Evidence That Constitutively Active Luteinizing Hormone/Human Chorionic Gonadotropin Receptors Are Rapidly Internalized[†,‡]

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ABSTRACT: The luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor, which belongs to the family of G-protein coupled receptors, plays an important role in gonadal steroidogenesis. Substitution of aspartic acid 556 of the LH/hCG receptor with glycine (D556G) creates a constitutively active receptor that activates adenylyl cyclase in the absence of hormone. To examine receptor internalization, human embryonic kidney cells (293 T) expressing wild type (WT) or D556G mutant receptors were incubated with [^{125}I]hCG and subsequently analyzed for cell surface bound and internalized radioactivity. Comparison of the rate constants of internalization of the D556G mutant and WT receptors revealed that the rate of internalization of the D556G mutant was five times greater than that of the WT receptor. Although the D556G receptor internalizes [^{125}I]hCG rapidly, a corresponding increase in [^{125}I]hCG degradation was not seen. The internalization of another constitutively active LH/hCG receptor (aspartic acid 556 to tyrosine) was also greater than that of the WT receptor. Internalization of receptor bound [^{125}I]hCG was inhibited by a hypertonic sucrose solution, confirming that the ligand enters the cell by receptor-mediated endocytosis. Furthermore, the constitutively active D556G and D556Y LH/hCG receptors utilize the arrestin dependent internalization pathway. These results suggest that the active state conformation of the constitutively active receptor is conducive to rapid internalization.

The luteinizing hormone (LH)[1] and human chorionic gonadotropin (hCG) receptor belongs to the large family of G-protein coupled receptors (*1*, *2*) Within this family the receptor falls into a subgroup that includes other glycoprotein hormone receptors such as the thyrotropin receptor and the follicle stimulating hormone receptor that have large N-terminal extracellular domains (*3*, *4*). Both the pituitary hormone, LH, and its placental counterpart hCG bind the receptor with high affinity (*5*). Binding of either hormone causes the heterotrimeric Gs-protein to exchange GDP for GTP and activation of adenylyl cyclase to yield cyclic AMP (*6*, *7*). The subsequent activation of protein kinase A by cyclic AMP leads to increased steroidogenesis in gonadal tissues (*8*). LH/hCG is also known to stimulate phospholipase C resulting in the hydrolysis of phosphatidylinositol in cells expressing the LH/hCG receptor (*9*−*12*).

Several mutations have been identified in the LH/hCG receptor that activate the receptor in the absence of LH or hCG (*13*−*24*). The first mutation identified was the substitution of aspartic acid in the sixth transmembrane domain with glycine (*25*−*27*). This mutant receptor was identified in males with gonadotropin-independent, male-limited precocious puberty and transfection of the mutant receptor cDNA into COS-7 cells showed that the receptor stimulated cyclic AMP production in the absence of LH or hCG (*26*). Subsequent studies showed that mutation of this aspartic acid to tyrosine also produces constitutive activation of the LH/hCG receptor, and this mutation has been identified in males with precocious puberty (*13*, *16*, *20*).

We have shown previously that mutation of conserved aspartic acid residue 556 in the sixth transmembrane domain of the rat LH/hCG receptor to glycine (D556G) also produces a constitutively active mutant receptor (*28*). Pulse chase studies showed that unlike the wild type (WT) receptors most of the mutant receptors were trapped in the endoplasmic reticulum (*28*). However, binding assays using intact cells showed that low levels of the constitutively active mutant receptors were present at the cell surface indicating that some of the mutant receptors were processed through the Golgi and trafficked to the cell surface (*28*). The mutant receptor at the cell surface binds hormone with high affinity (*28*).

Several studies have shown that the LH/hCG receptor undergoes internalization following binding of LH or hCG (*29*−*32*). We investigated whether the activated state of the constitutively active receptor is conducive to increased internalization compared to WT receptors. Our results show that the constitutively active D556G and D556Y LH/hCG receptors are internalized more rapidly than the WT receptor, suggesting that rapid internalization may be property of constitutively active receptors.

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[1] Abbreviations: LH, luteinizing hormone; hCG, human chorionic gonadotropin; WT, wild type; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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EXPERIMENTAL PROCEDURES

Materials

Human chorionic gonadotropin (3000 IU/mg, EEC No. 232-660-2) for nonspecific binding, NH_4Cl, Chloramine T, and 3-isobutyl-1-methylxanthine were purchased from Sigma. Leupeptin and sucrose were purchased from Boehringer Mannheim. The sodium salt of ^{125}I was purchased from ICN. All other chemicals used were reagents purified for use in molecular biology studies. The GeneEditor in vitro site-directed mutagenesis system was purchased from Promega. Highly purified hCG (CR 127) was a gift from the Center for Population Research, NICHD, National Institutes of Health, distributed through the National Hormone and Pituitary Program. The human cytomegalovirus promoter-driven vector, pCMV4, was a gift from Dr. D. Russell, University of Texas Southern Medical Center, Dallas, TX (*33*). Human embryonic kidney cells expressing the SV40 large T antigen (293 T cells) were a gift from Dr. G. P. Nolan, Stanford University, Stanford, CA. Arrestin-3, β-arrestin, and β-arrestin-(319−418) cDNAs were a gift from Dr. J. L. Benovic, Thomas Jefferson University, Philadelphia, PA.

Methods

Site-Directed Mutagenesis of the LH/hCG Receptor. Cloning of the WT LH/hCG receptor into the eukaryotic expression vector, pCMV4, and preparation of the D556G LH/hCG receptor has been described previously by our laboratory (*28*, *34*). The GeneEditor in vitro site-directed mutagenesis system from Promega was used to generate the D556Y mutation. The plasmid WT LH/hCGR-pCMV4 was alkaline denatured for 20 min at room temperature followed by annealing to the mutagenic oligo (CTCATCTTCACAT-ACTTCACGTGCA) and the Bottom strand selection oligo provided by Promega. The remaining procedure was performed as described in the GeneEditor protocol provided by Promega. The mutation was confirmed by dideoxy chain termination sequencing.

Cell Culture and Transfection. Cell culture and transfection were performed as described previously (*28*) except that all experiments were performed using cells grown in 10 cm plates. In Figures 1−5, and 8 and Table 1 the cells were transfected with 10 μg WT or 10 μg mutant (D556G or D556Y) LH/hCG receptor cDNA. In Figures 6, 7, and 9 and Table 2 the cells were transfected with cDNA quantities necessary to give equivalent expression of WT and mutant receptors at the cell surface. The concentrations of cDNAs added to the cells are given in the figure legends.

Receptor Bound [^{125}I]hCG Internalization Assay. Transfected 293 T cells were harvested with phosphate-buffered saline containing 1 mM EDTA (ethylenedinitrilotetraacetic acid), pH 7.4 (PBS-EDTA), and centrifuged at 250*g* for 5 min at room temperature. The cell pellets were resuspended in 8 mL assay medium (Waymouth's MB752/1 medium, 20 mM Hepes, 2.24 g/L $NaHCO_3$, 50 μg/mL gentamicin, and 0.1% bovine serum albumin (BSA), pH 7.4) maintained at 37 °C. Aliquots of cells were assayed for DNA content to ensure that equivalent numbers of WT and mutant receptor expressing cells were present in the internalization assay (*35*). To measure internalization, 0.2 mL of the cell suspension ((3−5) × 10^5 cells) was incubated with a saturating concentration of 100 ng/mL [^{125}I]hCG in a shaking water bath set at 50 strokes/min and 37 °C for 0−60 min. The [^{125}I]hCG was added immediately prior to the incubation. Highly purified hCG CR127 was iodinated by the chloramine T method to a specific activity of 30 000−50 000 cpm/ng as described previously (*36*). Nonspecific binding of [^{125}I]hCG was determined by incubating the cells with a 1000-fold excess unlabeled hCG. The internalization reactions were terminated by the addition of 2 mL of cold assay media, and the cells were pelleted at 250*g* for 5 min at 4 °C. The supernatant was removed, and the wash was repeated. The cell pellets were incubated with a low pH buffer (100 mM NaCl, 50 mM glycine, pH 3) at 4 °C for 5 min to remove cell surface bound [^{125}I]hCG. The cells were repelleted and the supernatant was saved. The cell pellets were then rinsed once with low pH buffer, and the supernatants were combined and counted in a γ-counter. The supernatant fractions represent [^{125}I]hCG that was bound to LH/hCG receptor on the cell surface. The cell pellets were also counted in a γ-counter and represent internalized [^{125}I]hCG. Using this procedure, more than 90% of the surface bound [^{125}I]hCG was removed (data not shown).

Determination of the Rate Constant of Internalization. To determine the rate of internalization, surface bound and internalized [^{125}I]hCG were measured at 5 min intervals during a 30 min incubation period as described above. The rate constant of internalization was calculated from the slope of the line generated by the first-order rate equation, $kt = 2.3 \log[(\text{surface bound plus internalized})/(\text{surface bound})]$ (*37*).

Inhibition of Receptor Bound [^{125}I]hCG Internalization. To determine the effect of temperature on internalization, 0.2 mL of cells ((3−5) × 10^5 cells) was incubated with 100 ng/mL [^{125}I]hCG at 37 °C with shaking for 10 min. A 2 mL volume of assay medium was added to the cells followed by centrifugation at 250*g* for 5 min at room temperature. The supernatant was aspirated and the washing procedure repeated. The cell pellet was resuspended in 0.2 mL of assay medium and incubated at 37 or 0 °C for 50 min in order to allow internalization to proceed. The cells were then centrifuged at 250*g* for 5 min at 4 °C and the media aspirated. The [^{125}I]hCG remaining at the cell surface was removed by incubation with the low pH buffer as described in the internalization assay.

In the experiments using sucrose, (3−5) × 10^5 cells were resuspended in 0.3 mL of 0.6 M sucrose. After the addition of [^{125}I]hCG (final concentration 100 ng/mL), the final sucrose concentration was 0.45 M. The cells were then incubated with shaking at 37 °C for 30 min. The surface bound [^{125}I]hCG was removed by incubation with low pH buffer as described in the internalization assay.

Internalization of Receptor. 293 T cells ((3−5) × 10^5) expressing WT or D556G receptors were incubated with 100 ng/mL of unlabeled highly purified hCG at 37 °C with shaking for 0, 5, and 60 min. Cell surface bound hCG was removed by incubation with low pH buffer as described earlier in the internalization assay. After the final acid wash, the cells were incubated with 100 ng/mL [^{125}I]hCG for 20 h at 4 °C. At the end of the incubation, 2 mL of cold assay medium was added and the cells were centrifuged at 2000*g* for 10 min at 4 °C. The cells were washed again, and the cell pellet was counted in a γ-counter.

Assay of Receptor Bound [^{125}I]hCG Degradation. Degradation of [^{125}I]hCG was determined by incubating (3−5) × 10^5 cells with 100 ng/mL [^{125}I]hCG at 37 °C for 15 min. The cells were washed twice with 1 mL of assay medium and centrifuged at 250*g* for 5 min at room temperature. The cells were resuspended in 0.2 mL of assay medium and incubated with shaking at 37 °C for 0, 1, 2, 3, or 4 h. At the end of the incubation, 0.8 mL of assay medium was added and the cells were centrifuged at 250*g* for 5 min at room temperature. The pellets were processed to determine cell surface and internalized [^{125}I]hCG as described earlier. The supernatants were saved for assay of acid soluble radioactivity. A 0.2 mL volume of Waymouth's MB 752/1 medium containing 2% BSA, 20 mM Hepes, 2.24 g/L $NaHCO_3$, and 50 μg/mL gentamicin was added to the supernatant followed by the addition of 0.3 mL of 50% TCA. After a 1 h incubation at 4 °C, the tubes were centrifuged at 2000*g* for 10 min at 4 °C. The supernatants were saved, and the pellets were resuspended in 0.5 mL of 10% TCA and centrifuged as above. The supernatants, which represent degraded [^{125}I]hCG, were combined, and radioactivity was measured in a γ-counter.

Inhibition of Receptor Bound [^{125}I]hCG Degradation by Leupeptin and NH_4Cl. To measure inhibition of receptor bound [^{125}I]hCG degradation, (3−5) × 10^5 cells were preincubated with 10 mM NH_4Cl, a lysosomotropic reagent, or 100 μM leupeptin, a lysosomal thiol protease inhibitor, at 37 °C for 1 h. After a 3 h incubation, cell surface, internalized, and degraded [^{125}I]hCG were measured as described in the degradation assay. The inhibitors, 100 μM leupeptin or 10 mM NH_4Cl, were present throughout the experiment.

[^{125}I]hCG Binding to 293 T Cells Expressing Wild-Type or Mutant LH/hCG Receptors. Transfected cells ((3−5) × 10^5) were incubated with increasing concentrations of [^{125}I]hCG (3−120 ng/mL) with shaking at 37 °C for 1 h. At the end of the incubation, 2 mL of assay medium was added to the tubes and centrifuged at 2000*g* at 4 °C for 10 min. The wash procedure was repeated, and the pellets were counted in a γ-counter. The equilibrium dissociation constant (K_d) and maximal binding capacity (B_{max}) were calculated from a Scatchard plot using the Ligand program (*38*). The DNA content of the cells was measured by the method of Burton (*35*).

Determination of the Rate Constant of hCG−Receptor Association, k_1. The rate constant of hCG−receptor association was measured from the slope of the line generated by the second-order equation, $k_1t = [2.303/(a - b)][\log b(a - x)/a(b - x)]$, where "*a*" is the concentration of [^{125}I]hCG, "*b*" is the concentration of receptors, and "*x*" is the amount of "*a*" bound in time "*t*" (*39*, *40*). The concentration of receptors was calculated from the maximal binding capacity (B_{max}) determined from the Scatchard plot of an [^{125}I]hCG binding assay as described above. The "*x*" value was determined by incubating cells with a saturating concentration of [^{125}I]hCG (100 ng/mL) at 37 °C for 5, 10, 20, and 30 min. The specifically bound [^{125}I]hCG was taken as the "*x*" value, and the concentration of [^{125}I]hCG (100 ng/mL) was used as the "*a*" value.

Assay of Cyclic AMP. Transfected cells (5 × 10^5) were incubated with 0.5 mM 3-isobutyl-1-methylxanthine in assay medium at 37 °C for 15 min followed by incubation with 0 or 100 ng/mL hCG at 37 °C for 30 min. At the end of the incubation, ice-cold ethanol was added to give a final concentration of 91% ethanol. The cells were incubated at −20 °C overnight and centrifuged at 2000*g* for 15 min at 4 °C. The supernatants were collected, and the ethanol was evaporated in a Savant Speed Vac at 65 °C. The remaining residue was resuspended in 1 mL of the assay buffer provided in the cyclic AMP [^{125}I] assay system from Amersham Life Sciences, and the assay was performed by following the manufacturer's instructions. The cell pellet was dissolved in 1 mL of 0.1 M NaOH, sonicated briefly, and assayed for protein content using the BCA protein assay reagent by Pierce.

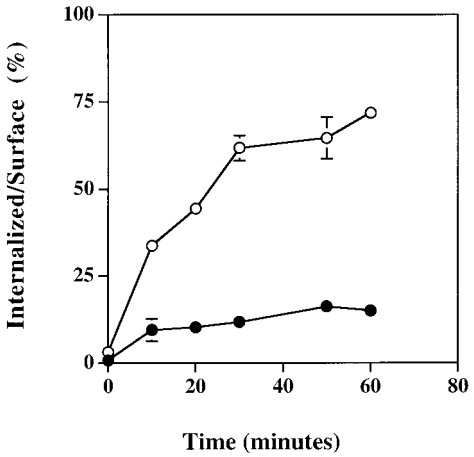
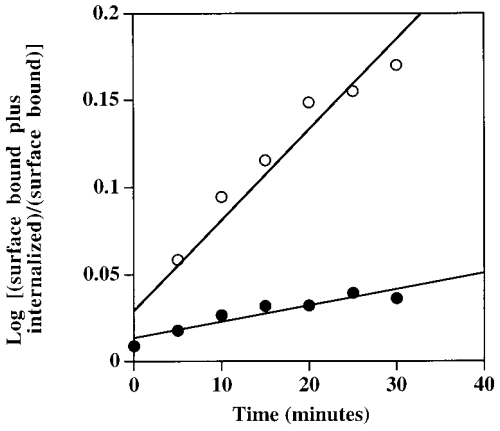


FIGURE 1: Internalization of [^{125}I]hCG bound to WT or D556G LH/hCG receptors. 293 T cells transiently transfected with 10 μg each of WT (●) or D556G (○) LH/hCG receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the absence (total binding) or presence (nonspecific binding) of 100 μg/mL unlabeled hCG for the times indicated. Internalized [^{125}I]hCG and cell surface [^{125}I]hCG were determined as described in the Methods section. Specific binding was determined by subtracting nonspecific binding from total binding. Internalized radioactivity was divided by cell surface bound radioactivity and plotted as a percentage versus time. Each point is the mean of triplicates ± SEM, and the experiment is representative of at least two experiments.

RESULTS

Internalization of [^{125}I]hCG by 293 T Cells Expressing Wild Type or D556G Receptors. Initial experiments were conducted to examine the internalization of the WT or constitutively active, D556G, LH/hCG receptor. To measure internalization, transiently transfected 293 T cells expressing WT or D556G receptors were incubated with a saturating concentration of [^{125}I]hCG at 37 °C for 0−60 min. At the end of the incubation, the cell surface associated and the internalized radioactivity were determined as described in the Methods section. Figure 1 shows that, for each incubation period, the ratio of internalized to cell surface [^{125}I]hCG was higher in cells expressing D556G receptor than in cells expressing WT receptor. After 10 min of incubation, there was 4-fold more internalization of D556G receptor bound [^{125}I]hCG than [^{125}I]hCG bound to WT receptor. At the end of the 60 min incubation, the internalization of the D556G receptor was 5-fold higher than the WT receptor.

The rate constants of internalization of WT or D556G receptors were then determined. Surface bound and internalized [^{125}I]hCG were measured again at 5 min intervals during



Receptor	Rate Constants (min^{-1})
WT	2.30×10^{-3}
D556G	1.15×10^{-2}

FIGURE 2: Determination of the rate constants of internalization of WT or D556G LH/hCG receptors. 293 T cells transiently transfected with 10 μg each of WT (●) or D556G (○) LH/hCG receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the absence (total binding) or presence (nonspecific binding) of 100 μg/mL unlabeled hCG for the times indicated. Internalized [^{125}I]hCG and cell surface [^{125}I]hCG were determined as described in the Methods section. Specific binding was determined by subtracting the nonspecific binding from the total binding. The graph was generated using the equation kt = 2.3 log[(surface bound plus internalized)/(surface bound)]. Each point is the mean of duplicates ± SEM.

the first 30 min, since internalization plateaued after this period (Figure 1). A plot of [^{125}I]hCG internalized by receptor versus incubation time showed that internalization the LH/hCG receptor followed first-order kinetics (data not shown). Internalization of other receptors has also been shown to be a first-order reaction (*41*). The rate constants of internalization of the WT and D556G receptors were determined from the slopes derived from Figure 2. The internalization rate constant of [^{125}I]hCG bound to the D556G mutant receptor was 5-fold higher than [^{125}I]hCG bound to the WT receptor (1.15×10^{-2} and 2.30×10^{-3} min^{-1}, respectively). The 5-fold increase in [^{125}I]hCG bound D556G receptor internalization over the WT receptor was reflected in the half-lives, which were 60 and 300 min, respectively ($t_{1/2} = 0.69/k$).

Inhibition of Internalization of [^{125}I]hCG by 293 T Cells Expressing Wild Type or D556G Receptors. To further characterize the internalization process, the internalization assay was performed under conditions known to block receptor mediated endocytosis. Exposure of cells to low temperatures is expected to block receptor internalization because internalization is an energy dependent process (*31*, *42*). Since binding of [^{125}I]hCG occurs more slowly at 0 °C than at 37 °C, 293 T cells expressing WT or D556G mutant receptors were incubated with [^{125}I]hCG at 37 °C for 10 min, to ensure that binding had occurred in cells that would subsequently be incubated at 0 °C. The 10 min incubation time period was chosen because, as shown in Figure 1, internalization of the D556G receptor continues to occur rapidly after 10 min. Therefore, it would be possible to show changes in endocytosis at different temperatures after the

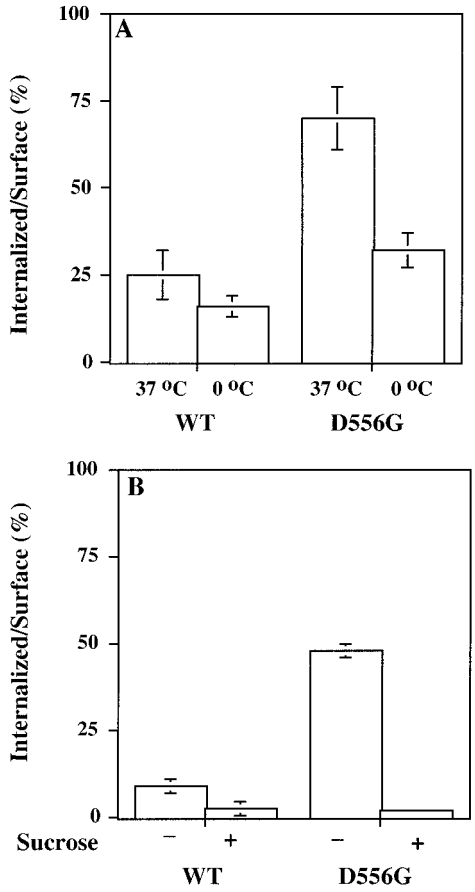


FIGURE 3: Inhibition of internalization of WT or D556G LH/hCG receptors. (A) 293 T cells transiently transfected with 10 μg each of WT or D556G LH/hCG receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C for 10 min, followed by washing and incubation at 37 °C (open bar) or 0 °C (gray bar) for 50 min. (B) 293 T cells transiently transfected with 10 μg each of WT or D556G LH/hCG receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG in the absence (open bar) or presence (gray bar) of 0.45 M sucrose at 37 °C for 30 min. For each inhibitor, nonspecific binding was determined by incubating the cells with 100 μg/mL unlabeled hCG. Specific binding was calculated by subtracting nonspecific binding from total binding. Internalized [^{125}I]hCG and cell surface [^{125}I]hCG were determined as described in the Methods section. Internalized radioactivity was divided by cell surface bound radioactivity and plotted as a percentage. Each column represents the mean ±SEM and is representative of three experiments for each treatment.

10 min incubation period. The cells were then washed to remove free [^{125}I]hCG and incubated at 37 or 0 °C for 50 min. Cell surface and internalized [^{125}I]hCG were determined as described in the Methods section. The results presented in Figure 3A show that the D556G receptor was internalized twice as rapidly at 37 °C compared to 0 °C. The corresponding change for WT was small and not significant, reflecting the low rate of internalization of the WT receptor.

In addition to low temperatures, exposure of cells to hypertonic solutions of sucrose prevents internalization by blocking clathrin-coated pit formation (*43*, *44*). To determine the effect of hypertonic media on internalization of [^{125}I]-hCG by WT or D556G receptors, 293 T cells expressing either receptor were incubated with 100 ng/mL [^{125}I]hCG in the presence of 0.45 M sucrose at 37 °C for 30 min. At the end of the incubation, the cells were treated as described in the Methods section to determine cell surface and internalized [^{125}I]hCG. Figure 3B shows that the incubation with sucrose

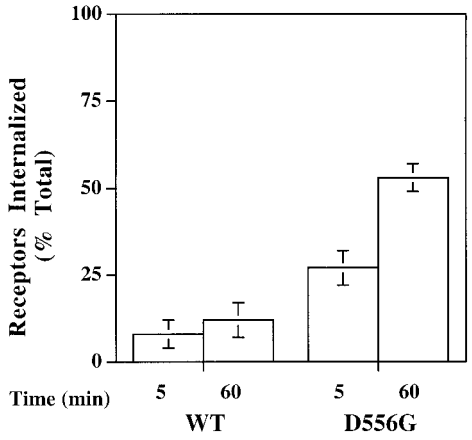


FIGURE 4: Loss of WT or D556G LH/hCG receptors in response to hCG. 293 T cells transiently transfected with 10 μg each of WT or D556G LH/hCG receptor cDNAs were incubated with 100 ng/mL of unlabeled highly purified CR127 hCG at 37 °C for 0, 5 (open bar), or 60 min (gray bar). After being washed with low pH buffer, the cells were incubated with 100 ng/mL [^{125}I]hCG at 4 °C for 20 h. Nonspecific binding was determined by incubating the cells with 100 μg/mL unlabeled hCG. Specific binding was calculated from the difference of total binding and nonspecific binding. The percentage of internalized receptors was determined by subtracting the radioactivity bound to cells in the presence of hCG for 5 or 60 min from the radioactivity bound to cells in the presence of hCG for 0 min. These values were then divided by radioactivity bound to cells in the presence of hCG for 0 min. Each column represents the mean ± SEM of triplicates and is representative of two experiments.

abolished WT and D556G receptor internalization. These results show that the internalization of WT and D556G LH/hCG receptors involves clathrin-coated pit formation.

Loss of Wild Type or D556G Receptors from the Cell Surface. The previous experiments examined the disappearance of [^{125}I]hCG rather than the loss of receptor from the cell surface. To determine specifically receptor loss, 293 T cells expressing WT or D556G LH/hCG receptors were incubated with unlabeled hCG at 37 °C for 0, 5, and 60 min. At the end of the incubation, the hCG bound to the cell surface was removed by incubation with low pH buffer as described in the Methods section. The cells were then incubated with [^{125}I]hCG at 4 °C overnight, and bound [^{125}I]hCG was determined. The results show that exposure of cells expressing D556G receptor to unlabeled hCG for 60 min resulted in a 2-fold increase in the percentage of receptors internalized compared to cells exposed for 5 min (Figure 4). The data corroborate the previous experiments, in which internalization of the WT and D556G receptors was measured by determining internalized [^{125}I]hCG.

Degradation of [^{125}I]hCG Internalized by Wild Type or D556G Receptors. Since the D556G mutant receptor is rapidly internalized, the degradation of [^{125}I]hCG bound to the D556G receptor was investigated. Cells expressing WT or D556G receptors were incubated with [^{125}I]hCG for 15 min. The free [^{125}I]hCG was removed by washing, and the cells were incubated for the times indicated in Figure 5. Degraded [^{125}I]hCG was measured in the TCA soluble fraction. Figure 5A shows that there is very little degradation of [^{125}I]hCG by cells expressing WT receptor. A small increase in degradation of [^{125}I]hCG was seen in cells expressing D556G receptor compared to cells expressing WT receptor (Figure 5B). However, the percentage of [^{125}I]hCG

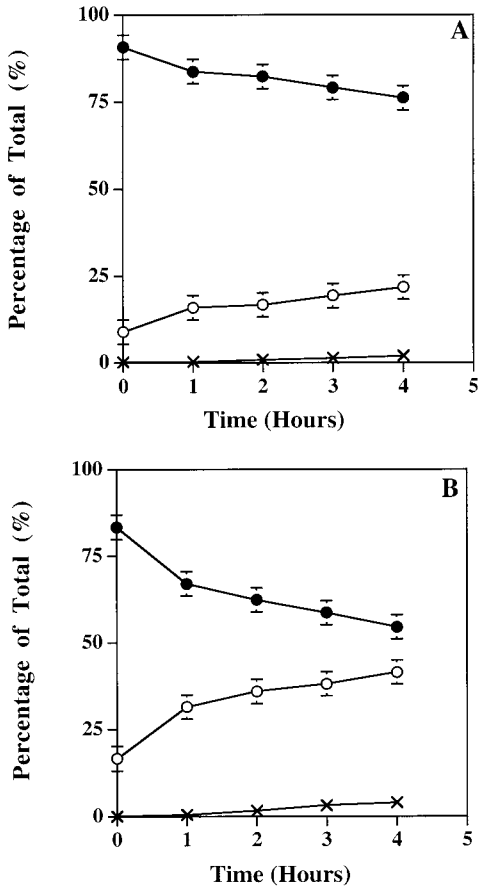


FIGURE 5: Degradation of [^{125}I]hCG internalized by WT (A) or D556G (B) LH/hCG receptors. 293 T cells transiently transfected with 10 μg each of WT (A) or D556G (B) LH/hCG receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C for 15 min followed by washing. The cells were then incubated at 37 °C for the times indicated in the figure. Nonspecific binding was determined by incubating the cells with 100 μg/mL unlabeled hCG. Specific binding was calculated by subtracting nonspecific binding from total binding. At the end of each incubation period, the cells were assayed for surface, internalized, and degraded [^{125}I]hCG as described in the Methods section. The radioactivity associated with the cell surface (●), internalized (○), or degraded (×) fractions were divided by the total of all three fractions. Each point represents the mean ± SEM.

degraded was low despite rapid internalization of [^{125}I]hCG by the D556G receptor.

Degradation of [^{125}I]hCG internalized by the WT receptor is inhibited by the lysosomotropic reagent, NH_4Cl (*30*), and the lysosomal thiol protease inhibitor, leupeptin (*45*). Cells expressing the WT or D556G receptors were incubated with [^{125}I]hCG in the presence or absence of NH_4Cl or leupeptin for 15 min. The cells were washed with assay medium and incubated for an additional 3 h in the presence or absence of NH_4Cl or leupeptin. Both NH_4Cl and leupeptin blocked the small fraction of [^{125}I]hCG degradation observed for both WT and D556G receptor expressing cells (Table 1). The results demonstrate that degradation of [^{125}I]hCG internalized by both the WT and D556G receptors was inhibited by agents that disrupt lysosomal function.

Internalization of [^{125}I]hCG by 293 T Cells Expressing Equal Numbers of Wild Type and D556G Receptors. We have shown previously that cell surface expression of the D556G receptor was only 20% that of the corresponding WT receptor expression despite transfection with equal amounts

Table 1: Inhibition of Degradation of [^{125}I]hCG Internalized by WT or D556G Receptors

		percentage of total (%)		
receptor	treatment	surface	internalized	degraded
WT	none	79 ± 1	19 ± 1	1.41 ± 0.01
	10 mM NH_4Cl	80 ± 2	20 ± 2	0.62 ± 0.04
	100 μM leupeptin	82 ± 4	18 ± 4	0.43 ± 0.16
D556G	none	59 ± 1	38 ± 1	3.10 ± 0.40
	10 mM NH_4Cl	61 ± 3	40 ± 2	0.52 ± 0.36
	100 μM leupeptin	61 ± 1	38 ± 1	0.98 ± 0.59

[a] Following a 1 h preincubation in the presence or absence of 10 mM NH_4Cl or 100 μM leupeptin, 293 T cells transiently transfected with 10 μg each of WT or D556G receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the presence or absence of the degradation inhibitors for 15 min. Unbound [^{125}I]hCG was removed by washing, and the cells were incubated in the presence or absence of the degradation inhibitors at 37 °C for 3 h. Nonspecific binding was determined by incubating the cells with 100 μg/mL unlabeled hCG. Surface associated, internalized, and degraded [^{125}I]hCG were measured as described in the Methods section. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The radioactivity associated with the cell surface, internalized, or degraded fractions were divided by the total of all three fractions. Each column represents the mean ± SEM.

of WT or D556G receptor cDNA (*28*). To establish that the increased internalization of the D556G mutant receptor is not due to decreased cell surface expression compared to WT receptor, we examined internalization of [^{125}I]hCG in cells expressing equal numbers of cell surface WT or D556G receptors. The number of WT receptors at the cell surface was reduced by transfecting 293 T cells with 1.75 μg rather than 10 μg of WT receptor cDNA. A reduction in cell surface WT receptors was confirmed by an [^{125}I]hCG binding assay. Figure 6 A shows that 293 T cells transfected with 1.75 μg of WT cDNA or 10 μg of D556G cDNA expressed comparable levels of cell surface receptors. The B_{max} values for the WT or D556G expressing cells were 1.9 and 2.3 fmol/μg DNA, respectively. The K_d values, 1.5×10^{-9} and 1.8×10^{-9} M, were also comparable for WT and D556G, respectively. Thus, by reducing the amount of WT receptor cDNA transfected, we were able to express comparable numbers of WT and D556G cell surface receptors.

Cells expressing comparable numbers of cell surface WT receptors and D556G mutant receptors were used for the internalization assay. The transfected cells were incubated with a saturating concentration of [^{125}I]hCG at 37 °C for 0−60 min. Cell surface and internalized radioactivity were separated as described in the Methods section. The results show that, at each incubation period, cells expressing D556G receptors internalized more [^{125}I]hCG than cells expressing WT receptors (Figure 6B). Thus, the increased internalization of the D556G receptors is not due to their reduced presence at the cell surface.

hCG-WT or hCG-D556G Receptor Association Rate Constants, k_1. To investigate the possibility that the D556G mutation might increase internalization due to an increase in the rate of association with hCG, we measured the association rate constants (k_1) of D556G receptor and WT receptor. The association of hormone with receptor is a bimolecular interaction (*39*, *46*, *47*). The association rate constants were determined as described in the Methods section and calculated from the slopes derived from Figure 7. The k_1 of the D556G receptor is 1.5-fold higher than the

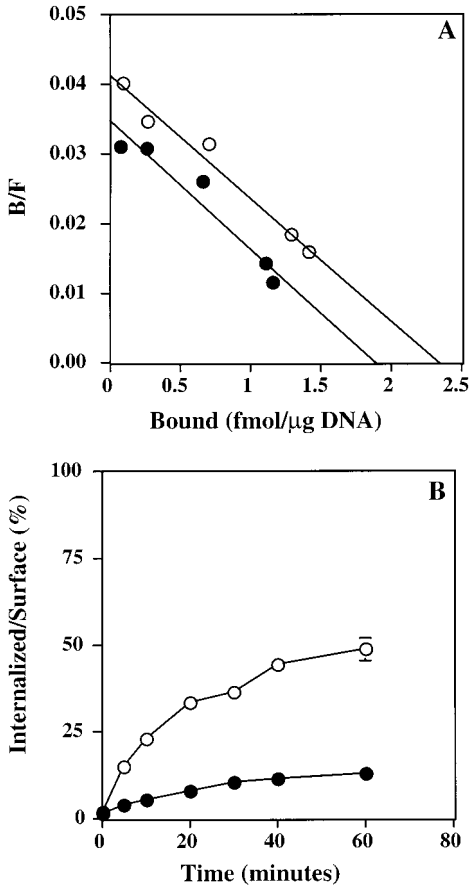
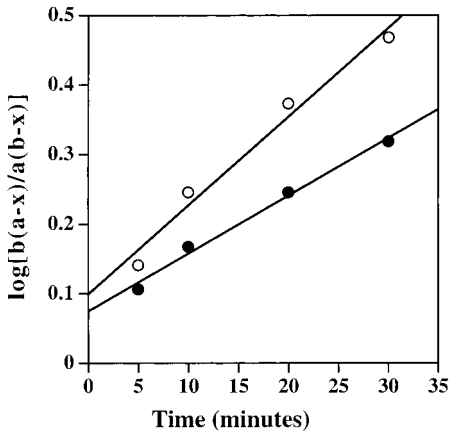


FIGURE 6: Internalization of [^{125}I]hCG by equal numbers of WT and D556G receptors. (A) 293 T cells transiently transfected with 1.75 μg of WT (●) cDNA or 10 μg of D556G (○) cDNA were assayed for [^{125}I]hCG binding as described in the Methods section. Each point is the mean of duplicates and is representative of five experiments (B/F = bound-to-free ratio). (B) 293 T cells transiently transfected with 1.75 μg of WT (●) cDNA or 10 μg of D556G (○) cDNA were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the absence (total binding) or presence (nonspecific binding) of 100 μg/mL unlabeled hCG for the times indicated. Internalized [^{125}I]hCG and cell surface [^{125}I]hCG were determined as described in the Methods section. Specific binding was determined by subtracting nonspecific binding from total binding. Internalized radioactivity was divided by cell surface bound radioactivity and plotted as a percentage versus time. Each point is the mean of triplicates ± SEM.

WT receptor (1.02×10^7 and 0.67×10^7 M^{-1} min^{-1}, respectively). Although k_1 of the D556G receptor is slightly higher than the WT receptor, it does not account for the 5-fold increase of D556G receptor internalization over WT receptor internalization.

Internalization of D556Y LH/hCG Receptor Bound [^{125}I]hCG. To determine if other constitutively active LH/hCG receptors were also more rapidly internalized than WT receptors, we mutated aspartic acid 556 to tyrosine, which has been shown to constitutively activate the human LH/hCG receptor (*13*, *16*, *20*). A comparison of the [^{125}I]hCG binding characteristics of cells transfected with 10 μg of WT, D556Y, or D556G receptor cDNAs are shown in Table 2. The K_d values of the D556Y and D556G receptors were similar to those of the WT receptor. Although the B_{max} value of cells expressing the D556Y receptor was similar to that of WT receptor expressing cells, the B_{max} value of D556G receptor expressing cells was lower. To compare the basal and hCG stimulated cyclic AMP levels of cells expressing



Receptor	Association Rate Constants ($M^{-1}min^{-1}$)
WT	0.67×10^7
D556G	1.02×10^7

FIGURE 7: Determination of the rate constants of hCG-WT receptor and hCG D556G receptor association, k_1. 293 T cells transiently transfected with 1.75 μg of WT (●) cDNA or 10 μg of D556G (○) cDNA were subjected to a binding assay or incubated with 100 ng/mL [^{125}I]hCG at 37 °C for 5, 10, 20, and 30 min as described in the Methods section. Nonspecific binding was measured by incubation in the presence of a 1000-fold excess of unlabeled hCG. Specific binding was calculated by subtracting nonspecific binding from total binding. The graph was generated using the equation $k_1t = [2.303/(a - b)][\log b(a - x)/a(b - x)]$, where "*a*" is the concentration of [^{125}I]hCG, "*b*" is the concentration of receptors and "*x*" is the amount of "*a*" binding in time "*t*". Each point is the mean of triplicates and is representative of two separate experiments.

Table 2: Binding and Cyclic AMP Production Properties of Cells Expressing WT, D556G, or D556Y LH/HCG Receptors

receptor	basal cAMP (fmol/μg of protein)	max cAMP[a] (fmol/μg of protein)	$K_d \times 10^9$ (M)	B_{max} (fmol/μg of DNA)
WT (10)[b]	N.D.[c]	N.D.	3.2	15.7
D556G (10)	N.D.	N.D.	1.8	2.3
D556Y (10)	N.D.	N.D.	2.0	12.9
normalized				
WT (1.75)	28 ± 4	3328 ± 438	1.5	1.9
D556G (10)	289 ± 5	4592 ± 432	1.8	2.3
D556Y (0.5)	300 ± 28	3813 ± 289	1.7	1.8

[a] In the upper half of the table, 293 T cells were transiently transfected with 10 μg of WT, D556G, or D556Y receptor cDNAs. The cells were assayed for [^{125}I]hCG binding as described in the Methods section, and the K_d and B_{max} values were calculated from a Scatchard plot using the program Ligand. To obtain normalized levels of WT, D556G, and D556Y cell surface receptors, 293 T cells were transiently transfected with 1.75 μg of WT receptor cDNA and 8.25 μg of empty pCMV4 vector, 10 μg of D556G receptor cDNA, or 0.5 μg of D556Y receptor cDNA and 9.5 μg of empty pCMV4 vector. The cells were assayed for [^{125}I]hCG binding and cyclic AMP production as described in the Methods section. The basal cyclic AMP values are the mean ± SEM of quadruplicates. The K_d and B_{max} values were calculated from the average of duplicate measurements. [b] Maximal cAMP refers to cAMP produced in response to a maximum stimulatory dose of hCG. [c] The numbers in parentheses refer to the μg of receptor cDNA transfected. [d] N.D. = not determined.

WT, D556G, or D556Y receptors, the number of receptors at the cell surface were normalized by transfecting with 1.75 μg of WT, 10 μg of D556G, or 0.5 μg of D556Y LH/hCG receptor cDNAs. As shown in Table 2, transfecting with these quantities of cDNAs produced cell populations with similar numbers of cell surface receptors as measured by B_{max} values. The basal levels of cyclic AMP were measured by radioimmunoassay and show that D556G and D556Y produce 10.3-fold and 10.7-fold more cyclic AMP than WT receptor in the absence of hCG (Table 2). Thus, mutation of aspartic acid 556 to tyrosine also constitutively activates the rat LH/hCG receptor. However, unlike the human LH/hCG receptor, the tyrosine mutant is not more constitutively active than the glycine mutant. In response to a maximal dose of hCG, both constitutively active mutants produce comparable levels of cyclic AMP. Since we have shown that receptor internalization is independent of receptor number (Figure 6), D556Y internalization was assessed in cells transfected with 10 μg of D556Y LH/hCG receptor cDNA and, therefore, high numbers of D556Y receptors. Internalization was measured by incubating cells expressing WT or D556Y receptors with a saturating concentration of [^{125}I]hCG at 37 °C for the times indicated in Figure 8. The internalized and cell surface associated [^{125}I]hCG were measured as described in the Methods section. Figure 8 shows that, at 30 and 60 min, the D556Y mutant receptor internalized 5-fold more [^{125}I]hCG than the WT receptor. These results suggest that increased internalization is a property of constitutively active receptors generated by mutations at position 556.

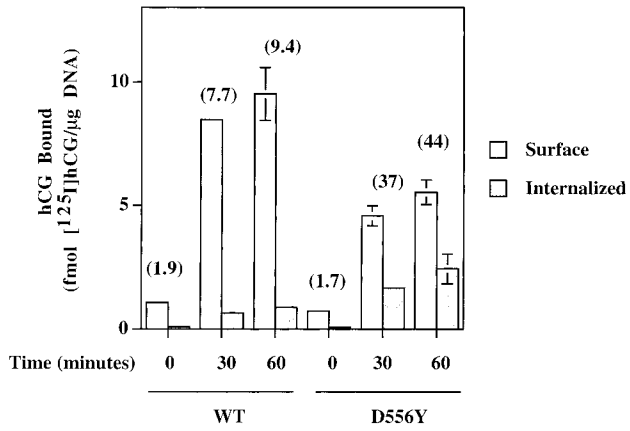


FIGURE 8: Internalization of [^{125}I]hCG bound to WT or D556Y LH/hCG receptors. 293 T cells transiently transfected with 10 μg each of WT or D556Y receptor cDNAs were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the absence (total binding) or presence (nonspecific binding) of 100 μg/mL unlabeled hCG for 0, 30, or 60 min. Internalized [^{125}I]hCG (gray bar) and cell surface [^{125}I]hCG (open bar) were determined as described in the Methods section. Specific binding was determined by subtracting nonspecific binding from total binding. Internalized and cell surface radioactivity were converted to femtomoles of [^{125}I]hCG bound and normalized to DNA. The numbers in parentheses represent internalized radioactivity divided by cell surface radioactivity expressed as a percentage. Each point is the mean of duplicates ± SE and is representative of four experiments.

Internalization of Constitutively Active Receptors in the Presence of Arrestins. The proposed pathway for WT LH/hCG receptor internalization involves phosphorylation of the cytoplasmic tail of the receptor followed by interaction with arrestins which bind to clathrin (*48*). To delve further into the mechanism of constitutively active LH/hCG receptor internalization, the effect of overexpression of arrestins was examined. These studies were performed with 293 T cells expressing equivalent numbers of WT, D556G, or D556Y

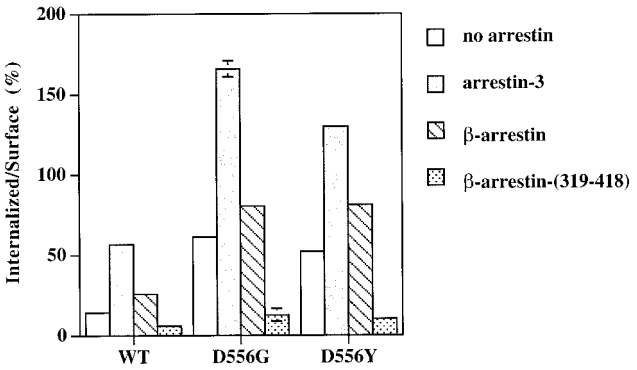


FIGURE 9: Internalization of [^{125}I]hCG bound to WT or constitutively active receptors in the presence of arrestins. For the WT receptor, 293 T cells were cotransfected transiently with 1.75 μg of WT receptor cDNA, 8.25 μg of empty pCMV4 vector, and 10 μg of arrestin-3, β-arrestin, β-arrestin-(319−418), or empty pCMV4 vector. For the D556G receptor, 293 T cells were cotransfected transiently with 10 μg of D556G cDNA and arrestin cDNA or empty pCMV4 vector as described for the WT. For the D556Y receptor, 293 T cells were cotransfected transiently with 0.5 μg of D556Y cDNA, 9.5 μg of empty pCMV4 vector and arrestin cDNA, or empty pCMV4 vector as described for the WT. The total cDNA transfected in all three groups was 20 μg. The transfected cells were incubated with 100 ng/mL [^{125}I]hCG at 37 °C in the absence (total binding) or presence (nonspecific binding) of 100 μg/mL unlabeled hCG for 60 min. Internalized [^{125}I]hCG and cell surface [^{125}I]hCG were determined as described in the Methods section. Specific binding was determined by subtracting nonspecific binding from total binding. Internalized radioactivity was divided by cell surface bound radioactivity and plotted as a percentage versus time. Each point is the mean of triplicates ± SEM, and the experiment is representative of three experiments.

LH/hCG receptors as described in the Methods section. Cells were cotransfected with their respective quantities of LH/hCG receptor cDNA and with 10 μg of arrestin-3, β-arrestin, or β-arrestin-(319−418). Arrestin-3 and β-arrestin are nonvisual mammalian arrestins and β-arrestin-(319−418) is a dominant negative arrestin which is unable to bind phosphorylated receptor (*49*). The cotransfected cells were incubated with a saturating concentration of [^{125}I]hCG at 37 °C for 1 h and processed for an internalization assay as described in the Methods section. As shown in Figure 9, arrestin-3 and β-arrestin increased the internalization of WT, D556G, and D556Y LH/hCG receptors. However, arrestin-3 was a much more potent stimulator of internalization than β-arrestin. A possible explanation for the heightened effect is that arrestin-3 may have a higher affinity than β-arrestin for the cytoplasmic domains of the LH/hCG receptor. The dominant negative mutant β-arrestin-(319−418) reduced the internalization of D556G and D556Y receptors to levels observed for WT receptor in the absence of arrestin-3 or β-arrestin. The dominant negative mutant also decreased WT receptor internalization but to a lesser extent than the constitutively active receptors. These results show that the constitutively active D556G and D556Y receptors utilize an arrestin-dependent pathway for internalization.

DISCUSSION

The results presented here describe the internalization of constitutively active LH/hCG receptors. Since the constitutively active receptor exists in an activated state, we reasoned that this property may have an effect on receptor turnover. The results show clearly that the D556G and D556Y receptors were internalized more rapidly than WT receptors. We calculated that the D556G receptor was internalized at a rate five times greater than the WT receptor. The reduced cell surface receptor density of the D556G receptor compared to the WT receptor does not appear to be the cause of the increased internalization of the D556G receptor. Reduction of the number of cell surface WT receptors to comparable levels of cell surface D556G receptors did not lead to increased WT receptor internalization (Figure 6). Furthermore, both the WT receptor and the constitutively active D556Y receptor were expressed at high levels on the cell surface (Table 2). However, the D556Y receptor is internalized more rapidly from the cell surface than the WT receptor (Figure 8). When the number of D556Y receptors at the cell surface were reduced, the percentage of receptors internalized was similar to that seen in cells expressing high numbers of cell surface D556Y receptors, further excluding the possibility that internalization is dictated by receptor density (Figures 8 and 9). We also investigated the possibility that the D556G mutation increased the rate of association of receptor with hCG and was the reason for the increased rate of internalization. A 1.5-fold increase in the rate of hCG association with D556G receptor over hCG association with WT receptor was observed. However, this increase does not appear to be large enough to account for the 5-fold increase in the rate of D556G receptor internalization over WT receptor.

The cellular processes involved in the internalization of the D556G receptor and the associated degradation of the ligand are the same as for the wild-type receptor. The internalization of both receptors was sensitive to hypertonic media, which disrupts clathrin-coated pit formation. The low level of [^{125}I]hCG degraded by cells expressing WT or D556G receptor was abrogated by a lysosomotropic agent and a lysosomal thiol protease inhibitor. Sensitivity to these agents suggests that internalization of the hormone bound receptors involves clustering of the receptors into clathrin-coated pits followed by endocytosis and transport to the lysosomes for degradation. Although the rate of internalization of [^{125}I]hCG by the D556G mutant receptor was higher than the WT receptor, a corresponding increase in degradation of the receptor bound [^{125}I]hCG was not observed. It appears that increased internalization does not necessarily lead to an increase in degradation. A previous study on D383N and R442H LH/hCG receptor mutants has shown that they have the same degradation rate as the WT receptor despite being internalized more slowly than WT (*50*). Alternatively, degradation of the D556G receptor may be slower than the wild-type receptor. The internalized WT or D556G receptors did not appear to undergo recycling (data not shown), which is in agreement with what others have reported (*32*, *51*, *52*).

While our manuscript was being submitted, Min et al. reported the internalization of the constitutively active D556Y LH/hCG receptor (*53*). These studies show that the basal cyclic AMP expressed as a percentage of maximal hCG stimulated cyclic AMP is 12.3% and 0.6% for cells expressing D556Y or WT LH/hCG receptors, respectively. In comparison, our results show that basal cyclic AMP as a percentage of maximal hCG stimulated cyclic AMP is 7.9% and 0.9% for cells expressing D556Y or WT receptors, respectively (Table 2). Both studies show conclusively that the tyrosine mutation constitutively activates the rat LH/hCG

receptor. We have shown that internalization of the D556Y receptor bound [^{125}I]hCG is almost 5-fold greater than WT receptor bound [^{125}I]hCG. In the study by Min et al., the D556Y receptor internalizes 3-fold more [^{125}I]hCG than the WT receptor. Although there is a small difference in the magnitude of increase in [^{125}I]hCG internalization, both studies independently show that cells expressing the constitutively active D556Y LH/hCG receptor internalize significantly more [^{125}I]hCG than cells expressing the WT LH/hCG receptor.

The underlying mechanism that drives the rapid internalization of the constitutively active receptor is not understood. However, several possibilities exist. Among these, one possibility is that rapid internalization is a consequence of the active state conformation of the constitutively active receptor. Computer modeling has shown that a constitutively active LH/hCG receptor has a different conformation than the WT receptor that is not bound to hormone (*54*). The previous finding that hCG linked to its receptor via a peptide sequence produces a constitutively active receptor suggests that the constitutively active mutant and hormone bound WT receptors have similar conformations (*55*). It has also been shown that the LH/hCG receptor needs to be in an active state to undergo internalization (*50*, *56*). Due to its active state conformation, the constitutively active receptor may reside in clathrin-coated pits prior to binding hCG. When the hormone binds to the constitutively active receptor, it may be rapidly internalized due to the location of the receptor in the coated pit, whereas the WT receptor would not aggregate in the coated pit until it binds hormone. Thus, the active state conformation of the constitutively active receptor is likely to play a role in the rapid internalization by the mutant receptor.

The phosphorylation state of the D556G and D556Y receptors may also provide an explanation for their rapid internalization. It has been shown that the WT LH/hCG receptor is phosphorylated in response to hormone binding and that phosphorylation of the receptor facilitates internalization through an arrestin- and dynamin-dependent pathway (*48*, *57*−*59*). Our studies now show that the constitutively active D556G and D556Y LH/hCG receptor mutants also utilize the arrestin-dependent internalization pathway (Figure 9). Since the constitutively active receptor exists in a semiactive state, it may be possible that the constitutively active receptors are partially phosphorylated prior to hormone binding. Upon binding hCG the constitutively active receptor might be phosphorylated to completion earlier than the WT receptor. Partial phosphorylation of the constitutively active receptor would facilitate interaction with arrestin and eventually, dynamin, resulting in more rapid internalization. Support for partial phosphorylation of constitutively active receptors comes from studies of constitutively active α2, β2-, and α-1b-adrenergic receptors, which exist in a phosphorylated state in the absence of ligand and are further phosphorylated in the presence of hormone (*60*−*62*). It has also been suggested for the LH/hCG receptor that the four serines in the cytoplasmic tail which undergo phosphorylation may be phosphorylated to different extents or by a hierarchical mechanism, further supporting the possibility of partial LH/hCG receptor phosphorylation (*48*). The phosphorylation state of the constitutively active receptor is likely to play an important role in its internalization.

Studies using deglycosylated hCG would argue against the possibility that it is the higher basal levels of cyclic AMP in cells expressing the D556G or D556Y receptors that lead to rapid internalization. Although capable of binding to the receptor, deglycosylated hCG fails to stimulate cyclic AMP production and slows the rate of receptor internalization (*56*). However, the absence of cyclic AMP does not appear to be the reason for decreased receptor internalization since the addition of 8-bromo-cyclic AMP did not lead to increased internalization of deglycosylated hCG (*56*). Additionally, the LH/hCG receptor mutant, D383N, is internalized more slowly than the WT receptor, despite stimulating cAMP production at a level that is comparable to that of the WT receptor (*50*). These observations would argue against the increased intracellular levels of cyclic AMP as a contributing factor in the rapid internalization of the D556G and D556Y receptors.

The results of this study and that by Min et al. show that three different mutations in the transmembrane domains of the LH/hCG receptor that constitutively activate the LH/hCG receptor also lead to increased internalization of [^{125}I]hCG. In contrast, two constitutively activating mutations, D142A and A293E, in the α-1b adrenergic receptor have been shown to have opposite effects on receptor internalization (*62*). The D142A mutant shows decreased internalization compared to the WT receptor, whereas the A293E receptor undergoes increased internalization compared to WT receptor. It would be of interest to determine if the analogous mutations in the LH/hCG receptor would also have opposite effects on receptor internalization.

In conclusion, the present study demonstrates that the constitutively active, D556G and D556Y LH/hCG receptors are rapidly internalized upon interaction with hCG. The rapid internalization may be the result of the constitutively active receptor existing in an active state prior to hormone binding. Furthermore, the constitutively active LH/hCG receptor could serve as an excellent model to examine the regulation of G-protein coupled receptor internalization.

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