Membrane Organization of Luteinizing Hormone Receptors Differs between Actively Signaling and Desensitized Receptors*

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Signaling by the luteinizing hormone/choriogonadotropin receptor (LHR) is of considerable interest because of its requirement for successful reproduction. Time-resolved phosphorescence anisotropy and fluorescence resonance energy transfer were used to investigate the organization of endogenous LHRs in porcine follicular membranes in two distinct signaling states, active and desensitized. Desensitized LHRs exhibited ~3-fold slower rotational correlation times compared with active LHRs (59 ± 4 and 21 ± 9 μs, respectively), suggesting that with agonist-dependent desensitization the receptors are organized into larger protein complexes. Incubation of membranes with inhibitors of LHR desensitization, such as neutralizing anti-arrestin antibodies, a synthetic peptide corresponding to the third intracellular loop of the LHR but not the corresponding scrambled peptide, or catalytically inactive ARNO, resulted in faster rotational diffusion times equivalent to those of actively signaling LHRs. Furthermore, desensitized LHRs exhibited a 2.4-fold increase in fluorescence resonance energy transfer between LHRs suggesting that the larger protein aggregates formed during desensitization contain more self-associated LHRs. These results indicate that agonist-dependent LHR desensitization precedes organization of LHRs at the cells surface into larger protein aggregates.

The luteinizing hormone/choriogonadotropin receptor (LHR) is a seven transmembrane-spanning receptor coupled to G-proteins, most typically the stimulatory guanine nucleotide-binding proteins, which activate adenyl cyclase to produce the second messenger signal, cAMP. Mechanisms involved in signal transduction by the LHR are of considerable interest because of the importance of the receptors in mammalian reproduction. In females, activation of the LHR regulates terminal follicular development and promotes ovulation and during pregnancy maintains progesterone production by the corpus luteum (2). In males, LHR signaling promotes testosterone production by Leydig cells (2). Signal transduction by the LHR to G, can be tempered or desensitized as a consequence of arrestin2 (β-arrestin1) binding to the receptor at the third intracellular (3i) loop (3). LHR desensitization occurs in ovarian follicles in response to the mid-cycle LH surge that promotes ovulation (4) and, based on serum progesterone levels, appears to occur in human corpora lutea in response to elevated levels of hCG during pregnancy (5).

The β2-adrenergic receptor has served as a model for desensitization of G-protein-coupled receptors (GPCRs). Following agonist activation, desensitization of the β2-adrenergic receptor is triggered by receptor phosphorylation catalyzed by a G-protein receptor kinase (6, 7). The G-protein receptor kinase phosphorylation sites on the β2-adrenergic receptor facilitate interaction of the receptor with non-visual arrestins (8). Arrestins sterically hinder the ability of the receptor to activate G-proteins and thus moderate signal transduction by GPCRs (9, 10). However, LHR phosphorylation is not obligatory for LHR desensitization (11–17); therefore, LHR desensitization is expected to involve other sorts of physical interactions with arrestin molecules. Consistent with this notion, we have shown recently that both recombinant visual arrestin1 and arrestin2 but not arrestin3 (β-arrestin2) bind with high affinity to a synthetic (unphosphorylated) peptide corresponding to the 3i loop of the receptor and promote LHR desensitization (18). Our recent data, using plasma membrane models of endogenously as well as heterologously expressed LHRs (18), show that LHR desensitization is triggered by agonist-dependent activation of ADP-ribosylation factor 6. ADP-ribosylation factor 6 activation results in the release from a plasma membrane docking site of a pool of arrestin2, which then binds to the active LHR, promoting receptor desensitization (19, 20). Activation of this cascade by the ADP-ribosylation factor 6 activator ARNO also promotes LHR desensitization (19). Similarly, inhibition of these reactions by catalytically inactive ARNO (21), antibodies that inhibit arrestin binding to GPCRs (3), or a synthetic LHR 3i peptide that competes with receptor for arrestin (22) blocks LHR desensitization.

Both the extent and rate of LHR desensitization in our membrane model are facilitated by incubation of membranes with 8% ethanol (23). We considered that ethanol most likely encouraged LHR desensitization by increasing the fluidity of the membrane and therefore the mobility of proteins and lipids in the membrane. We therefore tested the hypothesis that LHR desensitization required the formation of protein aggregates that contained self-associated LHRs. This hypothesis was supported by abundant evidence that the LHR, upon agonist binding, can form large protein aggregates (24–27), which are relatively immobile in the membrane (28–31). Utilizing a plasma membrane model of endogenously expressed receptors present...
in preovulatory follicles under conditions in which LHRs are “locked” in an active conformation and do not proceed to desensitization versus conditions in which LHRs become desensitized, we compared the aggregation state of the LHR when the receptor is active versus when the receptor is desensitized. Results indicate that agonist-dependent desensitization of endogenous LHRs in a plasma membrane model precedes, and is obligatory for, organization of LHRs into larger protein aggregates and that these protein aggregates contain self-associated LHRs.

MATERIALS AND METHODS
Preparation of Porcine Follicle Membranes—A membrane fraction enriched in adenyl cyclase activity was isolated from preovulatory-sized porcine ovarian follicles and stored at −70 °C (14). Membranes (100 μg of membrane protein) were incubated, unless otherwise indicated, for 40 min at 30 °C in a final volume of 40 μl in the presence of 25 mM Bis-tris propane, pH 7.2, 0.4 mM EDTA, 1 mM EGTA, 0.2 mg/ml creatine phosphokinase, 20 μM phosphocreatine, 5 mM MgCl₂, 1 mM cAMP, and either 1 mM ATP plus 10 μM GTP (+GTP) or 1 mM AMP-PNP (−GTP) in the presence of 10 μg/ml bovine serum albumin or hCG in 8% ethanol, as indicated. Membranes were then diluted to 1 ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.2, and pelleted by centrifuging for 15 min at 10,000 × g. Membrane pellets were then incubated at 4 °C in 100 μl of Hank’s balanced salt solution, pH 2.7, for 2 min to strip hCG off the receptor, the reaction mixture was neutralized with 2 μl Tris base, and membranes were repelleted and frozen at −70 °C. Membranes were then labeled with erythrosin isothiocyanate (ErITC) or tetramethylrhodamine isothiocyanate (TrITC) as described below. The source of receptors is as described previously (3).

Preparation of ErITC- and TrITC-derivatized hCG—Hormones were derivatized with ErITC or TrITC using a modification of methods described by Johnson and Holborow (32) that has been described in detail previously (28). The molar ratios for hCG derivatized with ErITC and TrITC and the concentration of protein in solution were determined spectrophotometrically. The hormone preparations used in these experiments had 1.2 mol of ErITC and of TrITC per mol of hCG. It has been previously shown that there is no effect of fluorescence conjugation on hormone biological activity (28). Prior to use, fluorophore-derivatized proteins were centrifuged at 130,000 × g for 5 min in a Beckman Airfuge (Beckman Instruments) to remove any protein aggregates that may have formed during storage at 4 °C.

Labeling Membranes with ErITC- and TrITC-derivatized hCG—Typically, 100 μg of porcine ovarian membrane proteins were suspended in 500 μl of phosphate-buffered saline and labeled with 10 nM ErITC- or TrITC-hCG at 37 °C for 30 min for each experiment. Membranes were then washed twice by centrifugation at 30,000 × g for 10 min in 10 ml of phosphate-buffered saline to remove unbound ligand. To verify that there were no nonspecific interactions between the membranes and the ErITC-hCG, some membranes were left untreared whereas other membranes were preincubated with excess hCG prior to labeling with the respective ErITC-derivatized hCG in some experiments. In both cases, there was no phosphorescence signal detected using the TPA apparatus described below.

TPA Measurements—After labeling with ErITC-derivatized hCG, plasma membrane preparations were deoxygenated for 15 min by purging with argon gas to eliminate phosphorescence quenching caused by O₂. The sample was then placed in a 5-mm Suprasil quartz cuvette (Helma Cells, Inc., Jamaica, NY) and inserted into a thermostated cuvette holder maintained at room temperature. The frequency-doubled 532 nm output of a Spectra-Physics DCR-11 Nd:YAG laser (Spectra-Physics, Albuquerque, NM) provided the excitation pulse for each experiment. The laser was operated at 10 Hz with a vertically polarized radially symmetric Gaussian transverse electromagnetic mode output of 0.19 mJ and a beam 1/e² radius of 3.6 mm. Beam diameter was measured at the sample. Phosphorescence emission from the sample was collected at 90° to the excitation axis and isolated with a 1-cm path length of 1 M Na₂Cr₂O₇ solution, a KV 550 color filter (Schott Glass Technologies, Inc., Duryea, PA), and a 3-mm-thick RG 665 filter. A rotating polarizer was placed in front of the photomultiplier tube to observe the intensity of phosphorescence with polarization parallel to and perpendicular to the exciting pulse. The phosphorescence signal was collected by a thermocooled cooled EMI 9816A photomultiplier tube. A fast gating circuit was used to turn the photomultiplier tube off during the high power Nd:YAG pulses (21). The output signal from the photomultiplier tube was amplified by a Tektronix 470 oscilloscope. The oscilloscope was further amplified by a 35-MHz bandwidth buffer amplifier and fed to a Nicolet 1270 signal averager equipped with a 20-MHz analog-to-digital converter. Phosphorescence decay traces from 4096 laser pulses were averaged for each polarization orientation, the channel width being 0.5 μs. After data acquisition was complete, data were downloaded for analysis and storage. Total phosphorescence emission was analyzed according to a multi-exponential decay model, and an apparent half-time for phosphorescence decay was calculated. Analysis of anisotropy decay yielded the initial anisotropy value R₀, the limiting anisotropy value R∞, and the rotational correlation time as well as the statistical uncertainties in these quantities (32).

Confocal Microscopy—Following membrane incubations to yield active or desensitized LHRs, removal of bound hCG, and binding TrITC-hCG receptors, membranes were resuspended in 50 μl of phosphate-buffered saline. The entire sample was placed in a quartz well slide, and the well was overlaid with a “00” glass coverslip and subjected to confocal microscopy with a Sarastro 2000 confocal microscope (standard TrITC filter set, aperture of 50 μm, laser power of 25 milliwatts, PMT voltage of 600 V, and magnification of 2000).

FRET Measurements—FRET between FITC- and TrITC-derivatized hCG was evaluated based on the reduced rate of irreversible photo bleaching of FITC fluorophores when TrITC fluorophores were present within less than ~100 Å, as previously described (30). For each treatment, data were collected from 20 membrane smears in which receptors were labeled with 0.5 μM FITC-hCG or 1.5 μM TrITC or with 0.5 μM FITC-hCG and 1.5 μM TrITC. Each sample was photobleached for 10 s during which time fluorescence emission was collected at 0.01-s intervals. Individual data traces were analyzed to yield the energy transfer efficiency (% E) for each treatment (34).

Statistics—Results were analyzed using Student’s t test to determine statistical significance (35) between treatments.

RESULTS
LHR Rotational Diffusion Time SloWs with LHR Desensitization—We showed previously, using an adenyl cyclase-enriched membrane fraction from porcine ovarian follicles, that LHR desensitization exhibits an absolute requirement for GTP (13, 14). Therefore, incubation of membranes with hCG but without GTP (+hCG −GTP) yields an active receptor which does not desensitize, whereas incubation with hCG plus GTP (+hCG + GTP) leads to receptor desensitization. Using the biophysical technique of time-resolved phosphorescence anisotropy (TPA), we measured the rotational diffusion of the LHR when it can actively transduce a CAMP response (membranes +hCG −GTP) versus when the receptor is desensitized (membranes +hCG + GTP). Following membrane incubations to generate active versus desensitized receptors, hCG was removed from the receptor by a low pH wash. Receptor was then bound with ErITC-derivatized hCG (in the absence of GTP) for rotational diffusion measurements of active and desensitized LHR. (Rotational diffusion of the inactive LHR could not be evaluated because LHRs were tagged with fluoroescently labeled hCG.) We showed previously that once desensitized, the LHR remains stably desensitized (in the absence of GTP) for at least 40 min (36).

Table I shows the initial anisotropy values R₀ and the limiting anisotropy values R∞ of active and desensitized LHRs obtained from raw data traces for a single experiment. Analysis of the anisotropic decay yields the rotational correlation time, which is a direct measurement of the in-membrane volume of the rotating protein complex. In general, the larger the complex the slower the rotational correlation time. The average rotational correlation time over five separate experimental days, each day involving four TPA measurements per sample, is shown in Fig. 1. Taken together, these results show that active LHRs exhibit a faster anisotropic decay and rotational correlation time than desensitized receptors. Desensitized LHRs therefore occupy a larger in-membrane volume than active receptors and are rotating more slowly than active LHRs. Consistent with this conclusion, the percent mobility (% M) of desensitized LHRs, as shown in Table I, is significantly (p <
Results are the mean ± S.D. (n = 8 for active and n = 4 for desensitized receptors) of initial anisotropy $R_0$ and limiting anisotropy at infinite time $R_{\infty}$. For details of membrane treatments, see legend to Fig. 1.

| Membrane treatment | Receptor → $G_s$ | $R_0^*$  | $R_{\infty}^*$ | % M (1 − $R_0/R_{\infty}$) |
|--------------------|-----------------|----------|----------------|--------------------------|
| hCG − GTP          | Active          | 0.0720 ± 0.0014 | 0.0547 ± 0.0008 | 24.0                      |
| hCG + GTP          | Desensitized    | 0.0697 ± 0.0011 | 0.0577 ± 0.0013 | 17.2                      |

* Results of $R_0$ versus $R_{\infty}$, p < 0.05 for active versus desensitized LHR.

Fig. 1. Rotational correlation times of active and desensitized LHR. Porcine follicular membranes were incubated (40 min) under conditions that promote only LHR activation (−GTP, +hCG) or LHR desensitization (+GTP, +hCG). hCG was then removed by a low pH wash, then ErITC-hCG was bound to membrane LHRs, and membranes were subjected to TPA as described under “Materials and Methods.” Results are the mean ± S.D. of four separate determinations of the rotational correlation times of active and desensitized LHRs. *p < 0.05 for active versus desensitized LHRs.

Fig. 2. Confocal microscopy of TrITC-hCG bound to active and desensitized LHRs. Active or desensitized LHRs were labeled with TrITC-hCG, as described under “Materials and Methods,” then subjected to confocal microscopy. For the rest of the details see legend to Fig. 1. -Fold magnification is indicated. Results are representative of two separate experiments.

LHR Desensitization and Oligomerization

To determine the time dependence of the reduction in rotational diffusion and formation of larger LHR aggregates, we measured the rotational diffusion of the LHR at 10-min intervals for 40 min in membranes incubated under conditions that do or do not promote LHR desensitization. We previously reported that LHRs in porcine follicle membranes are 80% desensitized within 10 min after agonist addition (23). Therefore, if receptor incorporation into larger protein complexes occurs prior to or coincident with desensitization, rotational correlation times should be slowed by 10 min. Results (Fig. 3) show that rotational correlation times for the LHR are slowed to 76 ± 20 µs by 10 min after agonist addition and remain slow for the next 30 min. These data suggest that formation of LHR aggregates is occurring with the same rapid time course as LHR desensitization. It is not apparent from these studies, however, whether LHR desensitization is required for or a consequence of LHR aggregation.

Complex Formation Can Be Blocked Using Antibodies, Proteins, or Peptide Sequences That Prevent LHR Desensitization—To determine whether LHR desensitization is necessary for receptor aggregation, we examined the rotational diffusion of the receptor when desensitization was inhibited. If desensitization is obligatory for LHR aggregation into larger protein complexes, we hypothesized that inhibiting desensitization should prevent LHR aggregation and result in comparatively fast rotational correlation times. Conversely, if desensitization occurs as a result of receptor aggregation, then inhibiting desensitization should not perturb the formation of LHR complexes and should produce long rotational correlation times indicative of receptor inclusion in large complexes.

We have previously shown that incubating membranes with various proteins or peptides that specifically block either the binding to or availability of arrestin2 to the LHR (3, 21, 22) abrogates LHR desensitization. These reagents which block LHR desensitization include neutralizing anti-arrestin antibodies, a synthetic peptide corresponding to the 3i loop of the LHR, and catalytically inactive (E156K) ARNO. As shown in Fig. 4, pretreatment of membranes with anti-arrestin antibodies (lane 6), the synthetic 3i peptide (lane 8), or catalytically inactive E156K ARNO (lane 14) followed by treatment with hCG and GTP led to faster rotational diffusion of the LHR. Rotational correlation times of these LHRs treated with hCG and GTP (lanes 6, 8, and 14) were not significantly different.
desensitized LHRs.

peptide and ARNO suggest that these reagents lead to formation of larger protein complexes, the mechanistic basis for this is not apparent.

FITC fluorescence decay between FITC-hCG- and TrITC-hCG-labeled receptors compared with only FITC-hCG-labeled receptors reflects the efficiency of energy transfer and can be quantitated (34). Energy transfer between FITC- and TrITC-labeled samples requires that these energy sources be within 100 Å of each other and is indicative of receptor dimers or oligomers (37). Results in Fig. 5 show that although there is energy transfer between active LHRs, reflecting the existence of some LHR self-association, LHR desensitization results in a 2.4-fold increase in FRET between LHRs. There was no measurable fluorescence signal from unlabeled plasma membrane preparations. These results suggest that the desensitized LHR aggregates contain more self-associated LHRs than actively signaling LHRs.

**DISCUSSION**

It is well established that for receptors that exhibit tyrosine kinase activity, agonist-dependent receptor dimerization is obligatory for the receptor to signal to downstream effectors (38). Until recently, GPCRs were believed to function as monomers, with a single receptor promoting the catalytic activation of linked G-proteins (39–41). However, recent studies have shown that many different GPCRs, including the β2-adrenergic receptor (42), δ- and κ-opioid (43, 44), dopamine D2 (45), calcium-sensing (46), somatostatin (47), and cholecystokinin receptors (48) exist at the cell surface as dimers or even higher oligomers. In some cases, receptor dimerization is constitutive and either not affected by agonist-dependent activation of the receptor, such as for the β2-adrenergic receptor (49) and chemokine receptor CCR5 (50), or reduced to monomers by agonist-dependent receptor activation, such as for the
δ-opioid (51) and cholecystokinin (48) receptors. In other cases, agonist activation of the receptor stimulates receptor dimerization or oligomerization, as for the gonadotropin-releasing hormone (52) and chemokine receptors (53, 54). However, to our knowledge, it is not known how receptor desensitization affects the oligomeric state of any GPCR.

Inactive LHR appears to exist at the cell surface either as a monomer or dimer. This conclusion is based on results from FRET studies between green fluorescent protein- and yellow fluorescent protein-tagged LHRs expressed in intact Chinese hamster ovarian cells, which show essentially no energy transfer between inactive LHRs (31). Exposure of LHR target cells to agonist has been shown to promote extensive LHR clustering, as detected by electron microscopy studies with ferritin-labeled LH (24). In addition, FRET studies in intact cells between green fluorescent protein- and yellow fluorescent protein-tagged LHRs showed that agonist treatment leading to LHR desensitization resulted in an energy transfer between LHRs, indicative of receptor self-association (31). However, it has not been possible previously to determine whether agonist-stimulated receptor aggregation was a consequence of LHR activation or desensitization.

Based on our observation that exposure of follicular membranes to 8% ethanol accelerates the rate and increases the extent of LHR desensitization (23), coupled with evidence that the receptor appears to aggregate under conditions that most likely lead to receptor desensitization (26, 29, 31), we hypothesized that conversion of the receptor from an active to a desensitized state was associated with oligomerization of the LHR into higher ordered structures. We used a plasma membrane model in which endogenous receptors are localized at the plasma membrane. Although we cannot determine whether the organization of active and desensitized LHRs on cells with an intact cytoskeleton differs from that inferred from our studies of the LHR in plasma membrane preparations, our cell-free model nevertheless affords us the unique opportunity to compare the membrane organization of actively signaling receptors that cannot desensitize versus desensitized LHRs. Moreover, this model allows us to study receptor desensitization as an event that is distinct from receptor sequestration and internalization. Additionally, LHR expression is at physiological levels in this membrane model; therefore, artifacts of receptor association upon receptor overexpression are nonexistent. Receptor solubilization is also not needed; therefore, artifacts introduced by detergents are avoided.

Our results demonstrate unequivocally that conversion of the LHR from an actively signaling module to one uncoupled from G, is intimately associated with the formation of larger protein aggregates containing more self-associated LHRs. In the absence of a good antibody probe to examine the rotational diffusion of unoccupied porcine LHRs on granulosa cell membranes, it is not possible to assess the rotational correlation time of the monomeric LHR and to then use this information to make direct estimates of the in-membrane size of complexes containing LHRs. However, if we assume that native LHRs on MA-10 Leydig cells treated with cytochalasin D minimally form receptor dimers upon binding of hCG, we can use the corresponding receptor rotational correlation time of −10 μs in the absence of a functional actin cytoskeleton (55) to estimate the size of structures formed by active and desensitized LHRs in porcine follicular membranes. Longer rotational correlation times observed for the desensitized LHRs are linearly related to the size of the structures containing the desensitized receptor and thus indicative of significantly larger complexes, which we estimate to have in-membrane volumes −6–10-fold greater than those of LHR dimers. Membrane treatments that block desensitization by blocking either arrestin2 release from its docking site or its binding to receptor also block formation of these higher order membrane aggregates. These results indicate that upon arrestin2 binding to receptor, the receptor-arrestin2 complex oligomerizes, forming a structure that is −3-fold less mobile, and thus larger, than that of the actively signaling receptor. This complex, again based on assumptions derived from the rotational correlation times for the LHR on MA-10 cells, has an −2-fold larger in-membrane volume than does the LHR dimer. The biochemical basis for LHR oligomerization is not known, although the desensitized receptor migrates on denaturing SDS-PAGE as a monomer (3, 19). Therefore, in contrast to the metabotropic glutamate or calcium-sensing receptors (41, 46, 55, 56), oligomerization of the LHR is unlikely because of formation of critical disulfide linkages between receptors.

These results suggest the intriguing hypothesis that it is the binding of arrestin2 to the 3i loop of the receptor that stimulates receptor oligomerization as well as clustering of the receptor into large, microscopically visible structures. These clusters, which appear from scanning confocal microscopy images to be larger for desensitized receptors than for actively signaling receptors, must be greater than −300 nm in diameter. Because single complexes of this size would be rotationally immobile, these images suggest that microscopically visible clusters contain many copies of independent, rotationally mobile complexes that are on the order of 10 nm in diameter. Possibly LHR oligomerization is triggered not by receptor-receptor interactions but rather by arrestin-arrestin interactions, or a combination of both. We conclude that receptor oligomerization occurs not on agonist-dependent receptor activation but rather on arrestin2-dependent receptor desensitization. This is the first report, to our knowledge, that defines the structural organization of a GPCR in the desensitized state.

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**Fig. 5. FRET of active and desensitized LHR.** Porcine follicular membranes were treated as indicated in legend to Fig. 1. The mean ± S.D. of FRET between FITC- and TrITC-hCG bound to desensitized (n = 2) and active (n = 4) LHRs is shown using procedures described under “Materials and Methods.”
