RESTRICTED LATERAL DIFFUSION OF LUTEINIZING HORMONE RECEPTORS IN MEMBRANE MICRODOMAINS

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Single particle tracking was used to evaluate lateral motions of individual FLAG-tagged human luteinizing hormone (LH) receptors expressed on Chinese hamster ovary (CHO) cells and native LH receptors on both KGN human granulosa-derived tumor cells and M17 human neuroblastoma cells before and after exposure to human chorionic gonadotropin (hCG). Compared to LH receptors on untreated cells, LH receptors on cells treated with 100 nM hCG exhibit restricted lateral diffusion and are confined in small, nanometer-scale, membrane compartments. Like LH receptors labeled with Au-hCG, LH receptors labeled with Au-deglycosylated hCG, an hCG antagonist, also exhibit restricted lateral diffusion and are confined in nanoscale membrane compartments on KGN cells treated with 100 nM hCG. LH receptor point mutants lacking potential palmitoylation sites remain in large compartments despite treatment with 100 nM hCG as do LH receptors on cells treated with cytochalasin D. Finally, both polarization homo-transfer fluorescence resonance energy transfer imaging and photon counting histogram analysis indicate that treatment with hCG induces aggregation of YFP-coupled LH receptors stably expressed on CHO cells. Taken together, our results demonstrate that binding of hCG induces aggregation of LH receptors within nanoscale, cell-surface membrane compartments, that hCG binding also affects the lateral motions of antagonist binding LH receptors, and that receptor surface densities must be considered in evaluating the extent of hormone-dependent receptor aggregation.

Signal transduction by luteinizing hormone1 (LH) receptor plays an essential role in the normal reproductive function of both male and female mammals by promoting ovulation, follicle maturation, corpus luteum formation and steroidogenesis. LH receptor signaling in response to saturating concentrations of LH or human chorionic gonadotropin (hCG) involves substantial changes in receptor lateral and rotational dynamics (1,2) as well as the association of LH receptors with membrane rafts (3). Fluorescence resonance energy transfer (FRET) techniques (4) and electron microscopy (5) indicate that functional hormone-receptor complexes can also become self-associated into dimers/oligomers following ligand binding. Previous experimental strategies have examined changes in receptor motions on collections of cells or large numbers of fluorescently-tagged molecules on single cells. It is now possible, however, to examine the lateral motions of individual LH receptors on living cells using microscope-based single particle tracking (SPT) techniques (6).

Although the mechanism involved in retention of hCG-occupied LH receptors in small membrane compartments is still unclear, exposure to saturating concentrations of hCG results in...
confinement of the majority of LH receptors within small cell-surface compartments. LH receptors remain within these compartments for comparatively long times and appear to diffuse pseudo-randomly before being captured within another compartment of similar size (7). Similar behavior has been described and analyzed by Kusumi and coworkers (6) for selected phospholipids and for transferrin receptors (8) and by Daumas and coworkers (9) for the μ opioid receptor. Daumas argues that the μ opioid receptor can both diffuse within the bulk membrane and exhibit confinement within a microdomain that itself diffuses slowly. Kusumi and coworkers (6) suggest that particles may be confined by proteins forming a barrier that is either continuous or discontinuous, leading to receptor diffusion within small membrane regions accompanied by intermittent escape from compartments and periods of unrestricted diffusion in the bulk membrane.

Our previous studies of LH receptor lateral and rotational diffusion suggest that actin microfilaments or membrane protein interactions with the cytoskeleton may provide organizing structures that restrict the lateral motions of receptors (1,2). However, the mechanism of hormone-initiated LH receptor confinement in small compartments has not previously been examined in detail at the single molecule level. In the present work, we compared the lateral dynamics of individual LH receptors that were either FLAG-tagged and identified using FLAG-specific antibodies with the diffusion of native receptors occupied by gold nanoparticle-hCG conjugates (Au-hCG) or Au-deglycosylated DG-hCG (Au-DG-hCG). The latter material is an hCG antagonist (10) that binds LH receptors with high affinity and can block hCG binding for more than 24 hrs (11). This allowed us to determine whether LH receptor motions are dependent on hCG concentration and limited to hCG-occupied receptors or, alternatively, whether hCG binding to a fraction of available receptors has global effects on LH receptor diffusion regardless of whether they have been activated by hCG. We also explored whether point mutations to palmitoylation sites on the LH receptor C-terminus, known to eliminate LH receptor translocation into membrane rafts (12) affected receptor confinement within small compartments and to what extent the actin cytoskeleton is responsible for the restriction of LH receptor lateral motions. Because slower lateral diffusion may result from extensive self-association of membrane proteins, polarization homo-transfer FRET (homoFRET) and photon counting histogram (PCH) analysis were utilized to evaluate increased oligomerization of YFP-coupled human LH receptors (YFP-LHR) stably expressed on individual, viable, CHO cells in response to increasing concentrations of hCG. Fluorescence correlation spectroscopy (FCS) was used to determine the equilibrium dissociation constant ($K_D$) of hCG binding to native LH receptors on KGN cells and to estimate the number of native LH receptors on these cells. FCS was also used estimate the number of YFP-LHR stably expressed on the CHO cells used in homoFRET and PCH analysis studies. Finally, we considered how changes in receptor cell surface density might contribute to the apparent aggregation of YFP-LHR stably expressed on CHO cells.

**EXPERIMENTAL PROCEDURES**

**Materials and cell culture** — CHO cells were maintained in high glucose Dulbecco’s Modification of Eagle’s Medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units of penicillin/ml, 100 μg streptomycin/ml (Gemini Bio-Products, Woodland, CA) and 1x MEM non-essential amino acid solution (Sigma-Aldrich, Inc., St. Louis, MO). KGN cells, a human granulosa-like tumor cell line developed by Dr. Yoshihiro Nishi and Dr. Toshihiko Yanase at Kyusyu University (13), were kindly provided by Dr. James Dias at the Wadsworth Center (New York State Department of Health, Albany, NY). KGN cells were maintained in DMEM/Ham’s F12 medium supplemented with 10% FBS, 2 mM L-glutamine and 100 units of penicillin/mL as previously described (13). M17 human neuroblastoma cells which express LH receptors (14) were purchased from ATCC (Manassas, VA) and maintained in Minimum Eagle’s Medium (MEM)/Ham’s F12 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS, 2 mM L-glutamine, 100 units of penicillin/mL, 100 μg streptomycin/mL (Gemini Bio-Products,
Woodland, CA), 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acid solution (Sigma-Aldrich, Inc., St. Louis, MO). All cells were grown in 5% CO2 at 37°C in a humidified environment. Genetecin (G418 sulfate) was purchased from Mediatech, Inc. (Manassas, VA). Monoclonal anti-FLAG antibody directed against the FLAG epitope tag was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). 40 nm gold colloid was obtained from Ted Pella, Inc. (Redding, CA). Highly purified hCG (Fitzgerald Industries, Inc., Concord, MA) was prepared in 1x PBS. Dr. George Bousfield, Wichita State University, kindly provided deglycosylated hCG used in single particle tracking studies on KGN and M17 cells. Deglycosylated hCG was prepared using modifications of the method described by Edge et al. (15). Rather than using a desalting column as described, deglycosylated protein was incubated in 0.5 M sodium acetate buffer, pH 6.0, overnight at 37°C to re-associate dissociated subunits and the heterodimer fraction was then isolated using Superdex 75 gel filtration chromatography. To monitor the extent of LH receptor signaling in M17 cells, levels of intracellular cAMP were assessed following exposure to hCG using a TiterFluor Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI).

**CHO cell lines expressing FLAG or YFP tags** — Dr. K.M. Menon from the University of Michigan kindly provided us with N-terminal FLAG-tagged LHR subcloned into the pFLAG vector (FLAG-LHR) (Sigma-Aldrich, Inc., St. Louis, MO). A stable cell line expressing FLAG-tagged palmitoylation-deficient LH receptors was prepared as previously described (12). For homoFRET, FCS and PCH experiments, we used a stable CHO cell line expressing human YFP-LHR at the C-terminus as previously described (16).

**Single particle tracking of individual LH receptors on live cells** — Lateral dynamics and the size of compartments accessed by individual FLAG-LHR on CHO cells or native LH receptors on KGN and M17 cells were evaluated using single particle tracking methods as described by Kusumi and coworkers (17). To identify FLAG-LHR, 40 nm nanogold particles were conjugated with a mixture of anti-FLAG monoclonal antibody and BSA at the lowest possible total protein concentration, typically 40 µg/mL, needed to stabilize the gold solution. To identify individual native LH receptors expressed on KGN or M17 cells, 40 nm gold particles were coupled to Au-hCG or Au-DG-hCG using the same protocol. For labeling cells, the ratio of antibody or hormone to BSA, typically 1:100 by weight, was selected to give 10 to 20 particles bound per cell. The binding of anti-FLAG antibody or hormone was specific: when cells were preincubated with a 10-fold excess of anti-FLAG antibody or Au-tagged hormone, no gold particles were detected on cells. Cells were labeled with Au-coupled probes for 1 hour at 4°C. After receptor labeling, cells were treated with 0.1 nM, 1 nM or 100 nM hCG for 1 hour at 37°C. Because the off rates for hCG and DG-hCG are on the order of 24-48 hours (11,18), it is unlikely that Au-tagged hormones were displaced from LH receptors by introduction of hCG on the timescale of these experiments. In some experiments, cells grown on coverslips placed in 60 mm2 petri dishes were pre-treated with 40 µg/mL cytochalasin D for 1 hour prior to labeling with gold-conjugated anti-FLAG antibody. Individual gold nanoparticles were imaged by differential interference contrast using a 1.4 N.A. 63x oil objective in a Zeiss Axiosvert 135 TV inverted microscope. Images were acquired using a Dage IFG-300 camera and were recorded for two minutes (3600 frames) at approximately 30 nm/pixel under the control of Metamorph software (Molecular Devices). Trajectories of individual gold particles were measured over time and then segmented into compartments by calculation of statistical variance in particle position (6,9,19). The variance of a particle’s position was calculated within windows of varying duration which were translated along the particle trajectory, producing a variance plot that exhibited peaks indicating inter-compartment boundaries. These results were analyzed using custom analysis programs to yield the compartment size and residence time for each particle. Effective macroscopic diffusion coefficients (D) were calculated as the square of the compartment diagonal divided by four times the residence time in the compartment as previously described (19). Data are presented either as the mean ± S.E.M. or mean ± S.D. as indicated in the figure legends. Significance was
assessed using Student’s t-test and p values are indicated (p < 0.05).

Polarization homo-transfer FRET measurements — We investigated aggregation of human LH receptors in response to 0.1 nM, 1 nM and 100 nM hCG using polarization homo-transfer FRET or energy migration FRET. Homo-transfer FRET assesses energy transfer between identical fluorescent donor and acceptor molecules by measuring fluorescence anisotropy as probe molecules are photobleached (20). Increasing anisotropy as probe molecules are progressively photobleached is indicative of FRET. CHO cells stably expressing YFP-LHR were grown to 50% confluence on sterile dishes and examined directly. Both FCS and PCH experiments were performed on a modified Nikon TE1000 inverted microscope equipped with a 100x, 1.25 NA, oil-immersion objective, an Omnicrome Melles-Griot multi-line air-cooled argon ion laser operating at 514.5 nm, two 570/32 nm bandpass filters, two PerkinElmer single photon counting modules (SPCM-AQR-14), an ALV-6010 digital hardware correlator and a Becker and Hickl GmbH PMS-400 multichannel photon counter as previously described (23). The $1/e^2$ radius of the excitation beam at the sample was experimentally determined to be 241 nm using aqueous rhodamine 6G. In live cell studies, the laser excitation beam was vertically positioned on the apical cell membrane by adjusting the objective z-position to maximize detector count rates and minimize diffusional correlation times ($\tau_0$) as described by Ries and Schwille (24). All FCS and PCH experiments were conducted at room temperature. In both FCS and PCH experiments samples were illuminated for approximately 10 sec before collection of data to allow for the irreversible photobleaching of immobile particles (25). FCS data were then collected for two consecutive 10 sec intervals, while PCH data were collected for 30 sec. During acquisition of FCS and PCH data the average fluorescence from the sample was relatively uniform as indicated by the avalanche photodiode signal readouts. Analysis of FCS data, including determination of $\tau_0$ and the normalized initial $g(\tau)$ were accomplished according to established procedures using Igor Pro 5.05A (24,26-28). Using FCS, the number of receptors per $\mu m^2$ was determined from the effective number of correlating particles detected in the illuminated area ($N_{eff}$) at saturating concentrations of fluorophore conjugated receptor-specific probe and the radius of the laser interrogation area. The $K_D$ for hCG was determined by fitting FCS data to a hyperbolic function describing a single-site binding model. For PCH, detected photons were accumulated into successive 1 $\mu s$ counting channels and rebinned into 9 $\mu s$ channels to
improve signal-to-noise. Rebinned photon counts were then simultaneously subjected to autocorrelation, pseudo-crosscorrelation and PCH. Weighted least-squares fitting was used to obtain estimates of $N_{eff}$, the average molecular brightness ($\varepsilon$) of detected particles and the out-of-focus emission ratio ($F$) as previously described (29-31).

**RESULTS**

Reduced lateral diffusion and increased compartmentalization of LH receptors follows treatment with hCG — Single particle tracking methods were used to examine the lateral diffusion of individual LH receptors and receptor compartmentalization in response to binding of hCG (Figure 1). Treating CHO cells expressing FLAG-LHR with 0.1 nM hCG did not have a statistically significant effect on either receptor diffusion (lower panel) or average size of compartments containing LH receptors (upper panel). However 1 nM hCG and 100 nM hCG significantly increased the number of receptors appearing in compartments of less than 100 nm in diameter to 62% and 91% of the total receptor population, respectively (Figure 2). Individual FLAG-LH receptors on cells treated with 100 nM hCG, also exhibited significantly slower lateral diffusion (Figure 1, lower panel).

To examine the lateral dynamics of native LH receptors on viable KGN granulosa-like tumor cells and M17 human neuroblastoma cells, 10-20 receptors per cell were tagged with Au-hCG. When KGN cells were exposed to 1 nM and 100 nM unlabeled hCG, Au-tagged LH receptors became confined in small membrane compartments of less than 100 nm diameter (Figure 1, upper panel). For cells treated with 0.1 nM hCG, 16% of hCG-occupied LH receptors on KGN cells were confined in these small compartments and this number increased to 48% when cells were treated with 100 nM hCG. The diffusion rate of individual LH receptors was also reduced from $0.7\pm0.2 \times 10^{-11} \text{cm}^2\text{sec}^{-1}$ to $0.3\pm0.1 \times 10^{-11} \text{cm}^2\text{sec}^{-1}$ after 100 nM hCG treatment (Table 1). We observed similar dose-dependent effects of hCG on the diameter of compartments accessed by hCG-occupied LH receptors on M17 neuroblastoma cells. With increasing concentrations of hCG, more Au-hCG occupied LH receptors were confined in small membrane compartments. Au-hCG occupied LH receptors on cells treated with 100 nM were located in smaller compartments with an average diameter of 131±18 nm. In M17 cells the average rate of diffusion was reduced from $0.9\pm0.3 \times 10^{-11} \text{cm}^2\text{sec}^{-1}$ to $0.4\pm0.1 \times 10^{-11} \text{cm}^2\text{sec}^{-1}$ after treatment with 100 nM hCG (Table 2).

Activation of LH receptors also resulted in increased levels of intracellular cAMP. There was a 1.6±0.2-fold and a 2.5±0.9-fold increase in cAMP response over basal levels, respectively, in response to treatment with 100 nM hCG and 20 nM forskolin.

Non-functional receptor complexes exhibit reduced lateral diffusion and increased compartmentalization in response to hCG — Differences in the effects of hCG on the lateral motions of FLAG-LHR and native receptors on KGN and M17 cells were anticipated, particularly at lower hormone concentrations. When cells are treated with lower concentrations of hCG, FLAG-tagged receptors are not necessarily occupied by hCG. In contrast, only Au-hCG-occupied receptors are examined in single particle tracking of receptors on KGN and M17 cells. To determine whether hCG binding to LH receptors produced global effects on LH receptor motions or, alternatively, affected only hCG-occupied receptors, native LH receptors on KGN cells were labeled with $10^{-20}$ Au-DG-hCG molecules as probes for non-signaling receptors. Compared to Au-hCG occupied LH receptors, Au-DG-hCG occupied LH receptors exhibited relatively faster lateral diffusion within somewhat larger 217±27 nm diameter regions. When KGN cells were treated with 100 nM hCG, Au-DG-hCG occupied receptors also exhibited significantly slower lateral diffusion and became confined in smaller compartments, although neither receptor lateral diffusion nor average compartment size were affected by hormone treatment to the same extent as seen for Au-hCG occupied receptors. The results from these experiments are summarized in Table 1.

Mutation of palmitoylation sites on LH receptors or disruption of actin microfilaments prevents hormone-mediated receptor confinement — We examined LH receptor lateral motions and confinement in small compartments on cells expressing LH receptors in which cysteine was mutated to serine at positions 621 and 622 (LHR-
C621, 622S), previously described as palmitoylation sites for the LH receptor (32,33). The average values for the compartment diameters accessed by either untreated or hCG-treated LHR-C621, 622S are approximately 200 nm (Table 3). In addition, FLAG-LHR on CHO cells treated with 40 μg/mL cytochalasin D, an actin-filament disruptor (34-37), exhibit fast lateral diffusion within large compartments both before and after exposure to 100 nM hCG. After treatment with cytochalasin D, hCG had no effect on the average diffusion coefficient or the average compartment diameter accessed by FLAG-LHR. This may be a result of the disruption of cell-surface compartments defined by cytoskeletonally-anchored proteins (38). Individual LHR-C621, 622S were also generally not confined in small compartments although their diffusion coefficients were slower than those of either untreated wild type LH receptors or LH receptors on cells treated with cytochalasin D (Table 3).

**Human LH receptors self-associate in response to increasing concentrations of hCG** – Aggregation of LH receptors in response to hormone treatment was assessed using CHO cells stably expressing YPF-LH receptors and both homo-transfer FRET confocal imaging and PCH analysis (39). In homo-FRET studies, fluorescence emission anisotropy was measured as YFP molecules were irreversibly photobleached. Progressive loss of YFP molecules via irreversible photobleaching reduces the likelihood that two YFP molecules are in close proximity to one another and available for resonance energy transfer. Thus the anisotropy of fluorescence emission will increase with increasing photobleaching of the YFP fluorophore. The average percent increase in anisotropy for untreated CHO cells expressing human YFP-LHR was 11%, 26% for cells treated with 0.1 nM hCG, 36% for cells treated with 1 nM hCG and 54% for cells treated with 100 nM hCG. FRET efficiencies (E), calculated as described by Rao and Major (40), increased from about 9% for untreated cells to over 30% in response to increasing concentrations of hCG (Table 4).

Similarly, PCH analysis suggests that YFP-LHR undergo aggregation after exposure to hCG. The average photon counts detected per molecule per sampling time, often referred to as ε (31,41) for YFP-LHR on live CHO cells was 0.07 ± 0.01 before treatment with hCG. After treatment with increasing concentrations of hCG, the ε of detected YFP-LHR rose significantly to 0.13-0.14 (Table 5) suggesting increased aggregation of LH receptors in response to binding of ligand.

Finally, FCS was used to determine the number of YFP-LHR per μm² on the surface of the stably transfected CHO cells and to compare that to native LH receptor density on KGN cells. Figure 4 shows the distribution of YFP-LHR densities on individual CHO cells and indicates that the majority of these cells have LH receptor densities ranging from 10 receptors μm² to 100 receptors μm². This was similar to the number of YFP-LHR observed during PCH analysis (Table 5). Also, on average, the density of LH receptors on KGN cells (Figure 5), which we estimate to be 86 receptors μm², is greater than the receptor density on the transfected CHO cells. These experiments also indicate that native LH receptors on KGN cells bind hCG with K_D of approximately 210 pM which is similar to previously determined values obtained using alternative methods (42). Using known estimates of CHO cell surface area (43), we calculate that each transfected CHO cell expresses approximately 33,000 total YFP-LHR.

**DISCUSSION**

We have previously shown that binding of saturating concentrations of hCG to rat LH-wt receptors results in redistribution of essentially all LH receptors from the bulk membrane into cholesterol-enriched membrane rafts (7) and that LH or hCG treatment cause a marked reduction in the fraction of mobile receptors (44). In contrast to methods used in these previous studies, single particle tracking techniques probe the lateral motions of individual LH receptors rather than a large population of receptors and so permit analysis of specific sub-populations of receptors exhibiting unique diffusive properties. Here we have shown that, when cells were treated with 100 nM hCG, a concentration sufficient to saturate available LH receptors, the average diffusion coefficient for individual receptors was approximately 10⁻¹² cm² sec⁻¹. This agrees with previous measurements of lateral diffusion for LH receptors on ovine luteal cells (45) and cell lines...
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stably expressing LH receptors covalently coupled to visible fluorescent proteins (46) where most, if not all, LH receptors binding either saturating concentrations of LH or hCG were laterally immobile on the timescale of photobleaching recovery experiments. These laterally immobile receptors had diffusion coefficients estimated to be less than $10^{-12}$ cm$^2$ sec$^{-1}$ (47), a practical detection limit for fluorescence photobleaching recovery measurements.

It appears that the extent of receptor occupancy by hormone is related to LH receptor retention in small compartments where individual particles exhibit slower diffusion. These should be considered distinct from membrane rafts (48) which are small microdomains within the membrane that may be transiently organized as proteins and small numbers of associated lipids or may be more persistent in the membrane (49). Hormone-treated LH receptors were not confined in small membrane compartments when treated with cytochalasin D, a disruptor of the actin-based cytoskeleton (34-37), suggesting that the compartments evaluated with single particle tracking methods are bounded by protein fences or cytoskeletonally-anchored protein networks that limit lateral diffusion of transmembrane proteins (6). It is also possible that the restriction of LH receptor lateral motions involves both lipid rafts and protein delineated networks nested in a hierarchical fashion as has recently been proposed (50).

Interestingly, individual LHR-C621,622S were generally not confined in small compartments. Nevertheless, their diffusion coefficients were slower than those of either untreated FLAG-LH receptors or FLAG-LH receptors on cells treated with cytochalasin D. Although palmitoylation-deficient mutant LH receptors are not found in rafts (12), they retain their ability to signal via cAMP (32,33) as dopamine D1 (51) and serotonin 4a (52) receptors with similar mutations. These results suggest that either prolonged LH receptor retention in small membrane compartments is not required for LH receptor-mediated signal transduction or, alternatively, that interactions of mutant receptors with the molecular contents of small compartments are more short-lived than for wild type receptors but still sufficient to initiate downstream signaling events.

Single particle tracking studies of endogenous LH receptors demonstrated that native hCG-occupied LH receptors on KGN human granulosa or M17 human neuroblastoma cells exhibited a dose-dependent increase in receptor compartmentalization in response to increasing concentrations of hCG which, in M17 cells, corresponded to increases in cAMP. The relationship between receptor compartmentalization and cAMP signals in these neuronal tissue may be important in aging where elevated LH levels are associated with decreased cognition in animal models (53). It may also suggest a role for deglycosylated hCG or other hCG antagonists in reducing compartmentalization of LH receptors, particularly if compartmentalization of LH receptors increases local concentrations of LH receptors and contributes to declines in cognition with aging.

However, the question of whether binding of hCG has global effects on the motions and compartmentalization of both hormone-occupied and unoccupied LH receptors remains unresolved. In KGN cells, DG-hCG-tagged LH receptors were confined in smaller compartments when cells were exposed to saturating concentrations of hCG (100 nM) suggesting that some non-functional hormone-receptor complexes may redistribute into small membrane compartments together with hCG-occupied receptors. It is important to note that high concentrations of DG-hCG alone do not alter the molecular motions of LH receptors or lead to accumulation of LH receptors into small membrane microdomains. The rotational diffusion coefficients for LH receptors remain fast when receptors are occupied by DG-hCG (54) and there is no evidence of raft localization or confinement of DG-hCG occupied LH receptors in small nanoscale membrane microdomains using SPT (55). Similarly, non-functional hormone receptor complexes formed by binding of hCG to non-signaling LH receptor mutants exhibit fast rotational diffusion (54), no increase in FRET in response to ligand (4) and are not confined in either raft domains or membrane microdomains examined using SPT (7). These results suggest that the slower lateral dynamics observed for Au-DG-hCG when cells are treated with hCG may result from a bystander effect on Au-DG-tagged receptors that may include direct interactions
between hCG-occupied LH receptors and the Au-DG-hCG occupied receptors similar to LH receptor trans-activation described by Ji and coworkers (56).

These studies do no resolve the extent to which LH receptors may exist as pre-aggregated dimers or low molecular weight oligomers prior to binding hormone. Analysis of such structures on living cells would require single molecule methods for evaluating, for example, single molecule measurements of FRET analogous to SPT measurements of lateral diffusion. Nevertheless, it is clear that there are hCG-dependent increases in the extent of LH receptor aggregation regardless of whether the receptor exists initially as a monomer, dimer or larger structure. Using homotrailer FRET techniques to evaluate interactions between stably expressed YFP-LHR, we show that hormone-treated LH receptors exhibit a higher degree of self-association than untreated LH receptors and that the extent of receptor interactions is dependent on hormone concentrations. Similar results were also observed in PCH analysis of changes in ε of YFP-LHR aggregation on CHO cells after hormone treatment.

The hCG-dependent increase in ε also argues against a conformational change in LH receptors leading to increased FRET. There have, in addition, been previous observations of FRET made using fluorescein isothiocyanate (FITC)-hCG and tetramethylrhodamine isothiocyanate (TrITC)-hCG as a donor/acceptor pair under conditions where the formation of large molecular weight complexes containing LH receptors was assessed. Using membrane preparations from porcine granulosa cells, the GTP-dependent progression from “active” LH receptors to “desensitized” LH receptors was accompanied by an increase in FRET efficiency from values less than 1% to over 10%. Increased FRET efficiency was accompanied by slower receptor rotational dynamics indicative of receptors within large molecular weight structures (57). Because both active and desensitized LH receptors in these studies were occupied by hCG, the absence of FRET between “active” receptors also suggests that FRET does not result from an agonist-stabilized receptor conformation.

Importantly, the surface density of stably-transfected LH receptors on these CHO cells was comparable to that of native LH receptors expressed by KGN cells. This suggests that CHO cells were not expressing unrealistically high levels of YFP-LHR. Nonetheless, evidence for some energy transfer between LH receptors expressed at physiologically-relevant numbers raises questions about the role of receptor density in receptor-receptor interactions. Figure 6 shows the extent E of spontaneous FRET arising from an increase in molecular density. As shown in Figure 6, the probability of energy transfer for receptors expressed at a uniform surface density of 20 receptors µm⁻² and a Förster critical distance (r₀) of 4.5 nm is less than 1%. The likelihood of receptor interactions, simply as a function of receptor density, increases with increased receptor expression. For acceptor molecules confined to a plane and having a surface density σ, the efficiency of transfer for given value of r₀ is shown to be 1-exp[−(2π²/3√3)σr₀²] (see Supplemental Material).

From estimates of spontaneous FRET shown in Figure 6, it seems likely those CHO cells stably expressing LHR-GFP with receptor densities that are 2-3 fold greater than the average number of receptors/cell may contribute the FRET efficiencies observed for untreated cells in this study. Substantial FRET will be observed even for homogeneously-distributed molecules if expressed at higher, but commonly encountered, levels. For instance, ErbB1 expressed at levels of 50,000-200,000 receptors per cell is monomeric. At receptor numbers greater than 500,000, approximately 30% of these receptors are in pre-formed dimers (58). Moreover, lateral compartmentalization can only increase the likelihood of apparent FRET. If an acceptor molecule with r₀ of 5 nm in a hetero-FRET pair or a donor molecule in homo-FRET is expressed at an average density of 500 µm⁻² but is confined in lateral compartments occupying 10% of the cell-surface, then an apparent FRET efficiency of 50% would arise solely from molecular crowding.

Nonetheless, these FRET results agree with previous studies showing increased interactions between LH receptors following hCG treatment. These studies have measured FRET between FITC- and TrITC-derivatized hormones or between LH receptors tagged with either visible fluorescent protein (VFP) donor or acceptor. VFP-tagged wild type rat LH receptors occupied by
either LH or hCG had FRET efficiencies of 13-17%. More importantly, most LH receptors interacted only after binding ligand (44) and only functional ligand-receptor complexes or constitutively active LH receptors showed evidence of such interactions (16). In the absence of ligand, values for energy transfer efficiency between stably expressed, VFP-tagged hLHR-wt receptors on individual cells were typically less than 1% while constitutively active receptors also appeared to be constitutively self-associated with values for energy transfer efficiency greater than 10% (16). These various FRET results agree with electron microscopy studies of rat granulosa cells performed by Luborsky and coworkers (59) where only hormone-treated cells showed evidence of receptor-receptor interactions and with immunofluorescence studies of the LH receptor on rat granulosa cells where large, punctate structures formed on the cell membrane following treatment with hCG (60).

Methodological differences may account, however, for a report by Tao et al. (61) suggesting that LH receptors may self-associate in the endoplasmic reticulum and remain constitutively associated when expressed at the plasma membrane. These studies were performed using immunoprecipitation of receptors following cell solubilization, an approach that has significant drawbacks (62), as well as by bioluminescence resonance energy transfer, BRET$^1$ and BRET$^2$, techniques. Similarly, Urizar et al. (63) have suggested that LH receptors are constitutively self-associated as has Guan et al. (64). As with co-immunoprecipitation studies of the LH receptor, BRET studies examine receptor interactions on large numbers of cells that are transiently transfected and can, under conditions where expression levels are high, lead to random receptor interactions (65) and non-physiological receptor aggregation simply due to molecular crowding (62).

In addition, the number of molecules expressed in individual cells following transient transfection may exhibit larger cell to cell variation than, for example, CHO cells stably expressing YFP-LHR. As an example, flow cytometric analysis of cells transfected with a membrane-targeted form of GFP show that about 17% of cells express detectable levels of GFP (66) and, when cells do express GFP, the amount per cell can vary over 100-fold. In a BRET experiment, signal comes from cells that express the donor/acceptor pair and, amongst this group of cells, a disproportionally high signal will arise from cells that highly express the BRET pair. Although reducing the amount of DNA used to transfect cells reduces protein expression and the average level of fluorescence within a cell population, this would not alter cell-to-cell differences in the number of expressed molecules or the disproportionate contribution to BRET signal by cells that over-express the proteins in question. The use of BRET pairs may also magnify this effect. The $r_0$ for the BRET pair used in BRET$^2$ experiments is 7.5 nm, the largest value reported for a resonance energy transfer pair used in biological studies (67), and, as shown in Figure 6, the effect of protein density on FRET efficiency is greater for energy transfer pairs with larger $r_0$.

Finally, ligand-induced receptor conformational changes may increase the likelihood of receptor interactions with the membrane cytoskeleton. Given the dynamic nature of the cytoskeleton, such molecular complexes can be expected to generate apparent compartmentalization of receptor motions over a range of times and distances. Presumably, hCG-induced formation of larger complexes of LH receptors, signaling molecules or other cytoplasmic proteins enhances interaction with elements of the cytoskeleton and reduces the apparent size of the compartments confining the receptors. Nonetheless, understanding the relationship between receptor-mediated signaling and LH receptor palmitoylation, aggregation and confinement in membrane rafts or larger membrane compartments will require more detailed examination of confined receptors and local signaling events, a process that will likely involve microscopic resolution of membrane microdomains and single molecule detection methods for identifying locally high concentrations of second messengers such as cAMP.
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REFERENCES

Abbreviations used in this manuscript; luteinizing hormone (LH), human chorionic gonadotropin (hCG), fluorescence resonance energy transfer (FRET), single particle tracking (SPT), gold nanoparticle-hCG conjugate (Au-hCG), Au-deglycosylated-hCG (Au-DG-hCG), homo-transfer FRET (homoFRET), photon counting histogram (PCH), YFP-coupled human LH receptor (YFP-LHR), fluorescence correlation spectroscopy (FCS), equilibrium dissociation constant (K_D), Dulbecco’s modification of Eagle’s Medium (DMEM), fetal bovine serum (FBS), Minimum Eagle’s Medium, (MEM), Genetin (G418 sulfate), FLAG-tagged LH receptors (FLAG-LHR), macroscopic diffusion coefficient (D), total intensity (s), fluorescence anisotropy (r), diffusional correlation time (τ_D), effective number of detected particles (N_eff), molecular brightness (ε), out-of-focus emission ratio (F), LH receptor point cysteine point mutants (LHR-C621, S622), homoFRET efficiency (E), fluorescein isothiocynate (FITC), tetramethylrhodamine isothiocynate (TRITC), Förster critical distance (r_0), visible fluorescent protein (VFP), bioluminescence resonance energy transfer (BRET).

FIGURE LEGENDS

Figure 1: Compartment sizes (upper panel) and diffusion coefficients (lower panel) for CHO cells expressing FLAG-LHR and for native LH receptors on KGN and M17 cells before and after treatment with 0.1 nM to 100 nM hCG. Data shown are the mean ± S.E.M. where data groups marked a, b, c, d, e and f differ significantly (p<0.05).

Figure 2: Distribution of compartment sizes accessed by FLAG-LH receptors stably expressed on CHO cells before (●) and after treatment with 0.1 nM hCG (○), 1.0 nM hCG (▼) or 100 nM hCG (▲).

Figure 3: Initial and final anisotropy measured before and after photobleaching of YPF coupled to LHR. Data shown are the mean and S.E.M. of at least 20 measurements on individual cells where data groups marked a,b,c,d,e and f differ significantly (p<0.01). The origin of the smaller S.E.M. for the difference (r_final−r_initial) relative to separate values of r_initial and r_final is explained in the text.

Figure 4: Distribution of stably expressed YFP-LHR surface-densities for 58 individual CHO cells as determined by fluorescence correlation spectroscopy.

Figure 5: Number of hCG molecules bound to LH receptors on KGN cells within the focused laser spot as determined by fluorescence correlation spectroscopy. KGN cells were treated with hCG and sequentially labeled with rabbit anti-hCG IgG, biotinylated anti-rabbit IgG and FITC-avidin as described in Materials and Methods. Each point (●) shown is the mean ± S.D. of measurements from at least 14 individual cells.

Figure 6: Degree of spontaneous homoFRET arising from various molecular surface densities. Values shown are for r_0 of 4.5 nm (◊), 6.0 nm (○) and 7.5 nm (■).
Table 1: Effects of hCG treatment on single particle tracking of individual human LH receptors on KGN cells using Au-hCG or Au-DG-hCG probes.\textsuperscript{(a)}

<table>
<thead>
<tr>
<th>Au probe</th>
<th>Treatment ([\text{hCG}])</th>
<th>Compartments\textsuperscript{(b)} per trajectory</th>
<th>(D_{0.1})\textsuperscript{(c)} ((10^{-11}\text{cm}^2\text{sec}^{-1}))</th>
<th>(D = L_r^2/4t)\textsuperscript{(d)} ((10^{-11}\text{cm}^2\text{sec}^{-1}))</th>
<th>Time\textsuperscript{(e)} (sec)</th>
<th>Compartment Size\textsuperscript{(f)} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au-hCG</td>
<td>None</td>
<td>5 ± 2</td>
<td>2.8 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>24 ± 21</td>
<td>186 ± 21\textsuperscript{1}</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>0.1</td>
<td>5 ± 2</td>
<td>2.2 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>24 ± 20</td>
<td>156 ± 17</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>1</td>
<td>6 ± 2</td>
<td>2.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>21 ± 17</td>
<td>148 ± 16\textsuperscript{2}</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>100</td>
<td>6 ± 2</td>
<td>2.0 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>20 ± 13</td>
<td>108 ± 11\textsuperscript{2}</td>
</tr>
<tr>
<td>Au-DG-hCG</td>
<td>None</td>
<td>4 ± 2</td>
<td>2.0 ± 0.2</td>
<td>1.6 ± 0.9</td>
<td>27 ± 26</td>
<td>217 ± 27\textsuperscript{1}</td>
</tr>
<tr>
<td>Au-DG-hCG</td>
<td>100</td>
<td>5 ± 1</td>
<td>2.1 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>25 ± 18</td>
<td>145 ± 20\textsuperscript{2}</td>
</tr>
</tbody>
</table>

\textsuperscript{(a)} Each data point shown is the mean ± S.E.M. from measurements on 10-15 individual cells.

\textsuperscript{(b)} The average number of compartments accessed by an individual LH receptor during a 2 min, 3600 frame image sequence.

\textsuperscript{(c)} \(D_{0.1}\): Diffusion coefficient within compartment calculated from the first two points of MSD vs. time plot as described by Daumas et al. (9).

\textsuperscript{(d)} \(D\) represents the diffusion coefficient within a compartment as calculated from compartment size \((L_r)\) and particle residence time \((t)\) as \(D = L_r^2/4t\) as described by Saxton (19).

\textsuperscript{(e)} Average particle residence time within a compartment.

\textsuperscript{(f)} The average size of an individual compartment was calculated as described by Daumas et al. (9) and Murase et al. (6). Values with subscript 2 differ significantly from values with subscript 1\((p<0.05)\).
Table 2: Effects of hCG treatment on single particle tracking of individual native LH receptors on M17 human neuroblastoma cells using Au-hCG or Au-DG-hCG probes.\(^{(a)}\)

<table>
<thead>
<tr>
<th>Au probe</th>
<th>Treatment [hCG]</th>
<th>Compartments per trajectory</th>
<th>(D_{0.1}) (\times 10^{-11}) cm(^2) sec(^{-1})</th>
<th>(D = L_r^2/4t) (\times 10^{-11}) cm(^2) sec(^{-1})</th>
<th>Time (sec)</th>
<th>Compartment Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au-hCG</td>
<td>None</td>
<td>5 ± 1</td>
<td>2.1 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>25 ± 18</td>
<td>219 ± 22</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>0.1</td>
<td>4 ± 1</td>
<td>2.1 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>28 ± 20</td>
<td>195 ± 26</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>1</td>
<td>4 ± 2</td>
<td>2.3 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>27 ± 21</td>
<td>143 ± 17</td>
</tr>
<tr>
<td>Au-hCG</td>
<td>100</td>
<td>5 ± 2</td>
<td>1.9 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>22 ± 16</td>
<td>131 ± 18</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Each data point shown is the mean ± S.E.M. from measurements on 10-15 individual cells.

\(^{(b)}\) The average number of compartments accessed by an individual LH receptor during a 2 min, 3600 frame image sequence.

\(^{(c)}\) \(D_{0.1}\): Diffusion coefficient within compartment calculated from the first two points of MSD vs. time plot as described by Daumas et al. (9).

\(^{(d)}\) \(D\) represents the diffusion coefficient within a compartment as calculated from compartment size \((L_r)\) and particle residence time \((t)\) as \(D = L_r^2/4t\) as described by Saxton (19).

\(^{(e)}\) Average particle residence time within a compartment.

\(^{(f)}\) The average size of an individual compartment was calculated as described by Daumas et al. (9) and Murase et al. (6). Values with subscript 2 differ significantly from values with subscript 1\((p<0.05)\).
Table 3: Effects of cytochalasin D treatment on single particle tracking of individual human FLAG-tagged LHR-C621, 622S and FLAG-LHR on CHO cells using an Au-anti-FLAG probe.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatments [hCG]</th>
<th>Compartments (a) per trajectory</th>
<th>$D_{0-1}$ (b) $(10^{-11} \text{cm}^2 \text{sec}^{-1})$</th>
<th>$D = L_r^2/4t$ (c) $(10^{-11} \text{cm}^2 \text{sec}^{-1})$</th>
<th>Time (d) (sec)</th>
<th>Compartment Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO FLAG-LHR</td>
<td>None</td>
<td>2 ± 1</td>
<td>3.6 ± 0.9</td>
<td>2.8 ± 0.5</td>
<td>25 ± 18</td>
<td>221 ± 80¹</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO FLAG-LHR</td>
<td>100 nM hCG</td>
<td>2 ± 1</td>
<td>2.8 ± 0.4</td>
<td>2.2 ± 0.4</td>
<td>29 ± 14</td>
<td>204 ± 66¹</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO FLAG-LHR  621,622S</td>
<td>None</td>
<td>3 ± 1</td>
<td>1.1 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>29 ± 12</td>
<td>204± 74¹</td>
</tr>
<tr>
<td>CHO FLAG-LHR  621,622S</td>
<td>100 nM hCG</td>
<td>3 ± 1</td>
<td>1.0 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>32 ± 1</td>
<td>195± 47¹</td>
</tr>
</tbody>
</table>

(a) Each data point shown is the mean ± S.E.M. from measurements on 10-20 individual cells.
(b) The average number of compartments accessed by an individual LH receptor during a 2 min, 3600 frame image sequence.
(c) $D_{0-1}$: Diffusion coefficient within compartment calculated from the first two points of MSD vs. time plot as described by Daumas et al. (9).
(d) D represents the diffusion coefficient within a compartment as calculated from compartment size ($L_r$) and particle residence time (t) as $D = L_r^2/4t$ as described by Saxton (19).
(e) Average particle residence time within a compartment.
(f) The average size of an individual compartment was calculated as described by Daumas et al. (9) and Murase et al. (6). Values with subscript 2 differ significantly from values with subscript 1 ($p<0.05$).
Table 4: Effects of hCG treatment on efficiency of homoFRET between YFP-LHR on CHO cells.\(^{(a)}\)

<table>
<thead>
<tr>
<th>Treatment [hCG]</th>
<th>(r_{\text{initial}})</th>
<th>(r_{\text{final}})</th>
<th>E (%)(^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.18 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>10%</td>
</tr>
<tr>
<td>0.1 nM</td>
<td>0.15 ± 0.01</td>
<td>0.19 ± 0.04</td>
<td>20%</td>
</tr>
<tr>
<td>1.0 nM</td>
<td>0.17 ± 0.05</td>
<td>0.23 ± 0.05</td>
<td>26%</td>
</tr>
<tr>
<td>100 nM</td>
<td>0.17 ± 0.04</td>
<td>0.26 ± 0.06</td>
<td>34%</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Data points shown are the mean ± S.D.
\(^{(b)}\) FRET efficiencies (E) were estimated from average initial and final anisotropy values shown in Figure 3 as \(\%E = (1 - r_{\text{initial}}/r_{\text{final}}) \times 100\).
Table 5: Effects of hCG treatment on PCH analysis of YFP-LHR on CHO cells.

<table>
<thead>
<tr>
<th>hCG (nM)</th>
<th>$N_{eff}^{(a)}$</th>
<th>$\varepsilon$ (kHz) $^{(b)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.5±2.6</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>1</td>
<td>13.6±3.3</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>10</td>
<td>13.4±5.6</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>100</td>
<td>15.2±4.2</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>1000</td>
<td>15.3±3.4</td>
<td>0.14±0.04</td>
</tr>
</tbody>
</table>

(a) Each data point shown is the mean ± SEM of measurements on at least 6 individual cells.
(b) Estimates of $\varepsilon$ were obtained using a detector bin time of 9 μs and F values from 0.5-0.7. Data points shown are the mean ± SD.
(c) Values were statistically different from cells not treated with hCG using students t-test (p<0.02).
Figure 2

![Graph showing the relationship between compartment size (nm) and the fraction of analyzed domains. The x-axis represents compartment size from 50 to 450 nm, while the y-axis represents the fraction of analyzed domains from 0.0 to 0.4. Multiple lines with different markers indicate different categories or conditions.]
Figure 3

Anisotropy (r)

[r initial](
[r final](
[r final - r initial](

Anisotropy (r)

[0.0](
[0.1](
[0.2](
[0.3](
[0.4](

[hCG] (nM)

0 0.1 1.0 100

a a ab
c cc
e e f
f

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Figure 4

LHR-YFP receptors/μm²

Number of cells

0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
>100

0 2 4 6 8 10 12 14 16 18
Figure 5

 Detected particles ($N_{eff}$) vs. hCG (nM)

- Vertical axis: Detected particles ($N_{eff}$)
- Horizontal axis: hCG (nM)

Data points and error bars are plotted on the graph.
Figure 6

Local receptor density (μm⁻²)

FRET (%)