Pleiotropic Effects of Substitutions of a Highly Conserved Leucine in Transmembrane Helix III of the Human Lutropin/Choriogonadotropin Receptor with Respect to Constitutive Activation and Hormone Responsiveness

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It has been shown previously that a naturally occurring mutation of the human LH/CG receptor (hLHR), which replaces L457 in helix III with arginine, results in a receptor that constitutively elevates basal cAMP but does not respond to human CG (hCG) with further cAMP production. In the present study, substitutions of L457 with several amino acids were examined. The constitutive activation of cAMP production was observed only when L457 was replaced with a positively charged residue. Although constitutive activation of the inositol phosphate pathway could not be detected when measuring inositol phosphate production, the use of a more sensitive reporter gene assay for protein kinase C activation revealed the constitutive activation of this pathway by the R- and K-substituted mutants. Therefore, L457 of the hLHR plays a key role in stabilizing the receptor in an inactive conformation. Molecular modeling shows that the insertion of R, K, or H at position 457 triggers the receptor transition toward an active state due to the proximity of an anionic amino acid, D578, in helix VI. These substitutions cause perturbations in helix III-helix VI and helix III-helix VII interactions that culminate in the opening of a solvent-accessible site in the cytosolic domains potentially involved in Gs recognition.

Interestingly, L457R was completely unresponsive and the K- and H-substituted L457 hLHR mutants were significantly blunted in their cAMP responses to hCG stimulation. Cells expressing L457R were also unresponsive to hCG with regards to increased inositol phosphate production. Other substitutions of L457 were identified, though, that selectively permit the hormonal stimulation of only one of the two signaling pathways. These results suggest a pivotal role for L457 in hormone-stimulated signal transduction by the hLHR. (Molecular Endocrinology 15: 972–984, 2001)

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INTRODUCTION

The LH/CG receptor (LHR) is a G protein-coupled receptor (GPCR) that plays a central role in reproductive physiology in both males and females. The carboxyl half of the receptor is composed of seven transmembrane (TM) helices connected by extracellular and intracellular loops. The amino acid sequences of the predicted TM regions of the LHR define it as a member of the large subfamily of rhodopsin-like GPCRs (1). Like the closely related FSH receptor (FSHR) and TSH receptor (TSHR), the LHR contains a relatively large amino-terminal extracellular domain composed of multiple leucine-rich repeats (2, 3). Leucine-rich repeats have been suggested to be involved in protein-protein interactions (4, 5) and, indeed, it has been shown that the high-affinity binding

of hormones to the LHR, FSHR, and TSHR occur via interactions with the extracellular domain (6–12). The interactions of LH or hCG with the extracellular domain of the LHR then presumably allow regions of the extracellular domain and/or hormone to interact with the carboxyl half of the receptor in a manner that stabilizes it in an active conformation (13–15). The LHR is known to stimulate both the cAMP and the inositol phosphate second messenger pathways (16–20). However, the activation of the inositol phosphate pathway is observed only under conditions of high LHR numbers and relatively high concentrations of hormone (18, 19). Whereas the physiological contributions of LH/CG-stimulated cAMP in male and female physiology are well established, the role of LH/CG-stimulated inositol phosphate production is less clear.

The LHR present in the ovaries and testes binds either LH, produced by the pituitary in postpubertal men and women, or the structurally related hormone hCG, produced by the placenta of pregnant females, and serves to regulate several key aspects of reproductive physiology and developmental biology. In nonpregnant females, LH is involved in the production of ovarian steroid hormones. The monthly midcycle surge of LH also mediates ovarian follicular maturation and ovulation. In males, the role of the LHR comes into play as early as fetal development. During that time, the LHR present on Leydig cells of the testes binds maternal hCG and stimulates the production of testosterone. Under the actions of this androgen, the external genitalia of the fetus differentiate into the male phenotype. After birth, the male is no longer exposed to maternal hCG and is not exposed to LH until the time of puberty. When puberty is reached, the hypothalamic-pituitary-gonadal axis matures, and the elevated levels of LH then stimulate testosterone production.

In recent years many naturally occurring loss-of-function and gain-of-function mutations of the hLHR have been described (see Ref. 21 for a review). The gain-of-function mutations have been identified in young boys with gonadotropin-independent precocious puberty, also called “testotoxicosis.” These individuals have been found to have heterozygous mutations of the hLHR that cause it to constitutively elevate basal cAMP levels. Therefore, in the face of prepubertal low levels of pituitary LH, the testes of these boys constitutively secrete testosterone, which then elicits the physiological changes accompanying male puberty.

At this writing, 14 independent naturally occurring activating mutations of the human (h)LHR have been identified (21, 22). All of them have been identified in the carboxyl half of the receptor, with many of them clustering in TM VI. This may reflect the importance of this region of the hLHR in activating Gs (23, 24). It should also be pointed out, however, that many earlier studies focused only on this portion of the gene when searching for mutations. Indeed, as more recent studies have sequenced the entire carboxyl half of the gene from individuals thought to have activating hLHR mutations, substitutions causing activation have been found in other helices as well.

We had previously reported the identification of an activating mutation of the hLHR in TM III (25). This mutation results in the substitution of an arginine in place of a highly conserved leucine. This leucine, designated Leu III.18 per the Baldwin model of the GPCR TM helices (26), is conserved in $\geq$70% of rhodopsin-like GPCRs. As one would predict, cells transfected with the L457R mutant exhibited elevated levels of basal cAMP. The basal levels of cAMP in cells expressing the activating mutant, while greater than the basal levels of cells expressing the wild-type hLHR, were less than the maximal levels of cAMP observed in cells expressing the wild-type hLHR incubated with a saturating concentration of hormone. However, whereas cells expressing most activating mutants will respond with a further increase in cAMP when challenged with hormone, cells expressing L457K are completely unresponsive to further cAMP stimulation by hCG addition (25).

The present studies were undertaken to determine the molecular basis for the constitutive activation of the cAMP pathway by hLHR(L457R) and its inability to respond further to hormone. Results presented support the model that Leu III.18 plays a conserved role in stabilizing the inactive state of GPCRs, but that constitutive activity requires both the disruption of the bonds stabilized by this conserved leucine and the introduction of specific residues that promote interhelical interactions stabilizing the active state of the receptor.

RESULTS

Previous studies had shown that substitution of Leu III.18 (L457) of the hLHR with arginine results in a receptor that constitutively stimulates the production of cAMP, but is unresponsive to further cAMP production by hCG addition (25). To determine the mechanisms underlying this peculiar phenotype, we studied additional mutants of the hLHR in which L457 was substituted with basically charged residues (arginine, lysine, or histidine), with an uncharged residue (alanine), or with a negatively charged residue (aspartate).

When transfected with maximal amounts of plasmid, the levels of cell surface receptors for L457K were higher than those of wild-type receptor. Those for L457R, L457H, and L457A were similar to the wild-type receptor, and those of L457D were much lower (Table 1). Since both basal and hormone-stimulated second messenger production can be dependent upon cell surface receptor number (27, 28), a correction for receptor expression must be taken into account. Because second messenger production was not linear with respect to receptor number over the range of receptor numbers in these experiments, it

Table 1. Cell Surface Receptor Levels in Cells Expressing hLHR(wt) or L457-Substituted Mutants

Receptor	K_d (nM)
hLHR(wt)	10.7 ± 4.2
hLHR(L457R)	12.6 ± 2.7
hLHR(L457K)	19.0 ± 5.6
hLHR(L457H)	11.0 ± 3.6
hLHR(L457A)	10.0 ± 1.2
hLHR(L457D)	2.7 ± 0.1

HEK 293 cells were transiently transfected with the cDNAs for the wild-type hLHR or the L457-substituted mutants shown. ^{125}I-hCG binding assays using a saturating concentration of hormone were then performed to determine the maximal amount of hormone binding. Data shown are the mean ± SEM of three independent experiments.

Table 2. Solvent-Accessible Surface (SAS) of the Cytosolic Extensions of Helices III and VI of hLHR(wt) and L457-Substituted Mutants Correlates with Observed Constitutive Activation

Receptor	Basal cAMP[a] (mutant basal/ wt basal)	Composite SAS[b] (Å^2)
hLHR(wt)	1.00	49
hLHR(L457R)	10.6 ± 1.6	247
hLHR(L457K)	7.0 ± 1.2	138
hLHR(L457H)	3.2 ± 0.3	106
hLHR(L457A)	0.8 ± 0.2	73
hLHR(L457D)	0.8 ± 0.1	38

[a] Basal cAMP data are presented as the ratio of basal cAMP in cells expressing a mutant relative to basal cAMP in matched cells expressing the same number of wild-type hLHRs. The data shown are the mean ± SEM of five to nine independent experiments.

[b] The composite SAS was calculated as the solvent accessible surface of residues W465 and I468 in helix III as well as I567 and K570 in helix VI based upon the *ab initio* model of the hLHR and L457-substituted mutants.

was not possible to correct a given response by dividing by the cell surface binding. Instead, we used an approach previously described (25, 29, 30) in which we deliberately varied the plasmid concentrations used in the transfections to yield cells with similar (no more than 2-fold different) numbers of cell surface receptors. In our experience, a 2-fold difference in receptor number (within the range of expression observed in these experiments) has not been of consequence. Therefore, although the absolute numbers of cell surface receptors varied from experiment to experiment, a given mutant was always compared within the same experiment to a wild-type control with matched numbers of cell surface receptors.

As shown in Table 2, cells expressing hLHR(L457R) exhibit approximately 10-fold elevation in basal cAMP levels as compared with cells expressing similar numbers of wild-type receptors, consistent with the constitutive activation of this signaling pathway (31). Cells expressing L457K also exhibited markedly increased basal levels of cAMP, suggesting that the introduction of a positive charge at codon 457 is required for constitutive activation of the cAMP pathway by the hLHR. Consistent with this conclusion is the observation that L457H cells showed slightly elevated levels of basal cAMP and that the basal levels of cAMP in L457A and L457D cells were not at all elevated.

To determine whether any of the L457-substituted hLHR mutants constitutively activated the inositol phosphate pathway, we measured basal inositol phosphate levels in cells expressing the wild-type hLHR and each of the mutants. We found no detectable increases in basal inositol phosphates in cells expressing any of the mutants (Table 3). It has been shown, however, that the measurement of basal inositol phosphate levels does not always reveal constitutive activation of this pathway due to the relatively poor sensitivity of this assay (32). Rather, constitutive activation of this pathway can more readily be observed using a more sensitive reporter gene assay indicative of C kinase activation (32). Using the C kinase-responsive reporter gene assay it was possible to detect small, but reproducible, increases in basal C kinase activity in cells expressing the K- and R-substituted mutants. Cells expressing L457K exhibited approximately 20% increase in activity and cells expressing L457R exhibited about 90% increase in activity. These results suggest that substitutions of hLHR(L457) with positively charged residues cause constitutive activation of both the cAMP and the inositol phosphate pathways.

Recently, a molecular modeling approach has been used to describe hLHR mutants that constitutively activate the cAMP pathway (33). Comparative molecular dynamics (MD) simulations were carried out on most of the naturally occurring activating and inactivating mutations discovered thus far by using an *ab initio* model of the hLHR (33). The constitutively active mutants were all predicted to have an augmented solvent exposure of the cytosolic domains. A good marker of this structural feature was found to be the solvent-accessible surface of W465 (SAS_{W465}). This theoretical descriptor was able to account for the structural differences between inactive and active hLHR forms, being lower than 32 Å^2 in inactive mutants and greater than 32 Å^2 in constitutively active mutants. SAS_{W465} values greater than 32 Å^2 were found to mark the opening of a crevice between the second and third intracellular loops (i2 and i3), respectively. The same computational approach has been applied to the molecular modeling of the L457 mutants presented in this work. The comparison between computer simulations and experiments strengthens the ability of SAS_{W465} to predict the functional behavior of the hLHR mutants. In fact, the average minimized structures of the R, K, and H constitutively active mutants are characterized by SAS_{W465} values above the threshold and an opening in the crevice between i2 an i3. The D and A mutants, which do not constitutively activate Gs, are characterized by the absence of an open crevice between i2 and

i3. However, for the L457A mutant, the theoretical descriptor SAS_{W465} does not properly describe the degree of solvent exposure of the cytosolic extensions of helices III, V, and VI. In fact, although the $SAS_{(W465)}$ value for the L457A mutant is above 30.0 $Å^2$, the L457A mutant does not exhibit an increased cytosolic exposure of helices III and VI. In the attempt to improve the predictive power of the theoretical descriptor, we determined that a composite SAS computed over a greater number of residues in the cytosolic extensions of helices III and VI facing the core of the helix bundle (*i.e.* W465 and I468 in helix III as well as I567 and K570 in helix VI) more effectively differentiates between the active and inactive forms of the L457-substituted mutants. The composite SAS is below 100 $Å^2$ in the wild-type hLHR and the L457D and L457A mutants, whereas it is greater than 100 $Å^2$ in the L457H, L457K, and L457R constitutively active mutants (Table 2).

Our data show that substitution of L467R in TM III of the hLHR with a positively charged residue stabilizes the hLHR in a conformation that can constitutively activate both the cAMP and the inositol phosphate pathways. The following experiments were performed to examine what effects substitutions of this highly conserved leucine residue have on hormone-stimulated second messenger production. Looking at the hCG responsiveness of the wild-type hLHR and the L457-substituted mutants, there was little (L457K, L457H) or no (L457R) hCG-mediated stimulation of cAMP in the mutants with a positively charged residue at III.18 (Fig. 1A). This is not because the basal levels of cAMP induced by these mutants were already maximal. As shown in Fig. 1B, the Rmax values of L457R, L457K, and L457H were about one-half that of cells expressing the same numbers of wild-type hLHR. On the other hand, the hCG responsiveness of cells expressing the alanine or aspartate-substituted mutants was completely normal (Fig. 1, A and B). Therefore, with regard to the cAMP second messenger pathway, there appears to be a correlation between the introduction of a positive charge at III.18, the induction of constitutive activity, and reduced hCG-mediated stimulation of cAMP.

The following experiments were performed to determine the basis for the lack of stimulation of cAMP production by hCG in cells expressing the L457R, L457K, or L457H. One possibility considered was that

Table 3. Basal Activation of the Inositol Phosphate/C Kinase Pathway in Cells Expressing hLHR(wt) or L457-Substituted Mutants

Receptor	Measurements of Inositol Phosphates[a] (mutant basal/wt basal)	Reporter Gene Activity[b] (mutant basal/wt basal)
hLHR(wt)	1.00	1.00
hLHR(L457R)	1.00	1.88 ± 0.28
hLHR(L457K)	1.00	1.21 ± 0.07
hLHR(L457H)	1.00	0.87 ± 0.11
hLHR(L457A)	1.00	1.16 ± 0.15
hLHR(L457D)	1.00	0.90 ± 0.11

[a] HEK 293 cells were transiently transfected with the indicated receptor cDNA. Using cells expressing matched numbers of cell surface receptors, the basal levels of inositol phosphates were assayed as described in *Materials and Methods*. No increases were detected in three independent experiments.

[b] HEK cells were transiently cotransfected with the indicated receptor cDNA and a C kinase-responsive reporter gene. Using cells expressing matched numbers of cell surface receptors, the basal luciferase activities were assayed as described in *Materials and Methods*. Data shown are the mean ± SEM of 6–13 independent experiments.

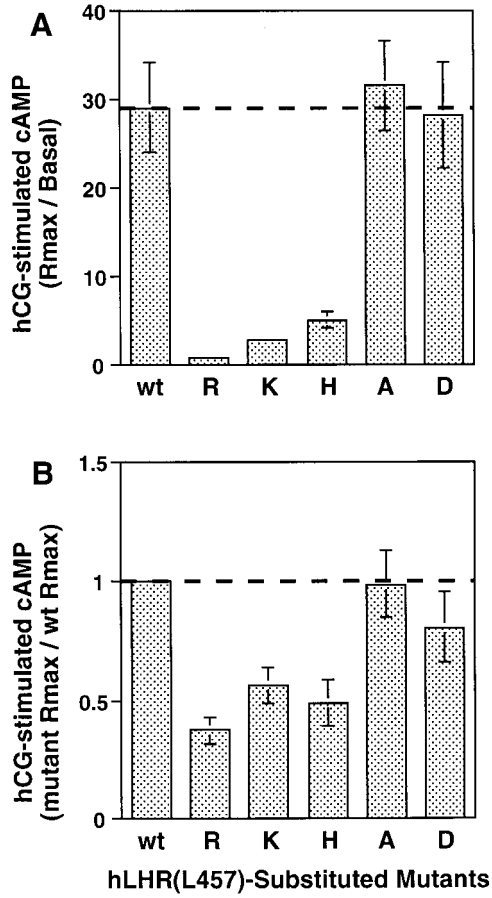

Fig. 1. Replacement of hLHR(L457R) with Positively Charged Residues Attenuates hCG-Stimulated cAMP Accumulation in Intact Cells

HEK293 cells were transiently transfected with the indicated hLHR construct to generate cells with matching numbers of cell surface receptors as described in *Materials and Methods*. Panel A, cAMP in response to a saturating concentration of hCG is presented as the ratio of Rmax to basal cAMP (*i.e.* the fold stimulation) in cells expressing a given receptor. The *dashed line* indicates the fold stimulation of the wild-type hLHR. Panel B, cAMP in response to a saturating concentration of hCG is presented as the Rmax in cells expressing a mutant relative to the Rmax in matched cells expressing the wild-type hLHR. Therefore, the Rmax of wild-type hLHR-expressing cells is defined as 1.0 and is shown by the *dashed line*.

these mutants might have a lower binding affinity for hCG. However, as shown in Table 4, binding assays to intact cells revealed similar binding affinities for all the L457-substituted mutants as compared with the wild-type hLHR. Another possibility considered was whether the R, K, and H mutants might internalize hCG more rapidly. If so, this may serve to terminate the signaling of cAMP production by the hormone-occupied receptor. As shown in Fig. 2, we found that cells expressing L457R, L457K, or L457H do internalize hCG at a faster rate than cells expressing the wild-type hLHR. In contrast, cells expressing L457A or L457D internalize hCG at a slower rate than cells expressing the wild-type receptor.

If the increased rate of hCG internalization by the R, K, and H-substituted L457 hLHR mutants was the cause of the attenuated hCG-stimulated cAMP production of cells expressing these receptors, then one would predict that membranes isolated from these cells would respond normally to hCG with increased adenylyl cyclase activity (since the membranes cannot internalize hCG). Therefore, membranes were isolated from cells expressing either the wild-type hLHR, or the R, K, or H-substituted L457 mutants and assayed for both basal and hCG-stimulated adenylyl cyclase activity (Fig. 3). The basal adenylyl cyclase activity of L457R membranes was elevated more than 2-fold above that of wild-type hLHR membranes. The basal activities of L457K and L457H membranes, however, showed little or no increase though. With regard to the hCG-stimulated cyclase activity in membranes containing the L457R-substituted hLHR mutants, the maximal response of L457K and L457H membranes in response to hCG was similar to that of the wild-type hLHR membranes (Fig. 3). However, L457R membranes showed no detectable increase in adenylyl cyclase activity in response to hCG. These results suggest that the increased rate of internalization of hCG by L457R cells cannot account for the inability of cells expressing this mutant to respond to hCG with increased cAMP production. Rather, there must be a structural modification of the L457R mutant *per se* that impairs its ability to stimulate adenylyl cyclase when occupied by agonist.

Table 4. Binding Affinities of hLHR(L457)-Substituted Mutants for hCG

Receptor	K_d (nM)
hLHR(wt)	1.42 ± 0.25
hLHR(L457R)	1.96 ± 0.51
hLHR(L457K)	2.20 ± 0.33
hLHR(L457H)	1.21 ± 0.15
hLHR(L457A)	1.51 ± 0.16
hLHR(L457D)	1.67 ± 0.42

HEK 293 cells were transiently transfected with the cDNAs for the wild-type hLHR or the L457-substituted mutants shown. ^{125}I-hCG binding assays were then performed utilizing intact cells to determine the apparent binding affinities. Data shown are the mean ± range of two independent experiments.

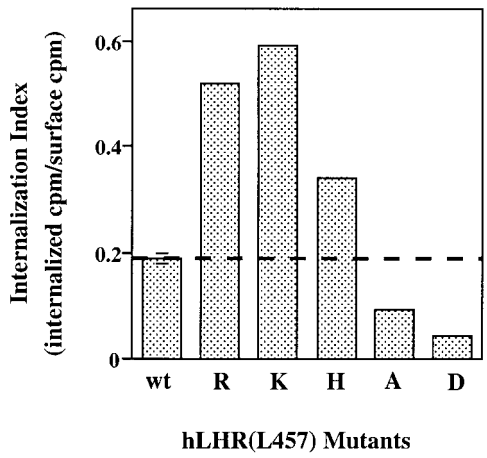

Fig. 2. Substitution of hLHR(L457) with Positively Charged Residues Causes the Mutant Receptors to Internalize hCG at a Faster Rate

293 cells were transiently transfected with either the wild-type (wt) hLHR or L457 mutants substituted with arginine (R), lysine (K), alanine (A), or aspartic acid (D). The internalization of ^{125}I-hCG was measured as described in *Materials and Methods*. Data are presented as an internalization index, which, under the conditions used, is proportional to the rate of internalization. Data shown are the mean ± range of two independent experiments. The internalization index of cells expressing the wild-type hLHR is noted with a *dashed line*.

Interestingly, cells expressing L457R, which do not respond to hCG with increased cAMP production, also do not respond to hCG with increased inositol phosphate production (Fig. 4). The L457R mutant, however, is the only mutant in which there is a correlation between hCG-stimulated cAMP and hCG-stimulated inositol phosphate responses. Cells expressing L457H responded to hCG with as great a fold stimulation of inositol phosphates as cells expressing the wild-type receptor (Fig. 4). This, one should note, is in contrast to hCG-stimulated cAMP production, which is blunted in the L457H cells (cf. Fig. 1). Similarly, cells expressing L457K responded to hCG with increased inositol phosphate production (albeit not as robustly as wild-type cells or L457R cells; see Fig. 4), whereas they showed little hCG stimulation of cAMP production (cf. Fig. 1). Cells expressing L457A also exhibited a moderate response to hCG with regard to inositol phosphate production (Fig. 4), in contrast to a fully normal response with regard to cAMP production (cf. Fig. 1). Interestingly, L457D cells did not show any detectable increases in inositol phosphates in response to hCG (Fig. 4) in spite of their responding normally to hCG with increased cAMP (cf. Fig. 1). Taken altogether, the data presented in Figs. 1 and 4 demonstrate that substitution of L457 of the hLHR with aspartate causes the receptor to respond to hCG with the selective stimulation of the cAMP pathway, whereas substitution with histidine causes the receptor to respond to hCG with the selective stimulation of the inositol phosphate pathway.

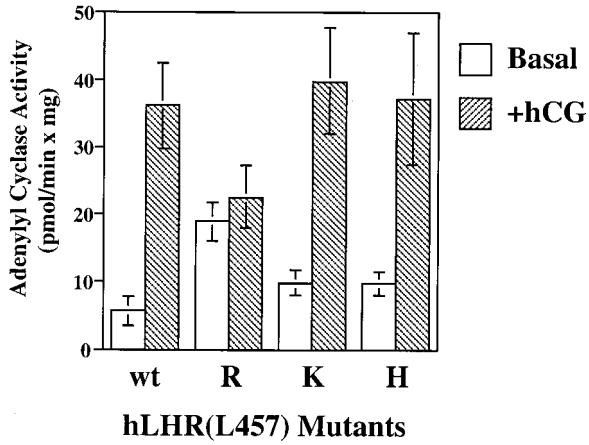

Fig. 3. Adenylyl Cyclase Assays Reveal Different Mechanisms Underlying the Lack of hCG Stimulation of cAMP by hLHR(L457) Mutants Substituted with Positively Charged Residues

293 cells were transiently transfected with either the wild-type (wt) hLHR or L457 mutants substituted with arginine (R), lysine (K), alanine (A), or aspartic acid (D) to match receptor numbers as described in *Materials and Methods*. Membranes were isolated and assayed for basal and hCG-stimulated adenylyl cyclase activity as described in *Materials and Methods*. Data shown are the mean ± SEM of four independent experiments.

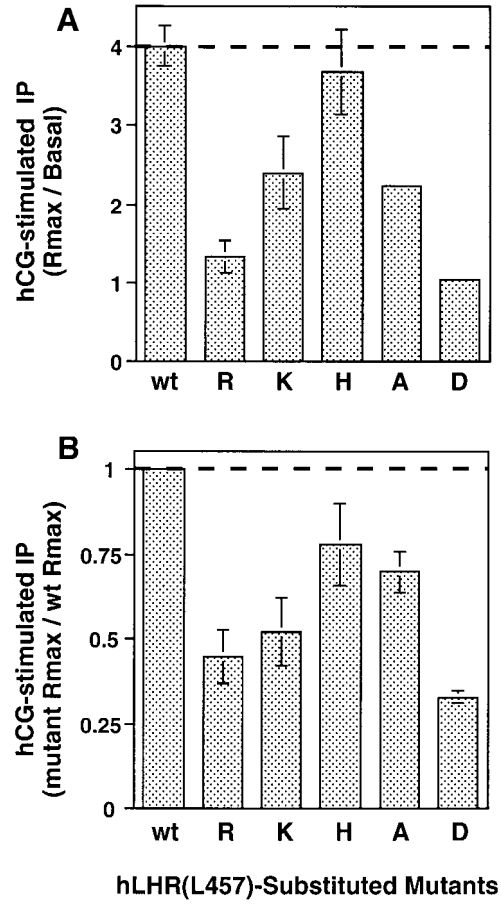

Fig. 4. Effects of Substitutions of hLHR(L457) on hCG-Stimulated Inositol Phosphate Production

HEK293 cells were transiently transfected with the indicated hLHR construct to generate cells with matching numbers of cell surface receptors as described in *Materials and Methods*. Panel A, The data for basal inositol phosphate production are presented as the ratio of basal inositol phosphates in cells expressing a mutant relative to basal inositol phosphates in matched cells expressing the wild-type hLHR. Therefore, basal inositol phosphates in wild-type hLHR-expressing cells are defined as 1.0 and are shown by the *dashed line*. Panel B, Inositol phosphates in response to a saturating concentration of hCG are presented as the ratio of Rmax to basal inositol phosphates (*i.e.* the fold stimulation) in cells expressing a given receptor. The *dashed line* indicates the fold stimulation of the wild-type hLHR.

DISCUSSION

Previous studies from our laboratory have identified an activating mutation of the hLHR in a young boy with gonadotropin-independent precocious puberty that resulted in the substitution of L457 with arginine (25). As with other activating mutations of the hLHR, cells expressing this mutant receptor exhibited elevated levels of basal cAMP when compared with cells expressing equivalent numbers of cell surface wild-type hLHR. Compared with other activating hLHR mutations, however, this L457R mutation causes a relatively higher fold increase in basal cAMP than most other mutants. Another interesting feature of this mutant is that, in spite of it causing constitutive activation of the cAMP pathway, it does not respond to hCG with further increases in cAMP even though the levels of basal cAMP are not as great as those in cells expressing the wild-type receptor that have been stimulated with hormone.

The revised ternary model for GPCR activation predicts that a given receptor exists in the plasma membrane in an equilibrium between inactive R state and an active R* state (see Ref. 1 for a review). The binding of agonist shifts the equilibrium toward the active state, thereby stabilizing this pool of receptor. Constitutively activating mutations of GPCRs are thought to also stabilize the active state of the receptor. Whether a mutation-induced R* conformation is identical to the agonist-induced R* conformation is not yet clear. This issue is further compounded by observations suggesting that there may be multiple activated states for a given GPCR, some intermediary in nature (designated R′) (1, 34–36). As such, any one of a number of intermediary R′ or fully active R* states may in turn be stabilized by a given mutation causing constitutive activation.

Leucine 457 of the hLHR represents a highly conserved leucine in TM III in GPCRs (26). The studies presented herein show that substitution of L457R of the hLHR with arginine, lysine, or histidine, but not with alanine or aspartate, causes the receptor to constitutively activate the cAMP pathway, with the greatest activation observed with an R substitution and the

least with the H substitution. We could not detect any elevations in inositol phosphate levels in cells expressing these mutants. However, since it was previously shown by Jinsi-Parimoo and Gershengorn (32) that whereas constitutive activation of the inositol phosphate/C kinase pathway by isoforms of the TRH receptor could not be detected by classical measurements of inositol phosphate production but could be readily discerned using a more sensitive C kinase-responsive reporter gene assay, we also examined activation of this pathway using the same C kinase-responsive reporter gene construct. Under these conditions, modest (20% and 90%) increases in basal C kinase activity were observed for cells expressing L457K and L457R, respectively. Although reports differ in the identification of G proteins mediating the stimulation of the inositol phosphate pathway by the LHR (16, 20), it is clear that Gs is not involved and it is likely that Gi is involved. Therefore, our results suggest that substitutions of L457 of the hLHR with positively charged residues stabilize the receptor in a conformation capable of constitutively activating both Gs and Gi.

Recently, a theoretical model for mutation-induced constitutive activation of the hLHR with regard to stimulation of cAMP production via Gs activation was proposed (33). The comparative MD analyses showed that the hLHR sites susceptible to activating mutations lie mainly at interhelical positions close to highly conserved amino acids. Constitutively active hLHR mutants were characterized by the opening of a crevice between i2 and i3 that allows solvent exposure of the intracellular extensions of helices III and VI. This presumably allows greater accessibility of Gs to the regions of the hLHR involved in G protein activation. In agreement with the conclusions of the modeling studies, a peptide corresponding to the juxtacytoplasmic region of helix VI has been shown to be able to activate Gs directly (23, 24).

Similar computer simulations on the L457-substitutions of the hLHR predict conformations of the R, K, and H-substituted mutants as being active (Table 1) and further suggest a mechanism underlying their constitutive activation. According to the theoretical model, L457 is close to D578 in helix IV (VI:16) as well as to N615 and N619 in helix VII (VII:17), where N619 belongs to the highly conserved NPXXY motif. The introduction of a positively charged amino acid at position 457 generates an attractive effect on D458 (VI:16), thus inducing perturbations in helix III-helix VI and helix III-helix VII interaction patterns. The local perturbations introduced by these amino acid replacements culminate in the opening of a solvent-accessible site in the cytosolic domains potentially involved in Gs recognition.

To provide further insight into the structural features of the L457 mutants, a new hLHR model has been built (F. Fanelli, manuscript in preparation) by comparative modeling using the recently determined crystal structure of rhodopsin (37). Preliminary computer simulations on the wild-type and the L457 mutants with this homology model agree with the *ab initio* model in that positively charged amino acids in position L457 are predicted to induce perturbations in helix III-helix VI and helix III-helix VII interactions. As shown in Fig. 5, these rearrangements result from the formation of new interactions between the replacement amino acid at III.18, on the one hand, and D578 (VI:16), N615 (VII:13), and N619 (VII:17), on the other. Similarly to the *ab initio* model, the homology model predicts that the local perturbations induced by these activating mutations enhance the solvent exposure of the cytosolic extensions of helices III and VI.

It is notable that, thus far, five GPCRs, the hLHR, hFSHR (30), human β_2-adrenergic receptor (hβ_2-AR) (30), rat m_1 muscarinic acetylcholine receptor (rm_1AchR) (38), and the C5a receptor (39), have been shown to be rendered constitutively active upon substitution of Leu III.18, suggesting that this residue may play a conserved role in stabilizing the inactive state of GPCRs. Notably, there is variability among these five GPCRs with respect to which amino acid replacements of Leu III.18 cause constitutive activation. The rm_1AchR and C5a receptor Leu III.18 substitutions were made in the context of alanine scanning mutagenesis where alanine substitutions were found to cause constitutive activation. For the hFSHR, hLHR, and hβ_2-AR, substitutions of Leu III.18 with arginine, lysine, histidine, alanine, and aspartate were examined. In terms of the cAMP pathway, constitutive activation of the hLHR was observed with arginine, lysine, or histidine substitutions (Table 1), but constitutive activation of the hFSH was observed only with an arginine substitution (30). Constitutive activation of the hβ_2-AR was unrelated to the insertion of positive residues since arginine, lysine, or alanine caused activation (in this case the histidine replacement was not examined and an aspartate substitution caused lack of cell surface expression) (30). The mutagenesis studies of Leu III.18 in these five GPCRs further complement other studies suggesting that helix III may serve as a critical component of the switch for GPCR activation (1, 39–43).

The wild-type LHR can activate both the cAMP and the inositol phosphate second messenger pathways (18, 44). Our studies show that substitution of L457 with arginine renders the hLHR unable to stimulate either the cAMP or the inositol phosphate pathway. However, the other hLHR(L457) substitutions examined herein showed no correlation between an attenuation of hCG-stimulated cAMP and hCG-stimulated inositol phosphate production. Notably, certain substitutions of L457 were found to confer selectivity with respect to the second messenger pathway stimulated by hCG occupancy. For example, in response to hCG, a histidine substitution of L457 causes activation of the inositol phosphate pathway, but not the cAMP pathway. In contrast, an aspartate substitution of L457 causes hCG-stimulated activation of the cAMP pathway, but not the inositol phosphate pathway. Previous

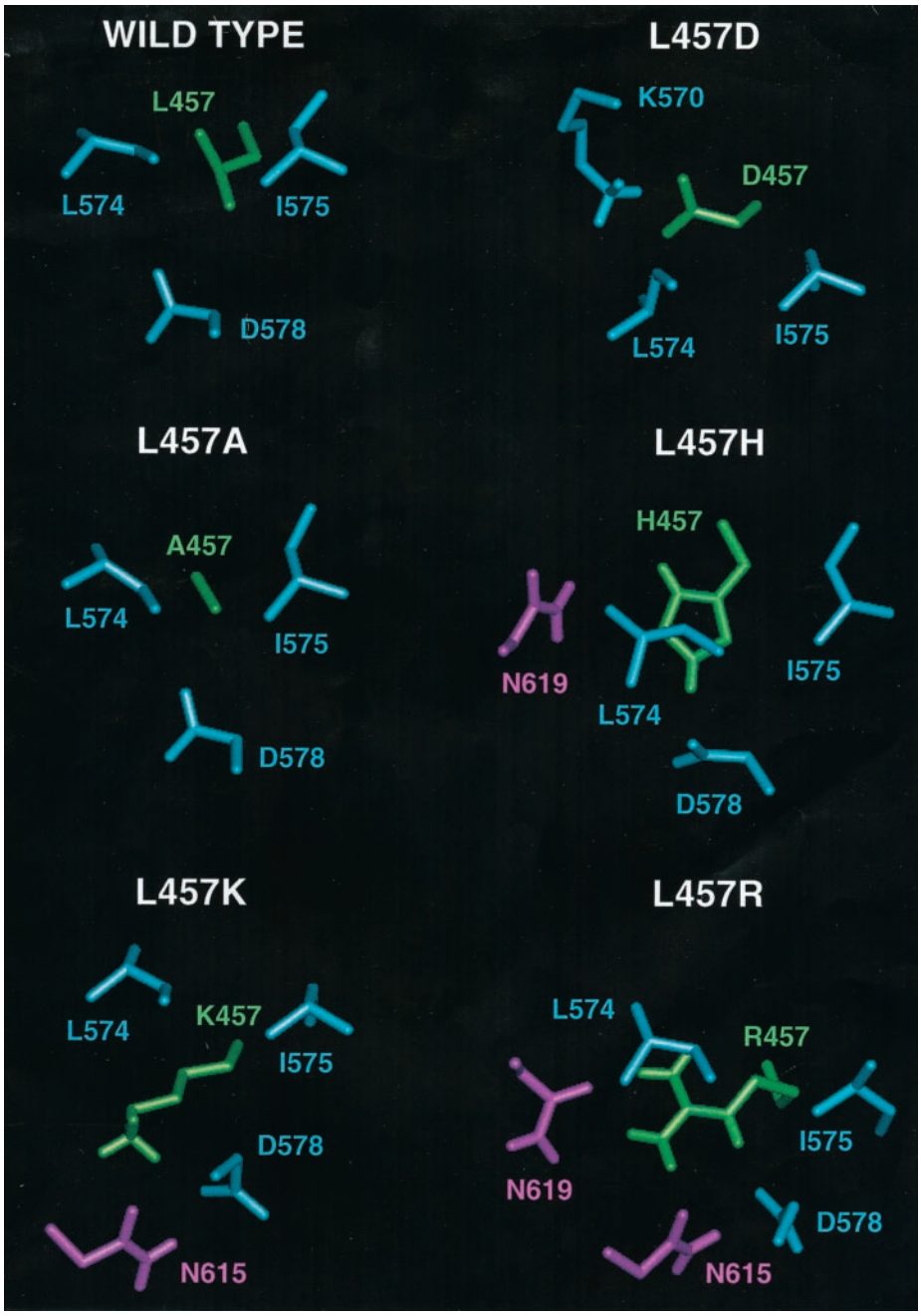

Fig. 5. Structural Environment of the Mutated Position 457 (III.18) in the Homology hLHR Model

Details of the amino acids that constitute the environment of position 457 are shown. The receptor is seen in a direction parallel to the membrane surface. Residues in helices III, VI, and VII are colored in *green, sky blue,* and *violet*, respectively.

studies by Ji and colleagues (45) showed that certain substitutions of K583 in the third extracellular loop of the rat LHR (rLHR) caused the receptor to respond to hCG with increased inositol phosphate production, but not cAMP production, suggesting a divergence in the signaling by the LHR to these two pathways. The present studies now show that not only can certain substitutions of the LHR cause selective agonist-induced activation of the inositol phosphate pathway, but also others can confer selective agonist-induced activation of the cAMP pathway. The ability of substitutions of L457 of the hLHR to abrogate the agonist stimulation of one or both second messenger pathways suggests a key role for this residue in mediating the hormonal activation of the hLHR.

The mutants in which a positively charged R, K, or H was inserted at position 457 were both constitutively active and unresponsive to further hormonal stimulation of cAMP production. It should be noted that although these mutants caused basal levels of cAMP to

be elevated, they were not elevated to the same magnitude as cells expressing the wild-type hLHR incubated with a saturating concentration of hormone. Therefore, the lack of hormonal responsiveness is not simply a consequence of the mutants causing maximal elevation of cAMP under basal conditions. Indeed, these observations suggest that the active states of constitutively active hLHR mutants are probably intermediate between the inactive state and the fully activated state stabilized by hormone. Further studies to explore the potential causes of the lack of hCG-stimulated cAMP production by these mutants revealed several interesting features. First, the lack of hCG responsiveness is not due to a decreased binding affinity of the mutants for hCG since the binding affinities of all were normal. Second, it was observed that cells expressing the R, K, and H-substituted L457 mutants all internalized hCG at a significantly higher rate than the wild-type hLHR. Since the internalized hormone-occupied LHR no longer stimulates cAMP production (46), the increased rate of internalization by these mutants could conceivably account for their decreased hCG responsiveness if internalization was the primary means of terminating the signaling to Gs. Indeed, when the rat LHR (rLHR) containing a mutation of Leu III.18 to arginine was examined, it was found to constitutively elevate basal cAMP production, to be unresponsive to hCG with regard to further cAMP increases, and to internalize hCG at a faster rate than the wild-type rLHR (47). When isolated membranes (which, by definition, are devoid of any internalization activity) derived from cells expressing either the wild-type or the mutant rLHR were examined for basal and hCG-stimulated cAMP production, it was found that the membranes with mutant rLHR had an elevated basal adenylyl cyclase activity (consistent with the mutant's constitutive activity), but responded similarly to wild-type rLHR membranes when incubated with hCG (47). These observations supported the hypothesis that the increased internalization of hCG by the rLHR mutant was responsible for the decreased ability of intact cells to respond to further hormonal stimulation of the cAMP pathway.

We, therefore, also measured the basal and hCG-stimulated adenylyl cyclase activity of membranes isolated from cells expressing the wild-type hLHR or the mutants L457R, L457K, or L457H. Although all three of these mutants, when analyzed in the context of intact cells, exhibited constitutively elevated levels of basal cAMP, only L457R showed a significant increase in basal cyclase activity above that of wild-type hLHR membranes. It is likely, however, that the inability to detect a measurable increase in the basal cyclase activity of the L457K and L457H membranes is due to the reduced sensitivity of the measurement of adenylyl cyclase activity in membranes as compared with cAMP accumulation in intact cells. Since the constitutive activity (as measured by cAMP accumulation) of the L457R mutant is greater than that of the L467K and L457H mutants, its increased basal activity, as determined in cyclase assays, may be more readily detectable. With regard to agonist-stimulated cyclase activity, the L457K and L457H membranes responded to hCG with increased adenylyl cyclase activity, suggesting that the inability of these mutants to respond in the context of intact cells is due to their more rapid internalization of hCG and/or to other properties of the receptors that are present in intact cells but not in membranes. However, the L457R membranes remained completely unresponsive to hCG stimulation. These data contrast with the observations reported on the rLHR containing the comparable substitution and suggest a different mechanism underlying the lack of hCG-stimulated cAMP production by cells expressing the human *vs.* rat LHR in which Leu III.18 is substituted by arginine.[1] The lack of hCG responsiveness of membranes containing hLHR(L457R) suggests that there is a modification of this mutant that makes it unable to further activate Gs when occupied by hCG. This change may reflect a structural alteration or posttranslational modification of the receptor and/or an association of the mutant receptor with other proteins. Whatever it may be, it must be preserved during the preparation of membranes from intact cells, and it prevents the receptor from undergoing the transition from one of constitutively active in an intermediate state to one of full activation stabilized by hormone.

It is intriguing to note that certain substitutions of Leu III.18 of the hFSHR also cause this receptor to exhibit little or no response to FSH with regard to further increases in cAMP (30). The demonstration that certain substitutions of Leu III.18 of either the hLHR or hFSHR cause decreases in hormone-stimulated activation of the cAMP pathway and/or inositol phosphate pathway suggests that Leu III.18 plays a key role in the transduction of hormone binding to the activation of G proteins in these two related GPCRs. Because substitutions of Leu III.18 of the hβ_2-AR receptor do not cause decreased hormone responsiveness (30), it suggests that the role of Leu III.18 in hormone-stimulated signal transduction may be restricted to the gonadotropin receptors, or possibly the glycoprotein hormone receptors if it is found to play a similar role in the closely related TSHR.

MATERIALS AND METHODS

Hormones and Supplies

Highly purified hCG was generously provided by Dr. A. Parlow and NIDDK's National Hormone and Pituitary Program. hCG was iodinated as described previously (48). The crude

[1] We have replicated the previously reported results showing that membranes expressing the rLHR containing a Leu III.18 to arginine substitution respond with increased adenylyl cyclase activity using the same conditions as those used to assay hLHR(L457R). Therefore, we conclude that the differences observed are indeed due to differences between the two species of receptor and not to methodological differences.

preparation hCG used for determining nonspecific binding in ^{125}I-hCG binding assays was obtained from Sigma (St. Louis, MO). ^{125}I-cAMP and cell culture media were obtained from the Iodination Core and the Media and Cell Production Core, respectively, of the Diabetes and Endocrinology Research Center of the University of Iowa. Tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD) and Corning, Inc. plasticwares were obtained from Fisher Scientific (Pittsburgh, PA).

Plasmids and Cells

The wild-type hLHR cDNA was kindly provided by Ares Advanced Technology (Ares-Serono Group, Randolph, MA) and was subcloned into pcDNA 3.1 (Invitrogen, San Diego, CA). Mutagenesis was performed using the PCR overlap extension method (49, 50). The entire region amplified by PCR, as well as the sites of ligation, were sequenced to ensure that there were no unintended mutations of the amplified cDNA. DNA sequencing was performed either within our laboratory or by automated sequencing within the DNA Core of the Diabetes and Endocrinology Research Center of the University of Iowa.

Human embryonic 293 cells were obtained from the American Type Tissue Collection (CRL 1573) and were maintained at 5% CO_2 in growth media consisting of high-glucose DMEM containing 50 μg gentamicin, 10 mM HEPES, and 10% newborn calf serum. For most experiments, cells were plated onto 35-mm wells that had been precoated for 1 h with 0.1% gelatin in calcium and magnesium-free PBS, pH 7.4. Cells were transiently transfected when they were 50–70% confluent following the protocol of Chen and Okayama (51) except that the overnight precipitation was performed in a 5% CO_2 atmosphere. Differing concentrations of plasmids were used to achieve the same cell surface expression of a given mutant with the wild-type hLHR. Cells were then washed with Waymouth's MB752/1 media modified to contain 50 μg gentamicin and 1 mg/ml BSA, after which fresh growth media were added. The cells were used for experiments 24 h later.

Standardization of Cell Surface Receptor Numbers

In all experiments where the signaling properties of cells expressing the hLHR(wt) were compared with cells expressing a mutant, 293 cells were transiently transfected with varying plasmid concentrations such that within each experiment the number of cell surface receptors for a given mutant was matched to cells expressing comparable numbers of wild-type receptors. For each experiment the ratio of ^{125}I-hCG binding to cells expressing a given mutant *vs.* the matched controls was determined, and the experiment was used only if the ratio was within the range of 0.5–2.0 (*i.e.* within a 2-fold difference). For the experiments measuring basal and hormone-stimulated cAMP production (Table 1 and Fig. 1) the means $\pm$ SEM of the ratios of cell surface ^{125}I-hCG binding to mutant *vs.* wild-type hLHR-expressing cells were the following: 0.98 $\pm$ 0.16 for L457R (n = 8); 1.30 $\pm$ 0.18 for L457K (n = 7); 0.92 $\pm$ 0.06 for L457H (n = 5); 1.19 $\pm$ 0.15 for L457A (n = 7); and 1.17 $\pm$ 0.15 for L457D (n = 9). For the experiments measuring basal (Table 2) and hCG-stimulated inositol phosphate production (Fig. 4) the means $\pm$ SEM of the ratios of cell surface ^{125}I-hCG binding to mutant *vs.* wild-type hLHR-expressing cells from three experiments were the following: 0.81 $\pm$ 0.13 for L457R; 0.97 $\pm$ 0.30 for L457K; 0.1.18 $\pm$ 0.10 for L457H; 1.09 $\pm$ 0.19 for L457A; and 1.19 $\pm$ 0.24 for L457D. For the experiments measuring basal C kinase-responsive luciferase activity (Table 2) the means $\pm$ SEM of the ratios of cell surface ^{125}I-hCG binding to mutant *vs.* wild-type hLHR-expressing cells were the following: 1.13 $\pm$ 0.11 for L457R (n = 9); 1.17 $\pm$ 0.12 for L457K (n = 11); 1.09 $\pm$ 0.12 for L457H (n = 11); 0.81 $\pm$ 0.08 for L457A (n = 13); and 0.96 $\pm$ 0.014 for L457D (n = 6). Therefore, the mutants were well matched to the controls and what little deviation existed varied equally between mutants being expressed $\leq$2-fold higher *vs.* $\leq$2-fold lower density than the wild-type hLHR.

Binding Assays to Intact Cells Expressing the hLHR

HEK 293 cells were plated onto gelatin-coated 35-mm wells and transiently transfected as described above. On the day of the experiment cells were placed on ice for 15 min and washed two times with cold Waymouth's MB752/1 containing 50 μg/ml gentamicin and 1 mg/ml BSA but no sodium bicarbonate. To determine the maximal binding capacity, the cells were then incubated overnight at 4 C in the same media containing a saturating concentration of ^{125}I-hCG (500 ng/ml) with or without an excess of unlabeled crude hCG (50 IU/ml). To determine the binding affinity, the cells were incubated overnight at 4 C with increasing concentrations of ^{125}I-hCG in the presence or absence of unlabeled hCG. To terminate the assay, the cells were placed on ice. The contents of each well were scraped into a plastic tube on ice and combined with a 1 ml wash using cold HBSS modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA. The tubes were centrifuged at 4 C and the pellets resuspended in 2 ml of the same wash media. After a second centrifugation, the supernatants were aspirated and the pellets counted in a γ counter. Apparent binding affinities were determined as the concentrations of ^{125}I-hCG yielding half-maximal binding as calculated by the DeltaGraph software Deltapoint (Monterey, CA) when the data were fit to a sigmoidal equation (52).

Measurement of cAMP or Inositol Phosphate Production

The levels of cell surface receptors were measured within the same experiment, and only those experiments in which the numbers of cell surface receptors for wild-type *vs.* mutant receptors differed by no more than 2-fold (see above) were used for second messenger analyses. HEK 293 cells were plated on gelatin-coated 35-mm wells and transfected as described above.

For cAMP determinations, cells were washed on the day of the experiment twice with warm Waymouth MB752/1 media containing 50 μg/ml gentamicin and 1 mg/ml BSA and placed in 1 ml of the same medium containing 0.5 mM isobutylmethylxanthine. After 15 min at 37 C, a saturating concentration of hCG (100 ng/ml final concentration) or buffer only was added, and the incubation was continued for 60 min at 37 C. The cells were then placed on ice, the media were aspirated, and intracellular cAMP was extracted by the addition of 0.5 N perchloric acid containing 180 μg/ml theophylline and then measured by RIA. All determinations were performed in triplicate.

For inositol phosphate determinations, after the transfection, the cells were washed with inositol-free DMEM containing 1% newborn calf serum, 20 mM HEPES, and 50 μg/ml gentamicin and placed in 1 ml of the same media supplemented with 2 μCi/ml of [2-^{3}H]myo-inositol for 24 h. On the day of the experiment, the cells were washed twice with warm Waymouth MB752/1 media modified to contain 50 μg/ml gentamicin, 1 mg/ml BSA, and 20 mM LiCl and then placed in this medium. After 15 min at 37 C, a saturating concentration of hCG (100 ng/ml final concentration) or buffer only was added, and the incubation was continued for 60 min at 37 C. To terminate the assay, the cells were placed on ice, and cold 0.5 N perchloric acid was added to each well. The inositol phosphates were extracted and assayed as described previously (53). All determinations were performed in triplicate.

Measurement of C Kinase Activation Using a C Kinase-Responsive Reporter Gene Assay

Plasmid containing a C kinase-responsive AP-1-fos-Luc reporter gene construct was a gift from Dr. Marvin Gershengorn (NIDDK/NIH). HEK 293 cells were plated onto gelatin-coated 35-mm wells and transiently cotransfected as described above with the C kinase-responsive reporter gene construct, pcDNA3.1/neo containing the cDNA encoding the hLHR(wt) or L457-substituted mutant, and empty pcDNA3.1/neo. The concentrations of plasmid encoding the wild-type and mutant receptors were varied so that a given mutant could be matched with a parallel set of cells expressing the same level of wild-type receptor (see above). Because luficerase activity was observed to be affected by the concentration of pcDNA3.1/neo in the 293 cells, empty vector was utilized to adjust the total concentration of pcDNA3.1/neo to be equal in all the cells.

Measurement of Adenylyl Cyclase Activity

The levels of cell surface receptors were measured within the same experiment, and only those experiments in which the numbers of cell surface receptors for wild-type *vs.* mutant receptors differed by no more than 2-fold (see above) were used to determine adenylyl cyclase activity. Membranes were prepared from 293 cells as described previously (23). Adenylyl cyclase assays, based on the procedure of Salomon (54), were performed as described previously (23) except that GTP was not added to the assay. This change was incorporated into the protocol because we observed a greater fold stimulation of adenylyl cyclase activity in response to hCG when the exogenous GTP was omitted.

Internalization of Receptor-Bound hCG

The hLHR-mediated internalization of hCG was measured following the protocol described by Ascoli and colleagues (55). Transiently transfected cells in 35-mm wells were preincubated in 1 ml Waymouth MB752/1 media containing 1 mg/ml BSA and 20 mM HEPES, pH 7.4, for 30 min at 37C. ^{125}I-hCG was then added to give a final concentration of 40 ng/ml (with or without an excess of unlabeled hCG to correct for nonspecific binding), and the cells were incubated for 9 min at 37 C. The cells were then washed twice with cold HBSS modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA. The surface-bound ^{125}I-hCG was released by incubating the cells on ice in 1 ml of cold 50 mM glycine, 150 mM NaCl, pH 3, for 4 min and rinsing them with 1 ml of the acidic buffer (56). The acid washes from each well were combined and counted to determine the amount of surface-bound ^{125}I-hCG. Each well of acid-treated cells was then solubilized in 0.5 N NaOH and counted to determine the amount of internalized radioactivity. The results of these experiments are expressed as an internalization index, which is defined as the ratio of internalized *vs.* surface-bound ^{125}I-hCG (57). Under the experimental conditions used herein, the internalization index accurately reflects the rate of internalization (55, 57). The rate of internalization is a first order rate constant (55, 57) and is, therefore, independent of the concentration of receptor or hormone. Therefore, for these experiments, no effort was made to standardize the number of cell surface receptors between the wild-type *vs.* mutant-expressing cells. In addition, a subsaturating concentration of hormone was used to conserve ^{125}I-hCG.

Molecular Modeling of the hLHR Mutants of L457

The initial structures of the L457 hLHR mutants were obtained by replacing the target amino acid in the wild-type hLHR input structure previously built after an *ab initio* approach (33). Energy minimization and molecular dynamics simulations of the mutants were performed using the program CHARMm (Molecular Simulations, Inc, Waltham, MA) following the computational protocol previously described (33).

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