THESIS

EVALUATING LUTEINIZING HORMONE RECEPTOR SIGNALING USING THE CYCLIC AMP REPORTER ICUE3

Submitted by

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ABSTRACT

EVALUATING LUTEINIZING HORMONE SIGNALING USING THE CYCLIC AMP REPORTER ICUE3

The luteinizing hormone (LH) receptor is a member of the G protein-coupled receptor family and the subfamily of glycoprotein hormone receptors. The LH receptor plays a vital role in normal development and in the function of the gonads. The LH receptor is expressed in interstitial cells, thecal cells, and granulosa cells and in cells making up the corpus luteum. Signal transduction by LH receptors is dependent on hormone activation of LH receptors and subsequent activation of G proteins. Evaluating the cyclic adenosine monophosphate (cAMP) level in response to binding of ligand to LH receptors depends on activation of adenylyl cyclase by G proteins and production of cAMP.

In this project, fluorescence resonance energy transfer (FRET) methods were used to evaluate cAMP levels in individual cells in response to binding of ligand to LH receptors. These studies used ICUE3, an EPAC-based reporter molecule for cAMP in cells. ICUE3 is an engineered molecule that contains a FRET donor and acceptor pair as well as a membrane-targeting sequence. FRET occurs when the donor fluorophore in an excited electronic state transfers its excitation energy to a nearby acceptor chromophore. In the absence of bound cAMP, the fluorescence donor in ICUE3 transfers energy to the fluorescence acceptor. When ICUE3 binds cAMP, the donor-acceptor distance increases and the FRET signal is reduced.

cAMP levels were evaluated in individual cells expressing ICUE3 and LH receptor under different conditions including exposure of cells to human chorionic gonadotropin (hCG). In

some experiments, CHO cells co-expressing ICUE3 and constitutively-active LH receptors, LH receptors yoked to a single-chain modified form of hCG (yLHR), were used to determine whether the presence of a constitutively-active receptor increased basal cAMP levels in CHO cells. Our results show that ICUE3 is a reliable reporter molecule that measures basal cAMP levels in untreated cells and is responsive to changes in intracellular cAMP levels in response to forskolin treatment or, in the presence of a functioning LH receptor, to hCG.

ACKNOWLEDGEMENTS

This completion of this project would not have been possible without the support of many people. I would first like to express my deepest gratitude to my parents, brothers and sisters for supporting me in my studies with their unconditional love.

I would also like to thank my advisor Dr. Deborah A. Roess who welcomed me to the lab and gave me the chance to pursue graduate studies at Colorado State University. I appreciate the guidance that she provided throughout my graduate study.

I have been honored to have Dr. George Barisas and Dr. Debbie C. Crans as members of my graduate committee. Their help has been invaluable to me in completing this project. I would like to thank all my friends who supported me in my academic work and members, both past and present, of the Roess/Barisas lab.

DEDICATION

This thesis is dedicated to my mother, who taught me that the best kind of knowledge is knowledge learned for its own sake. It is also dedicated to my father, who taught me that even the largest task can be accomplished if it is done one step at a time.

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CHAPTER I

INTRODUCTION

The goal of this study was to measure cyclic adenosine monophosphate (cAMP) levels individual cells in response to binding of ligand to luteinizing hormone (LH) receptors. cAMP is a second messenger that regulates many fundamental cellular functions including cell metabolism, cytoskeletal organization and transcriptional responses [1-3].

EPAC has been identified as a cAMP binding protein that mediates some aspects of cAMP signaling [4]. A modified form of EPAC with a fluorescence donor and acceptor at opposite ends of the molecule, ICUE3, can be used as a reporter for cAMP levels in cells. FRET measurements can be made in real time using hetero-transfer FRET methods. FRET signals depend on the ability of an excited FRET donor to transfer energy to a FRET acceptor. If FRET occurs, fluorescence emission from the excited donor will be reduced and acceptor emission will increase [5]. When using variants of green fluorescent protein (GFP), proximity of the fluorescent donor and acceptor within $\sim 0.09 \mu m^2$ is sufficient for FRET signals [6]. In this project, a constructed molecule containing a FRET donor and acceptor, ICUE3 [7], was used to evaluate changes in cAMP in cells expressing LH receptors.

LUTEINIZING HORMONE RECEPTOR STRUCTURE

Luteinizing hormone (LH) receptors play an essential role in reproductive function in both male and female mammals by enhancing ovulation, follicle maturation, corpus luteum formation, spermatogenesis and steroidogenesis. Signaling by LH receptor occurs in response to LH or human chorionic gonadotropin (hCG) which affects lateral and rotational dynamics of the LH receptor, causes fundamental changes in receptor conformation and increases receptor-

receptor interactions [8-10]. LH itself is released from the anterior pituitary together with follicle stimulating hormone. These glycoprotein hormones are members of the superfamily of cysteine-knot growth factors and work together to organize gonadal functions [11] including maturation of the testes and ovary, steroidogensis and gametogensis. In women, gonadotropins play important roles in regulating follicular growth, ovulation and progesterone production by luteal cells of the ovary [11]. In men, LH is needed for testosterone production by Leydig cells in the testes [11].

The LH receptor is a glycoprotein which consists of a single amino acid sequence encoded in DNA [12]. Figure 1 shows the amino acid sequence of the LH receptor and its structural homology to other glycoprotein hormone receptors [11]. The LH receptor can be divided into functional units including a seven membrane transmembrane/cytoplamic module that anchors the receptor in the plasma membrane. This portion of the receptor interacts with G proteins via sites in the transmembrane domains, intracellular loops and the receptor C-terminus and converts signal initiated by binding of hormone to the extracellular domain to receptor interactions with G proteins [12, 13]. Signal transduction leads, ultimately, to activation of adenylyl cyclase and production of cAMP. Two adjacent cysteines, Cys621 and Cys622, in the LH receptor C terminus provide additional membrane anchorage sites and are palmitoylated [14, 15]. The addition of palmitoylation creates a fourth intracellular loop that is used to couple ligand binding to intracellular signaling. The large extracellular domain of the LH receptor has approximately 341 amino acids and contains a single LH/hCG binding site [13]. Both LH and hCG can bind the receptor with high affinity. The extracellular domain of N-terminal is distinguished by leucine-rich repeats that are important for hormone binding to the receptor [16]. The N-terminus of the LH receptor also contains a signal sequence of 22 amino acids in the

human and 26 amino acids in the rat that is important for insertion of the LH receptor into the endoplasmic reticulum during translation.

LH receptors can be modified to form constitutively-active receptors. The yoked LH receptor (yLHR) was developed by Wu, Narayan and Puett [17] and is composed of a single chain hCG molecule formed from sequential α and β chains covalently coupled to the LH receptor amino acid sequence. To construct the complex, the entire coding sequence of the sequential α and β chains of hCG were followed by the first half of the C-terminal peptide (CTP) sequence which was then ligated to 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 [17]. Cells transfected with yLH receptor had elevated basal levels of cAMP suggesting that yLHR was constitutively active. The yLHR was also functional when expressed in transgenic mice which had elevated testosterone levels at 3 and 5 weeks of age [18].

G PROTEINS MEDIATE LH RECEPTOR SIGNALING TRANSDUCTION

Binding of ligand to the LH receptor results in activation of G proteins. G proteins are heterotrimers consisting of α, β and γ subunits. They are classified based on amino acid sequence similarities in their α subunits which are encoded into seventeen different genes [19]. Based on sequence homology, Gα are further designated as $G_{\alpha}i$, $G_{\alpha}q$, etc. [20]. The exchange of GDP for GTP on α subunits is responsible for the controlled dissociation-reassociation of α subunits and βγ subunits [21]. Both α subunit and βγ subunits are signaling molecules in their own right and modulate the activity of specific downstream effectors [22].

CYCLIC ADENOSINE MONOPHOSPATE (cAMP) AND EPAC

cAMP is a second messenger which activates cyclic nucleotide-regulated ion channels, protein kinase A (PKA) and binds EPAC. cAMP is produced by two classes of adenylyl cyclases (AC). The activity of transmembrane AC is regulated by heterotrimeric G proteins in response to hormone binding to extracellular domains on transmembrane receptors. This form of AC is tethered to the plasma membrane. Soluble AC is localized to microtubules, the nucleus, mitochondria and centrioles and is affected by calcium levels [23]. Following activation of adenylyl cyclase and production of cAMP, increases in intracellular cAMP affect a number of enzymes and cell function. As an example, cAMP regulates PKA activity. PKA in its inactive conformation has two regulatory subunits binding to two catalytic subunits [23]. The catalytic subunits dissociate following cAMP binding to the complex which results in the availability of an active enzyme [23]. cAMP also binds EPAC which is a guanine nucleotide exchange factor (GEF) [4]. The GEF EPAC1 contains a N- terminal regulated domain and a C-terminal catalytic domain that is characteristic of exchange factors from the Ras family of GTPases. The latter domain has a cAMP-binding site similar to that of PKA and a domain which mediates membrane attachment [4]. cAMP *in vitro* is required for the activation of EPAC [4]. EPAC is folded into an active conformation when cAMP levels are low in a way that prevents Rap binding due to steric hindrance.

REPORTER MOLECULES FOR cAMP ENGINEERED FROM EPAC

cAMP is the second messenger that regulates many fundamental cellular functions including metabolic, electrical, cytoskeletal, and transcriptional responses by cells [2]. EPAC has been identified as a cAMP binding protein that mediates cAMP signaling [4]. EPAC- based

reporter molecules can be used to measure cAMP levels by sandwiching the full length EPAC between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) which function as a fluorescence donor and a fluorescence acceptor, respectively. The reporters based on EPAC have been constructed so that fluorescence resonance energy transfer (FRET) from CFP to YFP occurs in the absence of cAMP. ICUE3 is similar to earlier biosensors formed from EPAC. ICUE3 has a substitution of circularly permuted YFP (vpVenus) for citrine and the addition of sequences which can be myristylated or palmitoylated and thus used to target ICUE3 to the plasma membrane [7].

MEASURING CFP to YFP FRET ON LIVING CELLS

The extent of energy transfer between CFP and YFP can be measured by FRET methods and is an accurate measurement of molecular proximity at 10-100 angstroms (Å). Because FRET depends on the inverse sixth power of intermolecular separation [24], FRET is a sensitive technique for investigating different biological phenomena that produce changes in molecular proximity. Selection of a FRET donor and acceptor pair involves adequate separation in excitation spectra for selective excitation of the donor fluorophore and an overlap (>30%) between the emission spectrum of the donor and the excitation spectrum for the acceptor. It is also desirable to separate emission spectra of the donor and acceptor to allow independent measurement of fluorescence from each fluorophore [25].

Fluorescence resonance energy transfer (FRET) can be accomplished using microscope-based imaging to detect changes in inter-chromophore distances on cell surfaces. This technique can be used to provide an accurate assessment of molecular proximity at an angstrom distance less than 100Å between the donor and acceptor pairs (Figure 3) and the efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation [24].

Acceptor photobleaching is one method that can be used to image FRET between CFP and YFP. CFP and modified citrine (YFP) make a good FRET pair since excitation of CFP at 440nm does not excite YFP. In FRET imaging, images of CFP, the donor fluorophore D, and YFP, the acceptor fluorophore, are obtained separately. After the fluorescence acceptor is photobleached completely, CFP and YFP are reimaged. 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 of YFP, minus the fluorescence of the donor before photobleaching of YFP, divided by the donor fluorescence after photobleaching 100. The efficiency of energy transfer is calculated using the following formula:

E% = 1- (D prebleach / D postbleach) x 100

It is also possible to perform FRET studies of ICUE3 using a microscope-based system which is described in Chapter II. These measurements are faster and more consistent than photobleaching-based methods for FRET.

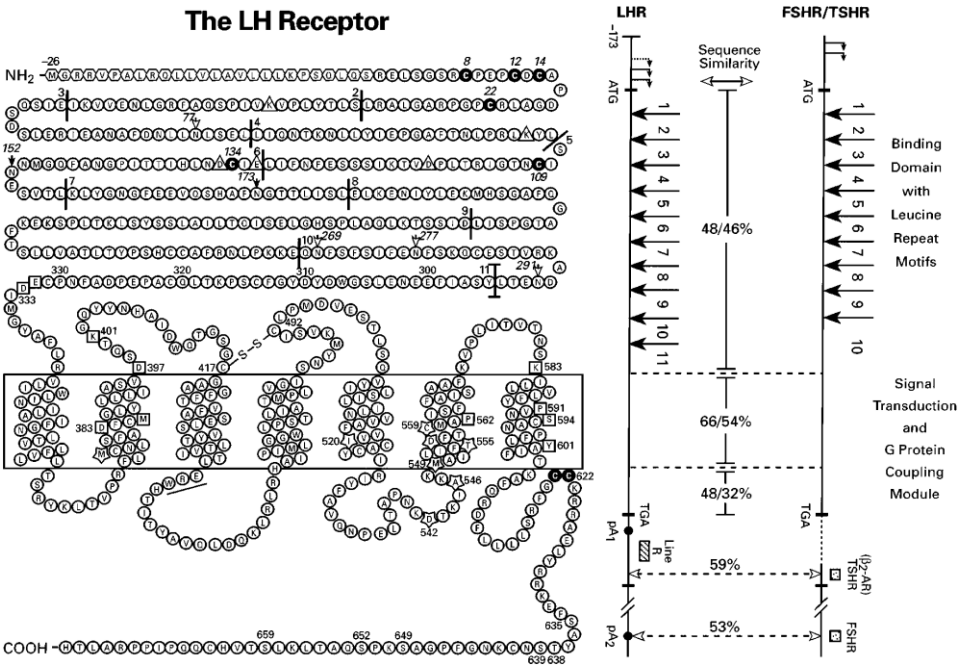

Figure 1: The amino acid sequence of luteinizing hormone (LH) receptors. The LH receptor is a single polypeptide G protein-coupled receptor with 699 amino acids. It has amino acid sequences that are similar to those seen in the thyroid-stimulating hormone receptor (TSHR) and the follicle stimulating hormone receptor (FSHR) as shown on the right. Adapted from Dufau [11].

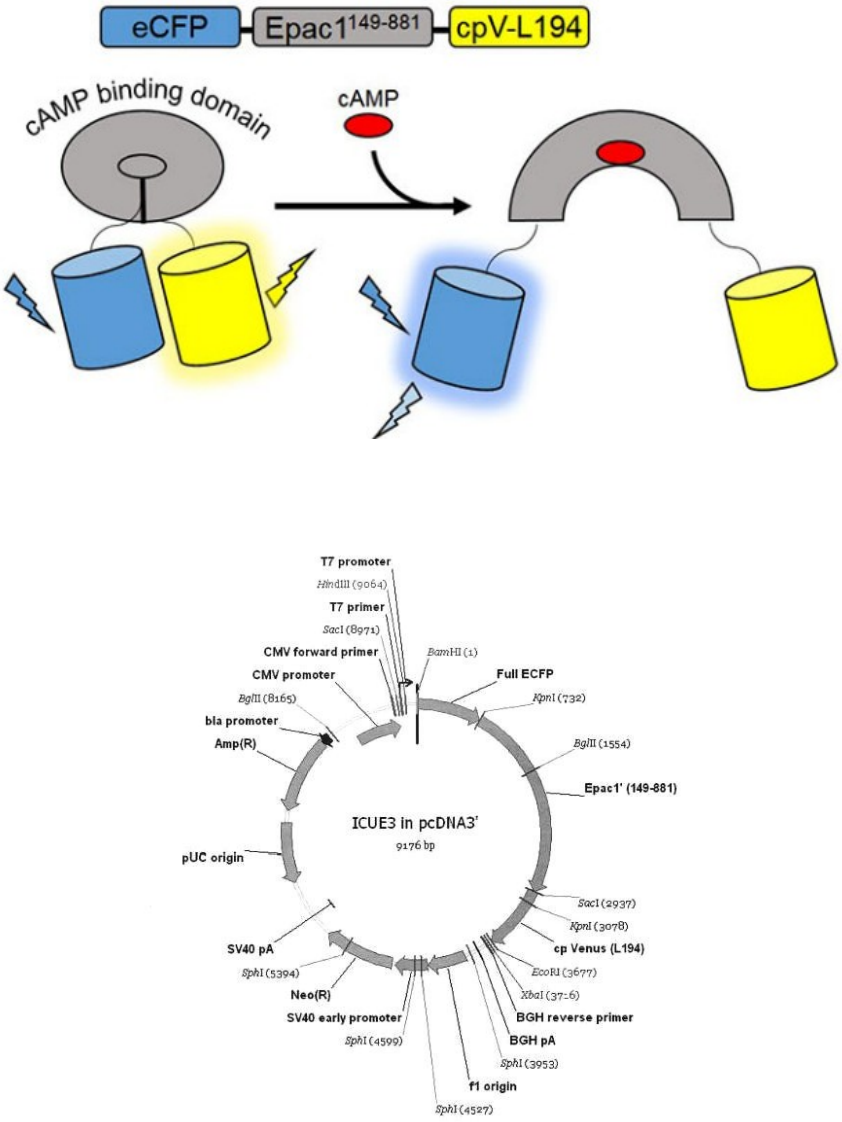

Figure 2. The domain structure of EPAC1 and EPAC2 chimeras (left) and the plasmid structure of ICUE3 which exhibits the best FRET responsive to cAMP (right image). The ICUE3 used in our experiments also has a membrane targeting sequence that is believe to be palmitoylated following gene expression. Adapted from. DiPilato et a. [26].

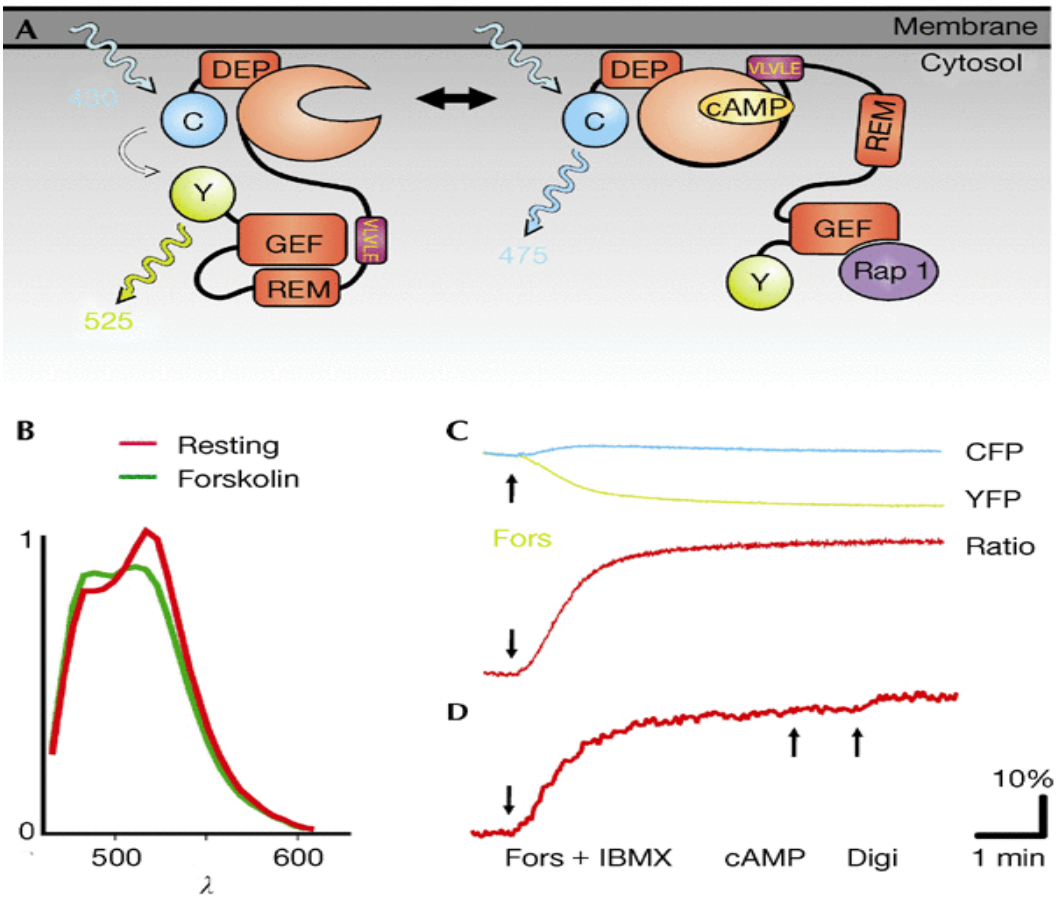

Figure 3. Heterotransfer FRET signal from ICUE in the absence (upper left) and presence (upper right) of cAMP. The lower left panel shows changes in FRET signal from ICUE in the absence of cAMP (red line) and the presence of cAMP (green line) YFP emission increases following CFP excitation occurs when cyan and yellow fluorophores are in close proximity to each other. A decrease in the FRET signal occurs when ICUE3 has bound cAMP due to a conformational change in cAMP-binding EPAC which separates the fluorescence donor and acceptor. The lower right panel demonstrate effects of adding compounds to cells expressing ICUE 3. (Adapted from Bos [27].

CHAPTER II

EVALUATING LUTEINIZING HORMONE SIGNALING USING THE cAMP REPORTER ICUE3

INTRODUCTION

The goal of this study was to measure cAMP levels in individual cells in response to binding of ligand to LH receptors. In this study we used ICUE3 to assess cAMP levels in CHO cells that expressed either ICUE3 alone or ICUE3 together with yLHR which is reported to be constitutively active [28].

MATERIALS AND METHODS

MATERIALS

Chinese Hamster Ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle medium (DMEM) was purchased from Corning Cellgro (Visalia, CA). Penicilin/streptomycin and L-glutamine solution were purchased from Gemini Bio-Products (West Sacramento, CA). FBS was purchased from Atlas Biologicals (Fort Collins, CO). 100X MEM non-essential amino acid solution, bovine albumin and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). Cyclic adenosine monophosphate (cAMP) and saponin were also purchased from Sigma-Aldrich (Milwaukee, WI). Human chorionic gonadotropin (hCG) was purchased from Fitzgerald Industries (Acton, MA). Forskolin was purchased from Enzo Life Sciences (New York, NY). Lipofectamine 2000 regent and OPTI-MEM reduced serum medium were purchased from Life

Technologies (Carlsbad, CA). Quantum R-PE and FITC MESF beads (molecules of equivalent soluble fluorophore) were purchased from Bangs Laboratories (Fishers, IN). Glass bottom cell culture dishes with 35mm diameter and 14mm diameter glass bottoms were purchased from In Vitro Scientific (Sunnyvale, CA). ICUE3 in pcDNA 3 was a gift from Lisa DiPilato at The School of Medicine of Johns Hopkins University. DNA for yLHR cloned in pcDNA 3 was a gift from Dr. Prema Narayan at Southern Illinois University.

ICUE3 PLASMID DNA AMPLIFECTION IN *E. coli (DH5α)*

3 μg of DNA expression plasmid was added to 30 uL *E. coli DH5α* which were heat shocked for 45 sec at 42°C and transferred to ice for 2 min with gentle mixing. L-broth media with DNA, 5uL ampicillin and cells were grown at 37°C with vigorous shaking (225 rpm) for one hour. *E.coli* were then plated together with DNA on L-broth agar with ampicillin and cells were grown overnight at 37°C. A single colony was selected from the plate and used to inoculate 2-5 ml of L-broth media. The colony was grown at 37°C with vigorous shaking (280 rpm) for 18 hours. Plasmid DNA was purified from *E. coli* using Qiagen Plasmid Mega and Giga kits. The DNA concentration was determined by running a 0.8% agarose gel and UV spectrophotometry at 260 nm.

TRANSFECTION OF CHO CELLS

Chinese Hamster Ovary (CHO) cells were grown in a $25cm^2$ culture flask in DMEM medium supplemented with 10% FBS, 2mM L-glutamine, 100 units of penicillin/ml, 100μg streptomycin/mL and 1% 1X MEM non-essential amino acid solution. All cells were maintained at 5% CO_2 and 37°C in a humidified environment. Before transfection, cells were incubated with

5mM EDTA for 5 minutes and 1.5ml cells were plated in a 35mm glass bottom petri dish. Cells grew to approximately 80%-90% confluence in Petri dishes in two days.

CHO cells were transiently transfected with 3.2μg of cAMP reporter ICUE3, kindly provided by Dr. Jin Zhang and Lisa DiPilato at Johns Hopkins University, Baltimore, USA, using Lipofectamine 2000 in accordance with Manufacturer's instructions. Two sterilized microcentrifuge tubes each containing 125 mL of OPTI-MEM medium were prepared. One tube contained 3.2 μg of ICUE3 and the other tube contained 6 μl of Lipofectamine 2000. The two microcentrifuge tubes containing ICUE3 and Lipofectamine 2000 were mixed together by drop by drop addition of diluted DNA to diluted Lipofectamine 2000 and held at room temperature for 5 min. The mixture was then was added to the cells in a 35 mm Petri dish which contained 1ml OPTI-MEM reduced serum medium. Transfection proceeded for at least 12 hours while maintaining cells in incubator with 5% CO_2 at 37ºC. In some experiments, CHO cells were transiently co-transfected with 3.2 μg ICUE3 and μg yLHR using the same procedure described for ICUE3 alone.

HETERO-FRET MEASURMENT USING DUAL EMISSION RAIO IMAGING

After cells were transient transfected with ICUE3 alone or ICUE3 and yLHR, cells were washed twice with and maintained in 1x phosphate buffered saline (PBS) PH 7.3 containing 0.1% BSA. Ratio imaging of CFP and YFP emission due to FRET as well as YFP emission with 488nm light used the procedure developed in Dr. Jin Zhang's laboratory [29]. Imaging FRET data were collected using a 1.4 N.A. 63X water objective in a Zeiss Axiovert 135 inverted microscope with ANDOR ixon EM+ camera controlled by METAFLUOR software. A neutral density filter was used to reduce the intensity of the arc lamp source. Emission ratios were obtained using a 436DF20 filter for CFP excitation, a 455 DRLP dichroic mirror, and two

emission filters (480DF40 for CFP emission and 535DF30 for YFP emission due to CFP→YFP energy transfer). All filters were obtained from Chroma Technology. Images were taken every 50s for up to 23 minutes. Dishes of cells were analyzed by selecting and focusing on cells that were neither too bright or too dim and moving the dishes from edge to edge in a zigzag manner to avoid repeat measurements. In some expermints PBS from the dishes was discarded, replaced by medium containing 50 μM forskolin or 100 nM hCG, and cells were incubated at room temperature for 15 min. Maintaining the same objective and camera settings, cells were photographed as described above. From these images, fluorscence emission for the entire cell was evaluated using the Image J software and analyzed for CFP emission and the FRET signal resulting from YFP emission with 436nm excitation. Fluorescent images were background-corrected by subtracting fluorescence intensities of backgrounds with no cells from emission intensities of the isolated cell membranes.

RESULTS

Initial experiments evaluated the ICUE3 signal from CHO cells before and after treatment with 50 uM forskolin. Forskolin is a diterpene produced by the Indian Coleus plant that reversibly activates adenylyl cyclase and increases intracellular levels of cAMP. Adenylyl cyclase activation by forskolin is unique because it does not require interaction with any major cell surface receptors [30]. We predicted that in the presence of forskolin, ICUE3 signal would be reduced due to binding of cAMP by the reporter molecule and separation of CFP and YFP fluorophores. As summarized in Table 1, 50 μM forskolin treatment increased the ratio of CFP emission:YFP emission by 1.33 ± 0.07 fold (n=21) when cells were exposed to 436nm light. An increase in the ratio of CFP emission relative to YFP emission, the FRET signal, reflects a decrease in transfer of energy from CFP to YFP. Figure 4 shows a representative trace with data

from a single cell. Introduction of 50 μM forskolin typically produced a large, rapid increase in the CFP:YFP ratio for cells exposed to 436nm light.

To verify results obtained with forskolin treatment, cells were also treated with saponin and cAMP (Figure 5). Saponin is a plant product that permits cAMP to enter cells readily. Treatment of cells with saponin and cAMP, caused a highly variable change in the CFP:YFP ratio. These experiments did not show a consistent increase in the ratio of CFP emission:YFP emission due to FRET, and thus were not conclusive, perhaps due to experimental conditions that reduced permeability of cAMP (data not shown). A data trace from one experiment is shown in Figure 5 although other data were noisy and inconsistent.

Finally, for comparison with subsequent experiments evaluating effects of hCG treatment on cells expressing both ICUE3 and yLH receptors, we examined effects of hCG on CHO cells expressing ICUE3 alone. To the best of our knowledge, there are no native LH receptors on CHO cells. As summarized in Table II, 100 nM hCG had only a modest effect on cAMP production causing a 1.04 ± 0.03 (n=18) increase in the CFP:YFP emission due to FRET.

We next co-expressed both ICUE3 and the yLHR in CHO cells as described in Materials and Methods above. The yLHR is reported to be a constitutively active when both beta and alpha subunits of hCG are linked in tandem to the LH receptor sequence. It is believed that this activity results from the close proximity of folded hCG to binding sites on the receptor [31-35]. To evaluate the activity of yLHR, we examined baseline levels of cAMP in cells expressing both the receptor and ICUE3 (1.25±0.08) and compared them to baseline ratios for CFP:YFP ratios for CHO cells expressing only ICUE3(1.01±0.06) and lacking a response to hCG as summarized in Table III. The presence of yLHR caused a decrease in the FRET signal and an accompanying

increase in the ratio of CFP:YFP emission indicating high cAMP levels in cells expressing yLHR.

Addition of hCG to cells expressing ICUE3 and yLHR caused a small increase (1.11±0.08-fold increase) in intracellular cAMP (Table IV and Figure 8) compared to a smaller increase in cAMP in cells expressing ICUE3 alone (1.04±0.03-fold increase in CFP:YFP ratio). This result is consistent with results presented by Wu et al. [28] who achieved only small increases in cAMP response with hCG treatment using yLHR-expressing cells when compared to responses in cell lines expressing the wild type LH receptor.

Forskolin treatment had a significant effect on cAMP in these cells (Table V). As shown in Figure 7 and, as seen for cells expressing ICUE3 alone, there was a rapid rise in intracellular cAMP following addition of forskolin although the overall magnitude of this increase in cAMP was less than that seen in cells that did not express yLHR (summaried in Table I).

We also used saponin treatment together with exogenous cAMP to determine whether addition activation of adenylyl cyclase was possible in cells expressing yLHR. These studies appeared to be more successful that experiments with ICUE3 alone and produced a 1.96±0.14 fold increase in CFP:YFP signal (Table VI). Table VII summarizes results obtained for CHO cells expressing only ICUE3 and for cells do-expressing ICUE3 with yLHR.

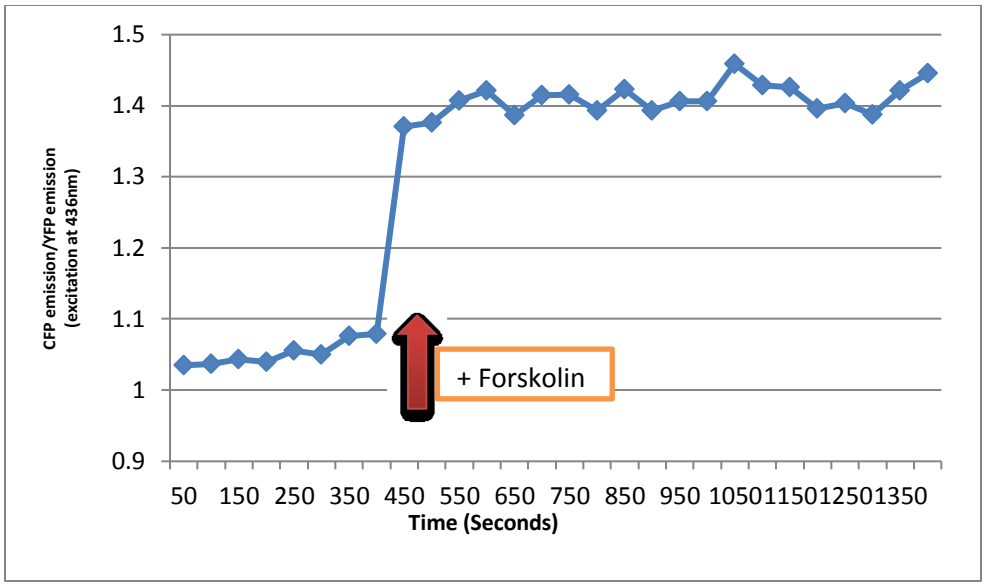

Figure 4: Effect of forskolin on CPF:YFP emission in cells expressing ICUE3 alone. This figure shows a representative data trace from a CHO cell expressing only ICUE 3. Upon addition of 50 μM forskolin, there is an increase in the ratio of CFP:YFP emission following excitation at 436 nm. Forskolin was added at the time indicated by the arrow and produced a rapid increase in intracellular cAMP as indicated by the increase in the ratio of CFP emission: YFP emission when cells were illuminated with 436nm light.

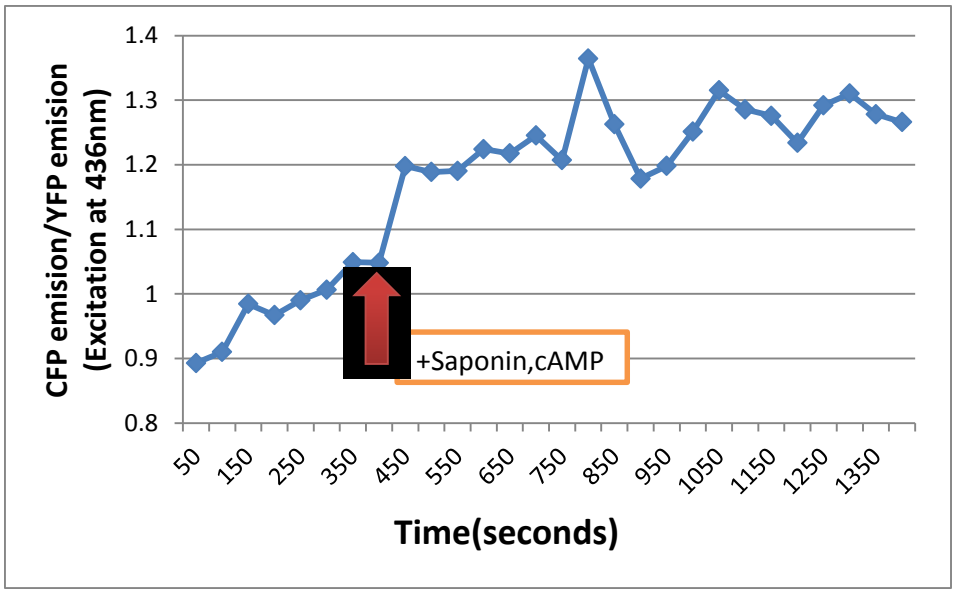

Figure 5: Effect of adding saponin and cAMP to CHO cells expressing ICUE3 alone. This representative data trace shows the effect of adding 0.05% saponin and 50μM cAMP to CHO cells expressing ICUE3. Saponin and cAMP were added at the time indicated by the arrow and produced a more gradual increase in CFP emission relative to YFP emission when cells were illuminated with 436 nm light.

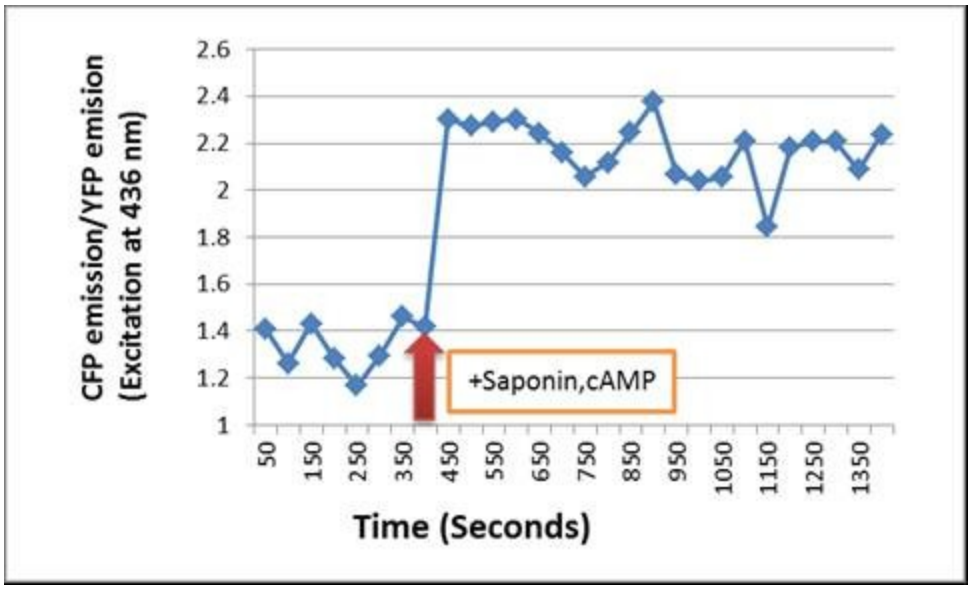

Figure 6: Effect of adding saponin and cAMP to CHO cells expressing ICUE3 and yLHR. This representative data trace shows the effect of adding 0.05% saponin and 50 μM cAMP to CHO cells expressing ICUE3. Saponin and cAMP were added at the time indicated by the arrow and produced a rapid increase in CFP emission relative to YFP emission when cells were illuminated with 436 nM light. The magnitude of the change in CFP:YFP emission was less than seen in cells expressing ICUE3 alone.

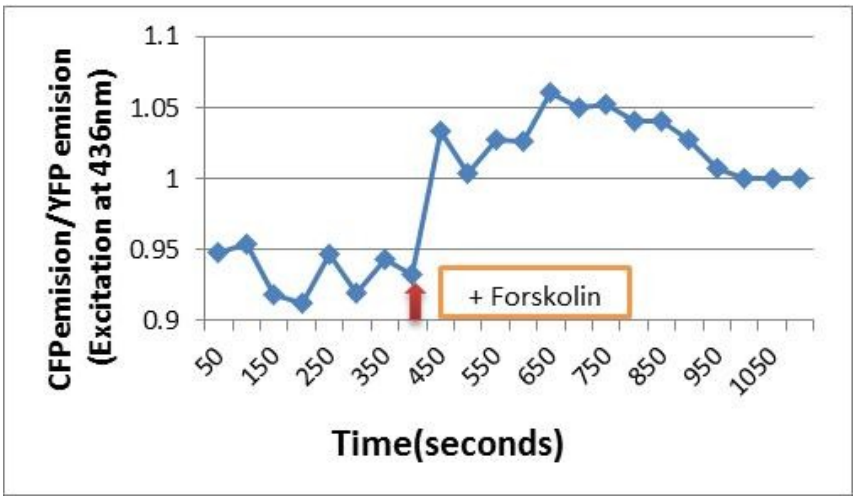

Figure 7: CHO cell showing the change in FRET signal in CHO cells expressing both ICUE 3 + yLHR following treatment with 50 μM forskolin. In response to 50 μM forskolin, the emission ratio of CFP:YFP was increased. Forskolin was added as indicated by the arrow and produced a rapid increase in intracellular cAMP similar to that seen in cells expressing ICUE3 alone.

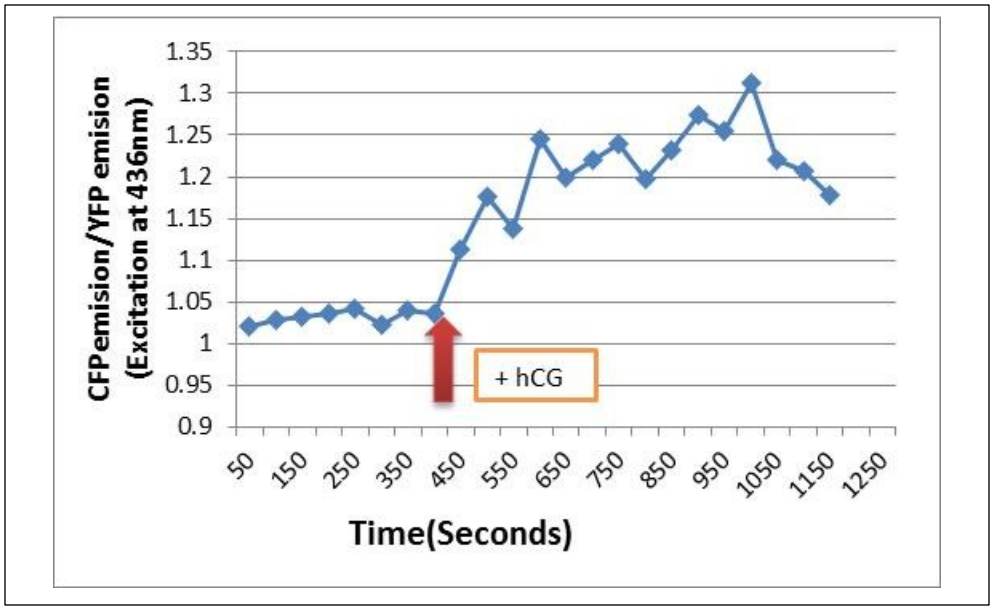

Figure 8: Effect of 100 nM hCG on a representative CHO expressing ICUE 3 and yLHR. A CHO cells expressing both ICUE3 and yLHR were treated with 100 nM hCG at the time indicated by the arrow. hCG treatment produced a gradual increase in intracellular cAMP as indicated by the increase in the ratio of CFP:YFP emission when cells were illuminated with 405nm light. The magnitude of this change was comparatively small which, as discussed in Result, may be due to higher basal levels of cAMP in cells expressing yLHR.

Table I: Effects of 50 μM forskolin treatment on CHO cells expressing ICUE3 alone[1].

CELL	ICUE3 ALONE	50 μM FORSKOLIN	FOLD-CHANGE IN SIGNAL
1	0.68±0.03	1.2±0.03	1.76
2	1.24±0.06	0.96±0.06	0.77
3	0.72±0.05	1.19±0.03	1.65
4	0.83±0.07	1.13±0.03	1.37
5	0.79±0.05	1.39±0.03	1.76
6	0.91±0.04	1.04±0.04	1.15
7	1.14±0.1	1.24±0.04	1.08
8	1.16±0.14	1.35±0.07	1.16
9	0.95±0.04	1.4±0.04	1.46
10	0.9±0.06	1.19±0.04	1.32
11	0.97±0.06	1.07±0.06	1.10
12	1.05±0.03	1.42±0.03	1.35
13	0.64±0.03	1.09±0.02	1.70
14	0.56±0.03	0.86±0.14	1.53
15	0.57±0.04	0.99±0.05	1.75
16	0.62±0.04	1.01±0.04	1.63
17	0.89±0.05	0.96±0.02	1.08
18	0.85±0.03	1.05±0.03	1.23
19	0.73±0.16	0.86±0.03	1.18
20	2±1	1.17±1.17	0.59
21	0.75±0.03	0.9±0.02	1.20
$\bar{x}$			1.33
S.D			0.32
SEM			0.07

[1]CHO cells were transfected with ICUE3 alone and exposed to 50 μM forskolin. Column 1 shows the cell being treated. Column 2 shows baseline values for the CFP:YPF ratio as discussed in Methods and Materials. Column 3 shows the CFP:YFP ratio following addition of 50 μM forskolin to CHO cells. Column 4 shows the "Fold-change in signal" which is the ratio of the signal following forskolin treatment: FRET baseline values for each cell.

Table II: Effects of 100 nM hCG on ICUE3 signal in CHO cells[1]

CELL	ICUE3 ALONE	100nM hCG	FOLD-CHANGE IN SIGNAL
1	0.96±0.05	0.96±0.05	1.00
2	0.96±0.04	0.94±0.04	0.98
3	0.59±0.03	0.68±0.05	1.16
4	0.53±0.02	0.56±0.05	1.06
5	0.78±0.03	0.66±0.03	0.85
6	1.17±0.05	1.26±0.14	1.07
7	1.03±0.05	1.16±0.13	1.13
8	0.76±0.04	0.95±0.1	1.25
9	1.17±0.06	1.05±0.04	0.90
10	0.95±0.04	1.26±0.07	1.32
11	1.26±0.03	1.15±0.04	0.91
12	1.16±0.02	1.33±0.04	1.14
13	1.29±0.16	1.2±0.05	0.93
14	1.08±0.13	0.87±0.04	0.80
15	2.11±0.06	2.12±0.08	1.00
16	2.15±0.06	2.04±0.1	0.95
17	0.55±0.03	0.6±0.06	1.09
18	0.52±0.04	0.56±0.05	1.07
$\bar{x}$			1.04
S.D			0.14
SEM			0.03

[1]CHO cells were transfected with ICUE3 alone and exposed to 100nM hCG. Column 1 shows the cell being treated. Column 2 shows baseline values for the CFP:YPF ratio as discussed in Methods and Materials. Column 3 shows the CFP:YFP ratio following addition of 100 nM hCG to CHO cells. Column 4 shows the "Fold-change in signal" which is the ratio of the FRET signal following hCG treatment: FRET baseline value for each cell.

Table III: Baseline CFP:YFP emission values for FRET signals for cells expressing ICUE 3 alone and for cells expressing ICUE 3 and yLHR. Baseline values for FRET signals from cells expressing ICUE3 were compared with those obtained from cells expressing yLHR which is a constitutively active form of the LHR receptor. As indicated below, the mean ratio of CFP:YFP emission increased in cells expressing yLHR which indicated higher levels of cAMP.

	Baseline of ICUE3	Baseline of ICUE3+yLHR
	0.68	0.93
	1.23	0.98
	0.71	1.09
	0.82	0.86
	0.79	0.91
	0.9	0.92
	1.12	0.8
	1.16	0.99
	0.95	0.99
	0.96	1.15
	0.59	1.09
	0.52	0.98
	0.78	1.32
	1.17	0.81
	1.02	0.88
	0.75	2.04
	0.95	1.41
	1.23	1.85
	1.21	1.56
	0.98	1.98
	1.72	1.46
	1.86	1.74
	0.98	1.52
	1.13	1.7
$\bar{x}$	1.01	1.25
S.D	0.32	0.39
SEM	0.06	0.08

Table IV: Effect of hCG on ICUE3 signal in cells expressing y-LHR[1]

CELL	ICUE3+yLHR	100nM hCG	FOLD-CHANGE IN SIGNAL
1	0.99±0.05	1.14±0.05	1.15
2	1.15±0.05	1.17±0.06	1.01
3	0.98±0.08	3.29±1.49	3.35
4	0.82±0.09	1.05±0.08	1.29
5	0.88±0.09	0.91±0.1	1.04
6	1.27±0.1	1.43±0.1	1.12
7	1.28±0.13	1.16±0.14	0.91
8	0.96±0.04	1.08±0.12	1.13
9	0.95±0.03	1.18±0.09	1.23
10	1.05±0.04	1.16±0.07	1.10
11	0.96±0.04	1±0.06	1.04
12	1.05±0.05	1.2±0.12	1.14
13	1.04±0.02	1.2±0.07	1.15
14	1.58±0.12	1.55±0.07	0.98
15	1.89±0.15	1.73±0.07	0.91
16	0.99±0.06	1.08±0.07	1.09
17	0.89±0.06	1.17±0.09	1.32
18	1.34±0.12	1.16±0.08	0.86
19	0.91±0.06	1.33±0.08	1.46
20	1.05±0.06	1.34±0.17	1.27
21	1.09±0.05	1.38±0.14	1.27
$\bar{x}$			1.11
S.D.			0.45
SEM			0.08

[1]CHO cells transfected with ICUE3 and yLHR were treated with 100nM of hCG. Column 1 shows the cell being treated. Column 2 shows baseline values for the CFP:YPF ratio as discussed in Methods and Materials. Column 3 shows the CFP:YFP ratio following addition of 100 nM hCG to CHO cells. Column 4 shows the "Fold-change in signal" which is the ratio of theFRET signal following hCG treatment: FRET baseline value for each cell.

Table V: Effect of 50 μM forskolin on CHO cells expressing both ICUE 3 and yLHR[1]

CELL	ICUE3+yLHR	100 μM FORSKOLIN	FOLD-CHANGE IN SIGNAL
1	0.93±0.02	1.04±0.03	1.10
2	0.98±0.05	0.95±0.15	0.97
3	1.09±0.06	0.78±0.05	0.72
4	0.86±0.05	0.97±0.04	1.12
5	0.91±0.08	0.79±0.05	0.86
6	0.92±0.05	0.92±0.16	0.98
7	0.8±0.03	0.78±0.15	0.97
8	0.99±0.05	0.89±0.04	0.90
9	0.91±0.06	1.05±0.06	1.14
10	0.71±0.06	0.94±0.06	1.29
11	0.7±0.06	0.93±0.06	1.30
12	0.92±0.06	1.16±0.06	1.24
13	0.86±0.02	0.97±0.11	1.13
14	0.95±0.04	1.13±0.09	1.18
15	1.04±0.02	1.03±0.08	0.98
16	0.8±0.01	0.8±0.09	1.00
17	0.98±0.02	0.86±0.08	0.88
18	0.88±0.06	0.83±0.05	0.94
19	0.79±0.07	1.22±0.05	1.54
$\bar{x}$			1.07
S.D.			0.19
SEM			0.04

[1]CHO cells were transfected with ICUE3 and yLHR and exposed to 50 μM of Forskolin, Column 1 shows the cell being treated. Column 2 shows baseline values for the CFP:YPF ratio as discussed in Methods and Materials. Column 3 shows the CFP:YFP ratio following addition of 50 μM forskolin to CHO cells. Column 4 shows the "Fold-change in signal" which is the ratio of the FRET signal following hCG treatment: FRET baseline value for each cell.

Table VI. Effect of treatment with saponin and cAMP on CHO cells expressing both ICUE3 and yLHR.

CELL	ICUE3+yLHR	0.05% SAPONIN+50 μM cAMP	**FOLD-CHANGE IN SIGNAL**
1	1.42±0.13	1.25±0.05	1.42
2	1.86±0.13	1.98±0.08	1.86
3	1.56±0.07	2.04±0.18	1.56
4	1.98±0.13	1.86±0.27	1.98
5	1.46±0.09	2.34±0.23	1.46
6	1.74±0.11	2.48±0.22	1.74
7	1.52±0.08	2.16±0.19	1.52
8	1.7±0.07	1.85±0.16	1.70
9	1.62±0.07	2.19±0.17	1.62
10	1.69±0.09	2.21±0.2	1.69
11	1.55±0.14	1.98±0.15	1.55
12	1.35±0.1	2.17±0.13	1.35
13	1.16±0.07	1.81±0.12	1.16
14	3.11±0.31	3.08±0.19	3.11
15	3.45±0.32	3.13±0.22	3.45
16	2.89±0.32	3.43±0.29	2.89
17	2.75±0.17	3.09±0.17	2.75
18	2.9±0.22	3.18±0.17	2.90
19	2.69±0.27	3.04±0.18	2.69
20	1.36±0.08	1.85±0.17	1.36
21	1.44±0.05	1.53±0.08	1.44
22	2.03±0.12	1.51±0.07	2.03
$\bar{x}$			1.96
ST			0.66
SEM			0.14

[1]CHO cells were transfected with ICUE3 and yLHR and treated with 0.05% saponin and 50 μM cAMP. Column 1 shows the cell being treated. Column 2 shows baseline values for the CFP:YPF ratio as discussed in Methods and Materials. Column 3 shows the CFP:YFP ratio following addition of saponin and cAMP to CHO cells. Column 4 shows the "Fold-change in signal" which is the ratio of the FRET baseline value:FRET signal following hCG treatment for each cell.

Table VII. Comparison of CHO cell responses to hCG, forskolin or saponin and cAMP when cells expressed ICUE3 alone or expressed both ICUE3 and the constitutively active yLHR[1].

	Cell line	Cell line
Treatment	ICUE3 alone	ICUE3 + yLHR
100 nM hCG	1.04 ± 0.03	1.11 ± 0.08
	(n=18)	(n=31)
50 μM Forskolin	1.33 ± 0.07	1.07 ± 0.04
	(n=21)	(n=19)
Saponin +cAMP	1.07± 0.04	1.96 ±0.14
	(n = 27)	(n=22)

[1]Values for CHO cells without LH receptors or expressing yLHR following treatment with either 100 nM hCG, 50 μM forskolin or saponin and cAMP. Values shown represent the mean of n samples as indicated.

DISCUSSION AND FUTURE DIRECTIONS

Together these results suggest that the ICUE3 reporter is a useful probe for evaluating cAMP levels in viable cells. Results from these various cell treatments were generally consistent with reported changes in cAMP evaluated using traditional biochemical assays such as colorimetric assays or assays requiring radiolabeled cAMP. The major advantage of ICUE3, however, is that this probe makes it possible to evaluate cAMP levels in real-time as cell conditions change and to evaluate changes in cAMP within a single cell using single cell imaging methods.

We also demonstrate here that a newer fluorescence microscope-based method developed by J. Zhang and coworkers [29] can provide information on relative changes in FRET between CFP and a modified variant of YFP which are indicative of cAMP changes. This approach has a number of benefits compared to, for example, fluorescence dequenching methods which we have used previously. Fluorescence dequenching of the fluorescent donor has been used to evaluate the efficiency of energy transfer between the fluorescent donor (CFP) and fluorescent acceptor (YFP). CFP and YFP fluorescence are imaged separately using filter sets for these visible fluorescent proteins that minimized the fluorescence contribution from CFP when imaging YFP [36]. YFP is then photobleached to approximately 10% of its initial fluorescence intensity and, following photobleaching, each cell is re-imaged using the same filter sets. Although both methods can be accomplished on single cells, the method used in this project which evaluates both CFP emission and YFP emission due to FRET is both faster and more reliable. This is largely due to technical issues encountered when photobleaching YFP which, inevitably, leads to some CFP photobleaching and produces an under-estimation of FRET efficiency.

ICUE3 transfected CHO cells showed comparatively large changes in FRET signal with addition of forskolin suggesting that these cells had comparatively low basal levels of intracellular cAMP and markedly more cAMP in response to forskolin. Expression of of yLHR in addition to ICUE3 increased the basal cAMP level in CHO cells as expected for a constitutively-active receptor [28]. Nevertheless, cAMP production in cells expressing yLHR increased with addition of forskolin which suggests that the presence of a constitutively-active receptor is not sufficient for maximum activation of adenylyl cyclase.

Effects of hCG on cells expressing ICUE3 alone and ICUE3 together with yLHR were consistent with previous observations. CHO cells appeared unresponsive to 100 nM hCG, a hormone concentration that maximally stimulates cAMP production in cells expressing either native LH receptor or stably transfected LH receptors [10]. There were modest effects of hCG on cAMP levels when cells expressed both ICUE3 and yLHR which again is consistent with previous results [28]. Wu et al. speculate that the limited hCG responsiveness of cells lines expressing yLHR results from reduced access for hCG to binding sites on the LH receptor. These binding sites are presumably occupied by the single stranded yoked hormone.

It was somewhat disappointing that saponin treatment together with cAMP did not produce a response in CHO cells expressing ICUE3 that was comparable to the forskolin response. The advantage of using saponin + cAMP is that a standard curve could be constructed that directly relates the FRET signal from ICUE3 to cell concentrations of cAMP. Another member of the Roess-Barisas group has performed similar experiments using a multi-laser flow cytometer with filter sets for isolating CFP emission, YFP emission due to FRET, and YFP emission due to direct laser excitation of YFP (G. Barisas, personal communication). The cells used in these experiments also expressed human LH receptors coupled to mCherry. Flow

cytometry permitted the cAMP signal to be related to LH receptor number and was checked for the relationship between ICUE3 levels which were assessed from direct excitation of YFP emission and compared to fluorescent bead standards. Treating cells with saponin and increasing concentrations of cAMP permitting group members to construct a standard curve relating ICUE3 signal to intracellular cAMP. Because thousands of cells can be sampled with this method in a relatively brief timeframe, flow cytometry may to be a more effective method with which to examine cAMP levels in hormone-responsive cells. Nevertheless, the microscope-based method used here remains a good choice for examining time-dependent changes in cAMP levels in single cells.

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LIST OF ABBREVIATIONS

AC: adenylyl cyclase

cAMP: cyclic adenosine monophosphate

CFP: cyan fluorescent protein

CHO: Chinese hamster ovary

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

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

DMEM: Dulbecco's modified minimum essential medium

%E: percent energy transfer efficiency

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

GPCR: G protein coupled receptor

GTP: guanosine triphosphate

hCG: human chorionic gonadotropin

hetero-FRET: heterotransfer fluorescence resonance energy transfer

ICUE3: indicator of cAMP using modified EPAC

LH: luteinizing hormone

LHR luteinizing hormone receptor

PBS: phosphate buffered saline

PKA: protein kinase A

TSH: thyroid-stimulating hormone or thyrotropin

vpVENUS: a circularly permuted form of YFP

yLHR: LH receptors yolked to a single chain modified form of hCG

YFP: yellow fluorescent protein

LHR luteinizing hormone receptor

PBS: phosphate buffered saline

PKA: protein kinase A

TSH: thyroid-stimulating hormone or thyrotropin

vpVENUS: a circularly permuted form of YFP

yLHR: LH receptors yolked to a single chain modified form of hCG

YFP: yellow fluorescent protein