DISSERTATION

DYNAMIC INTERACTIONS OF LUTEINIZING HORMONE RECEPTORS DURING SIGNAL TRANSDUCTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YING LEI ENTITLED DYNAMIC INTERACTIONS OF LUTEINIZING HORMONE RECEPTORS DURING SIGNAL TRANSDUCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DYNAMIC INTERACTIONS OF LUTEINIZING HORMONE RECEPTORS DURING SIGNAL TRANSDUCTION

There is increasing evidence that receptor-mediated signal transduction by G-protein coupled receptors can involve self-association and redistribution of plasma membrane receptors into specialized plasma microdomains or rafts. These membrane fragments are characterized by insolubility in cold Triton X-100 and localization to low density regions in sucrose gradients.

Here, we characterized the translocation of wild type rat and human luteinizing hormone (LH) receptors into rafts following the binding of human chorionic gonadotropin (hCG) via a sucrose gradient ultracentrifugation method. We have also examined constitutively active human LH receptors which, in contrast to wild type receptors, were self-associated in the absence of hormone with a significant fraction of the receptors localized in rafts.

Receptor localization in membrane rafts did not occur when cells were pretreated with 1% methyl-β-cyclodextrin (MβCD), a cholesterol sequestering agent that reduces membrane cholesterol and disrupts membrane rafts. Pretreatment of cells with MβCD also significantly decreased levels of hormone-induced intracellular cAMP in

wild type LHR, but not constitutively active receptors, and reduced the extent of receptor self-association as evaluated by fluorescence resonance energy transfer (FRET).

Translocation of LH receptors into rafts required a functional hormone-receptor complex. When CHO cells expressing FLAG-LHR-wt were treated with an hCG antagonist such as deglycosylated hCG, receptors remained in high density membrane fractions as did hCG-treated point mutated receptor LHR-K583R that has reduced responsiveness to hCG binding. On the other hand, extensive crosslinking of wild type LH receptors with anti-FLAG antibody and a second anti-mouse IgG, while elevating intracellular cAMP, did not drive LH receptors into the raft environment. Finally, we found that the LH receptor C-terminus and its ability to be palmitoylated might affect raft localization.

In conclusion, translocation of the LH receptor into specialized membrane microdomains may be important in hormone-mediated signaling and a characteristic of functional, hormone-occupied LH receptors. Nonetheless, under some conditions, the raft environment is not essential for cAMP-mediated signaling.

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BACKGROUND HISTORY

Introduction

Hypothalamic-pituitary-gonadal axis in reproduction

The regulation of luteinizing hormone (LH) release by the hypothalamic-pituitary-gonadal axis has been well characterized. In response to neural, as well as endocrine stimuli, the hypothalamus releases the decapeptide hormone gonadotropin releasing hormone (GnRH) into the hypophysial portal circulation. GnRH binds the GnRH receptor located on gonadotrophs in the anterior pituitary. Hormone binding to the GnRH receptor initiates both synthesis and release of LH into the systemic circulation. A high-affinity receptor for LH is expressed on the plasma membrane of cells in the gonads. Binding of LH to its receptor leads to the up-regulation of tissue-specific sex steroid synthesis and release (Conn et al., 1987). A negative feedback loop exists in which sex steroids from both the male and female gonads decrease secretion of GnRH from the hypothalamus and LH from the anterior

pituitary. This feedback loop helps regulate the levels of LH and the sex steroids very tightly in the body.

Role of the luteinizing hormone receptor in human reproduction

The receptor for luteinizing hormone and chorionic gonadotropin plays a key role in normal and abnormal reproductive physiology. In females, the LH receptor is found on granulosa and thecal cells in the follicle to produce androgen precursors necessary for estrogen synthesis, and on luteal cells to promote ovulation, corpus luteum formation and progesterone secretion. Human chorionic gonadotropin (hCG) produced by the placenta acts on the corpus luteum which secretes progesterone necessary for maintenance of pregnancy and promotes development of the fetal testes during the first trimester of pregnancy. In males, development, differentiation and activity of Leydig cells is dependent on LH and hCG acting through the LH receptor. The testicular Leydig cells produce testosterone that is responsible for normal growth and differentiation of the male genital tract which includes the epididymis, the vasa deferentia, the seminal vesicles, the prostate and the penis. At puberty and thereafter, testosterone is required for the development of secondary sex characteristics and for sperm production. In humans fetal androgen production is directed by maternal hCG, while after birth this process is controlled by the pituitary through the production of luteinizing hormone (McFarland et al., 1989; Shenker, 2002).

Activating and inactivating mutations have been identified in LH receptor genes,

which produce very different phenotypic effects (Themmen and Huhtaniemi, 2000). Dominant mutations that lead to constitutive activation of the LH receptor-mediated cAMP (cyclic adenosine monophosphate) signaling pathway appear to explain the pathophysiology of Familial Male-limited Precocious Puberty (FMPP). LH receptor-mediated effects, including testosterone production, are known to involve increased production of cellular cAMP. Intracellular cAMP accumulation triggered by unoccupied mutant receptors appears sufficient to cause Leydig cell hyperfunction, hyperplasia and even tumor formation. Loss-of-function mutations of the LH receptor gene can cause Leydig cell hypoplasia, male pseudohermaphroditism, and primary amenorrhea in females (Shenker, 2002). Apparently patients with defects in the LH receptor display aberrant sex differentiation and/or infertility.

Structure of LH and hCG

As previously mentioned, the LH receptor binds two hormones, LH and hCG, which are members of the glycoprotein hormone family that also includes follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). LH and hCG are heterodimeric proteins composed of one α and one β subunit that assemble by noncovalent interactions (Li and Starman, 1964; Dias, 1992; Xing et al., 2001). The α subunit is 92-96 amino acids long and is highly conserved for both LH and hCG. The β subunit is hormone specific for each member of the glycoprotein hormone family. The β subunit of LH is 117 amino acids in length, while the β subunit of

hCG is 145 amino acids (Ascoli and Segaloff, 1989). The first 114 amino acids of LH and hCG have 85% homology, but hCG differs from LH in that it has a 20 amino acid C-terminal extension (Pierce and Parsons, 1981; Sairam and Manjunath, 1983). All of the β subunits have 12 cysteine residues at highly conserved positions which form six disulfide bridges. Although both subunits are necessary for binding to receptor, the β subunit dictates receptor specificity.

Upon association of the α and β subunits, a conformational change occurs, which results in the active heterodimer (Ingham et al., 1976). Since free α subunit has little or no binding activity, it is the association of the α with the β subunit that results in the proper conformation for binding of the heterodimer to receptor. Based on results from studies using hybrid hormones consisting of various α and β subunits, it is believed that the α subunit is the driving force for the association of hormone with receptor but that the β subunit specifically limits the types of hormone-receptor interactions (Combarnous, 1992). The crystal structure of deglycosylated hCG has shown that each of its two different subunits has a similar topology (Lapthorn et al., 1994), with three disulfide bonds forming a cysteine knot, the same folding motif found in some protein growth factors (McDonald et al., 1991; Oefner et al., 1992; Schlunegger and Grutter, 1993). The heterodimer is stabilized by a segment of the β-subunit which wraps around the α-subunit and is covalently linked like a seat belt by the disulfide. This extraordinary feature appears to be essential not only for the association of these heterodimers but also for receptor binding by the glycoprotein hormones (Lapthorn et al., 1994; Bernard MP et al., 2004).

Recent studies of FSH have presented the crystal structure of a partially deglycosylated complex of human FSH bound to the extracellular hormone-binding domain of its receptor ($FSHR_{HB}$). The hormone is bound in a hand-clasp fashion to an elongated, curved receptor as shown in Figure 1. The buried interface of the complex is large and has a high charge density. The analysis suggests that all glycoprotein hormones bind to their receptors in this mode and that binding specificity is mediated by key interaction sites involving both the common α-subunits and hormone-specific β-subunits. On binding, FSH undergoes a concerted conformational change that affects protruding loops implicated in receptor activation. The FSH-$FSHR_{HB}$ complex forms dimers in the crystal and at high concentrations in solution. Such dimers may participate in transmembrane signal transduction (Fan and Hendrickson, 2005).

Carbohydrates attach to both α and β subunits at various points. There are up to four N-linked and three O-linked oligosaccharides representing 18-45% of the total hormone weight. The α subunit contains two sites for N-linked glycosylation (Bahl and Moyle, 1978) as does the β subunit of LH. In contrast, the β subunit of hCG contains two sites for N-linked glycosylation and four O-linked glycosylation sites on the 20 amino acid C-terminus (Winzler, 1973). Carbohydrate moieties are thought to be involved in stabilization of hormone conformation, regulation of hormone half-life in the circulation, secretion and uptake by cells (Sairam and Bhargavi, 1985; Matzuk et al., 1989; Petäjä-Repo et al., 1991). Carbohydrates attached to LH and hCG are also vital for physiologic responses such as normal receptor ligand interactions and

Figure 1. Crystal structure of human FSH bound to $FSHR_{HB}$. **a, b,** Ribbon diagram of the complex structure shown in two views related by a 90° rotation about the vertical axis. FSH α-chains and β-chains are in green and cyan, respectively. $FSHR_{HB}$ is in red. The observed N-linked carbohydrates at N52 and N78 of FSH-α, N7 and N24 of FSH-β, and N191 of $FSHR_{HB}$ are in yellow. Disulphide bonds are in black. (Fan and Hendrickson, 2005)

internal cell signaling. For example, the role hCG's carbohydrates play in the activation of internal cell signaling mechanisms such as adenylate cyclase has been studied. In these investigations, the serial removal of carbohydrate residues from hCG caused a progressive decrease in hCG-induced cyclic adenosine monophosphate (cAMP) accumulation, an internal signaling molecule (Chen et al., 1982) and thus function as an hCG antagonist. Compared with glycosylated hCG, fully deglycosylated hCG has been shown to have normal binding but reduced biological activity (Manjunath and Sairam, 1982). Although the role carbohydrates play in hormonal signaling is not well defined, it is believed that carbohydrates on LH and hCG help maintain hormone structure in a conformation that is required to activate LH receptors (Thotakura et al., 1990). How carbohydrates might play a role in receptor-mediated signaling remains unclear.

LH/hCG Receptor Structure

The LH receptor is a member of the G-protein-coupled receptor (GPCR) family (Wess, 1998; Gether, 2000; Shenker, 2002). Like the other members of the GPCR subfamily whose agonists are glycoprotein hormones (follitropin [FSH] and thyrotropin [TSH]), the LH receptor is characterized by a large, glycosylated N-terminal extracellular (EC) domain about 340 amino acids, which independently determines hormone binding affinity and specificity (Segaloff and Ascoli, 1993; Dufau, 1998). The rat LH receptor (rLHR) is a single polypeptide composed of 674

amino acid residues, which also contains seven transmembrane (TM) domains consisting of approximately 24 amino acids each, and a C-terminal intracellular tail of 70 amino acids (McFarland et al., 1989; Probst et al., 1992). Three extracellular loops and three intracellular loops act like the bridges that connect those transmembrane domains (Figure 2). The sequence of the human LH receptor (hLHR) is very similar to the rat receptor and consists of 699 amino acids (Jia et al., 1991).

Conservation of the transmembrane domains between species is high while the larger extracellular region and the cytoplasmic tail are less conserved (Segaloff and Ascoli, 1993). The amino acid sequence identity between the hLHR and the rLHR is approximately 88% in the extracellular domain, approximately 92% in the transmembrane domains, and approximately 69% in the C-terminal cytoplasmic tail (Ascoli et al., 2002). A number of orphan GPCRs with large EC domains containing leucine-rich repeats and other sequence similarity to the glycoprotein hormone receptors have been cloned from invertebrates as well as mammals, suggesting that this GPCR group is evolutionarily ancient (Shenker, 2002).

The EC domain is encoded by the first ten exons of the LH receptor gene and contains imperfect leucine-rich repeats of approximately 25 residues each and a short hinge region. The repeats are believed to be arranged in the shape of a horseshoe, with the parallel β strands and loops comprising an inner circumference that provides important contact sites for hormone binding (Moyle et al., 1995; Bhowmick et al., 1996; Zeng et al., 2001). The LH receptor protein sequence has six potential sites

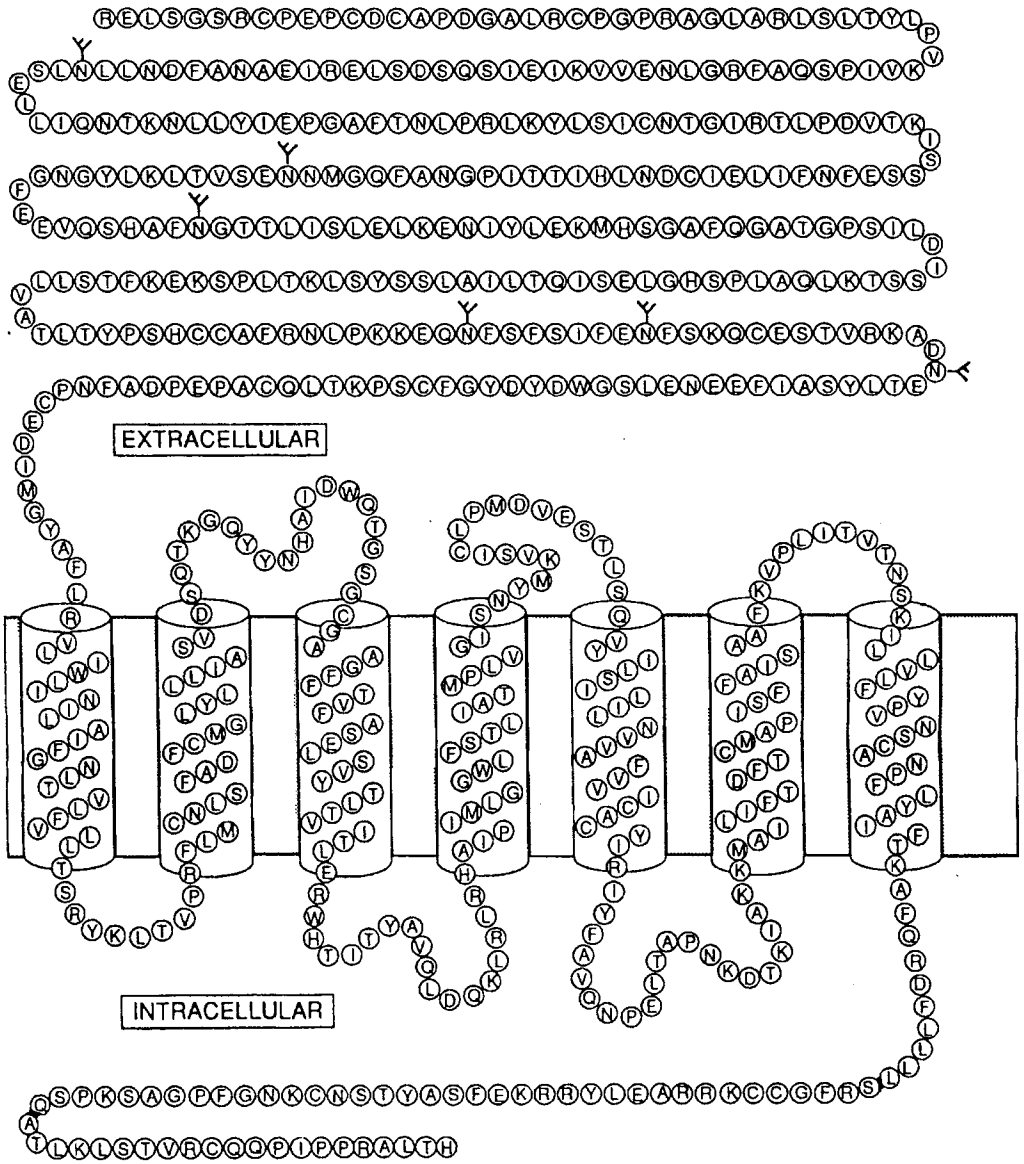

Figure 2. LHR amino acid sequence. (McFarland et al., 1989)

for N-linked glycosylation, all of which are found on the EC domain. Although it is known that the LH receptor contains N-linked carbohydrates, whether all six potential sites for carbohydrate attachment are used is still not clear (Dias, 1992; Ascoli et al., 2002).

Studies using synthetic peptides created to mimic sections of the natural extracellular domain and three exoloops have located four main sites for hormone-receptor interaction. These experiments showed that three regions in the extracellular domain and one in the third exoloop could interact with hormone (Roche et al., 1992). The leucine-rich repeat domain (LRD) in the extracellular domain, which is believed to have a primary role in ligand binding, is separated from TM domain by a "linker". The linker is usually thought to function as a "hinge" that enables the LRD to convey the ligand to the TM domain. The linker has also been proposed to couple the LRD directly to the TM domain and thereby enable changes in the conformation of the LRD to be transferred to the TM domain and vice versa (Vassart et al., 2004). In a model that describes the structure of the linker, the linker is thought to be much more than a hinge, and interactions of the linker with the ligand, the LRD, and the TM domain are thought to be important for ligand binding and signaling (Moyle et al., 2004).

Exon 11 encodes the transmembrane domain, seven membrane-spanning α-helical segments (TM1-TM7) that are connected by alternating extracellular (e1, e2, e3) and intracellular (i1, i2, i3) loops. There are 12 cysteine residues on the EC

domain and 13 in the transmembrane and cytoplasmic domains which are important for forming disulfide bridges. As with other GPCRs, the helical segments of the LH receptor are predicted to be arranged in a compact bundle with a central hydrophilic pocket (Baldwin et al., 1997; Ballesteros et al., 2001). Certain amino acid residues in the cytoplasmic tail and endoloops are important in relaying the signal between the receptor and intracellular signaling mechanisms. In many GPCRs, endoloop 2 and 3 along with parts of the cytoplasmic tail have been implicated in linking the receptors to signaling proteins (Gether, 2000). According to the experiments on mutated rat LH receptors, certain residues in the endoloop 2 appear to be important for proper localization of LH receptor and some other residues have been implicated in signaling and ligand-mediated internalization (Fernandez and Puett, 1997). The sixth transmembrane domain and third endoloop are thought to be critical for initiating intracellular signaling (Kudo et al., 1996; Abell and Segaloff D, 1997). For example, naturally occurring mutations in the sixth transmembrane domain at position 578 are associated with constitutive activation of LH receptor, which causes an increase in intracellular cAMP concentrations in the absence of hormone. It indicates that the amino acid is important for intracellular signaling of the LH receptor (Themmen and Huhtaniemi, 2000; Shenker, 2002).

Besides relaying the signal between the receptor and intracellular signaling mechanism, the LH receptor C terminus has also been implicated in modulation of receptor turnover. At two cysteine residues in the C-terminal tail, the LH receptor is palmitoylated, which is believed to provide two anchoring sites for the cytoplasmic

tail onto the plasma membrane. This may be related to the internalization of the receptor (Menon et al., 2004). The absence of palmitoylation increases the hCG-induced internalization of the receptor, although it has no effect on the intracellular trafficking of the receptor and the ability to stimulate cAMP production (Qanbar and Bouvier, 2003; Menon et al., 2004). In some studies of the intracellular tail, mutants have been constructed with progressive truncations of the cytoplasmic tail (Sanchez-Yague et al., 1992; Rodriguez et al., 1992). Results from these studies suggest that a region(s) between residues 616 and 631 of the rLHR is required for proper insertion and/or targeting of the receptor to the plasma membrane, and that rLHR-t631 exhibits faster rates of hCG-induced internalization as compared to the full-length receptor (Rodriguez et al., 1992). In addition, the C-terminal cytoplasmic tail of the LH receptor is necessary for agonist-induced desensitization (Sanchez-Yague et al., 1992).

At the present time, no structural data exist for LH receptors. Since the first crystal structure of a GPCR, bovine rhodopsin, became available in 2000 (Palczewski et al., 2000), three molecular models of the transmembrane domains of the LH receptor, with or without the extracellular and intracellular loops, have been built following *ab initio* or comparative modeling approaches by using the structural information inferred from multiple alignments of GPCR sequences and from the electron density maps of rhodopsin (Fanelli et al., 2004). In general, the length of the seven helices in the *ab initio* LH receptor model is in good agreement with that found in the rhodopsin structure. The biggest deviations between the wild type LH

receptor model and the rhodopsin structure are mainly due to the arrangements of helices 2 and 3 (Fanelli et al., 2001). These models provide an important source of information for designing new experiments aimed at elucidating structure-function relationships in glycoprotein hormone GPCRs and suggest novel receptor sites potentially susceptible to activating mutations. Combining the high-resolution structural information on rhodopsin with computer simulations constitutes a promising and powerful tool for gaining significant insight in our understanding of the molecular mechanism associated with function of these membrane proteins.

Signal transduction by LH receptors

The LH receptor is a member of the GPCR superfamily that transduces signal intracellularly in response to hormone binding. All GPCRs, including LH receptor and β-adrenergic receptor, share common structural features such as an extracellular domain, seven membrane-spanning α helical transmembrane domains, three exoloops, three endoloops, and a cytoplasmic tail (Probst et al., 1992). For GPCRs, the third endoloop, the second endoloop, and in some receptors, the cytoplasmic tail, are known to couple the receptor to G-protein (Gether, 2000). In the case of LH receptors, the lower portion of the sixth transmembrane domain has been proven to be critical in activation of adenylate cyclase (Abell and Segaloff, 1997). It is believed that agonist binding to GPCR results in a conformational change in the ligand binding domain. This causes secondary contacts with the extracellular loop regions of the

receptor and thus, receptor activation. Activation of the receptors apparently involves a counter-clockwise rotation of TM6 and movement of the cytoplasmic end of the helix away from TM3. Due to this rotation, amino acids previously buried within the membrane are now found in a more polar environment where they can interact with G proteins (Gether, 2000).

G proteins exist as inactive complexes containing three distinct subunits, α, β, and γ, prior to ligand binding as shown in Figure 3. In this inactive state, the α-subunit has GDP bound to it while the β and γ subunits help to anchor the heterotrimer in the inner leaflet of the plasma membrane. Receptor activation catalyzes the rate limiting release of GDP from the α-subunit and subsequent binding of GTP. Once GTP is bound, the α-subunit becomes activated and dissociates from both the activated receptor and the βγ-subunits. The activated state persists until GTP is hydrolyzed to GDP by the endogenous GTPase activity of the α-subunit. Upon hydrolysis of GTP to GDP, α-GDP reassociates with βγ. This cycle persists as long as agonist is available to bind receptor and as long as the agonist-bound receptor can activate G proteins (Stryer and Bourne, 1986).

The LH receptor was one of the first GPCRs shown to independently activate two G protein-dependent signaling pathways, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and the diacylglycerol (DAG)/protein kinase C (PKC) pathway (Herrlich et al., 1996; Spiegel, 1998; Ascoli et al., 2002). Activation of the LH receptor leads primarily to the activation of the G-protein G_s

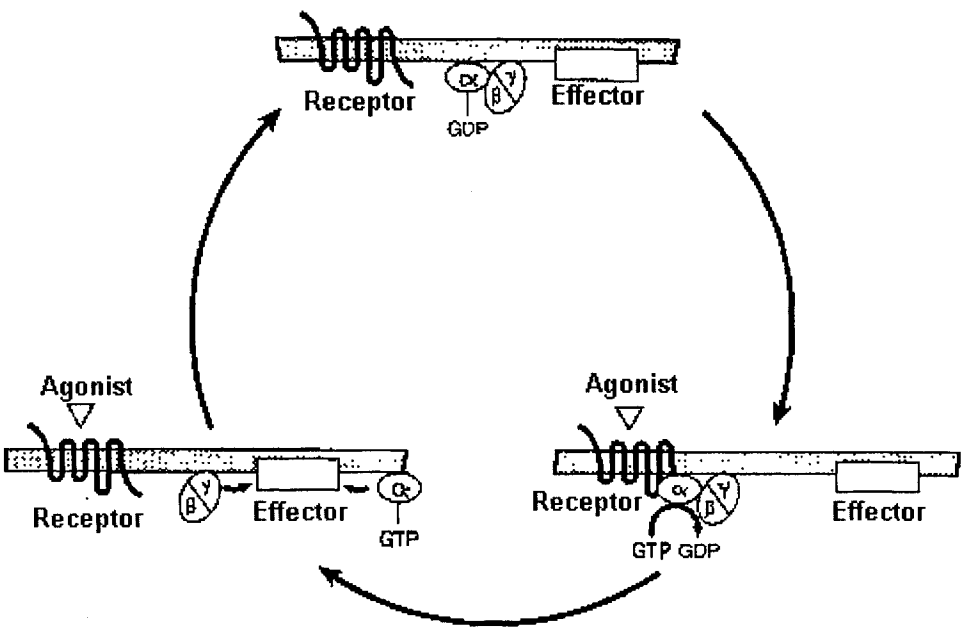

Figure 3. Diagram of the G protein-coupled receptor signaling showing the seven transmembrane domain receptor, the heterotrimeric G protein, the exchange of GDP for GTP upon activation of the G protein, dissociation of the G protein, activation of the effector molecule by the subunits, and the reassembly of the signaling proteins. (Spiegel, 1998)

(Dufau, 1998). Following dissociation from βγ, the α-subunit activates the membrane effector adenylate cyclase (AC). Activation of AC in turn converts adenosine triphosphate (ATP) to the second messenger molecule, cAMP. cAMP can then bind to the regulatory subunit of PKA and cause a release of the catalytic subunits (Yen et al., 1999). The active catalytic subunits of PKA can phosphorylate specific serine and threonine residues on ribosomal, nuclear and cytoskeletal proteins, some of which participate in the synthesis and secretion of steroids. In addition, the PKA pathway regulates the activity of specific enzymes necessary for the conversion of cholesterol to the sex steroids (Tang et al., 1998).

Ligand binding to the LH receptor under some conditions may also activate the PKC pathway. LH receptors found on the ovaries as well as receptors stably expressed in cultured cells exhibit an increase in free intracellular calcium and phosphoinostitide (PI) hydrolysis when activated with hCG (Gudermann et al., 1992; Dufau, 1998). *In vivo*, this occurs at the time of the LH surge when receptor density is high. This pathway may be important during pregnancy and the preovulatory LH surge. Upon receptor activation, the effector molecule phospholipase C (PLC) cleaves membrane lipid phosphatidylinositol biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 is released into the cytoplasm causing the release of sequestered calcium from the endoplasmic reticulum. DAG remains in the membrane and activates PKC which phosphorylates specific serine and threonine amino acid residues on target proteins and thereby regulates their action

(Yen et al., 1999). The hydrolysis of PI to IP_3 and DAG probably results from interactions of the LH receptor with the βγ-subunits released from G_s or G_i rather than from the release of the α-subunit of G_q/G_{11}.

However, the mechanism of activation for LH receptors is still poorly understood. A novel mechanism of intermolecular GPCR activation has been described recently (Figure 4). It shows that binding of hormone to one receptor can activate adenylate cyclase through its transmembrane bundle, intramolecular activation (*cis*-activation), as well as *trans*-activation through the transmembrane bundle of an adjacent receptor (Ji et al., 2002). The experiment, in which coexpression of a mutant receptor defective in hormone binding and another mutant defective in signal generation rescues hormone-activated cAMP production, has provided support to this theory.

Furthermore, the idea that GPCRs can exist and potentially function as dimers and/or higher oligomers is now generally accepted (Milligan et al., 2003). Although an increasing amount of data suggests that dimers represent the basic signaling unit for most, maybe not all, members of this receptor family, GPCR dimerization might also be necessary to pass quality-control checkpoints of the biosynthetic pathway of GPCRs. To date, this hypothesis has been demonstrated unambiguously for only a small number of receptors that must form heterodimers to be exported properly to the plasma membrane (referred to as obligatory heterodimers). However, increasing evidence suggests that homodimerization might have a similar role in the receptor maturation process for many GPCRs (Bulenger et al., 2005).

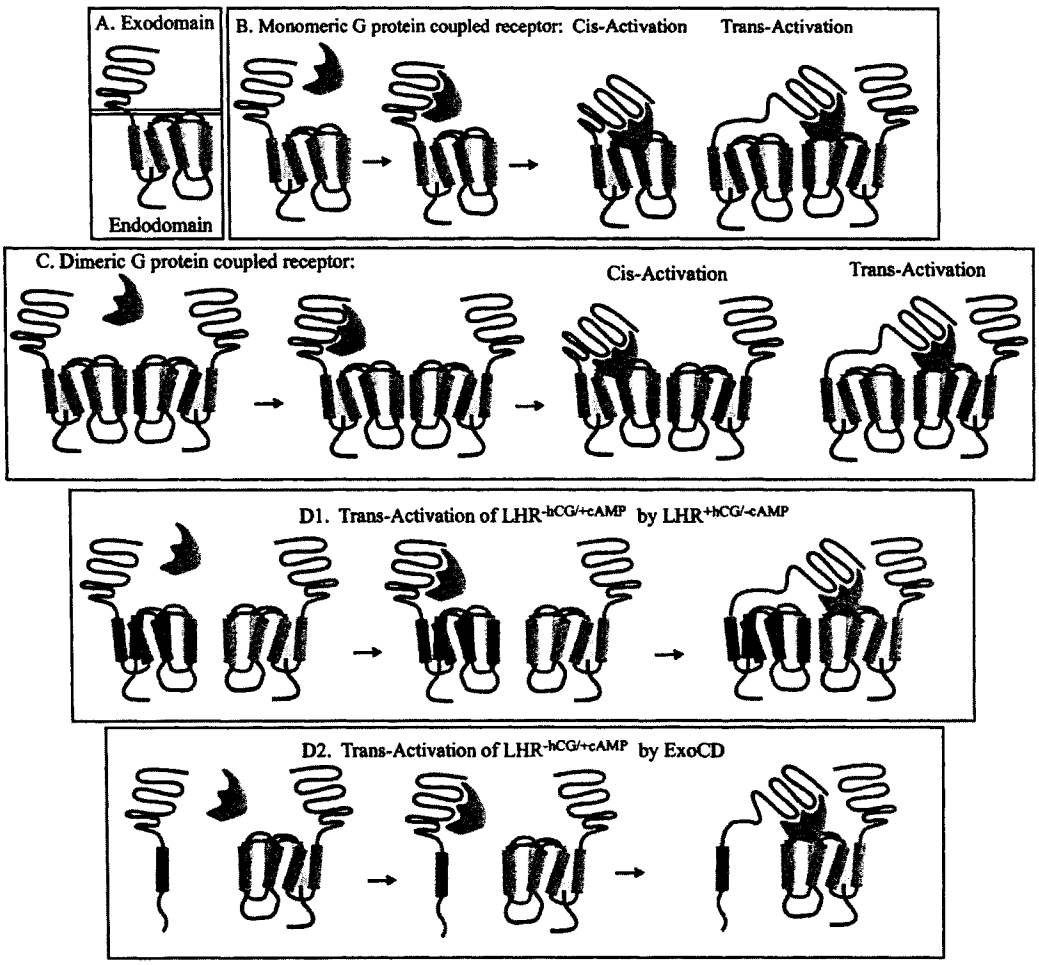

Figure 4. Hypothetical models for Monomeric and Dimeric *Cis*-Activation and *Trans*-Activation. (Ji et al., 2002)

Following signaling, there is a decrease in receptor responsiveness to repetitive or continuous stimulation, which is termed receptor desensitization (Ferguson, 2001). LH receptor desensitization occurs in ovarian follicles in response to the mid-cycle LH surge that promotes ovulation and, based on serum progesterone levels, appears to occur in human corpora lutea in response to elevated levels of hCG during pregnancy (Hunzicker-Dunn et al., 2003). LH receptors, in response to binding of ligand, can also undergo time-dependent changes in their functional status and remain within the plasma membrane. Like other GPCRs including the β-adrenergic receptor (β-AR), the LH receptor becomes less responsive, i.e., desensitized, within minutes after binding of hCG or LH. The LH receptor exhibits desensitization in response to treatment with saturating hormone concentrations, a process that has been observed *in vitro* in granulosa, luteal, and Leydig cells. Desensitization of the LH receptor is followed by a decrease in cellular cAMP even with the presence of LH. Once desensitized, hormone binding to LH receptors only minimally activates adenylate cyclase despite the fact that adenylate cyclase is still functional and can be activated by other means. Desensitization of LH receptors following brief exposure to hormone is initially characterized by uncoupling of the receptor from the signal transduction apparatus rather than by a decrease in receptor number (Roess and Smith, 2003).

The desensitization of GPCRs is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from

heterotrimeric G proteins in response to receptor phosphorylation, the internalization of cell surface receptors to intracellular membranous compartments, and the downregulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Ferguson, 2001). However, the inability of receptors to activate their respective G-protein appears to be the most important cause of receptor desensitization (Dohlman et al., 1991). The molecular mechanism for desensitization of β-AR has been examined in detail. Activation of β-AR by epinephrine binding leads to the activation of the cAMP/PKA signaling pathway and to the phosphorylation of the β-AR C-terminal tail and intracellular loops both by second messenger-dependent kinases as well as by G protein-coupled receptor kinases (GRKs). These phosphorylated sites can bind arrestin which prevents the receptor from interacting with other G proteins and thus serves to "uncouple" the receptor from the signaling machinery (Freedman and Lefkowitz, 1996).

In addition to uncoupling receptors from G proteins, arrestins also play an integral role in receptor sequestration. Receptor sequestration is defined as the movement of the agonist-occupied receptor from a site where the receptor is accessible to ligand to a location in which the receptor is no longer accessible. Receptor sequestration generally occurs after receptor phosphorylation and may be required for reactivation of GPCRs, including receptor dephosphorylation and recycling to the cell surface (Krueger et al., 1997).

Unlike β-AR, whose desensitization is dependent on receptor phosphorylation, desensitization of the LH receptor does not require phosphorylation (Lamm and Hunzicker-Dunn, 1994). This can be demonstrated by truncating the receptor's cytoplasmic tail to remove serine and threonine residues or mutating these amino acids to alanines. Both procedures abolished all detectable phosphorylation but do not affect desensitization (Hunzicker-Dunn et al., 1996).

Desensitization is followed by endocytosis of a number of GPCRs, including β-AR, which utilizes a clathrin-coated vesicle pathway. Interactions between clathrin and the receptor are mediated by arrestins. Upon recruitment and binding of β-arrestin to the phosphorylated β-AR, the receptor is sequestered in the clathrin-coated pits (Goodman et al., 1996).

Activated receptors that have been desensitized in response to ligand binding are said to have undergone homologous receptor desensitization. However, due to the non-specific action of GRKs and second messenger-dependent kinases, receptors that have not been activated may be phosphorylated and desensitized. This non-specific response is termed heterologous desensitization. Heterologous desensitization is probably most important in conditions where receptor occupancy and thus agonist concentrations are low (Freedman and Lefkowitz, 1996).

Role of plasma membrane structure in signal transduction

Membrane environment may also affect the LH receptor signaling, so it is helpful to know about plasma membrane structure. Singer and Nicolson proposed a model for plasma membrane structure in 1972 which, with revision, remains valid today (Figure 5). Their model, known as the fluid mosaic model, predicted that the plasma membrane was a viscous structure with dynamic organization. In Singer and Nicolson's model, phospholipid molecules, which make up the bulk of a cell's plasma membrane, orient themselves two-dimensionally. Furthermore, since phospholipids are amphipathic, they believed that a phospholipid's polar head group would contact aqueous environments while their long fatty acid chains would orient to exclude water and pack tightly to form the hydrophobic environment of the intra-membrane space of the lipid bilayer (Singer and Nicolson, 1972).

The fluid mosaic model also predicts the interaction of proteins with the lipid bilayer. Singer and Nicolson proposed two types of membrane proteins: peripheral and integral. Peripheral membrane proteins associate with the membrane by weak, noncovalent interactions. These proteins, such as cytochrome *c*, which is associated with the inner mitochondrial membrane, are easily removed from membrane and do not interact with the phospholipid's fatty acid chains. Integral membrane proteins span the entire plasma membrane bilayer. These proteins make intimate contact with both the hydrophobic and hydrophilic areas of the membrane and are much harder to dissociate from lipids. An example of an integral membrane protein is the LH

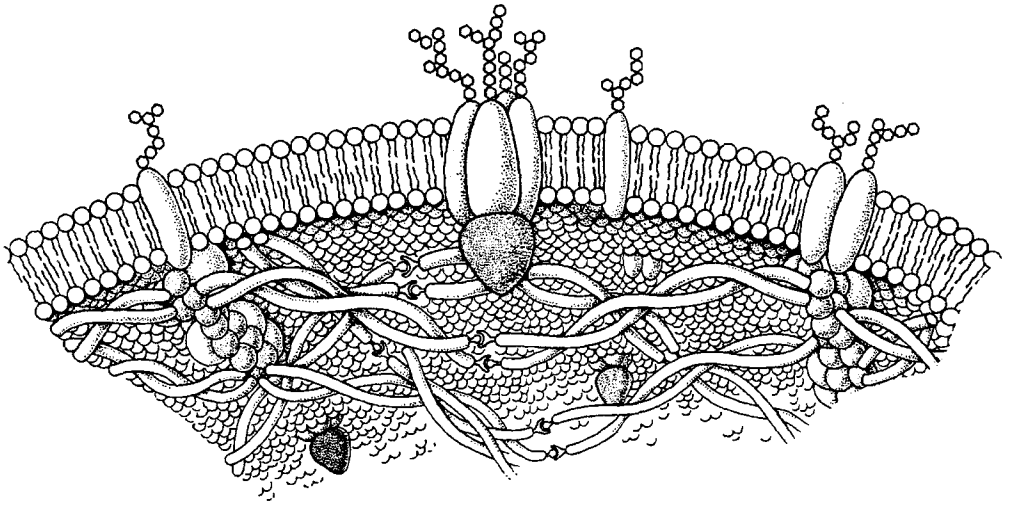

Figure 5. Plasma membrane structure showing proteins in the phospholipids bilayer. Modified from Stryer, 1988.

receptor that not only interacts with the plasma membrane via transmembrane domains but also has intracellular and extracellular domains. In this sea of lipids, integral membrane proteins, such as LH receptors, exhibit lateral motions as well as self-association during and after cell signaling. The nature of these motions is determined by how proteins interact with the lipid bilayer as well as by the extent of protein-protein interactions. Thus changes in lipid composition, clustering of proteins into large molecular weight complexes or associations between membrane proteins and the cytoskeleton will affect the movement of a given protein within the membrane, and furthermore the function of the protein.

Role of lipid rafts in signaling

Lipid rafts were conceived as functional lipid microdomains less than 17 years ago (Simons and van Meer, 1988; van Meer and Simons, 1988). Although Singer & Nicolson considered the possibility of small membrane domains in the fluid cell membrane bilayer, all models of cell membranes comprising domains were rather general and did not focus on specific biological functions that required domain formation (Edidin, 2003). Recently, the different components and organization of plasma membrane lipids has attracted more attention. Distinct plasma membrane fractions have been isolated from cells because of their unique densities. These fractions are characterized by proteins found within them such as glycosylphosphatidylinositol (GPI)-anchored proteins. Within the disordered phase

that constitutes the bulk of the lipid bilayer, membrane subdomains, i.e. lipid rafts, enriched in cholesterol and sphingolipids are thought to form a separate liquid ordered phase (Incardona and Eaton, 2000). Lipid rafts were initially identified based on their ability to form insoluble complexes after cell lysis in cold Triton X-100. They are thought to form by the self-association of sphingolipids which have long and mostly saturated hydrocarbon chains (Pralle et al., 2000). Upon ultracentrifugation of membrane fractions placed in sucrose gradients, the high cholesterol and sphingolipid content contained in rafts help them float to lower density sucrose bands and carry proteins from the membrane with them (Simons and Ikonen, 1997). Rafts are believed to be important as a "relay station" in intracellular signaling. Recently, G proteins and tyrosine kinase signaling proteins have been found associated with lipid rafts (Simons and Ikonen, 1997; Fessler et al., 2004; Allen et al., 2005).

Association of receptors with lipid rafts in response to hormone binding could bring receptors into contact with proteins necessary for cell signaling. In fact, signaling proteins were some of the first proteins to be isolated from lipid raft domains. For example, lipid rafts have recently been shown to promote the clustering of intracellular signaling proteins such as Src family kinases (Incardona and Eaton, 2000).

One of the best characterized examples of the role lipid rafts play in intracellular signaling is illustrated by the immunoglobulin receptor, FcεRI, found on mast cells and basophils. Upon cross-linking of FcεRI with antigen, the receptor undergoes

tyrosine phosphorylation by the Src family kinase Lyn. This phosphorylation is the first step in a signaling cascade utilized by mast cells and basophils during an allergic response. Eventually, these cells will release cytoplasmic vesicle contents including histamines. Initial studies of FcεRI cross-linking found an increased percentage of Lyn recovered in lipid rafts suggesting that membrane structure plays a critical role in linking signal molecules with FcεRI and initiating signal (Sheets et al., 1999). Furthermore, studies in which lipid rafts were disrupted with the cholesterol sequestering detergent methyl-β cyclodextrin (MβCD), showed a substantial reduction in tyrosine phosphorylation of FcεRI and its association with Lyn (Sheets et al., 1999). As we know, cholesterol is important for the formation and stability of lipid rafts where cholesterol molecules act as spacers between associating sphingolipids. Tightly packing sphingolipids and cholesterol are thought to form large clusters on the plasma membrane between regions occupied by unsaturated phosphatidylcholine molecules (Simons and Ikonen, 1997). Removal of cholesterol from the plasma membrane disrupts lipid rafts and the proteins that are thought to associate with them. MβCD has been shown to be non-toxic to living cells and sequesters cholesterol into water-soluble complexes. In addition, MβCD has been reported to be a rapid and efficient method to selectively remove cholesterol from the plasma membrane of living cells (Hansen, 2000). Depletion of cholesterol from lipid rafts not only disrupts the formation of rafts but also decreases the ability of receptor to associate with signaling proteins.

LH receptor organization in the plasma membrane

Although dimerization has long been recognized to be involved in the signal transduction of integral membrane receptors such as receptors for growth factors and cytokines, GPCRs were believed to act as monomers interacting with a single G protein (Heldin, 1995). However, in recent years, the view that GPCRs function as monomeric proteins has been challenged by biochemical, biophysical, and functional studies, which have increasingly suggested that GPCRs exist in cells as dimers or higher-ordered oligomers. In addition to homodimers of a given GPCR, specific heterodimerization between distinct GPCRs has also been documented. GPCR heterodimerization is of functional consequence. It has been shown to modulate the ligand binding, signaling and trafficking properties of some GPCRs, as well as regulating receptor phosphorylation and desensitization. In contrast, the functional role of GPCR homodimerization is still unclear (Tao et al., 2004).

Several lines of evidence suggest that active LH receptors are self-associated within large molecular weight structures following the binding of hormone. Electron micrographs of LH receptors on rat granulosa cells show large clusters of receptors that form only after binding of hormone (Luborsky et al., 1984) as does immunofluorescent labeling of rat receptors in granulosa cells (Amsterdam et al., 1980). Large clusters of wild type rat LH receptors tagged with green fluorescent protein (LHR-GFP) also form within minutes following binding of either LH or hCG to receptors on viable cells (Horvat et al., 1999). The presence of receptors in

physically large structures is also suggested by lateral diffusion studies of the LH receptor in luteal cells from sheep (Niswender et al., 1985) and rat (Roess et al., 1992) in which most LH receptors were laterally immobile. In time-resolved phosphorescence anisotropy (TPA) studies of receptor rotational diffusion, long rotational correlation times for the LH receptor on bovine and ovine plasma membranes are also consistent with the notion that LH receptors are present in large complexes of restricted mobility (Philpott et al., 1995). The aggregation of LH receptors may be indicative of the receptor's response to hormone binding. The functional hormone-receptor complexes exhibit significantly slower rotational dynamics than the complexes formed by hormone binding to non-functional receptors or by a non-functional ligand binding to a normally functioning receptor (Roess et al., 2000).

Rates of lateral diffusion for membrane proteins may reflect large extents of receptor aggregation

Lateral diffusion of membrane proteins occurs through Brownian motion. The rate of protein lateral diffusion in the plasma membrane not only depends on the size of the diffusing protein but the viscosity of the membrane as well. Microspectrophotometry was used in 1974 to determine the rate of lateral diffusion of rhodopsin, a photosensitive integral membrane protein found on frog disk membranes and mudpuppy rods (Poo and Cone, 1974). The calculated diffusion coefficient was

approximately the same as that for a 50kDa protein in oil with a viscosity of 1-6 poise. Unfortunately, microspectrophotometry was useful only for membranes that contain high concentrations of photosensitive molecules like rhodopsin.

The advent of laser-based instrumentation made it possible to examine protein organization in the plasma membrane. In 1974, Peters and coworkers developed an approach for measuring lateral diffusion of Band 3, a protein found on the plasma membrane of a red blood cell. They labeled Band 3 on erythrocyte membrane ghosts with a fluorescein-antibody and examined the antibody using a fluorescence microscope. Fluorophores found on half of a membrane ghost were bleached using light from a high energy excitation source with a 434nm interference filter in place. After 30 seconds a neutral grey filter was put in the light path to attenuate the light source by 98%. The fluorescence intensity of the bleached and unbleached portions of the membrane was monitored over time. The rate of lateral diffusion for Band 3 was 1000-fold slower than the diffusion coefficient for rhodopsin. It was later determined that Band 3 was laterally immobile due to the high concentration of cholesterol and saturated fatty acids in the membrane and due to the interactions between Band 3 and spectrin (Datta, 1987).

In 1976, Jacobson et al. (Jacobson et al., 1976), and Axelrod et al. (Axelrod et al., 1976) independently developed a technique for measuring lateral diffusion on single cells using a focused, symmetric, Gaussian laser beam. This method is known as spot fluorescence photobleaching recovery (FPR) (Figure 6). Briefly, low intensity

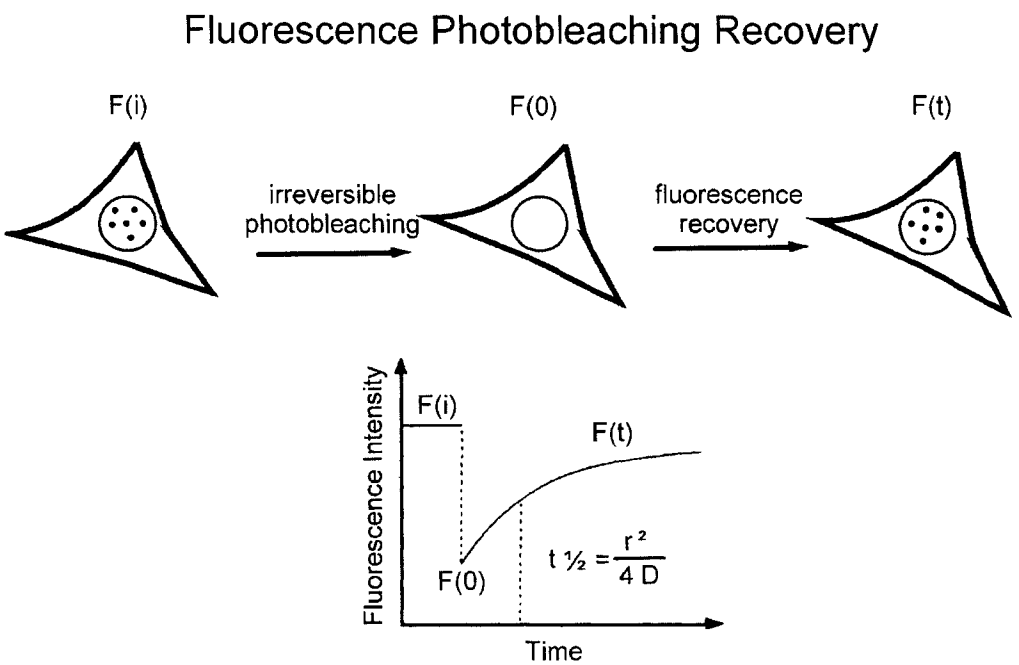

Figure 6. Schematic of spot fluorescence photobleaching recovery method and a representative data trace. The diffusion coefficient D is related to the fluorescence recovery half-time $t_{1/2}$.

laser beam is imaged onto the membrane of the cell as a small spot that is about 1/200 of the cellular diameter. Any protein labeled with a fluorescent marker will emit a fluorescent signal. The intensity from this spot is recorded as the initial fluorescent intensity $F_{(i)}$. The fluorophores in this spot are the exposed to a brief, high powered bleaching pulse that irreversibly photobleaches 50-75% of the fluorophores found within the spot. Fluorescence intensity decreases to $F_{(0)}$. Fluorescence from the spot is then monitored over time using the low intensity laser beam. As new unbleached fluorophores move into the spot, the fluorescence intensity increases $F_{(t)}$.

From this data trace two key pieces of information are calculated, first the fractional fluorescence recovery $f_k(t)$:

$$f_k(t) = [F(t) - F(0)] / [F(\infty) - F(0)]$$

where $F(\infty)$ is the fluorescence intensity at long times. The second piece of information obtained from a spot FPR trace is the diffusion coefficient. The recovery kinetics following the bleaching pulse are represented as

$$F(0) = (qP_0C_0/AK)(1-e^{-K})$$

Where F(0) is the initial bleaching fluorescence, q is the product of the quantum efficiencies of light excitation, emission and detection, P_0 is the laser power, C_0 is the initial fluorophore concentration, A is the beam attenuation during the recovery period and K is the bleaching constant which is proportional to bleaching time and beam intensity (Axelrod et al., 1976). Using this equation the recovery half time ($t_{1/2}$),

which is the time it takes for one-half of the unbleached fluorophores to diffuse back into the bleached area, can be determined. Once the $t_{1/2}$ is known the rate of diffusion D can be calculated using

$$D = (r^2/4t_{1/2})\gamma,$$

where r is the $1/e^2$ laser beam radius, γ is a constant dependent upon the degree of bleaching and beam profile and γ has a typical value of 1-2. Since the recovery half-time is proportional to the square of the laser beam radius, the kinetics of the recovery curve will change with the beam diameter.

Fluorescence Resonance Energy Transfer

Over the past decade, genetically encoded fluorescent proteins have become widely used as noninvasive markers in living cells. The development of fluorescent proteins, coupled with advances in digital imaging, has led to the rapid evolution of live-cell imaging methods (Chan et al., 2001). Fluorescence resonance energy transfer (FRET) is a biophysical technique widely used to determine interchromophoric distance relationships in biomolecules and supramolecular structure on cell surfaces. For instance, FRET is valuable in studying the interaction of LH receptors in the plasma membrane. This technique allows specific questions to be addressed such as do LH receptors self-associate upon hormone binding.

FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. The method was initially proposed in 1948 by Von Förster. FRET can be an accurate measurement of molecular proximity at Angstrom distances. The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation (Stryer and Haugland, 1967), making it useful over distances comparable with the dimensions of biological macromolecules. Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity (Berney and Danuser, 2003). When FRET is used as a contrast mechanism, colocalization of proteins and other molecules can be imaged with spatial resolution beyond the limits of conventional optical microscopy (Kenworthy and Edidin, 1998).

During the fluorescence process, a photon of energy is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption return to the ground state (S_0) by fluorescence emission. Other processes such as nonradiative decay, intersystem crossing, irreversible

photobleaching and FRET may also depopulate S_1. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the relative extent to which these processes occur.

Experimentally, FRET can be detected in several ways. Energy transfer causes quenching of donor fluorescence and sensitized fluorescence of the acceptor. It also reduces the donor lifetime and decreases the rate of irreversible photobleaching of the donor. Traditional energy transfer methods monitor the fluorescence emitted by the acceptor molecule following excitation of the donor molecule (Kenworthy and Edidin, 1998). More recently, another microscopic method has been developed for measuring energy transfer between membrane proteins based on the reduced rate of irreversible photobleaching of the donor molecule when acceptor molecule is within a critical distance. In addition, imaging FRET techniques have been developed to measure all of these events.

Imaging FRET combines digital immunofluorescence microscopy with FRET, a phenomenon that reports proximity between molecules on a length scale of 1-10 nm. It thus increases the resolution of conventional immunofluorescence microscopy to the molecular level and so allows one to quantitatively assess molecular proximity in intact cell membranes. Imaging FRET maps energy transfer between molecules of interest on a cell-by-cell basis, a substantial advance over some previous microscopic FRET methods, which measured the average FRET for a cell population. By comparing experimental FRET values with theoretical predictions for randomly

distributed molecules, imaging FRET measurements can be used to infer the organization of molecules, clustered or randomly distributed, in the cell membranes. The rate of energy transfer is inversely proportional to the sixth power of the distance, r, between the donor and acceptor. The efficiency of energy transfer E is defined with respect to r and R_0, the characteristic Förster distance for the donor and acceptor pair by

$$E = 1/ [1 + (r/R_0)^6].$$

For example, when $r = R_0$, E is 50%, and when $r = 2R_0$, E is 1.5%. The value of R_0 depends on the relative orientation of the donor and acceptor, the overlap interval between the emission spectrum of the donor and the excitation spectrum of the acceptor (Kenworthy and Edidin, 1998).

In our studies, cyan fluorescent protein (CFP), a variant of green fluorescent protein (GFP) cloned from jellyfish, was used as the donor molecule, and yellow fluorescent protein (YFP), another variant, was used as the acceptor molecule. The human LH receptor was cloned into vectors containing these fluorophores. Then the cell lines were made by transfecting both of the vectors hLHR-CFP and hLHR-YFP at 1:3 ratio. The resulting cells signaled internally via cAMP and were detectable upon interrogation at the proper exciting wave length. Energy transfer was detected as an increase in donor fluorescence (dequenching) after complete photobleaching of the acceptor fluorophore, if the hLHRs were less than 100 Å from each other (Patterson et al., 2000). The validity of using donor dequenching to quantify FRET depends on the fact that the only factor that can lead to a difference in donor fluorescence in the

presence and absence of acceptor is energy transfer. Images mapping FRET between labeled receptors were calculated from the increase in donor fluorescence after acceptor photodestruction by

$$E\% = [1 - (\text{CFP prebleach/CFP postbleach})] \times 100$$

after subtracting the dark current contribution. A diagram of the microscopic energy transfer method is shown in Figure 7.

Summary and statement of research goals

Here I have used imaging FRET and FPR to explore the organization of LH receptors and have used sucrose gradient ultracentrifugation to determine whether receptors are located in specialized plasma membrane microdomains (rafts) that may serve as signaling platforms. My basic hypothesis is that prior to binding of ligand, wild type LH receptors exist as isolated membrane proteins. Binding of ligand leads to dimerization or oligomerization of the functional receptor and translocation into membrane rafts. Nonfunctional receptors or nonfunctional hormone binding can not make these changes. In contrast, constitutively active LH receptors are self-associated even in the absence of hormone and localized in membrane rafts. Signaling by constitutively active receptors may not require rafts. In addition, the LH receptor C-terminus and its palmitoylation may play a role in rafts localization.

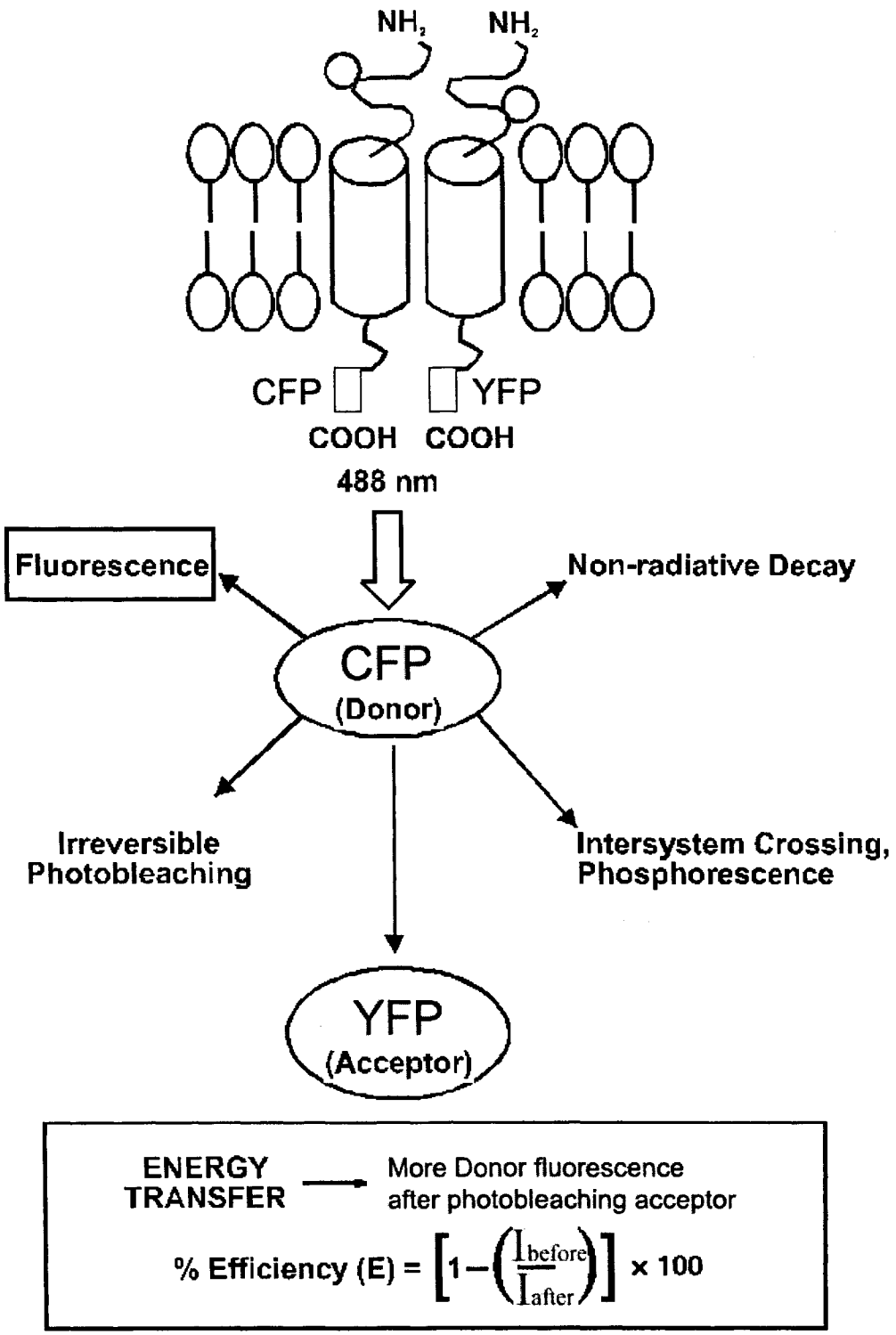

Figure 7. Method for single cell fluorescence energy transfer with %E equation.

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CHAPTER TWO

LUTEINIZING HORMONE RECEPTORS TRANSLOCATE TO SMALL PLASMA MEMBRANE MICRODOMAINS FOLLOWING BINDING OF HUMAN CHORIONIC GONADOTROPIN

Introduction

Several lines of evidence suggest that functional luteinizing hormone receptors (LHR) are clustered within high molecular weight structures following the binding of hormone. Electron micrographs of LHR on rat granulosa cells show large clusters of receptors that form only after binding of hormone (Luborsky et al., 1984) as does immunofluorescent labeling of rat receptors in granulosa cells (Amsterdam et al., 1980). Wild type rat LH receptors tagged with green fluorescent protein (LHR-GFP) aggregated within minutes following binding of either LH or hCG to receptors on viable cells (Horvat et al., 1999). This aggregation is accompanied by close interactions between individual receptors as indicated by comparatively high values for fluorescence energy transfer between receptors (Horvat et al., 2001). The presence of receptors in physically large structures has also been suggested by lateral

diffusion studies of the LH receptor in luteal cells from sheep (Niswender et al., 1985) and rat (Roess et al., 1992) in which most LH receptors were laterally immobile.

Because clustering of the rat LH receptor occurs within minutes and, upon microscopic inspection, involves the movement of diffusely distributed receptors into discrete membrane sites (Horvat et al., 2001), one question is whether receptors cluster at arbitrary sites on the plasma membrane or become confined in membrane microdomains. Membrane microdomains include so-called rafts which, because of their high cholesterol and sphingolipid content, "float" in sucrose gradients. Rafts are enriched, not only with sphingolipids and cholesterol (Brown and London, 1998) but with glycosylphosphatidyl-inositol (GPI)-anchored proteins (Ilangumaran and Hoessli, 1998). The lateral diffusion of specific membrane proteins within these microdomains is also reduced (Pralle et al., 1999; Pralle et al., 2000). Membrane domains, which on some cells may comprise a substantial fraction of the plasma membrane (Gidwani et al., 2001; Maxfield, 2002), can contain membrane proteins necessary for cell signaling such as G-proteins (Harder and Simons, 1997) and adenylate cyclase (Xiao and Devreotes, 1997). There is also evidence for transient protein targeting to membrane rafts including, as an example, regulators of G protein signaling (Hiol et al., 2003). Together, these observations have led to suggestions that rafts might serve as signaling platforms for G protein-coupled receptors and that this may by accomplished by targeting receptors to environments that favor receptor-mediated signaling.

An additional question raised by these observations is whether LHR function is dependent on receptor translocation into rafts. There are physical differences between functional hormone-receptor complexes, i.e., hCG- and LH-occupied wild type LH receptors, and those complexes that do not activate adenylate cyclase. As examples, binding of the hCG antagonist deglycosylated hCG (Keutmann et al., 1983) to rat LHR does not produce receptor self-association (Roess et al., 2000) or slow receptor rotational diffusion to the same extent observed for hCG-occupied LHR (Roess et al., 2000). These results indicate that larger receptor-containing structures do not form after receptor binding of deglycosylated hCG. Similarly, there is no self-association of rat LH receptors containing a substitution of arginine for lysine at amino acid 583 (LHR-K583R) which either partially (Fernandez and Puett, 1996) or fully (Ryu et al., 1996) eliminates the cAMP response to hCG. Thus, if rafts serve as signaling platforms for rat LH receptors, non-functional receptors may be excluded in some manner from these structures or, alternatively, lack some critical receptor feature needed to direct the hormone-occupied receptor to rafts.

To address these questions, we have isolated membrane fragments from hCG- or deglycosylated hCG-treated CHO cells expressing wild type rat LH receptors tagged at their N-terminus with the FLAG sequence and from hCG-treated CHO cells expressing a FLAG-tagged LHR containing a lysine-to-arginine mutation at position 583 (LHR-K583R). To further demonstrate the localization of functional LH receptors within rafts, we have treated cells with methyl β-cyclodextrin (MβCD) which can efficiently remove cholesterol from the plasma membranes of live cells

(Kilsdonk, 1995; Christian, 1997) and thus disrupts raft structure. To independently examine the effects of hCG on LH receptor motions within the plasma membrane, we have used single particle tracking methods to evaluate the average size of membrane microdomains accessed by the receptor as visualized microscopically on viable cells (Young et al., 2003). Finally, we examined whether disruption of membrane rafts is accompanied by altered signaling in CHO cells in response to hCG or affects LH receptor self-association, a characteristic of functional but not non-functional hormone-receptor complexes (Roess et al., 2000).

Materials and Methods

Materials

Dulbecco's modified Eagle's medium containing 4.5 g/L glucose was purchased from VWR. Geneticin was purchased from Invitrogen. HEPES and non-essential amino acids were purchased from Sigma-Aldrich (St. Louis, MO) as was methyl-β-cyclodextrin (MβCD). Fetal bovine serum (FBS) was purchased from Gemini Bio Products (Woodland, CA). hCG was purchased from Research Diagnostics Inc. (Flanders, NJ). Cellular cAMP was measured using a "Direct Cyclic AMP Correlate-EIA" kit (Assay Designs, Ann Arbor, MI) as per the Manufacture's instructions. Colloidal gold (40 nm) was purchased from Ted Pella, Inc. (Redding, CA).

Cell lines

To test whether the rat wild type LH receptor becomes associated with membrane rafts following binding of ligand, we generated a stable cell line expressing the FLAG-tagged receptor. Dr. K.J. Menon from the University of Michigan kindly provided us with N-terminal FLAG- tagged LHR subcloned into the pFLAG vector (Sigma, St. Louis, MO). Using the FLAG-LHR vector we made a mutation of lysine 583 to arginine (FLAG-LHR-K583R). CHO cells were stably transfected with 5 μg of the FLAG-LHR or K583R-FLAG-LHR vector using Lipofectamine-Plus (Gibco-BRL, Gaithersburg, MD) as per Manufacturer's instructions. Selection of stable clones expressing the FLAG-tagged receptors was based on the acquisition of geneticin (G418) resistance. Stable cell lines used in FRET experiments included cells expressing GFP-LHR-wt or YFP-LHR-wt alone and cells co-expressing GFP-LHR-wt and YFP-LHR-wt. Preparation of these cell lines has been described in detail previously (Horvat et al., 2001).

CHO cells lines were maintained in cell medium that included Dulbecco's modified Eagle media supplemented with 4.5g/mL glucose and containing 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin and 1x MEM non-essential amino acids (Sigma Chemical Co., St. Louis, MO) that was supplemented with 400μg/mL G418.

Isolation of plasma membrane rafts

Cells were incubated with either 100nM hCG or 100nM deglycosylated hCG (Keutmann et al., 1983), the kind gift of Dr. Henry Keutmann, or PBS for 1 hour at 37°C prior to cell lysis. To isolate membrane rafts from LHR-wt and LHR-K583R cells, 5 x 10^7 cells were washed two times with phosphate-buffered saline, pH 7.2 (PBS) and lysed for 5-10 minutes on ice in 1ml of a buffer containing 25mM MES, 150mM NaCl, 2mM EDTA, 20% glycerol, 0.25% Triton-X100, and protease inhibitors including aprotinin, leupeptin, EDTA, and PMSF (Roche). A low speed 300 x g spin was used to remove cell nuclei and large cell debris. 1mL of the supernatant from this spin, which contained plasma membrane fragments, was then combined with 1mL of 80% sucrose containing 0.25% Triton-X100 and protease inhibitors to produce a 40% sucrose solution. A discontinuous sucrose gradient from 10-80% was created with the sample in 40% sucrose layered within this gradient. The gradient was loaded into a Beckman SW-41 swinging bucket rotor and spun at 175,000 x g for 20 hours at 4°C. After the spin, eighteen 650μL fractions were carefully collected from the top of the gradient downward. A 50μL aliquot from each fraction was diluted 1:1 with 95% SDS and 5% β-mercaptoethanol. After separation of proteins from each fraction using SDS-PAGE and transfer of proteins to nitrocellulose, the LH receptor was identified using 30μg of an anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO). The amount of receptor in each fraction was measured using a Bio-Rad GS-800 calibrated densitometer. The sucrose concentration in each fraction was determined using a Bausch and Lomb

refractometer. In some experiments, cells were pretreated for 1 hr at 37°C with 1% or 2% methyl-β- cyclodextrin (MβCD) in serum-free Dulbecco's modified Eagle medium containing high glucose prior to incubation with hCG or PBS or with 100nM anti-FLAG antibody for 45 min at room temperature followed by a second 45 min incubation with 1 μM anti-mouse IgG (Sigma). MβCD neither binds nor inserts in the plasma membrane of cells but rather extracts cholesterol by including it in a central non-polar cavity of cyclic oligomers of glucopyranoside in α-1,4-glycosidic linkages (Ilangumaran and Hoessli, 1998). At low concentrations, it is non toxic to cells and does not compromise cell integrity (Sheets et al., 1999).

Single particle tracking of FLAG-LHR-wt receptors on individual cells

Lateral dynamics and the size of domains accessed by individual FLAG-LHR-wt were evaluated using single-particle tracking methods as described by Kusumi and coworkers (Dietrich et al., 2002). 40 nm nanogold particles were conjugated with the lowest possible concentration of anti-FLAG monoclonal antibody (mAb) needed to stabilize the gold solution and were then incubated with CHO cells expressing FLAG-LHR-wt receptors. The anti-FLAG-gold concentration, typically 15μg/mL, was then further reduced by addition of 1% BSA in PBS until there were approximately 1-4 gold particles per cell. This binding was specific for FLAG-tagged receptors; when cells were preincubated with a 10-fold excess of anti-FLAG antibody, no anti-FLAG-gold particles were detected on cells. In some

experiments, cells were treated with 100nM hCG for 1 hr after labeling of receptors with anti-FLAG mAb or were pre-treated with 1% MβCD for 1 hr prior to labeling with anti-FLAG antibody.

Individual nanoparticles were imaged by differential interference contrast with a 1.4 N.A. 63x objective in a Zeiss Axiovert 135 microscope. Images were acquired using a Dage IFG-300 camera and were recorded for 2 min (3600 frames) at approximately 30 nm/pixel under the control of Metamorph software from Universal Imaging. The trajectories for individual gold particles were segmented into domains by calculation of statistical variance in particle position over times using a procedure similar to that developed by a number of investigators (Murase et al., 2004; Daumas et al., 2003; Saxton, 1997). The variance of a particle's position can be calculated within windows of varying duration. These windows are translated along the particle trajectory, producing a variance plot that exhibits peaks that indicate inter-domain boundaries. These results can be analyzed to yield the domain size and residence time for each particle. Effective macroscopic diffusion constants were calculated as the square of the domain diagonal divided by four times the residence time in the domain as previously described (Saxton, 1997).

Results

Functional but not non-functional LH receptors appear in membrane rafts following binding of hCG

After isopycnic centrifugation of plasma membrane fractions from CHO cells expressing FLAG-LHR-wt, unoccupied LH receptors were found in sucrose fractions with relatively high densities (Figure 8). Over 90% of FLAG-LHR-wt receptors were localized in sucrose fractions 10-15 where sucrose concentrations ranged from approximately 36-56%. Following treatment of cells with 100nM hCG, there was marked change in the distribution of receptors to lower density sucrose fractions. Over 80% of hCG-treated FLAG-LHR-wt receptors consistently appeared in fractions 3-7 which, on average, contained 14-26% sucrose and over 90% of the receptors appeared in less dense sucrose fractions than did untreated receptors.

The translocation of LH receptors from high to low density membrane fractions required functional hormone-receptor complexes; LHR from CHO cells expressing FLAG-LHR-K583R treated with hCG or FLAG-LHR-wt cells treated with deglycosylated hCG remained associated with higher density sucrose fractions (Figure 9) despite the presence of bound ligand. Crosslinking of FLAG-LHR-wt receptors using an anti-FLAG antibody either alone or together with an excess of a secondary polyclonal anti-mouse antibody (Figure 9) also had no significant effect on the distribution of FLAG-LHR-wt within the sucrose gradient.

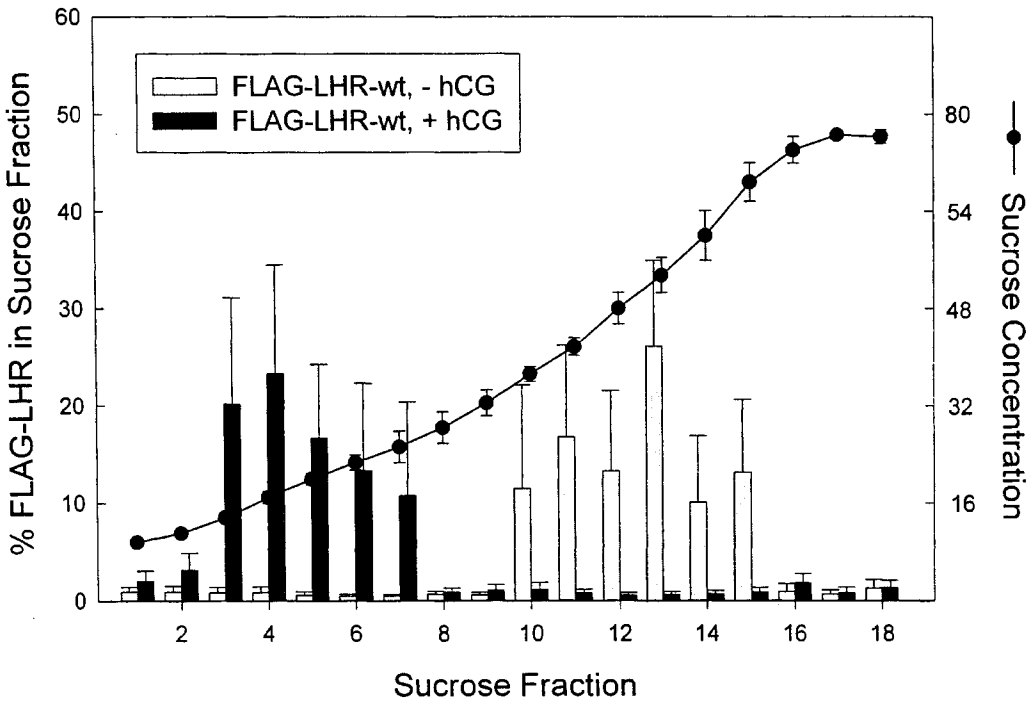

Figure 8. Wild type rat LH receptors are localized in high density membrane fractions before exposure to hCG and appear in low fractions containing lower sucrose concentrations after treatment of CHO cells with 100nM hCG.

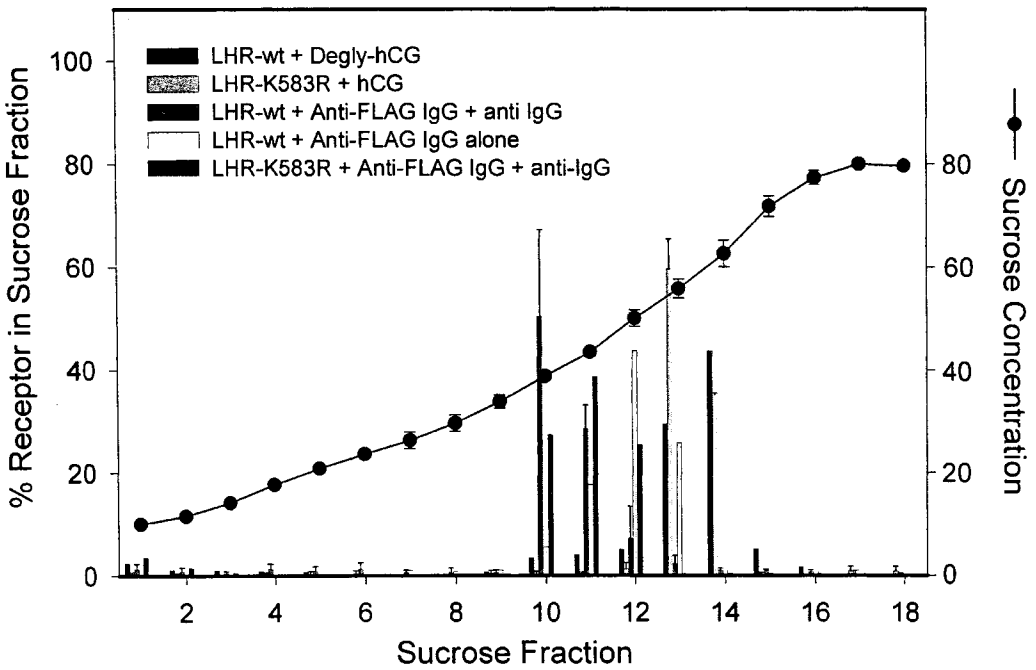

Figure 9. Translocation of LH receptors into buoyant membrane fractions required functional LH receptors. Neither wild type rat LH receptors treated with 100nM deglycosylated hCG, LHR-K583R treated with hCG or antibody treated (anti-FLAG antibody alone) or extensively crosslinked LHR-wt (anti-FLAG antibody followed by excess anti-mouse IgG) were found in plasma membrane rafts.

Depleting membrane cholesterol disrupts membrane rafts and reduces LH receptor self-association in response to hCG treatment

Preincubation of cells for 30 minutes with 1% MβCD disrupted membrane rafts containing the LH receptor. Over 93% of hCG-treated FLAG-LHR-wt were found in fractions 10-12 following exposure of cells to 1% MβCD (Figure 10) which was essentially unchanged from the distribution of unoccupied FLAG-LHR-wt receptors on CHO cells treated with MβCD (data not shown) and to the distribution of unoccupied receptors shown in Figure 8. Interestingly, disruption of LHR-containing rafts with MβCD did not affect caveolae. Western blots prepared in tandem with those used to identify FLAG-LHR-wt were probed with an anti-caveolin antibody from Santa Cruz Biotechnology. Caveolin remained broadly distributed in lower density sucrose fractions under all experimental conditions (Figure 10) suggesting that LH receptors are associated with membrane microdomains that are distinct from caveolin-containing membrane regions.

Disruption of membrane rafts also reduced cell signaling in response to hormone. It has been suggested that plasma membrane rafts may serve as signaling platforms, effectively concentrating receptors and other plasma membrane molecules necessary for initiating down-stream signaling events. If this were correct, disruption of membrane rafts should reduce or eliminate selective signaling events dependent on this membrane micro-environment. We examined accumulation of cAMP in response

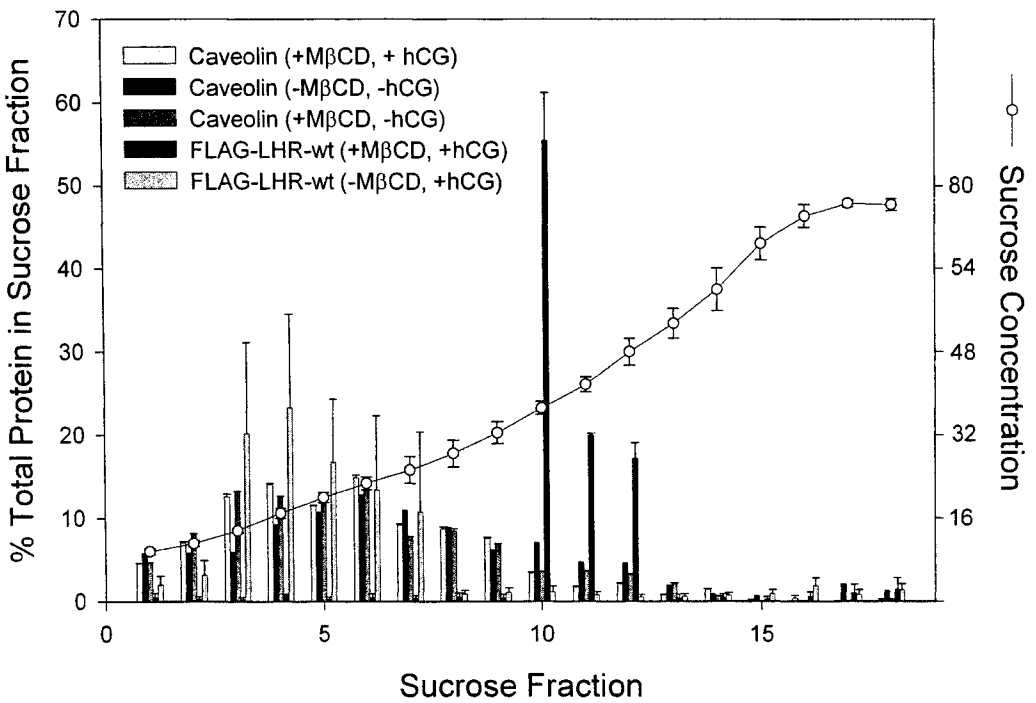

Figure 10. Disruption of plasma membrane rafts by extraction of cholesterol from the plasma membrane eliminated translocation of FLAG-LHR-wt into low density sucrose fractions. In addition, anti-caveolin antibody was used to identify caveolin in sucrose fractions. The distribution of caveolin in fractions 1-10 remained relatively constant in untreated cell samples and in samples exposed to hCG or MβCD.

to hCG in cells pretreated with either 1% or 2% MβCD. As shown in Figure 11, pretreatment of cells with 1% MβCD, reduced hCG-mediated increases in intracellular cAMP to basal levels. Interestingly, extensive crosslinking of LHR-wt caused a significant increase in cAMP production (Figure 11) even though this antibody-induced form of receptor self association was not sufficient to drive receptor translocation from the bulk membrane to rafts (Figure 9).

Single particle tracking of hCG-occupied FLAG-LHR-wt receptors demonstrates trapping of receptors in small membrane compartments

To independently assess the localization of hCG-treated FLAG-LHR-wt in membrane microdomains, single particle tracking methods were used. This technique identifies individual LH receptors on the surface of viable cells and tracks their motions over approximately two minutes. The centroid for a 40 nm gold particle attached to an individual receptor can be identified visually on video obtained from each experiment and its motions can be quantitatively described. As shown in Figure 12, gold particles bound to FLAG-LHR-wt exhibited distinctive motions in the presence and absence of hCG. hCG treatment reduced the size of compartments containing FLAG-LHR-wt from 230 ± 79 to 86 ± 36 nm (Table 1). Although the average residence time for receptors within microdomains and the number of microdomains, 6 ± 2 and 5 ± 2, accessed by the receptor in two minutes did not differ significantly for untreated and hCG-treated cells, respectively, an individual receptor's

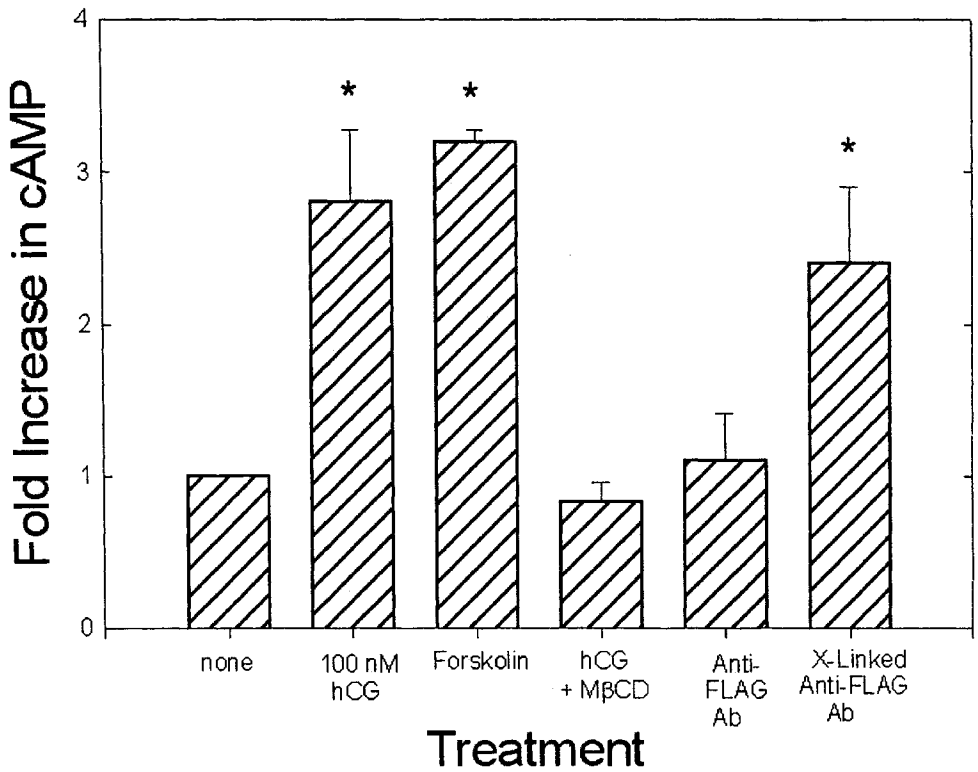

Figure 11. There was an approximately 3-fold increase in cAMP in response to cell treatment with either 100nM hCG or Forskolin. 1% MβCD significantly reduced cAMP levels in hCG-treated samples to basal levels. Although exposure of FLAG-LHR-wt to monoclonal anti-FLAG antibody had no effect on cAMP levels, crosslinking of the receptor with an excess of anti-mouse IgG elevated cAMP levels to values comparable to those of hCG-treated cells.

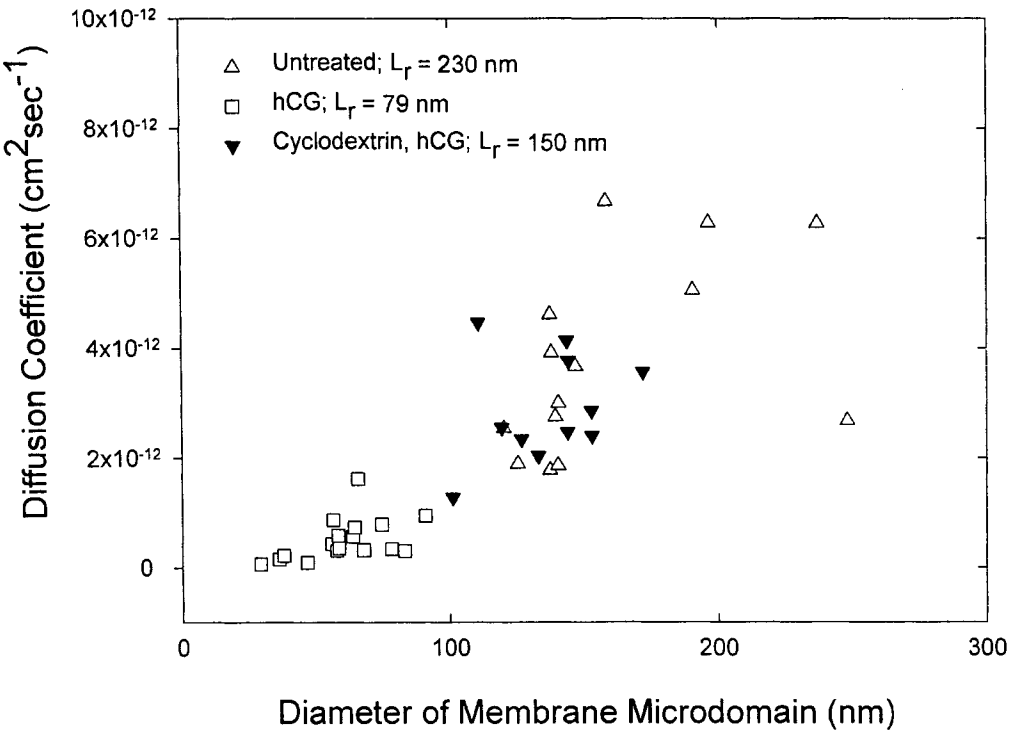

Figure 12. Single particle tracking of individual FLAG-LHR-wt receptors labeled with gold-anti-FLAG antibody.

Table 1. Tracking of individual anti-FLAG-gold particles bound to FLAG-LHR-wt on CHO cells.

Pretreatment	Hormone	Number of particles analyzed	Number of domains/ 2min trajectory	D_{0-1}[a] ($10^{-11}cm^2sec^{-1}$)	$D = L_r^2/4t$ [b] ($10^{-11}cm^2sec^{-1}$)	Time[c] (sec)	Domain Diameter (d)[c]
none	none	20	6.4 ± 1.6	6.8 ± 3.8	1.2 ± 0.8	16.3 ± 13.4	230 ± 79
none	hCG	20	5.2 ± 1.7	2.9 ± 1.1	0.12 ± 1.8	19.5 ± 10.8	86 ± 36
MβCD	hCG	8	5.0 ± 1.2	4.8 ± 2.8	0.32 ± 0.8	18.9 ± 4.7	150 ± 12

[a] D_{0-1}: Diffusion coefficient of the first 2 points (Daumas et al., 2003)

[b] D presents the diffusion coefficient within a domain as described by Saxton (Saxton, 1997).

[c] The average diameter of an individual domain was calculated as described by Daumas et al. (Daumas et al., 2003) and Murase et al. (Murase et al., 2004).

[d] Average time for residence within a domain

rate of diffusion D within each domain was reduced by a factor of ten following hCG treatment. Figure 13 illustrates these results for untreated and hCG treated cells and shows that as receptors access progressively larger membrane microdomains, their rate of diffusion within those microdomains increases.

Because MβCD pre-treatment largely reversed the effects of hCG on compartment size and receptor lateral diffusion, it seems plausible that compartments retaining hCG-treated receptors are rafts. As shown in Table 1 and illustrated in Figure 12, MβCD pre-treatment results in a significant increase in the size of membrane compartments containing LHR-wt. The size of these compartments does not differ significantly from those accessed by untreated FLAG-LHR-wt receptors and, together with results from sucrose density gradient centrifugation experiments, suggests that these unbound receptors are residing in the bulk membrane rather than rafts.

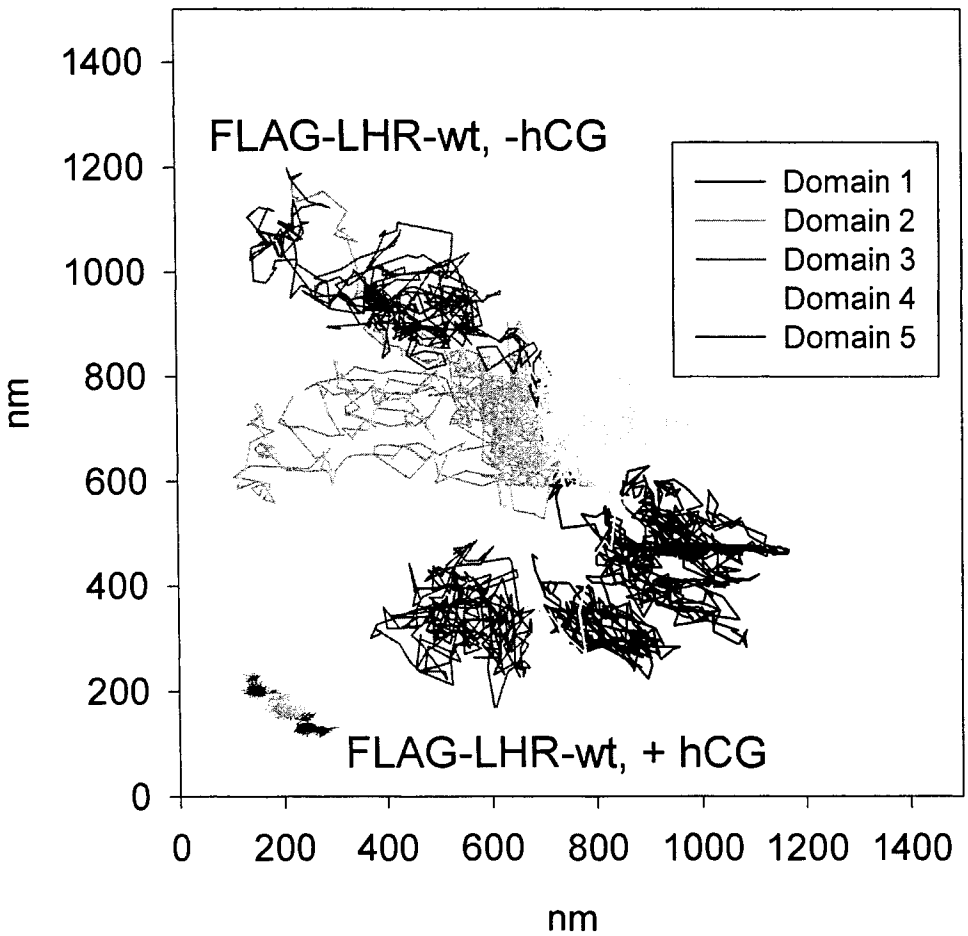

Figure 13. Representative trajectory for a single FLAG-LHR-wt receptor on an untreated cell or a cell treated with hCG.

Discussion

Our results demonstrate that wild type LHRs, if occupied by a functional ligand, are capable of moving from the bulk membrane into small nm-diameter plasma membrane compartments with characteristics of so-called rafts. The translocation of LHR to low density membrane regions appears to be characteristic of the functional hormone-receptor complex and is not observed when either the LHR is non-functional or when a hormone antagonist has bound to the wild type receptor. Moreover, the regions within which hormone-treated LHR-wt receptors are confined are small. Translocation of receptors into rafts is also implicated in receptor-mediated signaling as demonstrated by the decrease in hCG-mediated cellular levels of cAMP when rafts were disrupted.

The appearance of receptors within low density, detergent-insoluble membrane fractions occurs upon binding of hormone agonist to LHR. This is similar to results of Bramley and Ryan (Bramley and Ryan, 1978) who separated membrane fragments from homogenated ovaries of superovulated ewes on continuous sucrose gradients. They isolated two distinct membrane fractions that displayed hCG binding and, as a result, suggested that there might be two populations of LH receptors present on granulosa cells. They also characterized selected proteins in "heavy" and "light" membrane fractions containing LHR including adenylate cyclase which, interestingly, associated with the "heavy" receptor population.

The mechanism involved in targeting of LHR to rafts is not clear. Proteins

found in plasma membrane rafts include transmembrane proteins with attached lipid groups including, most commonly, glycosyl phosphatidylinositol (GPI) or palmitate (Brown and London, 2000). There is evidence for palmitoylation of the LH receptor at two cysteines located in the receptor's intracellular carboxy terminal (Kawate et al., 1997). Although mutations to the palmitoylated cysteines did not affect cAMP production, the mutated receptors were internalized more quickly than wild type receptors, apparently through an arrestin-mediated pathway (Munshi et al., 2001). Point mutations to palmitoylation sites on the LH receptor C terminus also eliminate LHR-wt translocation into rafts (Roess et al., manuscript under review). However, it is not known whether there is reversible palmitoylation of the wild type LHR or whether the binding of ligand alters equilibrium between palmitoylated and non-palmitoylated LH receptors. This appears to be the case for β-adrenergic receptors, G-protein coupled receptors structurally related to LHR, which undergo palmitoylation/depalmitoylation cycling with binding of agonist favoring receptor depalmitoylation (Loisel et al., 1996). If palmitoylation were necessary for targeting of a ligand-activated G protein-coupled receptor to rafts, depalmitoylation would be predicted to reduce the likelihood of finding, for example, β-adrenergic receptors in rafts. However, the role for palmitoylation in receptor-mediated signaling (Qanbar and Bouvier, 2003) and raft localization is generally unclear and may prove ultimately to be receptor-specific.

Receptor aggregation, either together with receptor palmitoylation or independently, may increase the affinity of the LH receptor for rafts. Signaling by

multi-chain receptors such as T-cell and B-cell antigen receptors are present in the bulk membrane prior to crosslinking by multivalent antigens (Dykstra et al., 2003; Harder, 2001). Crosslinking may shift the equilibrium distribution for membrane proteins within the lipid bilayer and favor interactions between proteins and more ordered lipid microdomains such as rafts as has been demonstrated for lipid-anchored proteins within artificial lipid monolayers (Dietrich et al., 2001). In our hands, LH receptor self-association (Roess et al., 2000) as well as raft localization are characteristic of functional hormone-receptor complexes. One could hypothesize that agonist-induced association between LH receptors causes translocation of receptors to rafts. Interestingly however, presumably random interactions between LHR receptors following antibody crosslinking are not sufficient to drive receptor translocation to rafts. Although targeting of proteins to rafts can be initiated by antibody crosslinking of, for example, T-cell and B-cell antigen receptors (Dykstra et al., 2003; Harder, 2001) and the Type 1 Fcε receptor on rat basophilic leukemia cells (Young et al., 2003), this was not the case for antibody-crosslinked LH receptors, suggesting that LH receptors required binding of a hormone agonist and potentially specific receptor conformations for targeting of activated receptors to rafts.

It appears that membrane rafts may serve as signaling platforms for the LH receptor although the specific signaling event(s) are uncertain. Spatial coordination of key signaling proteins in lipid rafts may provide a rapid, efficient and specific mechanism for promoting signal transduction from extracellular to intracellular mechanisms while also preventing cross-talk between pathways (Moffett et al., 2000;

Oh, 2001). As an example, Type I Fcε receptors form, at a minimum, receptor dimers that are tyrosine phosphorylated by Lyn and then colocalize with Lyn in membrane rafts (Young et al., 2003). Other downstream signaling molecules also appear within rafts. Oh and Schnitzer have shown that G_i and G_s are targeted to, and concentrated in, lipid rafts (Oh, 2001) and some, but not all, isoforms of adenylate cyclase have been found associated with caveolae in membrane rafts (Rybin et al., 2000; Ostrom et al., 2000; Ostrom et al., 2001). If rafts serve to concentrate these proteins, cholesterol depletion and raft disruption may disperse raft-associated proteins and reduce the likelihood of protein-protein interactions necessary for cell signaling. Indeed, MβCD treatment reduced FRET between GPI-anchored proteins (Varma and Mayor, 1998) and signal transduction by these proteins (Stulnig, 1997). Other studies have shown that cholesterol is important for lipid raft formation and that its depletion decreases signal transduction efficiency (Sheets et al., 1999; Huby et al., 1999).

Single particle tracking studies provide some insight into the nature of LH receptor-containing structures. Upon binding ligand, the LH receptor becomes largely confined within small compartments with an average diameter of 86 nm. For the most part, the receptor remains within these regions for comparatively long times and appear to diffuse pseudo-randomly before being captured within another compartment of similar size. Similar behavior has been described and analyzed by Kusumi and coworkers (Murase et al., 2004) for selected phospholipids and for transferrin receptor (Sako Y and Kusumi, 1994) and by Daumas et al. (Daumas et al.,

2003) for the μ opioid receptor, a G protein-coupled receptor involved in pain responses. Daumas argues that μ opioid receptor motions reflect its diffusion within the bulk membrane followed by confinement within a domain that itself diffuses slowly and suggests that this confinement is due to interactions with the confining molecules. Alternatively, Ritchie et al. (Ritchie et al., 2003) suggest that these interactions may be with proteins forming a continuous barrier (fences) or discontinuous protein barrier (pickets). Fences or pickets can confine and limit receptor diffusion within small membrane regions while still permitting intermittent escape from a compartment zone followed by faster diffusion in the bulk membrane. Our previous studies of LH receptor lateral diffusion using fluorescence photobleaching recovery methods suggest that actin microfilaments may provide fences or organizing structures for pickets that restrict the lateral motions of the receptor (Roess et al., 1988). Nonetheless, one caveat in interpreting these results is that the compartments occupied by hCG-occupied LH receptors should not be equated *a priori* with structures identified in biochemical studies such as plasma membrane rafts. The relationship of biochemically-identified rafts to membrane compartments visualized via single particle tracking remains a topic of active debate.

Finally, disruption of the membrane rafts reduced, but did not completely eliminate, LH signaling. This result is reasonable if hormone-mediated signaling is most efficient within rafts where higher concentrations of downstream signaling molecules exist. Nonetheless, some signaling proteins remain available within the bulk membrane, albeit at reduced concentrations, where they are capable of relaying a

productive signal. The questions that remain are what role receptor aggregation plays in signaling and raft localization, whether small membrane compartments accessed by hCG-occupied receptors are the same structures isolated in low density sucrose fractions, and whether rafts are essential for LH receptor function. These questions seem likely to be resolved only if it can be demonstrated that forcing an LH receptor, either with or without ligand, into the raft environment, can produce a downstream signal and if some, or all, of the components involved in LH-receptor mediated signaling can be localized with LH receptors in the same membrane compartments. Alternatively, raft localization may be a convenient, but not essential method, for concentrating membrane proteins involved in signal transduction.

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CHAPTER THREE

CONSTITUTIVELY-ACTIVE HUMAN LH RECEPTORS ARE SELF-ASSOCIATED AND PRESENT IN PLASMA MEMBRANE RAFTS IN THE ABSENCE OF HORMONE

Introduction

Functional luteinizing hormone (LH) receptors are critical to fertility in both males and females. In females, the LH receptor is found on granulosa and thecal cells in the follicle and on luteal cells. In males, the receptor is found on Leydig cells. Binding of LH from the anterior pituitary results in signaling cascades leading to follicle maturation, steroidogenesis or spermatogenesis. Mutations in the human luteinizing hormone (hLH) receptor aspartic acid residue at position 578 are associated with constitutive activation of Gs by the receptor (Abell et al., 1998) as well as with naturally-occurring pathologies, such as Familial Male-limited Precocious Puberty (FMPP) and Leydig cell adenomas (Liu et al., 1999).

Although details of G protein-coupled receptor signaling through intracellular

mediators are increasingly well-characterized, membrane events involved in signaling are less understood, including receptor interactions such as receptor dimer or oligomerization or interactions with other membrane proteins. Several lines of evidence suggest that functional LH receptors, i.e., receptors that have bound hormone and are actively transducing signal, are associated within large molecular weight structures following the binding of hormone. Electron micrographs of LHR on rat granulosa cells show large clusters of receptors that form only after binding of hormone (Luborsky et al., 1984) as does immunofluorescent labeling of rat receptors in granulosa cells (Podesta et al., 1986). Large clusters of wild type rat LH receptors tagged with green fluorescent protein (LHR-GFP) also form within minutes following binding of either LH or hCG to receptors on viable cells (Horvat et al., 1999). The presence of receptors in physically large structures is also suggested by lateral diffusion studies of hormone-treated LH receptors on luteal cells from sheep and rat in which most LH receptors were laterally immobile (Roess and Smith, 2003).

The LH receptor within these membrane clusters appears to be self-associated. Rat LH receptors become self-associated upon binding of either LH or hCG (Roess and Smith, 2003) and when desensitized in plasma membrane preparations from porcine granulosa cells (Hunzicker-Dunn et al., 2003). Tao et al. (Tao et al., 2004) have used immunoprecipitation methods to show that some human LH receptors stably expressed in 293 cells exist as receptor dimers or oligomers and that the relative amounts of these receptor structures increases upon binding of hCG.

Receptor self-association may also be accompanied by a redistribution of the LH receptor within the plasma membrane. Upon binding of ligand, rat LH receptors partition into high buoyancy membrane fractions that can be isolated via density gradient centrifugation (Roess and Smith, 2003). Because of their high lipid content, these specialized membrane microdomains or rafts are found in membrane fractions with low density and "float" in sucrose gradients. The outer leaflet of the raft membrane is enriched with sphingolipids and cholesterol as well as glycosylphosphatidyl-inositol (GPI)-anchored proteins and can limit the lateral diffusion of specific membrane proteins (Ediddin, 2003). In addition to sequestering receptors, such domains may serve "signaling platforms" for a diverse group of signaling molecules (Simons and Toomore, 2000) as well as G protein-coupled receptors such as the rat LH receptor (Smith et al., in preparation) and the gonadotropin releasing hormone (GnRH) receptor (Navratil et al., 2003). The presence of membrane microdomains with higher affinity for activated, signaling receptors than the bulk membrane could also explain, at least in part, why receptor clustering occurs within minutes and, upon microscopic inspection, involves the movement of diffusely distributed LH receptors into discrete membrane locations (Horvat et al., 1999). In single particle tracking experiments of rat LH receptors on viable cells, the size of compartments accessed by hCG-treated receptors is reduced by over 60% (Smith et al., in preparation). Thus, the question raised by these various observations is whether constitutively-active hLHRs, but not wild type receptors, are self-associated in the absence of hormone, are restricted in their motions

within the plane of the membrane and localized in membrane rafts involved in receptor-mediated signaling.

Materials and Methods

Materials

Dulbecco's modified Eagle medium containing high glucose was purchased from Irvine Scientific, Santa Ana, CA. Geneticin was purchased from GIBCO, Grand Island, NY. Non-essential amino acids were purchased from Sigma-Aldrich, St. Louis, MO. Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). hCG was purchased from Research Diagnostics Inc. (Flanders, NJ). Methyl-β-cyclodextrin (MβCD) and the FLAG vector were purchased from Sigma-Aldrich (St. Louis, MO). CFP and YFP vectors were purchased from Clontech. Vectors containing human LHR (LHR-wt) receptors or receptors with mutations D578G, D578H or D578Y were gifts from Dr. Andrew Shenker. Intracellular cAMP was measured using a TiterFluor cAMP EIA kit obtained from Assay Designs, Ann Arbor, MI.

Preparation and maintenance of CHO cells expressing visible fluorescent proteins (VFP) or epitope tags

Stable CHO cell lines expressing wild type human LHR receptors or receptors with mutations, D578G, D578H or D578Y, associated with constitutive activation, were coupled to one of the visible fluorescent proteins, either enhanced cyan fluorescent protein (CFP) or enhanced yellow fluorescent protein (YFP), at their C terminus using N-terminal Protein Fusion Vectors pECFP-C1 (6900-1) and pEYFP-C1 (6006-1), respectively. To prepare these cell lines, vectors were constructed for LHR-wt-CFP and -YFP, LHR-D578G-CFP and -YFP, LHR-D578H-CFP and -YFP, and LHR-D578Y-CFP and -YFP as previously described for transfection of rat LHR coupled to GFP (Horvat et al., 1999). CHO cells in 60 mm dishes at 40-80% confluence were transfected with 5μg DNA in 20μL lipofectamine. DNA was added as either a single vector or, to accomplish co-transfection of two vectors, at a 1:3 ratio of CFP:YFP. After 3-4 weeks, clones expressing both CFP and YFP were selected using fluorescence microscopy. To determine whether LHRs partitioned into membrane rafts, stable CHO cell lines were prepared expressing either LHR-wt or LHR-D578H coupled to the FLAG epitope at the receptor N terminus and were maintained in CHO cell medium (Roess and Smith, 2003).

Imaging analysis of FRET using fluorescence dequenching

To evaluate the effects of hCG treatment on energy transfer efficiency, flasks containing 3-4 x 10^6 cells were selected. The medium was discarded and cells were removed from the flask using PBS containing 5mM EDTA, washed with 12mls of PBS, and spun down. The cell pellet was resuspended in 500 μL of PBS alone or in PBS containing 100nM hCG. The cells were then incubated at 37°C for 1 hour, washed once and resuspended in PBS for FRET measurements. FRET between hLHR-CFP and hLHR-YFP was evaluated on individual cells by measuring the intensity of the plasma membrane localized fluorescence donor CFP in the presence and absence of a fluorescence acceptor YFP (Llopis et al., 2000). More intense signals from CFP, the fluorescence donor, after photobleaching of YFP, the fluorescence acceptor, were indicative of energy transfer from fluorescence donor to acceptor. For this donor-acceptor pair, Förster's r_0 is calculated to be 56Å (Patterson et al., 2000) and energy transfer occurs to a measurable extent only when the donor and acceptor are separated by distances less than about 100Å. FRET measurements were made using a Zeiss Axiovert 135 microscope or a Zeiss Axiovert 200 microscope, Omega Optical filter sets for imaging of CFP and YFP and Metamorph software from Universal Imaging. Before photobleaching YFP, CFP and YFP were imaged separately using a Princeton Instruments1300YHS ICCD camera. After photobleaching YFP for 5 minutes using a mercury arc lamp source and an Omega Optical XF1074 filter, YFP and CFP were imaged again. Five minute

exposure to 525nm light was sufficient to bleach essentially all YFP signal. The intensity of CFP signals before and after YFP photobleaching was then compared. After subtracting the background signal from each image and correcting for the small extent of donor bleaching during the bleaching of acceptor, energy transfer efficiency was calculated using %E= (donor fluorescence after photobleaching - donor fluorescence before photobleaching / donor fluorescence after photobleaching) x 100.

Isolation of plasma membrane rafts

Cells were incubated with either 100nM hCG or PBS for 1 hour at 37°C prior to cell lysis. As previously described (Roess and Smith, 2003), 1mL cell lysate was combined with 1ml of 80% sucrose and the sample was layered at 40% sucrose within a discontinuous sucrose gradient from 10-80%. After centrifugation at 175,000 x g for 20 hours at 4°C, fractions were collected from the top of the gradient downward and an aliquots from each fraction were diluted 1:1 in Laemmli SDS buffer. After separation of proteins using SDS-PAGE and transfer to nitrocellulose, the LH receptor was identified using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). In some experiments, cells were pretreated for 1 hr at 37°C with 1% MβCD in serum free DMEM media prior to incubation with hCG or PBS.

Single particle tracking of FLAG-LHR-wt receptors on individual cells

Lateral dynamics and the size of domains accessed by individual FLAG-LHR-wt were evaluated using single-particle tracking methods as described by Kusumi and coworkers (Dietrich et al., 2002) and Smith et al. (Smith et al., in preparation). 40 nm nanogold particles were conjugated with the lowest possible concentration of anti-FLAG monoclonal antibody (mAb) and then incubated with CHO cells expressing FLAG-LHR-wt receptors at concentrations typically less 15μg/mL to produce 1-4 gold particles per cell. The trajectories for individual gold particles were segmented into domains by calculation of statistical variance in particle position over times using a procedure similar to that developed by a number of investigators (Murase et al., 2004; Daumas et al., 2003; Saxton, 1997). Results were analyzed to yield the domain size, the residence time for each particle and the effective macroscopic diffusion constants as described by Saxton (Saxton, 1997).

Results

hLHR-wt become self-associated and translocate into membrane rafts following treatment with hormone

We used fluorescence dequenching of the fluorescence donor and imaging methods to examine conditions in which wild type human LHR were self-associated. This FRET method has a number of advantages, the most important being that all

measurements of fluorescence emission from the fluorescence donor are accomplished on the same cell. To perform these experiments, CHO cells were stably cotransfected with both CFP- and YFP-coupled LH receptors which is reported to decrease immature or misfolded hLHR and increase cell membrane expression (Tao et al., 2004). We imaged CFP and YFP fluorescence separately using fluorescence filter sets for these visible fluorescent proteins that minimized the fluorescence contribution from CFP when imaging YFP (Llopis et al., 2000) and that, in subsequent steps, permitted photobleaching of YFP only. Following photobleaching, each cell was reimaged using the same filter sets. As shown in Figure 14, after photobleaching of YPP, an increase in CFP dequenching and thus a brighter CFP image was obtained when there was energy transfer between the visible fluorescence proteins.

We used two methods to analyze data from these studies (Figure 15). In both methods, background fluorescence was subtracted from the four images obtained in each individual experiment. This involved selection of a cell-free site on the complete image and subtraction of the average intensity of that site from the complete image. This typically produced images of cells on a dark background. The first method used for analysis of fluorescence donor intensity averaged fluorescence intensity from the entire cell before and after photobleaching to calculate energy transfer efficiency. Figure 15 shows donor fluorescence from a representative cell before and after photobleaching of the fluorescence acceptor. We compared this method with one in which only fluorescence from the periphery of the cell was used.

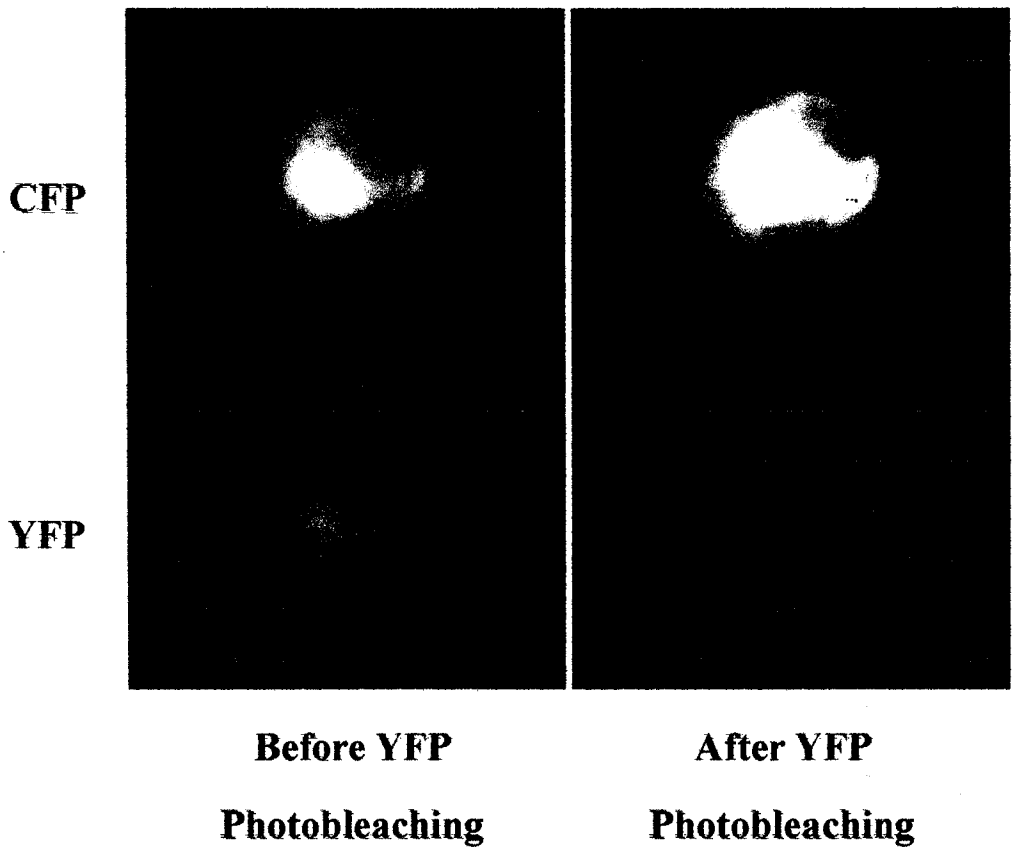

Figure 14. After photobleaching of YFP, an increase in CFP dequenching and thus a brighter CFP image occurred when there was energy transfer between the visible fluorescence proteins. In this case, the energy transfer efficiency is 24%.

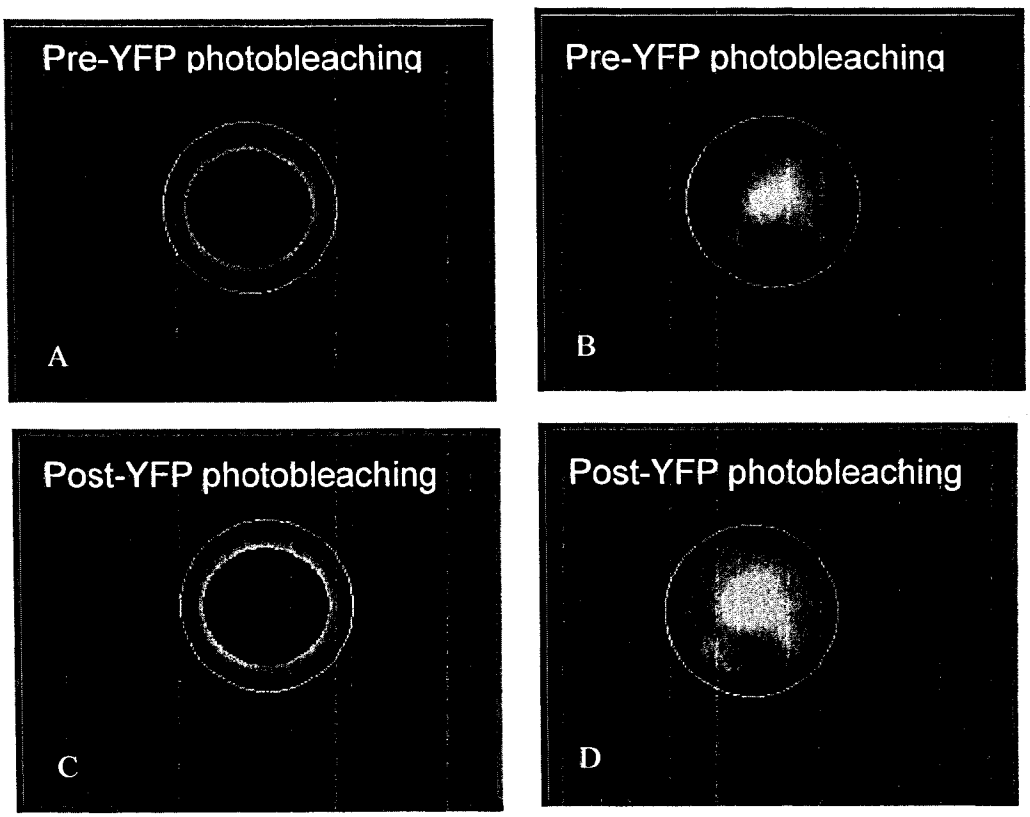

Figure 15. Different analysis methods of imaging FRET.

In the example shown in Figure 15, the average intensity before photobleaching from the entire cell as compared to "plasma membrane only" was reduced from 1530 cps to 487 cps. After photobleaching, values increased to 1753 cps and 560 cps. Energy transfer efficiency (%E) was not statistically different (12.7% and 12.9%, respectively) using these two methods for analysis of FRET. More generally, examining only the plasma membrane of each of the cells lines used for FRET analysis produced a decrease in the mean and S.D. of 2.3 ± 1.4% for %E (data not shown) which was not significant while reducing the overall number of photon counts used to evaluate FRET.

A summary of results using the whole cell imaging method is shown in Table 2. In the absence of ligand, values for energy transfer efficiency between LHR-wt receptors were, on average, less than 1%. Energy transfer efficiency increased significantly to 11.5 ± 1.2 % following exposure of LHR-wt to 100 nM hCG. Since energy transfer occurs to a measurable extent only when the CFP (donor) and YFP (acceptor) are separated by distances less than about 100Å (Patterson et al., 2000), FRET results indicate that wild type human LHRs, if occupied by a functional ligand, undergo receptor self-association.

LHR-wt receptors also translocated from the bulk plasma membrane into high buoyancy membrane fractions (rafts) following ligand treatment (Figure 16 and upper panel of Figure 17). Following isopycnic centrifugation of plasma membrane fractions from CHO cells, over 95% of unoccupied receptors were found fractions

Table 2. Efficiency of Fluorescence Energy Transfer between Wild Type LH receptors and Constitutively Active Receptors

Cell Line	Ligand	Treatment	%Efficiency	n
hLHR-wt	none	none	0.8 ± 1.3	17
	100nM hCG	none	11.5 ± 1.2	24
hLHR-DG	none	none	15.3 ± 1.9	11
	100nM hCG	none	11.1 ± 2.5	14
hLHR-DH	none	none	13.8 ± 1.6	39
	100nM hCG	none	13.0 ± 1.3	23
	none	1% MβCD, 45 min	8.0 ± 1.4	15
hLHR-DY	none	none	11.7 ± 2.2	14
	100nM hCG	none	14.1 ± 4.3	11

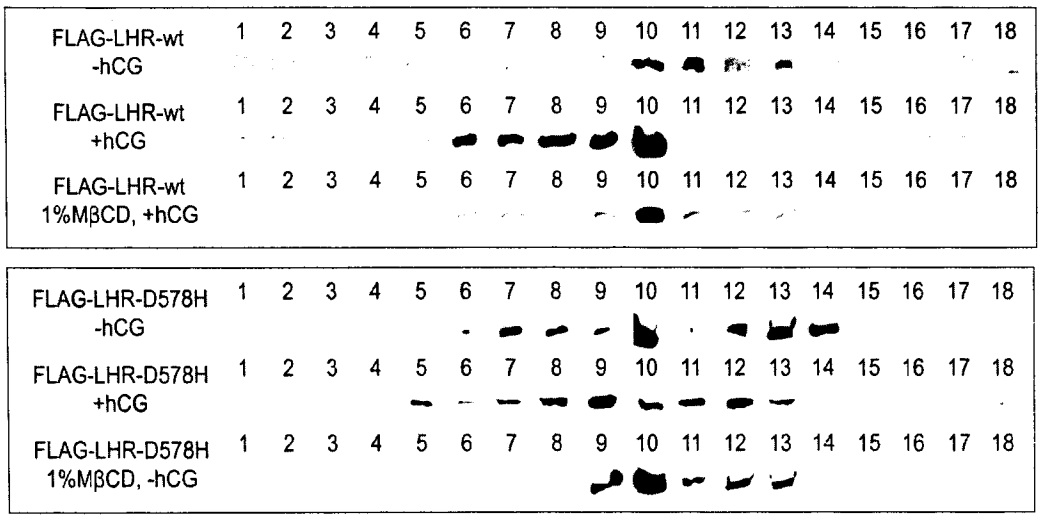

Figure 16. Representative western blots obtained from cell samples expressing FLAG-LHR-wt receptors (upper panel) and constitutively active human LH receptors LHR-D578H (lower panel) separated on sucrose density gradients.

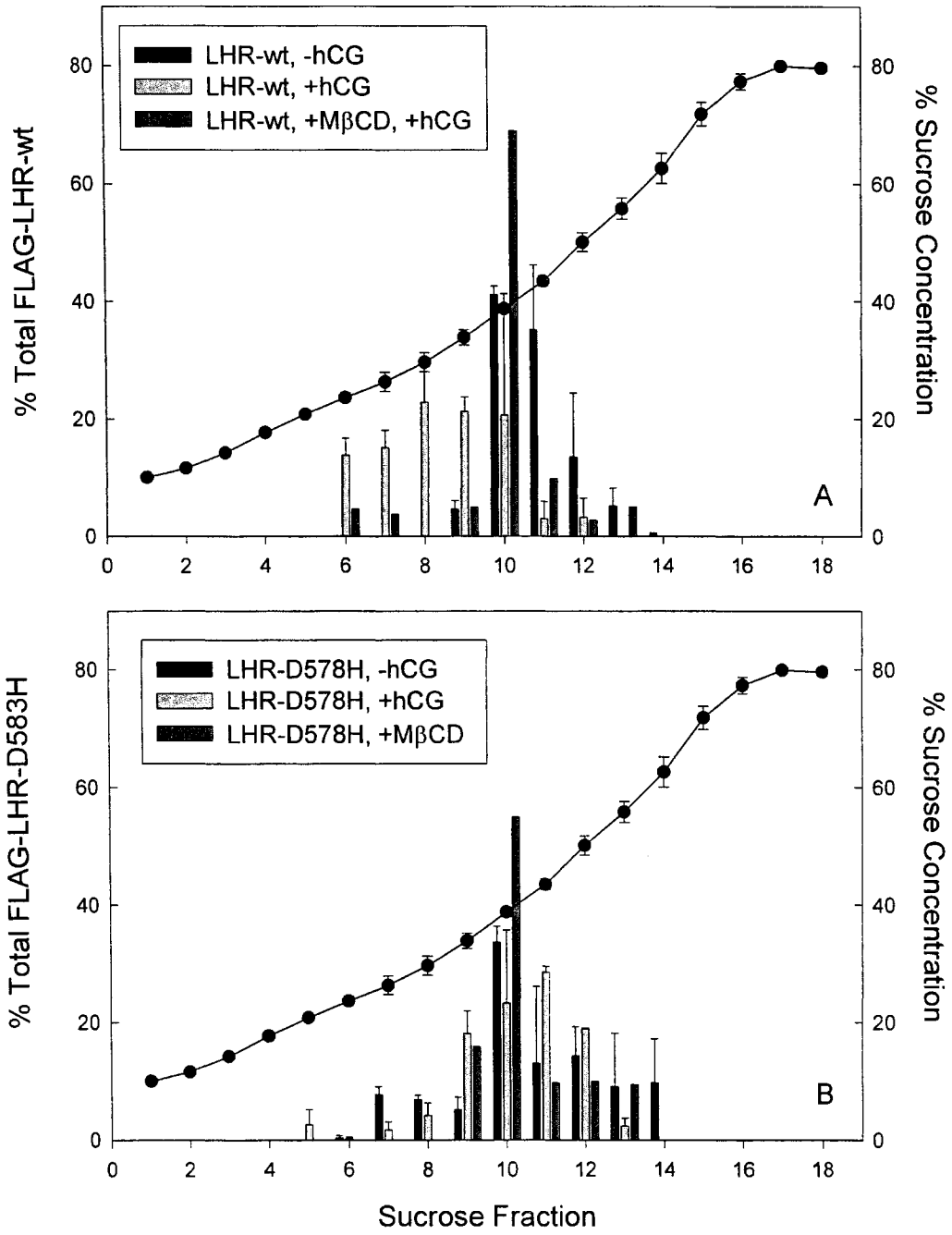

Figure 17. Bar graphs for LHR-wt and LHR-D578H in different treatment with sucrose concentrations and fractions of receptors.

containing 39-56% sucrose. About 75% of hCG-treated FLAG-hLHR-wt consistently appeared in sucrose fractions containing 24-34% sucrose and thus had "floated" to lower sucrose densities during centrifugation. The remaining receptors were found in fractions containing higher sucrose concentrations. To further demonstrate the presence of hormone-treated LH receptors within rafts, we treated cells with 1% MβCD, a cholesterol sequestering reagent that is efficient in removing cholesterol from the plasma membranes of live cells and that disrupts raft structure (Figure 16 and the upper panel of Figure 17). At low concentrations MβCD extracts membrane cholesterol by placing it in a central non-polar cavity of cyclic oligomers of glucopyranoside in 1, 4 glycosidic linkages (Ilangumaran and Hoessli, 1998) without affecting cell viability (data not shown). Pretreatment of CHO cells with MβCD followed by exposure to 100nM hCG reduced the relative number of receptors in fractions containing less than 34% sucrose from 75% to 13%, presumably by disrupting membrane microdomains.

Constitutively-active human LHR are self-associated in the absence of hormone

In contrast to wild type human LH receptors, constitutively active receptors in the absence of hormone exhibited relatively high values for fluorescence energy transfer efficiency that ranged from about 11-15% (Table 2). Hormone treatment had no statistically significant effect on FRET values. Following treatment of cells with 100nM hCG, energy transfer between LHR-D578Y increased from 11.7 ± 2.2 % to

14.1 ± 4.2% while energy transfer efficiency for cells expressing LHR-D578H decreased slightly. The absence of any statistically significant effect suggests that there was little, if any, overall change in the extent of receptor association when constitutively active receptors were occupied by ligand.

MβCD treatment disrupts plasma membrane rafts and reduces the extent of energy transfer between FLAG-LHR-D578H receptors but does not affect cAMP signaling

To examine localization of LHR-D578H in rafts, the receptor was epitope-tagged on the N terminus using FLAG. Western blots of sucrose gradient fractions obtained from isopycnic ultracentrifugation showed approximately 20-25% of FLAG-LHR-D578H associated with sucrose fractions containing 24-34% sucrose before and after binding of hCG (Figure 17, lower panel). As expected, MβCD treatment shifted the constitutively active receptor to lower buoyancy membrane fractions, which was consistent with disruption of membrane rafts (Figures 16 and 17, lower panel). Because it has been suggested that plasma membrane rafts may concentrate receptors and other plasma membrane molecules necessary for the transduction of a productive ligand-mediated signal, we examined whether disruption of membrane rafts reduced or eliminated cAMP signaling by FLAG-LHR-D578H cells. Interestingly, there was no decrease in cell signaling via cAMP following MβCD treatment (Table 3) suggesting that localization of LHRs to rafts may not be

Table 3: cAMP responsiveness of cells expressing FLAG-LHR-wt and FLAG-LHR-D578H[1]

Cell Line	Treatment	Fold Increase over basal cAMP levels	n
LHR-wt	None	1.0[a]	9
LHR-wt	100 nM hCG	1.6 ± 0.1[b]	9
LHR-D578H	None	2.1 ± 0.3[b]	9
LHR-D578H	1% MβCD	2.9 ± 0.6[b]	6

[1] CHO cells expressing LHR-wt were assayed for cAMP expression following treatment with 100nM hCG or following pretreatment of cells expressing LHR-D578H with MβCD. Results are expressed as the mean of the relative increase in cAMP ± S.E.M. compared to basal levels of cAMP in untreated CHO cells expressing LHR-wt receptors.

a,b Means with different superscripts differ ($p<0.03$).

required for receptor signaling. There was, however, a significant decrease in the extent of receptor self association from an average of 14% to 8% in MβCD-untreated cells (Table 2).

A more detailed analysis of this decrease in FRET efficiency is presented in Figure 18 which shows individual FRET values for both untreated and MβCD-treated cells. The efficiency of energy transfer for untreated and MβCD-treated cells varied on a cell-to-cell basis over a fairly large range. When LHR-D578H cells were treated with MβCD, higher values for FRET efficiency were absent as reflected in the left shift in accumulated FRET values. One explanation for this result is that there are two populations of self-associated, constitutively-active receptors on LHR-D578H cells with more extensive receptor self-association occurring in membrane rafts. The persistence of self-associated receptors in the bulk membrane following cell treatment with MβCD may be sufficient to maintain adenylate cyclase activation and elevated levels of cAMP in cells.

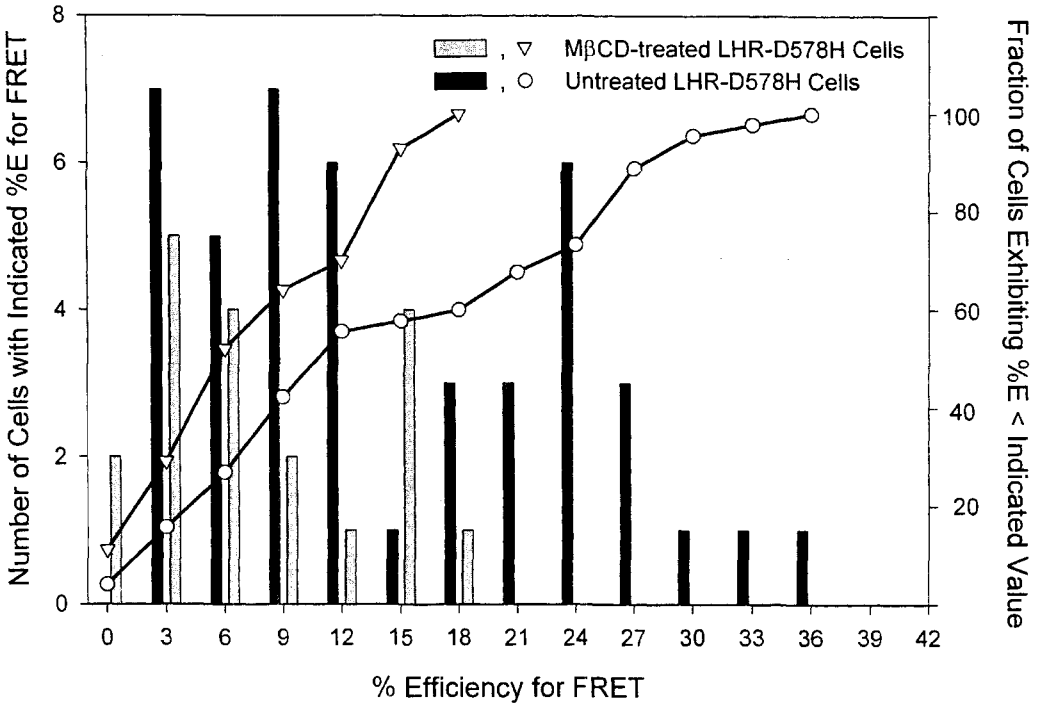

Figure 18. Individual FRET Measurements for LHR-D578H cells +/- CD.

Single particle tracking of hCG-occupied FLAG-LHR-wt receptors demonstrates "trapping" of receptors in small membrane compartments

To independently assess the localization of hCG-treated FLAG-LHR-wt in membrane compartments, single particle tracking methods were used. This technique identifies individual LH receptors on the surface of viable cells and tracks their motions over approximately two minutes. The centroid for a 40 nm gold particle attached to an individual receptor can be identified visually on video obtained from each experiment and its motions can be quantitatively described. hCG treatment reduced the average diameter of compartments containing human FLAG-LHR-wt approximately 2-fold from 225 ± 44 nm to 134 ±5 nm as well as the diffusion coefficient for receptors within compartments (Figure 19). For both unoccupied and hormone-treated FLAG-LHR-D578H, the compartments containing receptors were small with domain diameters, L_r, of 120 ± 20 nm and 100 ± 39 nm, respectively. Diffusion coefficients within the compartments ($0.19 \times 10^{-11} cm^2 sec^{-1}$ and $0.15 \times 10^{-11} cm^2 sec^{-1}$, respectively) were comparable to those obtained for hCG-treated FLAG-LHR-wt ($0.23 \times 10^{-11} cm^2 sec^{-1}$).

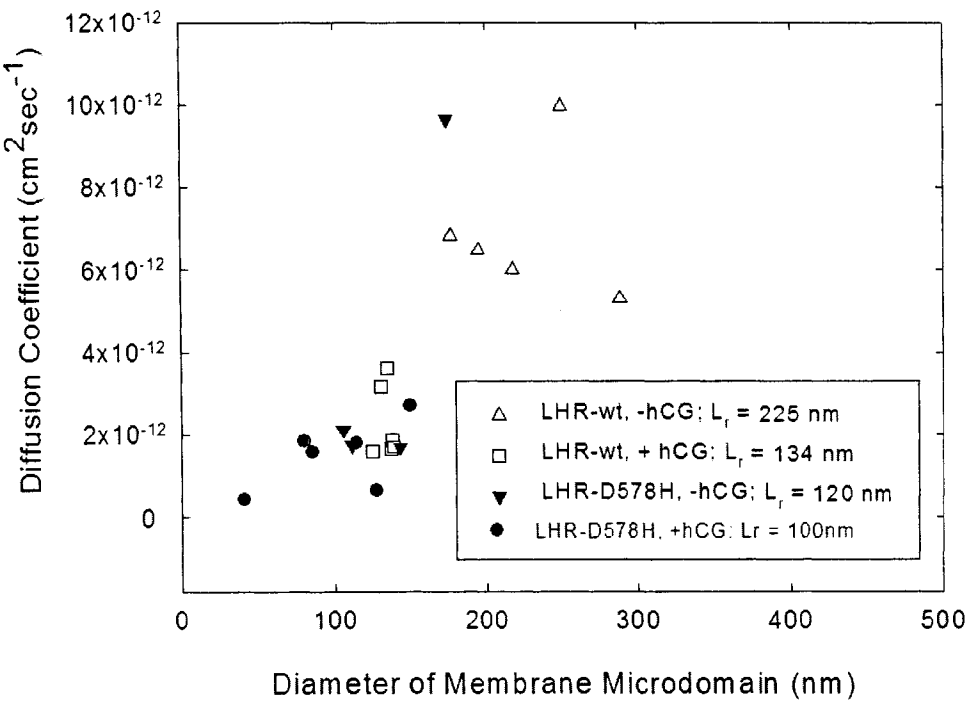

Figure 19. Single particle tracking of individual FLAG-LHR-wt and FLAG-LHR-D578H receptors labeled with gold-conjugated anti-FLAG antibody.

Discussion

In addition to exhibiting unregulated signaling, constitutively-active LH receptors have characteristics of hormone-treated, actively signaling wild type human receptors and rat LH receptors (Smith et al., in preparation). These receptors are self-associated, present in small membrane compartments on viable cells and isolated within plasma membrane rafts. The extent of receptor self-association is characteristic of wild type rat (Horvat et al., 2001) and human LH receptors treated with a high concentration of hCG with values for energy transfer efficiency ranging from 11-15%. The LHR-D578H receptor also appears to be distributed into sucrose fractions with densities comparable to those in which hormone-treated rat (Smith et al., in preparation) or human LHR-wt receptors are found.

Unlike constitutively active LHR-D578H receptors, human wild type receptors undergo translocation into rafts upon binding of ligand. In addition to rat and human LH receptors, there are a number of examples of plasma membrane receptors that are transiently associated with rafts during signaling. As examples, the β-adrenergic receptor becomes transiently associated with lipid rafts together with nitric oxide synthase and adenylate cyclase (Ostrom et al., 2004) and requires the raft environment for adenylate cyclase activity by Gs. The Type I Fcε receptor is also transiently associated with lipid rafts and exhibits increased signaling capability in that environment. Baird and coworkers suggest that, although the Type I Fcε receptor appears to be associated with Lyn in the bulk membrane, Lyn has low activity under

these conditions (Young et al., 2005). Rather, it is translocation of Type I Fcε receptors together with Lyn into the raft environment together as well as aggregation of the Fcε receptor that promotes the coupling of Type I Fcε receptors with Lyn phosphorylation. There are also examples of constitutive association of proteins with rafts including gonadotropin releasing hormone receptor, a G protein-coupled receptor (Navratil et al., 2003).

Factors driving translocation of either the wild type receptor into membrane rafts or residence of constitutively-active receptors within these structures are not known. Although the possibility remains that factors regulating LH receptor association with rafts differ for wild type and LHR-D578H, these studies indicate that association of LH receptors with rafts does *not* involve direct interactions between glycosylated hCG and raft components. While binding of hCG to the wild type receptor is necessary for translocation of the receptor into raft domains, the increase in association of the LHR-D578H with raft domains following addition of hormone is small, approximately 6% of the total receptor number. Moreover, studies of a rat LH receptor with a point mutation in the sixth transmembrane domain (Smith et al., in preparation) suggest that these receptors, which exhibit little if any capacity to signal, remain localized in bulk membrane fractions despite binding of hCG.

On the other hand, conformational differences between the wild type and constitutively active receptor may be sufficient to promote constitutive interactions between the receptor and raft components. An appropriate conformation for the

constitutively active receptor and acquisition of a similar conformation by the hormone-occupied wild type receptor may increase receptor affinity for raft-associated proteins. It is also possible that a critical receptor conformation is needed for association of ligand-activated receptors with other non-receptor raft components and it is these interactions that result in retention of the receptor in rafts.

Alternatively, structural modification of receptors such as lipidation could cause preferential localization of the receptor in the cholesterol and spingomyelin-rich outer leaflet that defines the raft environment. There is evidence of palmitoylation of the wild type rat LH receptor upon binding of hormone (Menon et al., 2004) and both the constitutively active human LH receptor and the human wild type receptor contain the palmitoylation motif. Menon and coworkers (Kawate and Menon, 1994; Munshi et al., 2001) have suggested that rat LH receptor depalmitoylation may be involved in internalization of the receptors: wild type receptors, which are more extensively palmitoylated, are also more rapidly internalized than a less palmitoylated, constitutively-active mutant. Whether palmitoylation is a dynamic process, occurring in response to, for example, binding of ligand, is not known although there are examples of other G-protein coupled receptors for which this process is dynamic (Qanbar and Bouvier, 2003). Moreover, palmitoylation of other membrane proteins such as influenza hemagglutinin (Melkonian et al., 1999) and Fyn (Webb et al., 2000) is necessary for retention in low density membrane fractions.

In these studies, a minimum of two events appear to be necessary for signaling by

the LH receptor. First, productive signaling by a hormone occupied receptor appears to involve receptor-receptor interactions that produce comparatively high positive values for energy transfer efficiency. Second, migration to the raft environment or retention in small membrane compartments may also play a role in signaling. Although we anticipated that disruption of lipid rafts would impair signaling by constitutively active LH receptors, as it did for rat LHR-wt receptors (Smith et al., in preparation), this was not the case. Rather, receptor-mediated cAMP accumulation by the constitutively-active receptor was not affected by cholesterol depletion although it did reduce the extent of receptor-receptor interaction. Thus, it remains possible that the key event in signaling by the constitutively-active receptor, in contrast to the wild type receptor, is establishing and maintaining receptor interactions rather than their membrane microenvironment.

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CHAPTER FOUR

CHIMERIC GNRH-LH RECEPTORS AND LH RECEPTORS LACKING C-TERMINUS PALMITOYLATION SITES DO NOT LOCALIZE TO PLASMA MEMBRANE RAFTS

Introduction

Binding of luteinizing hormone (LH) or its placental counterpart, human chorionic gonadotropin (hCG), to the LH receptor (LHR) in gonadal tissues, results in the increased production of cAMP and steroidogenesis (Menon and Gunaga, 1974). The LH receptor is a G-protein-coupled receptor (GPCR) and a member of the glycoprotein hormone receptor subfamily that includes follitropin (FSH) receptor and thyrotropin (TSH) receptor. These receptors are characterized by large, glycosylated N-terminal extracellular (EC) domains which bind hormone with high affinity and specificity (Segaloff and Ascoli, 1993; Dufau, 1998). In addition, these single-strand receptors have seven transmembrane (TM) domains and a C-terminal intracellular tail which, in the case of the LH receptor, has about 70 amino acids (McFarland et al., 1989; Probst et al., 1992).

The LH receptor C-terminus has been implicated in modulation of receptor function and turnover. Progressive truncation of the C terminus suggests that a region(s) between residues 616 and 631 of the rLHR is required for proper insertion and/or targeting of the receptor into the plasma membrane. Truncation of the LH receptor at position 631 in the C terminus slows receptor internalization following binding of hormone (Sanchez-Yague et al., 1992; Rodriguez et al., 1992). Palmitoylation at cysteine residues 621 and 622, in addition to providing anchoring sites for the cytoplasmic tail in the plasma membrane, may also affect receptor turnover: mutations in these palmitoylation sites enhance the rate of ligand-induced receptor internalization although other studies have reported no effect on the intracellular trafficking of the receptor (Qanbar and Bouvier, 2003; Menon et al., 2004).

The C-terminus of the LH receptor may also affect localization of the receptor in membrane microdomains. These specialized membrane microdomains, sometimes called rafts, have high lipid content and "float" in sucrose gradient (Tsui-Pierchala et al., 2002). GnRH receptors, which are also coupled to G-proteins, have an abbreviated C terminus with, at most, three amino acids (Stojilkovic et al., 1994). These receptors are constitutively localized in membrane microdomains with high buoyancy in sucrose gradients (Navratil et al., 2003). In contrast, rat LH receptors are found in the bulk membrane prior and only translocate into plasma membrane rafts upon binding of hormone agonists (Roess and Smith, 2003). Palmitoylation of other membrane proteins, in addition to targeting proteins to the plasma membrane

(Pallavi and Nagaraj, 2003), is involved in raft localization of, for example, the Src family kinase Fyn and influenza hemagglutinin within the membrane. Inhibition of Fyn palmitoylation blocked Fyn localization to rafts (Webb et al., 2000), and association of influenza hemagglutinin with rafts required all three of its palmitoylated cysteine residues (Melkonian et al., 1999).

Here, we explore whether the full-length LH receptor C terminus, when coupled to GnRH receptors, altered the distribution of these chimeric receptors within the membrane and whether this distribution is affected by mutations to cysteine residues at positions 621 and 622 or truncation of the C-terminus at position 631. We also compared the membrane distribution of these chimeric receptors with that of a full-length LHR with mutations to cysteines at positions 621 and 622 that eliminated receptor palmitoylation.

Materials and Methods

Materials

Geneticin and Dulbecco's modified Eagle medium containing high glucose was purchased from Mediatech, Inc (Herndon, VA). Non-essential Amino Acids and penicillin-streptomycin solution were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). hCG was purchased from Research Diagnostics Inc. (Flanders, NJ).

D-Ala-GnRH, FLAG vector and anti-FLAG M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA) and Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA).

Construction of plasmids and cell lines

We mutated cysteines in the rat LH receptor at positions 621 and 622 to serines using the QuikChange site-directed mutagenesis kit from Stratagene and manufacturer's instructions. Using PCR, GnRH receptors were coupled to the full length rat LH receptor C-terminus tail (amino acids 604-674; GnRHR-LHR-C_{full}), the rat LH receptor truncated C-terminus tail (amino acids 604-631; GnRHR-LHR-C_{t631}), or the rat LH receptor C-terminus with C621S and C622S mutations (GnRHR-LHR-$C_{C621,622S}$). Stable CHO cell lines were constructed expressing mutated rat LH receptors or chimeric GnRH receptors coupled to FLAG epitope on their N-terminus. To prepare these cell lines, vectors were constructed for FLAG-rLHR-C621,622S, FLAG-GnRHR-LHR-C_{full}, FLAG-GnRHR-LHR-C_{t631} and FLAG-GnRHR-LHR-$C_{C621,622S}$. Then 8μg DNA and 20μL of Lipofectamine 2000 were added to CHO cells that were 90-95% confluent in 60 mm polystyrene plates. After 3-4 weeks, clones expressing FLAG were selected based on membrane fluorescence following cell labeling Cy3 conjugated anti-FLAG antibody.

For fluorescence photobleaching recovery measurements of receptor lateral

diffusion, cell lines were constructed expressing receptor coupled to either green fluorescence protein or yellow fluorescence protein on their C-terminus as previously described for GFP-LHR (Horvat et al., 1999). Clones expressing GFP- or YFP-coupled proteins on their membrane were selected for expression. Transfected cell lines were maintained in CHO cell medium that included Dulbecco's modified Eagle media supplemented with 4.5mg/mL glucose, 10% FBS, 100U/mL penicillin, 100U/mL streptomycin and 1x MEM non-essential amino acids (Sigma Chemical Co., St. Louis, MO) that was supplemented with 400μg/mL G418. In some experiments, an Assay Designs cAMP kit (Ann Arbor, MI) was used to evaluate the ability of either chimeric receptors or mutated LH receptors to signal following binding of hormone.

Isolation of plasma membrane rafts

Cells were incubated with 100nM hCG, D-Ala or PBS for 1 hour at 37°C prior to cell lysis. To isolate membrane rafts, 3 x 10^7 cells were washed two times with phosphate-buffered saline, pH 7.2 (PBS) and lysed for 5-10 minutes on ice in 1ml of a buffer containing 25mM MES, 150mM NaCl, 2mM EDTA, 20% glycerol, 0.25% Triton-X100, 5mM N-ethyl maleimide and protease inhibitors including aprotinin, leupeptin, EDTA, and PMSF (Roche). The product of cell lysis, which contained plasma membrane fragments, was then combined with 1ml of 80% sucrose. A discontinuous sucrose gradient from 10-80% was created and the sample was layered at 40% sucrose, loaded into a Beckman SW-41 swinging bucket rotor and spun at

175,000x g for 20 hours at 4°C. After centrifugation, eighteen fractions, each containing 640μL fractions, were collected from the top of the gradient downward and diluted in SDS buffer. After separation of proteins using SDS-PAGE and transfer to nitrocellulose, the FLAG-tagged LHR or FLAG-tagged chimeric receptors were identified using 30μg of an anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO).

Spot fluorescence photobleaching recovery measurement

A description of the method used for measurements of GFP-tagged LH receptor lateral diffusion has been previously published (Horvat et al., 1999). The microscope objective used in this method was a Zeiss 40x objective of NA 0.65. Standard Zeiss filter and dichroic mirror sets for GFP or YFP fluorescence were used to examine fluorescence from cells under coverslip in well slides. An attenuated Coherent Radiation Innova 100 argon ion laser beam was focused to a spot of 0.41μm 1/e2 radius on the cell membrane. Bleaching and probe beam powers were 1.4mW and 1.7μW, respectively, at 488nm. Data were acquired at 50msec/point for 20 sec before, and for 30 sec after, a 150msec bleaching pulse and were analyzed to yield a diffusion coefficient D and an estimate of the relative number of mobile receptors based on the extent of fluorescence recovery (%R) after photobleaching.

Results and Discussion

LHR-C621,622S receptors do not translocate into membrane rafts upon binding hCG

Protein palmitoylation, in addition to anchoring proteins in the plasma membrane (Pallavi and Nagaraj, 2003), may affect the localization of these proteins in membrane microdomains. We have previously shown that LH receptors, upon binding of ligand, translocate to membrane microdomains (Roess and Smith, 2003). In addition, individual rat LHR identified with 40nm gold particles remain confined to these small membrane regions for comparatively long times and exhibit slow lateral diffusion. Treatment of cells with methyl-β-cyclodextrin disrupts membrane microdomains containing hormone-occupied LHRs and increases the average rate of receptor lateral diffusion (Smith et al., manuscript in preparation).

To determine whether palmitoylation of the receptor had an effect on receptor localization in membrane microdomains, we constructed receptors, originally described by Menon and coworkers, which contained mutations to both of the two possible palmitoylation sites on the LHR C-terminus (Kawate and Menon, 1994; Kawate et al., 1997). We confirmed that receptors with these mutations retained the ability to signal via cAMP, as previously reported (Kawate and Menon, 1994; Kawate et al., 1997), and, in fact, produced levels of cAMP that were comparably to wild type receptors (data not shown). Nevertheless, FLAG-tagged LHR-C621,622S did not translocate into membrane rafts following binding of hormone as shown in Figure 20.

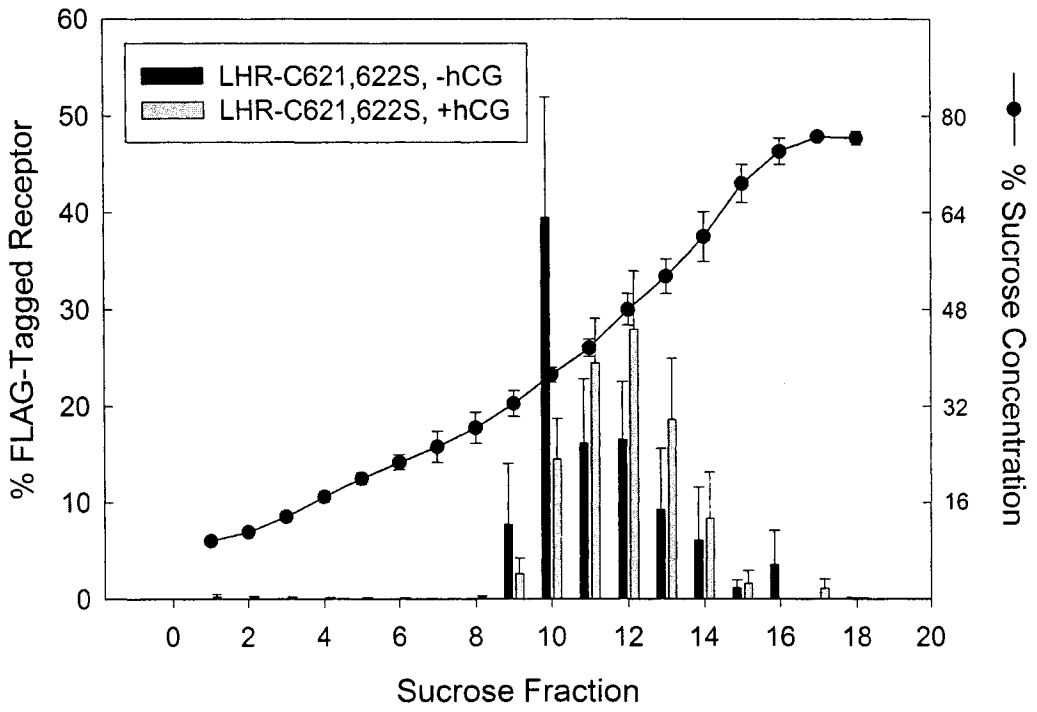

Figure 20. Rat LH receptors with mutations at position 621 and 622 in the receptor's C-terminus (LHR-C621,622S) are localized in high density membrane fractions before and after treatment of CHO cells with 100nM hCG.

Both in the absence and presence of hormone, LHR-C621,622S appeared in fractions containing approximately 30-60% sucrose. This contrasts with hCG-treated wild type LHR which consistently appears in low density membrane fractions containing 15-25% sucrose (Roess and Smith, 2003).

Chimeric GnRHR-LHR are located in the bulk membrane and do not translocate into rafts

The murine GnRH receptor is constitutively localized in membrane rafts (Navratil et al., 2003). The localization of GnRH receptors in this microenvironment is critical for signaling by the GnRH receptor to ERK and c-Fos suggesting that these microdomains may function as organizing structures that concentrate proteins involved in signal transduction. For all practical purposes, the mammalian GnRH receptor lacks an intracellular C-terminus although the C-terminus appears to have been lost during evolutionary development (Sealfon et al., 1997). A recent study of the sea lamprey GnRH receptor showed that the full length receptor with a C-terminus of approximately 120 amino acids was able to effectively activate the inositol phosphate (IP) signaling pathway (Silver et al., 2005). Although progressive truncation of the receptor's C-terminus significantly reduced signaling efficiency, signaling via IP was restored in lamprey GnRH receptors that complete lacked a C-terminus.

The LH receptor C-terminus has also been implicated in receptor function. Progressive truncation of the C-terminus affects targeting of the receptor to the membrane (Rodriguez et al., 1992) and, if the receptor is expressed in the membrane, the rate of receptor desensitization (Sanchez-Yague et al., 1992). As discussed earlier, palmitoylation of the C-terminus is implicated in the rate of receptor internalization (Qanbar and Bouvier, 2003; Menon et al., 2004).

To determine whether the C-terminus of the LH receptor also played a role in raft localization, we constructed several chimeric receptors using the rat GnRH receptor as a parent molecule and adding various forms of the LH receptor C-terminus. As shown in Figure 21, addition of an LH receptor C-terminus lacking palmitoylation sites at positions 621 and 622, resulted in a receptor that appeared in low density membrane fractions consistent with receptor localization in the bulk membrane. Treatment of GnRHR-LHR-$C_{C621,622S}$ with a GnRH agonist, D-ala-GnRH, which causes aggregation of the wild type GnRH receptor (Horvat et al., 2001), did not cause translocation of the receptor to rafts. Similarly, GnRH receptors coupled to the full-length LH receptor C-terminus (GnRHR-LHR-C_{full}) were also localized in the bulk membrane (Figure 22). Interestingly, with truncation of the receptor C-terminus, approximately 3% of GnRHR-LHR-C_{t621} consistently appeared in lower density fractions 4-6 which suggests raft localization of some receptors.

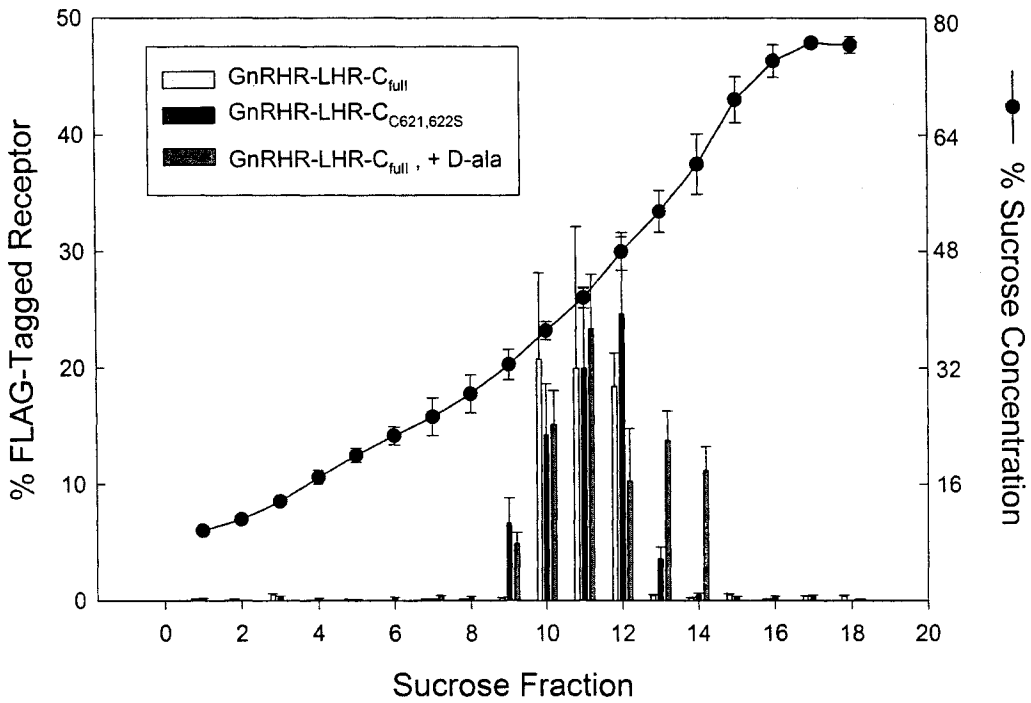

Figure 21. Chimeric GnRHR-LHR receptors with either the full-length LH receptor C-terminus or a C-terminus containing mutations to position 621 and 622 were located in high density sucrose fractions. Binding D-Ala-GnRH to GnRHR-LHR-C$_{full}$ did not cause translocation of the receptor into membrane rafts.

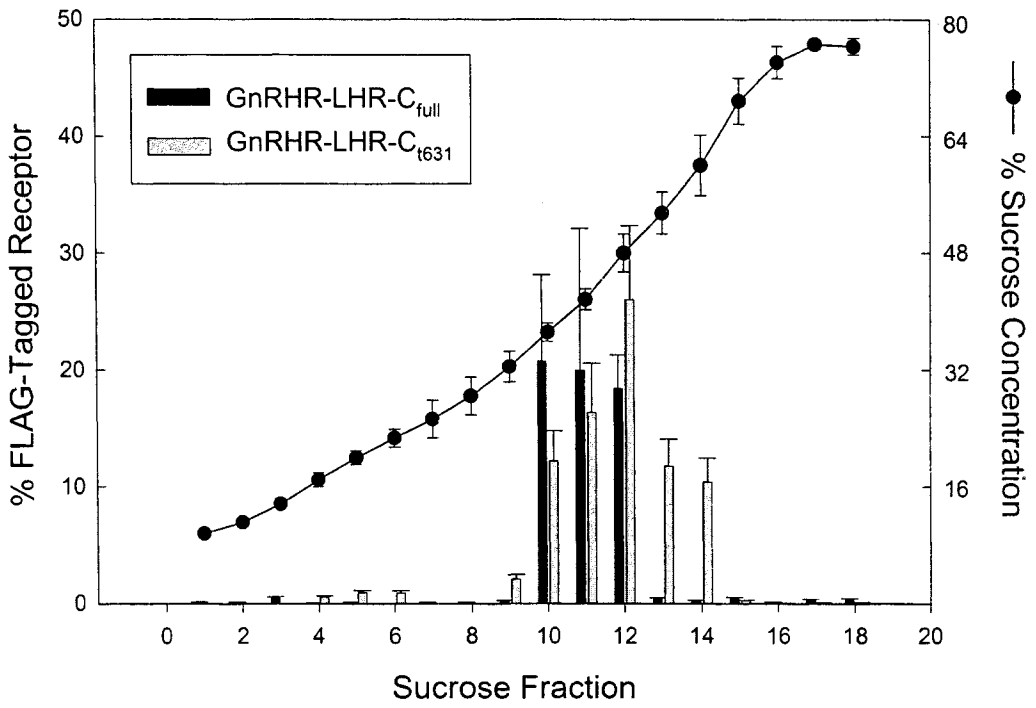

Figure 22. GnRHR-LHR-C_{full} chimeric receptors were located in membrane fractions containing high sucrose concentrations. Only with truncation of the LHR C-terminus at position 631 did approximately 3% of the GnRHR-LHR-C_{t631} consistently appear in fractions 4-6.

Chimeric receptors had larger mobile fractions than self-associated wild type GnRH receptors

The outer leaflet of the membrane in rafts is enriched with sphingolipids and cholesterol as well as glycosylphosphatidyl-inositol (GPI)-anchored proteins. This raft environment can limit the lateral diffusion of membrane proteins. Tracking of gold-tagged LH receptors demonstrates that hCG binding to the rat LHR restricts receptors to small membrane domains and limits the rate of lateral diffusion for the particle within that domain as well as the rate of diffusion for the receptor over long times (Smith et al, manuscript in preparation). If the diffusion of large numbers of receptors is evaluated using spot fluorescence recovery after photobleaching (FPR) methods, confinement of receptors in rafts or other small microdomains is also likely to result in a reduction in the fluorescence recovery after photobleaching (%R).

We used spot FPR methods to evaluate the extent of fluorescence recovery after photobleaching and compared these values for receptors that located in the bulk membrane with values for receptors known to be raft localized. As shown in Table 4, values for diffusion coefficients did not vary significantly for any of the receptors studied. However, fluorescence recovery for wild type LH receptors in the absence of hormone treatment was 52 ± 2% which was significantly higher than the value, 15 ± 2% for wild type receptors treated with hCG. This large reduction in the fraction of mobile receptors is likely to be due to a number of factors which are not mutually

Table 4. A comparison of lateral diffusion measurements for the wild type GnRH receptor and GnRHR-LHR chimeric receptors and for wild type LHR-GFP and wild type LHR labeled with TrITC-hCG[1].

Cell Line	D ($10^{-9}cm^2sec^{-1}$)	%R	n	Raft localization
GnRHR-LHR_{full}-YFP	0.9 ± 0.3	72 ± 3[a]	31	no
GnRHR-LHR_{t631}-YFP	0.6 ± 0.1	76 ± 2[a]	45	no
GnRHR-GFP	1.0 ± 0.2	55 ± 4[b]	30	yes*
LHR-GFP	0.6 ± 0.1	52 ± 2[b]	67	no**
LHR-wt + TrITC-hCG	0.9 ± 0.3	15 ± 2[c]	19	yes**

[1] Lateral diffusion characteristics, D and %R, for wild type and chimeric receptors. Measurements were made using spot fluorescence photobleaching recovery methods. Values are expressed as the means ± S.E.M.

[a],[b],[c] Means bearing different subscripts differ ($p<0.05$).

* Navratil et al., 2003

** Roess and Smith, 2003

exclusive including anchoring of receptors by cytoskeletal components (Roess et al., 1988; Roess et al., 1997), extensive self-aggregation of hormone-occupied receptors (Horvat et al., 1999; Horvat et al., 2001) and translocation into membrane rafts (Roess and Smith, 2003). Similarly, values for %R for GnRHR-LHR-C_{full} and GnRHR-LHR-C_{t631}, 72± 3% and 76± 4%, respectively, were significantly larger than the value for %R for wild type GnRH receptors (55± 4%) which also localized in membrane rafts (Navratil et al., 2003).

Together these studies demonstrate a role for the LH receptor C-terminus in regulating the localization of the receptor within the membrane. It appears that palmitoylation may be important in driving receptor translocation into rafts but whether raft localization occurs as a consequence of the affinity of palmitate for raft-associated lipids or whether this is due to a structural change in the receptor itself, is not clear. Addition of the large C-terminus to GnRH receptors also appears to actively exclude the GnRH receptor from rafts.

One lingering question is whether raft domains are important in signaling by the LH receptor. Although Navratil et al. have demonstrated that constitutive localization of the GnRH receptor in low density membrane microdomains is necessary for GnRH signaling to ERK (Navratil et al., 2003), LHR with point mutations in the receptor's C-terminus that prevented palmitoylation, retained the ability to signal via cAMP following hCG treatment but did not translocate to rafts. This is consistent with other results suggesting that disruption of rafts by

methyl-β-cyclodextrin reduces, but does not completely eliminate, either receptor self-association or signaling. Thus the raft environment, while enhancing signaling efficiency, is not essential to the production of cAMP in response binding of hormone by receptor.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

LH receptor function is critically important for male and female reproductive success. These LH receptors signal through G-proteins and participate in ovulation, regulation of sex steroid synthesis, and maternal recognition of pregnancy in humans. We have investigated LH receptor organization in membrane receptor-mediated intracellular signaling using several different approaches. FLAG tagged wild type receptors and receptors containing point mutations that affect receptor function have been used to explore relationship between receptor function and lipid raft localization. Imaging FRET methods have been used to demonstrate receptor self-association under various conditions. Finally, we have used MβCD treatment to disrupt rafts and to explore the relationship between signaling and raft localization. These studies have provided insight into the sequence of events that occur following hormone binding and that lead to initiation of signal transduction.

Wild type LH receptors undergo self-association following hormone binding

Our data suggested that wild type LH receptors are not self-associated until they have bound hormone but that constitutively active receptors are self-associated even in the absence of hormone. MβCD not only disrupted rafts but also reduced self-association of LH receptors. Furthermore, we can speculate that rafts may promote higher degrees of LH receptors self-association. Nonetheless, the role of receptor self-association in signaling is not fully resolved.

As we know, LH receptors bind their hormones at the N-terminal extracellular exo-domain, whereas the hormone signals are generated in the membrane-associated endo-domain. Therefore, the liganded exo-domain modulates the endo-domain to generate a hormone signal. In the case of dimeric receptor complex, it is unclear, however, whether only one or both of the two endo-domains need to be activated for signal generation, and particularly whether the liganded exo-domain is capable of intramolecularly activating its cognate endo-domain (*cis*-activation) and/or intermolecularly activating the endo-domain of the unliganded receptor (*trans*-activation). The studies of LH receptor signaling in coexpression of a mutant receptor defective in hormone binding and another mutant defective in signal generation should be undertaken, which may provide more evidence for the activation mechanism.

Wild type human LH receptors translocate into rafts

After isopycnic centrifugation of plasma membrane fractions from CHO cells expressing FLAG tagged wild type rat or human LH receptors, unoccupied LH receptors were found in membranes with higher densities. Following treatment of cells with 100nM hCG, there was a shift in the distribution of receptors in various sucrose fractions with the consistent appearance of receptors in lower density fractions (rafts). However, CHO cells expressing FLAG-LHR-K583R treated with hCG or FLAG-LHR-wt cells treated with deglycosylated hCG had LH receptors remained associated with higher density membrane fractions despite the presence of bound ligand and despite overexposure of western blots probed with anti-FLAG antibody. In addition, constitutively active human LH receptors showed association with lower density membrane fractions before and after binding of hCG. As expected, MβCD treatment shifted the LH receptors to lower buoyancy membrane fractions, which was consistent with disruption of membrane rafts. Interestingly, extensive crosslinking of FLAG-LHR-wt receptors with anti-FLAG antibody and a second anti-mouse IgG, while elevating intracellular cAMP, did not drive LH receptors into the raft environment.

The above results still did not tell us if receptors associate before or after entering rafts and the effects this has on signal transduction. However, considering that the exclusion of LHR-K583R from rafts could be related to the mutant's inability to self-associate and disruption of rafts did not eliminate receptors self-association

completely, we can speculate that self-association might be a preliminary step for entry into rafts. In addition, rafts are believed to be important as a "relay station" in intracellular signaling. In fact, signaling proteins were some of the first proteins to be isolated from lipid raft domains. Recently, G proteins, tyrosine kinase signaling proteins and β-arrestin have been found associated with lipid rafts. To study further the role lipid rafts play in signal transduction, immunoprecipitation studies could be undertaken. Immunoprecipitation of the receptors could allow us to confirm that LH receptors and signaling proteins are co-localized to lipid rafts. We can also check to see which signaling proteins are associated with LH receptors such as β-arrestin. Since they may not be essential to cAMP-mediated signaling, lipid rafts could be more involved in other functions such as desensitization.

Role of LH receptor C-terminus in membrane localization and receptor aggregation

The LH receptor C-terminus has been implicated in modulation of receptor function and turnover. Other evidence suggests that the C-terminus of the LH receptor may also affect localization of the receptor in membrane microdomains. To investigate whether the LH receptor C-terminus plays a role in raft localization and whether the C-terminus palmitoylation is important, we constructed three kinds of chimeric receptors (GnRHR-LHR-C_{full}, GnRHR-LHR-C_{t631} and GnRHR-LHR-$C_{C621,622S}$), and a mutated rat LH receptor (LHR-C621,622S). Compared with that

GnRH receptors are constitutively localized in membrane microdomains (rafts), none of those chimeric receptors were associated with rafts except very small amount of GnRHR-LHR-C_{t631}. LHR-C621,622S also lost its ability to translocate into rafts upon hormone binding. This data suggests that the LH receptor C-terminus and its palmitoylation are important for raft localization. On the other hand, spot FPR experiments showed that GnRHR-LHR-C_{full} and GnRHR-LHR-C_{t631} receptors are less immobilized than wild type GnRH receptors. We can speculate that LH receptor C-terminus may also play a role in receptor aggregation.

In conclusion, these studies have moved us towards a better understanding of the interactions of LH receptors in the plasma membrane. The sequence of events and mechanisms for self-association of LH receptors upon hormone binding and their movement into lipid rafts is still not understood. However, it can now be definitively stated that wild type LH receptors, if occupied by a functional ligand, are self-associated and capable of moving into specialized, low density plasma membrane microdomains (rafts). The translocation of LHR appears to be characteristic of the functional hormone-receptor complex and is not observed when either the LHR is non-functional or when a hormone antagonist has bound to the wild type receptor. Translocation of receptors into rafts is also implicated in receptor-mediated signaling as demonstrated by the decrease in hCG-mediated cellular levels of cAMP when rafts were disrupted. In the contrast, constitutively-active human LHR are self-associated in the absence of hormone with a significant fraction of the receptors localized in rafts. Treatment of constitutively-active human LHR with hCG does not significantly

increase receptor self-association or raft localization. And signaling by constitutively active receptors is not affected by raft disruption. The LH receptor C-terminus may affect raft localization and the palmitoylation of C-terminus is also very important. These findings will present numerous research opportunities to investigate how signaling in lipid rafts might be involved from gonadal cancers to birth control mechanisms.

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LIST OF ABBREVIATIONS

LH:	Luteinizing hormone
GnRH:	Gonadotropin releasing hormone
hCG:	Human chorionic gonadotropin
cAMP:	Cyclic adenosine monophosphate
FMPP:	Familial male-limited precocious puberty
FSH:	Follicle stimulating hormone or follitropin
TSH:	thyroid stimulating hormone or thyrotropin
$FSHR_{HB}$:	Hormone-binding domain of follitropin receptor
GPCR:	G protein-coupled receptor
EC:	Extracellular domain
rLHR:	Rat luteinizing hormone receptor
hLHR:	Human luteinizing hormone receptor
TM:	Transmembrane domain
LRD:	Leucine-rich repeat domain
GTP:	Guanosine triphosphate
GDP:	Guanosine diphosphate
PKA:	Protein kinase A

PKC:	Protein kinase C
DAG:	Diacylglycerol
AC:	Adenylate cyclase
ATP:	Adenosine triphosphate
PI:	Phosphoinostitide
PLC:	Phospholipase C
PIP_2:	Phosphatidylinositol biphosphate
IP_3:	Inositol 1,4,5-triphosphate
β-AR:	β-adrenergic receptor
GRK:	G protein-coupled receptor kinase
GPI:	Glycosylphosphatidylinositol
MβCD:	Methyl-β cyclodextrin
TPA:	Time-resolved phosphorescence anisotropy
FPR:	Fluorescence photobleaching recovery
FRET:	Fluorescence resonance energy transfer
CFP:	Cyan fluorescent protein
GFP:	Green fluorescent protein
YFP:	Yellow fluorescent protein
CHO:	Chinese hamster ovary
G418:	Geneticin
MHC II:	Major histocompatibility complex class II
HLA:	Human leukocyte antigen