THESIS

EVALUATING LUTEINIZING HORMONE RECEPTOR FUNCTION USING THE CYCLIC AMP REPORTER PROBE ICUE1

Submitted by

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ABSTRACT

EVALUATING LUTEINIZING HORMONE RECEPTOR FUNCTION USING THE CYCLIC AMP REPORTER PROBE ICUE1

Luteinizing hormone receptors (LHR) are G protein-coupled membrane protein receptors. Mechanisms involved in initiation of signal transduction by luteinizing hormone (LH) receptors are important and they have been under active investigation because they play a vital role in regulating key events in mammalian reproduction. Evaluating cAMP levels in response to hormone treatment is usually used to demonstrate LH receptor activation and has historically relied on biochemical methods. ELISA, colorimetric assays and other techniques have been used to evaluate cAMP levels. ICUE1 is an Epac-based cAMP reporter which undergoes conformational changes upon binding cAMP. Unlike traditional biochemical assays, ICUE1 combined with FRET techniques is capable of real-time monitoring of cAMP levels in individual cells.

In this project, Epac reporters have been used to evaluate LH receptor activity in cells expressing ICUE1 only and in cells expressing ICUE1 and constitutively-active LH receptors. For the investigation of constitutively active LH receptors, CHO cells were co-transfected with DNA of ICUE1 and yoked LH receptor and they were expressed on the cell membrane. Our results show that ICUE1 probe is a useful tool for evaluating cAMP levels in real-time using single cell imaging methods. Hormone treatment of CHO cells

expressing constitutively active LH receptors show that cAMP levels measurable increase than the basal level in the cell. Similarly, treatment of these cells with forskolin cusese an increase in cAMP levels due to the increase in adenylate cyclase activity.

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BACKGROUND HISTORY

INTRODUCTION

Luteinizing hormone-choriogonadotropin (LH) receptor plays an important role in successful reproduction in mammals. Proper function of LH receptor is necessary for ovulation to occur in females and for Leydig cell development and function in males. In addition, LH receptors are necessary for the maintenance of early pregnancy by maintaining elevated levels of progesterone (Ascoli et al., 2002). Therefore, it is important to fully understand the biology of LH receptor and, in particular, to understand the underlying mechanisms of receptor activation, signal transduction and regulation of receptor-mediated signaling. In addition, the presence of the LH receptors in extragonadal tissues implicates possible effects of gonadotropins and their receptors in both physiological and disease processes outside of reproduction.

HYPOTHALAMIC-PITUITARY-GONADAL AXIS

The gonadotropins play a central role in a highly regulated system known as the hypothalamic pituitary gonadal (HPG) axis as shown in Figure 1. Hypothalamic pituitary gonadal axis is a critical part in the regulation and development of a number of organ systems such as the reproductive system. In females, the brain and gonads are able to regulate reproduction by controlling the uterine and ovarian cycles. Maintaining

homeostasis of these hormones is carried out by feedback mechanisms at all levels in the axis. The HPG axis is regulated by a number of G protein-coupled receptors (GPCRs), including the GnRH, LH and FSH receptors.

The hypothalamus produces the decapeptide hormone gonadotropin-releasing hormone (GnRH) into the hypophysial portal circulation. GnRH is not diluted in the systemic circulation before it reaches the target cells making it a rapid and efficient signal from the brain (Knobil and Neill, 1998). GnRH binds its G protein-coupled receptor (GnRHRs) located on gonadotrope cells in the anterior lobe of the pituitary gland and, in response to hormone binding, the anterior pituitary synthesizes and releases the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the peripheral circulation. The gonadotropins then affect their target organs (the gonads) where specific LH and FSH receptors are expressed. The gonadotropins LH and FSH work together to regulate functions of ovary and testes including gametogenesis and steridogenesis. In the gonads, LH and FSH bind their high-affinity G protein coupled receptors leading to the up-regulation of tissue-specific synthesis and release of the sex steroid hormones testosterone, estrogen and progesterone (Conn et al., 1987). The gonadal sex hormones from both females and males exert negative feedback at the level of hypothalamus affecting GnRH secretion and, at the level of the pituitary, affecting gonadotropin secretion. Thus this feedback loop helps regulate the levels of LH, FSH and the sex steroids tightly in the body.

LH receptors are expressed in gonadal and extragonadal organs, although high-affinity receptors are expressed primarily on the plasma membrane of cells in the gonads.

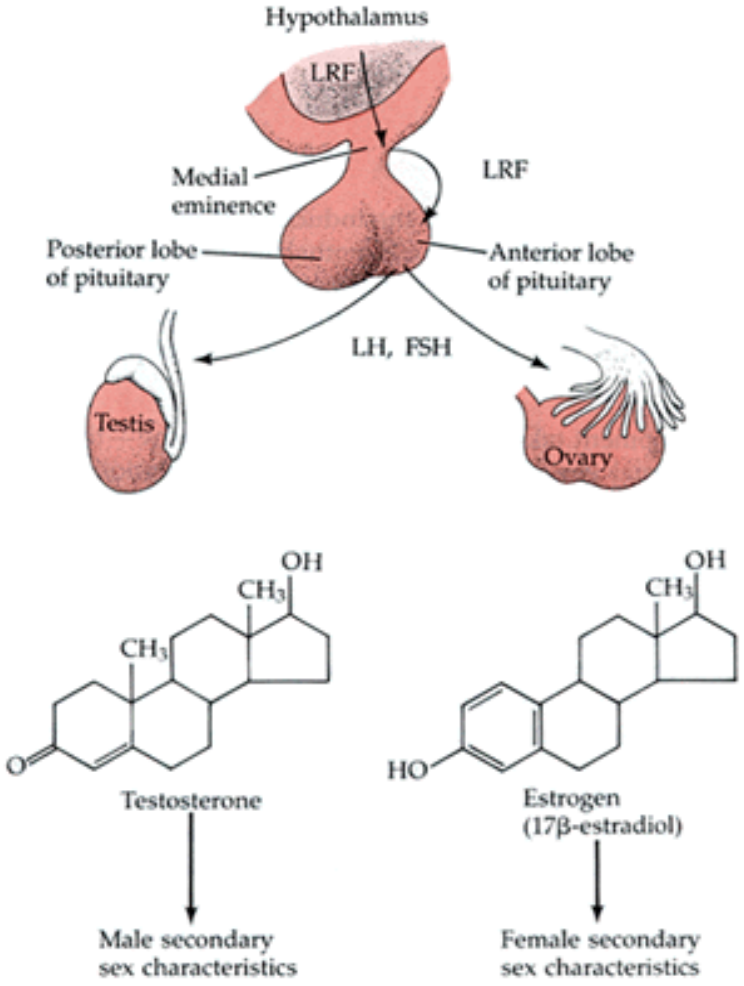


Figure 1 Hypothalamic-pituitary-gonadal axis in mammals (Gilbert 2010)

(Adapted from Scott F. Gilbert 2010, Sinauer Associates)

ROLE OF LUTEINIZING HORMON RECEPTOR IN REPRODUCTION

Functional LH receptors are necessary to maintain normal reproductive function in mammals. The principle physiological functions of LH receptor are found in its actions in the growing follicles and luteal cells of the ovary, and in Leydig cells of the testes (Ascoli et al., 2002, Menon et al., 2004). In females, the LH receptors are expressed in interstitial cells, differentiated granulosa cells and theca cells in the growing follicle which produce and synthesis estrogen from androgen precursors. On luteal cells, LH receptors are involved in promoting ovulation, corpus luteum formation and progesterone secretion. On granulosa and thecal cell in the follicle, the LH receptor stimulates maturation of the follicle and steroidogenesis. In males, LH receptor regulates the development, differentiation and function of testicular Leydig cells, which leads to the secretion of testosterone. Testosterone is responsible for the growth and differentiation of the male genital tract including the epididymis, the vasa differentia, the seminal vesicle, the prostate and the penis (Shenker, 2002).

In the hypothalamus, GnRH is released episodically in pulses. Similarly, LH is secreted in pulsatile manner. The nature of LH release is important to fully understand LH receptor-mediated signaling because excessive release of LH may result in down regulation of the number of active receptors on the surface of target cells (Knobil and Neill, 1998). In females, the positive feedback loop between estrogen and luteinizing hormone helps to prepare the follicle in the ovary for ovulation and the uterus for implantation. Gonadotropins are released cyclically and characterized by a surge prior to ovulation. High levels of estrogen in the blood stimulate the preovulatory LH surge and these events result in ovulation. After the egg is released, the LH surge initiates

luteinization process, the formation of the corpus luteum from the mature ovarian follicle (Knobil and Neill, 1998). The LH surge also stimulates the conversion of granulosa cells into luteal cells and increases the enzymes needed for progesterone synthesis by the ovary to inhibit the hypothalamus and the anterior pituitary, thus stopping the positive feedback loop. LH causes an increase in the synthesis of progesterone mainly by stimulating cholesterol transport and activating the cholesterol side chain cleavage enzyme P450. The corpus luteum (CL) was first named from the Latin *corpus* (body) and *luteum* (yellow) to describe the yellow-colored tissue that fills the cavity of the ruptured ovulatory follicle (Knobil and Neill, 1998). The corpus luteum is necessary and required for the maintenance of pregnancy by providing high levels of progesterone. If conception occurs, stimulating factor such as chorionic gonadotropin produced by the placenta in primates and horses acts to prolong the life of the corpus luteum. Conversely, if conception does not occur, progesterone production decreases allowing the hypothalamus to restart GnRH secretion. The activation of hypothalamic pituitary gonadal axis in both males and females during puberty also causes the development of the secondary sex characteristics.

In women, hCG is synthesized by the syncytiotrophoblastic cells of the placenta and secreted as early as one day after embryo implantation (Jaffe et al., 1969). In the same way, in horses, equine choiogonadotropin (eCG) is secreted by chorion-derived uterine endometrial cells (Knobil and Neill, 1998). During the first trimester of gestation, hCG levels reach their peak, stimulating the corpus luteum to maintain the secretion of enough estrogen and progesterone needed to maintain pregnancy (Catt and Dufau, 1991). However, as the placenta grows, it becomes able to produce enough progesterone for

maintenance of pregnancy and hCG production consequently diminishes (Knobil and Neill, 1998).

Studying LH receptor signaling mechanisms is interesting because naturally-occurring mutations in the receptor can result in human disease. A large number of mutations of LH receptor genes have been identified producing different phenotypic effects (Themmen and Huhtaniemi, 2000). The dominant mutations lead to constitutive activation or inactivation of LH receptor-mediated cyclic adenosine monophosphate (cAMP) signaling pathway (Chan, 1998). These constitutively activating mutations may cause familial male-limited precocious puberty (Fanelli, 2000), while the inactivating mutations can lead to Leydig cell hypoplasia (Chan, 1998). LH receptor-mediated effects, including testosterone production, are known to cause an increase in the cellular cAMP production. Accumulation of intracellular cAMP triggered by unoccupied mutant receptors appears sufficient to cause Leydig cell hyperfunction, hyperplasia and even tumor formation. Mutations of LH receptor genes may also cause pseudohermaphroditism in males and primary amenorrhea in females (Shenker, 2002). Apparently, defects in LH receptor genes display aberrant sex differentiation and/or infertility.

STRUCTURE OF LH AND hCG

As previously mentioned, LH receptor binds two hormones, LH and hCG which are members of the glycoprotein hormone family that includes follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). The glycoprotein hormones, LH and hCG are heterodimeric proteins composed of one α and one β-subunits that assemble

by noncovalent interactions (Li and Starman, 1964; Dias, 1992; Xing et al., 2001). The α-subunit is highly conserved for both LH and hCG and has 92 – 96 amino acids. The β-subunit is specific for each member of the glycoprotein hormone family. The β subunit for LH has 117 amino acids while that for hCG has 145 amino acids (Ascoli and Segaloff, 1989). The first 114 amino acids of LH and hCG have 85% homology, but hCG differs from LH in that it has a 20 amino acid C-terminal extension (Pierce and Parsons, 1981; Sairam and Manjunath 1983). All of the β subunits of the glycoprotein hormone family have 12 cysteine residues at highly conserved positions which form six disulfide bridges. The β subunit dictates the receptor specificity although both subunits are necessary to bind the receptor. The nonvovalent interactions between α and β-subunits of hCG are stabilized by a segment of the β-subunit that wraps around the α-subunit forming a unique "seat belt" arrangement. This unusual structural feature appears to be essential to hold the two subunits together, and also for receptor binding of the glycoprotein hormone (Lapthorn et al., 1994; Bernard MP et al., 2004). Both α and β-subunits are members of the superfamily of cysteine-knot growth factors that include nerve growth factor (NGF), transforming growth factor-β (TGF- β), and platelet-derived growth factor- β (PDGF- β). Receptor binding involves a number of residues that were identified by different methods including chemical modification, site-directed mutagenesis, and the use of synthetic peptides in competitive inhibition studies (Lapthorn et al., 1994).

Upon binding of α and β-subunits of the hormone to the receptor, a conformational change occurs resulting in the formation of the active heterodimer (Ingham et al., 1976). The α-subunit has a little or no binding activity. Therefore, the association of the α-subunit with the β-subunit results in a proper conformation for

binding of the heterodimer to the receptor. Based on results from studies using hybrid hormones consisting of various α and β-subunits, it is believed that α-subunit is the driving force for the association of hormone with receptor, but the β-subunit specifically limits the types of hormone-receptor interactions (Combarnous, 1992). The crystal structure of deglycosylated hCG has shown that each of its two different subunits has a similar topology (Lapthorn et al., 1994; Oefner et al., 1992; Schlunegger and Grutter, 1993).

There are up to four N-linked and three O-linked oligosaccharides representing 18-45% of the total hormone weight, that attach to either the α or β-subunits at various points. For LH there are two sites for N-liked glycosylation on the α-subunit as well as on the β-subunit (Bahl and Moyle, 1978). In contrast, the β-subunit of hCG contains two sites for N-linked glycosylation and four sites for O-linked glycosylation on the 20 amino acid C-terminal tail (Winzler, 1973). The attachment of carbohydrates to LH and hCG is believed to be vital for physiological responses such as normal receptor-ligand interactions and internal cell signaling. For example, carbohydrates attached to hCG play a role in the activation of internal cell signaling mechanisms such as adenylyl cyclase, and it is also believed the carbohydrates on LH and hCG help maintaining hormone structure in a conformation that is needed to activate LH receptors (Thotakura et al., 1990), although, it remains unclear as to the role of carbohydrates in hormone-mediated signaling.

The LH receptor binds LH as well as hCG. In a study comparing the functionality of LH and hCG, fluorescence resonance energy transfer (FRET) was measured between LH and hCG molecules labeled with fluorescein and rhodamine, where the observed

FRET was higher between LH receptors that bind the fluorescent hCG molecules than the receptors that bind the LH molecules (Roess et al., 2000).

STRUCTURE OF THE LH/hCG RECEPTOR

Luteinizing hormone receptor (LHR), also called luteinizing hormone/chorio-gonadotropin receptor is a single polypeptide transmembrane receptor composed of 699 amino acid residues encoded by a single gene located on the short arm of the chromosome 2 (2P21) (Rousseau-Merck et al., 1990, Jia et al., 1991) (Figure 2).

The human and rat LH receptor genes are approximately 80 kb in size and each gene contains 10 introns and 11 exons (Koo et al., 1991, Tsai-Morris et al., 1991). Cloning, sequencing and expression of complementary DNA (cDNA) for the rat and procine LH receptors were reported in two papers in 1989 (McFarland et al., 1989, Loosfelt et al, 1989). It was established that the LH receptor is a polypeptide chain that binds glycoprotein hormones (McFarland et al., 1989, Probst et al., 1992) and a member of subfamily A, the rhodopsin/β2-adrenergic receptor-like subfamily of G protein-coupled receptors (GPCRs). Shortly after, the cloning of cDNA for human LH receptor was reported (Jia et al., 1991, Minegishi et al., 1990).

GPCRs are the largest class of membrane receptors with over 1000 members (Fredriksson and Schioth, 2005, Lefkowitz, 2007). Recently, a large number of drugs used in clinical practice can directly or indirectly affect GPCR activity (Tyndall and Sandilya 2005, Jacoby et al., 2006).

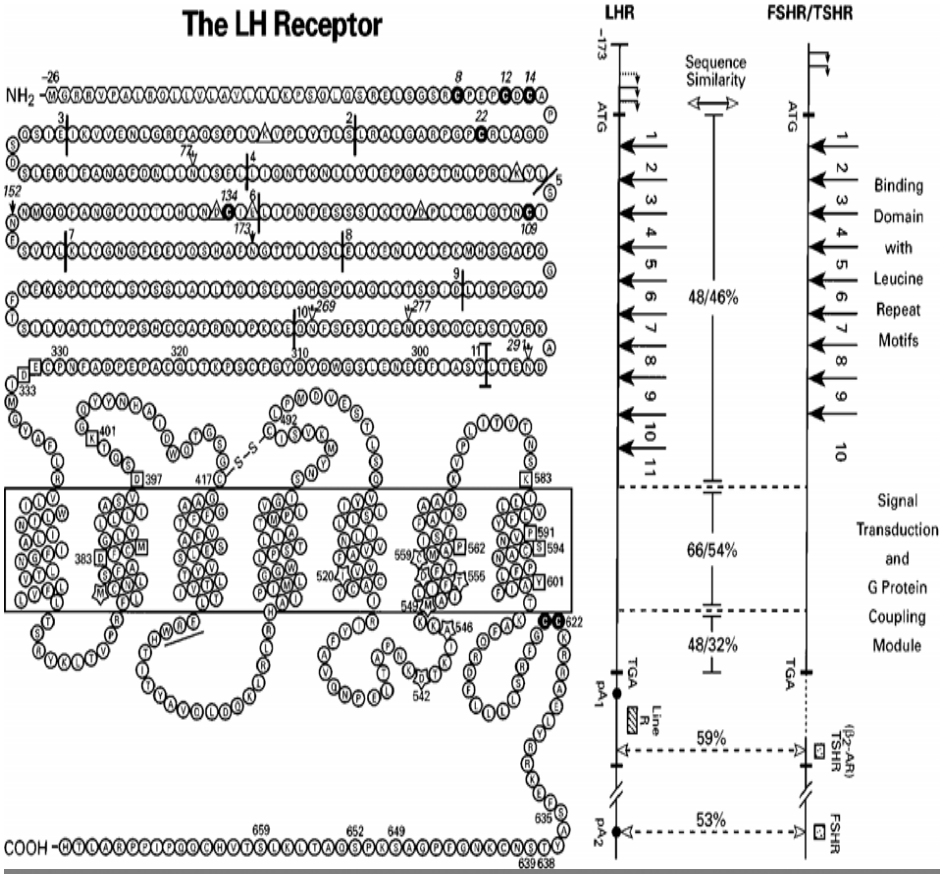


Figure 2: The amino acid sequence of LH receptor and the nucleotide similarities between the regions of the glycoprotein hormone receptor sequences (Dufau, 1998). (adapted from Dufau ML, 1998 *Annu Rev Physiol* 60:461-496)

LH receptors are found in the ovary, testis and extragonadal tissues such as the uterus. Transmembrane receptors are specialized integral membrane proteins that serve an important role in the communication between the intracellular and extracellular environments, as well as in signal transduction. Both luteinizing hormone (LH) and chorionic gonadotropins such as human chorionic gonadotropin (hCG) are members of the glycoprotein hormone family and interact with LHR (Roess et al., 2000).

LHR is a 93 KDa glycoprotein, and a member of the G- protein-coupled receptor family (Wess, 1998; Gether, 2000; Shenker, 2002). Like the other members of the GPCR subfamily, the LH receptor can be divided into three well-defined domains (Figure 3). The first domain is a long, heavily glycosylated N-terminal extracellular domain (about 340 amino acid), which is the part of the receptor on the extracellular face of the membrane. The extracellular domain is known as the ligand-binding domain because its primary function is to recognize and bind a specific ligand with high affinity (Segaloff and Ascoli, 1993; Dufau, 1998). This domain can be divided into three distinct regions-an N-terminal cysteine-rich region, a leucine-rich motif region, and a C-terminal cysteine-rich region or the hinge region (Ascoli et al., 2002). The glycoprotein hormone receptor family (LH/hCG receptor, FSH receptor and TSH receptor) make up their own subfamily of GPCRs that is characterized by the presence of a large N-terminal extracellular domain containing several leucine-rich repeats (Ascoli et al., 2002). For this reason, the glycoprotein hormone receptors were renamed the leucine-rich repeat-containing GPCR (LGR) family (Nishi et al., 2000).

The second domain is a highly conserved serpentine region containing seven hydrophobic transmembrane α-helices and it represents the transmembrane domain of the

receptor. Each seven spanning segment consists of 25-35 amino acids and these membrane-spanning segments are linked by three intracellular and three extracellular loops (McFarland et, al., 1989; Probst et al., 1992). Because of their characteristic structure, GPCRs are also known as seven transmembrane TM receptors, and this nomenclature is perhaps more accurate since GPCRs can also interact with signaling molecules other than GPCRs (Szidonya et al., 2008).

The third domain is the intracellular C-terminal cytoplasmic domain which is shorter consisting of about 70 amino acids. The role of the intracellular domain is to relay the signal by communication, via protein-protein interaction, with effecter proteins, or through the enzymatic activity of this domain which is usually a tyrosine kinase activity. It also plays a role in receptor desensitization (Sanches-Yague et al., 1992) and receptor internalization (Rozell et al., 1998).

In rat and human LH receptor, the extracellular domain also contains six conserved consensus sites (AsnXxxSer/Thr) for N-linked glycosylation, however increasing evidence suggests that these carbohydrate chains are not needed for binding hormone or for signal transduction. Although, it is thought that the N-linked carbohydrates of LH receptor may play a facilitative role with the chaperone protein, calnexin, in aiding the folding and trafficking of glycoproteins out of the endoplasmic reticulum (Ascoli et al., 2002). Interestingly, a single N-linked carbohydrate on the α-subunit of hCG is necessary for signal transduction (Matzuk et al., 1989, Sairam 1989). The extracellular and intracellular domains of the receptor are linked by a linker region. (Weiss and Schlessinger, 1998).

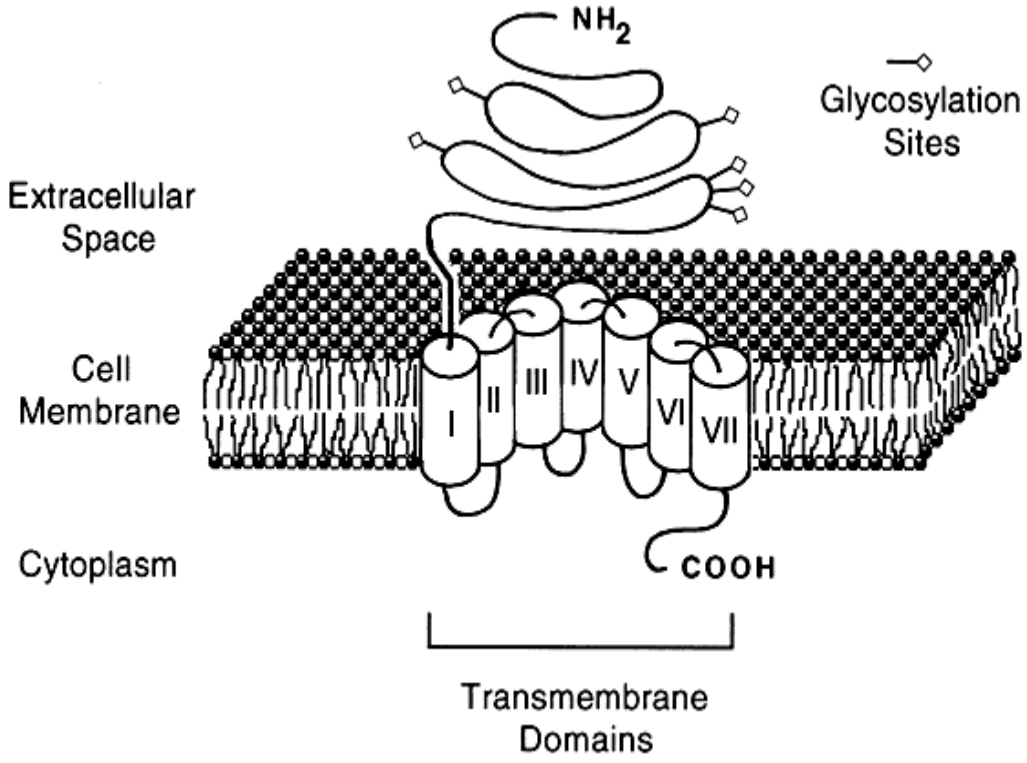


Figure 3. Luteinizing hormone receptor structure (Segaloff et al., 1990).

(Modified from Segaloff DL et al., 1990 *Recent Prog Hormone Res* 46:261–303.)

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

A number of naturally occurring mutations in the human LH receptor gene can lead to different reproductive disorders. For instance, loss-of-function mutations in human LH receptor prevent hCG binding and/or signal transduction. These mutations display some degree of constitutive activation and they provided valuable information about mechanisms of signal transfer and G protein coupling (Ascoli et al., 2002)

SIGNAL TRANSDUCTION MEDIATED BY LH RECEPTOR

As previously mentioned the LH receptor is a member of the GPCR superfamily and was one of the first GPCRs known to demonstrate dual coupling- the ability to independently activate two G proteins-dependent signaling pathways. The LH receptor interacts with the heterodimeric ($\alpha\beta\gamma$) G protein. This interaction leads to the activation of the effector proteins, adenylate cyclase (AC) as well as phospholipase C (PLC), as shown in Figure 4. The LH receptor interacts primarily with the G protein Gs, although it has been shown to interact with Gq/11, G13 and Gi (Dufau, 1998) (Figure 4).

All GPCRs, including LH receptor transduce intracellular signals in response to hormone binding and they have common structural features, an extracellular domain, seven spanning α helical domain, three exoloops, three endoloops and a cytoplasmic tail (Probst et al., 1992). The second endoloop, the third endoloop, and in some receptors, the cytoplasmic tail is known to couple the receptor to G-proteins, a process called *cis-activation* (Gether, 2000) (Figure 5). Recently, a new mechanism for receptor activation of the glycoprotein receptors has been proposed, this mechanism is called *trans-activation* (Ji et al., 2002). *Trans-activation* occurs when a ligand-occupied exodomain on one receptor interacts with the signaling domain of an adjoining receptor.

The LH receptor binds both LH and hCG with high affinity on its extracellular domain. Agonist binding to GPCRs causes a conformational change in the ligand binding domain leading to a rotation in the sixth transmembrane domain which is critical in activation of adenylate cyclase (Abell and Segaloff, 1997), and this triggers the activation of the receptor. It is thought that the intracellular loop, particularly the 3i loop, forms contact sites for intraction with G proteins such as Gs (Dufau, 1998). A model of the hCG-LHR-Gs signaling complex is illustrated in Figure 6.

G proteins prior to ligand binding are found as inactive complexes of three distinct subunits, α, β and γ (Figure 6). In this inactive state, α subunit has GDP bound, whereas β and γ subunits help to anchor the heterodimer in the inner leaflet of the plasma membrane. Binding and activation of the receptor catalyzes the release of GDP from the α subunit and subsequent binding of GTP. Once GTP is bound, the α subunit becomes active and dissociates from the receptor and the active βγ complex. The α subunit remains

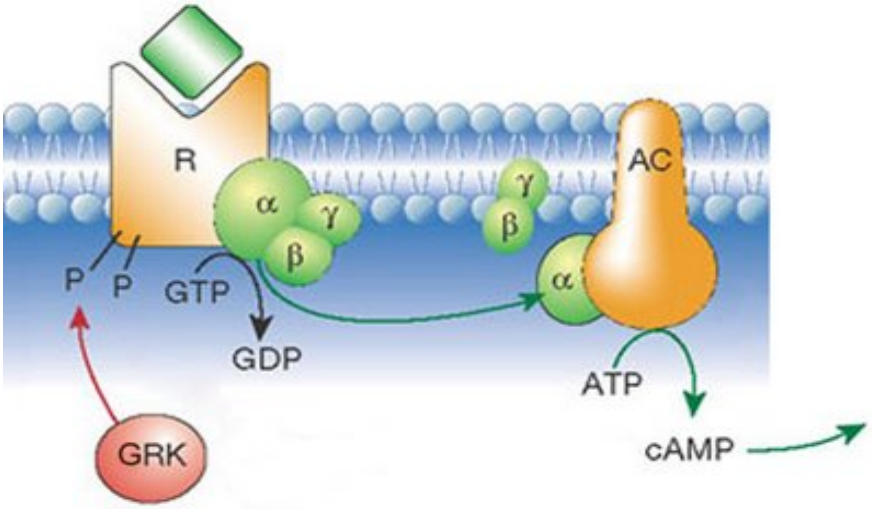


Figure 4. LHR activation of G_s and adenylate cyclase (AC)

(Adapted from Peter H. Raven, George B. Johnson, Jonathan B. Losos, and Susan R. Singer, Biology (7th edition), McGraw-Hill Co. NY, Chapter 7).

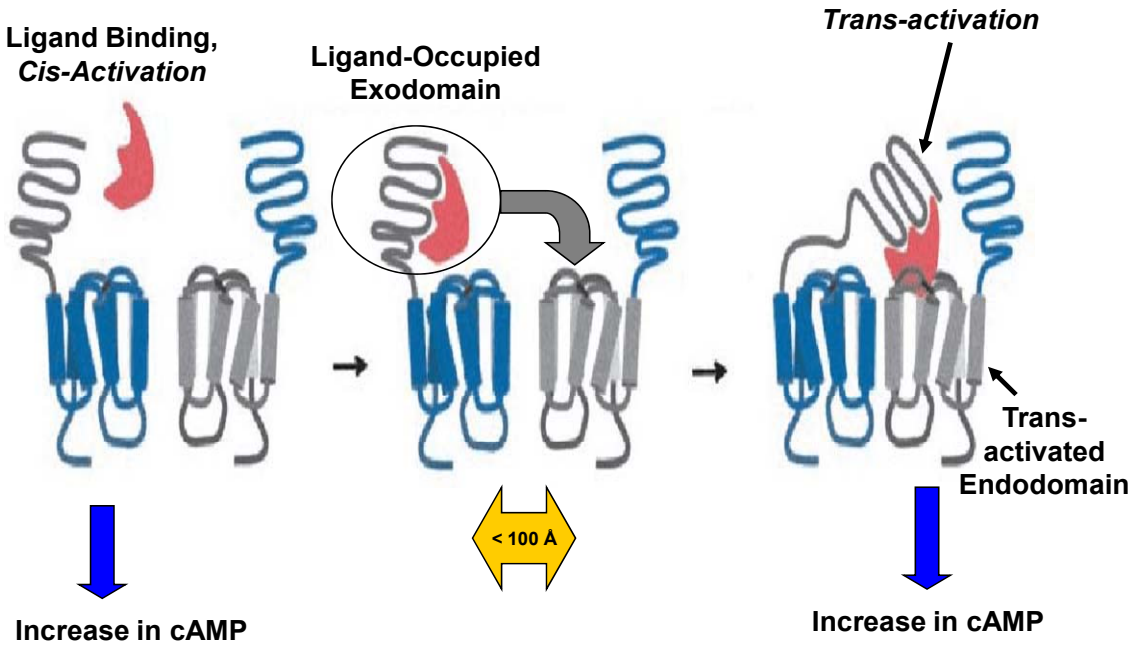


Figure 5. Cis-activation of the luteinizing hormone receptor. Hormone binding to the LH receptor exodomain results in activation of the receptor's endodomain which consists of the seven transmembrane sequences, the extracellular and intracellular loops and the long cytoplasmic tail (Ji et al., 2002)

(Adapted from Ji I et al., 2002 *Molecular Endocrinology* 16:1299-1308)

in the active state until GTP is hydrolyzed to GDP by the action of the endogenous GTPase of the α subunit. Upon hydrolysis of GTP to GDP, α-GDP reassociates with βγ complex. This cycle persists as long as the agonist is available to bind receptor and as long as the agonist-bound receptor can activate G proteins (Stryer and Bourne, 1986).

LH receptor is one of the GPCRs that independently activate two G protein-dependent signaling pathways, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and the diacylglycerol (DAG)/protein kinase C (PKC) pathway (Herrlich et al., 1996; Spiegel, 1998; Ascoli et al., 2002). Activation of LH receptor leads first to the activation of G-protein Gs (Dufau, 1998). After the dissociation of the active α subunit from βγ complex, it activates the membrane effector adenylyl cyclase (AC). Upon the activation of AC, adenosine triphosphate (ATP) is converted into the second messenger molecule cAMP. CAMP then can bind to the regulatory subunit of PKA and causes the release of the catalytic subunit (Yen et al., 1999). The active catalytic subunit of PKA can phosphorylate specific serine and threonine residues on ribosomal, nuclear and cytoskeletal proteins, some of which participate in the synthesis and secretion of steroids. Furthermore, the PKA pathway regulates the activity of specific enzymes necessary for the conversion of cholesterol to the sex steroids (Tang et al., 1998).

Ligand binding to the LH receptor under some conditions may also activate the PKC pathway. hCG binding to LH receptors on the ovaries as well as receptors stably-expressed in cultured cells exhibit an increase in free intracellular calcium and phosphoinostitide (PI) hydrolysis (Gudermann et al., 1992; Dufau, 1998).

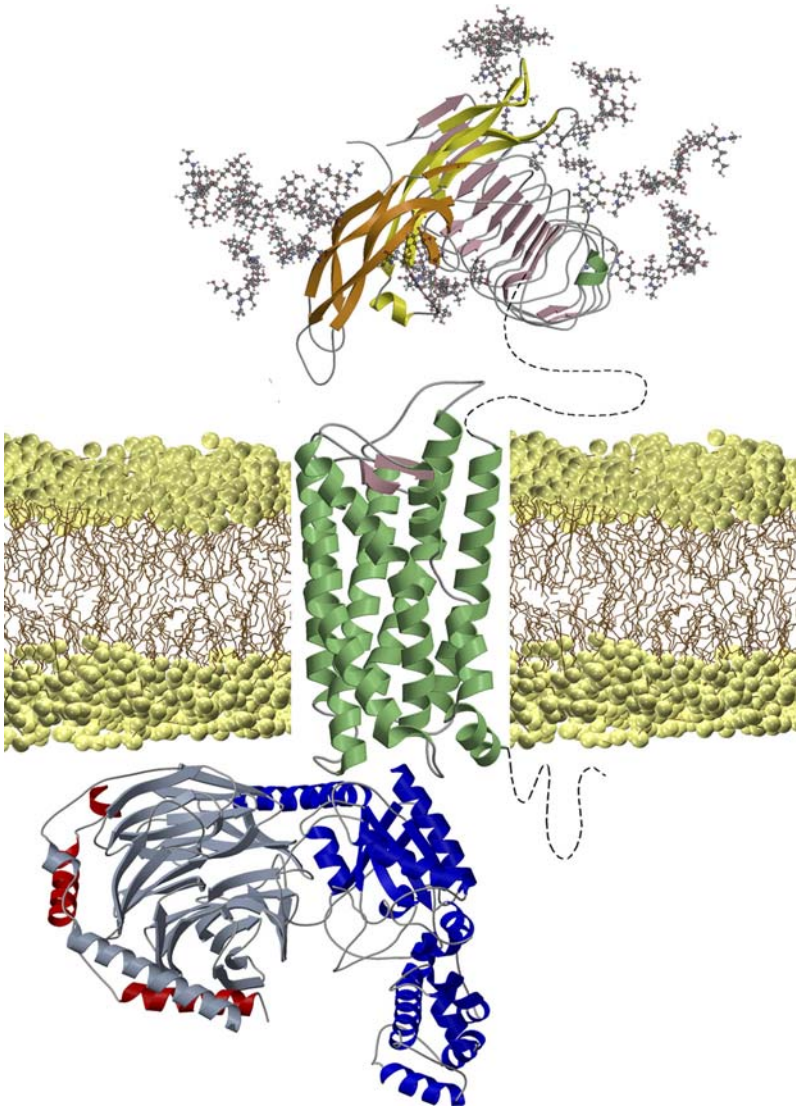


Figure 6: Schematic representation of the hCG-LHR-Gs complex for illustrative purpose only. Some of the regions are based on structural data and molecular modeling. This illustration shows a complex containing six proteins: the heterodimeric hormone, hCG, bound to the LHR-ECD and the transmembrane of LHR bound to the heterotrimeric G protein (Puett et al., 2007).

(Adapted from Puett D et al., 2007 *Mol and Cell Endocrinology* 260-262:126-136)

Upon receptor activation, the effector molecule phospholipase C (PLC) cleaves the membrane lipid phosphatidylinositol biphosphate (PIP2) to inositol 1, 4, 5-triphosphate (IP3) and 1, 2 diacylglycerol (DAG). IP3 is then released into the cytoplasm causing the release of sequestered calcium from the endoplasmic reticulum. DAG remains at the membrane and activates PKC which phosphorylates specific serine and threonine amino acid residues on target proteins and thereby regulates their action (Yen et al., 1999). The hydrolysis of PI to IP3 and DAG probably results from the interaction of the LH receptor with the βγ subunits released from Gs or Gi rather than from the release of α subunit.

The mechanism of LH receptor activation is sill poorly understood. However, a novel mechanism of intermolecular GPCR activation has been described. It shows that binding of ligand to one receptor is enough to activate adenylyl cyclase through its transmembrane bundle, intramolecular activation (cis-activation), as well as trans-activation through the transmembrane bundle of the adjacent receptor (Ji et al., 2002). This theory has been supported by an experiment in which coexpression of a mutant receptor defective in hormone binding and another mutant defective in signal generation rescues hormone-activated cAMP production.

GPCRs exist and potentially function as dimers and/or higher oligomers (Milligan et al., 2003). Although an increasing amount of data suggests that dimers represent the basic signaling unit for most members of this receptor family, GPCR dimerization might also be necessary to pass quality-control checkpoint of the biosynthetic pathway of GPCRs. To date, this hypothesis has been demonstrated for only a small number of receptors that must form heterodimers (obligatory heterodimers) to be exported properly

to the plasma membrane. However, increasing evidence suggests that homodimerization might have a similar role in the receptor maturation process for many GPCRs (Bulenger et al., 2005).

Following signaling, there is a decrease in the receptor responsiveness to repetitive or continuous stimulation, which is termed receptor desensitization (Ferguson, 2001). Like other GPCRs, the LH receptor becomes desensitized (less responsive) within minutes after binding of hCG or LH. LH receptor desensitization is followed by a decrease in cellular cAMP even in the presence of LH. Once desensitized, hormone binding to LH receptor only minimally activates adenylyl cyclase inspite of the fact that adenylyl cyclase is still functional and can be activated by other means. Desensitization of LH receptor following brief exposure to hormone is initially characterized by coupling of the receptor from the signal transduction apparatus rather than by a decrease in receptor number (Roess and Smith, 2003).

The desensitization of GPCRs is the consequence of a combination of different mechanisms. These mechanisms include the coupling of the receptor from heterodimeric G proteins in response to receptor phosphorylation, the internalization of cell surface receptors to intracellular membranous compartments and the down regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as the lysosomal and plasma membrane degradation of pre-existing receptors (Ferguson, 2001). The ability of receptors to activate their respective G protein, however, appears to be the most important cause of receptor desensitization (Dohlman et al., 1991).

In addition to the uncoupling receptors, arrestins also play an important role in receptor sequestration. Receptor sequestration is defined as the movement of the ligand-

occupied receptor from a site where the receptor is accessible to ligand to a location in which the receptor is no longer accessible. Receptor sequestration occurs after the phosphorylation of the receptor and it may be required for reactivation of GPCRs (Krueger et al., 1997).

LH RECEPTOR ORGANIZATION IN THE PLASMA MEMBRANE

GPCRs are believed to act as monomers when interacting with a single G protein, although the receptor dimerization has long been recognized to be involved in the signal transduction of integral membrane receptors such as receptors for growth factors and cytokines (Heldin, 1995). Recently, the view that GPCRs function as monomeric proteins has been challenged by biochemical, biophysical and functional studies, which have suggested that GPCRs exist in the cells as dimers ao higher oligomers. Specific heterodimerization between distinct GPCRs has been documented in addition to the homodimerization of a certain GPCR. GPCR heterodimerization is of functional consequence, whereas the functional role of GPCR homodimerization is still unclear (Tao et al., 2004).

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

(Horvat et al., 1999). The theory of the presence of receptors in large clusters has been also suggested by lateral diffusion studies of the LH receptors in luteal cells in sheep (Niswender et al., 1985) and rat (Roess et al., 1992) in which most LH receptors were laterally immobile. The LH receptor aggregation may indicate the response of the receptor to hormone binding. The functional hormone-receptor complexes show significantly slower rotational dynamics than the complexes formed by hormone binding to non-functional receptors or by a non-functional ligand binding to a normally functioning receptor (Roess et al., 2000)..

CONSTITUTIVELY ACTIVE LH RECEPTORS

Mutants of LH receptors that occur naturally have been identified including constitutively active LH receptors with continuous activation even in the absence of ligand. There are two ways to obtain constitutively active LH receptors in *vitro*; mutations in LH receptor at position 578 are associated with constitutively active receptor (Lei et al., 2007). The first human mutant LH receptor was Asp578Gly (Shenker et al., 1993) and it has been identified in males with precocious puberty, and it is considered the most common mutant of LH receptor (Themmen and Huhtaniemi, 2000). This mutation results in constitutive activation of Gs by the receptor (Abell et al., 1998) in the absence of LH or hCG. Furthermore, other mutations in human luteinizing hormone receptor (hLHR) at the aspartic acid residue at position 578 are associated with constitutive receptor activation and naturally-occurring pathologies such as familial male-limited precocious puberty (Shenker et al., 1993) and Leydig cell adenomas (Liu et al., 1999).

It has been hypothesized that the LHR-D578G mutant of LH receptor induced activation of adenylate cyclase using the same mechanism as the hCG-activated wild-type receptor (Lee et al., 2004). Under normal conditions, hCG binds to the wild type LH receptor and the receptor activates Gαs which, in turn, activates adenylate cyclase whose action is to convert ATP to cAMP (Dufau, 1998; Ascoli et al., 2002). To determine whether G-protein-coupled receptors interact with the C-terminal region of Gα subunits, corresponding C-terminal minigenes were used (Gilchrist et al., 2001). The C-terminal region of Gα subunits has been utilized to compare the induction of cAMP by wild-type LH receptor (wt-LHR) and LHR-D578G receptors. The results showed that different mechanisms were involved in elevating the basal cAMP induced by the activating mutations of LH receptor and increasing the cAMP levels induced by the wild-type receptor activated by hCG binding (Lee et al., 2004).

FRET measurements were used to investigate whether constitutively-active LH receptors were also constitutively associated with one another in the absence of ligand (Lei et al., 2007). In this experiment, wild type LH receptor and mutants of LH receptor at position 578 and constructed vectors attaching these receptors to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) were used. Using fluorescent acceptor bleaching and imaging methods, the results showed that stably-expressed constitutively active receptors exhibited 11-15% FRET efficiency, while for wild type LH receptor the efficiency was less than 1%. This suggested that constitutively active LHRs, unlike wild type LH receptors, were self-associated in the absence of ligand. Interestingly, FRET efficiency values between constitutively active receptors did not change with the addition of hCG. Furthermore, the constitutively active LH receptors

were generally located in high buoyancy membrane fractions; so-called plasma membrane rafts (Lei et al., 2007).

Another form of constitutively active LH receptors is the yoked LH receptor (YLHR) that was first developed by Chengbin Wu and Prema Narayan (Narayan and Puett, 1996). The yoked LH receptor is composed of a single chain hCG molecule covalently coupled to the LH receptor. To construct the complex, the entire coding sequence of yoked hCG were generated followed by the first half of the C-terminal peptide (CTP) sequence. This was ligated with the receptor sequence containing the second half of the CTP sequence upstream of the coding sequence for the mature receptor. The ligated product was subcloned into the BamHI site of the eukaryotic expression vector pcDNA3 (Wu et al., 1996). Both COS-7 and HEK 293 cell lines were generated and transfected with wild type LH receptor or yoked LH receptor to investigate receptor expression in transfected cells. Western blot analysis with an antibody against CTP showed that the yoked LH receptor complex was expressed in transfected cells (Lei et al., 2007). Cells transfected with yoked LH receptor were unable to bind significant amounts of exogenous hormone (Lei et al., 2007) but, nonetheless, had elevated basal levels of cAMP suggesting that yoked LH receptor was constitutively active. To investigate further, ^{125}I-hCG was added to COS-7 cells transfected with either wild-type LH receptor or yoked LH receptor cDNA (Lei et al., 2007). No significant amounts of exogenous hCG bound yoked LH receptor was expressed in COS-7 cells.

The yoked LH receptor was also functional *in vivo*. The effects of yoked LH receptor expressed in transgenic mice were investigated. The results detected an elevated testosterone levels in male mice expressing yoked LH receptor at 3 and 5 weeks of age.

There was also a decrease in testicular weight and serum levels of LH and follicle stimulating hormone (Coonce et al., 2009). The mRNA levels were also reported for insulin-like growth factor binding protein 3 and they were up-regulated in 3- and 5-week-old yoked LH receptor mice, and the mRNA levels for several germ cell-specific proteins were up-regulated at 5 weeks of age in both wild type and yoked LH receptor mice (Coonce et al., 2009).

STRUCTURE OF cAMP

cAMP is an important ubiquitous intracellular second messenger that has many cellular effects and regulates many fundamental biological processes (Beavo and Brunton, 2002, Gao and Zhang, 2010) (Figure 7). For instance, cAMP is involved in immune function (Torgersen et al., 2002), regulation of insulin secretion (Holz, 2004) and wide variety of metabolic, cytoskeletal and transcriptional responses (Gao and Zhang, 2010).

cAMP is produced in cells by a family of enzymes localized at the plasma membrane called adenylyl cyclases. These enzymes are capable of catalyzing ATP to cAMP (Cooper, 2003). Adenylyl cyclases are activated after the activation of GPCRs that stimulate Gs proteins and are inhibited by GPCRs that stimulate Gi proteins. cAMP is hydrolyzed by specific phosphodiesterases which terminate its action (Soderling and Beavo, 2000; Houslay and Adams, 2003).

cAMP exerts its cellular action via activation of three different kinds of effectors: cAMP-dependent protein kinases (PKA) (Taylor et al., 1990; Tasken and Aandahl, 2004), cyclic nucleotide-gated channels (CNGC) (Finn et al., 1996; Biel et al., 1999) and exchange proteins directly activated by cAMP (Epac) (Bos, 2003). Activation of all these.

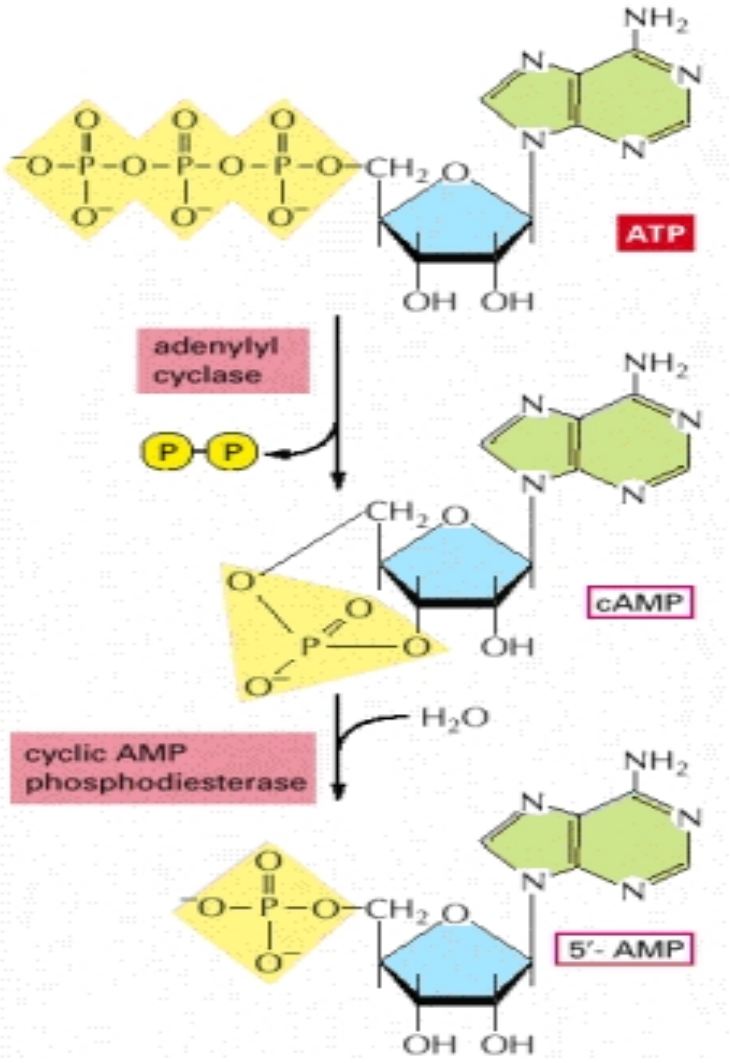


Figure 7: The chemical structure of the cAMP second messenger.

proteins can be used to monitor changes in the intracellular cAMP concentrations

FLUORESCENT RESONANCE ENERGY TRANSFER (FRET)

Over the past few decades, the development of new genetically encoded fluorescent labels coupled with the advances in fluorescence microscopy have made FRET the most useful and powerful technique for studying the physical interactions between membrane receptors in viable cells (Chan et al., 2001; Shekar and Periasamy, 2003). Fluorescence reasonance energy transfer (FRET) is a microscopic imaging technique that includes hetero-FRET and homo-FRET. FRET is widely used to determine the interchromophoric distance relationships in biomolecules and supramolecules on cell surfaces. For example, FRET is valuable in evaluating the interactions between LH receptors in the plasma membrane.

FRET is a distance-dependent interaction between the electronic excited states of two fluorescent molecules in which the energy is transferred nonradioactively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) without emission of photon. During the fluorescence process, a photon of energy is supplied by an external source such as a laser and absorbed by the fluorophore.

FRET was first proposed by Von Forster in 1948. This technique can be an accurate measurement of molecular proximity at an angstrom distance less than 100^{o}A between the donor and acceptor pairs, and the efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation (Stryer and Haugland, 1967; Lakowicz, 1999) making it a powerful technique for studying a variety of phenomena that produce changes in molecular proximity (Berney and Danuser, 2003). FRET

methods permit colocalization of proteins and other molecules to be imaged with spatial resolution beyond the limits of conventional optical microscopy (Kenworthy and Edidin, 1998).

Hetero-FRET

Hetero-FRET refers to the FRET between two different fluorescent proteins while homo-FRET refers to FRET between two identical fluorescent proteins. In hetero-FRET, excitation of a fluorescence donor such as CFP leads to energy transfer to fluorescence acceptor such as YFP and emission by that acceptor when a donor- acceptor pair is in close proximity at distance less than 100^{o}A (Figure 8). Acceptor photobleaching is one method that can be used to image FRET between CFP and YFP. Cyan and yellow fluorescent proteins make a good FRET pair since excitation of CFP at 440nm does not excite YFP. FRET imaging is a sequential procedure in which images of CFP (the donor florophore D) and YFP (the acceptor florophore) are obtained separately. Then YFP is photobleached for approximately 5 minutes. During photobleaching, the fluorescent acceptor (YFP) is irreversibly photobleached. Finally, after the fluorescent acceptor is photobleached completely, CFP and YFP are imaged again. When there is FRET between the two fluorescent proteins, the fluorescence signal from the donor increases after the acceptor has been photobleached and the intensity of CFP before and after YFP photobleaching can be used to evaluate energy transfer efficiency (%E). %E is calculated as fluorescence of the donor after photobleaching YFP, minus the fluorescence of the donor before photobleaching YFP, divided by the donor fluorescence after

photobleaching times 100. The efficiency of energy transfer is calculated using the following formula:

$$E\% = 1 - (D_{prebleach} / D_{postbleach}) \times 100$$

ICUE1, AN Epac-BASED cAMP REPORTER

As fluorescence techniques have improved, the development of fluorescent sensors, including a cAMP sensor, has been important for monitoring real-time signaling events in living cells. FRET is one of the fluorescent techniques that has been used with a number of different FRET-based indicators to monitor the intracellular Ca2+, kinase activities, protein-protein interactions, and cAMP levels (Zhang et al., 2002). As previously mentioned, cyclic AMP is a second messenger that regulates many cellular functions via different kinds of effectors including protein kinase A (PKA) and Epac, exchange proteins directly activated by cAMP (Zhang et al., 2004). Epac is a family of proteins that are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 (de Rooij et al., 1998). Rap GTPases cycle between an inactive GDP-bound state and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. Rap proteins are involved in many biological processes, most importantly, the regulation of cell adhesion through integrins and cadherins (Bos, 2003) (Figure 9).

The GEF Epac1 is composed of a C-terminal catalytic domain, characteristic of Ras family GTPase exchange factors, and an N-terminal regulatory domain. The N-terminal regulatory domain contains a cAMP-binding site similar to those of protein kinase A (PKA). In addition, it has a DEP domain which mediates the membrane attachment (de Rooij., et al, 1998; Rehmann et al., 2003). Zhang et al. have constructed

several cAMP mediators that report intracellular cAMP levels and Epac activation by sandwiching the full-length Epac1 between CFP and YFP (Zhang et al., 2004) (Figures 10, 11). Elevations in cAMP decrease FRET between CFP and YFP and increase the ratio of cyan-to yellow emissions by 10-30% in living mammalian cells (Figure-12). This response can be reversed by removing cAMP-elevating agents or abolished by mutating the critical residue responsible for cAMP binding. Targeting of the reporter to the plasma membrane, where cAMP is produced in response to the activation of β-adrenergic receptor, has been used to demonstrate a faster cAMP response at the membrane than in the cytoplasm and mitochondria (Zhang et al., 2004).

In this project, the Epac reporter has been used to evaluate LH receptor activity in cells expressing constitutively active LH receptors or when receptor activation occurs via *cis-activation* of LH receptor pairs.

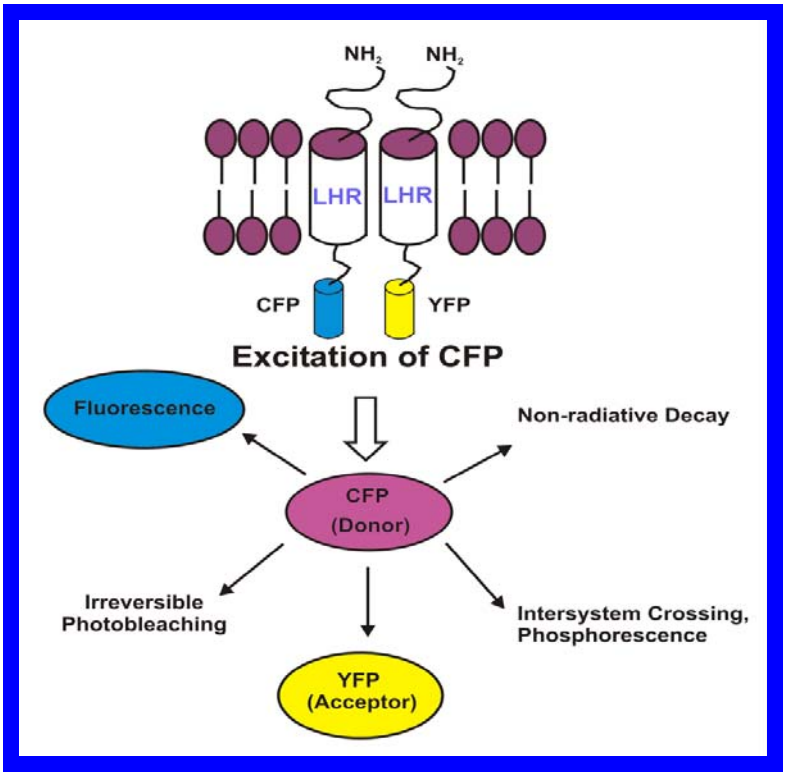


Figure 8. Hetero-FRET between CFP-LHR and YFP-LHR. Activation of the fluorescence donor CFP results in energy transfer to the YFP acceptor when these molecules are less than 100Å apart. When energy transfer is occurring between CFP and YFP, photobleaching of the YFP acceptor, using a mercury arc lamp or other light source, results in an increase in fluorescence from the CFP donor.

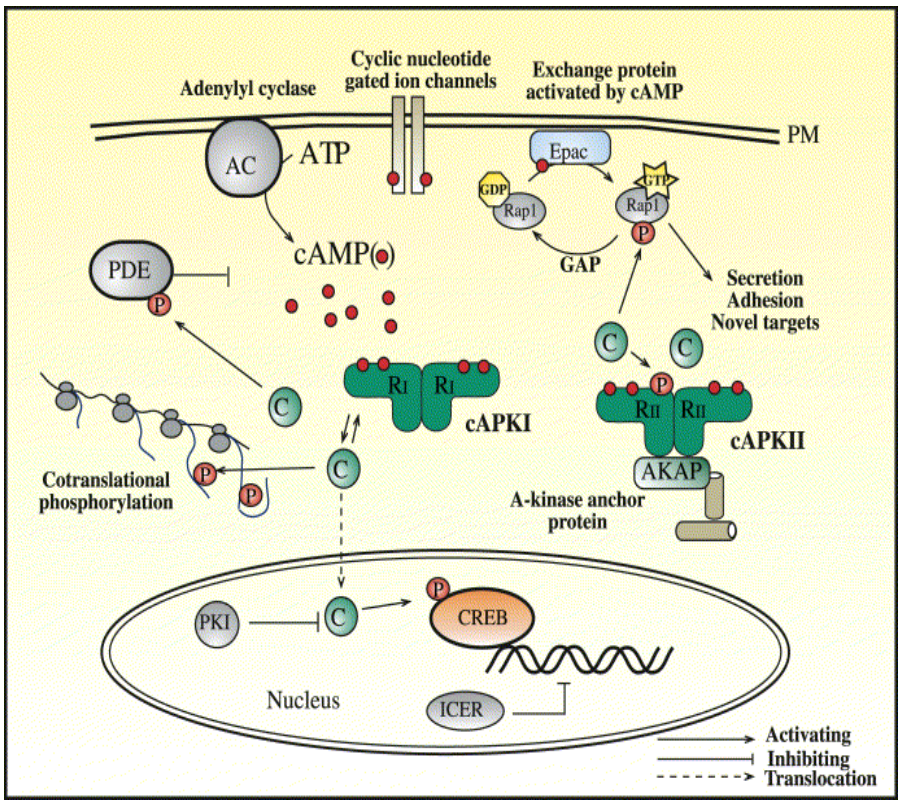


Figure 9: Epac is an exchange protein activated by cAMP. This molecule can be targeted to the plasma membrane using a membrane-targeting amino acid sequence and modified to include CFP and YFP. In the absence of cAMP, these fluorophores are close to one another and energy transfer from CFP to YFP occurs. When cAMP binds to Epac, a conformational change in Epac results in physical separation of CFP and YFP and a reduction in energy transfer efficiency (Kopprud et al., 2003)

(Adapted from Kopprud et al. 2003. FEBS Lett 3;546(1):121-6)

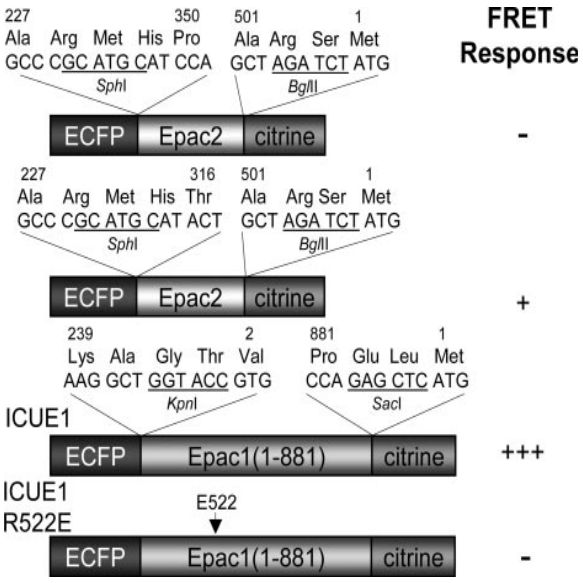


Figure 10: Domain structure and comparison of FRET responses for Epac-based cAMP reporters. Sandwiched between ECFP and citrine are truncated forms of Epac2, full-length Epac1 with or without R522E mutation, with R522 corresponding to R279 in Epac1. The construct that produced the highest FRET response in the absence of cAMP was designated as ICUE1 (Dipilato et al., 2004)

(Adapted from Dipilato L. et al., 2004. PNAS 101:16513-16518)

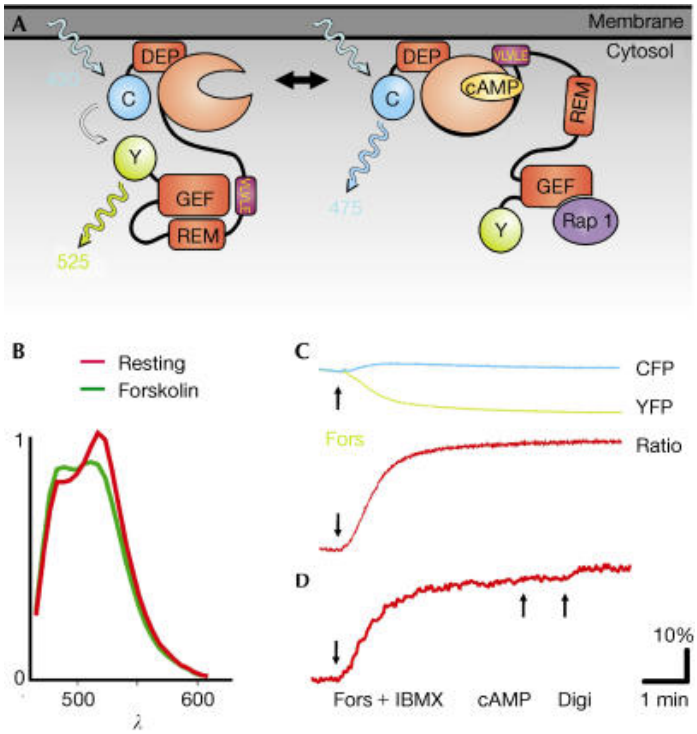


Figure 11: An Epac-based cAMP reporter has been engineered to undergo a decrease in FRET in the presence of cAMP. This decrease in FRET occurs between enhanced cyan (ECFP) and citrine fluorescent proteins due to the conformational change of Epac when binding cAMP (Bos 2003)

(Adapted from Bos et al. 2003 *Nat Rev Mol Cell Biol* 4(9): p. 733-8)

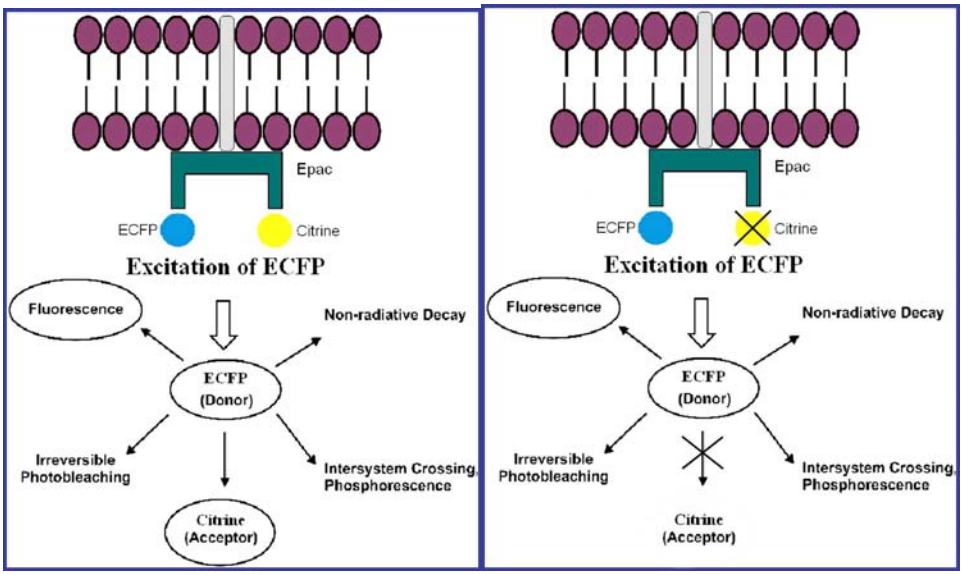


Figure 12: FRET between ECFP and citrine on Epac. In the absence of cAMP, ECFP and citrine are close together and values for energy transfer efficiency are high. The extent of energy transfer is evaluated by comparing ECFP fluorescence before and after irreversible photobleaching of citrine on Epac. When these molecules are close, photobleaching of citrine results in an increase in fluorescence emission from ECYP (Zhang et al., 2004).

(Adapted from Zhang J.F. et al., 2004. *J Chromatogr B Analyt Technol Biomed Life Sci* 804(2): p. 413-20)

CHAPTER II

EVALUATING LUTEINIZING HORMONE RECEPTOR FUNCTION USING THE CYCLIC AMP REPORTER PROBE ICUE1

INTRODUCTION

Levels of cAMP during LH signaling are used to assess G protein-coupled receptor activation by ligand. cAMP levels have been studied using several methods including ELISA techniques, colorimetric cAMP assays, radioimmunoassay and other methods. Recently, cAMP probes associated with fluorescent resonance energy transfer methods have been introduced to study cell signaling.

The goal of this project was to evaluate cAMP levels in cells expressing either wild type and constitutively active LH receptors using a FRET-based cAMP reporter. ICUE1 protein exchanger was used as an Epac-based cAMP reporter. This molecule undergoes a conformational change upon binding cAMP that reduces FRET between the fluorescent donor and acceptor that are integral components of this reporter molecule.

Hetero FRET between ECFP and EYFP on ICUE1 was performed using an imaging FRET method to detect a conformational change in ICUE1 upon binding cAMP. CHO cells were used and cell lines were prepared that transiently expressed ICUE1 alone or ICUE1 and Yoked LHR (YLHR).

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium (DMEM) containing high glucose was purchased from Irvine Scientific, Santa Ana; CA. Genticin (G418) sulfate was purchased from Mediatech, Inc. (Manassas, VA). Non-essential amino acid solution was purchased from Sigma-Aldrich, Inc., St. Louis, MO. Fetal bovine serum (FBS) was purchased from Invetrogen (Carlsbad, CA). L-glutamine, penicillin and streptomycin were purchased from Gemini Bioproducts, Woodland, CA. Intact highly pure hCG was purchased in 1x PBS from Fitzgerald Industries, Inc., Concord, MA. Forskolin was purchased from Sigma-Aldrich, Inc., St. Louis, MO.

Cell culture

The DNA for ICUE1 cloned in pcDNA 3 was a gift from Lisa DiPilato at The Johns Hopkins University School of Medicine. The DNA for the yoked LH receptor cloned in pcDNA 3 was a gift from Dr. Prema Narayan at Southern Illinois University.

Untransfected Chinese hamster ovary (CHO) cells were maintained in untransfected CHO cell medium that was made of 450ml DMEM containing high glucose, 50ml fetal bovine serum, 5ml non–essential amino acids solution, 5ml L-glutamine and 5 ml penicillin–streptomycin solution. Transfected CHO cell medium was made with all of the ingredients used for the untransfected CHO cell medium with the addition of 0.2g / 500ml G418 (geneticin).

Vector Preparation (DNA Amplification)

For amplification, DNA was transformed into *E.Coli*, DH5α, which readily takes up the DNA when heat shocked at 42^{o}C. Cells were then incubated on ice for 35 minutes. *E.Coli* and DNA mixture was then plated on L-broth agar plates with ampicillin. The plates are then left to grow overnight at 37^{o}C. Mono-clones were picked up and inoculated in 3 ml L-broth medium with ampicillin. The colonies are then grown at 37^{o}C on a shaker for about 16 hours. The DNA was extracted from *E.Coli* according to the Qiagen mini-prep protocol. The DNA samples were detected by running 0.8% agarose gel stained with ethidium bromide to determine whether the extracted DNA was the correct size. DNA concentration was determined by using Smart Spec 3000 (Bio-Rad).

Cell Transfection

Chinese hamster ovary (CHO) cells were cultured in 2mL untransfected CHO cell medium in 35 x 10mm dishes at 37^{o}C. When cells were at about 40-50% confluency, CHO cells were transfected with 2.5 μg ICUE1 DNA in 6.25 μl Lipofectamine 2000 according to the Invitrogen Lipofectamine 2000 protocol. ICUE1 was added as either a single vector, or to accomplish co-transfection of two vectors, ICUE1 + YLHR or ICUE1 + LHR-wt. After transfection, CHO cells were maintained in 2ml transfected CHO cell medium.

Imaging analysis of FRET using Fluorescence dequenching

To evaluate the cAMP levels in cells transfected with ICUE1 reporter alone or with ICUE1 and YLHR, flasks containing 3-4×10 CHO cells were selected. The medium

was discarded and cells were removed from the flask using 5mM EDTA, washed with 12mls of untransfected CHO cell medium. Cells then were plated on 35×10mm Petri dish and incubated for 12-24 hours. After incubation cells were transfected with ICUE1 plasmid and incubated again for 24 hours. The cells were removed from the Petri dish using 5mM EDTA and washed with 2mls of transfected CHO cell medium. The cells suspension (1ml) was plated in 2-Well Lab-Tek II#1.5 Chambered Coverglas slides. Cells then were incubated for 12-24 hours depending on the cells density before they were used for FRET measurement. After incubation, cells were washed twice with PBS or BSS, placed in 1ml PBS or BSS and imaged within 20 minutes.

For hCG treatment, cells in each chamber were treated with 100nM hCG suspended in PBS. After the CHO transfected media were removed from the chambers, cells were washed twice with PBS and suspended in 1ml PBS + 100nM hCG and incubated for 30-45 minutes before imaging. All images were initiated within 20 minutes. For treatment with forskolin, cells in each chamber were treated with 20nM suspended in 1ml PBS. After the cells were washed twice with PBS, cells were suspended in 20nM forskolin + 1ml PBS and imaged directly.

In cells that were co-transfected with ICUE1 and YLHR, cells were transfected with ICUE1 as mentioned previously, incubated for 24-48 hours and then transfected with YLHR and left to incubate for 24 hours before they used for FRET measurement.

Results

Different values of %E were obtained from CHO cells expressing ICUE1 alone, CHO cells expressing ICUE1 and treated with 100nM forskolin, CHO cells expressing

both Yoked LH receptor and ICUE1, CHO cells expressing both Yoked LH receptor and ICUE1 and treated with 100nM hCG, and CHO cells expressing both Yoked LH receptor (YLHR) and ICUE1 and treated with 100nM forskolin. Representative experiments are shown in Figure 13 and 14. Untreated CHO cells expressing ICUE1 cells had an average FRET efficiency of 19% which suggested that the basal levels of cAMP were low in these cells and that there was no significant conformational change in ICUE1 due to cAMP binding.

When CHO cells expressing ICUE1 were treated with 100nM forskolin, energy transfer efficiency was markedly reduced to 4.5%. This is consistent with forskolin effects on adenylate cyclase activity and increased cellular levels of cAMP. A representative experiment is shown in Figure 15.

We evaluated the activity of yoked LHR in CHO cells. YLHR has been reported to be constitutively active in the absence of exogenous hCG. In CHO cells that express both ICUE1 and YLHR, FRET efficiency decreased to 10.5% suggesting that yoked LHR has activity in CHO cells and that the basal levels of cAMP are higher than normal. A representative experiment is shown in Figure 16.

When CHO cells expressing ICUE1 and YLHR were treated with 100nM of exogenous hCG, FRET efficiency decreased significantly to 5.2%. This decrease suggests that exogenous treatment with hCG results in an increase in the cAMP levels and thereby a decrease in FRET efficiency. A representative experiment is shown in Figure 17.

Finally, we evaluated the FRET efficiency in CHO cells expressing ICUE1 and YLHR and treated with 100nM forskolin. Forskolin treatment causes maximal activation

of adenylate cyclase and this resulted in an increase in cAMP levels in the cells and FRET efficiency of 4%. A representative experiment is shown in Figure 18. A Summary of these results is shown in Table 1 and Figure 19.

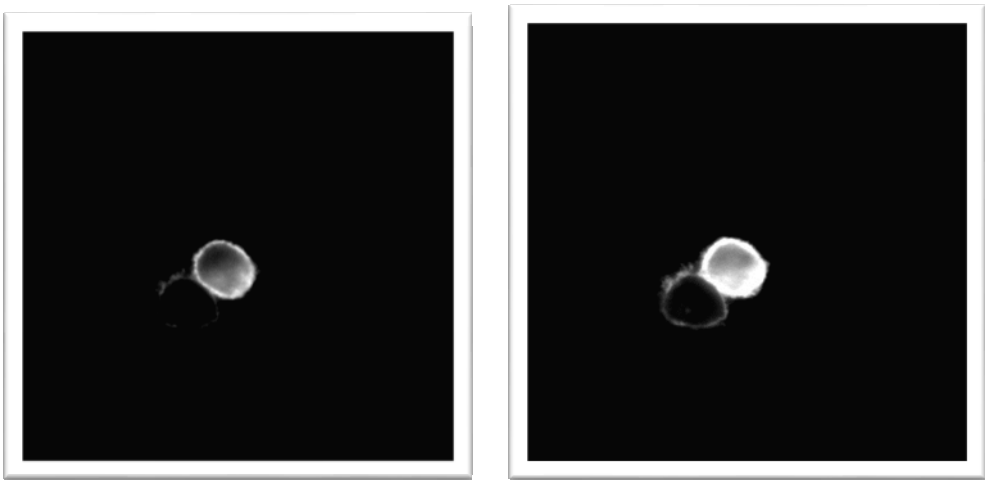
Discussion

We used fluorescence dequenching of the fluorescent donor to evaluate the efficiency of energy transfer between the fluorescent donor (CFP) and fluorescent acceptor (YFP). This FRET method has a number of advantages, the most important being that all measurements of fluorescence emission from the fluorescence donor are accomplished on the same cell. To perform these experiments, CHO cells were transiently transfected with both CFP- and YFP-coupled LH receptors which is reported to increase cell membrane expression (Tao et al., 2004). We imaged CFP and YFP fluorescence separately using fluorescence filter sets for these visible fluorescent proteins that minimized the fluorescence contribution from CFP when imaging YFP (LIopis et al., 2000) and that, in subsequent steps, permitted photobleaching of YFP only. Following photobleaching, each cell was reimaged using the same filter sets.

ICUE1 transfected CHO cells showed high energy transfer efficiency suggesting that these cells produce the basal levels of intracellular cAMP. %E in these cells was 19% in cells that express ICUE1 DNA and are treated with 100nM forskolin, the energy transfer efficiency was low indicating that cAMP production was high because of the activation of adenylate cyclase in response to forskolin treatment. %E in this case was 4.5% In CHO cells that were co-transfected with ICUE and YLHR (the constitutively active LHR), the energy transfer efficiency was 10.5%, and when cells were treated with

100nM hCG the %E was reduced to 4%. This indicates that hCG treatment of these cells causes a significant increase in the production of intracellular cAMP, a result that differs from the results of Narayan et al who showed that hCG treatment had no further effect on receptor function in CHO cells, although it had a measurable activation on LHR with an increase in intracellular cAMP in human emberyonic kidney 293 cells (Narayan et al., 2002). Treatment of these cells with 100nM forskolin results in a reduction of energy transfer efficiency to5.2% and this also indicated that the production of cAMP in these cells was high.

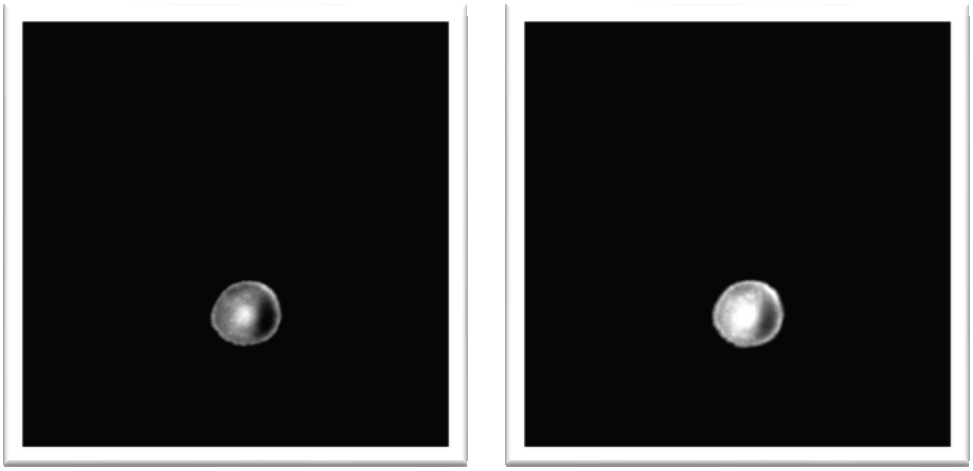
Together these results suggest that the ICUE1 reporter is a useful probe for evaluating cAMP levels in viable cells. Results from these various cell treatments were consistent with reported changes in cAMP evaluated using other, traditional biochemical assays such as colorimetric assays or assays requiring radiolabeled cAMP. The major advantage of ICUE1, however, is that this probe makes it possible to evaluate cAMP levels in real-time as cell conditions change and to evaluate these changes in cAMP within a single cell using single cell imaging methods.



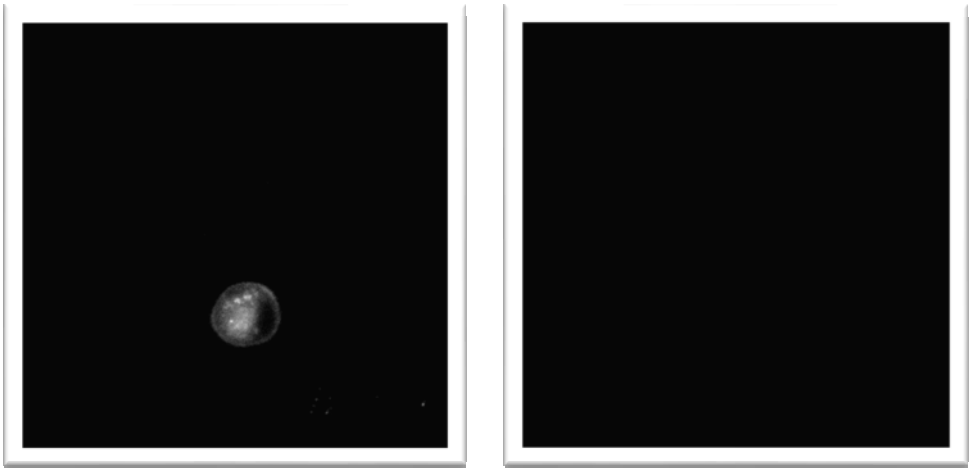
CFP before photobleaching CFP after photobleaching

YFP before photobleaching YFP after photobleaching

Figure 13. Hetero-FRET of a CHO-ICUE1 cell expressing ICUE1 showed CFP fluorescence increased to 21% after photobleaching YFP.



CFP before photobleaching CFP after photobleaching



YFP before photobleaching YFP after photobleaching

Figure 14. Hetero-FRET of a CHO-ICUE1 cell showed CFP fluorescence increased to19.4% after photobleaching YFP.

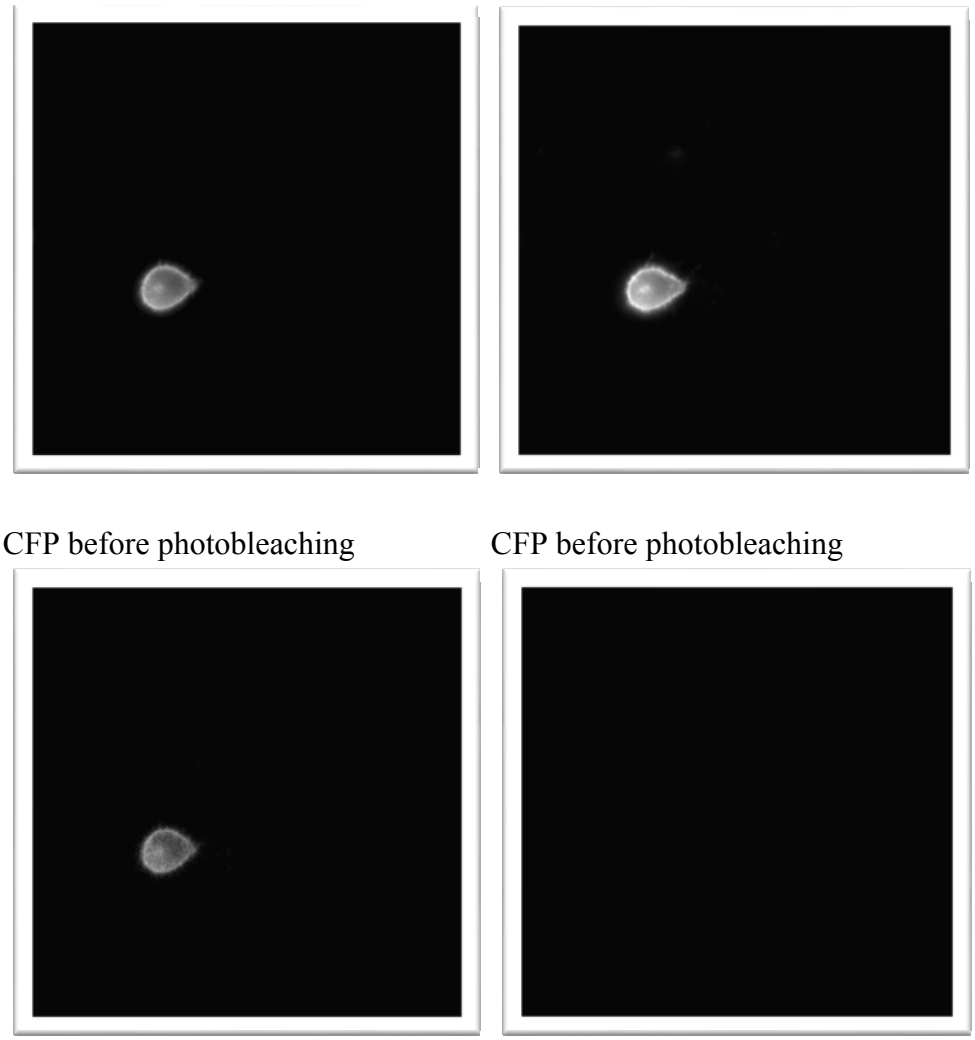
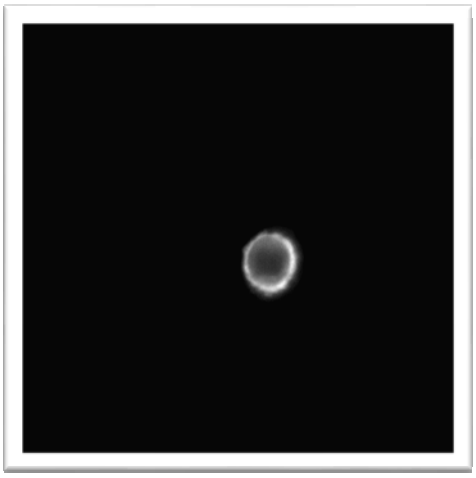
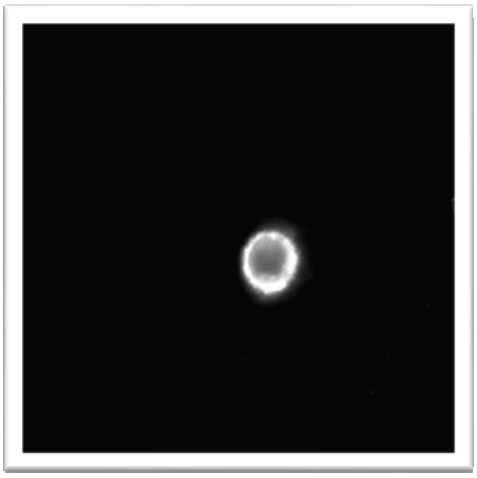


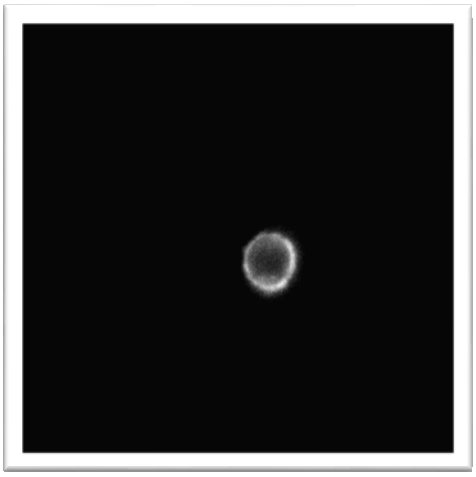
Figure 15. Hetero-FRET of a CHO-ICUE1 cell treated with 100nM forskolin for 5 minutes showed that the increase of CFP fluorescence was only 3.2% after photobleaching YFP.



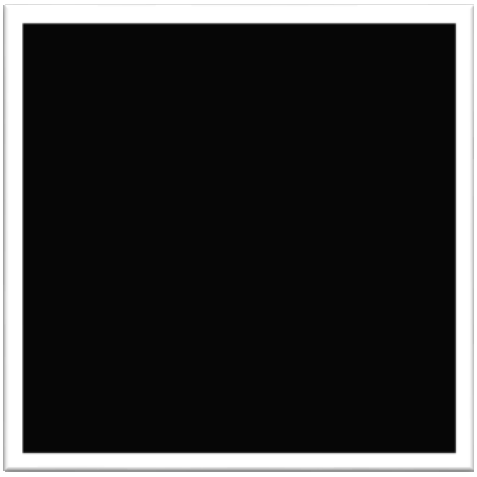
CFP before photobleaching



CFP after photobleaching

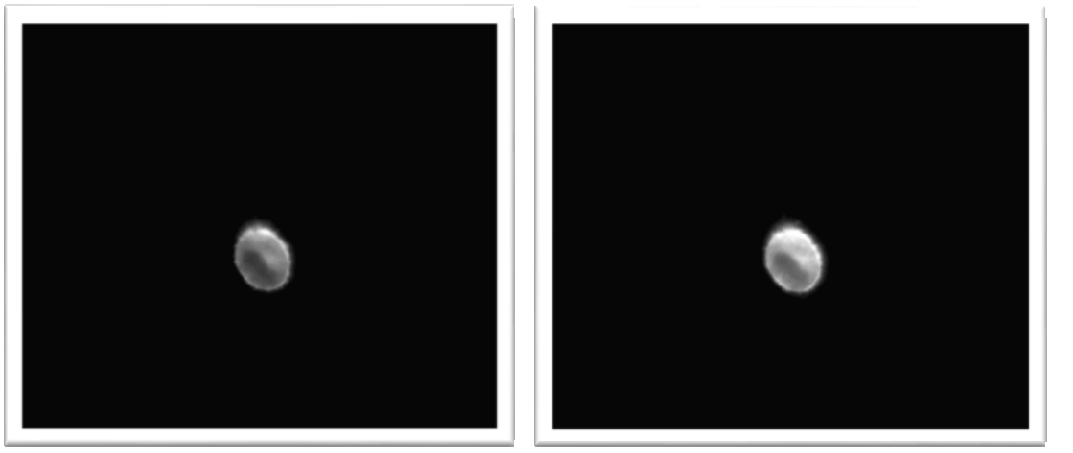


YFP before photobleaching

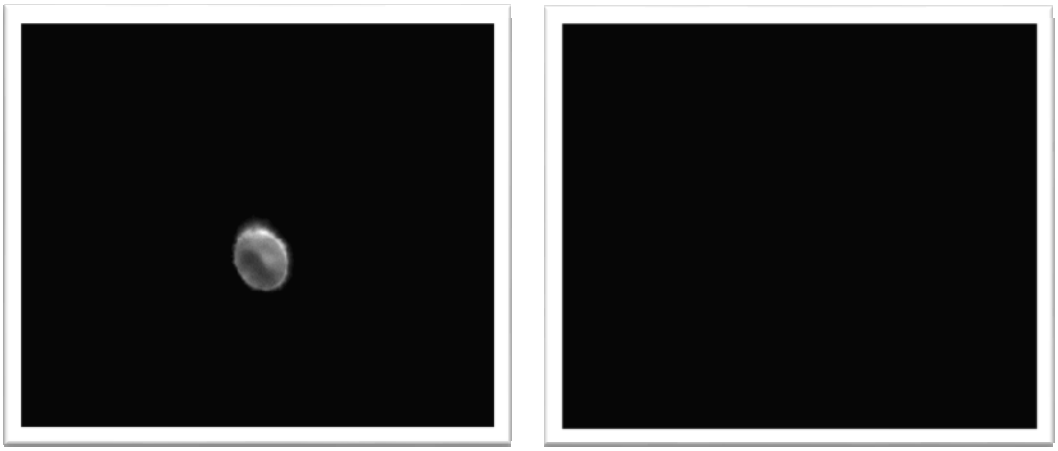


YFP after photobleaching

Figure 16. Hetero-FRET of a CHO-ICUE1-YLHR cell showed that an increase of CFP fluorescence and %E 12.4 % after photobleaching YFP.

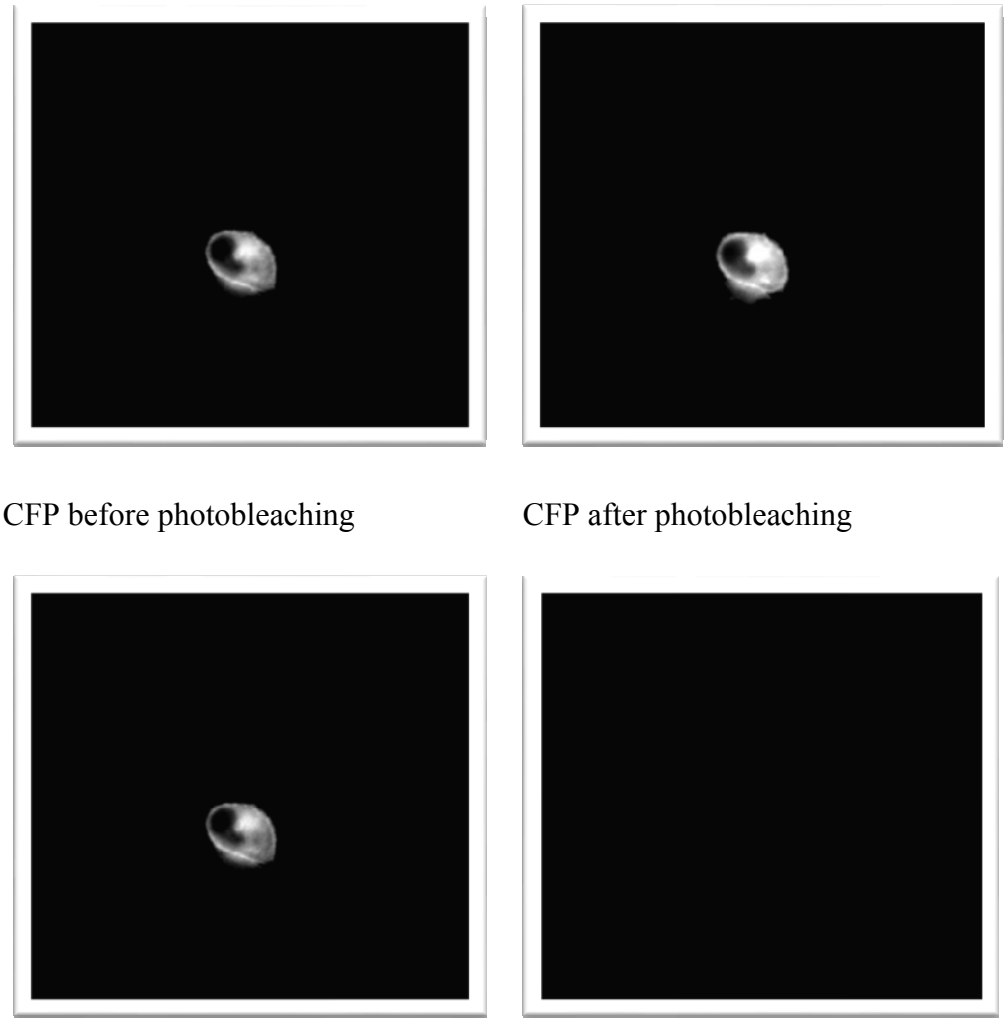


FP before CCFP before photobleaching CFP after photobleaching



YFP before photobleaching YFP after photobleaching

Figure 17. Hetero-FRET of a CHO-ICUE1-YLHR cell treated with 100nM forskolin for 5 minutes showed that an increase of CFP fluorescence and %E 4.8% after photobleaching YFP.



CFP before photobleaching CFP after photobleaching

YFP before photobleaching YFP after photobleaching

Figure 18. Hetero-FRET of a CHO-ICUE1-YLHR cell treated with 100nM hCG for 45 minutes showed an increase in CFP fluorescence and %E 3.7% after photobleaching YFP.

Table I. Summary of ICUE1 results

Sample	% Energy Transfer Efficiency	% standard deviation	n
CHO-ICUE1	19%	1.9%	12
CHO-ICUE1 treated with 100nM forskolin	4.5%[a]	1.3%	7
CHO-ICUE1-YLHR	10.5% [a]	1.6%	8
CHO-ICUE1-YLHR Treated with 100nM forskolin	5.2% [a]	1.2%	6
CHO-ICUE1-YLHR Treated with 100 nM hCG	4% [a]	1.3%	7

Values with the superscript a are different from CHO-ICUE1 (untreated) cells using a paired-test ($p<0.001$)

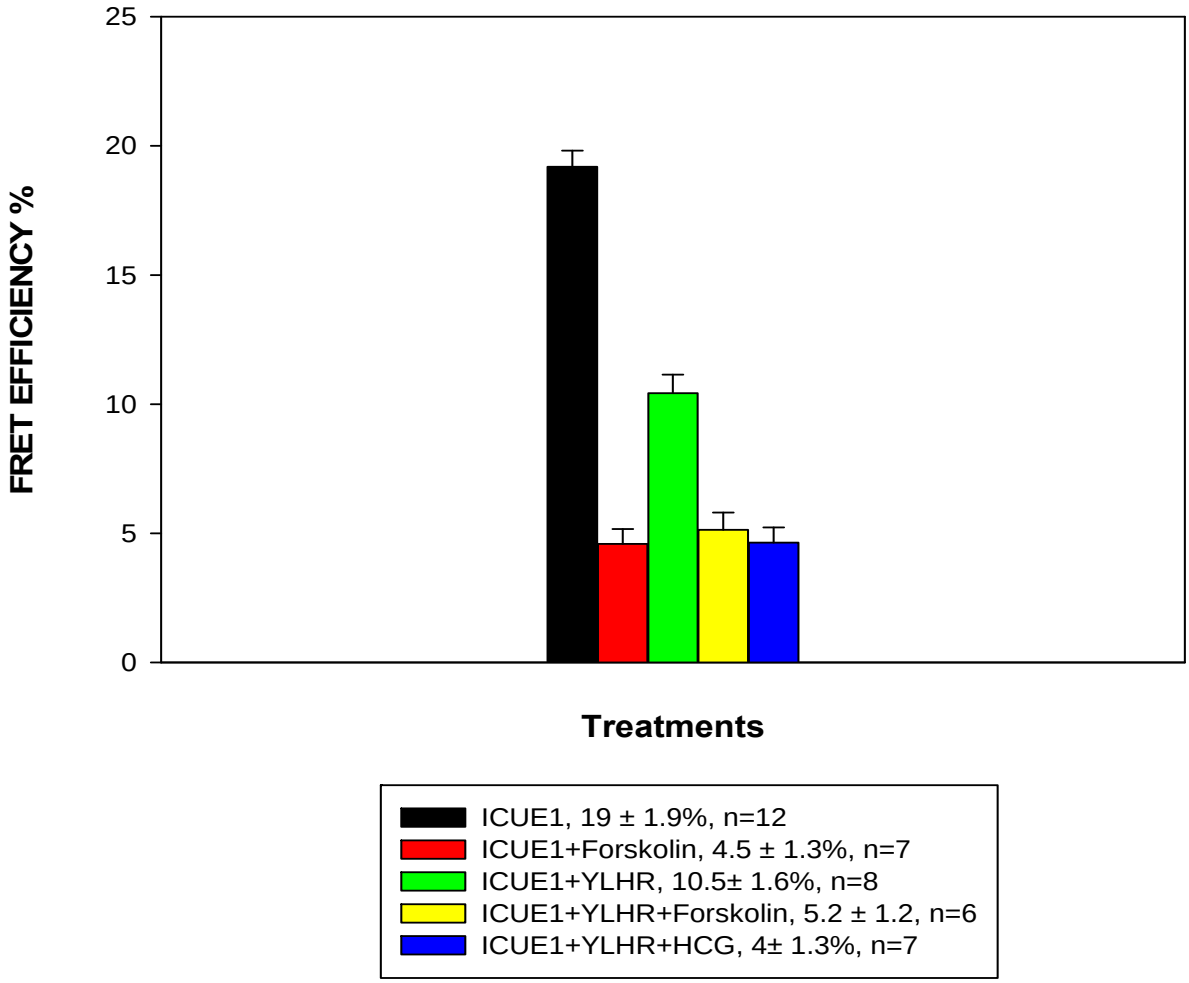


Figure 19 Energy transfer efficiency in CHO-ICUE1 and CHO-ICUE1-YLHR. There is a significant decrease in FRET in hCG and forskolin-treated cells suggesting that these treatments lead to increase cAMP production in these cells.

CONCLUSIONS AND FUTURE DIRECTIONS

LH receptor function is critically important for male and female reproductive success. These LH receptors signal through G proteins and participate in ovulation, regulation of sex steroid synthesis, and maternal recognition of pregnancy in mammals. We have investigated LH receptor function in signal transduction using the cyclic AMP reporter probe, ICUE1. Imaging FRET methods have been used to demonstrate receptor function under various conditions. This technique has provided insight into the sequence of events that occur following hormone binding and that lead to initiation of signal transduction.

Our data suggested that FRET efficiency in CHO cells expressing the exchange protein ICUE1 is high indicating that the level of cAMP in these cells is low. On the other hand, treatment of these cells with 100nM forskolin causes a maximal activation of the intracellular cAMP and causes an increase in FRET efficiency. FRET efficiency in CHO cells that were co-transfected with both ICUE1 and YLHR was lower than that in CHO cells expressing ICUE1 only. In CHO cells expressing both ICUE1 and YLHR FRET efficiency decreased in with forskolin treated cells and also decreased measurably in hCG treated cells. Future experiments should focus on evaluating FRET efficiency in cells that are co-transfected with ICUE1 DNA and Wt-LHR with and without exogenous hormone treatment.

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LIST OF ABBREVIATIONS

AC: adenylate cyclase

ACTH: adrenocorticotropic hormone

Asn: aspargine

ATP: adenosine triphosphate

BSS: hank's balanced salt solution

Ca^{+2}: calcium

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CFP: cyan fluorescent protein

CHO: chinese hamster ovary

CL: corpus luteum

CTP: c terminal peptide

Cys: cysteine

$D_{postbleach}$: donor fluorescent after acceptor photobleaching

$D_{prebleach}$: donor fluorescent before acceptor photobleaching

DAG: 1,2-diacylglycerol

DMEM: dulbecco's modified minimum essential medium

%E: energy transfer efficiency

ECFP: enhanced cyan fluorescent protein

eCG: equine chorionic gonadotropin

EDTA: ethylenediamineetetraacetic acid

Epac: exchange protein activated by cAMP

FBS: fetal bovine serum

FRET: fluorescent resonance energy transfer

FSH follicle-stimulating hormone or follitropin

G418: genticin

Gi: inhibitory G protein

Gs: stimulatory G protein

GDP: guanosine diphosphate

GEF: guanine exchange factor

GFP: green fluorescent protein

GnRH: gonadotropin releasing hormone

GnRHR: gonadotropin releasing hormone receptor

GPCR: G protein coupled receptor

GTP: guanosine triphosphate

hCG: human chorionic gonadotropin

hetero-FRET: heterotransfer fluorescent resonance energy transfer

hLHR: human luteinizing hormone receptor

homo-FRET: homotransfer fluorescent resonance energy transfer

HPG: hypothalamic-pituitary-gonadal

ICUE1: indicator of cAMP using Epac

IP3: inositol 1,4,5-triphosphate

LH: luteinizing hormone

LHR luteinizing hormone receptor

LRP: leucine-rich repeat

mRNA: messenger ribonucleic acid

PBS: phosphate buffered saline

PDGF-β: platelet-derived growth factor beta

PIP2: phosphatidylinositol 4,5-biphosphate

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipaase C

Ser: serine

TGF-β: transforming growth factor beta

Thr: threonine

TSH: thyroid-stimulating hormone or thyrotropin

Wt: wild type

YFP: yellow fluorescent protein