# Constitutive Activation of G Protein-Coupled Receptors as a Result of Selective Substitution of a Conserved Leucine Residue in Transmembrane Helix III

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**Whereas numerous mutations of the human lutropin receptor (hLHR) and human TSH receptor (hTSHR) have been shown to cause constitutive activation of these receptors, it has been suggested that either the hFSHR as a whole, or the i3/TM VI region of the hFSHR, is less susceptible to mutation-induced constitutive activation. However, as shown herein, substitution of a highly conserved leucine residue in transmembrane III (TM III) of the hFSHR (Leu III.18) with arginine causes a 5-fold increase in basal cAMP in transfected cells, consistent with a strong constitutive activation of the hFSHR. Interestingly, this mutant is unresponsive to further hormonal stimulation. Substitutions of hFSHR(L460) with lysine, alanine, or aspartate show that only arginine causes constitutive activation. However, all result in decreased FSH responsiveness, suggesting a role for L460 in FSH-stimulated cAMP production by the hFSHR.**

**Because Leu III.18 is highly conserved in rhodopsin-like G protein-coupled receptors (GPCRs), we tested the effects of substitution of the comparable leucine in the human $\beta_2$-adrenergic receptor (h$\beta_2$-AR). Substitution of L124 in the h$\beta_2$-AR with arginine, lysine, or alanine resulted in constitutive activation as evidenced by increased basal levels of cAMP that could be attenuated by an inverse agonist. In all cases, isoproterenol-stimulated cAMP was unaffected.**

**Taken altogether, our data support a model whereby Leu III.18 may play a general role in GPCRs by stabilizing them in an inactive state. Constitutive activation may arise by both a disruption of Leu III.18 as well as the introduction of a specific residue that serves to stabilize the active state of the receptor. (Molecular Endocrinology 14: 1272–1282, 2000)**

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## INTRODUCTION

The human follitropin receptor (hFSHR) is a member of the superfamily of rhodopsin-like G protein-coupled (GPCRs). Within this superfamily, the hFSHR, human LH receptor (hLHR), and human TSH receptor (hTSHR) are highly related and are collectively known as the glycoprotein hormone receptors. They are characterized by the presence of a large extracellular domain that binds hormone with high affinity (see Refs. 1–3 for reviews).

In recent years many naturally occurring constitutively activating mutations of both the hTSHR (4) and hLHR (5) have been described. Those in the hTSHR have been identified in patients with hyperfunctioning thyroid adenomas and thyroid carcinomas and those in the hLHR have been seen in young boys with gonadotropin-independent precocious puberty (5) or Leydig cell tumors (6). Only one putative constitutively activating mutation of the hFSHR has been reported, a D567G substitution in the carboxyl portion of the third intracellular loop (i3) (7). This mutation was originally identified in a hypophysectomized male who exhibited normal testes volume after testosterone treatment, thus suggesting the patient carried an activating mutation of the hFSHR. In the initial report, the observed increase in basal cAMP in cells transfected with hFSHR(D567G) was quite low ($<$2-fold). Subsequent studies from two other laboratories were unable to demonstrate any elevation of basal cAMP in cells transfected with hFSHR(D567G) (8, 9). Therefore, it may be that the D567G substitution is a nonfunctional polymorphic mutation in the hFSHR, and a different, as yet unidentified, mutation may be causing the phenotype of the patient in the original study. Alternatively,

the D567G mutation may cause a relatively small constitutive activation of the hFSHR that may be difficult to observe in a reproducible manner.

Other mutations in transmembrane (TM) VI of the hLHR that are known to cause constitutive activation of the hLHR were introduced by Kudo *et al.* (8) into the comparable residues of the hFSHR, and these, too, were found to be unable to induce constitutive activation of this receptor. These observations have led to the speculation that the hFSHR may be in a more highly constrained conformation and less susceptible in general to mutation-induced constitutive activation than the highly related hLHR and hTSHR. Alternatively, it is possible that the roles of TM VI and i3 in maintaining an inactive state of the hFSHR may be different from that of the hLHR and hTSHR. To address this question, we chose to introduce a mutation into the hFSHR in a region other than TM VI and i3 that would, based upon results obtained with the hLHR, be predicted to cause constitutive activation. The residue chosen for mutagenesis of the hFSHR was based on a previously reported mutation of L457 in TM III of the hLHR that was shown to cause constitutive activation (10). This leucine is conserved not only in the glycoprotein hormone receptors, but in >70% of all rhodopsin-like GPCRs and is defined as Leu III.18 by Baldwin *et al*. (11) in their model of GPCR transmembrane helices. Given its highly conserved nature, the strong constitutive activation observed in the hLHR(L457R) mutant, and the location of the leucine in a region other than TM VI and i3, we undertook the present studies to investigate the effects of substitution of the comparable TM III leucine (L460) within the hFSHR.

Our studies show that the substitution of L460 in TM III of the hFSHR does indeed cause a robust increase in basal levels of cAMP, consistent with it being constitutively active. The data presented further show that substitution of the comparable leucine residue in the human $\beta_2$-adrenergic receptor (h$\beta_2$-AR) also causes constitutive activation. Leu III.18 is situated in a region of TM III postulated to be involved as a general activating switch region for GPCRs (12). Our data support this model and further implicate Leu III.18 as serving a conserved role in GPCRs in maintaining them in an inactive state. However, our data further suggest that constitutive activation only arises from a combination both of disruption of Leu III.18 as well as the introduction of specific residues that presumably stabilize the receptor in an active state.

## RESULTS

Most rhodopsin-like GPCRs contain a highly conserved leucine in TM III which, per the numbering by Baldwin *et al*. (11), is designated III.18. The alignments of the sequences surrounding Leu III.18 in several representative GPCRs are illustrated in Fig. 1. Based upon the activating L457R mutation of the hLHR (10), we substituted the comparable TM III leucine of the hFSHR, L460, with arginine. HEK 293 cells were transiently transfected with cDNAs encoding either the wild-type or mutant hFSHR. All four of the mutants were expressed well on the cell surface. In a typical experiment in which cells were transfected with a maximal concentration of plasmid, the cell surface binding of $^{125}$I-hFSH was 1–2 ng/$10^6$ cells for the wild type, L457R, L457A, or L457D hFSHR, and 4–5 ng/$10^6$ cells for L457K. Experiments were performed in which the concentrations of plasmid used for transfection

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hLHR | 439 | C | S | T | A | G | F | F | T | V | F | A | S | E | L | S | V | Y | T | L | T | V | I | T | L | E |
| hFSHR | 442 | C | D | A | A | G | F | F | T | V | F | A | S | E | L | S | V | Y | T | L | T | A | I | T | L | E |
| hβ2AR | 106 | C | E | F | W | T | S | I | D | V | L | C | V | T | A | S | I | E | T | L | C | V | I | A | V | D |
| brho | 110 | C | N | L | E | G | F | F | A | T | L | G | G | E | I | A | L | W | S | L | V | V | L | A | I | E |
| r5HT2 | 126 | C | A | I | W | I | Y | L | D | V | L | F | S | T | A | S | I | M | H | L | C | A | I | S | L | D |
| hSST1 | 130 | C | R | L | V | L | S | V | D | A | V | N | M | F | T | S | I | Y | C | L | T | V | L | S | V | D |
| rAT1a | 101 | C | K | I | A | S | A | S | V | S | F | N | L | Y | A | S | V | F | L | L | T | C | L | S | I | D |
| $m_1$ACHR | 98 | C | D | L | W | L | A | L | D | Y | V | A | S | N | A | S | V | M | N | L | L | L | I | S | F | D |
| hC5aR | 109 | C | S | I | L | P | S | L | I | L | L | N | M | Y | A | S | I | L | L | L | A | T | I | S | A | D |
| | | C | | | | | | | | | | | | | | S | | | | L | | | I | S | | D |
| | | | | | | | | | | | | | | | | | | | | * | | | L | A | | E |

**Fig. 1.** Examples of GPCRs Containing the Conserved Leu III.18

The TM III leucine, which is conserved in >70% of rhodopsin-like GPCRs and is designated Leu III:18 in the Baldwin model (44), is highlighted in the sequences of several representative GPCRs. The alignment of the sequences shown is based on the model for rhodopsin-like GPCRs proposed by Baldwin (44), and the position of consensus residues as determined by Baldwin are indicated *below* the alignments. The sequences shown flank Leu III:18, but are not intended to delineate the boundaries of TM III.

were deliberately modified to yield the same numbers of cell surface receptors in the wild-type *vs.* mutant hFSHR-expressing cells. Figure 2 shows a representative experiment in which both basal and FSH-stimulated cAMP production were analyzed. Cells expressing hFSHR(L460R) exhibited increased levels of basal cAMP, consistent with the mutant being constitutively active. Interestingly, although the basal cAMP levels in hFSHR(L460R) cells were not as great as hFSHR(wt) cells stimulated with a saturating concentration of FSH, the hFSHR(L460R) cells exhibited little increase in cAMP in response to increasing concentrations of FSH (Fig. 2). Therefore, the mutation of L460 to arginine caused both constitutive activation of the hFSHR as well as impaired responsiveness to FSH. In the course of seven independent experiments measuring cAMP under basal conditions and in response to a saturating concentration of FSH, we observed an average increase in basal cAMP production by the L460R-expresssing cells of 5.2-fold when compared with cells expressing comparable numbers of cell surface wild-type receptors (Fig. 3A). Furthermore, whereas cells expressing the wild-type hFSHR responded to a saturating concentration of hFSH with an average 35-fold increase in cAMP levels above basal, cells expressing L460R responded to hFSHR with only a 1.8-fold increase in cAMP production (Fig. 3B).

To determine whether the constitutive activation of hFSHR(L460R) was due to the introduction of a positive charge in this site, we prepared and characterized additional L460-substituted mutants of the hFSHR.

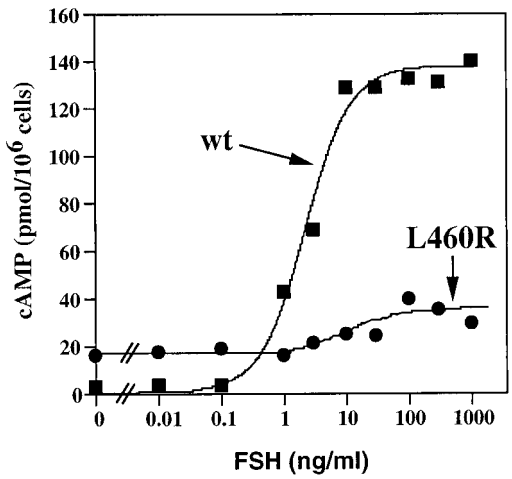


**Fig. 2.** Substitution of L460 in TM III of the hFSHR with Arginine Results in Constitutive Activation of cAMP Production Concomitant with Decreased FSH-Stimulated cAMP Production

293 cells were transiently transfected using conditions to yield comparable numbers of cell surface hFSHR(wt) and hFSHR(L460R). cAMP production was assayed under basal conditions and in response to increasing concentrations of FSH. Data shown are from a representative experiment. In this experiment, both wild-type and L460R cells bound 1.2 ng $^{125}$I-hFSHR/$10^6$ cells.

These consisted of mutants in which either a positively charged lysine, a neutral alanine, or a negatively charged aspartic acid were introduced at codon 460. As seen in Fig. 3A, the basal levels of cAMP were increased only in cells expressing hFSHR(L460R). Interestingly, the basal levels of cAMP in hFSHR(L460K) cells were similar to hFSHR(wt) cells, demonstrating that the constitutive activity of the hFSHR(L460R) mutant is not due simply to the introduction of a positive charge at this site.

The data presented thus far suggest that, within the context of the amino acids examined (arginine, lysine, alanine, and aspartic acid), constitutive activation of the hFSHR by substitution of L460 is restricted to the introduction of an arginine. If one examines the ability of the different mutants to respond to FSH with increased cAMP production, however, a different picture emerges. As shown in panel B of Fig. 3, cells expressing hLHR(L460) mutants containing arginine, lysine, alanine, or aspartic acid are blunted in their response to hFSH, as indicated by the decreased Rmax of cells expressing each of the mutants. The Rmax of L460R cells is approximately 25% that of cells expressing wild-type hFSHR; however, this is due primarily to the elevated levels of cAMP due to this mutant's constitutive activity, as opposed to the ability of the cells to respond to FSH (cf. panels A, B, and C of Fig. 3). The fold increases in cAMP elicited by FSH in L460K, L460A, and L460D cells are greater than that of the L460R cells, but they are still far less than observed for cells expressing the wild-type hFSHR (Fig. 3C). Thus, the substitution of L460 of the hFSHR with any of the four residues examined results in a marked attenuation of FSH responsiveness.

One possible cause of the decreased FSH responsiveness of cells expressing arginine-, lysine-, alanine-, or aspartic acid-substituted L460 mutants could be a decreased binding affinity of the mutants for FSH. This possibility was examined directly by determining the binding affinities of the cell surface form of each of the mutants. As shown in Table 1, the L460A and L460D mutants bound FSH with the same affinity as the wild-type receptor, while the L460R and L460K mutants bound with a similar or slightly higher affinity as the wild-type receptor. For each mutant, therefore, we can clearly exclude a decrease in binding affinity as a cause for the decreased FSH-stimulated cAMP production.

We also considered the possibility that the decreased cAMP production in response to FSH by cells expressing the L460 mutants might be due to increased rates of internalization of FSH by the mutant receptors. That is, if the cell surface mutant receptor were internalized faster upon agonist binding, then one could envision that the cellular response might be terminated sooner, thus attenuating it. As shown in Fig. 4, cells expressing hFSHR(L460R) or hFSHR(L460K) do exhibit a greater internalization index (which is proportional to the rate of internalization) than cells expressing the wild-type receptor. These

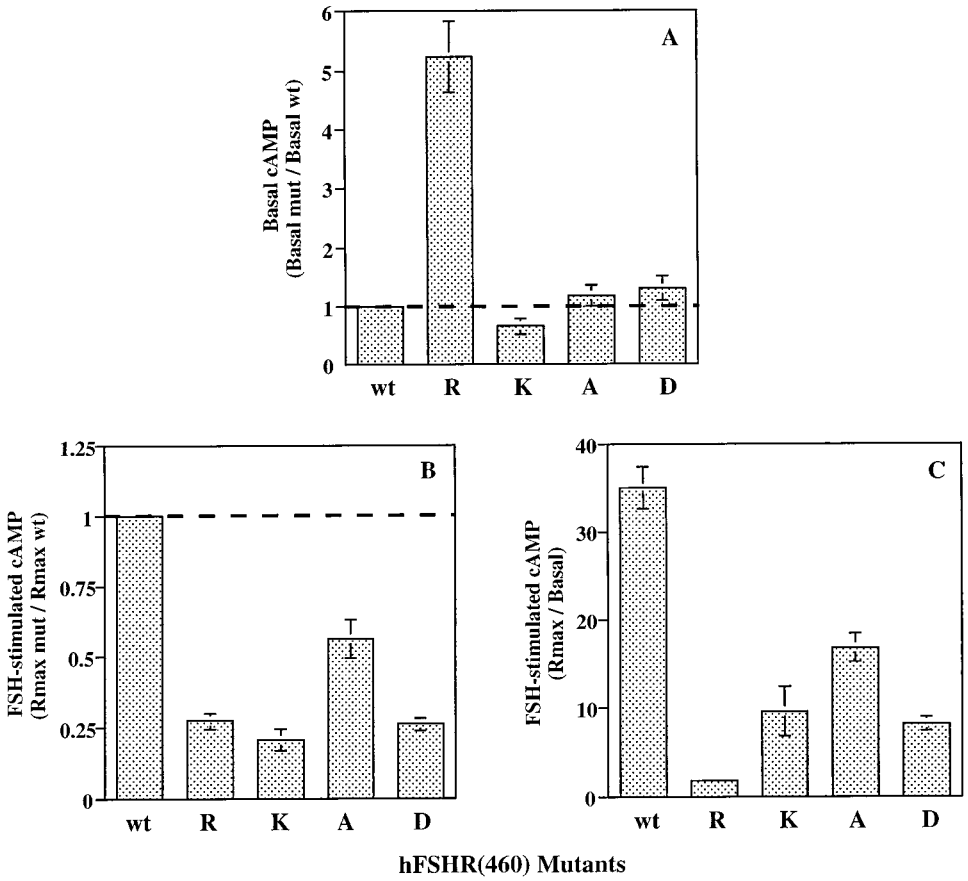


**Fig. 3.** Differentiation between Constitutive Activation *vs*. Attenuation of FSH-Stimulated cAMP Production in hFSHR(L460)-Substituted Mutants

hFSHR cDNA constructs were prepared in which L460 was substituted with either arginine (R), lysine (K), alanine (A), or aspartic acid (D). 293 cells were transiently transfected such that within each experiment the numbers of cell surface receptors for a given mutant were matched to cells expressing comparable numbers of wild-type receptors. For each experiment the ratio of $^{125}$I-hFSH 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). Over the course of several experiments, the means ± SEM of the ratios of cell surface $^{125}$I-hFSH binding to mutant- *vs*. wild-type hFSHR-expressing cells were the following: 0.94 ± 0.10 for L460R (n = 7 experiments); 0.82 ± 0.07 for L460K (n = 3 experiments); 0.94 ± 0.17 for L460A (n = 6 experiments); and 1.05 ± 0.12 for L460D (n = 5 experiments). Therefore, the mutants were matched well to the controls and what little deviation existed varied equally between mutants being expressed at ≤2-fold higher *vs*. 2-fold lower density than the wild-type hFSHR. The ranges of the binding values within each of the series were the following: L460R cells *vs*. wild-type were 1.26 ± 0.15 ng *vs*. 1.50 ± 0.27 $^{125}$I-hFSHR/$10^6$ cells (n = 7 experiments). L460K cells *vs*. wild-type were 2.18 ± 0.42 ng *vs*. 2.73 ± 0.64 $^{125}$I-hFSHR/$10^6$ cells (n = 3 experiments). L460A cells *vs*. wild-type were 1.66 ± 0.26 ng *vs*. 2.14 ± 0.49 $^{125}$I-hFSHR/$10^6$ cells (n = 6 experiments). L460D cells *vs*. wild-type were 2.58 ± 0.24 ng *vs*. 2.61 ± 1.16 $^{125}$I-hFSHR/$10^6$ cells (n = 5 experiments). Panel A, The data for basal cAMP production are presented as the ratio of basal cAMP in cells expressing a mutant relative to basal cAMP in matched cells expressing the wild-type receptor. Therefore, basal cAMP in wild-type hFSHR-expressing cells is defined as 1.0 and is shown by the *dashed line*. Panel B, cAMP in response to a saturating concentration of FSH is presented as the Rmax in cells expressing a mutant relative to the Rmax in matched cells expressing the wild-type receptor. Therefore, Rmax of wild-type hFSHR-expressing cells is defined as 1.0 and is shown by the *dashed line*. Panel C, cAMP in response to a saturating concentration of FSH is presented as the ratio of Rmax to basal cAMP (*i.e.* the fold stimulation) in cells expressing a given receptor.

data would be consistent with the decreased responsiveness of these cells to FSH. However, neither hFSHR(L460A) cells nor hFSHR(L460D) cells exhibited a greater internalization index in spite of their reduced responsiveness to FSH. Indeed, the hFSHR(L460D) cells exhibited a decreased internalization index. Therefore, an increased rate of internalization of FSH cannot account for the decreased FSH responsiveness seen in all four of the different hFSH(L460) mutants.

The data thus far show that, like the hLHR (10), substitution of Leu III.18 of the hFSHR results in constitutive activation and in reduced responsiveness to agonist. To test whether the effects of substitution of

**Table 1.** Binding Affinities of hFSHR(L460) Mutants for hFSH

| Receptor | $K_d$ (nM) |
|---|---|
| hFSHR(wt) | 1.02 ± 0.42 |
| hFSHR(L460R) | 0.50 ± 0.29 |
| hFSHR(L460K) | 0.37 ± 0.11 |
| hFSHR(L460A) | 1.04 ± 0.47 |
| hFSHR(L460D) | 0.84 ± 0.17 |

293 cells were transiently transfected with the cDNAs for the wild-type hFSHR or the L460-substituted mutants shown. $^{125}$I-hFSH binding assays were then performed utilizing intact cells to determine the apparent binding affinities. Data shown are the mean ± range of two independent experiments. The $K_d$ of 1.02 nM for hFSHR(wt) corresponds to 28.6 ng/ml.

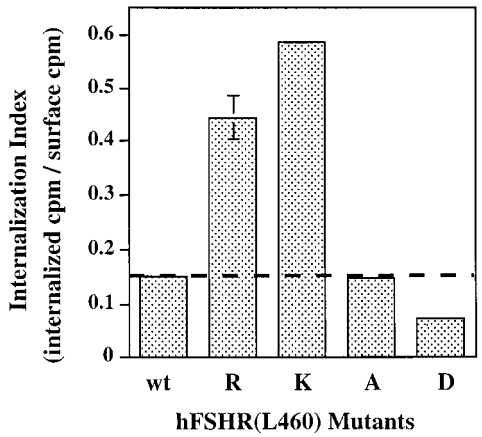


**Fig. 4.** Lack of Correlation between Rates of Internalization of FSH and Decreased Responsiveness to FSH by L460-Substituted hFSHR Mutants

293 cells were transiently transfected with either the wild-type (wt) hFSHR or L460 mutants substituted with arginine (R), lysine (K), alanine (A), or aspartic acid (D). The internalization of $^{125}$I-FSH 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 hFSHR is noted with a *dashed line* for reference purposes.

Leu III.18 are restricted to the gonadotropin receptors or whether they would be applicable to other rhodopsin-like GPCRs, we chose to examine the h$\beta_2$-AR. Toward this end, the equivalent residue (L124) in the h$\beta_2$-AR was substituted with arginine, lysine, alanine, or aspartate and the mutant receptors were transiently expressed in 293 cells. Because h$\beta_2$-AR(L124D) was poorly expressed at the cell surface, the signaling properties of this mutant were not examined.

Figure 5 summarizes the results of several experiments examining the basal and isoproterenol-stimulated levels of cAMP in cells expressing the L124-substituted h$\beta_2$-AR mutants as compared with cells expressing comparable numbers of cell surface wild-type h$\beta_2$-AR. Cells expressing h$\beta_2$-AR(L124R) exhibited a 9-fold elevation of basal cAMP production relative to cells expressing the wild-type receptor (Fig. 5A). Thus, the effects of the L124R substitution in the h$\beta_2$-AR on basal cAMP production were similar to those observed for hFSHR(L460R) (see Figs. 2 and 3) and hLHR(L457R) (10). Interestingly, unlike the hFSHR, cells expressing lysine and alanine substitutions of Leu III.18 also displayed elevated basal cAMP production relative to the wild-type receptor (Fig. 5A). Furthermore, the cells expressing L124R, L124K, or L124A h$\beta_2$-AR mutants each responded to isoproterenol with a Rmax comparable to cells expressing the wild-type receptor (Fig. 5B). Therefore, unlike the hLHR (10) or the hFSHR (Figs. 2 and 3), in which substitution of Leu III.18 impairs hormone-stimulated cAMP production, substitution of Leu III.18 in the h$\beta_2$-AR does not affect agonist-stimulated cAMP production.

Unlike the hLHR and hFSHR, the h$\beta_2$-AR is known to have inverse agonists, agents that reduce the elevated basal activity observed in cells overexpressing the wild-type h$\beta_2$-AR and in cells expressing a constitutively active h$\beta_2$-AR mutant (13, 14). Therefore, we tested the effects of ICI 118,551 on cells expressing either the wild-type or the constitutively active TM III L124 mutants. As shown in Fig. 6, the addition of 10 $\mu$M ICI 118,551 resulted in approximately 50% inhibition of basal cAMP production by cells expressing any of the three L124-substituted h$\beta_2$-AR mutants. These observations further confirm that these mutants behave as constitutively active receptors.

## DISCUSSION

The revised ternary model for GPCR activation predicts that the receptors exist in an equilibrium between an inactive R and an activated R* state (14–16). Thus, even in the absence of agonist, an increased number of cell surface receptors for a given GPCR would be predicted to cause (and indeed does cause) an increase in the basal level of second messenger production. Activation of the GPCR pool by agonist occurs as a consequence of the preferential binding of agonist to R*, thus shifting the equilibrium toward the activated state. Our understanding of the activation of GPCRs has been further expanded by the discoveries in recent years of constitutively active forms of GPCRs. Thus, it has been found that some mutations can stabilize a GPCR in the R* state. Cells expressing these mutants exhibit increased levels of second messenger production in the absence of any agonist, as compared with cells expressing comparable levels of wild-type receptors, and the increased basal levels of second messenger can be attenuated by an inverse agonist. 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

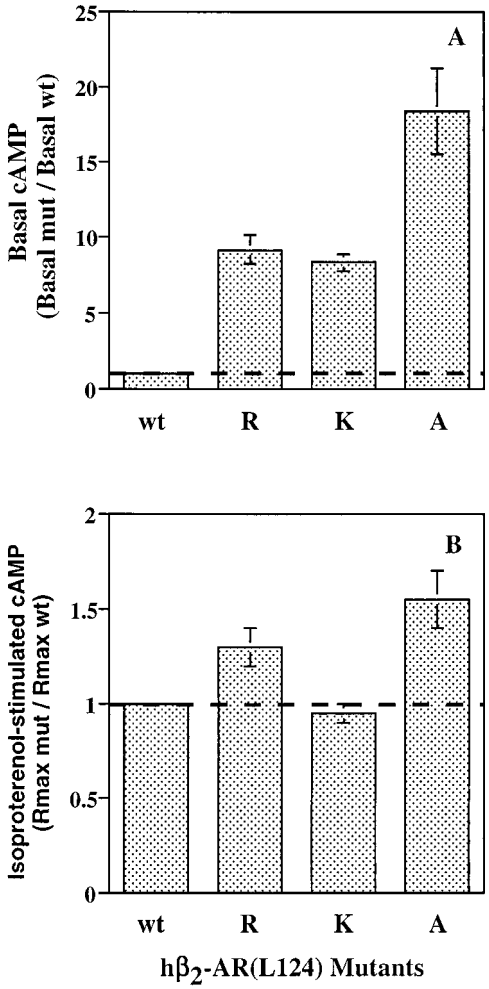


**Fig. 5.** Substitution of Leu III.18 in the h$\beta_2$-AR(L124R) Results in Constitutive Activation and Normal Responsiveness to Agonist

h$\beta_2$-AR cDNA constructs were prepared in which L124 was substituted with either arginine (R), lysine (K), or alanine (A). 293 cells were transiently transfected such that within each experiment the numbers of cell surface receptors for a given mutant were matched to cells expressing comparable numbers of wild-type receptors. Panel A, The data for basal cAMP production are presented as the ratio of basal cAMP in cells expressing a mutant relative to basal cAMP in matched cells expressing the wild-type receptor. Therefore, basal cAMP in wild-type h$\beta_2$-AR-expressing cells is defined as 1.0 and is shown by the *dashed line*. In these experiments, isoproterenol caused a 24.5 ± 5.3-fold increase in the basal levels of cAMP in the wild-type h$\beta_2$-AR-expressing cells. L124R cells binding 156 ± 29 fmol pindolol/$10^6$ cells and L124K cells binding 188 ± 33 fmol pindolol/$10^6$ cells were matched to wild-type receptor-expressing cells binding 167 ± 32 fmol pindolol/$10^6$ cells (n = 4 experiments). L124A cells binding 54.4 ± 10.0 fmol pindolol/$10^6$ cells were matched to wild-type-expressing cells binding 49.1 ± 8.7 fmol pindolol/$10^6$ cells (n = 3 experiments). Panel B, cAMP in response to a saturating concentration of isoproterenol is presented as the ratio of Rmax of cells expressing a given mutant to the Rmax of cells expressing the wild-type h$\beta_2$-AR. Therefore, the response of the wild-type h$\beta_2$-AR is defined as 1.0 and is shown by the *dashed lines*. L124R cells binding 131 ± 24 fmol pindolol/$10^6$ cells and L124K cells binding 163 ± 37 fmol pindolol/$10^6$ cells were matched to wild-type receptor expressing cells binding 137 ± 21 fmol pindolol/$10^6$ cells. L124A cells binding 43.9 ± 9.6 fmol pindolol/$10^6$ cells were matched to wild-type-expressing cells binding 45.8 ± 18.2 fmol pindolol/$10^6$ cells. Data shown are the mean ± range of two experiments.

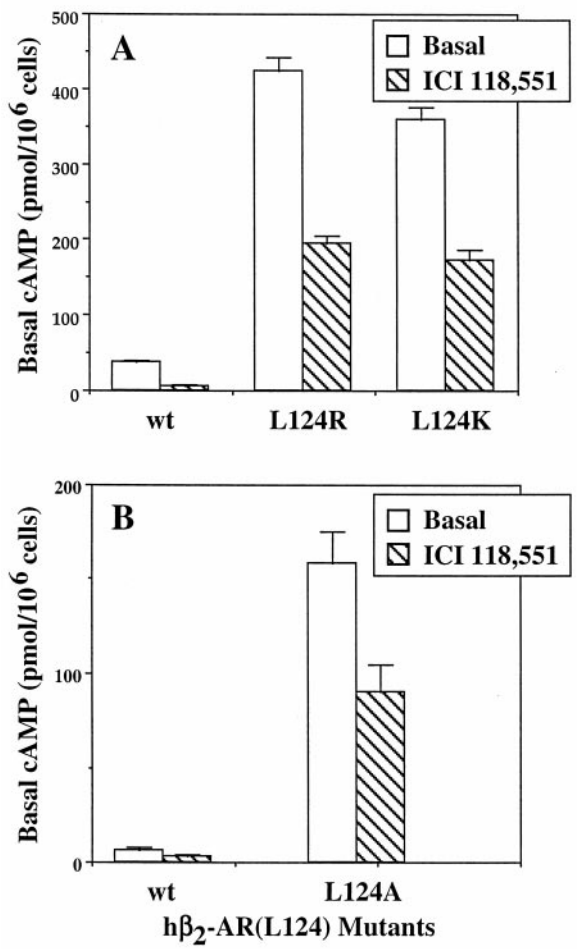


**Fig. 6.** Effects of the Inverse Agonist ICI 118,551 on Basal cAMP Production by 293 Cells Expressing Constitutively Active h$\beta_2$- AR(L124) Mutants

Basal cAMP production measured in the absence or the presence of the inverse agonist was determined in 293 cells expressing the wild-type or L124-substituted h$\beta_2$-AR. The data shown are from one experiment representative of two independent experiments. In this experiment, L124R cells binding 206 fmol pindolol/$10^6$ cells and L124K cells binding 238 fmol pindolol/$10^6$ cells were matched to wild-type receptor-expressing cells binding 227 fmol pindolol/$10^6$ cells. L124A cells binding 74 fmol pindolol/$10^6$ cells were matched to wild-type-expressing cells binding 72 fmol pindolol/$10^6$ cells.

activated states for a given GPCR, some intermediary in nature (designated R′) (16–18). 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.

The FSHR, LHR, and TSHR form a subset of related GPCRs within the superfamily of rhodopsin-like receptors (1–3). In contrast to the hFSHR, numerous activating mutations of both the hLHR and hTSHR have been reported (see Refs. 5 and 4, respectively, for reviews). Furthermore, some mutations within i3 or TM VI of the hLHR that are known to cause constitutive activation have been reported to be without effect when introduced into the hFSHR (8, 9). These observations have led to the speculation that the hFSHR may be more resistant to mutation-induced constitutive activation than the hLHR or hTHSR. However, because the previous mutations that were ineffective in inducing constitutive activation of the hFSHR were located in i3 or TM VI, we chose to examine whether a mutation elsewhere could induce constitutive activation in this receptor. Previous studies had identified a leucine-to-arginine substitution in TM III of the hLHR that results in constitutive activity of the hLHR (10). Because this leucine is highly conserved in the superfamily of rhodopsin-like GPCRs (designated Leu III.18 per the numbering system of Baldwin *et al*. (11), and because this mutation is one of the more robust constitutively activating mutations of the hLHR (10), substitution of the comparable leucine in the hFSHR was chosen as a means to test whether the hFSHR could be made constitutively active.

As shown herein, a L460R substitution in TM III of the hFSHR causes an increase in basal levels of cAMP in cells expressing the mutant hFSHR, consistent with the mutation causing constitutive activity. It should be noted that because there are no known inverse agonists for either the hLHR or the hFSHR, verification of constitutive activation of these receptors by the attenuation of increased basal activity by an inverse agonist cannot be ascertained. Consequently, constitutive activation of the gonadotropin receptors has been defined routinely by an observed increase in basal levels of cAMP in transfected cells under conditions where the cell surface expression of the mutant receptor is comparable to (or in some cases less than) that of the wild-type receptor (5, 7, 19). Our results clearly show that the hFSHR is not entirely resistant to mutation-induced constitutive activation. Of four amino acid substitutions tested (arginine, lysine, alanine, and aspartate), constitutive activation of the hFSHR was only observed by substitution of Leu III.18 with arginine. Therefore, the introduction of a positive charge (*i.e.* lysine) into the III.18 position is not sufficient for conferring constitutive activity.

Having previously shown that a L460R substitution of the hLHR causes constitutive activation (10) and now showing that the comparable L457R substitution of the hFSHR causes constitutive activation, we wished to explore the generality of the substitution of Leu III.18 in causing constitutive activation of GPCRs. Toward this end, we investigated the effects of substituting L124 of the h$\beta_2$-AR. Indeed, substitution of L124 with arginine, lysine, or alanine caused constitutive activation of the h$\beta_2$-AR, as determined by increased levels of basal cAMP in transfected cells that could be attenuated by the inverse agonist ICI 118,551, properties previously shown to be associated with constitutively activating mutants of the h$\beta_2$-AR (13, 14). While our studies were in progress, two other reports were published showing that substitutions of Leu III.18 in the rat $m_1$ muscarinic acetylcholine receptor ($rm_1$AChR) and the C5a receptor (C5aR) cause constitutive activation (12, 20). Both studies used alanine scanning mutagenesis and thus were not focusing on Leu III.18 *per se*. However, for both GPCRs, it was found that one of several alanine substitutions that resulted in constitutive activation included that of the Leu III.18. As of this writing, therefore, constitutive activation by substitution of Leu III.18 has now been shown for five different GPCRs (the hLHR, hFSHR, h$\beta_2$-AR, $rm_1$AChR, and C5aR) which couple to at least two different second messenger pathways. Of these, however, the amino acid requirements for conferring constitutive activation were only examined in the hFSHR and the h$\beta_2$-AR. Whereas any of three amino acids tested (arginine, lysine, alanine) replacing Leu III.18 of the h$\beta_2$-AR resulted in constitutive activation, only one (arginine) of four amino acids tested (arginine, lysine, alanine, and aspartate) replacing Leu III.18 of the hFSHR resulted in constitutive activation. Consequently, alanine scanning mutagenesis of TM III of the hFSHR would not have identified Leu III.18 as a residue whose substitution would cause constitutive activation.

The first report of a mutation causing constitutive activation of a GPCR showed that substitution of an alanine residue in the third intracellular loop of the $\alpha_{1B}$-AR with any other amino acid resulted in constitutive activation of the inositol phosphate second messenger pathway (21). It has since been reported that any residue substituted in place of an aspartate within the highly conserved E/DRY motif at the cytoplasmic end of TM III also results in constitutive activity of this receptor (22). Substitution of these two residues does not, therefore, appear to be selective in that any other residue introduced in their place confers constitutive activity. This has led to the hypothesis that constitutive activation of GPCRs results from the disruption of interhelical bonds that stabilize the receptor in an inactive state. However, in many other studies examining constitutive activation of GPCRs (including the hLHR) as a result of different amino acid substitutions of a given residue, there appears to be a selectivity as to which newly introduced residues can confer constitutive activity (see Refs. 23–26 for examples). In these cases, as with Leu III.18 of the hFSHR, some, but not all substitutions of a given residue result in constitutive activation. The hypothesis that constitutive activation arises solely as a result of disruption of interhelical bonds that stabilize the receptor in an inactive state cannot fully account for these observations. Rather, in these cases, a more likely hypothesis

is that constitutive activity arises by the disruption of interhelical bonds stabilizing the inactive state as well as formation of other bonds involving the newly introduced residue, which can stabilize an active state of the receptor. It should further be considered that whereas different substitutions causing constitutive activation may stabilize the same activated state, it is also possible that different substitutions impart different interhelical interactions, which in turn stabilize different activated states. An additional point that needs to be taken into consideration is that in the saturation mutagenesis studies of the $\alpha_{1B}$-AR by Cotecchia and colleagues (21, 22) it was observed that the wild-type receptor did not exhibit any basal inositol phosphate production above that seen in mock transfected cells. This lack of basal activity by the wild-type receptor is unusual and may contribute to the lack of selectivity reported in these studies.

Molecular modeling of the GPCR helices based upon the known structure of rhodopsin predicts that Leu III.18 lies approximately midway in the third transmembrane helix between the predicted cytoplasmic and extracellular boundaries and that it faces inward toward the crevice formed by the transmembrane helices (11). Studies using different experimental approaches have suggested that both the ligand-induced and mutation-induced activation of GPCRs involves the movement of helices III and VI relative to each other (16, 27–30). Based on the data from these studies, the hypothesis has been put forth that helices III and VI serve as switches for GPCR activation (12). The conserved nature of Leu III.18, coupled with its position in a region postulated to be a switch region for GPCR activation, suggests that it may serve a conserved function in GPCRs by being one of several residues on this face of TM III that serve to help stabilize the receptor in an inactive conformation. Interestingly, residues in TM III of the TSHR predicted to lie on the same face as Leu III.18 have also been shown to cause constitutive activation. For example, naturally occurring mutations resulting in the substitution of S505 with either arginine or asparagine or the substitution of S509 with alanine result in TSHR that constitutively activates the cAMP pathway (31–33).

Whereas the L460R substitution in the hFSHR causes constitutive activation, it also renders the receptor unresponsive to further hormonal stimulation, in spite of the fact that the mutant binds hFSH with the same affinity as the wild-type receptor. Because cells expressing the mutant hFSHR have elevated levels of basal cAMP, the ability of the mutant to activate cAMP synthesis is at least partially intact. Therefore, it is likely that the impairment in FSH-stimulated cAMP production by hFSHR(L460R) is restricted to the transduction of agonist binding to cAMP accumulation. Whether this impairment is at the level of the receptor itself or reflects a postreceptor event remains to be elucidated. Interestingly, substitutions of hFSHR(L460) with either lysine, alanine, or aspartic acid also resulted in decreased FSH-stimulated cAMP. Unlike the arginine substitution, however, they did not also cause constitutive activation. Although we cannot yet conclude whether or not the different substitutions mediate decreased hormonal responsiveness by the same mechanism, the data thus far suggest that Leu460 may play a critical role in FSH-stimulated cAMP production by the hFSHR. It has previously been shown that the comparable L457R mutant of the hLHR, which is constitutively active, is also relatively unresponsive to further hormonal stimulation (10). However, as shown herein, when Leu III.18 in the h$\beta_2$-AR was substituted with arginine, lysine, or alanine, the resulting mutants were both constitutively active and fully responsive to additional stimulation by isoproterenol. Therefore, in contrast to its apparent role in stabilizing the inactive state of GPCRs, the role of Leu III.18 in hormone-stimulated cAMP production is not universal among all GPCRs. Rather, it appears to be restricted to the gonadotropin receptors (or possibly even the glycoprotein hormone receptors if the TSHR is found to behave similarly).

One possible mechanism by which substitutions of L460 in the hFSHR might lead to an impairment of FSH-stimulated cAMP production in intact cells is the potential increased internalization of the hormone-receptor complex by the mutants. Indeed, when Leu III.18 of the rat LHR (rLHR) is substituted with arginine, the resulting receptor internalizes hCG at a much faster rate than the wild-type receptor (34). Interestingly, although cells expressing this rLHR mutant do not respond to hCG with increased cAMP, isolated membranes derived from these cells are fully responsive to hCG (34). These data suggest that, for the rLHR, mutation of Leu III.18 to arginine does not impair hCG-induced coupling of the receptor to Gs, but rather it induces a faster rate of internalization of the hormone-receptor complex, thus terminating the signal. However, for the hFSHR, our data clearly show a lack of correlation between decreased FSH-stimulated cAMP production and increased rates of internalization of the hormone-receptor complex in cells expressing Leu III.18-substituted hFSHR mutants. Thus, whereas arginine, lysine, alanine, and aspartate substitutions of hFSHR(L460) resulted in decreased hFSH responsiveness, only the mutants with arginine and lysine substitutions internalized hCG at a faster rate. Whether the correlation between decreased hormonal responsiveness and increased rates of internalization in the rLHR and the lack thereof in the hFSHR are due to a difference between species (*i.e.* human *vs.* rat) and/or LHR *vs.* FSHR remains to be determined.

In summary, these studies unequivocally show that the hFSHR can be made constitutively active. The observations that substitutions of Leu III.18 cause constitutive activation of several GPCRs, but that the substitutions rendering constitutive activity are receptor specific, suggest that Leu III.18 contributes to maintaining rhodopsin-like GPCRs in an inactive state. However, specific substitutions are required to stabilize an active conformation. Lastly, Leu III.18 also is

involved in the agonist-mediated activation of the cAMP pathway of the gonadotropin receptors, but not other GPCRs. Further studies identifying the rearrangements of interhelical bonds that occur as a result of the substitutions of Leu III.18 should provide a more detailed understanding for the mechanisms of mutation-induced *vs.* hormone-induced activation of the gonadotropin receptors.

## MATERIALS AND METHODS

### Plasmids and Cells

The wild-type hFSHR cDNA was kindly provided by Ares Advanced Technology (Ares-Serono Group, Randolph, MA), and the h$\beta_2$-AR was obtained from the American Type Culture Collection (ATCC CRL 57536, Manassas, VA). Each was subcloned into pcDNA 3.1 (Invitrogen). Mutagenesis was performed using the PCR overlap extension method (35, 36). The entire region amplified by PCR, as well as the sites of ligation, was sequenced to ensure the fidelity of the mutant 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 a culture medium consisting of high- glucose DMEM containing 50 $\mu$g 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. For competition binding experiments on cells expressing the h$\beta_2$-AR, cells were plated on uncoated 100-mm dishes. Cells were transiently transfected when they were 50–70% confluent following the protocol of Chen and Okayama (37) except that the overnight precipitation was performed in a 5% $CO_2$ atmosphere. Cells were then washed with Waymouth's MB752/1 media modified to contain 50 $\mu$g gentamicin and 1 mg/ml BSA, after which fresh growth media were added. The cells were used for experiments 24 h later.

### Binding Assays to Intact Cells Expressing the hFSHR

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 washed two times with warm Waymouth's MB752/1 containing 50 $\mu$g/ml gentamicin and 1 mg/ml BSA. To determine the maximal binding capacity, the cells were then incubated 1 h at 37 C in the same media containing a saturating concentration of $^{125}$I-hFSH (500 ng/ml final concentration) with or without an excess of unlabeled PMSG (480 IU/ml final concentration). To determine the binding affinity, the cells were incubated with increasing concentrations of $^{125}$I-hFSH in the presence or absence of unlabeled PMSG. The assay was finished by washing the cells three times with cold HBSS modified to contain 50 $\mu$g/ml gentamicin and 1 mg/ml BSA. The cells were then solubilized in 100 $\mu$l of 0.5 N NaOH and transferred to plastic test tubes with cotton swabs. Apparent binding affinities were determined as the concentrations of $^{125}$I-hFSH yielding half-maximal binding as calculated by the Deltapoint software (DeltaGraph, Monterey, CA) when the data were fit to a sigmoidal equation (38).

Admittedly, the binding of $^{125}$I-hFSH was not performed under equilibrium conditions. Although ideally the binding of hormone should be performed at 4 C, conditions in which no internalization occurs, the binding of $^{125}$I-hFSH at 4 C results in unacceptably high levels of nonspecific binding. The conditions described (1 h at 37 C), however, were found to yield reasonable levels of nonspecific binding (*i.e.* $<$20% of total counts per min bound). In contrast to hFSH binding to the hFSHR, hCG binding to the hLHR can readily be measured at 4 C. Therefore, we compared the binding of $^{125}$I-hCG overnight at 4 C *vs.* 1 h at 37 C to cells expressing the hLHR(wt) or hLHR(L457R) mutant, which like the hFSHR(L460R) mutant internalizes hormone at a faster rate. Although the absolute numbers differed between the two assay conditions, the relative amount of binding to wild-type *vs.* mutant receptor was the same. By extrapolation, we feel it reasonable to assume that the relative amounts of wild-type *vs.* mutant hFSHR determined by the bindings at 1 h at 37 C are a good approximation.

### Binding Assays to Intact Cells Expressing the h$\beta_2$-AR

To determine cell surface receptor numbers, bindings were performed to intact cells. Dishes were placed on ice for 10 min and subsequently washed twice with ice-cold Waymouth's MB752/1 medium supplemented with 1 mg/ml BSA and 50 $\mu$g/ml gentamicin but lacking bicarbonate. Dishes were incubated overnight with a saturating concentration of [$^{125}$I]-pindolol (200 pM, final concentration) and either cold buffer A (150 mM NaCl, 20 mM HEPES, 2.1 mM ascorbic acid, pH 7.4) to measure total binding, a saturating concentration of propranolol dissolved in cold buffer A (1 $\mu$M, final concentration) to measure nonspecific binding, or CGP 12177 dissolved in cold buffer A (0.3 $\mu$M, final concentration) to determine cell surface expression. Dishes were incubated overnight in the dark at 4 C. The following day, cells were scraped, rinsed, and transferred into 12 $\times$ 75 tubes. Bindings were terminated by rapid filtration over filters (Whatman, Clifton, NJ) preincubated for 1 h in 3% BSA dissolved in PBS. Filters were washed five times with iced 1% BSA in PBS, dried, and counted for 1 min with a $\gamma$ counter. All points were in duplicate. Specific binding was calculated as the difference between total and nonspecific binding. Cell surface binding was calculated from the percentage of total binding displaced by the hydrophilic ligand CGP-12177.

### Measurement of cAMP Production

In each experiment in which cAMP production was measured, the levels of cell surface receptors were measured within the same experiment. Only those experiments in which the numbers of cell surface receptors for wild-type *vs.* mutant receptors differed by no more than 2-fold were used for cAMP analyses. 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 MB752/1 media containing 50 $\mu$g/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 hormone was added (hFSH at 100 ng/ml final concentration, isoproterenol at 1 $\mu$M final concentration, or ICI 118, 551 at 10 $\mu$M, final concentration) or buffer only and the incubation was continued for 60 min at 37C. 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 $\mu$g/ml theophylline and then measured by RIA. All determinations were performed in triplicate.

### Internalization of hFSH

The hFSHR-mediated internalization of hFSH was measured following the protocol described by Ascoli and colleagues (39). 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 37 C. $^{125}$I-hFSH was then added to give a final concentration of 40 ng/ml (with or without an excess of PMSG 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 $\mu$g/ml gentamicin and 1 mg/ml BSA. The surface-bound $^{125}$I-hFSH 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 (40, 41). The acid washes from each well were combined and counted to determine the amount of surface-bound $^{125}$I-hFSH. 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-hFSH (42). Under the experimental conditions used herein, the internalization index accurately reflects the rate of internalization (39, 42). The rate of internalization is a first order rate constant (39, 42) and is, therefore, independent of the concentration of receptor or hormone. Therefore, for these experiments no effort was made to standardize the numbers 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-hFSH.

### Hormones and Supplies

Purified hFSH and PMSG were provided by NIDDK's National Hormone and Pituitary Program and A. F. Parlow. hFSH was iodinated following the procedure described for the iodination of hCG (43). $^{125}$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 plasticwares (Corning, Inc., Corning, NY) were obtained from Fisher Scientific (Pittsburgh, PA). [$^{125}$I]-pindolol was purchased from NEN Life Science Products (Boston, MA). ICI 118,551 and CGP 12177 were from RBI (Natick, MA). General chemicals, including (-) isoproterenol, were purchased from Sigma (St. Louis, MO).

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