DISSERTATION

ORGANIZATION OF RECEPTORS FOR LUTEINIZING HORMONE AND GONADOTROPIN RELEASING HORMONE DURING SIGNAL TRANSDUCTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY REGINA D. HORVAT ENTITLED ORGANIZATION OF RECEPTORS FOR LUTEINIZING HORMONE AND GONADOTROPIN RELEASING HORMONE DURING SIGNAL TRANSDUCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ORGANIZATION OF RECEPTORS FOR LUTEINIZING HORMONE AND GONADOTROPIN RELEASING HORMONE DURING SIGNAL TRANSDUCTION

Mechanisms involved in signal transduction by the luteinizing hormone (LH) and gonadotropin releasing hormone (GnRH) receptors are of considerable interest because of their importance in mammalian reproduction. Studying the organization of these receptors in the plasma membrane during signaling provides insight into protein interactions during a signaling response. This organization can be probed using biophysical methods.

Using fluorescence energy transfer and fluorescence photobleaching recovery techniques, we have shown that LH receptors are found self-associated within slowly diffusing complexes whose size depends on whether LH or human chorionic gonadotropin (hCG) occupies the receptor. We have also created an intrinsically-fluorescent and functional LH receptor and measured its lateral diffusion in the absence and presence of ligand. Prior to binding of ligand the fluorescent receptor existed as a membrane protein whose diffusion coefficient was about $16 \pm 3.5 \times 10^{-10} cm^2 sec^{-1}$. Upon binding of either LH or hCG, the rate of receptor lateral diffusion slowed and the fraction of mobile receptors decreased. Protein complexes containing the LH receptor formed in response to ligand

binding were stable in the membrane while the receptor was desensitized. Non-responsive LH receptors remained self-associated and present in protein complexes. When receptors were again hormone-responsive, they exhibited fast lateral diffusion and were diffusely distributed in the membrane. Thus complexes that form in response to hormone binding must dissociate before the receptor can again respond to ligand.

We also examined whether another G protein-coupled receptor, the GnRH receptor, self-associated in response to ligand binding. Lateral diffusion of an intrinsically-fluorescent GnRH receptor depended on the concentration of the applied ligand. However, only upon the addition of an agonist did we see a dose-dependent decrease in fraction of mobile receptors which was accompanied by an increase in the energy transfer efficiency between agonist-occupied GnRH receptors. From these data we have postulated a mechanism for LH receptor and GnRH receptor activation as well as a mechanism for LH receptor desensitization, each which involve the formation of protein complexes containing receptors that are self-associated.

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To my parents,
Helen and Donald Horvat

TABLE OF CONTENTS

CHAPTER FIVE

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

BACKGROUND HISTORY

INTRODUCTION

THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Reproduction in mammalian species is regulated very tightly by a number of hormones including luteinizing hormone (LH), a pituitary gonadotropin. In males, LH regulates the development and function of Leydig cells. In females, LH regulates follicular development by acting on theca cells to produce androgen precursors necessary for estrogen synthesis and on follicles to promote ovulation. Although relatively low, pulsatile levels of LH are characteristic of much of the estrous cycle, a mid-cycle surge of LH, characterized by $\geq$ 10-fold increase in serum LH levels, is necessary to induce ovulation. After ovulation, a glandular tissue forms from the ruptured follicle and is called the corpus luteum due to its yellowish color. The corpus luteum secretes the hormone progesterone, which maintains uterine wall during the luteal phase of the estrous cycle and in early pregnancy.

LH synthesis and secretion are regulated by the hypothalamus. In response to neural (Conn *et al.*, 1987) as well as endocrine stimuli (Conn *et al.*, 1987), the hypothalamus releases the deca-peptide hormone Gonadotropin Releasing Hormone (GnRH) into the hypophysial portal circulation. GnRH binds the GnRH receptor located on the gonadotrophs in the anterior pituitary (Conn *et al.*, 1987). Hormone binding to the GnRH receptor initiates both the synthesis and release of LH (Conn *et al.*, 1987) 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 regulation of tissue-specific sex steroid synthesis and release. Estrogen, progesterone, and androgens, are synthesized in the gonads in response to LH. Besides the effects on gametogenesis and secondary sex characteristics (Conn *et al.*, 1987), these steroids also have a negative effect on the release of the gonadotropins from the anterior pituitary. Steroids directly suppress the release of LH at the level of the anterior pituitary and indirectly affect LH levels by decreasing GnRH release from the hypothalamus.

STRUCTURE OF LH AND hCG

LH is a member of the glycoprotein hormone family that includes follicle-stimulating hormone (FSH) and thyroid-stimulation hormone (TSH). These pituitary glycoproteins consist of an α- and β-subunit which interact non-covalently to form a heterodimer (Li and Starman, 1964). In a given species the α-subunits of LH, FSH and TSH are identical, consisting of a polypeptide chain with 92-96 amino acids containing 10 half-cysteine residues that form 5 disulfide bridges (Combarnous, 1992). The β-subunits are unique for

each hormone and are important in binding specificity by the respective receptors. The β-subunits vary in size. LH has one of the larger β-subunits containing 117-121 residues (Combarnous, 1992). All of the β-subunits have 12 cysteine residues at highly conserved positions which form six disulfide bridges (Combarnous, 1992).

Upon association of the α- and β-subunits, a conformational change occurs that 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).

Another membrane of the glycoprotein hormone family containing LH and FSH, is human chorionic gonadotropin (hCG) which is synthesized by the placenta of the human female. This hormone binds LH receptors with high-affinity. Like LH and FSH, hCG consists of a common α subunit and a hormone-specific β subunit. The first 114 amino acids of LH and hCG are 85% homologous, but hCG differs from LH in that it has a 20 amino acid C-terminal extension (Pierce and Parsons, 1981; Sairam and Manjunath, 1983).

The glycoprotein hormones contain oligosaccharides. These sugars participate in receptor binding, secretion, intracellular stability and half-life in circulation (Petäjä-Repo *et al.*, 1991). There are up to four N-linked and three O-linked oligosaccharides representing

18-45% of the total hormone weight (Combarnous, 1992). The α subunit contains two sites for N-linked glycosylation (Bahl and Moyle, 1978) as does the β subunit of LH. Interestingly, 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). When LH and hCG are deglycoslyated, they bind to the LH receptor with higher affinity but activation of adenylyl cyclase by the LH receptor and the subsequent steroidogenesis is significantly decreased or eliminated altogether (Matzuk *et al.*, 1989).

SIGNALING BY LH AND GnRH RECEPTORS

Receptors for LH and GnRH are part of G protein-coupled receptor (GPCR) superfamily, mediating their actions through the activation of G proteins and downstream effectors like adenylyl cyclase (AC) and phospholipase C (PLC) (Spiegel, 1998; Conn *et al.*, 1995; Dohlman *et al.*, 1991; Kaiser *et al.*, 1997; Loosfelt *et al.*, 1989). The GPCR share common structural features. They all have an extracellular domain which contains ligand-binding sites, seven membrane-spanning α helical transmembrane (TM) domains that are connected by alternating extracellular (1e, 2e, 3e) and intracellular loops (1i, 2i, 3i), and an intracellular cytoplasmic tail (Probst *et al.*, 1992). Portions of 3i and the sixth transmembrane region (TM6) are thought to be important for G-protein coupling for most GPCR's (Gether, 2000) including the LH receptor (Abell and Segaloff, 1997) and the GnRH receptor (Schulz *et al.*, 1999). It is believed that agonist binding to GPCR results in a conformational change of the ligand binding domain. This causes secondary contacts with the extracellular loop regions of the receptor and thus, receptor activation (Gether, 2000). Activation of the

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

G proteins consist of 3 subunits: α, β, and γ (Stryer and Bourne, 1986). G proteins exist in an inactive state as a heterotrimer with GDP bound to the α subunit as shown in Figure 1. 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 (Stryer and Bourne, 1986). 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, the α subunit bound to GDP reassociates with the βγ subunit reforming the G protein. This cycle persists as long as agonist is available to bind receptor and as long as the agonist-bound receptor can activate G proteins.

Signaling by the LH receptor involves two distinct signaling pathways, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and the diacylglycerol (DAG)/protein kinase C (PKC) pathway (Spiegel, 1998). Activation of the LH receptor leads primarily to the activation of the G-protein G_s (Dufau, 1998). Following dissociation, the α subunit activates the membrane effector protein 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 protein kinase A (PKA) and cause a release of the catalytic subunits (Yen *et al.*, 1999). The active catalytic subunits of PKA can phosphorylate specific serines and

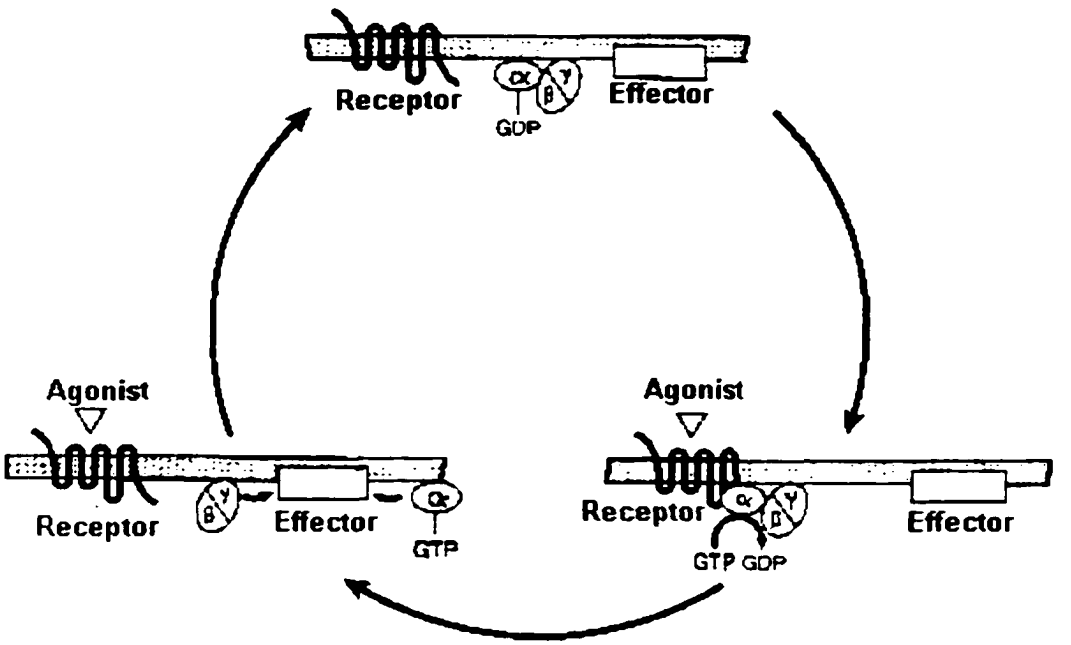

Figure 1. Diagram of the G protein-coupled receptor signaling showing the seven transmembrane domain receptor, the heterotrimeric G protein, the exchange of GDP for GTP upon activation of the G protein, dissociation of the G protein, activation of the effector molecule by the subunits, and the reassembly of the signaling proteins. (Spiegel, 1998)

threonine 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, such as 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4isomerases (3β-HSD) (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 (Dufau, 1998; Gudermann *et al.*, 1992). *In vivo,* this only occurs at the time of the LH surge when receptor density is high. Upon receptor activation the effector molecule phospholipase C (PLC) cleaves membrane phospholipids to inositol 1,4,5 triphosphate (InsP3) and 1,2 diacylglycerol (DAG) (Yen *et al.*, 1999). InsP3 is released into the cytoplasm causing the release of sequestered calcium from the endoplasmic reticulum. DAG remains in the membrane and activates PKC (Yen *et al.*, 1999). The hydrolysis of PI to InsP3 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} (Dufau, 1998).

Primary signaling by the GnRH receptor occurs through the DAG/PKC signaling pathway (Kaiser *et al.*, 1997). Agonist binding to the receptor leads to the activation of the G-proteins G_q/G_{11}. Dissociation of the α-subunits of G_q/G_{11} activates PLC and causes increases in free calcium and activation of PKC. PKC causes increased synthesis of LH β-mRNA and LH. Release of intracellular calcium not only enhances PKC activation but also activates calmodulin which leads to the releases of stored α subunit and LH (Conn *et al.*, 1995).

REGULATION OF SIGNAL TRANSDUCTION

Following signaling, there is a decrease in receptor responsiveness to repetitive or continuous stimulation which is termed receptor desensitization. Although desensitization of the GPCR may involve attenuation of either receptor function, G-protein function, or the downstream effector, 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 the β-adrenergic receptor (β-AR), a member of the GPCR superfamily, 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-dependant kinases as well as by G protein-coupled receptor kinases (GRK's) (Freedman and Lefkowitz, 1996). 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 (Krueger *et al.*, 1997b). Receptor sequestration generally occurs after receptor phosphorylation and may be required for reactivation of GPCR, including receptor dephosphorylation and recycling to the cell surface (Krueger *et al.*, 1997b).

Although desensitization of the LH receptor may be necessary for internalization of the hormone-receptor complex (Amsterdam *et al.*, 1980), desensitization of the LH receptors initially occurs without any change in the number of receptors on the plasma membrane (Amsterdam *et al.*, 1980; Meduri *et al.*, 1996; Rao *et al.*, 19977; Schwall and Erickson, 1984). Desensitization is followed by endocytosis of a number of GPCR's, including β-AR, which utilizes a clathrin-coated vesicle pathway (Krueger *et al.*, 1997a). Interactions between clathrin and the receptor are mediated by arrestins. Recent studies show that arrestin-1 and arrestin-3, but not visual arrestin, are clatherin adaptors and bind clathrin (Goodman *et al.*, 1997). Upon recruitment and binding of β-arrestin to the phosphorylated β-AR, the receptor is sequestered in the clatherin-coated pits. In contrast to these short-term events following agonist-receptor activation, long-term desensitization of GPCR's (> 1 h) results from receptor down-regulation involving a pathway other than that used for receptor recycling.

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 also 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).

Activation of the LH receptor with saturating concentrations of LH or hCG leads to signaling and desensitization. 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 (Hunzicker-Dunn *et al.*, 1996). Both procedures abolished all detectable phosphorylation (Hunzicker-Dunn *et al.*, 1996) but do not affect desensitization (Hunzicker-Dunn *et al.*, 1996). Desensitization was reduced from ~ 85% at 60 min after activation in cells containing the wild type LH receptor to ~ 65% in cells with phosphorylation mutated LH receptor (Wang *et al.*, 1997). Other receptors including the angiotensin type I, serotonin type II, cholecystokinin and secretin receptors (Vouret-Craviari *et al.*, 1995; Roettger *et al.*, 1995) also undergo receptor desensitization that is independent of apparent phosphorylation.

THE ROLE OF RECEPTOR SELF-ASSOCIATION IN SIGNALING

It has been well established that receptor dimerization is required for signaling by many protein tyrosine kinase receptors including a number of growth factor receptors. Ligand-induced receptor dimerization increases the number of the kinase domains on the intracellular tail leading to more efficient transphosphorylation of tyrosine residues of adjoining receptor (Weiss and Schlessinger, 1998).

Dimerization of several G protein-coupled receptors, including the β_2-adrenergic receptor (Hebert *et al.*, 1996) and the M3 muscarinic receptor (Maggio *et al.*, 1993), also appears to be obligatory for ligand-mediated signal transduction. In addition, non-functional β_2-adrenergic receptors can be rescued via dimerization (Hebert *et al.*, 1996). Although this

dimerization event is poorly understood, a dimerization motif, $\underline{L}_{46}$RCP$\underline{G}$PRA$\underline{G}$LAR$\underline{L}_{58}$, with the critical residues underlined, has been proposed (Lemmon *et al.*, 1992). This sequence has been found in the sixth transmembrane domain (TM6) of the β_2-adrenergic receptor and is believed to be necessary for receptor-receptor interactions (Hebert *et al.*, 1998). When chimeric receptors formed from the α_{2c}-adrenergic receptor and the M3 muscarinic receptor contain this domain, they will dimerize (Maggio *et al.*, 1993). Moreover, incubation of receptor-expressing cells with a 20 amino acid peptide containing the TM6 sequence inhibits β_2-adrenergic receptor dimerization and blocks ligand-mediated signal transduction by the receptor (Hebert *et al.*, 1998).

It is not known whether signal transduction by the LH receptor or the GnRH receptor involves receptor dimerization. Electron microscopy studies by Luborsky (Luborsky *et al.*, 1984) demonstrate extensive association of LH receptors into clusters following exposure of rat luteal cells to high concentrations of ovine LH. Similarly, immunofluorescence studies of the LH receptor on rat granulosa cells demonstrates the presence of large, punctate structures on the cell membrane following ligand binding (Amsterdam *et al.*, 1980). In time-resolved phosphorescence anisotropy 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 physical size of receptor-containing complexes may be indicative of the receptor's response to hormone binding. Functional hormone-receptor complexes, *i.e.*, those capable of activating adenylyl cyclase, exhibit significantly slower rotational dynamics than do complexes formed by hormone binding to non-functional receptors or by a non-functional ligand binding

to a normally functioning receptor (Roess *et al.*, 2000a). These several lines of evidence suggest that LH receptors exist as protein aggregates containing multiple copies of the receptor, however, it does not demonstrate that receptors are found as receptor dimers or oligomers.

Dimerization of the GnRH receptor has long been thought to be an early event in signal transduction (Conn *et al.*, 1982a and b). Early studies focusing on GnRH receptor signaling have concentrated on the possibility that the GnRH receptor must self-associate to transduce a second messenger signal. However, the GnRH receptor lacks the dimerization sequence proposed for β-AR within its TM6 domain. In early fluorescent microscopy studies using fluorescent-GnRH, the receptor could be seen to undergo patching, capping and internalization (Hazum *et al.*, 1980). Studies by Conn and coworkers have suggested that the molecular weight of the GnRHR when unbound is about 60 kDa but when bound by agonist increases to 136 kDa as determined by radiation inactivation (Conn and Venter, 1985). Using receptor iodination it was determined that this increase is possibly due to the incorporation of receptors into protein complexes that contain at least one other receptor (Janovick and Conn, 1996a). Further evidence that the GnRHR must undergo self-association to activate a signaling response comes from physically crosslinking multiple receptors. Binding of a single antagonist to the GnRHR does not elicit a signaling response nor does a 12-15 Å GnRH-antagonist dimer. However, if the distance between the two antagonists is increased to a distance of 150 Å, allowing for the crosslinking of two receptors, the antagonists can be converted to agonist and thus the formation of a dimer is sufficient to stimulate a response (Conn *et al.*, 1982a).

ORGANIZATION OF PROTEINS IN THE PLASMA MEMBRANE

Membrane proteins, such as the LH or GnRH receptors, exhibit lateral motions in the plane of the membrane 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, as shown in Figure 2, will affect movement of a given protein within the membrane.

To understand protein motions in the membrane, it is helpful to examine plasma membrane structure. The plasma membrane of a cell defines the boundaries between extracellular and intracellular by controlling the movement of materials into and out of the cell. In 1972, Singer and Nicolson proposed a model for the organization of the plasma membrane which they called the fluid mosaic model (Singer and Nicolson, 1972). The fluid mosaic model describes the plasma membrane as a two dimensional matrix of proteins organized within a sea of lipids.

The lipid bilayer is formed from membrane lipids which are generally amphipathic. One end of the lipid contains a polar group, such as a phosphate, amine, or alcohol, while the remainder of the molecule is hydrophobic with long hydrocarbon chains (Singer and Nicolson, 1972). Lipids are organized in an aqueous environment according to hydrophobic effects (Gorter and Grendel, 1925). The polar group found at the head of the lipid allows for the

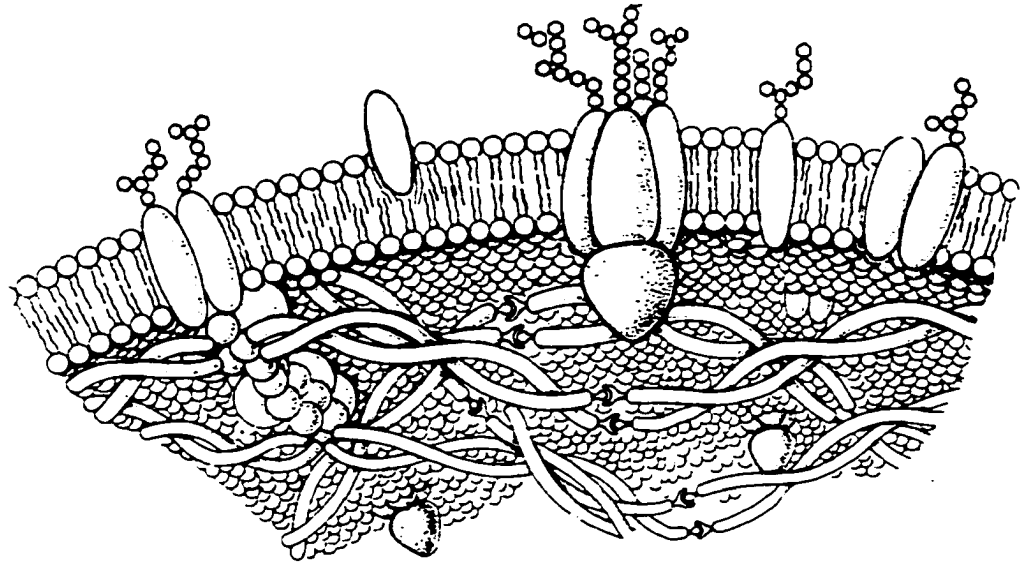

Figure 2. Diagram of the plasma membrane showing protein organization in the phospholipid bilayer. Modified from Stryer, 1988.

hydrophilic interactions with the extracellular as well as intracellular environments, while the lipid tails pack tightly to form the hydrophobic environment of the intra-membrane space of the bilayer.

Within the lipid bilayer are integral and peripheral proteins. Peripheral proteins interact with the membrane via weak non-covalent interactions (Singer and Nicolson, 1972). These proteins are easily extracted from the membrane using mild treatments, such as an increase in ionic strength, and, once dissociated from the lipid bilayer, they are relatively soluble in aqueous buffers. These proteins are generally not associated with the hydrophobic regions of the bilayer (Singer and Nicolson, 1972). One example of a peripheral membrane protein is cytochrome *c* which is associated with the inner mitochondrial membrane.

Integral membrane proteins represent 70-80% of membrane proteins (Singer, 1974) with a smaller percentage actually spanning the entire lipid bilayer. Proteins that span the entire bilayer can serve as receptors, junctions and connections between cells. Integral membrane proteins are much harder to extract from the membrane. Removing them from the lipid bilayer requires harsher methods such as detergent extraction. Freeze fracture electron microscopy and spectroscopic observations indicated that many proteins were found embedded within the leaflet of the membrane and generally existed as globular structures containing numerous α-helices.

MEASURING LATERAL DIFFUSION OF MEMBRANE PROTEINS

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. Microspectrophotometery was used in 1974 to determine the rate of lateral diffusion of rhodopsin, a photosensitive integral membrane protein found on frog optical disk membranes and mudpuppy visual rods (Poo and Cone, 1974). Poo and Cone using microspectrophotometery were able to determined the rate of lateral diffusion for rhodopsin using equation 1,

$$D = (0.69bL^2)/\pi^2 t_{1/2} \quad (1)$$

where L is the diameter of the disk membrane, b is a factor to account for the irregular disk boundary, and t1/2 is the half-time in which the pigment distribution becomes uniform. Using this microspectrophotometery, the measured rate of rhodopsin lateral diffusion was calculated to be $3.5 \times 10^{-9} cm^2 sec^{-1}$ in frog optical disk membranes and $3.9 \times 10^{-9} cm^2 sec^{-1}$ in mudpuppy visual rods. The calculated diffusion coefficient was approximately the same as that for a 50 kDa protein in oil with a viscosity of 1-6 poise. Unfortunately, microspectrophotometry was useful only for membranes that contain high concentrations of photosensitive molecules such as 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 ghost 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 from the bleached and unbleached portions of the membrane was monitored over time. The rate of lateral diffusion for Band 3 was calculated to be $3.0 \times 10^{-12} cm^2 sec^{-1}$ (Poo and Cone, 1974), 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 (Jacobson *et al.*, 1976), and Axelrod (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 3). Briefly, a low intensity 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 fluorescence intensity ($F_{(i)}$). The fluorophores in this spot are then 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 $F_{(t)}$.

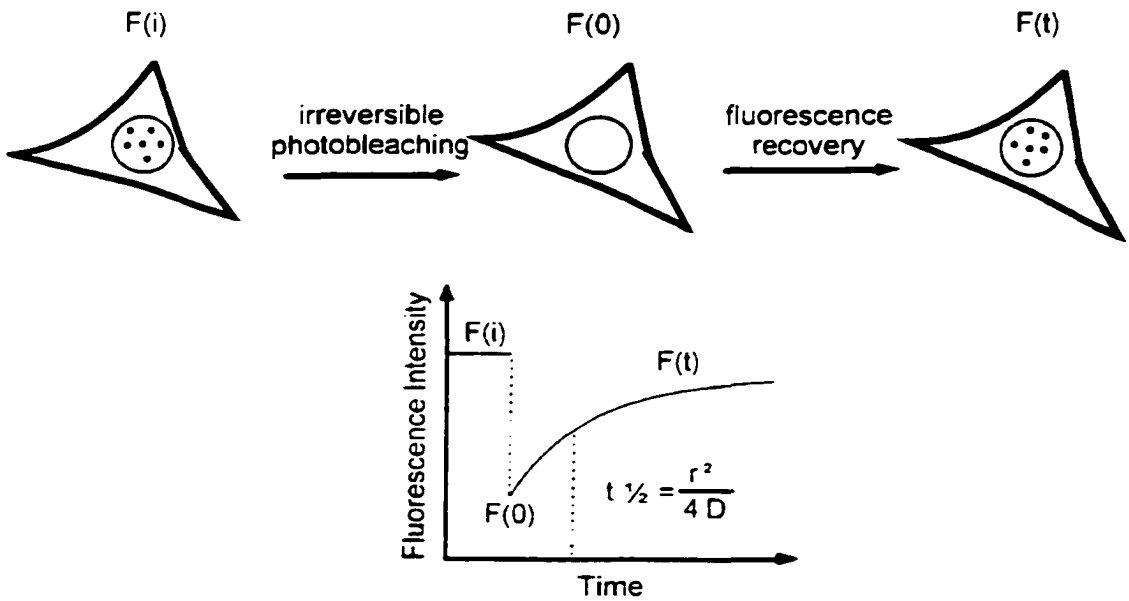

Figure 3. Schematic of spot fluorescence photobleaching recovery method and a representative data trace. The diffusion coefficient D is related to the fluorescence recovery half-time $t_{1/2}$.

From this data trace two key pieces of information are calculated, first the fractional fluorescence recovery $f_k(t)$ given as equation 2,

$$f_k(t) = [F(t)- F(0)] / [F(\infty)-F(0)] \quad (2)$$

where $F(\infty)$ is the fluorescence intensity at very 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 in Equation 3,

$$F_{(0)} = (qP_0C_0/AK)(1-e^{-K}) \quad (3)$$

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 equation 3 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 Equation 4,

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

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.

There are, however, limitations to traditional spot FPR. Because of the small beam interrogation area, the fluorescent signal to noise ratios can be very low. To increase the size of the interrogation area a number of methods have been proposed including using a Ronchi ruling pattern for photobleaching or, alternatively, using an interference pattern for photobleaching. McConnell and coworkers have used a Ronchi ruling to generate a two-dimensional fringe pattern focused onto a flat sample and measured the diffusion of fluorescent molecules from the dark areas to the light areas (Smith and McConnell, 1978; Smith *et al.*, 1981). Due to the two-dimensionality of the generated pattern, the Ronchi ruling was not appropriate for measuring diffusion of molecules found on the membrane of spherical cells.

Our laboratory has developed a method for measuring lateral diffusion of receptors on the entire surface of a single cell (Barisas *et al.*, 1997). An interference fringe pattern is generated within the optical path of a conventional FPR instrument as shown in Figure 4. Figure 5 shows the optical components involved in the generation of the interference pattern. The interferometer contains 5 optical mirrors and one cubic beam splitter to produce two identical beams that will intersect at a distance d and the optical paths of the two beams are superimposed. Due to the small angle of intersection θ, about 3mrad, of the two beams, θ can be calculated as $\theta = x/d$, where x is the beam spacing at the interferometer. Knowing θ, the distance between the generated fringes (s) can be calculated with Equation 5,

$$S = \lambda/m t\theta \qquad (5)$$

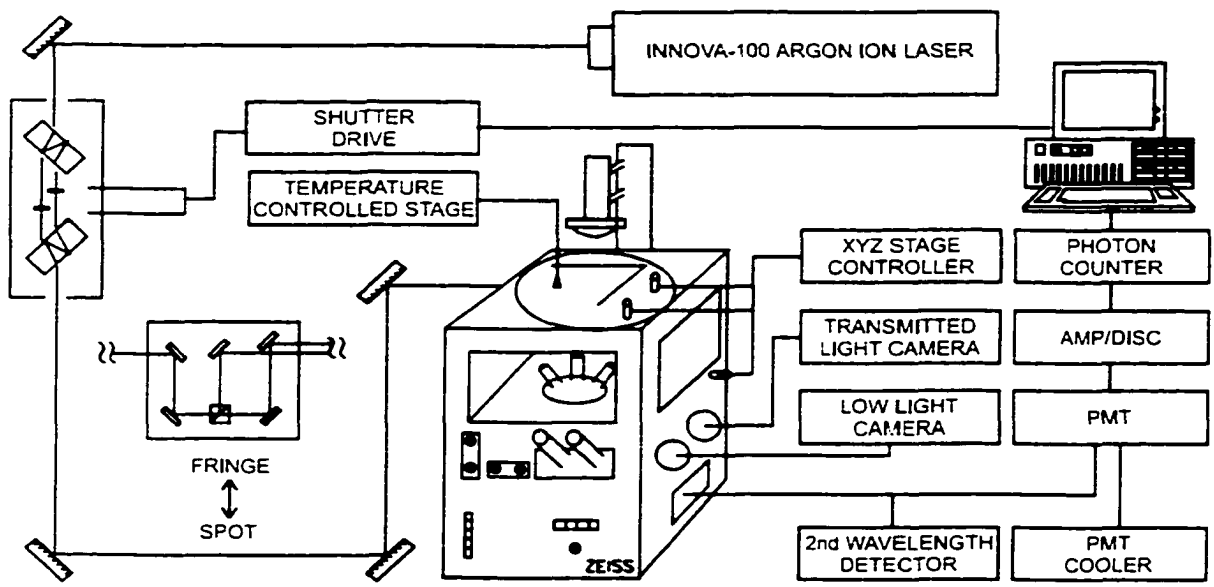

Figure 4. Diagram of the instrumentation used for spot and fringe fluorescence photobleaching recovery measurements.

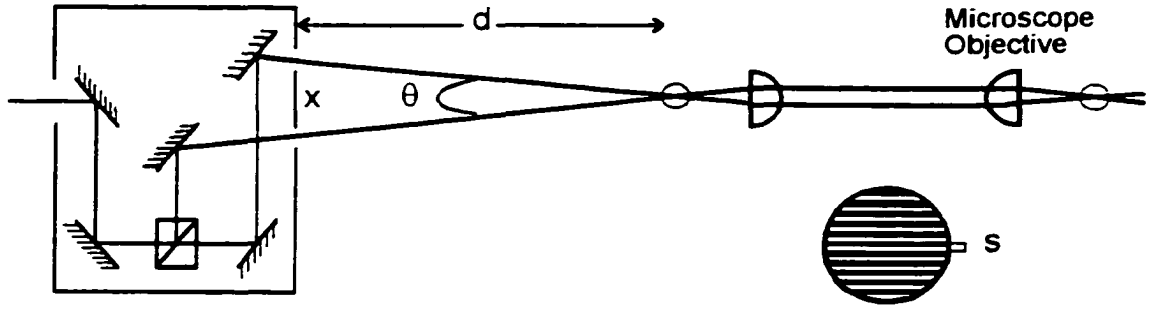

Figure 5. The interferometer used for fringe pattern generation. Consists of five mirrors and one cubic beam splitter. Fringe spacing s can be determined using the distance from interferometer to beam crossing d, beam spacing at the interferometer x, wavelength λ, and the angle of beam intersection θ.

where, λ is the wavelength of the laser light, m is the power of the microscope objective and t is the laser tube factor. Shown in Figure 6 is a schematic representation of interference fringe FPR. Fluorescent tagged molecules found within the illuminated regions are irreversibly photobleached with the high powered bleaching pulse. Following the bleaching pulse, fluorophores found within the darkened areas can diffuse into the illuminated areas. This technique interrogates the entire cell surface and therefore can be performed on individual round cells or flat planar samples.

MEASURING FLUORESCENCE ENERGY TRANSFER BETWEEN SELF-ASSOCIATED RECEPTORS

Fluorescence resonance energy transfer can be used to evaluate the proximity of membrane proteins. Förster proposed the theory for fluorescence energy transfer in1948. He proposed that energy could be transferred between dipole-dipole pairs where the rate of energy transfer was dependent on the inverse of the sixth power of the distance between the donor and acceptor pair. Fluorescent donor and acceptor pairs are chosen such that the donor emission spectra overlaps the excitation spectra of the acceptor. The most commonly used pair of donor and acceptor molecules is fluorescein and rhodamine respectively. Fluorescein is maximally excited by light at 488nm and emits maximally at 518nm. If rhodamine is within approximately 100 Å it can be excited by the energy transferred from the fluorescein and will emit light maximally at 580nm.

Traditional energy transfer methods monitor the fluorescence emitted by the acceptor

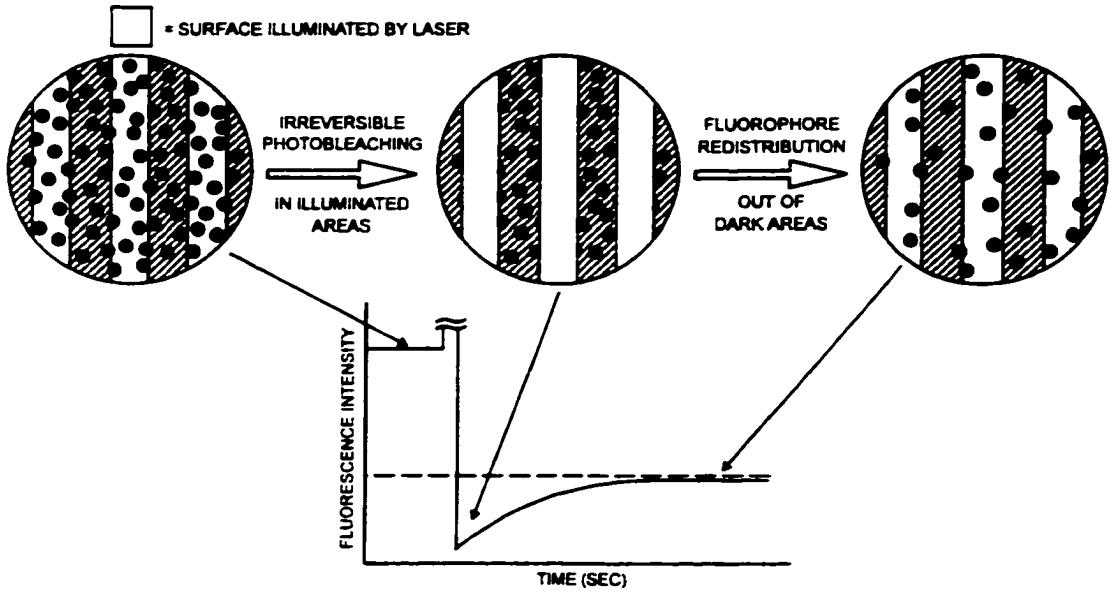

Figure 6. Schematic of the fringe fluorescence photobleaching recovery method and representation of a recovery trace. The large circle represents a cell interrogated by an interference pattern of alternating light and dark areas. The small darkened circles represent fluorescently-labeled membrane proteins which can diffuse from the darkened areas into the illuminated areas.

molecule following excitation of the donor molecule. We have developed a new microscopic method 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. For fluorescein and rhodamine, the distance at which 50% of the fluorescein fluorescence is quenched by rhodamine is 56Å (Chan *et al.*, 1979).

In our experiments, measurements are made on cellular proteins labeled with donor only and proteins labeled with donor and acceptor present simultaneously in a ratio of one donor molecule to three acceptor molecules (Young *et al.*, 1994). Photobleaching of the donor molecule is accomplished by irradiating the samples with a high powered beam at the maximal excitation wavelength, 488nm for fluorescein. The rate of irreversible photobleaching of the fluorescent donor is measured on the samples labeled with donor alone (k_D) and on samples labeled with donor plus acceptor ($k_{D\text{-}A}$). Fluorescence energy transfer provides a competing deactivation pathway for the donor excited states so that irreversible photobleaching is slowed in the presence of an acceptor molecule. Energy transfer will only occur if the donor and acceptor molecules are within a close proximity. Percent of energy transfer efficiency %E can be calculated from the rate constants of photobleaching using equation 6.

$$\%E = [1-(k_{D\text{-}A}/k_D)] \times 100 \qquad (6)$$

A diagram of the microscopic energy transfer method is shown in Figure 7. The microscopic energy transfer method provides greater sensitivity and enhanced signal to noise ratio (Matko *et al.*, 1993; Young *et al.*, 1994).

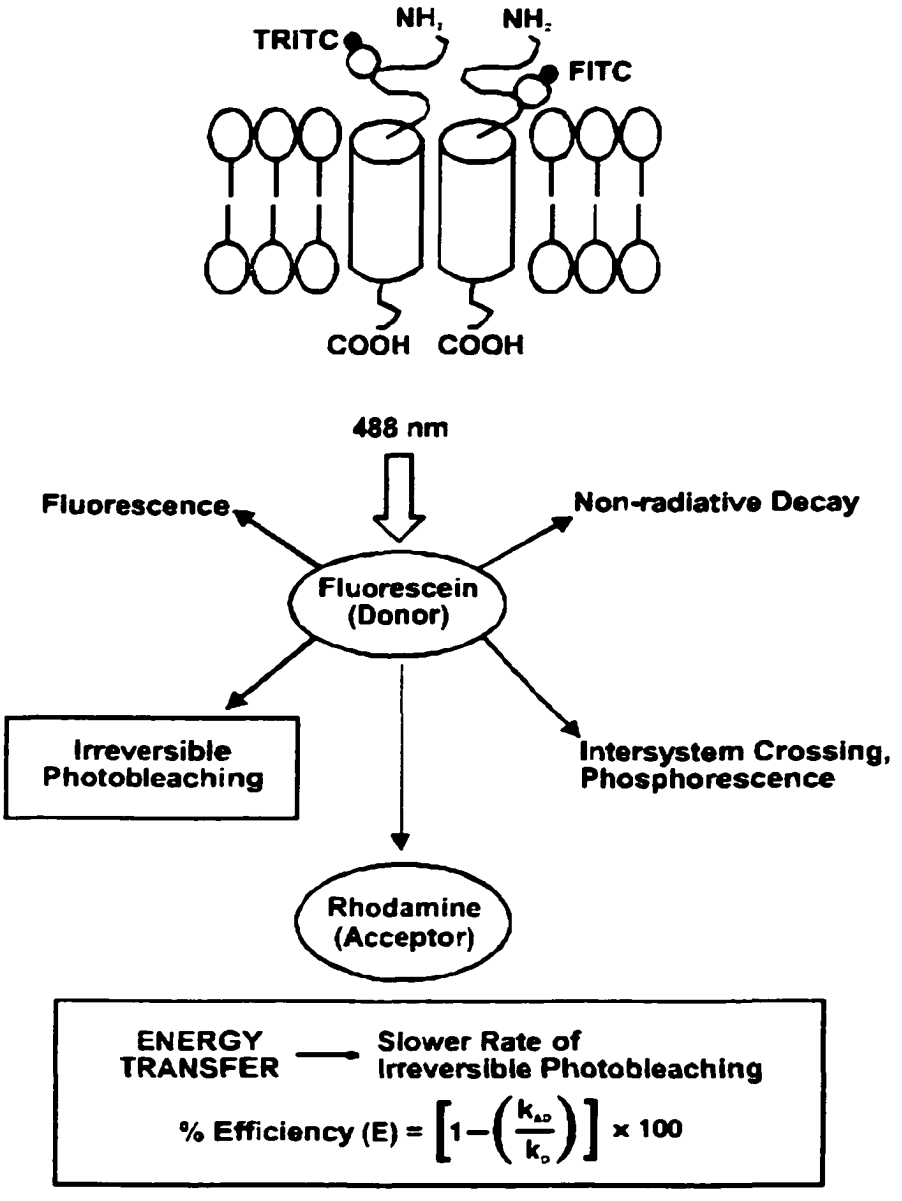

Figure 7. Diagram of fluorescence energy transfer. The fluorescence donor molecule, fluorescein, is excited by 488nm light. Excited electrons in fluorescein can return to the ground state through several pathways including irreversible photobleaching (k_D). If an acceptor molecule is within about 100Å of fluorescein, a competing pathway is introduced and the rate of irreversible photobleaching will slow (k_{D-A}). Percent energy transfer efficiency can be determined from rates of photobleaching using the equation above.

SUMMARY AND GOALS OF THIS RESEARCH

Here we have used biophysical methods to examine the organization of the LH and GnRH receptors at times when these receptors are hormone responsive and at times when the LH receptor is incapable of transducing a second messenger response. Our basic hypothesis is that prior to binding of ligand both the LH and GnRH receptors exist as isolated membrane proteins. Binding of ligand to either receptor leads to dimerization or oligomerization of the receptor. Following receptor self-association the receptors may undergo interactions with peripheral proteins necessary for the activation of a G-proteins and in the case of LH receptors, receptor desensitization. The interactions between receptors and non-receptors proteins can be effectively determined by examining the lateral diffusion of the receptors using fluorescence photobleaching recovery (FPR) and fluorescence energy transfer (FET) to examine receptor-receptor association.

Submitted to *Endocrinology*, January 2000

CHAPTER TWO

LUTEINIZING HORMONE RECEPTORS ARE SELF-ASSOCIATED IN THE PLASMA MEMBRANE

INTRODUCTION

The nature of luteinizing hormone (LH) receptor interactions with other membrane proteins during signal transduction is not well understood. Several lines of evidence suggest that LH receptors exist in discrete complexes in the plasma membrane following hormone binding. Elegant electron microscopy studies by Luborsky *et al.* (Luborsky *et al.*, 1984) demonstrate that extensive association of LH receptors into clusters containing multiple copies of the LH receptor occurs following exposure of rat luteal cells to high concentrations of ovine LH. Similarly, immunofluorescence studies of the LH receptor on rat granulosa cells demonstrates the presence of large, punctate structures on the cell membrane following ligand binding (Amsterdam *et al.*, 1980). In time-resolved phosphorescence anisotropy 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 physical size of receptor-containing complexes may be indicative of the receptor's response to hormone binding: functional hormone-receptor complexes, *i.e.* those capable of activating adenylate cyclase, exhibit significantly slower rotational dynamics than do complexes formed by hormone binding to non-functional receptors or by a non-functional ligand binding to a normally functioning receptor (Roess *et al.*, 2000a).

Nonetheless, the question of whether liganded LH receptors are intimately self-associated, forming receptor dimers or oligomers following binding of hormone, has not been resolved. Such interactions have been suggested by electron microscopy studies of this receptor which show grouping of hormone-conjugated ferritin molecules. However, the diameter of ferritin molecules used to image LH receptors is about 24 nm (Luborsky *et al.*, 1984), approximately 3-fold greater than the diameter of the hormone itself (Anderson *et al.*, 1979). Thus these studies fail to distinguish actual receptor oligomerization from simple concentration of receptors into small membrane microdomains. Similarly light microscopy results showing fluorescent macroscopic clusters containing LH receptors (Amsterdam *et al.*, 1980; Podestá *et al.*, 1983) can arise either from receptor oligomers or from restriction of receptors to specific small domains.

Because there is evidence that minimally dimeric structures may be necessary for function of G protein-coupled receptors such as the δ-opioid receptor (Cvejic and Devi, 1997) and β_2-adrenergic receptor (Hebert *et al.*, 1996), the question of whether the functional LH

receptor self-associates is of interest. We have approached this question by first examining the differences in the lateral dynamics of the wild type rat LH receptor and a non-functional LH receptor expressed in human embryonic kidney 293 cells. The non-functional receptor contains a single point mutation in lysine 583 located in the third extracellular loop which is involved in signal transduction (Fernandez and Puett, 1996; Ryu *et al.*, 1996). In cells stably expressing the mutant receptor, the cAMP response to hCG is either eliminated completely [9] or reduced by over 75% (Fernandez and Puett, 1996). Because large, slowly diffusing complexes may contain multiple copies of the LH receptor, we have examined receptor-receptor interactions on individual cells using fluorescence resonance energy transfer techniques (Young *et al.*, 1994). The results presented here suggest that functional, but not non-functional, LH receptors are self-associated and present in slowly diffusing complexes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium was purchased from Irvine Scientific, Santa Ana, CA. Gentamicin and geneticin were purchased from GIBCO, Grand Island, NY. HEPES was purchased from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum was purchased from Summit Biotechnology, Fort Collins, CO. Ovine LH (oLH; NIH 28) and hCG (CR-127) were obtained from the National Hormone and Pituitary Program, NIDDK, Baltimore, MD. Tetramethylrhodamine isothiocyanate (TrITC) and fluorescein isothiocyanate (FITC) were purchased from Molecular Probes, Eugene, OR.

Cell culture

Dr. Tae Ji from the Department of Chemistry at the University of Kentucky kindly provided 293 cells stably transfected with the wild type LH receptor (LHR-wt) or with an LH receptor modified in position 583 by substitution of lysine with arginine (LHR-K583R) (Ryu *et al.*, 1996). Untransfected 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% horse serum (Summit Biotechnology, Ft. Collins, CO), 100 U penicillin, 1000 μg/mL streptomycin and 10 mM HEPES, pH 7.4. LHR-wt cells and LHR-K583R cells were maintained in the same medium supplemented with 400 μg/mL geneticin.

Preparation of TrITC- and FITC-derivatized hormones

Hormones were derivatized with FITC or TrITC using a modification of methods described by Brinkley *et al.* (Brinkley, 1992) and described in detail elsewhere (Young *et al.*, 1994). Briefly, hormones were dissolved in phosphate buffered saline (1.9 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 0.15 M NaCl, PBS) containing 50 mM sodium borate, pH 9.3. Protein concentrations were determined spectrophotometrically at 280 nm. A 5-fold molar excess of TrITC or FITC was added to the protein solutions and the mixtures were kept at 4°C for 18 h in the dark. After quenching with 1 M Tris, the fluorophore-derivatized hormones were separated from the unreacted free dye on a Sephadex G-25 column. After extraction of remaining free dye with n-butanol and extensive dialysis, the molar ratios of dye to hormone were determined spectrophotometrically. Hormone preparations used in these experiments had 1.0-1.5 moles of TrITC or FITC per mole of oLH or hCG. It has been previously shown that

there is no effect of fluorophore conjugation on hormone biological activity (Niswender *et al.*, 1985). Prior to use, all fluorophore-derivatized proteins were centrifuged at 130,000 x g for 10 min in a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA) to remove any protein aggregates that may have formed during storage at 4°C.

Labeling cells with fluorophore-derivatized hormones

Before labeling with TrITC- or FITC-derivatized hormones, cells were incubated in balanced salt solution containing 0.1% NaN_3 at 37°C for 30 min to prevent hormone internalization (Niswender *et al.*, 1985). Typically, 2-5 x 10^6 cells in 1 mL of balanced salt solution were labeled with 1 μM TrITC-derivatized oLH or hCG for each fringe photobleaching recovery experiment. For fluorescence energy transfer measurements, four groups, each containing 1 x 10^6 cells, were labeled and examined. One group of cells was not labeled. The remaining groups were labeled with a total hormone concentration of 2.0μM. Cells were labeled with fluorescent donor alone were incubated with 1.5μM unlabeled hormone, either LH or hCG, and 0.5μM of the same hormone derivatized with FITC. Cells labeled with fluorescence donor and acceptor, were incubated with 1.5μM TrITC- and 0.5μM FITC-derivatized hormone. Cells labeled with fluorescence acceptor were treated with 1.5μM TrITC-derivatized hormone and 0.5μM unlabeled hormone. The 3:1 ratio of fluorescent donor to acceptor has been shown previously to produce optimal signal (Young *et al.*, 1994). Following labeling for 1 hr at 37°C, cells were then washed two times by centrifugation at 300 x g for 3 min in balanced salt solution to remove any unbound ligand. In some lateral diffusion and fluorescence energy transfer experiments, cells were pretreated with 40 μg/mL

cytochalasin D for 30 min at 37°C prior to cell labeling.

Fringe fluorescence photobleaching recovery measurements

The optical system for performing fringe fluorescence photobleaching recovery measurements and the method used for data analysis have been described previously (Munnelly *et al.*, 1998). The microscope objective used in these studies was a Zeiss 40x objective of NA 0.65. Standard Zeiss filter and dichroic mirror sets for FITC and TrITC fluorescence were used. Cells were examined under coverslip on well slides while temperature was maintained by a thermoelectrically cooled/heated thermal stage with a temperature range of 0°C to 40°C. For fringe measurements, the region illuminated at the sample has a $1/e^2$ radius of about 18 μm and the photometer acceptance region is large enough to encompass the entire cell. The fringe spacing used in these experiments was 2.3 μm. Because of the large interrogated area, 1.3 W in the bleaching pulse and 3 mW in the probe beam were used. Unadjusted raw data were represented directly in terms of the various parameters associated with a given measurement including the prebleach and immediate postbleach fluorescence levels, the extent M of fluorophore mobile on the timescale of the experiment, and a function representing the recovery kinetics in terms of a decay half-time. These parameters were evaluated directly by a nonlinear least-squares procedure and, from the measured time $t_{1/2}$ at which fluorescence recovery was half-complete and from the known optical parameters, the desired diffusion coefficient D was evaluated.

Single-cell fluorescence energy transfer

Fluorescence energy transfer between FITC- and TrITC-derivatized LH or hCG was evaluated based on the reduced rate of irreversible photobleaching of FITC fluorophores when TrITC fluorophores were present (Young *et al.*, 1994). Slower rates of fluorescence decay for cells labeled with the FITC fluorescence donor and TrITC fluorescence acceptor (D+A) than for cells labeled with FITC only (D) were indicative of energy transfer from fluorescence donor to acceptor and occurred only when the donor and acceptor were separated by distances less than R_o, a characteristic of the specific donor/acceptor pair. For FITC and TrITC this distance is 56Å (Chan *et al.*, 1979).

To perform these experiments, we used a fluorescence microscope photometer based on the inverted-configuration Zeiss Axiomat microscope and associated components used for fringe fluorescence photobleaching recovery measurements at room temperature. Fluorescence excitation was provided by a Coherent Radiation Innova 100 argon ion laser operating under light control at 488 nm. The intensity of the laser radiation focused on the cell was 15-20 mW and this was held constant between measurements on cells labeled with FITC-derivatized LH or hCG only or on cells labeled with FITC- plus TrITC-derivatized hormone. The $1/e^2$ Gaussian spot diameter was 18 μm. Donor fluorescence from FITC was isolated with a standard fluorescein filter set together with a short pass fluorescein-selective filter to remove red tetramethylrhodamine fluorescence. This combination was highly effective in rejecting TrITC fluorescence: TrITC-LH or -hCG labeled cells gave very low fluorescence signals using the fluorescein-selective filter set which were indistinguishable from those of unlabeled cells.

Signals from cells labeled with either FITC-LH or hCG only or with FITC- and TrITC-LH or hCG were approximately 4-fold higher than background levels. In individual experiments cells were identified and centered in the microscope field. At time zero, an electronically controlled shutter was opened to allow laser radiation to impinge on the cell. Simultaneously, a computer program was activated to record the output of the photomultiplier measuring membrane fluorescence. Data were collected at 0.01 sec intervals for 10 sec. Typically, about 20 cells in each sample were photobleached in this manner. The data traces were analyzed to give the energy transfer efficiency (%E) as has been described in detail previously (Young *et al.*, 1994).

Statistical analysis of data

In photobleaching recovery and fluorescence energy transfer experiments, diffusion coefficients and energy transfer efficiencies were obtained through curve fitting appropriate mathematical models to experimental data sets. These data sets contained hundreds of points and fitting is accomplished using the Marquardt algorithm (Marquardt, 1963). Since each of the many observations in a single measurement provides independent information on the parameter of interest, the standard error of the parameter was calculated at the same time as the fitted parameter itself. However, because any real data set has some systematic deviation from a model representing the parent experiment, these standard errors calculated during the curve fitting procedure almost certainly *overestimate* the reliability of parameters. We thus present the uncertainties of a fitted parameter x as $\langle x \rangle \pm 2s$ where s is the standard error of the mean of a set of three to four complete, independent determinations of x. Uncertainties in quantities

such as percent efficiency of energy transfer which involve parameters obtained in at least three separate experiments were calculated by standard propagation of errors methods. Decisions as to whether parameters differ significantly between multiple treatment groups were made using single classification analysis of variance methods (SigmaStat, Jandel Scientific, San Rafael, CA).

RESULTS

LH receptors are laterally mobile at 37 °C following binding of LH or hCG

We examined the lateral diffusion of the LH receptor expressed on LHR-wt and LHR-K583R cells using fringe photobleaching recovery techniques. The entire surface of cells was interrogated with a interferometrically-generated fringe pattern (Munnelly *et al.*, 1998). This permitted us to obtain lateral diffusion information from a large population of LH receptors in a single measurement. Because 293 cells exhibited low levels of autofluorescence when excited by 514 nm light, we also performed fringe FPR experiments on untreated cells transfected with the appropriate form of the LH receptor, summed and averaged approximately 40 data traces from individual cells and then subtracted the average background signal from individual traces obtained when LH receptors were labeled with TrITC-derivatized hormones. Representative data are shown in Figure 8. Using this protocol, lateral diffusion coefficients D for LH- and hCG-occupied receptors on the various cell types ranged from 2.1-4.5 x $10^{-10}$$cm^2sec^{-1}$ at 37°C (Table I). There was a significant difference between the diffusion coefficients for LH- and hCG-occupied receptors on LHR-wt cells: oLH-occupied

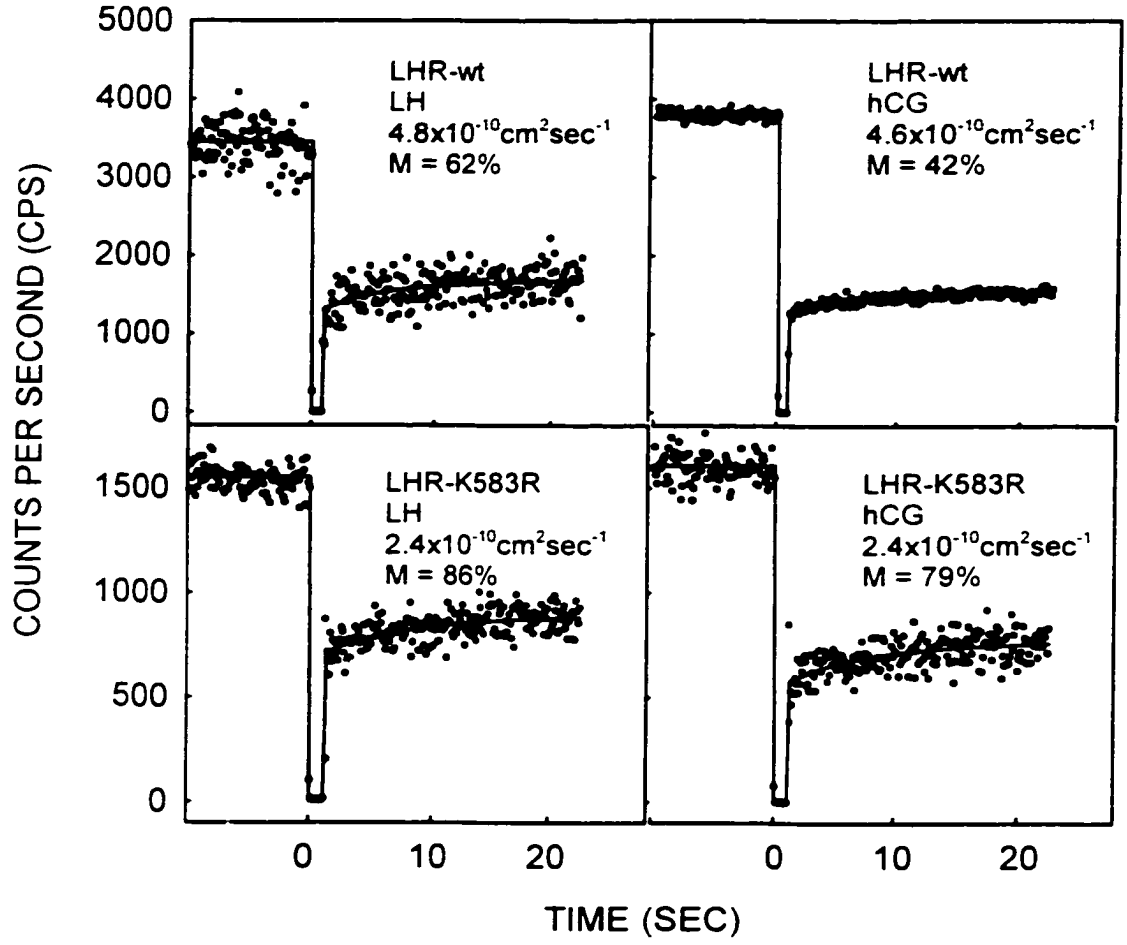

Figure 8. Data traces from fringe fluorescence photobleaching recovery measurements at 37°C following binding of either TrITC-LH or TrITC-hCG to LH receptors on LHR-wt or LHR-K583R cells. The fluorescence contribution due to cellular autofluorescence has been removed from these traces as described in Experimental Procedures.

TABLE 1

LATERAL DIFFUSION COEFFICIENTS (D) AND MOBILE FRACTIONS (%M) FOR LH RECEPTORS ON LHR-wt AND LHR-K583R CELLS AT 37°C

Cells	Ligand	Treatment	D ($10^{-10}cm^2sec^{-1}$)	%M
LHR-wt	oLH	none	3.2 ± 1.0^a	69 ± 13^a
	oLH	CD	4.6 ± 0.5^b	50 ± 5^b
	hCG	none	4.5 ± 1.5^b	43 ± 3^b
	hCG	CD	1.8 ± 0.9^c	66 ± 13^a
LHR-K583R	oLH	none	2.1 ± 0.3^c	94 ± 11^c
	oLH	CD	2.4 ± 1.2^c	95 ± 2^c
	hCG	none	2.8 ± 1.0^{ca}	71 ± 10^a
	hCG	CD	2.1 ± 0.4^c	73 ± 1^a

[1] Diffusion coefficients and mobile fractions and the S.D. associated with these values were calculated from 35-66 measurements on individual cells.

[2] Values for diffusion coefficients and mobile fractions were analyzed by one way ANOVA and means were separated using least significant differences criteria Values for D or for %M with different superscripts were different ($p<0.01$).

receptors on LHR-wt cells had a diffusion coefficient of 3.2±1.0 x $10^{-10}cm^2sec^{-1}$ while that of hCG-occupied receptors was 4.5±1.5 x $10^{-10}cm^2sec^{-1}$. There was no difference between the diffusion coefficients for hormone-occupied receptors on LHR-K583R cells.

Fractions of receptors mobile at 37°C were significantly larger for LH-occupied receptors than for hCG-occupied receptors on LHR-wt and LHR-K583R cells. Following binding of LH to receptors on LHR-wt cells, %M was 69±13. This value decreased to 43 ± 3 following binding of hCG to the wild type receptor. On LHR-K583R cells, %M was 94% and 71% for receptors binding LH and hCG, respectively. After binding either LH or hCG, there were fewer receptors mobile on LHR-wt cells than on LHR-K583R cells.

Differences in the diffusion characteristics for LH receptors on LHR-wt and LHR-K583R cells were not the result of differences in receptor number. Prior to initiating each fluorescence photobleaching recovery experiment on an individual cell, we measured fluorescence counts per second (cps) from the area illuminated by the attenuated argon ion laser. The means and S.D. for counts from 20-40 experiments on ten separate days were 2,934±1,650 cps/cell on LHR-wt cells compared to 3,055±1,726 cps/sec on LHR-K583R cells. These values do not differ significantly.

Disruption of microfilaments increased the fraction of mobile receptors for hCG but not LH-occupied receptors on LHR-wt cells at 37 °C

Cytoskeletal components can affect the motions of LH receptors in some cell systems.

The most pronounced effects on protein motions have been observed with cytochalasin D-treated ovine luteal cells where disruption of microfilaments increased the rate of LH receptor lateral diffusion (Roess *et al.*, 1988) and the fraction of mobile receptors (Roess *et al.*, 1988). On MA-10 cells, cytochalasin D treatment resulted in faster rotational diffusion of the receptor (Roess *et al.*, 1997). To determine whether lower values for the mobile fraction on LHR-wt cells were due to restriction of receptor lateral diffusion by microfilaments, cells were treated with cytochalasin D for 1 hr prior to labeling of cells for fluorescence photobleaching recovery measurements. Cytochalasin D treatment significantly affected the measured rate of receptor lateral diffusion and the fraction of mobile receptors on LHR-wt but not LHR-K583R cells (Table I). Following treatment with cytochalasin D, the fractions of mobile hCG-occupied receptors on LHR-wt cells increased from 43±3% to 66±13% while the fraction of mobile LH-occupied receptors decreased.

Energy transfer occurs between receptors on LHR-wt cells

We then used single cells fluorescence energy transfer methods to evaluate whether different extents of receptor self-association accompanied diiferent fractions of mobile receptors. Representative data traces showing fluorescence energy transfer between LH- and hCG-occupied receptors are presented in Figure 9. As summarized in Table II, energy transfer efficiency was significantly higher for hCG-occupied receptors as compared to LH occupied receptors, 17% and 13%, respectively, on LHR-wt cells. On LHR-K583R cells, energy transfer efficiency between hCG-occupied receptors was 5% and thus considerably lower than for hCG-occupied receptors on LHR-wt cells. There was essentially no measurable energy transfer

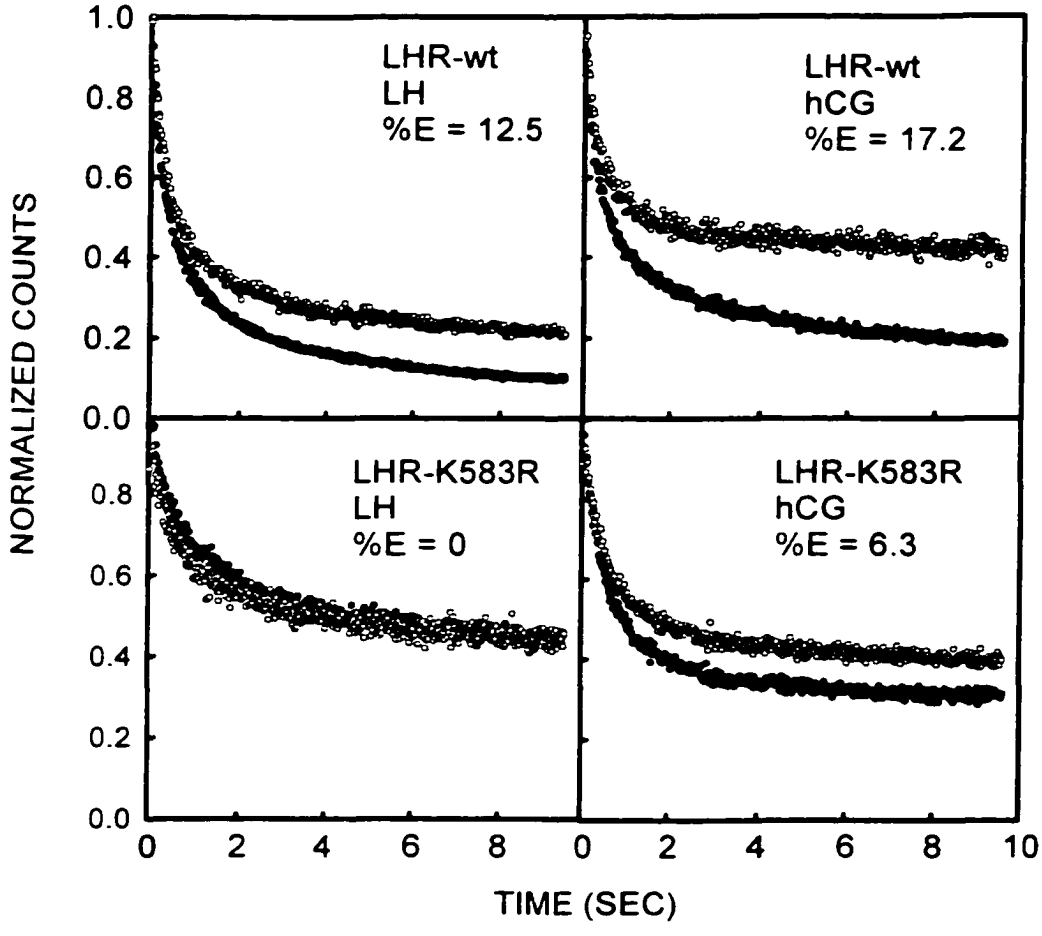

Figure 9: Representative data traces from measurements of fluorescence energy transfer at room temperature between LH receptors on LHR-wt or LHR-K583R cells. The upper trace in each panel is the fluorescence decay from cells labeled with fluorescent donor and fluorescence acceptor (○). The lower trace in each panel is from cells labeled with fluorescence donor only (●). Calculated values for energy transfer efficiency are shown in the upper right hand corner of each panel. On LHR-K583R cells, the rates of fluorescence decay did not differ indicating that there was no measurable energy transfer between LH receptors.

TABLE 2

FLUORESCENCE ENERGY TRANSFER EFFICIENCY BETWEEN LHR-wt RECEPTORS [1]

Cell line	Treatment	% Energy transfer efficiency	
		LH	hCG
LHR-wt	none	12.8 ± 1.7^{a}	17.2 ± 2.7^{bc}
	cytochalasin D	19.2 ± 1.9^{c}	16.8 ± 1.7^{b}
LHR K583R	none	0.3 ± 0.4^{d}	5.1 ± 5.1^{e}
	cytochalasin D	2.7 ± 3.8^{dc}	0^{d}

[1] Data collected by Dr. Heidi Munnelly.

[2] LH receptors was assessed on individual cells labeled with fluorescent LH or hCG at a molar ratio of acceptor- to donor-labeled hormone of 3 to 1.

[3] Cells were pre-incubated with 40 μg/mL cytochalasin D for 30 min at 37°C prior to addition of fluorescent hormones.

[4] Values for energy transfer efficiency were analyzed by one way ANOVA and means were separated using least significant differences criteria. Values with different superscripts were different ($p<0.05$).

between LH-occupied receptors on LHR-K583R cells.

Significant effects of cytochalasin D treatment on fluorescence energy transfer between LH receptors were observed only for LH-occupied receptors on LHR-wt cells (Table II). On these cells energy transfer efficiency between LH-occupied receptors increased from 13% to 19%. This increase in energy transfer efficiency, and thus the extent of receptor self-association, occurred under conditions where there was also a significant decrease in the fraction of mobile receptors.

DISCUSSION

We have measured comparatively large values for fluorescence energy transfer between LH or hCG bound to LH receptors on LHR-wt cells. This suggests that functional LH receptors are self-aggregated into dimers or oligomers and resolves uncertainty as to whether grouping of receptors in electron microscopy studies (Luborsky *et al.*, 1984) and fluorescence microscopy studies (Amsterdam *et al.*, 1980; Podestá *et al.*, 1983) arises simply from restriction of receptors to specific small domains or, as we have demonstrated here, from receptor self-association. These results are also consistent with the appearance of large, fluorescent clusters of wild type rat LH receptors on CHO cells where the receptor is expressed as a green fluorescent protein construct (Horvat *et al.*, 1999). Fluorescence energy transfer, whether via spectroscopic methods or flow cytometric techniques (Trón *et al.*, 1984), has proved to be a sensitive method for detecting molecular associations in the plasma membrane. The characteristic Förster distance R_0 for the fluorescein-rhodamine pair used in these studies

is approximately 56Å (Chan *et al.*, 1979) and the radius of hCG has been estimated to be 35 Å (Anderson *et al.*, 1979). Thus, values for energy transfer efficiency of 12-18% are reasonable for LH receptor dimers or oligomers within protein clusters on the plasma membrane (Chan *et al.*, 1979).

The lateral dynamics of wild type LH receptors on LHR-wt cells were typical of those measured for other membrane proteins including, for example, the major histocompatibility complex class I antigens (Edidin and Zuniga, 1984) and lymphocyte membrane glycoproteins (Leuther *et al.*, 1981). The diffusion coefficient measured for both LH- and hCG-occupied receptors was also similar to that measured for LH-occupied native LH receptors on ovine luteal cells (Niswender *et al.*, 1985) and rat luteal cells (Roess *et al.*, 1992a). However, in contrast to these other cell types where hCG-occupied receptors appeared laterally immobile, hCG-occupied receptors on LHR-wt cells exhibited measurable rates for lateral diffusion. These results for hCG-occupied receptors differed from those previously reported in which the hCG-occupied LH receptor was laterally immobile on luteal cells membranes at 37°C (Niswender *et al.*, 1985; Roess *et al.*, 1992a) with values for fluorescence recovery after photobleaching of less than 20%. Nonetheless, binding of either LH or hCG to receptors on LHR-wt cells affected both the diffusion parameters and fluorescence energy transfer efficiency between hormone-occupied receptors. LH-occupied wild type receptors had larger mobile fractions and less energy transfer between receptors than did the hCG-occupied receptors. Together with studies showing slower rotational diffusion following binding of hCG and thus presumably large complexes that on LHR-wt cells (Roess *et al.*, 2000a), these results suggests that receptor-containing structures formed following binding of LH or hCG differ.

The differences between the lateral diffusion of functional hormone receptors complexes on LHR-wt cells and non-functional hormone-receptor complexes on LHR-K583R cells raise questions as to whether receptor-receptor interactions may be necessary for signal transduction. The non-functional LH receptors on LHR-K583R cells were highly mobile, exhibited little or no inter-receptor energy transfer and, in rotational diffusion studies (Roess *et al.*, 2000a), had fast rotational correlation times which were consistent with small complex sizes. In contrast, functional receptors on LHR-wt cells have slow rotational correlation times (Roess *et al.*, 2000a) and exhibit substantial energy transfer between receptors. Thus, microaggregation of LH receptors of receptors on LHR-wt cells may accompany, or be required for, productive signal transduction as been proposed as a early event in the function of another structurally-related hormone receptor involved in reproductive function. Janovick and Conn, using lactoperoxidase-conjugated hormones to iodinate proximal gonadotropin-releasing hormone (GnRH) receptors, have demonstrated that agonist, but not antagonist, binding to the GnRH receptor results in formation of GnRH microaggregates and have identified non-receptor proteins in the vicinity of the receptor. We have directly demonstrated such microaggregates in studies of fluorescence energy transfer between GnRH receptors (Horvat *et al.*, 2000). GnRH agonists increase energy transfer efficiency between receptors in a dose-dependent fashion but binding of a GnRH antagonist results in no energy transfer between receptors. Lateral diffusion of the GnRH receptor also depends on whether the receptor has bound GnRH agonist or antagonist. There is a significant decrease in the fraction of mobile receptors in response to agonist but not antagonist binding. The picture that emerges from Janovick and Conn's work (Janovick and Conn, 1996a), as well as from our biophysical studies (Horvat *et al.*, 2000), is that hormone-responsive GnRH receptors appear to cluster in structures that

contain both self-associated receptors and non-receptor proteins and that are sufficiently large to contain a large population of laterally immobile receptors.

Disruption of microfilaments also affected the ability of the functional receptor on LHR-wt cells to form aggregated structures. When the influence of microfilaments on receptor organization was removed, a decrease in the fraction of mobile LH-occupied receptors on LHR-wt cells was accompanied by an increase in the extent of energy transfer between receptors. Interestingly, diffusion characteristics and energy transfer between LH-occupied receptors following cytochalasin D treatment did not differ significantly from those of untreated, hCG-occupied receptors. These data suggest that there may be limits to both the extent of energy transfer efficiency and the relative number of mobile receptors on LHR-wt cells. They also suggest that microfilaments interact in a differential fashion with the complexes formed following binding of LH and hCG. In the case of LH-occupied receptors, the cytoskeleton restricts receptor-receptor interactions. For hCG-occupied receptors, the cytoskeleton restricts the lateral diffusion of a significant fraction of the hCG-occupied receptor population. Lastly, interactions between the hormone-receptor complex may be important in receptor function: treating LHR-K583R cells with cytochalasin D did not affect either receptor lateral dynamics or increase receptor-receptor interactions.

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CHAPTER THREE

INTRINSICALLY FLUORESCENT LUTEINIZING HORMONE RECEPTOR DEMONSTRATES HORMONE-DRIVEN AGGREGATION

INTRODUCTION

The luteinizing hormone receptor (LHR) is a seven-transmembrane domain receptor (McFarland *et al.*, 1989) that, upon binding of either luteinizing hormone (LH) or human chorionic gonadotropin (hCG), initiates signal transduction including activation of adenylyl cyclase. Following binding of hormone, the receptor appears to exist in large molecular weight structures (Philpott *et al.*, 1995) that may include other non-receptor proteins (Roess *et al.*, 1998). One long-standing question is whether these large structures containing the LH receptor exist in the membrane prior to receptor interactions with hormone or, alternatively, whether these structures form only following binding of ligand.

In principle, the arrangement of LH receptors in the plasma membrane might be examined using a high-affinity monoclonal anti-receptor antibody that neither interferes with

subsequent hormone binding nor promotes receptor crosslinking. Unfortunately it has proven to be surprisingly difficult to develop such an antibody. Thus alternative methods for fluorescently visualizing the unoccupied LH receptor have been explored. Here, we report the development of an intrinsically-fluorescent fusion protein of LH receptor and enhanced green fluorescent protein that retains many of the properties of native LH receptors. Using rLHR cDNA, the generous gift of Dr. Deborah Segaloff at the University of Iowa, we subcloned the full-length receptor cDNA into enhanced EGFP- N_2 vector (Clontech , Palo Alto, CA) as a SacI/EcoRI fragment. Deep Vent Polymerase (New England Biolabs, Beverly, MA) was used in a polymerase chain reaction to amplify a fragment beginning at the intrinsic ScaI site (ACTATAACCACGCCATAGAC) of the LH receptor and ending at the receptor 3'-end removing the stop codon and replacing it with an in-frame BamHI site (underlined) at the 3'-end (CGGGATCCAACGCTCTCGGTGGTATGG). The plasmid was then digested with ScaI and BamHI to remove a portion of the LHR receptor containing coding sequence, the stop codon, and the 3' untranslated region. The PCR product was digested with BamHI and ligated into ScaI and BamHI plasmid. The final fusion protein DNA sequence consisted of rLHR, a spacer sequence of the amino acids IHRPVAT, and enhanced GFP. The ScaI/BamHI fragment was confirmed by cDNA sequencing by Macromolecular Resources (Fort Collins, CO).

CHO cells were transfected with 1 μg of the rLHR-GFP vector using Lipofectamine-Plus (Gibco-BRL, Gaithersburg, MD) as per manufacturer's instructions. Selection of clones stably expressing rLHR-GFP was performed as described previously for murine gonadotropin-releasing hormone receptor fused to GFP (Nelson *et al.*, 1998). To assess the relative amount of rLHR-GFP in the plasma membrane we evaluated the colocalization of rLHR-GFP with

other membrane glycoproteins using fluorescent Concanavalin A (Con A). CHO cells expressing the fusion protein were plated onto sterile Lab-tek Chambered Coverglass (Nalge Nunc International, Naperville IL) overnight prior to staining with 10μg/mL of ice cold Alexa 594-Con A from Molecular Probes (Eugene, OR) in ice-cold phosphate buffered saline (PBS), washed twice in ice-cold PBS and imaged in PBS. Confocal fluorescence images were acquired on a Sarastro 2000 confocal laser-scanning microscope (Molecular Dynamics, Sunnyvale, CA) in an epifluorescence mode. rLHR-GFP receptors were interrogated using the 488nm line of a krypton-argon ion laser while Alexa 594-Con A was interrogated with the 568nm line. A 565nm dichroic mirror was used to separate red/green fluorescence, a 515-545nm band-pass filter was used to isolate the green fluorescence from enhanced GFP and a 600nm long-pass filter was used to isolate the Alexa 594-Con A signal. GFP colocalization with Alexa 594 was quantitated using ImageSpace software (Molecular Dynamics). Colocalization was expressed as the fraction of total green fluorescence falling within the boundaries of the mask delineating red fluorescence from Alexa Con A. Using this procedure, a total of twelve cells were analyzed. GFP fluorescence colocalizing with Alexa 594 was 54±4% of the total fluorescence signal from rLHR-GFP suggesting that approximately half of rLHR-GFP molecules were located in the plasma membrane. In addition, the plasma membrane of transfected CHO cells was visible as a bright ring on the cell boundary while the remaining fluorescence was distributed uniformly throughout the cytoplasm.

To determine whether rLHR-GFP were functional, production of cAMP in response to ovine LH (AFP-5551B, National Hormone and Pituitary Program, Bethesda, MD) or hCG (Research Diagnostics, Inc., Flanders, NJ) was evaluated per manufacturer's instructions using

a TiterFluor cAMP EIA kit (Perkin-Elmer Biosystems, Norwalk, CT). To reduce well-to-well variations in measured levels of cAMP, 96-well plates coated with anti-rabbit IgG were obtained from Pierce (Rockford, Il) and were substituted for those provided in the TiterFluor cAMP kits.

As shown in Figure 10, increasing concentrations of both LH and hCG increased cAMP production by CHO cells expressing rLHR-GFP. Background cAMP levels from non-transfected cells and from CHO cells expressing rLHR-GFP were 3.1±1.4 and 3.4±1.6pmol/10^6 cells, respectively. Maximum levels of cAMP were obtained when cells were incubated with 10nM LH or hCG and represented a 3-5 fold increase in cAMP above basal levels. These results suggest that rLHR-GFP is competent in signal transduction.

Hormone binding also caused aggregation of the rLHR-GFP on the plasma membrane. Prior to hormone binding, cell membrane fluorescence was distributed uniformly throughout the plasma membrane as shown in the left panel (A) of Figure 11. Microscopic images from hormone-treated CHO cells shown in Figure 11 showed appreciable patching of rLHR-GFP 1 hr following introduction of 10nM LH (panel B) and hCG (panel C). Our results are consistent with electron microscopy studies of the LH receptor demonstrating hormone-induced receptor aggregation. Luborsky *et al* (1984b) reported hormone-dependent aggregation of rat LH receptors on rat luteal cells while Ghinea *et al* have shown that LH receptors have a diffuse distribution on the cell surface prior to ligand binding and that hormone binding causes redistribution of the receptors into coated pits and coated vesicles as a prelude in internalization of the hormone-receptor complexes (Ghinea *et al.*, 1992).

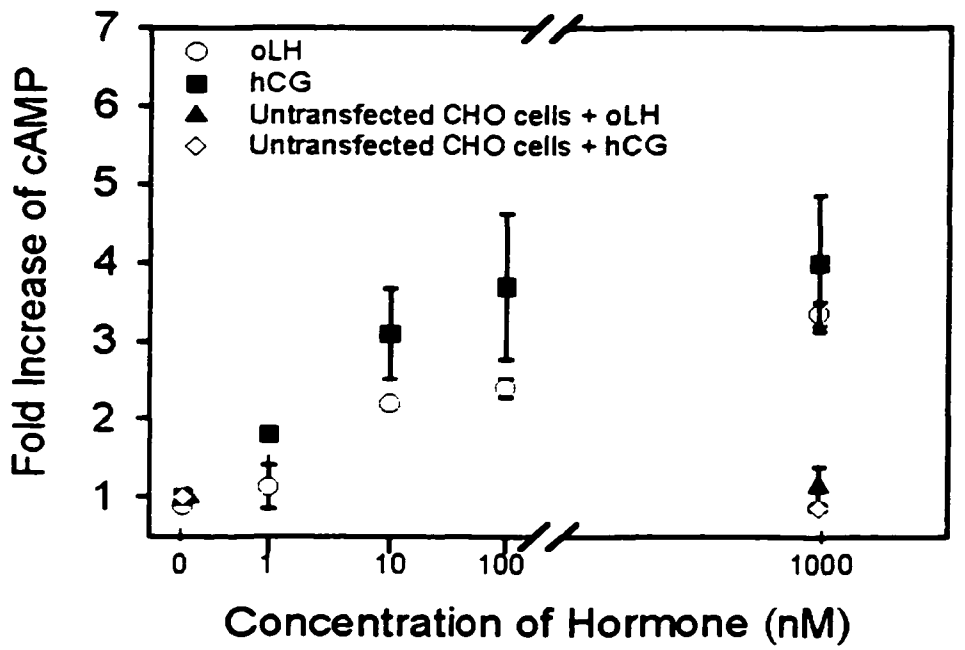
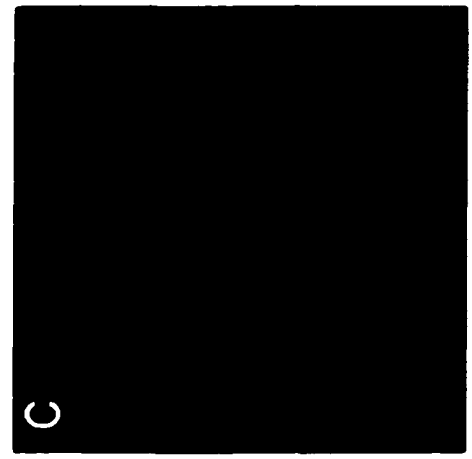
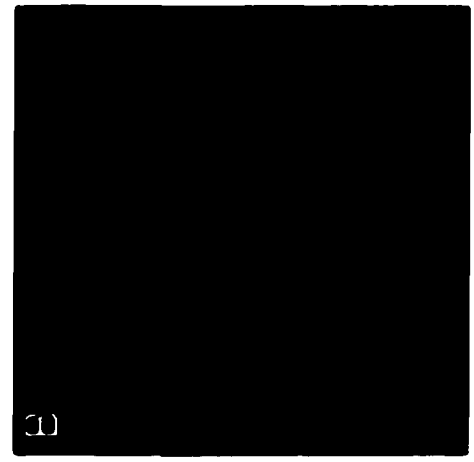
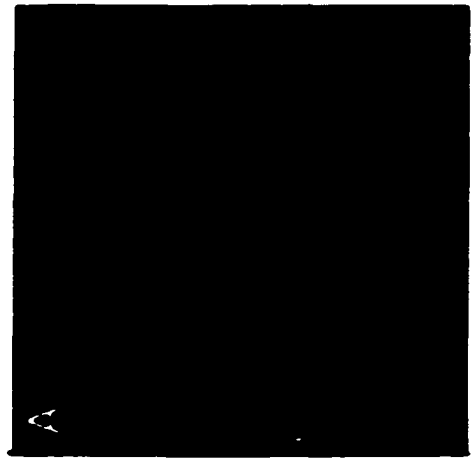


Figure 10. Treating CHO cells expressing rLHR-GFP with increasing concentrations of either LH or hCG resulted in a dose-dependent increase in intracellular cAMP. There was no increase in cAMP in CHO cells that did not express rLHR-GFP.

Figure 11. Fluorescence images from rLHR-GFP obtained using a Zeiss Axiovert microscope. Panel A shows fluorescence from rLHR-GFP prior to binding of ligand. Panel B shows small fluorescent patches formed after exposure to oLH for 1 hour. Panel C shows fluorescence from rLHR-GFP after a 1 hour exposure to hCG.







Because hormone binding produced marked changes in receptor distribution and caused the appearance of small, punctate fluorescent patches on the plasma membrane, we used fluorescence photobleaching recovery (FPR) methods to determine whether ligand binding to the rLHR-GFP receptor was also accompanied by changes in receptor lateral diffusion. The equipment and methods used for performing spot FPR measurements at 37°C have been described previously in detail (Barisas *et al.*, 1997). An attenuated Coherent Radiation Innova 100 argon ion laser beam was focused to a spot of 0.41μm $1/e^2$ radius. Bleaching and probe beam powers were 1.4mW and 1.7μW, respectively, at 488nm. Data were acquired at 50 msec/point for 20 sec before, and for 30 sec after, a 150 msec bleaching pulse. FPR measurements were processed as previously described (Barisas *et al.*, 1997). CHO cells expressing rLHR-GFP were incubated with hormone for 1 hour prior to the lateral diffusion measurements. These measurements were completed within 30 minutes and were made on three separate days. As shown in Figure 12, panel A, the lateral diffusion coefficient of the unoccupied rLHR-GFP was $16.0 \pm 3.5 \times 10^{-10} cm^2 sec^{-1}$. This diffusion coefficient is comparable to that of the diffusion coefficients of GFP-fused β-adrenergic receptor (Barak *et al.*, 1997). Treatment of cells with either LH or hCG slowed lateral diffusion of the receptor significantly. Exposure to 10nM LH reduced the receptor lateral diffusion coefficient to $6.8 \times 10^{-10} cm^2 sec^{-1}$ and decreased values for fluorescence recovery after photobleaching from 54±13% for the unoccupied receptor to 27 ± 1%. Treatment with hCG essentially immobilized rLHR-GFP, reducing values for fluorescence recovery to about 16%. This indicates that most hCG-receptors have a diffusion coefficient less than $1 \times 10^{-12} cm^2 sec^{-1}$ (Sheetz *et al.*, 1980). Because FPR measurements were completed within 1 - 1 ½ hr following addition of hormone, it is also likely that these slower diffusion coefficients were obtained from a receptor population that was

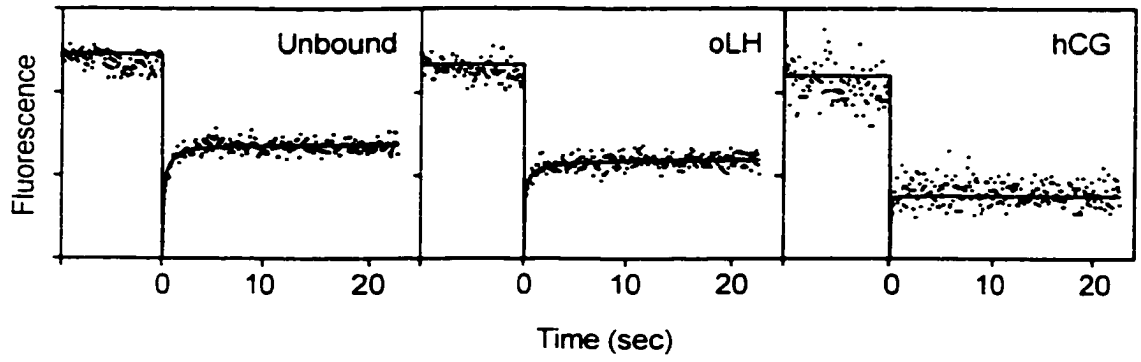


Figure 12. Representative lateral diffusion traces for rLHR-GFP obtained using spot fluorescence photobleaching recovery methods prior to binding of ligand (left panel), after a 1 hour incubation with oLH (middle panel), and after a 1 hour incubation with hCG (right panel).

desensitized by hormone exposure (Sanchez-Yague *et al.*, 1992)

Conventional fluorescence microscopic images of rLHR-GFP receptors show proteins uniformly dispersed in the plasma membrane of CHO cells. However, the value for fluorescence recovery, 54%, is lower than those values reported for fusion proteins of GFP with the β-adrenergic receptor (Barak *et al.*, 1997) and with GnRH (Nelson *et al.*, 1998). LH receptor may thus have a heterogeneous distribution on the plasma membrane with some receptors located in membrane domains or other structures where motions of LHR-GFP are severely limited. The motions of LHR in these various environments would then be averaged by spot FPR into a single low value for fluorescence recovery. Other methods such as single particle tracking techniques (Kusumi *et al.*, 1993) will help resolve this question by examining the movement of individual receptors.

Binding of ligand to LHR-GFP causes redistribution of receptors into small, fluorescent clusters. These hormone-occupied receptors have diffusion coefficients that are dependent on whether LH or hCG has bound the receptor. This was also the case for native LH receptors on rat luteal cells (Roess *et al.*, 1992b) and on ovine luteal cells (Niswender *et al.*, 1985) for where hCG-occupied LH receptors were laterally immobile while LH-occupied receptors exhibited a diffusion coefficient similar in magnitude to that of LH-occupied rLHR-GFP. The receptor's membrane environment, as determined by the expressing cell type, apparently also affects receptor diffusion. The wild-type LH receptor expressed in 293 cells exhibited diffusion coefficients that did *not* vary according to the hormone bound (Horvat and Roess, 1997). However, the fraction of mobile hCG-occupied receptors was significantly lower than that of

LH-occupied receptors. Experiments are now underway to express the rLHR-GFP in 293 cells and thus test whether the type of the expressing cell is a significant determinant of receptor dynamics. In conclusion, the rLHR-GFP fusion protein is of great interest because it combines many of the properties of native LH receptors in luteal tissue with intrinsic fluorescence. The expression of biologically active and fluorescent form of the LH receptor makes it possible to examine the physical distribution of the receptor in the plasma membrane as well as movement of the receptor within cell organelles in response to ligand binding.

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CHAPTER FOUR

LUTEINIZING HORMONE RECEPTORS ARE PRESENT IN SLOWLY DIFFUSING COMPLEXES DURING RECEPTOR DESENSITIZATION

INTRODUCTION

Like other G-protein coupled receptors including the β-adrenergic receptor (Freedman and Lefkowitz, 1996), the LH receptor can become less responsive after initial binding of ligand (Hunzicker-Dunn and Birnbaumer, 1981; Hunzicker-Dunn *et al.*, 1979). This may be important *in vivo* where the preovulatory surge of LH results in a time-dependent decrease in a subsequent response to hormone (Bockaert *et al.*, 1976) but only if hormone doses are sufficient to cause ovulation (Hunzicker-Dunn and Brinbaumer, 1975). The initial step in LH receptor desensitization involves "uncoupling" the receptor from signal transduction mechanisms, a change in receptor function that can occur without a concurrent change in

receptor number. This "uncoupling" process may be required for subsequent internalization of the receptor and receptor down-regulation (Dix *et al.*, 1982; Wang *et al.*, 1997).

Despite considerable effort by a number of investigators, little is known about the molecular mechanisms by which LH receptor desensitization occurs. Desensitization for the β-adrenergic receptor has served as the model for most G-protein coupled receptors (Lefkowitz *et al.*, 1990). However, unlike the β-adrenergic receptor, receptor phosphorylation is not required for desensitization (Hunzicker-Dunn and Lamm, 1994; Wang *et al.*, 1997). Nonetheless, LH receptor desensitization may also involve physical interactions between the receptor and other proteins including β-arrestin (Mukherjee *et al.*, 1999), and/or segregation of the receptor into membrane domains or membrane rafts containing proteins needed for signaling.

One model for receptor function involves the lateral movement of LH receptors into membrane regions or domains that contain molecules needed for cell signaling and subsequent receptor desensitization. Glaser and coworkers (Yang and Glaser, 1995) have suggested that activation of molecules involved in signal transduction may be accompanied by carefully orchestrated protein-protein associations that result from step-wise movement of molecules into and out of biologically-functional protein complexes. Within these complexes, proteins involved in signal transduction, including G-protein coupled receptors, could be clustered in microdomains within the plasma membrane. This seems to be the case for G-protein coupled receptors on *Dictyostelium* which exist in highly specialized membrane domains that include adenylyl cyclase and phosphodiesterase (Xiao and Devreotes, 1997). Lateral diffusion of the

receptor may also play a important role in the signaling process. Hunzicker-Dunn has suggested that LH receptors previously exposed to hormone must diffuse into new membrane domains before becoming hormone responsive again (Hunzicker-Dunn *et al.*, 1996).

To test whether the organization of LH receptors within the plasma membrane is substantively affected by the functional state of the receptor, we have examined the lateral motions of the receptor and fluorescence energy transfer between receptors following brief exposure to either LH or hCG. These studies made use of the fluorescence properties of enhanced green fluorescent protein (GFP) and its red-shifted variant, yellow fluorescent protein (YFP). Both these proteins were coupled to the rat LH receptor at its C terminus and stably expressed either singly or together in CHO cells. We have previously shown that hormone binding to GFP-LHR-wt, which is successfully transported to the plasma membrane, results in cAMP accumulation and slower GFP-LHR-wt lateral diffusion (Horvat *et al.*, 1999). Here we have examined both LHR lateral diffusion and receptor-self association over the time course of receptor desensitization. Receptor-expressing cells were exposed briefly to either LH or hCG which was removed by washing cells with a low pH buffer. This treatment has been shown to reduce receptor responsiveness without damaging the ability of receptors to interact with hormone and without reducing the number of receptors on the plasma membrane (Dix *et al.*, 1982). Then, in addition to assessing receptor lateral dynamics and self-association during receptor desensitization, we examined whether the desensitized receptor was contained in complexes whose diffusive properties were characteristic of the hormone used to desensitize the LH receptor. Together with fluorescence images of green fluorescent protein over the time course of receptor desensitization, it appears that desensitized LH receptors are self-associated.

Thus, receptors exist with slowly diffusing and stable complexes whose properties are hormone-specific and which must dissociate before the receptor can again respond to fresh hormone.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium and Dulbecco's modified Eagle medium containing high glucose were purchased from Irvine Scientific, Santa Ana, CA. Geneticin was purchased from GIBCO, Grand Island, NY. HEPES and non-essential amino acids were purchased from Sigma Chemical Co., St. Louis, MO. Horse serum was purchased from Summit Biotechnology (Fort Collins, CO) and fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). Ovine LH (oLH; NIH 28) was obtained from the National Hormone and Pituitary Program, NIADDK, Baltimore, MD and hCG was purchased from Research Diagnostics Inc. (Flanders, NJ). Tetramethylrhodamine isothiocyanate (TrITC) and erythrosin isothiocyanate (ErITC) were purchased from Molecular Probes, Eugene, OR.

Cell lines

Dr. Tae Ji kindly provided 293 cells stably transfected with the wild type rat LH receptor (LHR-wt cells). These cells, as well as untransfected 293 cells, were maintained in Dulbecco's modified Eagle's medium containing 10% horse serum, 100 U pencillin, 1000

μg/mL streptomycin and 10 mM HEPES, pH 7.4. Medium for LHR-wt cells was supplemented with 400 μg/mL geneticin. Untransfected Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle media supplemented with 4,500 mg/mL glucose and containing 10% FBS, 100U/mL penicillin, 1000µg/mL streptomycin and 1XMEM non-essential amino acids.

CHO cells stably transfected with the GFP-LHR-wt construct were prepared as described previously (Horvat *et al.*, 1999) and maintained in CHO cell medium containing 200µg/mL geneticin. CHO cells expressing YFP-LHR-wt alone or together with GFP-LHR-wt were prepared using the same strategy described in detail for GFP-LHR-wt (Horvat *et al.*, 1999). The full-length receptor cDNA for rLHR, a gift from Dr. Deborah Segaloff, was subcloned into enhanced EYFP-N2 vector (Clontech , Palo Alto, CA). A fragment of the LHR beginning at the intrinsic ScaI site (ACTATAACCACGCCATAGAC) and ending at the receptor 3'-end was removed, thus removing an intrinsic stop codon, and replaced with an in frame BamHI site (underlined) at the 3'-end (CGGGATCCAACGCTCTCGGTGGTATGG). This fragment was amplified by polymerase chain reaction. The PCR product was digested with BamHI, as was the eYFP-N2plasmid, and ligated into ScaI and BamHI plasmid. The final fusion protein DNA sequence consisted of rLHR, a spacer sequence of the amino acids IHRPVAT, and enhanced YFP. The ScaI/BamHI fragment was confirmed by cDNA sequencing by Macromolecular Resources (Fort Collins, CO). CHO cells expressing YFP-LHR-wt alone were transfected with 1 μg of the rLHR-YFP vector using Lipofectamine-Plus (Gibco-BRL, Gaithersburg, MD) as per manufacturer's instructions. CHO cells co-expressing both fusion proteins were transfected with 0.4 mg of GFP-LHR-wt and 1.2mg of YFP-LHR-wt.

After overnight culture, transfected cells were transferred into 150 mm plates and cultured with CHO cell medium containing 600 mg/mL G418 (Gemini Bioproducts, Woodland CA) for seven days. At this time, cells were washed with PBS and cloned by limiting dilution in 96 well plates (ISC BioExpress, Kaysville, UT) where then were incubated for two weeks before selection of fluorescent colonies and expansion of those colonies.

Preparation of TrITC or ErITC-derivatized hormones

Hormones were derivatized with ErITC or TrITC using a modification of methods described by Brinkley *et al.* (Brinkley, 1992) and described in detail elsewhere (Young *et al.*, 1994). The molar ratios of dye to hormone were determined spectrophotometrically. Hormone preparations used in these experiments had 1.0-1.5 moles of ErITC or TrITC per mole of oLH or hCG. Prior to use, all derivatized hormones in PBS were centrifuged at 130,000 x g for 10 min in a Beckman Airfuge (Beckman Instruments Inc., Palo Alto, CA) to remove any protein aggregates that may have formed during storage at 4 °C.

LH receptor desensitization

LH receptors were desensitized using a protocol that has been described in detail by others (Sanchez-Yague *et al.*, 1992). Briefly, CHO cells or 293 cells expressing LH receptors were incubated for 30 min with 10 nM LH or hCG at 37 °C and then treated for 5 min at 4 °C with 50 mM glycine, 100 mM NaCl, pH 3.0, balanced salt solution (BSS) to remove hormone bound to the LH receptor. The cells were centrifuged at 300 x g, and resuspended in fresh BSS.

Following hormone treatment, receptor number was unchanged and receptors were able to rebind hormone. This was confirmed by measuring the phosphorescent signal from erythrosin-isothioyanate-labeled hormones bound to LH receptors on LHR-wt cells. Cells were incubated with 10 nM ErITC-hCG or ErITC-LH, for 1 hour and washed two times by centrifugation at 300 x g for 3 min in BSS to remove any unbound ligand. The sample was then deoxygenated for 15 min by purging with argon gas to eliminate phosphorescence quenching caused by O_2 and placed in a 5-mm Suprasil quartz cuvette (Helma Cells, Inc., Jamaica, NY) which was inserted in a thermostatted cuvette holder. The frequency-doubled 532 nm output of a Spectra-Physics DCR-11 Nd:YAG laser was used to excite ErITC. The laser was operated at 10 Hz with a vertically polarized TEM 00 output of 0.19mJ and a beam $1/e^2$ radius restricted to 2.5 mm at the sample. Phosphorescence emission from the sample was collected by a cooled EMI 9816A photomultiplier tube, amplified by a Tektronix 476 oscilloscope and a 35 MHZ bandwidth buffer amplifier, and fed to a Nicolet 12/70 signal averager equipped with a 20 MHZ analog-to-digital converter. After data acquisition was complete, the data were downloaded into a Pentium II microcomputer (Philpott *et al.*, 1995).

The untreated cell sample had phosphorescent cps/10^5 cells of 0.5±3. Labeling cells with ErITC-oLH or ErITC-hCG increased cps/10^5 cells to 5.8±0.4 and 6.2±0.1, respectively. Treating cells with low pH buffer removed hormone from the receptor as indicated by a decrease in the phosphorescent signal to baseline values (0.53-0.57 cps/10^5 cells). Rebinding ErTIC-LH or ErITC-hCG to LH receptors at 1 hour and 5 hours removal of hormone with low pH buffer increased the phosphorescence signal to 4.6±1.5-6.2±0.1 cps/10^5 cells which did not differ significantly from signals measured following initial binding of either ErITC-LH or

ErITC-hCG. To verify that there were no non-specific interactions of the ErITC-hormones with human kidney 293 cells, 293 cells that did not contain expression vectors for the LH receptor were treated with 10 nM ErITC-rLH or -hCG. To determine whether binding of the ErITC-derivatized hormones was specific, cells were preincubated with excess oLH prior to labeling with ErITC-oLH in some experiments. In both cases, there was no detectable phosphorescence signal from the cell sample.

The extent of LH receptor desensitization was evaluated by measuring cellular cAMP levels following binding of fresh hormone using a TiterFluor cAMP EIA kit (Perkin-Elmer Biosystems, Norwalk, CT) as per the manufacture's instructions. To reduce well-to-well variations in measured levels of cAMP, 96-well plates coated with anti-rabbit IgG were obtained from Pierce (Rockford, Il) and were substituted for those provided in the TiterFluor cAMP kits.

Spot and fringe fluorescence photobleaching recovery measurements

The optical system for performing fringe fluorescence photobleaching recovery measurements and the method used for data analysis have been described in detail (Munnelly *et al.*, 1996). The microscope objective used in these studies was a Zeiss 40x objective of NA 0.65. Standard Zeiss filter and dichroic mirror sets for FITC and TrITC fluorescence were used. Cells were examined under coverslip on well slides while temperature was maintained by a thermoelectrically cooled/heated thermal stage with a temperature range of 0 °C to 40 °C. For spot measurements, an attenuated Coherent Radiation Innova 100 argon ion laser beam was

focused to a spot of 0.41μm $1/e^2$ radius. Bleaching and probe beam powers were 1.4mW and 1.7μW, respectively, at 488nm. Data were acquired at 50 msec/point for 20 sec before, and for 30 sec after, a 150 msec bleaching pulse. For fringe measurements, the region illuminated at the sample has a $1/e^2$ radius of at least 18μm and the photometer acceptance region large enough to encompass the entire cell. The fringe spacing used in these experiments was 2.3μm. Because of the large interrogated area, 1.3 W in the bleaching pulse and 3mW in the probe beam were used. Unadjusted raw data were represented directly in terms of the various parameters associated with a given measurement including the prebleach and immediate postbleach fluorescence levels F_- and F_o, the extent M of fluorophore mobile on the timescale of the experiment, and a function representing the recovery kinetics in terms of a decay half-time. These parameters were evaluated directly by a nonlinear least-squares procedure and from the measured time $t_{1/2}$ at which fluorescence recovery was half-complete and from the known optical parameters, the desired diffusion coefficient was evaluated.

Single cell fluorescence energy transfer

Fluorescence energy transfer (FET) between GFP- and YFP-LHR was evaluated based on the reduced rate of irreversible photobleaching of GFP fluorophores when YFP fluorophores were present (Young *et al.*, 1994). Slower rates of fluorescence decay for cells expressing both GFP-LHR and YFP-LHR acceptor (D+A) than for cells expressing GFP-GnRHR only (D) were indicative of energy transfer from fluorescence donor to acceptor and occurred only when the donor and acceptor were separated by distances less than about 100Å for this donor and acceptor pair which have an r_0 of 51 Å (Barisas, unpublished results). FET measurements were

made using a fluorescence microscope photometer based on the inverted-configuration Zeiss Axiomat microscope and associated components used for spot fluorescence photobleaching recovery measurements. Fluorescence excitation was provided by a Coherent Radiation Innova 100 argon ion laser operating under light control at 488 nm. The intensity of the laser radiation focused on the cell was 45μW and this was held constant between measurements on LHR-GFP cells or on LHR-GFP/YFP expressing cells. The $1/e^2$ Gaussian spot diameter was 0.41μm. Donor fluorescence from GFP was isolated with a standard fluorescein filter set together with a short pass fluorescein-selective filter to remove yellow fluorescence contributed by YFP-LHR-wt. This combination was highly effective in rejecting YFP fluorescence. In individual experiments, cells were identified and centered in the microscope field. At time zero, an electronically controlled shutter was opened to allow laser radiation to impinge on the cell. Simultaneously, a computer program was activated to record the output of the photomultiplier measuring membrane fluorescence. Data were collected at 0.01 sec intervals for 10 sec. Typically, about 20 cells in each sample were photobleached in this manner. Cells expressing LHR-YFP alone produced signals that did not differ significantly from those of untransfected CHO cells using the fluorescein-selective filter set. Signal from CHO cells expressing LHR-GFP or LHR-GFP/YFP was greater than 8-fold higher than background levels. The data traces were analyzed to give the energy transfer efficiency (%E) as has been described in detail previously (Young *et al.*, 1994).

Fluorescence imaging of GFP-LHR-wt

GFP-LHR-wt fluorescence from individual cells was acquired on a Zeiss Axiovert microscope equipped with a 1.4 NA oil immersion condenser and a 1.3 NA 63x Plan-Neofluar objective. An 100W arc lamp was used to excite the sample and a standard FITC filter set was used to isolate the green (GFP) signal. Fluorescent images were obtained using a Dage MTI digital camera using an integration time of 30 sec and exported into Metamorph imaging software (Universal Imaging, West Chester, PA) where they were pseudo-colored. The images were then exported to Adobe PhotoShop for further image processing and printed on a Kodak color printer. A minimum of ten cells for each sample were imaged and analyzed.

RESULTS

Binding of oLH or hCG to receptors on GFP-LHR-wt or LHR-wt cells desensitizes the receptor within 1 hour.

To determine how long LH receptors on CHO and 293 cells remained desensitized following brief hCG exposure, cAMP levels were measured in response to subsequent hormone challenge. Cells expressing GFP-LHR-wt or LHR-wt were incubated for 30 minutes at 37°C with 10 nM hCG and then washed with a low pH buffer to remove the bound hormone as described in Materials and Methods. At one hour intervals following removal of hormone, cells were challenged with 10 nM hCG for an additional hr at 37°C. As shown in Figure 13, initial treatment of cells expressing either GFP-LHR-wt (Panel A) and LHR-wt (Panel B)

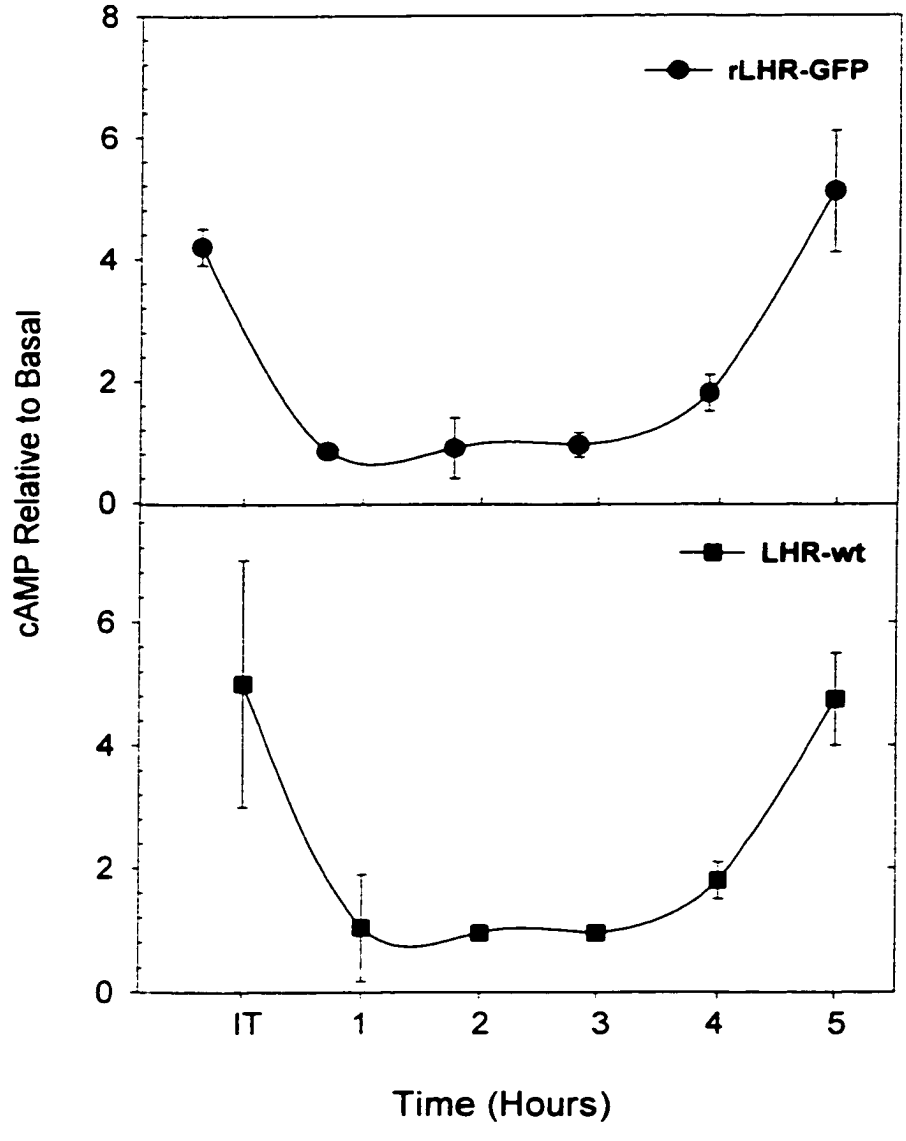


Figure 13. The time course for LH receptor signaling for CHO cells expressing rLHR-GFP (●) and HEK 293 cells expressing wild type LHR (■) following receptor desensitization. GFP-LHR-wt or LHR-wt were initially treated (IT) with hCG for 1 hr prior to measuring intracellular cAMP. Hormone was then removed from the receptor using low pH buffer. At the indicated times (1-5 hrs), intracellular cAMP levels were measured following a 1 hr incubation of cells with 10 nM hCG. In response to initial treatment with hCG, there was a 4-6 fold increase in intracellular cAMP. After desensitization of the receptor, subsequent hormone treatment for 1 hr had no effect on intracellular cAMP until 5 hours following removal of desensitizing hormone. Results are the average and S.D. for 4 experiments performed in triplicate.

resulted in a 5-7 fold increase in cAMP over basal levels. Following removal of hCG from these receptors, there was no increase in intracellular cAMP over basal levels in response to hormone challenge for four hours. After 5 hrs, cells responded to hormone challenge with an increase in cAMP to levels comparable to those seen following initial exposure to hCG.

Lateral diffusion of desensitized GFP-LHR-wt receptors on CHO cells is slow

Prior to binding of ligand, GFP-LHR-wt exhibited a $1.6 \pm 0.3 \times 10^{-9} cm^2 sec^{-1}$ diffusion coefficient D with a high fraction of mobile receptors (%M = 58±4%). After desensitization of the receptor by LH, D was reduced more than 3-fold to $4.4 \pm 1.0 \times 10^{-10} cm^2 sec^{-1}$ and fluorescence recovery decreased to 26±3% (Figure 14). Between two to four hours following desensitization of the receptor, lateral diffusion became progressively faster and the extent of fluorescence recovery increased. However, only when receptors were again hormone responsive were the rate of receptor lateral diffusion and the fraction of laterally mobile receptors comparable to values measured on untreated cells. Receptors desensitized by hCG were initially laterally immobile. The fraction of mobile receptor dropped to 16% at one hr following hormone treatment indicating that most receptors were laterally immobile (Golan and Veatch, 1980). Between 2 and 4 hrs following hormone treatment, the extent of fluorescence recovery after photobleaching, and thus the relative fraction of mobile receptors, increased, reaching 42% at 4 hrs. Nonetheless, diffusion coefficients for hCG-desensitized receptors were significantly slower than those of LH-desensitized receptors at each time point. As was the case for receptors desensitized by LH, diffusion coefficients for hCG-desensitized receptors were again comparable to those of untreated receptors at 5 hrs.

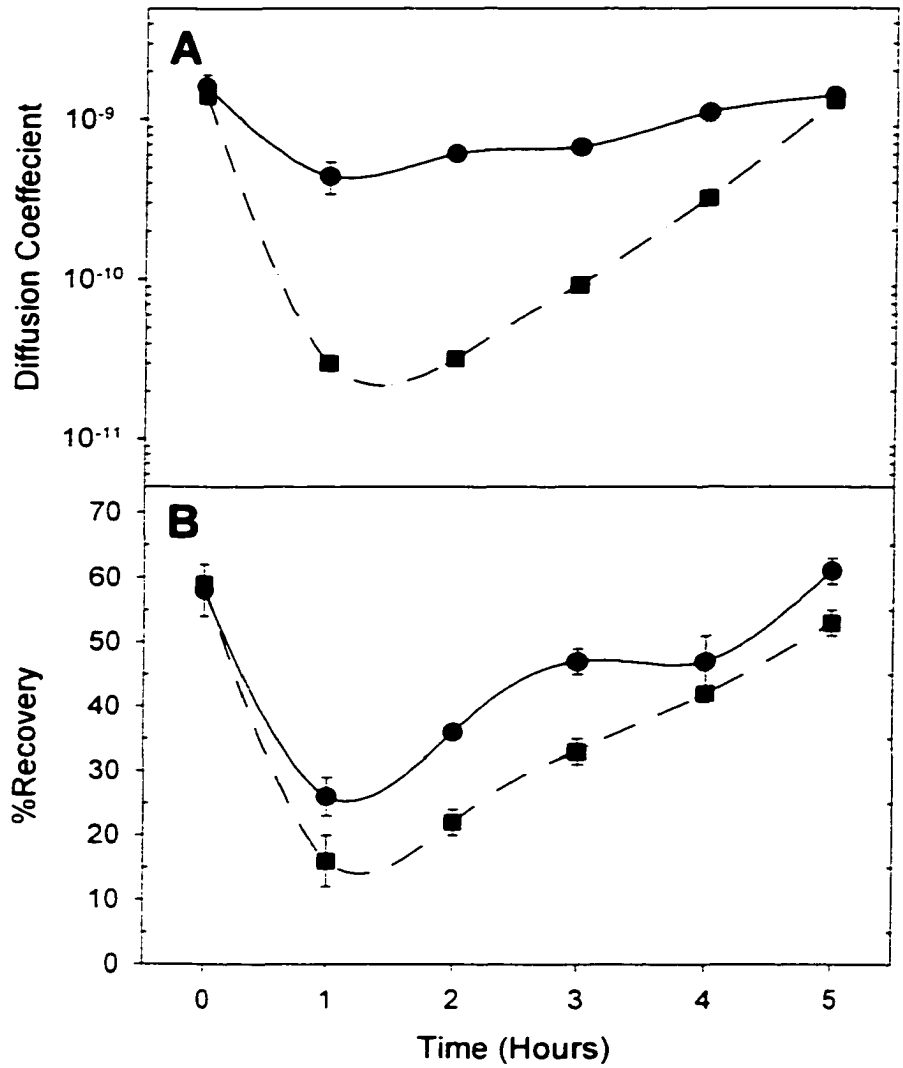


Figure 14. Lateral diffusion of rLHR-GFP prior to signaling and during oLH- (●) and hCG-induced (■) receptor desensitization. Panel A shows the diffusion coefficient (D) for the GFP-LHR-wt over the time course of receptor desensitization. Panel B shows the mobile fraction (%M) of GFP-LHR-wt over the time course of receptor desensitization. Receptors desensitized by either ligand exhibited significantly slower D 1 hour following desensitization. D increased over the next 4 hours until D was indistinguishable from that of untreated LHR. %M decreases upon binding of hormone. %M increases over time until 5 hours when %M did not differ significantly from untreated receptor. The magnitude of the decrease in D and %M in response to receptor desensitization was dependent on the hormone used to desensitize the receptor.

Hormone specific complexes are formed following desensitization of LH receptors

We then examined desensitized LHR-wt receptors on 293 cells using fringe fluorescence photobleaching recovery methods following treatment with either LH or hCG. We had previously shown using fringe FPR that the fraction of mobile receptors following binding of TrITC-LH- or TrITC-hCG differed significantly (Horvat and Roess, 1997). Although the diffusion coefficients for hCG- and LH-occupied receptors on LHR-wt cells were 3-5 x $10^{-10}cm^2sec^{-1}$, the fraction of mobile LH receptors, as indicated by %mobility (%M), was significantly higher following binding of LH (69 ± 13%) when compared to hCG-occupied receptors (43 ± 3%). As shown in Table 4, 1 hr following washing of cells with low pH buffer, values for %M always reflected the hormone initially used to desensitize receptors regardless of whether LH or hCG was later used as the fluorescent probe. After 5 hrs values for %M were characteristic of the fluorescent ligand bound to the receptor and, thus, were independent of the hormone used initially to desensitize the receptor. When cells were not treated with hormone prior to acid washing and then labeled with TrITC-LH or TrITC-hCG, the diffusion coefficients and mobile fractions for these hormones did not differ significantly from those of hormone-occupied LH receptors on LHR-wt cells that had not received low pH treatment. These data suggest that the rebinding of ligand to a desensitized receptor does not induce formation of a new receptor-containing structure but rather that the complexes are stable and characteristic of the hormone used to desensitize the receptor.

TABLE 3

LATERAL DIFFUSION CHARACTERISTICS FOR LHR-wt RECEPTORS DURING AND FOLLOWING oLH- OR hCG-INDUCED RECEPTOR DESENSITIZATION

Desensitizing Hormone	FPR Probe	Time after Desensitization	D ($x10^{10}cm^2sec^{-1}$)	%M	cAMP (Relative to Basal)
none	TrITC-LH	1 hr	3.1 ± 0.1^a	72 ± 6^b	4.8 ± 0.4^d
		5 hr	2.0 ± 0.6^a	82 ± 10^b	5.2 ± 0.6^d
	TrITC-hCG	1 hr	2.2 ± 1.5^a	49 ± 2^c	5.7 ± 0.1^d
		5 hr	2.9 ± 0.2^a	45 ± 7^c	4.6 ± 0.1^d
LH	TrITC-LH	1 hr	1.3 ± 0.6^a	81 ± 5^b	1.1 ± 0.2^e
		5 hr	1.4 ± 0.8^a	87 ± 4^b	6.2 ± 0.7^d
	TrITC-hCG	1 hr	3.1 ± 0.7^a	83 ± 9^b	0.9 ± 0.1^e
		5 hr	2.3 ± 0.3^a	41 ± 7^c	5.8 ± 0.4^d
hCG	TrITC-LH	1 hr	2.7 ± 0.2^a	$55 \pm 3^{b,c}$	1.3 ± 0.3^e
		5 hr	2.1 ± 0.5^a	78 ± 7^b	6.5 ± 0.6^d
	TrITC-hCG	1 hr	2.3 ± 0.8^a	49 ± 7^c	1.4 ± 0.3^e
		5 hr	2.1 ± 0.6^a	48 ± 3^c	5.7 ± 0.8^d

[a], [b], [c] Diffusion coefficients and mobile fractions are expressed as the mean ± SD. Values with superscripts differ significantly ($p<0.05$).

[d], [e] Fold increase of intracellular cAMP during and following oLH- or hCG-induced receptor desensitization. Values are expressed as the mean fold increase ± SD. Values with superscripts differ significantly ($p<0.05$).

Fluorescence energy transfer between GFP-LHR-wt and YFP-LHR-wt

We have previously shown that LHR-wt receptors on 293 cells are self-associated following binding of LH or hCG (Roess *et al.*, 2000b). To investigate whether these associations occur in response to hormone binding and persist during receptor desensitization, we measured the fluorescence energy transfer between hCG-treated LH receptors coupled to GFP and YFP. Prior to binding of hCG there was no significant energy transfer between GFP-LHR-wt and YFP-LHR-wt fusion proteins as indicated by a no energy transfer (Figure 15). After desensitization of the receptor by 10 nM hCG, %E increased to 18.2 ± 0.5% at 1 hr. %E decreased while the receptor was desensitized but did not reach 0 until the receptors were again responsive to hCG challenge.

Imaging of Desensitized GFP-LHR-wt

To determine whether self-associated GFP-LHR-wt were present in microscopically visible clusters during receptor desensitization, CHO cells were imaged before and after brief hormone treatment. We have previously shown that both LH and hCG binding to GFP-LHR-wt caused aggregation of the GFP- LHR-wt (Horvat *et al.*, 1999). Here, as shown in Figure 16, fluorescence from GFP was distributed uniformly in the plasma membrane prior to hormone binding. After treatment of CHO cells with 10nM hCG for 30 minutes followed by hormone removal using low pH buffer, there was significant patching of GFP-LHR-wt. The apparent size of these structures was largest 1 hr following hormone treatment. Over the next three hours, the apparent size of clusters containing GFP-LHR-wt was progressively smaller.

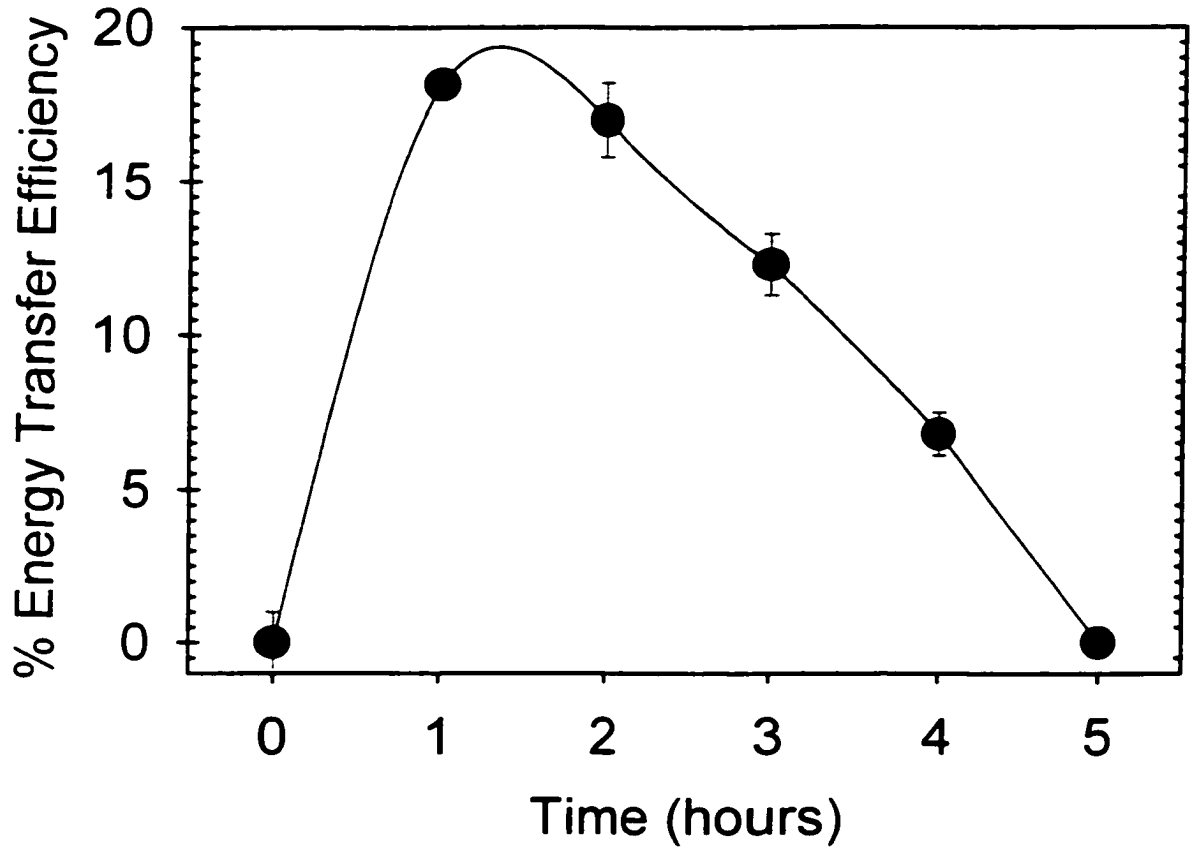
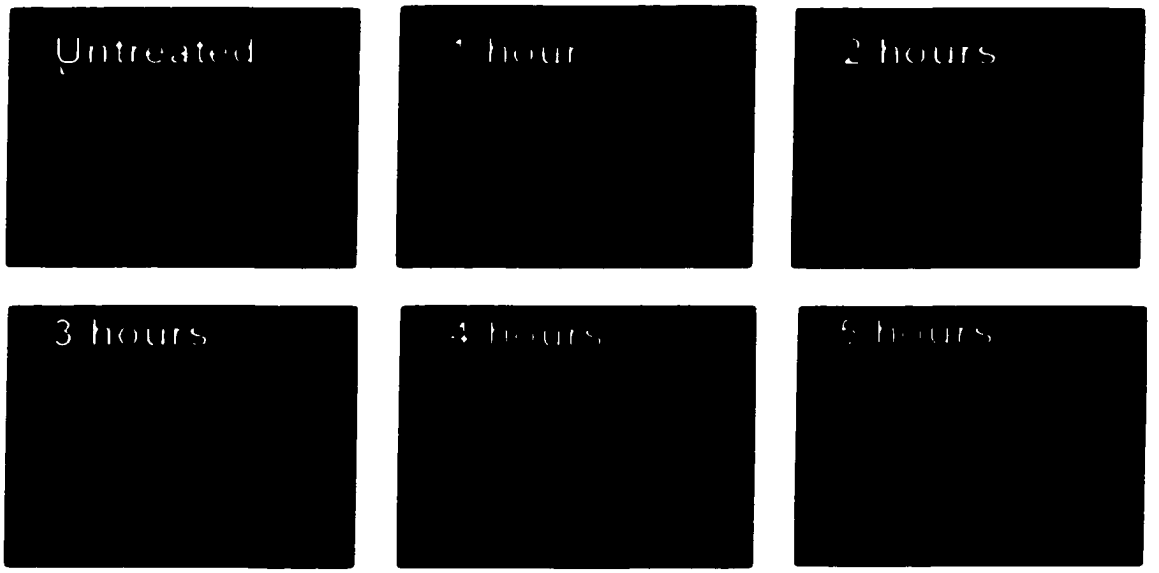


Figure 15: Percent energy transfer efficiency (%E) between LH receptor fusion proteins before (0 hour) and after receptor desensitization (1-5 hours) with 10nM hCG. At 0 and 5 hrs when receptors are hormone responsive, there is no significant energy transfer between LH receptors. When receptors are not hormone responsive there is significant energy transfer between LH receptors. The results are the average and S.D. of 60 measurements on individual cells.

Figure 16: Fluorescence images of GFP-LHR-wt prior to (untreated) and during the time course of receptor desensitization (1-5 hours). When the receptors were capable of signal transduction, fluorescence was diffusely distributed over the membrane (untreated, 5 hours). When receptors are unable to transduce signal, fluorescence from GFP was distributed in discrete patches. At 1 and 2 hours the patches are larger than those seen 3 and 4 hours after hormone-induced desensitization.



By 5 hrs, the receptors were again uniformly distributed over the cell surface.

DISCUSSION

Desensitization of the LH receptor is associated with slower receptor lateral diffusion and a decrease in the fraction of mobile receptors. At early times following treatment with LH or hCG, the diffusion coefficients measured for desensitized GFP-LHR-wt were similar to those measured for LH and hCG-occupied receptors on ovine (Niswender *et al.*, 1985) and rat (Roess *et al.*, 1992a) luteal cells. In spot FPR measurements of LH receptors on ovine luteal cells, most hCG-occupied receptors were immobile on the cell surface, exhibiting less than 20% fluorescence recovery after photobleaching (Niswender *et al.*, 1985). The few mobile LH receptors occupied by hCG had a 7-fold slower diffusion coefficient than did LH-occupied receptors (Roess *et al.*, 1988). Although more ovine LH-occupied receptors were mobile, fluorescence recovery after photobleaching was only about 30% (Niswender *et al.*, 1985), which is considerably less than the 60% fluorescence recovery typical of most membrane proteins (Jacobson, 1983).

Large complexes containing the desensitized receptor, as reflected in the diffusive properties of the desensitized receptor, were formed in response to initial hormone treatment. Prior to binding of hormone, GFP-LHR-wt appeared to be monomeric receptor that was diffusely distributed throughout the plasma membrane. The receptor formed dimers or oligomers only after hormone binding. In addition, binding of fresh hormone, either LH or hCG, did not alter the diffusive properties of the desensitized receptor. The mobile fractions

for receptors desensitized by LH or hCG were characteristic of the desensitized complex regardless of which hormone bound to the receptor at one hour. These results suggest that interactions between the LH receptor and, as examples, proposed lectin receptors (Anderson *et al.*, 1979) or the cytoskeleton (Roess *et al.*, 1998), may occur as the receptor-containing complex is forming. Once formed, physical constraints limit new protein-protein interactions and thus binding of fresh hormone to the desensitized receptor has no affect on the fraction of mobile receptors.

Although the rate of LH receptor lateral diffusion was dependent on whether LH or hCG was used to desensitize the receptor, the magnitude of the cAMP response and the duration of receptor desensitization was not affected by the hormone used to desensitize the receptor. For both LH- and hCG-desensitized receptors, there was no response to hormone binding within one hour following initial hormone exposure which is consistent with *in vitro* studies by Hunzicker-Dunn and coworkers who have demonstrated in a cell-free system that LH receptors on pig Graafian follicles are fully uncoupled from adenylate cyclase within 30 minutes (Bockaert *et al.*, 1976). Similarly, Segaloff and coworkers have shown in intact cells that rat wild type LH receptor expressed in 293 cells is desensitized within one hour (Rodriquez *et al.*, 1991).

Resensitization of the GFP-LHR-wt and LHR-wt occurred within about 5 hours. This is significantly slower than resensitization of the β-adrenergic receptor which occurs within 15-20 minutes following removal of hormone agonist with low pH buffer (Lefkowitz, 1998). Although LH receptor desensitization has been studied *in vivo*, it cannot be examined

independent of down-regulation of receptor number (Conti *et al.*, 1977) and degradation of mRNA transcripts (Camp *et al.*, 1991; Peng *et al.*, 1991). Thus, *in vivo* the reappearance of functional LH receptors on the plasma membrane is slow with times varying from 72 hours (Conti *et al.*, 1976) to 7 days (Conti *et al.*, 1977) depending on cell type and involves receptor replenishment through a very different mechanism than is involved in LH receptor resensitization in this study.

Dimerization or oligomerization occurs in response to hCG binding and persists while the LH receptor is non-responsive. Hebert *et al.*(1996) have suggested that dimerization of the β-adrenergic receptor is essential for receptor signaling. Non-functional receptors can be rescued by antibody-induced receptor dimerization (Hebert *et al.*, 1998) which is mediated by a dimerization sequence in the sixth transmembrane domain (Hebert *et al.*, 1996). There are probably critical differences between the extent of LH receptor and β-adrenergic receptor self-association. The β-adrenergic receptor is forming small homodimers (Hebert *et al.*, 1996) which are detected as discrete punctate structures less than 1 μM in diameter using fluorescence microscopy (von Zastrow, 1992). In contrast, the LH receptor, which lacks this dimerization sequence in its TM6 domain, is present in larger clusters. Luborsky has observed clusters of about 10-20 receptors on rat granulosa cells using electron microscopy following binding of high concentrations of LH (Luborsky *et al.*, 1984). In immunofluorescence studies of LH receptors on rat granulosa cells, there are large, punctate receptor aggregates on the cell membrane following hCG binding (Amsterdam *et al.*, 1980) under conditions in which the receptor was desensitized. Amsterdam *et al* (1980) suggests aggregation of the receptor may be required for desensitization of the receptor and subsequent receptor internalization. As is

the case for β-adrenergic receptor, self-association of LH receptors may be required for receptor signaling. Rat LH receptors containing a single point mutation are able to bind hormone but do not signal or have measurable levels of fluorescence energy transfer between receptors in response to binding of LH or hCG (Roess *et al.*, 2000b). The components of the large molecular weight complexes formed during receptor desensitization are not known. Structures containing LH receptors exhibit very slow rotational dynamics in time-resolved phosphorescence anisotropy studies of receptor rotational diffusion on ovine and bovine luteal cell membranes (Philpott *et al.*, 1995). These larger complexes form only when the hormone-receptor pair is functional, i.e., capable of activating adenylate cyclase, and exhibit significantly slower rotational dynamics than do complexes formed by hormone binding to non-functional receptors or by a non-functional ligand binding to a normally functioning receptor (Roess *et al.*, 2000a). There are indications that these complexes contain proteins other than LH receptors. LH receptors on bovine luteal cell plasma membranes are associated with a family of non-receptor proteins (Meiklejohn *et al.*, 1997). On membranes from porcine granulosa cells, a number of signaling molecules including β-arrestin must also be available for desensitization of the receptor (Mukherjee *et al.*, 1999).

We speculate that ligand binding to LH receptors may also be associated with a redistribution of receptors in the membrane into small membrane domains where signaling and/or receptor desensitization can occur. Although the sequence of events following binding of hormone to receptor is not clear, LH receptor dimerization or oligomerization, is arguably the initial step in signal transduction. This could be followed by the movement of LH receptors into membrane regions containing proteins required for signaling including, for example, G

proteins, effector proteins, and proteins for desensitization. Alternatively, the signaling could occur outside of membrane domains and be followed by movement of receptors into membrane domains containing proteins necessary for desensitization of the receptor. In addition to interactions with β-arrestins, clustering of receptors into very large complexes may, as Amsterdam *et al.* have suggested , interfere with receptor response to hormone (Amsterdam *et al.*, 1980). In either case, one would predict that the fraction of immobile receptors would increase upon desensitization of the receptor which we, in fact, observe. Dissociation of the receptor from complexes needed for signaling and/or desensitization would result in an increase in the fraction of mobile receptors and the average diffusion coefficient for the receptor population. However, there is no productive signaling until a sufficiently large population of freely diffusing receptors and/or molecules necessary for signal transduction were again available outside specialized membrane domains.

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CHAPTER FIVE

CHARACTERIZATION OF AN INTRINSICALLY FLUORESCENT GnRH RECEPTOR AND EFFECTS OF LIGAND BINDING ON RECEPTOR LATERAL DIFFUSION

INTRODUCTION

Since the elucidation of the primary amino acid sequence of gonadotropin releasing hormone (GnRH) over 25 years ago, a clear picture of the key role of this molecule in controlling reproductive function in mammals has emerged. The pulsatile discharge of GnRH from hypothalamic neurosecretory neurons not only stimulates, but is obligatory for, the synthesis and secretion of luteinizing hormone (LH) and, to a lesser extent, follicle stimulating hormone (FSH) from the anterior pituitary gland (Hamernik and Nett, 1988; Gharib *et al.*, 1990). Not surprisingly, changes in GnRH secretory rates are a primary regulator of gonadotropin secretion (Gharib *et al.*, 1990). Similarly, changes in the number of pituitary

receptors for GnRH have also been implicated as an important mechanism underlying the regulation of gonadotropin secretion (Crowder and Nett, 1984; Sealfon *et al.*, 1990; Hamernik *et al.*, 1995). Thus changes in pituitary content and secretion of LH may depend not only on changes in GnRH availability but also on the number of GnRH receptors (GnRHR) available for binding and consequently the responsiveness of gonadotropes to GnRH (Wise *et al.*, 1984). With recent availability of cDNAs encoding GnRHR (Tsutsumi *et al.*, 1992; Reinhart *et al.*, 1992; Eidne *et al.*, 1992; Chi *et al.*, 1993; Kakar *et al.*, 1993), researchers have found that changes in the number of GnRHR are correlated with concomitant fluxes in steady state concentrations of mRNA for the GnRHR (Hamernik *et al.*, 1995; Turzillo *et al.*, 1995; Wu *et al.*, 1994; Kaiser *et al.*, 1997; Turzillo *et al.*, 1994), thus implicating transcriptional regulation as an important component underlying the number of GnRH receptors in the pituitary gland. Consistent with this, we have found that the promoter of the GnRHR gene is hormonally regulated in transgenic mice (McCue *et al.*, 1997).

Not only has the GnRHR cDNA proven to be a probe for gene expression but it has also allowed investigations of structure-function relationships that exist in this protein. In this regard, the cDNA for the murine GnRHR predicts a protein containing seven hydrophobic amino acid domains and suggests membership of the GnRHR in the G-protein coupled receptor superfamily (Tsutsumi *et al.*, 1992), a classification that is consistent with the proposed mechanism(s) of action (Kaiser *et al.*, 1997; Sealfon *et al.*, 1997). The predicted GnRHR structure is, however, somewhat unusual in that it possesses an extremely short intracellular carboxyl terminus of only 1 to 2 amino acids. This structural difference alone raises interesting questions as to the functional domains of this molecule required for trafficking, signal

transduction and internalization. In fact, significant progress has been made in dissecting functional domains of the GnRHR (Kaiser *et al.*, 1997; Sealfon *et al.*, 1997); however, large gaps remain in our understanding of this molecule. In particular, the absence of any antibodies capable of recognizing the GnRHR *in situ* has precluded any analysis of the GnRHR in the unbound state. Thus it has been difficult to monitor the rate of receptor biosynthesis, its trafficking through various intracellular compartments, and its behavior in the plasma membrane. Nonetheless, these steps have been implicated as important regulatory points for controlling the number of GnRH receptors availability for hormone binding and signal transduction (Tsutsumi *et al.*, 1993; 1995).

As an alternative to immunological detection of the GnRHR, we have generated a fusion protein consisting of codon-optimized, "enhanced" green fluorescent protein (GFP) derived from *Aqueorea victoria* (Chalfie *et al.*, 1994) and the murine GnRHR. This approach was selected based on several considerations. First the fusion of GFP to a number of different proteins has not interfered with protein function or affected the fluorescent properties of GFP (Tarasova *et al.*, 1997; Barak *et al.*, 1997; Hampton *et al.*, 1998; Htun *et al.*, 1996; Girotti and Banting, 1996; Wacker *et al.*, 1997). Second, since the fusion protein is intrinsically fluorescent, there are no problems of substrate availability to an enzyme marker. Finally, since GFP fluoresces *in vivo*, the use of this molecule as a fluorescent "tag" permits real-time imaging of the fusion protein and observations of regulation and trafficking in living cells (Tarasova *et al.*, 1997; Barak *et al.*, 1997; Hampton *et al.*, 1998; Htun *et al.*, 1996; Girotti and Banting, 1996; Wacker *et al.*, 1997).

In addition to allowing intracellular observations, an intrinsically fluorescent GnRHR also allows the application of various laser-optical methods, such as fluorescence photobleaching and fluorescence energy transfer, to study the behavior of this receptor in the plasma membrane in both the bound and unbound state. In fact, just such an approach was used to measure the lateral diffusion of a fusion protein consisting of the β-adrenergic receptor and GFP by fluorescence photobleaching recovery techniques (Barak *et al.*, 1997). Interestingly, the β-adrenergic receptor, which could be regarded as a prototype for signal transduction by G-protein coupled receptors, had a fast and apparently unrestricted lateral diffusion in the unbound state (Barak *et al.*, 1997). Herein, we report that a C-terminal fusion of GFP or yellow fluorescent protein (YFP) to the coding region of the GnRHR results in a fusion protein that is appropriately transported to the plasma membrane of mammalian cells and, for the first time, allows for direct observation of the lateral diffusion and aggregation GnRHR in the ligand and non-ligand bound state.

MATERIALS AND METHODS

Chemicals

Unless otherwise noted, all enzymes were from New England Biolabs (Beverly,MA). All other reagents were the highest grade available.

Preparation of murine GnRHR-GFP and GnRHR-YFP fusion vectors

Using Deep Vent Polymerase (New England Biolabs), a C-terminal fusion of the

murine GnRHR (GnRHR) was generated by polymerase chain reaction (PCR) of a cDNA containing the full coding sequence of the mGnRHR in pBSK (7), using a T3 specific primer (AATTAACCCTCACTAAGGG) and a gene-specific primer which eliminates the stop codon and substitutes a BamHI restriction enzyme site (underlined) at the 3' end (CAAGGATCCCCAAAGAGAATACCCATATATG) to create an in-frame restriction site. The PCR product was then digested with EcoRI and BamHI and ligated into pEGFP-N_2 or pEYFP-N_2 vectors (Clonetech, Palo Alto,CA) cut with the same enzymes. This resulted in a fusion protein consisting of the mGnRHR, a spacer sequence of amino acids IHRPVAT, and EGFP or EYFP. The identity of the fusion cDNA was confirmed by sequencing (Macromolecular Resources, Fort Collins, CO).

Cell Culture

Cells were maintained in high glucose Dulbecco's Modification of Eagle Media (DMEM; Mediatech, Herdon,VA) containing 2nM glutamine, 100U penicillin/ml, 100µg streptomycin/ml, 10% fetal bovine serum (FBS) (Gemini Bioproducts, Calabasas, CA) and 1x non-essential amino acids(Gibco BRL, Gaithersburg, MD) in a 5% CO_2, 37°C humidified environment.

Construction of stable cell lines expressing GnRHR-fusion proteins

αT3 cells were transfected using Lipofectamine-Plus (Gibco-BRL) per the manufacturer's instructions with 8 µg of mGnRHR-GFP. Cos-7 and CHO cells were

transfected using Lipofectamine (Gibco-BRL) and 16μg mGnRHR-GFP. Sixteen hours after transfection the media was changed and G-418 (Mediatech) was added to a final concentration of 500μg/ml. After two weeks selection in G-418, the cells were trypsinized, diluted 1:100 incomplete media plus G-418, plated in 150mm plates and incubated until single colonies became visible. Colonies were transferred into 24 well plates in the same media using sterile applicator swab and grown to confluency. A sample from each colony was examined under mercury arc lamp illumination using a 20x objective on a Nikon Optiphot microscope and fluorescein-selective filter set. The clones expressing the most intense fluorescence in each cell line were used in the subsequent studies.

0.4mg of mGnRHR-GFP and/or 1.2mg of mGnRHR-YFP were transfected into CHO cells using Lipofectamine Plus Reagent (Life Technologies, Rockville MD) in a 35mM plate (ISC BioExpress, Kaysville UT). After overnight culture, the cells were transferred into a 150 mm plate and cultured with 600mg/mL G418 (Gemini Bioproducts, Woodland CA) for seven days, washed with PBS, and cloned by limiting dilution in 96 well plates (ISC BioExpress). Wells were examined with epifluorescence for fluorescent cells. Those cells expressing fluorescence were expanded and used for studies.

Confocal Imaging

To assess the cellular localization of GnRHR-GFP, the αT3, CHO, and Cos-7 cell lines expressing fusion protein were plated in complete medium onto No.1 coverslips for 16-24 hours prior to the imaging studies. Coverslip with dispersed, isolated cells were stained with 10μg/mL of ice-cold Alexa 594 Concanavalin A (Con A; Molecular Probes, Eugene, OR) in

phosphate buffered saline (PBS). The coverslips were rinsed twice with ice cold PBS and fixed with 4% freshly prepared formaldehyde for 5 minute. After a further two washes with ice-cold PBS, the coverslip were mounted in 40% glycerol and the edges were sealed with rubber cement. Images were acquired with a Sarastro 2000 confocal laser-scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA) in an epifluorescence mode. The 488nm and 524nm lines of an argon ion laser were used to excite the sample, a 595nm dichroic mirror was used to separate red/green fluorescence, a 535nm long-pass filter was used to isolate the GFP signal and a 600nm long pass filter was used to further isolate the red Con A signal. A 50μm pinhole was used. Images were saved on a optical disk, exported to Adobe Photo Shop for image processing and printed on a Kodak color printer.

Colocalization of red and green signal was quantified using ImageSpace Software (Molecular Dynamics) and "masks" of the Alexa Con A. The extent of Alexa Con A and GnRHR-GFP colocalization were expressed as the percentage of total green fluorescence within a single cell using the measurement routine in ImageSpace Software provided for confocal microscope. One mask was generated utilizing the red fluorescent signal that encompassed the entire cell to measure the total green fluorescence and a second mask was generated utilizing the red fluorescence that demarcated the inner limit of the membrane and measured the amount of green fluorescence that was not co-localized with the membrane marker. The amount of fluorescence that co-localized with the membrane marker was determined as the difference of total fluorescence minus the internal fluorescence. All results were expressed as a percentage of the total fluorescence for each cell. A minimum of twelve cells from each cell line were analyzed.

Scatchard analysis

Approximately 200,000 cells/well from the αT3 and αT3 GnRHR-GFP cell lines and 100,000 cells/well of the CHO cells expressing GnRHR-GFP, GnRHR-YFP and GnRHR-GFP/GnRHR-YFP were plated in 24 well plates (Falcon) and cultured overnight. Concentrations of freshly prepared (^{125}I)-D-Ala-6 GnRH; (specific activity 1.5mCi/pmol) between 4.5nM and 20pM in 150μL of ice cold complete media were then added to each well in the presence or absence of 260nM of unlabeled D-Ala-6 GnRH. Cells were incubated on ice for 4 hours and then washed twice with 1 ml of 170mM NaCl, 3mM KCl, 10mM Na_2HPO_4, and 4mM K_2HPO_4, pH 7.4 (PBS) containing 2 mg/mL bovine serum albumin (BSA). The cells were lysed in 100 ml of 2% SDS (sodium dodecyl lauryl sulfate) and counted on a Apex Automatic Gamma Counter (Micromedic Systems Inc., Horsham PA). Specific counts were determined as total cpm bound less the cpm bound in the presence of 40 pmol of D-Ala GnRH. Independent experiments were conducted and the pooled data were analyzed by a non-linear regression using GraphPad Prism Software (GraphPad Software Inc, San Diego,CA). A one site model provided the best fit of the data.

Transient Transfection of mGnRHR-GFP.

CHO cells stably expressing the mGnRHR-GFP were plated at approximately 2 x 10^5 cells/well in 6 well plates in complete media and incubated overnight. The following morning cells were transfected for 5 hours in serum free DMEM with 5μl of Lipofectamine (Gibco BRL) and a reporter vector consisting of a 1600bp of the human alpha gonadotropin subunit

promoter fragment fused luciferase in pGL3 (Promega, Madison, WI). To control for transfection efficiency, the cells were co-transfected with 250ng of a Rous Sarcoma Virus-β-galactosidase construct (Duval *et al.*, 1997). After 5 hours of transfection, 1 ml of media containing 20% FBS, 200 U/ml penicillin and 200 mg/ml streptomycin was added and the cells incubated overnight. The following morning, 0, 0.01-1000nM GnRH, 1000nM GnRH plus 100nM Antide were added to triplicate wells and incubated a further 4 hours. The cells were then washed two times with ice-cold PBS and lysed in 200 ml of 25mM glyclyglycine, 15mM $MgSO_4$, 1mM dithiothreitol (freshly added), and 1% Triton X-100, pH 7.8 and the lysate cleared by centrifugation at 16,000x g for 2 minutes. Two microliters of the lysate were read in 100µl of luciferase assay buffer (Promega, Madison WI) and an additional two microliters were read in 200µl of Galacto-Light Assay Buffer (Tropix, Bedford MA) as per manufacturers instructions in a Turner 20-d luminometer. All results were normalized as luciferase value/ β-galactosidase value.

cAMP assays

Approximately 2 x 10^6 CHO cells expressing GnRHR-GFP were incubated with 0.01-100nM GnRH, D-ala, or Antide in a total volume of 500µl in PBS for 1 hour at 37°C. Reactions were terminated by the addition of 500 µL of ice-cold 10% trichloroacetic acid and centrifugation at 300xg for 5 minutes to remove insoluble material. The supernatant was then extracted 3 times with 3 ml of diethyl ether and the aqueous phase dried under nitrogen. The residue was resuspended in a 50nM sodium acetate buffer pH 6.2 (PE Biosystems, Norwalk,

CN) and cAMP was assayed using the TiterFluor Dual Range cAMP enzyme-linked immunoassay using goat anti-rabbit IgG coated plates (Pierce, Rockford, Il). Fluorescence was determined from a standard curve generated at the same time as the assay.

Fringe and spot fluorescence photobleaching recovery of GnRH-GFP lateral diffusion.

To assess the effect of GnRH, D-ala or Antide on the GnRHR receptor dynamics, various concentrations of GnRH, D-ala or Antide were added for 5 minutes prior to the acquisition of data and remained in the cell medium for the duration of the experiment which was approximately 45 minutes. The equipment and methods used for performing spot and fringe fluorescence photobleaching recovery (FPR) measurements have been described in detail elsewhere (Munnelly *et al.*, 1998). In these studies, all measurements were performed at 37^{0}C using a Zeiss Axiomat-based instrument and a Zeiss 40x microscope objective. For spot FPR measurements on individual cells, a Coherent Radiation Innova 100 argon ion laser interrogated an area of the cell with a $1/e^2$ radius of 0.41μm. The laser provided power of 53μW in the bleaching beam and 0.2μW in the probe beam at 488nm. For the fringe measurements, the fringe spacing was approximately 2.2μm and, at the sample, the illuminated area had a $1/e^2$ radius of 29μm. The photometer acceptance region was large enough to encompass the entire cell. In fringe fluorescence photobleaching recovery measurements, higher total powers of 11mW in the bleaching pulse and 33μW in the probe beam were used because of the larger illuminated area. Fluorophore bleaching times were 400 msec and 150 msec in fringe and spot FPR measurements, respectively. In an individual FPR experiment, data was acquired for 20 sec prior to fluorophore bleaching and for 30 sec postbleach at a rate

of 50 msec/point. FPR data were processed on-line. The unadjusted raw data were represented directly in terms of the various parameters associated with a given measurement including the prebleach and immediate postbleach fluorescence levels, the extent of fluorescence recovery after photobleaching, and an appropriate function in the fringe and spot modes to represent the recovery kinetics in terms of a decay half-time $t_{1/2}$. To reduce the contribution of cell autofluorescence, unlabeled, non-transfected cells were examined in parallel experiments on at least 20 cells under the same conditions used for cells expressing GFP-GnRH receptors. Individual data points were summed and averaged and this average was subtracted from data obtained on receptor-expressing cells. The diffusion coefficient was evaluated in background subtracted data making use of the time $t_{1/2}$ at which fluorescence recovery was half-complete and known optical parameters. Because the observed fluorescence values in spot and fringe FPR measurements are obtained by photon counting, Poisson statistics apply and each point is assigned a statistical weight inversely proportional to its own magnitude. For data in perfect agreement with the models, a reduced χ^2 value equal to the number of points taken minus four is expected. We accepted only individual experiments where reduced χ^2 was no more than twice this ideal value.

Spot fluorescent energy transfer between GnRHR-GFP and GnRHR-YFP

Fluorescence energy transfer between GFP- and YFP-GnRHR was evaluated based on the reduced rate of irreversible photobleaching of GFP when YFP was present (Young *et al.*, 1994). Slower rates of fluorescence decay for cells expressing the GFP-GnRHR and YFP-GnRHR acceptor (D+A) than for cells expressing GFP-GnRHR only (D) were indicative of

energy transfer from fluorescence donor to acceptor and occurred only when the donor and acceptor were separated by distances less than R_o a characteristic of the specific donor/acceptor pair, for GFP and YFP this distance is 51 Å (Barsias, unpublished data). To perform these experiments, we used a fluorescence microscope photometer based on the inverted-configuration Zeiss Axiomat microscope and associated components used for spot fluorescence photobleaching recovery measurements. Fluorescence excitation was provided by a Coherent Radiation Innova 100 argon ion laser operating under light control at 488nm. The intensity of the laser radiation focused on the cell was 45μW and this was held constant between measurements on GnRHR-GFP cells or on GnRHR-GFP/YFP cells . The $1/e^2$ Gaussian spot diameter was 0.41μm. Donor fluorescence from GFP was isolated with a standard fluorescein filter set together with a short pass fluorescein-selective filter to remove yellow fluorescence from the YFP-tagged GnRHR. This combination was highly effective in rejecting YFP fluorescence. In individual experiments cells were identified and centered in the microscope field. At time zero, an electronically controlled shutter was opened to allow laser radiation to impinge on the cell. Simultaneously, a computer program was activated to record the output of the photomultiplier measuring membrane fluorescence. Data were collected at 0.01 sec intervals for 10 sec. Typically, 20 cells in each sample were photobleached in this manner. Cells expressing GnRHR-YFP alone produced signals that did not differ significantly from those of unlabeled cells when examined with a fluorescein-selective filter set. Signal from cells expressing GnRHR-GFP or GnRHR-GFP/YFP was greater than 4-fold higher than background levels. The data traces were analyzed to give the energy transfer efficiency (%E) as has been described in detail previously (Young *et al.*, 1994).

Statistics

Normalized luciferase and cAMP data were analyzed by Dunnett's T test (SAS, SAS Institute, Cary, NC). FPR data were analyzed by ANOVA using SigmaStat (Jandel Scientific, San Rafel, CA). If the F-test was significant ($p<0.05$), means were separated using the least significant difference (LSD) criterion.

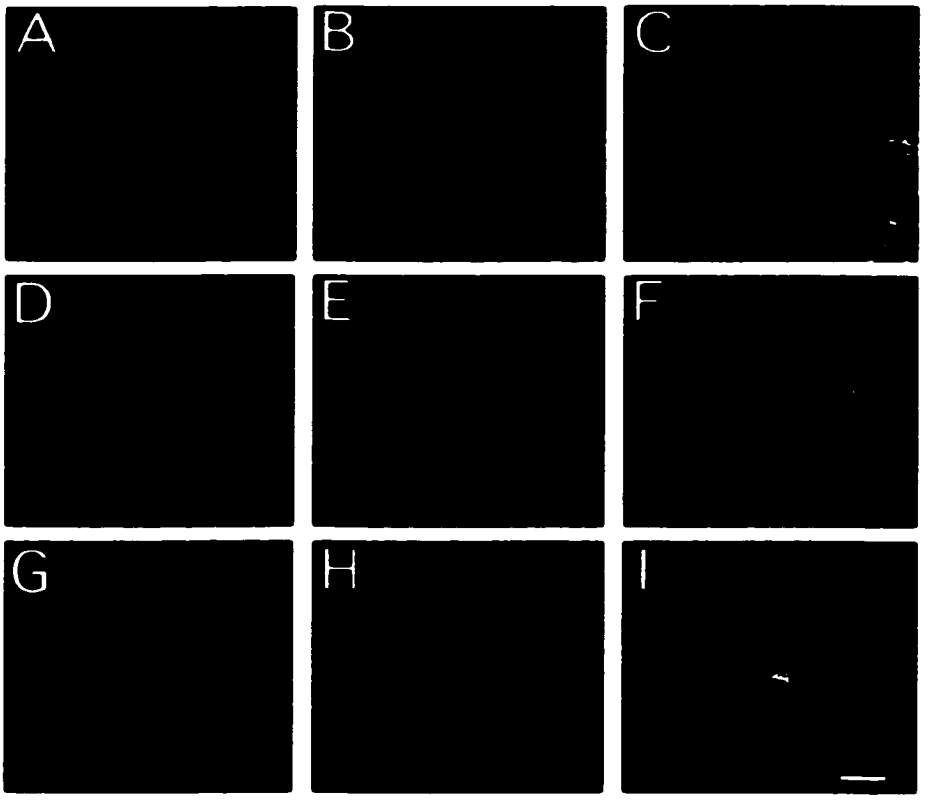
RESULTS

The GnRHR-GFP fusion protein was trafficked to the plasma membrane of mammalian cells

Confocal laser scanning microscopy was used to determine if the green signal resulting from the GnRHR-GFP fusion protein co-localizes with that of an Alexa concanavalin A (ConA). This red fluorescent derivative of concanavalin which to the plasma membrane of each of the 3 cell lines constructed and is shown as the isolated red signal in Figure 17, panels A, D, and G. A similar distribution of the green fluorescent signal resulting from the GnRHR-GFP is seen as the green signal in panels B, E, and H. Shown in panels, C, F, and I are the superimposition of the two signals, the overlap of the red and green is shown in yellow. Panel A-c are form a Cos-7 GnRHR-GFP cell line, panels D-F are from an αT3 cell line and G-I are from a CHO GnRHR-GFP cell line.

The percent of the fusion protein (green signal) that co-localized with the Alexa Con A mask (red signal) varied from 38.5 ± 12.5% in αT3 to 82.6 ± 7.3% in the CHO cell line. The

Figure 17: Scanning laser confocal microscopy images of cell lines expressing GnRHR-GFP. In panels A, D, and G are the signals from Alexa concanavalin -A. Panels B, E, and H are the green fluorescence signals resulting from the GnRHR-GFP fusion protein. In panels C, F, and I is the result of superimposition of the red and green fluorescent signals shown in yellow. Panels A-C are images obtained from Cos-7 GnRHR-GF cells. Panels D-F and G-I are images obtained from GnRHR-GFP expressing αT3 cells and CHO cells, respectively. The bar in panel I is 5μm for each image. Overall magnification is 1700x for all panels.



expression level of the GnRHR-GFP in αT3 and CHO cells was quite uniform; however, expression in the Cos-7 was generally much lower and much more variable. For this reason, subsequent analyses were confined to the αT3 and CHO expressing cell lines. Also, it is important to point out that the pattern of protein expression differs markedly from that of unfused GFP. When GFP alone was expressed, it was primarily found intracellularly with a high percentage of the protein apparently localized to the nucleus with the remainder distributed throughout the cytoplasm. A similar distribution has been reported previously in Cos-7 cells (Moriyoshi *et al.*, 1996). Similar results were determined for the GnRHR-YFP and the GnRHR-GFP/GnRHR-YFP expressing cell lines.

GnRHR-fusion proteins bound hormone with an affinity similar to the wild-type GnRHR and was capable of signal transduction

To determine if the GnRHR-fusion proteins displays binding kinetics similar to wild-type GnRH, binding studies were performed on non-transfected αT3 cells, which normally express the GnRHR (Kaiser *et al.*, 1997; Windle *et al.*, 1990), and the CHO and αt3 cell line expressing the GnRHR-GFP. Iodinated des-Gly^{10},D-ala^{6}-GnRH-N-ethyl amide bound to GnRHR-GFP with a K_d of 0.8 nM, a value similar to that calculated for the wild-type GnRHR-GFP in non-transfected αT3 cells. The number of receptors present in the plasma membrane of the three cell types, αT3, CHO and Cos-7, was however, more variable. We found approximately 13,300 and 20,800 receptors per cell in the CHO and αT3 GnRHR-GFP cell lines, respectively. Binding of des-Gly^{10}, D-ala^{6}-GnRH-N-ethylamide to wild type CHO cells was non-detectable.

CHO cells expressing GnRHR-YFP had a Kd of 1.3 nM similar to that of cells expressing the GnRHR-GFP alone. The number of receptors per cells present was 31,000. CHO cells co-expressing the GnRHR-GFP and -YFP receptors had a higher Kd, 4.4 nM, but also had significantly more receptors per cell (300,000 receptors/cell).

To determine if GnRHR-GFP was capable of signal transduction, a plasmid containing approximately 1600bp of proximal promoter of the human α subunit gene was fused to the cDNA encoding luciferase was tested for GnRH responsiveness after transient transfection into CHO GnRHR-GFP cell lines. This 1600bp promoter has previously been shown to be responsive to GnRH (Kaiser *et al.*, 1997; Bokar *et al.*, 1988; Hamernik *et al.*, 1992). Consistent with this, treatment of CHO GnRHR-GFP cells with increasing concentrations of GnRH led to a dose-dependent increase in the expression of luciferase. Furthermore, the addition of 100 nM Antide, a competitive inhibitor of GnRH, effectively blocked GnRH stimulation of the human α subunit promoter (data not shown). Thus GnRHR-GFP not only bound hormone with high affinity similar to wild type but was also capable of transducing a GnRH signal to the level of gene expression. To further confirm that the fusion receptors were capable of signal transduction, intracellular concentration of cAMP in the CHO GnRHR-GFP, GnRHR-YFP and GnRHR-GFP/-YFP cell lines were measured one hour after administration of increasing concentrations (0, 0.01nm-100nM) of GnRH, D-ala-6-GnRH, or Antide (Figure 18). Addition of GnRH and D-ala^6-GnRH to the cells resulted in a dose-dependent increase in intracellular cAMP concentration with an ED_{50} similar to the K_d of the wild type GnRH receptor (Kaiser *et al.*, 1997). Antide, did not elicit a second messenger response even at the highest concentrations in any of the cell lines (Figure 18). Consistent with the absence of

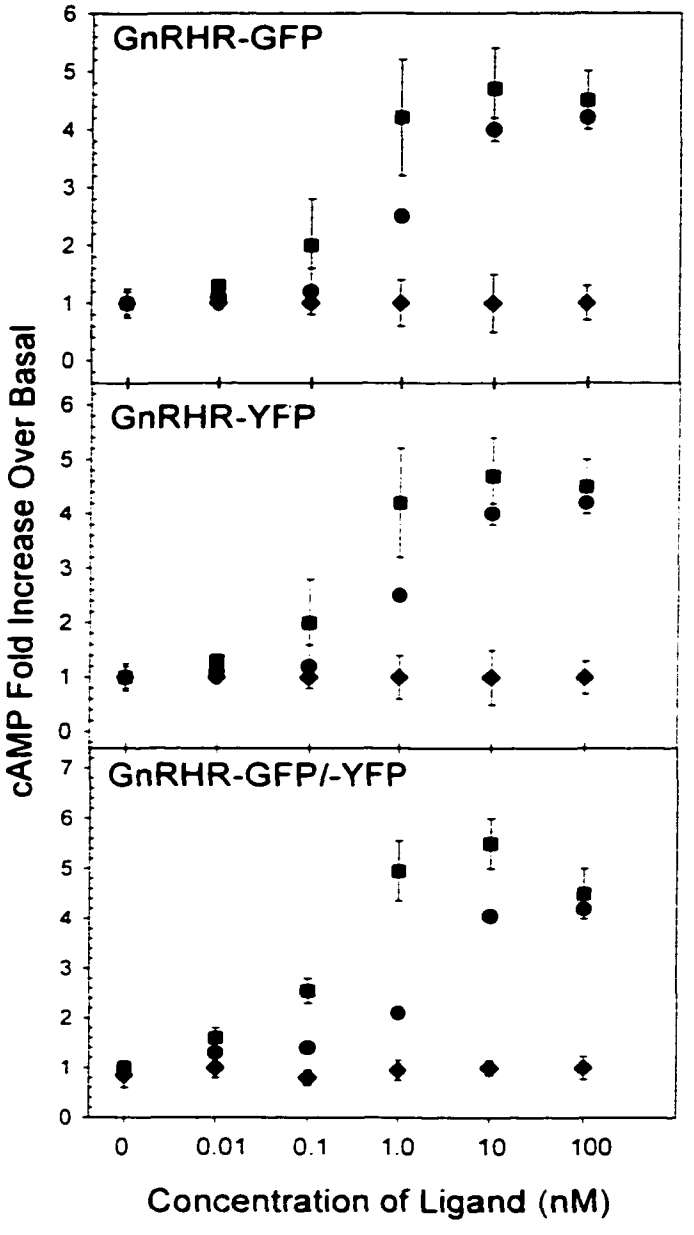


Figure 18: CHO cells expressing the different GnRHR-fusion proteins were capable of transducing a dose-dependent second messenger response following binding of agonist, GnRH (●) or D-ala-6-GnRH (■). There was no increase in response to binding of antagonist Antide (♦) to the GnRHR-fusion proteins. Values are expressed as the fold-increase in cAMP over the untreated control and represented as the mean ± SD of four measurements performed in triplicate.

GnRHR, cAMP concentrations in the untransfected CHO cells were not affected by GnRH (data not shown).

Lateral Diffusion of GnRH-GFP

To determine whether the GnRHR-GFP was laterally mobile in the membrane, we examined its membrane dynamics in GnRHR-GFP expressing αT3 and CHO cells before and after binding of GnRH and Antide using spot and fringe FPR methods. The unbound GnRHR-GFP receptor expressed in αT3 cells displayed a lateral diffusion coefficient of approximately 12 -16 $x10^{-10}cm^2sec^{-1}$ whether measured by spot (Table 5) or fringe (Table 6) FPR. Furthermore, the estimates of the mobile fraction (%M) using either spot (79%) or fringe (76%) suggested that the majority of the receptors were laterally mobile. Similar values were obtained with the CHO GnRHR-GFP cell line (Table 5 and 6) and the Cos-7 cell line (data not shown). To examine the effect of ligand binding on diffusion of the receptor, GnRHR-GFP were treated with 1 μM GnRH for 5 minutes prior to and during FPR measurements which were completed within 45minutes. The binding of GnRH not only slowed the rate of receptor lateral diffusion almost 3-fold to approximately $3.8 \times 10^{-10}cm^2sec^{-1}$ based on both spot (Table 5) and fringe FPR (Table 6) but also reduced the mobile fraction from about 80% to 50%. Incubation of cells with 1 μM Antide reduced the rate of GnRHR-GFP lateral diffusion to values similar to those of the GnRH occupied receptors. In contrast, the addition of Antide had little effect on the fraction of mobile receptors which remained in the range of 75%. The effects if GnRH and Antide on membrane dynamics of GnRHR-GFP in CHO cells were similar to those observed in αT3 cell lines (Table 5 and 6).

TABLE 4

LATERAL DIFFUSION CHARACTERISTICS OF GnRHR-GFP IN αT3 AND CHO CELLS USING SPOT FPR.

	Lateral Diffusion $D(cm^2/sec)x10^{-10}$	Mobile fraction (%M)	N[a]
GnRHR-GFP αT3			
Unbound	12.0 ± 6.0^b	79 ± 7^d	16
1μM GnRH	3.8 ± 0.2^c	52 ± 11^e	15
1μM Antide	3.8 ± 0.8^c	76 ± 3^d	16
GnRHR-GFP CHO			
Unbound	12.0 ± 2.0^b	76 ± 6^d	52
1μM GnRH	6.1 ± 0.2^c	55 ± 1^e	29
1μM Antide	6.0 ± 0.3^c	76 ± 1^d	30

[a] Number of cells for which measurements were obtained.

[b,c] Values are expressed as the mean D ± SD. Values with superscripts differ significantly ($p<.05$).

[d,e] Values are expressed as the mean %M ± SD. Values with superscripts differ significantly ($p<0.05$).

TABLE 5

LATERAL DIFFUSION CHARACTERISTICS OF GnRHR-GFP IN αT3 AND CHO CELLS USING FRINGE FPR

	Lateral Diffusion $D(cm^2/sec)x10^{-10}$	Mobile fraction (%M)	N[a]
GnRHR-GFP αT3			
Unbound	13.0 ± 2.0[b]	76 ± 7[d]	46
1μM GnRH	3.8 ± 1.0[c]	44 ± 3[c]	35
1μM Antide	5.3 ± 1.0[c]	76 ± 4[d]	29
GnRHR-GFP CHO			
Unbound	16.0 ± 1.7[b]	91 ± 4[d]	47
1μM GnRH	6.6 ± 0.7[c]	61 ± 7[c]	45
1μM Antide	6.4 ± 0.4[c]	76 ± 7[d]	47

[a] Number of cells for which measurements were obtained.

[b,c] Values are expressed as the mean D ± SD. Values with superscripts differ significantly ($p<0.05$).

[d,e] Values are expressed as the mean %M ± SD. Values with superscripts differ significantly ($p<0.05$).

We then examined whether the rate of GnRHR-GFP lateral diffusion was influenced by concentration of available ligand. We measured the lateral dynamics of the GnRHR-GFP using spot FPR in the presence of increasing concentrations (0.01 nM - 100 nM) of agonist (GnRH and D-ala) and antagonist (Antide). In these studies, the unoccupied receptor exhibited a diffusion coefficient D of 18.5 ± 3.2 $x10^{-10}cm^2sec^{-1}$. D decreased significantly when cells were incubated with 0.1 nM GnRH and continued to slow in a concentration-dependent manner until D reached 3.5 ± 1.5 $x10^{-10}cm^2sec^{-1}$ at 10 nM GnRH. Increasing the concentration of GnRH had no further effect. Incubating cells with D-ala-6-GnRH produced a similar dose-dependent decrease in D, however, the dose-response curve was left-shifted. The presence of the antagonist Antide slowed receptor diffusion in a concentration-dependent manner and resulted in diffusion coefficients similar to those observed with D-ala-6-GnRH treated cells.

Dose-dependent effects of GnRH and D-ala-6-GnRH on the fraction of mobile receptors were also observed (Figure 19). Binding of 1.0 nM GnRH to the GnRHR-GFP reduced the mobile fraction to 61 ± 1%. At a concentration of 0.01 nM, D-ala-6-GnRH decreased the mobile fraction from 71 ± 3% to 61 ± 2%. Both hormones maximally decreased the fraction to 53% at concentrations of 100 nM and 1 nM, respectively. The fraction of mobile Antide-occupied receptors was never significantly affected by ligand and ranging from 63-73%.

Energy transfer occurs between agonist-occupied GnRHR

The biological function of some serpentine receptors, including the β-adrenergic

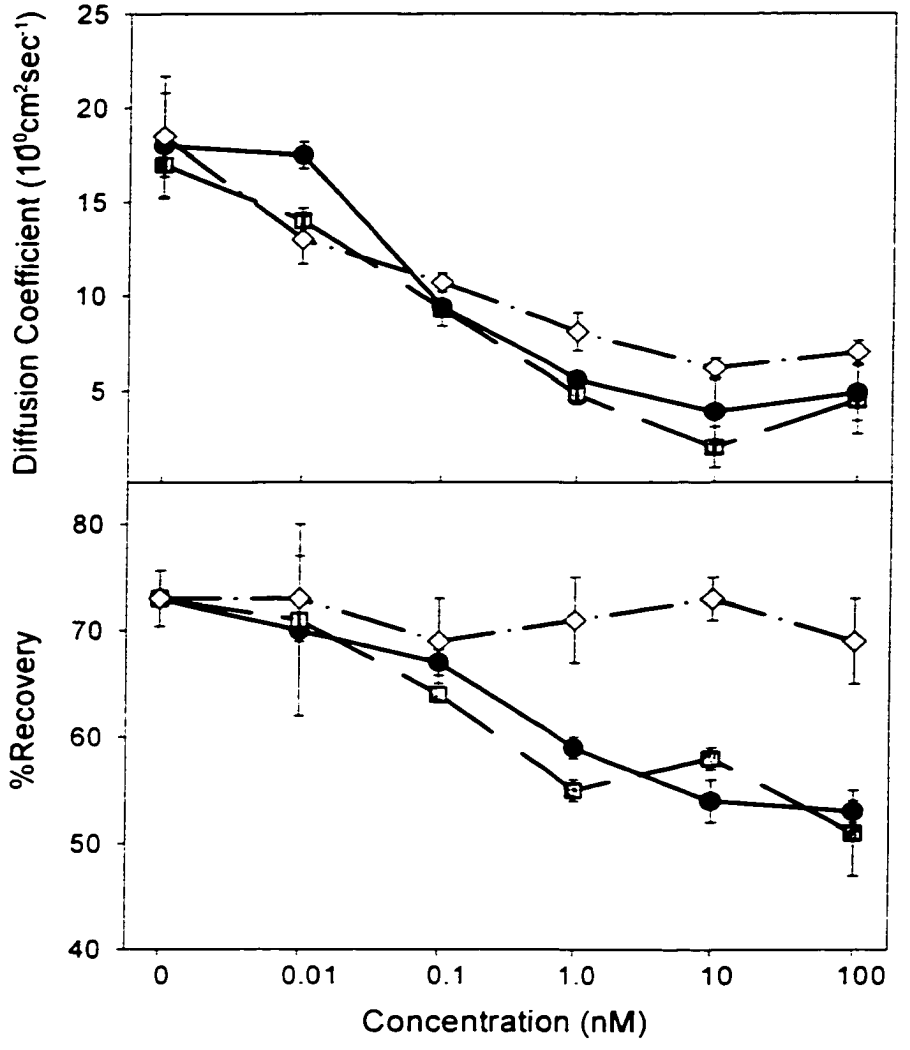


Figure 19: GnRHR-GFP undergoes a dose-dependent decrease in the diffusion coefficient D when bound by either GnRH (●), D-ala-6-GnRH (■), or Antide (♦). However, % recovery (% R) decreases only when receptors are occupied by GnRH (●) or D-ala-6-GnRH (■). The fraction of mobile receptors *does not* decrease when receptors are occupied by Antide (♦) at concentrations as high as 100nM.

receptor (Hebert *et al.*, 1996), M3 muscarinic receptor (Maggio *et al.*, 1993), and LH receptor (Roess *et al.*, 2000b), is accompanied by receptor self-association. As proposed by Janovick and Conn (1996b), the immobilization of a significant percentage of the receptors may be due to the ability of agonist-occupied receptors to undergo self-association, while the antagonist-occupied receptor can not. To determine whether this was the case, we measured energy transfer efficiency (%E) between GFP- and YFP-GnRHR. Representative data traces showing fluorescence energy transfer between unoccupied, GnRH- and Antide-occupied receptors are presented in Figure 20.

There was no significant energy transfer between GnRH receptors prior to the introduction of ligand, as shown in Figure 21. However, increasing concentrations of GnRH resulted in a maximum value for %E of 16 ± 1% at a concentration of 10 nM GnRH. Similarly increasing concentrations of D-ala produced a left-shifted dose-response curve for %E, reaching a maximum of 17 ± 1 % when cells were incubated with 1.0nM D-ala-6-GnRH Antide had no significant effect on energy transfer efficiency at any concentration. Thus, binding of the GnRHR with agonist but not antagonist results in receptor association into structures containing receptor dimers or oliogmers.

DISCUSSION

Gonadotropin releasing hormone is released in a pulsatile fashion by the hypothalamus and binds to specific, high-affinity receptors located on gonadotrope cells in the anterior pituitary gland (Gharib *et al.*, 1990; Wise *et al.*, 1984). The binding of GnRH to its cognate

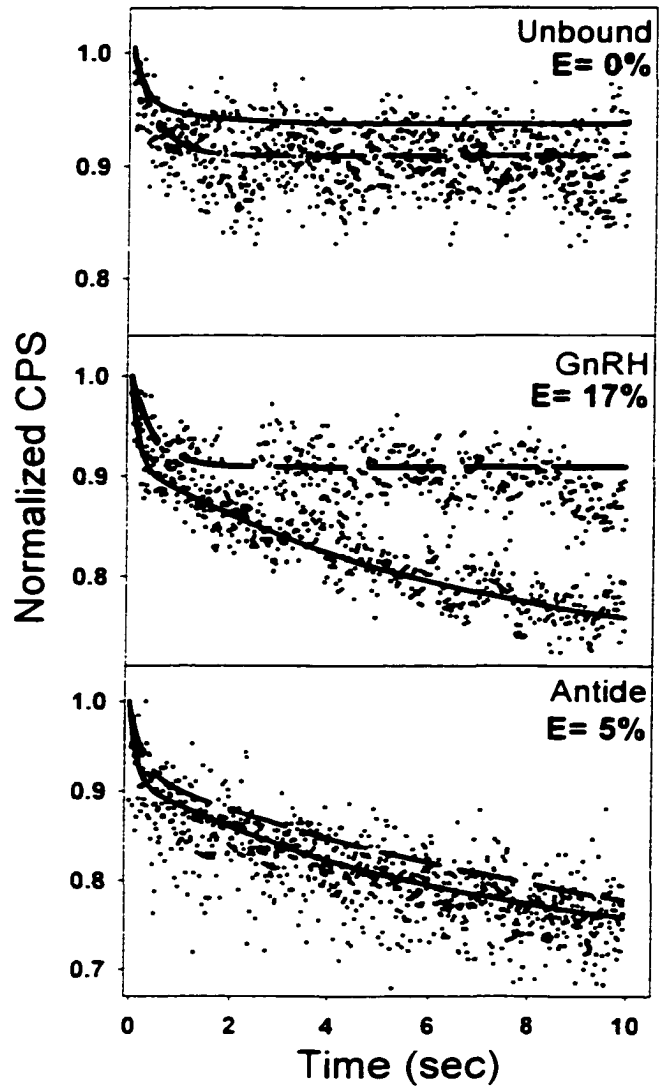


Figure 20: Representative data traces from measurements of fluorescence energy transfer at room temperature between GnRHR-fusion proteins. Fluorescence decay from CHO cells expressing GnRHR-GFP alone (—) and CHO cells co-expressing GnRHR-GFP and -YFP fusion proteins (– –) are shown. Calculated values for energy transfer efficiency are shown in the upper right hand corner of each panel. The top panel represents untreated CHO cells, the middle panel represents CHO cells treated with 10nM GnRH and the bottom panel are CHO cells treated with 10nM Antide. There is no significant energy transfer between receptors on untreated cells or Antide-treated cells. Treatment 10nM GnRH results in 17 ± 1% energy transfer efficiency.

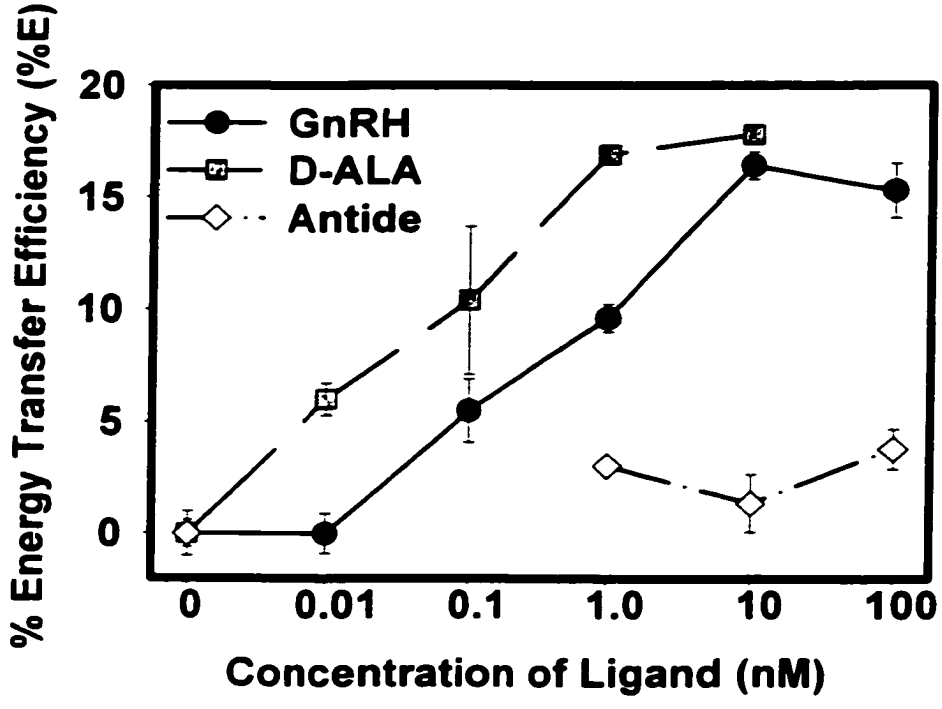


Figure 21: Increasing concentrations of either GnRH (•) or D-ala-6-GnRH (■) results in a dose-dependent increase in percent energy transfer efficiency (%E). The highest concentrations of antagonist Antide (♦) had no significant effect on %E. Increasing concentrations of agonist leads to the dose-dependent increase in energy transfer between GnRHR.

receptor not only stimulates but is obligatory for the synthesis and secretion of luteinizing hormone from the pituitary. Accordingly, much effort has been expended toward understanding the physiological consequences of regulation of GnRH and the GnRHR. With respect to GnRHR regulation, the greatest breakthrough in recent years has arguably been the initial cloning of the cDNA encoding for the GnRHR. Since that time, GnRHR cDNAs have been isolated from a number of species and have proven invaluable in studying the regulation of the GnRHR at the level of gene expression (Hamernik *et al.*, 1995; Turzillo *et al.*, 1995; Wu *et al.*, 1994; Turzillo *et al.*, 1994). In addition, the availability of these cDNAs has allowed investigators to begin to identify functional domains of the GnRHR that are involved in ligand binding, signal transduction, and internalization.

The protein predicted by the initial GnRHR cDNA isolated appears to be highly conserved across mammalian species and contains the 7 hydrophobic amino acid domains that are characteristic of members of the G-protein coupled receptor superfamily (Sealfon *et al.*, 1997). Consistent with this, the GnRHR is thought to be coupled to the $G_{q\alpha}$ and or the $G_{11\alpha}$-subunits of the G-protein complex (Hsieh and Martin, 1992; Stanislaus *et al.*, 1997). Despite the conservation of the 7 putative transmembrane domains characteristic of G-protein coupled receptors, there are several unique features of the mammalian GnRHR. Perhaps most striking is the absence of an intracellular carboxyl terminus (Sealfon *et al.*, 1997), a region which has been implicated in the activity of other members of this class of receptor (Barak *et al.*, 1997). This distinction alone raises questions as to potentially unique structure-function relationships in the GnRHR and, in fact, much progress has been made in this area (Kaiser *et al.*, 1997; Sealfon *et al.*, 1997). Unfortunately, significant gaps remain in our understanding of the

GnRHR. Of particular concern has been the absence of antibodies capable of recognizing the native receptor in situ thus precluding any analyses of the GnRHR in the unbound state. One approach that has been utilized to circumvent this problem has been to use a heterologous epitope "tag" fused in frame to the GnRHR cDNA (Neill *et al.*, 1997). As an alternative to epitope tagging, we have constructed an intrinsically fluorescent GnRHR consisting of an in frame fusion to GFP to the carboxyl terminus of the murine GnRHR. This fusion protein has, for the first time, allowed us to study the dynamics of not only the occupied GnRHR but also the unbound GnRHR.

Clearly, the utility of any "tagged" molecule depends on the degree to which the tagged molecule recapitulates the behavior of the wild-type form. Based on multiple lines of evidence, we suggest that the GFP fusion receptor colocalizes with a plasma membrane marker, Con A (Tarasova *et al.*, 1997). This cellular distribution of GnRHR-GFP stands in marked contrast to unfused GFP which is cytosolic and nuclear (Moriyoshi *et al.*, 1996). Second, Scatchard analysis indicated that the binding characteristics of the fusion protein are similar to the native or wild-type GnRHR expressed by αT3 cells. Third, using a transient expression system paradigm, we found that GnRH treatment of CHO cells stably expressing GnRHR-GFP led to a dose-dependent increase in activity of the promoter from the human α subunit, a well established GnRH-responsive promoter (Hamernik *et al.*, 1992; Andersen *et al.*, 1990). Additionally, the GnRH responsiveness mediated by GnRHR-GFP was blocked by the competitive GnRH antagonist Antide (Bagatell *et al.*, 1993). Finally, GnRH led to a dose-dependent accumulation of cAMP following the introduction of hormone that was also inhibited by Antide. Thus, an intrinsically fluorescent GnRHR was appropriately transported

to the membrane, could bind ligand with an affinity similar to the native receptor and was capable of signal transduction. Given the ability of the GFP fusion receptor to recapitulate these central characteristics of GnRH receptors, we feel that this approach may represent a useful avenue for addressing a number of basic questions regarding the biology of both the occupied and unoccupied GnRHR. Accordingly, we have used laser-based optical methods based on fluorescence photobleaching recovery (FPR) to examine the lateral dynamics of both the bound and unbound forms of the GnRHR-GFP in the plasma membrane of gonadotropes and non-gonadotrope cell lines.

Using both spot and fringe FPR methods we found that the rate of unbound GnRHR-GFP lateral diffusion in the membrane of αT3, CHO and Cos-7 cells was approximately $1.2x10^{-9}cm^2sec^{-1}$. With notable exception of rhodopsin, this rate of receptor lateral diffusion is 10-fold faster than that of most membrane proteins (Jacobson *et al.*, 1987). It is important to remember, however, that many of the values reported for lateral diffusion of membrane receptors are determined after the binding of a fluorescent ligand, not in the unbound state. In fact, the rate of lateral diffusion of the unbound GnRHR-GFP is similar to another G-protein coupled receptor (β-adrenergic receptor), also examined in the unbound form as a GFP fusion molecule (Barak *et al.*, 1997). Thus, at least in the unbound state, it would not appear that the length of the carboxyl terminus significantly impacts the rate of lateral diffusion of G-protein coupled receptors. Interestingly, the rate of diffusion of GnRHR-GFP after binding high concentrations of GnRH is similar to that of the ligand bound LH receptor (Horvat and Roess, 1997), which has an extensive intracellular carboxyl tail, suggesting that the length of the intracellular terminus may not have a significant impact on lateral diffusion even in the bound

form of the receptor. Clearly, one caveat of this interpretation is whether the GFP itself may act as an intracellular C-terminus and thus affect the mobility of the receptor. We have attempted to address this concern by examining lateral diffusion of the wild type GnRHR in αT23 cells using a rhodamine conjugated D-lys^{6}-GnRH as the fluorophore. While the rate of lateral diffusion using the rhodamine-labeled agonist (approximately 0.21 x10^{-9}cm^{2}sec^{-1}) was similar to that observed for the bound form of the GFP-GnRH receptor, the data were highly variable likely reflecting dissociation of the labeled ligand from the receptor during the course of the FPR measurements.

It appears that the binding of ligand to the GnRHR results in two distinct and separable events. First , there is a reduction in the rate of lateral diffusion of the receptors. Second, there is a decrease in the relative number of mobile receptors. There would seem to be several plausible explanations for these post-receptor binding changes. It is possible that binding of GnRH to the GnRHR may result in micro-aggregation of the receptors into structures which based on the magnitude of the decrease in diffusion coefficient, may be considerably larger than receptor dimers. The concentration dependent decrease in the rate of receptor diffusion paralleled the observed increase in energy transfer. Janovick and Conn (Janovick and Conn, 1996a) have suggested that aggregation of the GnRHR in pituicytes occurs as an early step in GnRH signaling. In a similar vein, aggregation of ligand-occupied LH receptors into large molecular weight complexes has been observed in stably transformed 293 cells (Roess and Munnelly, 1997; Roess and Philpott, 1996; Roess *et al.*, 1992a).

One potential concern with the present data is whether the effects of GnRH binding on

receptor dynamics (*i.e.* lateral diffusion) resulted from extensive internalization of hormone-occupied receptor complexes. We view this as unlikely for several reasons. First, given the relatively slow rate of internalization reported for the GnRH receptor of approximately 0.4-0.7% of total binding per minute (Arora *et al.*, 1995), significant internalization would not be expected in the 30-45 minute time frames of FPR data collection. Second, the total green fluorescent signal from GnRHR-GFP CHO cells reflect only a minimal contribution from intracellular sources. Third, spot FPR techniques are based on focusing a laser source to probe only the plasma membrane such that internal fluorescence contributes minimally to the measurements (Axelrod *et al.*, 1976). Finally, the total fluorescence signal from cells treated with GnRH did not diminish over time and was similar to the fluorescence signal obtained from untreated CHO cells expressing mGnRHR-GFP. Thus, we suggest that internalization was not a major factor contributing to the decrease in the mobile fraction of mGnRHR-GFP in the presence of GnRH.

Like GnRH, the binding of Antide led to a reduction in the rate of lateral diffusion of mGnRHR-GFP in αT3 and CHO cells. Antide did not, however, influence the fraction of mobile receptors. This striking difference suggests that these two distinct post-receptor binding events reflect a fundamental difference in the behavior of the agonist vs. antagonist occupied receptor. This finding is, in fact, compatible with Janovick et al. (Janovick *et al.*, 1993) who found differential orientation of photo affinity-labeled GnRH agonists and antagonists in the GnRHR. Since the reduction in the rate of GnRHR-GFP lateral diffusion was similar with GnRH and Antide it would appear that at least this event may simply reflect ligand binding irrespective of agonist and antagonist. In contrast, the reduction in the mobile fraction and

increase in %E of GnRHR observed only with agonist (*i.e.* GnRH) presumably reflects additional interactions between receptors and proteins necessary for signal transduction, internalization, or partitioning of the receptor into discrete membrane domains (Jacobson *et al.*, 1987). If correct, the differential membrane dynamics of the GnRHR-GFP may prove an useful adjunct in the evaluation of GnRH agonists and antagonists. Additionally, an intrinsically fluorescent GnRHR could also be extremely useful in evaluating mutations of the GnRHR that may affect processes such as trafficking, internalization, or receptor cycling.

CHAPTER SIX

CONCLUSIONS

Signal transduction by members of the heterotrimeric G protein-coupled family of receptors (GPCR) is critical in numerous biological systems. These receptors participate in nervous system and endocrine function as well as mediating sensory functions including perception of light, odor and taste.

We have investigated the effects of ligand binding on two GPCRs that are involved in reproduction. We have shown that the LH receptor and the GnRH receptor have similar responses following binding of hormone agonists. Using spot and fringe fluorescence photobleaching recovery methods, we have demonstrated that, in the absence of ligand, intrinsically fluorescent LH or GnRH receptors are mobile with fast lateral diffusion in the plasma membrane. These receptors appear to be monomers since upon binding of ligand, the rate of receptor lateral diffusion becomes slower, probably due, at least in part, to extensive receptor self-association. Interestingly, agonist-occupied receptors that did not signal did not form receptor dimers or oligomers.

From these data, we propose a general mechanism for signal transduction by LH and GnRH receptors. These GPCR's exist as isolated membrane proteins that diffuse freely in the plasma membrane. Upon binding of ligand, the receptors undergo a conformational change that exposes aggregation sites on one or more of the receptor's transmembrane domains. Interactions between receptors via these sites results in the formation of receptor aggregates. During receptor self-association, or perhaps as a result of receptor self-association, other proteins involved in signaling become associated with the receptor. Thus there is a complex formed that contains both receptors and non-receptor proteins. These complexes are sufficiently large to exhibit significantly slower rates of lateral diffusion than does the monomeric receptor. In addition, a large fraction of these complexes become laterally immobile. A schematic representation of this mechanism is shown in Figure 22.

The situation for non-functional receptors is interesting. GnRH receptors bound with antagonist and non-functional LH receptors have diffusion coefficients similar to those of their functional counterparts and significantly slower diffusion than unoccupied receptors. This suggests that the *bound* receptor is present in a complex containing other proteins. However, within these complexes, the non-signaling receptor has not formed receptor dimers or oligomers. Although the specific proteins present in these aggregates are not known, it is reasonable to believe that these complexes either do not contain the full complement of proteins required for signaling or that proteins necessary for signaling to do not have sufficiently high affinity for the monomeric receptor to interact productively. Thus, the ability of GnRH and LH receptors to transduce signal appears to require receptor self-association. This is in agreement with a number of studies in which GPCRs form dimers in response to ligand binding. For

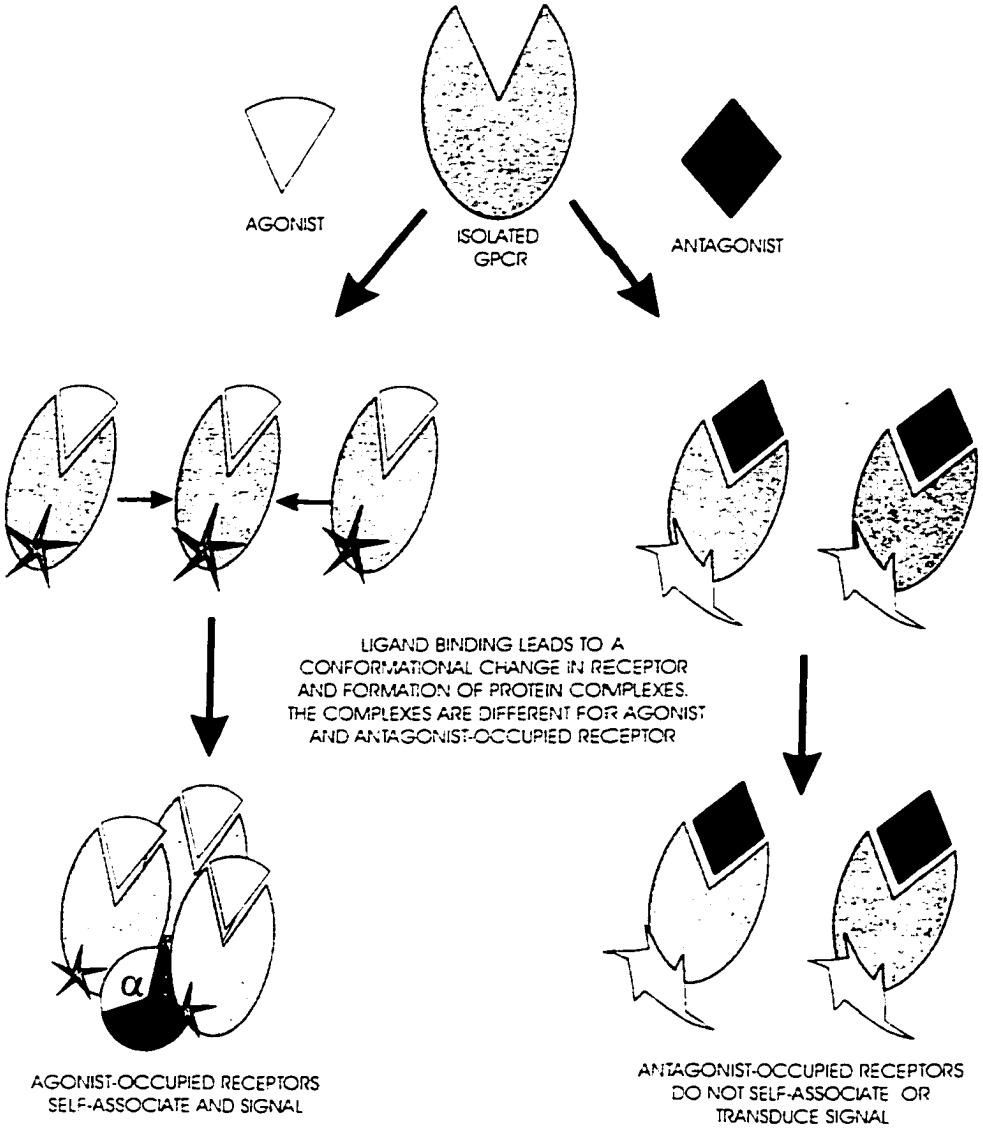


Figure 22: Proposed mechanism for the initiation of G protein-coupled receptor (GPCR) signal transduction. Introduction of ligand to receptor initiates a conformational change (★) in the receptor which leads to the formation of protein complexes which slow diffusion. Only binding of agonist brings about a conformation necessary for receptor self-association and thus initiation of signal transduction.

example homodimers have been demonstrated for the β-adrenergic receptor and the δ-opioid receptor (Cvejic and Devi, 1997; Hebert *et al.*, 1996). In addition dimerization of M_3 muscarinic receptors containing two reciprocal non-functional mutations can fully rescue functionality (Maggio *et al.*, 1993).

Nonetheless, there should be further investigation of the role of GnRH and LH receptor self-association. To this end, we have constructed GnRH and LH receptors that contain a small amino acid epitope tagged to the N-terminus of the receptor. This HA or FLAG epitope is recognized by commercial bivalent antibodies and monovalent F_{ab} fragments. Therefore it should be possible to crosslink two receptors using bivalent antibody and determine if simple receptor dimerization is sufficient to initiate signaling. If this is the case, there should be no signaling by binding of monovalent F_{ab} fragments of the anti-epitope antibody to the receptor.

For LH receptors, protein complexes may also be involved in regulating signaling. We have observed that LH receptors become desensitized to further hormone-initiated signaling following the formation of large complexes. Renewed receptor responsiveness requires dissociation of self-associated receptors and dispersion of the receptor-containing complexes. Shown in Figure 23 is a proposed mechanism for LH receptor desensitization and resensitization. In the absence of ligand, the LH receptor is an isolated membrane protein that exhibits fast lateral diffusion. Upon binding ligand, the LH receptor, like other GPCR (Gether, 2000), undergoes reorganization of its transmembrane helices. Sites are now available for receptor dimerization or oligomerization and association of the receptor with G proteins. This is accompanied by movement of the receptor into a membrane microdomain containing

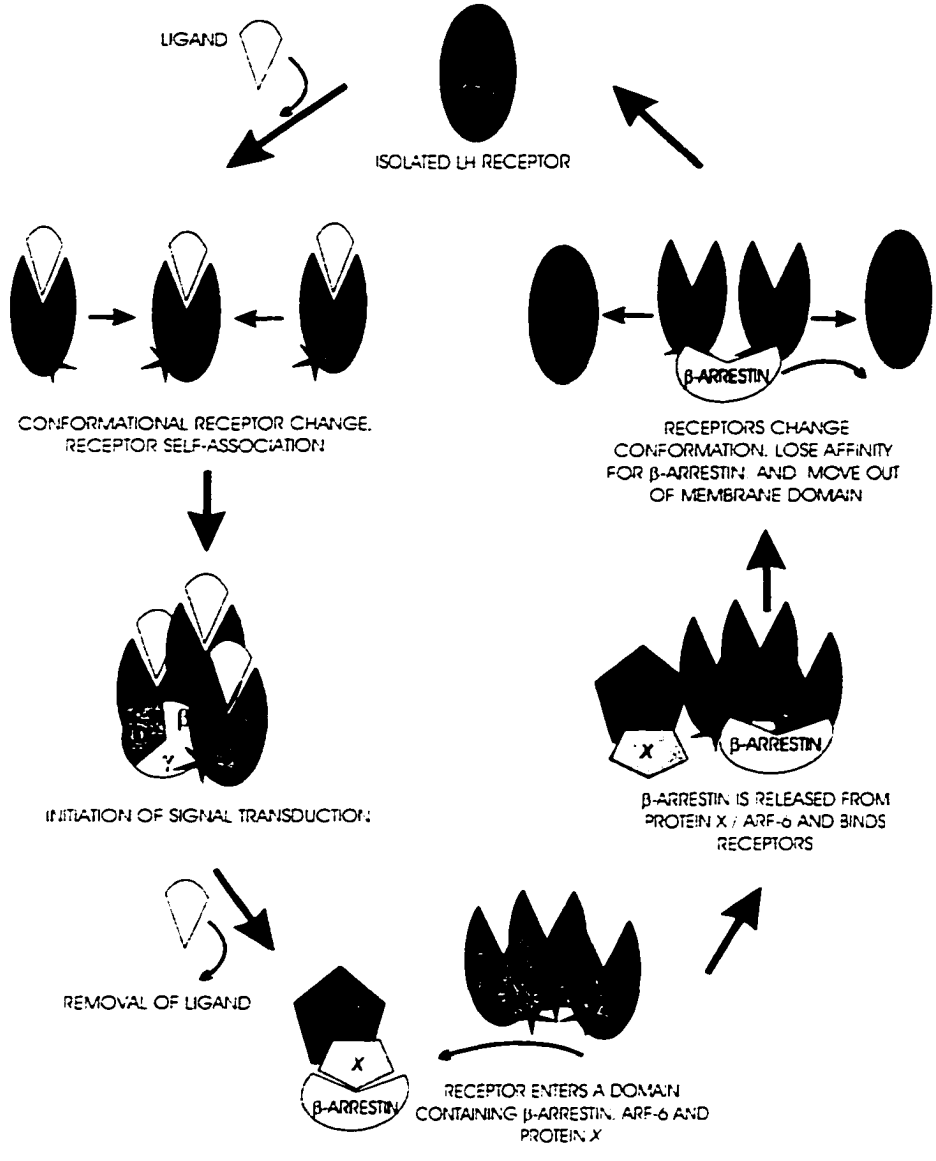


Figure 23: Proposed mechanism for LH receptor desensitization. Binding of LH or hCG leads to receptor self-association and initiation of signal transduction. Low pH buffers remove ligand. LH receptor-containing complexes are targeted to a membrane domain or membrane rafts containing proteins necessary for receptor desensitization including ARF-6, protein X and β-arrestin. Receptors interact with ARF-6 resulting in the release of β-arrestin from protein X. The activated receptor binds β-arrestin. Over time the receptors lose affinity for β-arrestin due to a change from an active to an inactive conformation. The inactive receptors can move out of the membrane domain and are distributed uniformly over the cell membrane.

proteins necessary for receptor desensitization including ARF-6, Protein X, and β-arrestin (Dr. Mary Hunzicker-Dunn, personal communication). Interaction between ARF-6 and LH receptors cause the release of β-arrestin from Protein X which is attached to ARF-6. β-arrestin may be binding at previously unavailable amino acids located in the receptor's transmembrane domains or to sites in the intracellular domain. Interestingly, there is evidence that β-arrestin does not bind to LH receptors in a 1:1 ratio (Dr. Mary Hunzicker-Dunn. personal communication). Rather multiple receptors must be self-associated before β-arrestin will bind. Once desensitized, individual receptors undergo a conformational change that causes them to loose their affinity for β-arrestin. Upon release from β-arrestin, the receptors move out of the membrane domain and redistribute themselves uniformly in the plasma membrane. They are then capable of binding hormone and initiating a hormone-induced response.

Recently, there has been increased speculation that the plasma membrane contains small domains with specialized functions. A number of plasma membrane receptors including the IgA receptor (Rietvild and Simons, 1998), EGF receptor (Waugh *et al.*, 1999), and the tissue factor receptor (Rietvild and Simons, 1998) are preferentially distributed into discrete domains, or into membrane "rafts". Membrane rafts are detergent-insoluble membrane fragments that exhibit low buoyancy following isopycnic centrifugation. These membrane regions typically contain high concentrations of sphingomyelin and cholesterol as well as membrane proteins involved in cell signaling such as G-proteins (Rietvild and Simons, 1998). However, it is not known whether the composition of these rafts is comparatively stable or whether it changes in response to events such as the introduction of ligand-occupied receptors. It is possible that binding of hormone to LH receptors not only drives receptors into rafts but

initiates the recruitment of other proteins involved in cell signaling or receptor desensitization into the rafts. The movement into rafts can be tested by examining whether LH receptors are present in plasma membrane rafts before and after binding of hormone. Then, membrane proteins in rafts can be screened using antibody probes. A number of antibodies against proteins known to be important in signal transduction including G-protein subunits, adenylate cyclase, ARF-6, and β-arrestin are available. Changes in the composition of rafts before and after the binding of hormone to receptor would suggest that proteins can move into and out of these structures in an orchestrated fashion.

It is also possible that LH receptors move into specialized membrane domains with lipid properties that differ from those of membrane rafts. Movement of individual receptors into, and retention of receptors within, such microdomains can also be visualized in single cells using nanovid or single particle tracking microscopy (Zhang *et al.*, 1993). In this approach, the gold particles conjugated to antibodies or ligands can be visualized using video-enhanced light microscopy and the trajectory of individual particles is computed from a series of video images. Nanovid microscopy has revealed that membrane proteins can be stationary in the membrane or can undergo simple diffusion, restricted diffusion, unidirectional motion over comparatively long distances or "obstacle-impeded" motion (Cherry, 1992). In addition a given membrane protein can move in the plane of the membrane using a combination of characteristic protein motions (Kusumi *et al.*, 1993). These studies of receptor self association and membrane domains, together with the development of new biophysical methods to examine membrane proteins in living cells, should considerably advance our understanding of GPCR function.

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LIST OF ABBREVIATIONS

oLH :	Ovine luteinizing hormone
hCG:	Human chorionic gonadotropin
GnRH:	Gonadotropin releasing hormone
GPCR:	G protein-coupled receptor
AC:	Adenylyl cyclase
DAG:	Diacylglycerol
GTP:	Guanosine triphosphate
GDP:	Guanosine diphosphate
LHR:	Luteinizing hormone receptor
GnRHR:	Gonadotropin Releasing Hormone Receptor
β-AR:	β_2-Adrenergic Receptor
LHR-wt:	293 human embryonic kidney cells with an expression vector for the wild type rat LH receptor
LHR-K583R:	293 human embryonic kidney cells with an expression vector for the rat LH receptor with a single amino acid point mutation at Lysine 583 to Arginine
FPR:	Fluorescent photobleaching recovery
FET:	Fluorescence resonance energy transfer

TrITC:	Tetramethylrhodamine isothiocyanate
FITC:	Fluorescein isothiocyanate
ErITC:	Erythroscein isothiocyanate
BSS:	Balanced salt solution
PBS:	Phosphate buffered saline
cAMP:	Cyclic adenosine monophosphate
GFP:	Green fluorescent protein
YFP:	Yellow fluorescent protein
CHO:	Chinese hamster ovary
rLHR-GFP:	Chinese hamster ovary cells expressing the rat LH receptor-green fluorescent protein fusion protein
GFP-LHR-wt:	Chinese hamster ovary cells expressing the rat LH receptor-green fluorescent protein fusion protein
YFP-LHR-wt:	Chinese hamster ovary cells expressing the rat LH receptor-yellow fluorescent protein fusion protein
GnRHR-GFP:	Fusion protein of the C-terminus of the murine GnRHR to the N-terminus of GFP
GnRHR-YFP:	Fusion protein of the C-terminus of the murine GnRHR to the N-terminus of YFP
ARF-6:	ADP-ribosylation factor: isotype 6