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Desensitization of Gs-Coupled Receptor Signaling by Constitutively Active Mutants of the Human Lutropin/Choriogonadotropin Receptor

HIROMITSU SHINOZAKI, VIKTOR BUTNEV, YA-XIONG TAO, KOK LONG ANG, MARCO CONTI, AND DEBORAH L. SEGALOFF

Department of Physiology and Biophysics (H.S., V.B., Y.-X.T., D.L.S.), The University of Iowa, Iowa City, Iowa 52242; and Department of Gynecology and Obstetrics (K.L.A., M.C.), Stanford University, Stanford, California 94305

Activating mutations of the human lutropin/choriogonadotropin receptor (hLHR), a Gs-coupled receptor, have been identified in young boys with gonadotropin-independent precocious puberty (testotoxicosis). The properties of these mutants have typically been characterized in heterologous cells transfected with recombinant mutant receptor and compared with those expressing wild-type (wt) receptor. The affected individuals, however, are heterozygous and, therefore, express wt receptor in addition to the mutant receptor. The present studies were undertaken to determine what effects, if any, coexpression of a constitutively active hLHR might have on hLHR(wt). HEK 293 cells were cotransfected with hLHR(wt) and hLHR(L457R), a mutant that we have previously shown to be both constitutively active and unresponsive to further hormonal stimulation as determined in both intact cells and isolated membranes. When coexpressed at submaximal concentrations, L457R does not decrease the cell surface expression of hLHR(wt). Coexpression of L457R, however, causes an attenuation of human choriogonadotropin-stimulated cAMP production by hLHR(wt). We show that this attenuation is caused by an activation of the phosphodiesterase (PDE)4D3. Additional experiments demonstrate that the coexpression of L457R with the human β_2-adrenergic receptor causes an attenuation of isoproterenol-stimulated cAMP and that other activating mutations of the hLHR also induce PDE activation. Taken together, these data demonstrate that the activation of PDE is a compensatory mechanism common to hLHR constitutively active mutants and that cellular responses to agonists that stimulate Gs-coupled receptors may be blunted in tissues expressing these activating mutants. This novel desensitizing effect of constitutively active hLHRs on hormone-stimulated cAMP production has not been noticed before and would typically not be detected because of the routine inclusion of PDE inhibitors in experiments determining cAMP accumulation. Importantly, however, this mechanism of desensitization would be expected to occur in a physiological context in which PDE inhibitors are not present and thus may influence hormonal signaling in cells expressing the activating hLHR mutant. (*J Clin Endocrinol Metab* 88: 1194–1204, 2003)

THE HUMAN LUTROPIN/CHORIOGONADOTROPIN receptor (hLHR) belongs to the family of rhodopsin-like G protein-coupled receptors (GPCRs). The hLHR is expressed primarily in the gonads, where it plays a pivotal role in both male and female reproductive physiology. On a cellular level, the binding of either pituitary LH or placental choriogonadotropin (CG) to the hLHR on gonadal target cells causes the stimulation of Gs, which in turn activates adenylyl cyclase, resulting in increased intracellular concentrations of cAMP. In heterologous cells expressing recombinant hLHR, LH and CG can readily be shown to stimulate cAMP production. Under certain circumstances (*i.e.* high receptor density and elevated concentrations of hormone), phospholipase C activity is increased as well (1–5). Although the roles of cAMP in the physiology of LH and CG actions are well established, it is not yet clear what physiological roles, if any, the latter pathway has in the actions of LH and CG.

In recent years, several naturally occurring constitutively active mutations of the hLHR have been identified in young boys with gonadotropin-independent precocious puberty, also called testotoxicosis (6, 7). The characterization of activating mutants of the hLHR and other GPCRs has typically been performed by analyzing cells expressing only the mutant receptor and comparing them to cells expressing only the wild-type (wt) receptor. Given the heterozygous expression of activating hLHR mutations in boys with testotoxicosis, we thought it important to ascertain whether the presence of an activating hLHR affects the expression or activity of hLHR(wt) when coexpressed. Toward this end, the present studies were undertaken to examine the coexpression of hLHR(wt) with a constitutively active hLHR mutant in transfected cells. For the mutant receptor, we chose to use hLHR(L457R), a naturally occurring activating mutation of the hLHR that has unique characteristics that would aid in the interpretation of the coexpression studies (8, 9). This mutant, in which a highly conserved leucine in transmembrane domain 3 is substituted with arginine, displays relatively strong constitutive activity compared with the wt receptor. However, cells expressing L457R do not respond to further hCG stimulation even though the constitutively elevated basal concentrations of cAMP are not maximal. On the basis of experiments using membranes derived from L457R-expressing cells, we postulate that the lack of hormone responsiveness of L457R is due to an inherent structural alteration of this mutant that impedes the translation of hormone binding into the activation of Gs. Thus, the basal

Abbreviations: CG, Choriogonadotropin; GPCR, G protein-coupled receptor; h, human; hβ_2-AR, hβ_2-adrenergic receptor; LHR, lutropin/CG receptor; PDE, phosphodiesterase; wt, wild-type.

levels of cyclase activity in membranes from cells expressing L457R are elevated compared with membranes from cells expressing hLHR(wt), consistent with the constitutive activation of Gs by L457R. However, hCG stimulation of L457R membranes does not cause a greater activation of cyclase, despite the fact that the constitutively high basal cyclase activity is not as high as that in hCG-stimulated membranes from hLHR(wt) cells (9). One can take advantage of these properties of hLHR(L457R) to determine the effects of the constitutive activity of L457R on the hormone-stimulation of hLHR(wt) when the two receptors are coexpressed because the hormone-stimulated cAMP in the cotransfected cells would, therefore, reflect only the hormone stimulation of the wt receptor.

The results presented show that the presence of hLHR(L457R) attenuates the ability of hLHR(wt) to respond to hCG with increased cAMP production and that this effect is the result of the constitutive stimulation of phosphodiesterase (PDE) activity, specifically PDE4D3, by L457R. It is further shown that the coexpression of hLHR(L457R) with the hβ_2-adrenergic receptor (hβ_2-AR) also attenuates isoproterenol-stimulated cAMP production. We propose, therefore, that a cAMP-dependent increase in PDE activity by mutant receptors that constitutively activate Gs serves to tightly regulate intracellular cAMP levels. This compensatory increase in PDE activity in target cells attenuates the hormone-stimulated cAMP production by Gs-coupled receptors expressed in those cells.

Materials and Methods

Hormones and supplies

Highly purified hCG was purchased from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health and Dr. A. F. Parlow. hCG was iodinated following the procedure described for the iodination of hCG (10). ^{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. (Grand Island, NY), and Corning plasticwares were obtained from Fisher Scientific (Pittsburgh, PA).

Agarose-conjugated anti-FLAG M2 antibody was purchased from Sigma (St. Louis, MO), and 9E10 anti-myc monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PDE4 (anti-4D monoclonal antibody) was a generous gift from Pfizer, Inc., Central Research Division (Groton, CT).

Plasmids and cells

The cDNA encoding the hLHR was a generous gift of Ares Advanced Technology (Ares-Serono Group, Randolph, MA). This and all other constructs used were subcloned into pcDNA3.1/neo (Invitrogen, San Diego, CA). FLAG-hLHR(wt) and *myc*-hLHR(L457R) were designed by placing the epitope tags between the signal peptide and amino terminus of the mature protein using standard PCR strategies (11, 12). Preliminary experiments showed that the insertion of the epitope tags at the amino terminus of the hLHR had no effect on cell surface expression, hormone binding, or hormone-stimulated second messenger production. The cDNA encoding the hβ_2-AR and HEK 293 cells were obtained from the American Type Tissue Collection (Rockville, MD). The hβ_2-AR in pcDNA3.1/neo has been previously described (13).

Cell culture and transfections

The 293 cells were maintained at 5% CO_2 in a culture medium consisting of DMEM containing 50 μg/ml gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells were transfected at a 60–80% confluence using the transient transfection procedure of Chen and Okayama (14), except that the overnight precipitation was performed in a 5% rather than 2.5% CO_2 atmosphere. After 18–20 h, the cells were washed with Waymouth's MB752/1 media modified with 50 μg/ml gentamicin and 1 mg/ml BSA, and then fresh growth medium was added. The cells were used for experiments 24 h later.

For experiments addressing the effects of coexpression of receptors, we first transfected cells with a given construct at various plasmid concentrations and measured hormone binding to intact cells to determine cell surface receptor levels. Plasmid concentrations for the two receptors were then chosen such that, when transfected individually, equal but submaximal cell surface levels of each receptor were expressed. Cells were then transfected with either of the two receptors individually or with both together using the predetermined plasmid concentrations. The total amount of plasmid used in the transfections was held constant with the use of empty vector.

^{125}I-hCG binding to intact cells

The levels of cell surface hLHR were determined by ^{125}I-hCG binding assays to intact cells. The 293 cells were plated onto gelatin-coated 35-mm wells and transiently transfected as described above. On the day of the experiment, cells were washed two times with warm Waymouth's MB752/1 media modified to omit the sodium bicarbonate and to contain 50 μg/ml gentamicin and 1 mg/ml BSA. The cells were then incubated 1 h at room temperature in the same media containing a saturating concentration of ^{125}I-hCG (final concentration, 500 ng/ml) with or without an excess of unlabeled hCG (final concentration, 50 IU/ml). The assay was finished by washing the cells three times with cold Hanks' Balanced Salt Solution modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA. The cells were then solubilized in 100 μl of 0.5 N NaOH, transferred to plastic test tubes with cotton swabs, and counted in a γ-counter.

Measurement of cAMP production

The 293 cells were plated on gelatin-coated 35-mm wells and transfected as described above. On the day of the experiment, cells were washed twice with warm Waymouth's MB752/1 media modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA and placed in 1 ml of the same medium to which buffer, hCG (final concentration, 500 ng/ml), or isoproterenol (final concentration, 1 μM) was added. The incubation was then continued for 120 min at 37 C. Unless otherwise noted, the incubations did not contain any PDE inhibitors. Where indicated, isomethylbutylxanthine (0.5 mM) or rolipram (20 μM) was included. The cells were then placed on ice, the media was 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.

Measurement of internalization of hCG

Internalization of hCG by the LHR was measured using the protocol described by Ascoli and colleagues (15). Transiently transfected cells in 35-mm wells were preincubated in 1ml warm Waymouth's MB752/1 media modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA for 30 min at 37 C. ^{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 rate of internalization is a first-order rate constant (15, 16) and is, therefore, independent of the concentration of receptor or hormone. Therefore, a subsaturating concentration of hormone was used to conserve ^{125}I-hCG.] The cells were then washed twice with cold Hanks' Balanced Salt Solution 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 (17). 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 (16). Under the experimental conditions used herein, the internalization index accurately reflects the rate of internalization (15, 16).

Determination of PDE activity

Transfected 293 cells in 35-mm dishes were washed two times with warm Waymouth's MB752/1 media modified to contain 50 μg/ml gentamicin and 1 mg/ml BSA and incubated in this media for 15 min at 37 C with no additions, hCG (final concentration, 100 ng/ml), or forskolin (final concentration, 90 μM). The cells were placed on ice; from this point on, all procedures were carried out at 4 C, and all buffers contained Complete EDTA-free Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN) as well as the phosphatase inhibitors 50 mM sodium fluoride and 1 mg/ml mycrocystin. Cells were washed twice with cold PBS (pH 8.0) and collected in the same. The cells were recovered by centrifugation and resuspended in homogenization buffer [20 mM Tris HCl (pH 8.0), 1 mM EDTA, 0.2 mM EGTA, and 10 mM sodium pyrophosphate] at 2–4 $\times$ 10^5 cells/ml. Cells were homogenized by forcing them through a 25-gauge needle 10 times, and the homogenate was clarified by centrifugation. PDE activity was measured in the homogenates by determining the rate of degradation of [^{3}H]cAMP as described by Conti and colleagues (18). Where indicated, rolipram (final concentration, 20 μM) was added to the reaction mixture.

Western blots to determine activation of PDE subtypes

The 293 cells were transfected under conditions that yielded comparable low or high levels of cell surface expression of hLHR(wt) and hLHR(L457R). The cells were then washed and lysed as described above for the determination of PDE activity. In this case, however, the cell extracts were diluted 4-fold into a 4$\times$ stock of SDS sample buffer containing reducing agents, boiled for 5 min, and resolved by SDS-PAGE. The samples were electrophoretically transferred to a polyvinylidene difluoride membrane and probed with antibodies specific to PDE4A, PDE4B, and PDE4D. The results using the PDE4D-specific monoclonal antibody are shown because only these gave positive results.

Cell surface biotinylation and Western blotting to examine the coexpression of cell surface receptors

HEK 293 cells were transfected as described above with FLAG-hLHR(wt) alone, *myc*-hLHR(wt) alone, or the two constructs together such that the cell surface expression (as determined by binding assays to intact cells) of each receptor alone was submaximal and equal to the other. The cell surface proteins were biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate (Vector Laboratories, Inc., Burlingame, CA) exactly as described by Min and Ascoli (19). The cells were then lysed by incubating them for 30 min on ice in lysis buffer [0.15 M NaCl, 20 mM HEPES, 5 mM EDTA (pH 7.4) containing 1% Nonidet P-40, 4 mg/ml dodecyl-β-D-maltoside, 0.8 mg/ml cholesterol hemisuccinate]. From this step on, all procedures were carried out at 4 C, and all solutions contained Complete EDTA-free Protease Inhibitor Cocktail (Roche Molecular Biochemicals). The extracts were clarified by centrifugation, and equal amounts of protein from the different cell lysates were then used for partial purification of the hLHR by chromatography on wheat germ agglutinin-agarose as described previously (20). The eluted fraction was then subjected to immunoprecipitation using either agarose-conjugated anti-FLAG M2-agarose affinity gel or 9E10 monoclonal anti-*myc* conjugated to protein G. After a 3-h incubation with antibody at 4 C, the resins were extensively washed and then boiled for 3 min in SDS sample buffer to elute the absorbed proteins. Equal volumes of the immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions, transferred to polyvinylidene difluoride membranes, and then probed by Western blotting with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Inc.) and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analyses

Data were analyzed statistically using the program InStat by GraphPad Software, Inc. (San Diego, CA).

Results

To examine the potential effects of the activating hLHR mutant L457R on hLHR(wt), 293 cells were transiently cotransfected with cDNAs encoding hLHR(wt) alone, hLHR(L457R) alone, or the two receptors together. One way by which we monitored the cell surface expression of the receptors was by ^{125}I-hCG binding assays to intact cells. We reasoned that if both receptors were expressed in the cotransfected cells, we would observe the binding capacity of the cotransfected cells to be the sum of that of the cells transfected individually. When cells were transfected with maximal concentrations of each receptor plasmid alone, the cells bound 15–25 ng ^{125}I-hCG per 10^6 cells, and the binding capacity of the cotransfected cells was no greater than that of the cells transfected with each receptor separately. Therefore, we deliberately chose concentrations of each plasmid that, when expressed alone, would yield submaximal (in the range of 2–5 ng ^{125}I-hCG bound per 10^6 cells) and equal levels of cell surface receptor. Under these conditions, the binding capacity of the cells coexpressing both receptors was indeed the sum of that observed in cells expressing hLHR(wt) or hLHR(L457R) alone (Fig. 1). To determine the effects of hLHR(L457R) on the cell surface expression of hLHR(wt), 293 cells were transfected with submaximal concentrations of plasmids encoding FLAG-tagged hLHR(wt) alone, *myc*-tagged hLHR(L457R) alone, or the two together. In preliminary experiments, it was determined that epitope tags placed at the N terminus of the mature hLHR do not affect cell surface expression, hormone binding, or signal transduction properties of the receptor (data not shown). The transfected cells were biotinylated to label cell surface proteins, lysates were immunoprecipitated with either anti-FLAG antibody or anti-*myc* antibody, and the immunoprecipitates were visualized on Western blots probed with streptavidin. Using this protocol, the receptor visualized on Western blots corresponds to cell surface receptor. As shown in Fig. 1, the levels of cell surface L457R were unaffected by the coexpression of hLHR(wt) and, similarly, the levels of cell surface hLHR(wt) were unaffected by the coexpression of L457R. It can be concluded, therefore, that when hLHR(L457R) and hLHR(wt) are coexpressed at submaximal levels, both are expressed at the cell surface and the levels of each are unaffected by the expression of the other receptor.

We then sought to determine whether the activity of hLHR(wt) was affected by the coexpression of hLHR(L457R). Toward this end, we cotransfected cells with a submaximal concentration of hLHR(wt) plus increasing (but submaximal) concentrations of hLHR(L457R) and then examined basal and hCG-stimulated cAMP production. Importantly, the cAMP assay was performed in the absence of any inhibitors of PDE activity to better mimic the normal physiological context. As shown in Fig. 2 and as we have shown previously (8, 9), basal levels of cAMP in cells expressing solely hLHR(L457R) are elevated approximately 10-fold compared with the basal levels of cAMP in cells expressing only hLHR(wt). Furthermore, although the basal levels of cAMP in L457R cells are not as great as those of hCG-stimulated hLHR(wt) cells, the L457R cells do not respond to hCG with further cAMP production. In contrast, cells expressing only

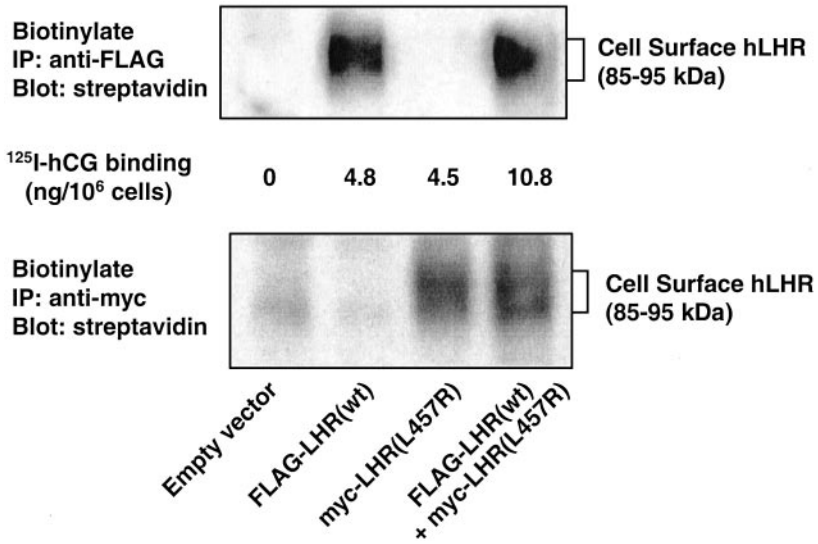


FIG. 1. Both hLHR(wt) and hLHR(L457R) are expressed at the cell surface when coexpressed at submaximal levels. HEK 293 cells were transiently transfected to yield cells expressing submaximal and equal levels of FLAG-hLHR(wt) or *myc*-hLHR(L457R), as determined by hCG binding to intact cells, or the two together. ^{125}I-hCG binding to intact cells expressing the different hLHR constructs is shown. A parallel group of cells was biotinylated to label cell surface proteins, and the extracts were precipitated with anti-FLAG (*top*) or anti-*myc* (*bottom*) antibodies as described in *Materials and Methods*. The immunoprecipitates were resolved by SDS-PAGE and Western blots probed with horseradish-peroxidase-conjugated streptavidin. Only the relevant portions of the gels are shown. Results shown are from one representative experiment.

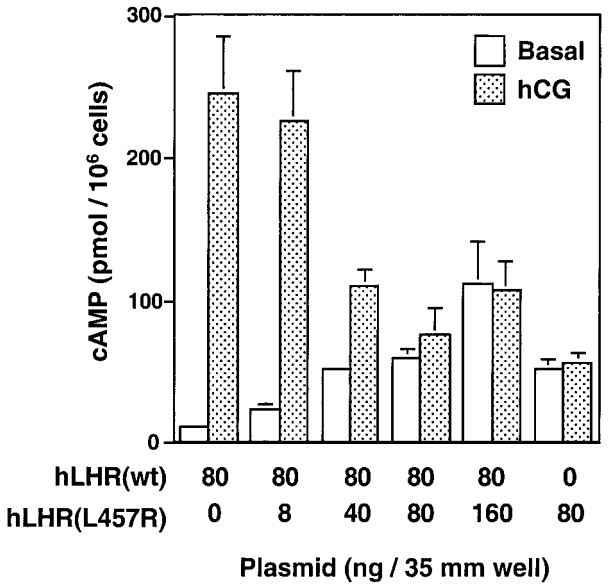


FIG. 2. Attenuation of hCG-stimulated cAMP production by hLHR(wt) caused by coexpression of the constitutively active and hormone unresponsive hLHR(L457R). 293 Cells in 35-mm wells were transiently transfected with the indicated submaximal amounts of plasmid encoding hLHR(wt) or hLHR(L457R). The total amount of plasmid in each group was held constant with empty vector. For comparison purposes, a maximal amount of plasmid per 35-mm well would be 2.4 μg. Cells were incubated 2 h at 37 C in the absence or the presence of a saturating concentration of hCG. The media was aspirated, and intracellular cAMP was extracted and measured by RIA. Results shown are the mean ± SEM of three independent experiments.

hLHR(wt) exhibit a robust hCG-mediated stimulation of cAMP production. As cells are transfected with increasing concentrations of hLHR(L457R) in addition to hLHR(wt), there is an increase in the basal levels of cAMP, consistent with the activating nature of the L457R mutant (Fig. 2). In addition, however, an attenuation of hCG-stimulated cAMP is also observed. Because hLHR(L457R) is constitutively active but unresponsive to hCG (9), the hCG-stimulated cAMP observed in cells coexpressing hLHR(L457R) and hLHR(wt) reflects the stimulation of the wt receptor only. Therefore, the data presented in Fig. 2 suggest that the coexpression of hLHR(L457R), which is constitutively active and intrinsically unresponsive to hCG, attenuates the ability of the wt receptor to respond normally to hCG. This attenuation must be related to the activity of the hLHR(wt) and not its cell surface expression because we have already demonstrated that the coexpression of hLHR(L457R) does not decrease the cell surface expression of hLHR(wt) (Fig. 1).

It has been shown previously that the rates of internalization of hormone-occupied constitutively active mutants of the hLHR are faster than those of the wt receptor (19). Therefore, we considered the possibility that L457R might be driving the internalization of hLHR(wt), thus potentially decreasing its responsiveness to hCG. We examined this possibility by transfecting cells with different ratios (using submaximal concentrations) of hLHR(wt) and hLHR(L457R) and then measuring the internalization index, which, under the experimental conditions used, accurately reflects the rate of internalization (15, 16). As shown in Table 1, the internalization index for cells expressing only hLHR(L457R) is 2.4-fold greater than that for cells expressing only hLHR(wt). Using the internalization indices of cells expressing only hLHR(L457R) or only hLHR(wt), we then calculated the predicted internalization index for cells expressing different ratios of the two receptors. If the internalization of the two receptors were occurring independently of each other, we would predict that the experimental determination of the internalization index would match the predicted internalization index. In contrast, if the L457R mutant were causing the wt receptor to be internalized faster, then we would expect the experimentally derived internalization index for cells expressing both receptors to be greater than the predicted value. The results of these experiments indicate no statistical differences between the predicted and the experimentally derived internalization indices for the cells coexpressing different ratios of hLHR(L457R) and hLHR(wt) (Table 1). These data suggest that the internalization of hCG-occupied hLHR(wt) is not affected by the coexpression of hLHR(L457R).

TABLE 1. The coexpression of hLHR(L457R) with hLHR(wt) does not increase the rate of internalization of hLHR(wt)

% wt/% L457R	Predicted internalization index	Experimental internalization index
100/0		0.160 ± 0.002
33/67	0.235 ± 0.024	0.217 ± 0.014
50/50	0.274 ± 0.024	0.261 ± 0.007
67/33	0.313 ± 0.024	0.337 ± 0.012
0/100		0.388 ± 0.010

HEK 293 cells were transfected with submaximal amounts of hLHR(wt) or hLHR(L457R) cDNAs at the indicated ratios such that the total amount of cDNA was constant. The experimental internalization index was determined as described in *Materials and Methods* and is presented as the mean ± SEM of three experiments. The predicted internalization index was calculated assuming that the wt and the L457R receptors internalized independently of each other and are based, therefore, on the ratios of the two receptors. Analyses of the data utilizing an unpaired *t* test revealed no statistically significant differences between the predicted and experimental internalization indices.

The possibility was also considered that hLHR(L457R), by constitutively elevating intracellular cAMP levels, might be stimulating one or more cAMP-dependent PDEs. To examine this possibility, we reexamined the cAMP production in cells coexpressing hLHR(L457R) and hLHR(wt) in the absence *vs.* the presence of PDE inhibitors. When the experiments were performed in the presence of isomethylbutylxanthine, a relatively nonspecific inhibitor of PDEs, no attenuation of hCG-stimulated cAMP production was observed in the cells coexpressing hLHR(L457R) and hLHR(wt) (data not shown), supporting the hypothesis that this attenuation is mediated by increased degradation of cAMP. Because the cAMP-activated PDE4 family of PDEs are the predominant PDEs expressed in 293 cells, we compared the basal and hCG-stimulated cAMP levels in cells expressing the mutant and wt receptors alone or together in the absence of any PDE inhibitor (Fig. 3, *top*) or in the presence of rolipram, an inhibitor specific for the PDE4 family of PDEs (Fig. 3, *bottom*). Although the inhibition of PDE4 activity by rolipram generally caused the intracellular accumulation of cAMP in the cells to be much higher, the overall properties of cells expressing hLHR(wt) alone or hLHR(L457R) alone are the same regardless of the presence or absence of the PDE inhibitor (Fig. 3, compare *top* and *bottom panels*). Thus, in either case, the basal levels of cAMP in hLHR(wt) cells are relatively low, and they are robustly stimulated by hCG, whereas the basal levels of cAMP in hLHR(L457R) cells are elevated and are not further increased by hCG. An important difference arising from the absence or presence of rolipram is observed, however, in the cells coexpressing hLHR(wt) and hLHR(L457R). In the absence of rolipram, the hCG-stimulated cAMP in cells coexpressing hLHR(wt) and hLHR(L457R) is significantly attenuated compared with the hCG-stimulated cAMP in cells expressing only hLHR(wt) (Fig. 3, *top*). In contrast, in the presence of rolipram, the hCG-stimulated cAMP in cells coexpressing the two receptors is completely normal (Fig. 3, *bottom*). The ability of rolipram to abolish all attenuating effects of L457R on hLHR(wt) indicates that it is an increase in PDE4 activity that is mediating the attenuating effects of L457R.

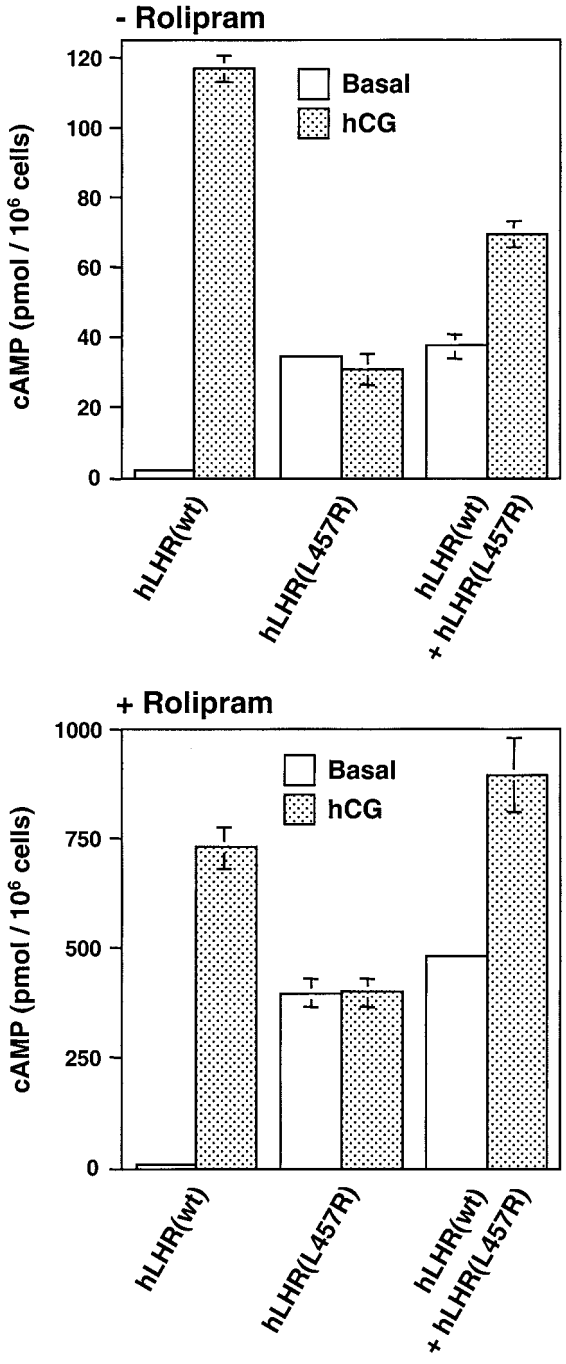


FIG. 3. The attenuation of hCG-stimulated cAMP production by hLHR(wt) caused by the coexpression of hLHR(L457R) is not observed when PDE4 activity is inhibited. 293 Cells were transiently transfected to yield cells expressing submaximal but equal levels of either hLHR(wt) or hLHR(L457R) or a combination of both. Cells were incubated 2 h at 37 C in the absence or the presence of a saturating concentration of hCG and in the absence (*top*) or presence (*bottom*) of the PDE4 inhibitor rolipram (20 μM). The media was aspirated, and intracellular cAMP was extracted and measured by RIA. Results shown are the mean ± range of two independent experiments. Cells expressing hLHR(wt), hLHR(L457R), or hLHR(wt) plus hLHR(L457R) bound 2.25 ± 0.75, 2.45 ± 0.65, and 5.10 ± 0.07 ng ^{125}I-hCG per 10^6 cells, respectively.

To confirm this, we examined the PDE activity of cells expressing hLHR(wt) and hLHR(L457R) individually and together, again using submaximal concentrations of receptors (Fig. 4A). Extracts from cells transfected with hLHR(wt) exhibited the same level of basal PDE activity as those from cells transfected with empty vector. About 50% of the basal PDE activity in hLHR(wt) cells can be attributed to PDE4 activity because rolipram inhibited the basal PDE activity to this extent. Treatment of the hLHR(wt) cells with hCG caused an approximately 1.6-fold increase in PDE activity that was rolipram sensitive, suggesting that the increased cAMP synthesis in hLHR(wt) cells in response to hCG stimulates PDE4 activity. In marked contrast to hLHR(wt) cells, the basal levels of PDE activity in hLHR(L457R) cells are significantly elevated. In fact, the basal levels of PDE activity in the L457R cells are comparable to the hCG-stimulated levels of PDE activity in hCG-treated hLHR(wt) cells. Because cAMP levels in hCG-treated L457R cells are no greater than those of L457R cells under basal conditions, it is not surprising that the addition of hCG to the L457R cells did not cause any further increase in PDE activity. Importantly, the increase in basal PDE activity in L457R cells is not observed when rolipram is present, suggesting that the elevated basal cAMP levels in L457R cells cause PDE4 activity to be elevated. Finally, the data in the *top panel* of Fig. 4 also show that the basal and hCG-stimulated levels of PDE activity in cells coexpressing hLHR(wt) and hLHR(L457R) are the same as those in cells expressing only hLHR(L457R). Thus, when constitutively active hLHR(L457R) is coexpressed with hLHR(wt), the basal levels of PDE are elevated compared with hLHR(wt) alone, and this elevation is inhibited by the inclusion of rolipram. The addition of hCG to the cells coexpressing hLHR(wt) and hLHR(L457R) does not increase PDE activity further.

To determine the potential maximal effect of L457R on PDE activity, the above experiment was repeated under conditions in which hLHR(wt) and hLHR(L457R) were each expressed maximally (Fig. 4, *bottom*). Under these conditions, approximately 2.4-fold increase in basal PDE activity is observed in hLHR(L457R) cells compared with hLHR(wt) cells. This increased basal level of PDE activity in L457R cells is inhibited by rolipram, and it is similar to the level of PDE activity observed in hCG- or forskolin-treated hLHR(wt)

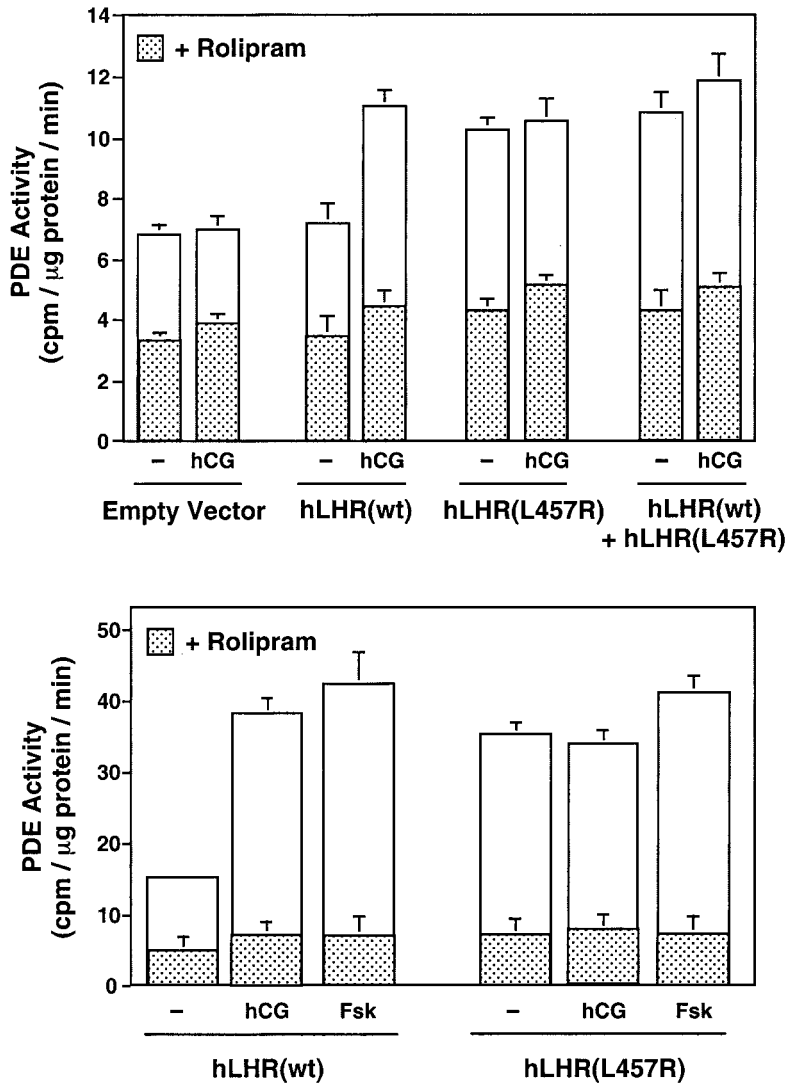


FIG. 4. hLHR(L457R) causes a rolipram-dependent increase in PDE activity in cells. *Top*, 293 Cells were transiently transfected to yield cells expressing submaximal and comparable levels of either hLHR(wt) or hLHR(L457R) or a combination of both. Cells were incubated with hCG (final concentration, 100 ng/ml) for 15 min at 37 C, and then lysates were prepared and analyzed for PDE activity in the absence or the presence of rolipram (20 μM). PDE activity is expressed as the counts per minute of [^{3}H]cAMP degraded per microgram protein per minute, where the specific activity of the [^{3}H]cAMP was 500 cpm/pmol. Results shown are the mean ± SEM of four independent experiments. Cells expressing hLHR(wt), hLHR(L457R), or hLHR(wt) plus hLHR(L457R) bound 3.9 ± 0.3, 4.4 ± 0.3, and 8.5 ± 1.7 ng ^{125}I-hCG per 10^6 cells, respectively. *Bottom*, 293 Cells were transiently transfected with maximal concentrations of plasmids encoding hLHR(wt) or hLHR(L457R). Cells were incubated with hCG (final concentration, 100 ng/ml) or forskolin (Fsk; 90 μM) for 15 min at 37 C, and then lysates were prepared and analyzed for PDE activity in the absence or presence of rolipram (20 μM). Results shown are the mean ± SEM of three independent experiments. hLHR(wt) cells and hLHR(L457R) cells bound 17.2 ± 0.6 and 27.3 ± 0.3 ng ^{125}I-hCG per 10^6 cells, respectively. Note the different scale of the y-axis compared with the *top panel*.

cells. Indeed, hCG or forskolin addition to the L457R cells caused little or no further increase in PDE activation.

Taken together, the data presented thus far show that hLHR(L457R), even when expressed at relatively low levels, causes an increase in basal PDE4 activity and that this increase in PDE4 activity accounts for the ability of L457R to attenuate hCG-stimulated cAMP by hLHR(wt) when coexpressed together. To examine this in more detail, we performed Western blots in which extracts from cells expressing hLHR(wt) *vs.* hLHR(L457R) were probed with antibodies specific for subtypes of PDE4. No clear signals were observed with antibodies to PDE4A or PDE4B. However, as shown in Fig. 5, specific bands corresponding to PDE4D were readily detected in the 293 cells. In these experiments, extracts were prepared from cells that expressed comparable submaximal levels of receptor (Fig. 5A) or comparable maximal levels of receptor (Fig. 5B). The results of both were similar. Thus, in the 293 cells, the anti-PDE4 antibody detected bands whose molecular weights of 93–95 kDa and 100 kDa are consistent with PDE4D3 (PDE4 family, gene product D, splice variant 3) and PDE4D5 (PDE4 family, gene product D, splice variant 5), respectively. It has been shown previously that the activation of either enzyme is associated with a decrease in mobility on SDS polyacrylamide gels and that this is due to the phosphorylation of the enzyme (21). The phosphorylation of both PDE4D5 and PDE4D3 is clearly detected in the cells transfected with empty vector that have been treated with forskolin, but not, as would be expected, with hCG. Under these conditions, all the PDE4D5 appears activated, whereas only a portion of PDE4D3 changes mobility. In cells expressing hLHR(wt), both PDE4D5 and PDE4D3 are in the dephosphorylated form under basal conditions. Upon stimulation with either hCG or forskolin, there is a shift in all the PDE4D5 and most of the PDE4D3 to the active form (similar to the forskolin-treated cells transfected with only empty vector). Thus, the increased cAMP accumulation due to forskolin activation of adenylyl cyclase or due to hCG activation of hLHR(wt) leads to activation of both PDE4D3 and PDE4D5 activity. There are several interesting features of the cells expressing the activating hLHR(L457R) mutant that can be determined from this experiment. First, unlike the cells transfected with empty vector or hLHR(wt) and incubated under basal conditions, the L457R cells under basal conditions exhibit a slower migrating band of PDE4D3 consistent with its partial activation. There is not a readily discernable shift in the mobility of PDE4D5, however, in the L457R cells under basal conditions. As would be expected, the addition of hCG to the L457R cells shows no further activation of PDE4D3 above that already observed under basal conditions, and it does not appear to activate PDE4D5. (The lack of effect of hCG on PDE4D3 or PDE4D5 phosphorylation in the L457R cells is consistent with the unresponsiveness of this activating mutant to hCG.) The addition of forskolin to the L457R cells does, however, cause a greater activation of PDE4D3, and it causes an activation of PDE4D5.

Taken together, the data presented thus far demonstrate that the constitutively active mutant hLHR(L457R) causes an increase in the basal levels of PDE activity, specifically that of PDE4D3. To determine whether this effect is unique to the L457R mutant or is a more general phenomenon common to other constitutively active hLHR mutants, we measured the PDE activity in cells expressing two other naturally occurring hLHR activating mutants, T577I (Refs. 22 and 23) and D578Y (Refs. 24 and 25; Fig. 6). Similar to cells expressing L457R, cells expressing either of these two other activating mutants also exhibited a marked increase in basal PDE activity that was sensitive to rolipram inhibition.

Finally, to determine whether the hormonal stimulation of other GPCRs that activate Gs would also be attenuated by an activating hLHR mutant, we examined the responsiveness of the hβ_2-AR when coexpressed with hLHR(L457R). 293 Cells were transfected with hLHR(L457R) alone, hβ_2-AR alone, or

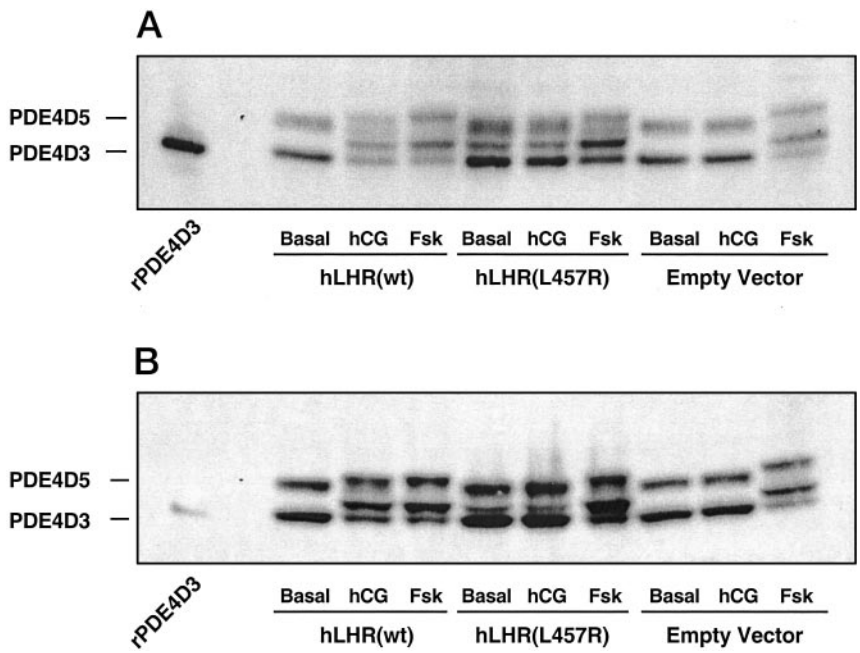
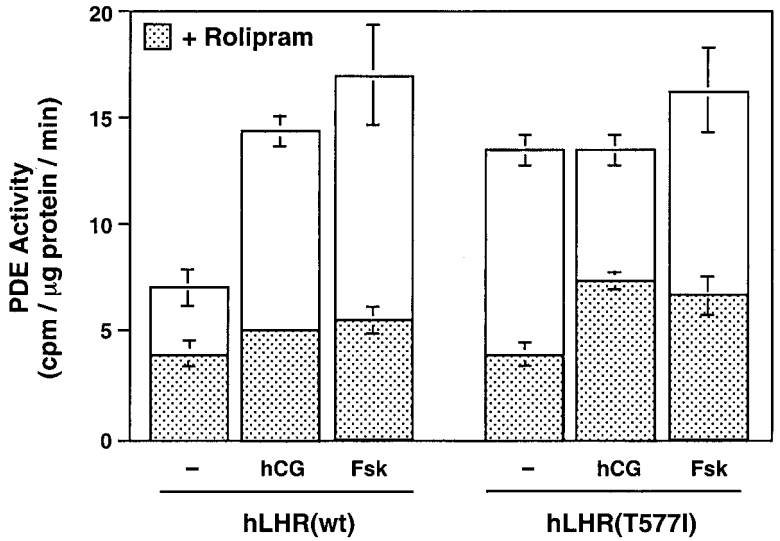


FIG. 5. hLHR(L457R) causes the phosphorylation of PDE4D3 under basal conditions. The 293 cells were transiently transfected to yield submaximal (A) or maximal (B) levels of hLHR(wt) or hLHR(L457R). Cells were incubated with hCG (final concentration, 100 ng/ml) or forskolin (Fsk; 90 μM) for 15 min at 37 C, and then lysates were prepared and resolved by SDS-PAGE. Western blots were probed with antibodies specific to PDE4D. rPDE4D3 refers to recombinant PDE4D3 that was included as an internal control. A, hLHR(wt) and hLHR(L457R) cells bound 6.0 and 3.3 ng ^{125}I-hCG per 10^6 cells, respectively, and 110 μg protein was applied per lane. B, hLHR(wt) and hLHR(L457R) cells bound 24.0 and 17.1 ng ^{125}I-hCG per 10^6 cells, respectively, and 55 μg protein was applied per lane. Results shown are from one representative experiment and depict only the relevant portions of the gels. Similar results were obtained with two different PDE4D-specific antibodies.



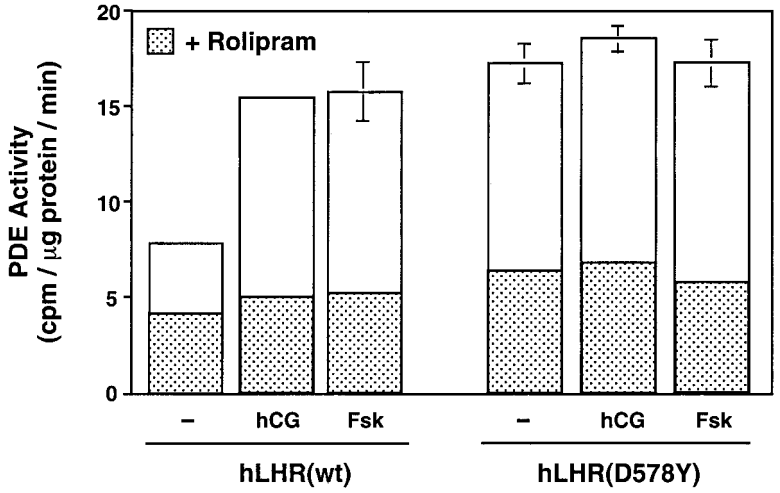


FIG. 6. Other constitutively active hLHR mutants stimulate PDE activity. The 293 cells were transiently transfected with a maximal concentration of plasmid encoding hLHR(T5771) (*top*) or hLHR(D578Y) (*bottom*) and submaximal concentrations of hLHR(wt) that would yield cells expressing comparable levels of cell surface receptors as the mutant. Cells were incubated with hCG (final concentration, 100 ng/ml) or forskolin (Fsk; 90 μM) for 15 min at 37 C, and then lysates were prepared and analyzed for PDE activity in the absence or the presence of rolipram (20 μM). PDE activity is expressed as the counts per minute of [^{3}H]cAMP degraded per microgram protein per minute, where the specific activity of the [^{3}H]cAMP was 500 cpm/pmol. Results shown are the mean ± range of two experiments (*top*) or mean ± SEM of three experiments (*bottom*). *Top*, hLHR(wt) cells and hLHR(T5771) cells bound 14.6 ± 3.2 and 11.2 ± 1.4 ng ^{125}I-hCG per 10^6 cells, respectively. *Bottom*, hLHR(wt) cells and hLHR(D578Y) cells bound 16.3 ± 0.9 and 15.1 ± 0.6 ng ^{125}I-hCG per 10^6 cells, respectively.

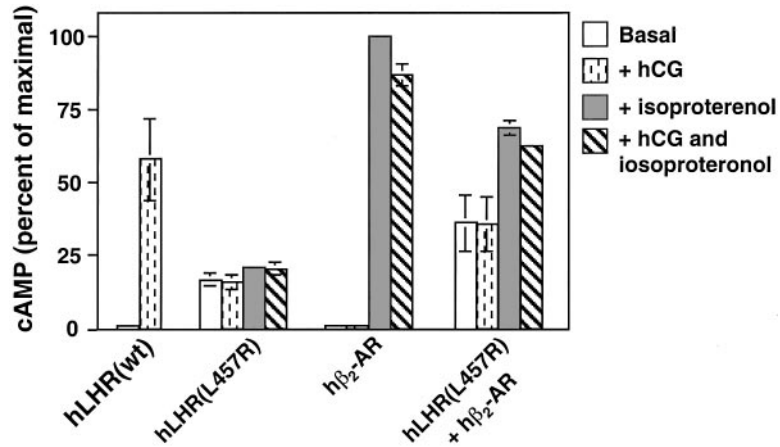


FIG. 7. Coexpression of hLHR(L457R) with the hβ_2-AR attenuates isoproterenol-stimulated cAMP production. 293 Cells were transiently transfected with submaximal concentrations of plasmid (240 ng of each plasmid per 35-mm well) encoding hLHR(wt) alone, hβ_2-AR alone, or hLHR(wt) plus hβ_2-AR. Total concentrations of plasmid were held constant with empty vector. Cells were incubated 2 h at 37 C with buffer only, hCG (final concentration; 100 ng/ml), isoproterenol (1 μM), or hCG plus isoproterenol. The media was aspirated, and intracellular cAMP was extracted and measured by RIA. Results shown are the mean ± range of two independent experiments.

the two receptors together, and cAMP production was measured in the absence of any PDE inhibitors. As would be expected, cells expressing hLHR(L457R) alone or hLHR(L457R) plus hβ_2-AR exhibited elevated levels of basal cAMP (Fig. 7). Although cells expressing the hβ_2-AR alone exhibited a robust stimulation of cAMP production in response to isoproterenol, the response to isoproterenol in cells expressing the hβ_2-AR and hLHR(L457R) was only 67% of that observed in the cells expressing the hβ_2-AR only. Therefore, the constitutive elevation of cAMP and concomitant activation of cAMP-dependent PDE activity by hLHR(L457R) causes the attenuation of the hormone-stimulated cAMP pro-

duction by another Gs-coupled receptor that is present in the same cell.

Discussion

In the past few years, a number of naturally occurring hLHR mutations have been described (6, 7). The gain-of-function mutations of the hLHR result in gonadotropin-independent precocious puberty in boys heterozygous for a given mutation (6). The phenotypes of these mutants are typically determined by transfecting heterologous cells with the recombinant mutant hLHR and comparing them to cells expressing comparable numbers of cell surface hLHR(wt). As such, these mutants have been shown to cause an increased activation of Gs in the absence of hormone as determined by increased basal levels of intracellular cAMP in intact cells incubated with PDE inhibitors (6, 7) or by increased basal adenylyl cyclase activity in isolated membranes (9). Activating hLHR mutants are, however, expressed physiologically in a heterozygous state. Until now, however, no studies had been performed to determine what effects, if any, an activating hLHR mutant might have on hLHR(wt) coexpressed in the same cell. Toward this end, the present studies were performed. Importantly, for these studies we chose to examine cAMP accumulation in the absence of PDE inhibitors, thus approximating a more physiological context. Under these conditions, we show that coexpression of an activating hLHR mutant with the hLHR(wt) has attenuating effects on hormone-stimulated cAMP by the wt receptor due to stimulation of PDE activity caused by the increased cAMP elicited by the activating mutant.

Our studies took advantage of a unique activating hLHR mutant, L457R, which is both constitutively active and hormone unresponsive (8, 9). Because L457R binds hCG with normal affinity and because membranes isolated from L457R-expressing cells display elevated basal levels of adenylyl cyclase activity but no hCG responsiveness, it appears that the L457R mutation somehow renders the receptor intrinsically unable to translate the binding of hormone into further activation of Gs. Therefore, if one coexpresses L457R with hLHR(wt) and examines hCG-stimulated cAMP production, only the cAMP resulting from the activation of the wt receptor is detected. As shown herein, when hLHR(wt) and hLHR(L457R) are coexpressed in 293 cells under conditions in which each is submaximally expressed, both receptors are expressed at the cell surface, and their levels are unaffected by the presence of the other receptor. Under these conditions, however, the presence of L457R attenuates the hCG-stimulated cAMP production elicited by hLHR(wt) as determined in the absence of PDE inhibitors. Our data show that this attenuation is due to an increase in basal PDE4D3 (PDE4 family, gene product D, splice variant 3) activity that is caused by the increased intracellular cAMP resulting from the constitutively active L457R mutant. Significantly, the attenuating effects of L457R on hLHR(wt) are abolished when cells are incubated with PDE inhibitors. Therefore, the increase in PDE activity resulting from the constitutively active mutant is sufficient to account for all attenuating effects of the mutant on hCG-stimulated cAMP by hLHR(wt).

The intracellular levels of cAMP are very tightly regulated, and only small changes in concentrations are sufficient to elicit biological responses. Ultimately, the concentrations of cAMP are regulated by both the synthesis of cAMP by adenylyl cyclase and the hydrolysis of cAMP by PDEs. In recent years, 21 genes encoding cyclic nucleotide PDEs have been identified (for a recent review, see Ref. 26). The gene products have been classified into 11 families of PDEs, in which within a given family there may be different gene products and splice variants of the gene products. Of particular relevance are PDE3 and PDE4, both of which are cAMP PDEs and both of which are activated by cAMP (27–29). These two families differ in their tissue distribution, however, with PDE3 being expressed primarily in platelets and adipocytes. PDE4 is the largest family of PDEs and is much more widely distributed. Of particular relevance to the hLHR, PDE4 is expressed in various endocrine cells (including those of the ovaries and testes, in which the LHR is expressed) as well as 293 cells used for heterologous expression experiments (30, 31). It has been shown that the activation of PDE4 by hormones that stimulate cAMP production involves an activation of preexisting enzyme by a protein kinase A-mediated phosphorylation as well as an increase in PDE4 mRNA levels and *de novo* synthesis of PDE4 protein (21, 29, 32). Studies in which PDE4 levels have been manipulated suggest that PDE4 activation controls the intensity of the intracellular cAMP signal and perhaps its duration (18).

In the present studies, we show that both PDE4D5 and PDE4D3 splice variants of PDE4 are activated by hCG or forskolin stimulation of cells expressing hLHR(wt). Activation of both forms of PDE4D is also observed in L457R cells incubated with forskolin. However, only PDE4D3 appears to be activated in the L457R cells under basal and hCG-stimulated conditions. It should be noted that one would expect there to be no hCG-stimulated PDE activation in L457R cells because this mutant does not respond to hCG with a further increase in cAMP (8, 9). The lack of stimulation of PDE4D5 in the L457R cells may be due to the fact that the intracellular levels of cAMP in these cells, although elevated compared with basal levels of hLHR(wt) cells, are lower than those of hLHR(wt) cells challenged with hCG or forskolin and lower than the L457R cells incubated with forskolin. The stimulation of PDE4D3 in the basal and hCG-treated L457R cells may, therefore, reflect a greater sensitivity of PDE4D3 than PDE4D5 to cAMP-mediated phosphorylation. Alternatively, it is possible that the activation of PDE4D3 at low cAMP concentrations is due to the binding of this PDE, but not PDE4D5, to an AKAP scaffold protein that brings it in close proximity to protein kinase A (33, 34).

Whereas the hormone occupancy of a GPCR allows for regulated activation and desensitization of the receptor within a given time frame, mutation-induced constitutive activation of a GPCR results in the activation of the receptor in an unregulated manner. Previous studies by Conti and colleagues (35) have shown that activating mutations of the hTSH receptor, similar to hormone-occupied wt hTSH receptor, cause an activation of PDE4D3. Although we initially documented this phenomenon in the context of the hLHR(L457R) mutant, we demonstrate that other activating mutants of the hLHR also induce PDE4D3 activity. Therefore, the activation of PDE4D3 and/or other cAMP-specific

PDEs is likely to be a general property of GPCR mutants that constitutively activate Gs. Presumably, this autoregulatory mechanism may help protect the cells from levels of intracellular cAMP that would be deleterious to the cell. It is important to note that although the elevated levels of cAMP resulting from the activating hLHR mutant increase PDE activity, which in turn degrades cAMP and decreases intracellular cAMP levels, the concentrations of intracellular cAMP in cells expressing activating hLHR mutants nonetheless remain higher than basal. Therefore, the increased PDE activity serves to attenuate, but not block, the elevation in cAMP caused by the constitutive activation of Gs by the hLHR mutant. In addition to Gs-coupled GPCRs activating PDE activity, it has also been shown that constitutively active Gsα, as naturally occurring in GH-secreting adenomas (36) or as expressed in transgenic mice (37), also similarly cause an increase in PDE activity.

Of particular significance, the data presented demonstrate that when the activating hLHR(L457R) mutant is coexpressed with hLHR(wt), it exerts an attenuating effect on the hormone-stimulated cAMP levels produced by the wt receptor. Our studies show that this attenuation is due to an activation of PDE4D3 caused by the elevated levels of cAMP resulting from the constitutive activation of Gs by hLHR(L457R). The increased PDE activity elicited by the activating L457R mutant would be expected to attenuate the activity of other Gs-coupled receptors as well. Indeed, we show that coexpression of hLHR(L457R) with the hβ2-AR results in an attenuation of isoproterenol-stimulated cAMP. Thus, an activating hLHR mutant present in a heterozygous individual would be expected to cause a constitutive elevation of basal cAMP and concomitantly an attenuation of the hormone-stimulated cAMP mediated by hLHR(wt) and any other Gs-coupled receptor expressed in the same cell. With regard to the LH/CG responsiveness of Leydig cells in boys with gonadotropin-independent precocious puberty, the data are incomplete to make a clear assessment. Earlier studies on boys with familial gonadotropin-dependent precocious puberty have shown that some boys, although exhibiting elevated levels of testosterone, responded to exogenously administered hCG with further increases in testosterone (38). Although it is presumed in retrospect that the underlying cause of their disease was an activating mutation of the hLHR, this is not known. In boys with gonadotropin-independent precocious puberty in which an activating hLHR gene mutation has been confirmed, only one boy to our knowledge was tested for hCG responsiveness. This boy, who is heterozygous for the hLHR(L457R) mutation, was unresponsive to hCG stimulation (8). Whether a similar lack of response would be observed in other boys with activating hLHR mutations remains to be determined. Although the lack of hCG responsiveness of the boy heterozygous for the L457R mutation would be consistent with our *in vitro* observations, one cannot exclude the possibility that the strong activating nature of this mutation and/or other factors may have contributed to his lack of further hCG responsiveness. With respect to females heterozygous for activating hLHR mutations, their reproductive functioning appears to be unimpaired (6).

It remains possible that an attenuation of hormone-stimulated cAMP due to an activating hLHR mutation may not necessarily be translated into decreased hormone-stimulated steroid production. Ultimately, the degree of coupling within a given cell between the levels of cAMP and the downstream events mediated by cAMP determines whether or not a given decrease in cAMP would cause a comparable decrease in the cAMP-stimulated pathways. It is important to bear in mind that hLHR-expressing testicular and ovarian gonadal cells possess a large excess of spare LHRs (39, 40). In these cells, submaximal concentrations of cAMP are sufficient to elicit maximal steroid production (hence, why a relatively low level of constitutive activity of an activating hLHR mutant can have major physiological consequences). Consequently, LHR-expressing gonadal cells can tolerate a certain extent of down-regulation of LHRs and a concomitant decrease in LH/CG-stimulated cAMP, without an accompanying decrease in LH/CG-stimulated steroid production (39, 40). It is possible, therefore, that although a heterologously expressed activating hLHR mutant may cause an attenuation of LH/CG-stimulated cAMP in these cells, the decrease in cAMP may not be sufficient to cause a decrease in steroid biosynthesis. Ideally, this question would best be addressed by examining the effects hLHR-activating mutants coexpressed with hLHR(wt) in gonadal cells. Unfortunately, the relatively low levels of endogenous LHR expression in primary cultures of Leydig cells or differentiated granulosa cells preclude the use of such cells for these studies. We have attempted to use MA-10 Leydig tumor cells that express undetectable levels of endogenous LHR to express recombinant hLHR and/or hLHR(L457R) (Ref. 41). Unfortunately, the levels of recombinant hLHR expressed in the MA-10 cells were found to be much lower than those observed in transfected 293 cells. Therefore, until such time that we can achieve a more robust expression of recombinant hLHR in cultured gonadal cells, we cannot directly examine the effects of activating hLHR mutants on PDE activity and on the hormone responsiveness of coexpressed hLHR(wt) in gonadal cells.

In summary, our studies in 293 cells demonstrate that the coexpression of activating hLHR mutants with hLHR(wt) causes an attenuation of the hormone-cAMP response mediated by hLHR(wt) and that this is caused by an increase in PDE activity induced by the constitutively elevated levels of cAMP produced by the activating mutant. The potential physiological consequences of this attenuation may be determined by the extent of coupling between intracellular cAMP levels and downstream signaling events in target cells and warrant further investigation.

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Address all correspondence and requests for reprints to: Deborah L. Segaloff, Ph.D., Department of Physiology and Biophysics 5-470 Bowen Science Building, The University of Iowa, Iowa City, Iowa 52242. E-mail: deborah-segaloff@uiowa.edu.

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