we would expect the rate of internalization of the free rLHR-D556Y and rLHR-L435R to be the same as the rate of internalization of the agonist-occupied rLHR-wt. Moreover, agonist binding to rLHR-D556Y or rLHR-L435R would not be expected to result in an increase in the rate of internalization. This is clearly not the case. The rate of internalization of the free rLHR-D556Y is the same as that of rLHR-wt (Table III), and although the rate of internalization of the free L435R mutant is faster than that of the free wild type receptor, it is still much slower than the rate of internalization of the agonist-occupied rLHR-wt (Tables II and III). Likewise, agonist binding not only increases the rate of internalization of the rLHR-wt, but it also increases the rate of internalization of the D556Y and L435R mutants (Tables II and III). In fact, the rate of internalization of the agonist-occupied constitutively active mutants is 3–20-times faster than the rate of internalization of the agonist-occupied rLHR-wt (Table II). These results clearly show that, in terms of internalization, the rLHR conformations induced by the D556Y or L435R mutations are not equivalent to the conformation induced by agonist binding to rLHR-wt. Moreover, we can also conclude that the rLHR conformations induced by the binding of agonist to the D556Y or L435R mutants are also different from each other and different from that induced by the binding of agonist to the rLHR-wt.

One reason why there may be a correlation between agonist-induced activation and agonist-induced internalization of some GPCRs is that agonist-activated GPCPs are known to be better substrates for the phosphorylation catalyzed by G protein-coupled receptor kinases. The G protein-coupled receptor kinase-phosphorylated GPCRs exhibit an enhanced affinity for nonvisual arrestins, and nonvisual arrestins are known to act as adapter molecules bridging the G protein-coupled receptor kinase-phosphorylated GPCR and clathrin (10, 48–51). Thus, the phosphorylation of many GPCRs, including the $\beta_2$-adrenergic receptor and LHR, is known to be necessary for the agonist-induced internalization of these receptors (3, 52). It is thus interesting to note that constitutively active mutants of (at least some) GPCRs exhibit a high level of basal phosphorylation (53, 54). It is not known, however, if their basal rate of internalization is faster than that of their wild type counterparts. Conversely, while we have shown here that at least one constitutively active mutant of rLHR (i.e. rLHR-L435R) has a faster basal rate of internalization than rLHR-wt (Table III), we do not know if this mutant also exhibits a high level of basal phosphorylation. Experiments are currently under way to determine if this is the case. Since we know that mutation of the serine residues phosphorylated in the agonist-activated rLHR impairs internalization (3, 15), it will also be of interest to determine if the mutation of these sites in rLHR-L435R returns the enhanced rate of internalization detected in the absence or presence of hCG to that detected in rLHR-wt.

Last, since we know that the LHR-mediated internalization of hCG is involved in the termination of the actions of hCG (7), we suggest that the enhanced rate of internalization of hCG displayed by rLHR-D556Y and rLHR-L435R limits their activity. In fact, the lack of hCG responsiveness in cells expressing rLHR-L435R (a receptor that can bind hCG with the same affinity as rLHR-wt; cf. Table I) seems to be due to the extremely fast rate at which this receptor internalizes the bound hCG. This statement is supported by the finding that hCG responsiveness is restored when the cells are lysed and signal transduction is measured in a subcellular fraction (i.e. membranes) that cannot internalize the bound hormone (Table IV).

It remains to be determined if this same explanation is applicable to intact cells expressing other naturally occurring or “designed” constitutively active mutants of the LHR that bind hCG but fail to respond with an increase in cAMP accumulation (22, 39).

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REFERENCES
