UNIVERSITY OF CALIFORNIA, SAN DIEGO

cAMP Signaling in the Gonadotropes

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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2012

The Thesis of Debra Ming-Yi Yeh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California, San Diego

2012

Dedication

This work is dedicated to my family, friends, and partner who have supported me unconditionally through all my endeavors.

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LIST OF ABBREVIATIONS

α-GSU	alpha glycoprotein subunit
AP1	activating protein 1
CRE	cAMP response element
CREB	cAMP response element binding
Egr1	early growth response protein 1
ERK	extracellular signal-regulated kinase
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
PACAP	pituitary adenylate cyclase-activating polypeptide
PKA	protein kinase A
PKC	protein kinase C
SRF	serum response factor

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ACKNOWLEDGEMENTS

I would like to thank my committee members for taking the time to evaluate my thesis. I have had the privilege of taking courses and serving as a teaching assistant to both Dr. Golden and Dr. Coleman and have experienced first hand their steadfast dedication to science and education. I would like to thank Dr. Coss for being an amazing mentor for the last 2 ½ years. Without her patience, knowledge, and guidance, I would not be where I am today.

I would also like to thank past and current members of my laboratory, specifically Raj Gaddameedi, Lacey Lindaman, Changchuan Xie, Arpi Hambarcyan, Patricia Pepa, and Jenny Liang, who have all helped me and made me laugh along the way.

Lastly, I want to thank the Endocrine Society for supporting my research.

ABSTRACT OF THE THESIS

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GnRH differentially regulates the expression of LHβ and FSHβ in the same anterior pituitary gonadotrope cell. The mechanisms that allow gonadotrope cells to decode the GnRH signal are not well understood but it has

been postulated that differential accumulation of secondary messengers cAMP and Ca^{2+} may play a role. Targets of cAMP in the gonadotropes have not been elucidated. Thus, studying cAMP signaling will provide insight into GnRH regulation of LHβ and FSHβ. cAMP is known to activate PKA. PKA is necessary for LHβ and FSHβ induction by GnRH, however, is only sufficient for FSHβ. The only known target of PKA, CREB, does not play a role in FSHβ induction. Moreover, PKA effect does not map to GnRH-regulated response elements on the FSHβ promoter. Another hormone called PACAP is known to selectively increase intracellular cAMP. However, the response to PACAP maps to the GnRH responsive AP1 site of the FSHβ promoter. PACAP can induce AP1 proteins, c-Fos and c-Jun, which are necessary for induction of FSHβ. Induction of c-Fos by PACAP requires PKA, ERK1/2, and p38 MAPK pathways and maps to the SRF site, which is also necessary for induction by GnRH. PACAP activates ERK, which leads to the phosphorylation of ELK and its interaction with SRF. A dominant negative form of Epac, a novel cAMP target in the gonadotrope, can abrogate induction of c-Fos by PACAP, and thus Epac may bridge cAMP production to MAPK pathway activation, identifying a point of crosstalk between PACAP and GnRH signaling pathways.

I

Introduction

Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is a cooperative system of endocrine glands comprised of the hypothalamus, anterior pituitary, and gonads. The axis and its secreted hormones are critical for the regulation of development, menstrual cycle, and reproductive fitness. At the apex, a scattered group of neurons release a decapeptide called gonadotropin-releasing hormone (GnRH) in a pulsatile fashion. These neurons originate in the nasal septum in early development and eventually migrate to the pre-optic area of hypothalamus extending its axons to innervate at the median eminence (1). GnRH is secreted into the hypophyseal portal system, which is a blood vessel system connecting the hypothalamus and the anterior pituitary. GnRH binds a seven transmembrane G-protein coupled receptor at the surface of gonadotrope cells in the anterior pituitary. The binding triggers a series of signaling cascades that leads to the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the gonadotropes. These hormones then travel through the blood and target the gonads—the ovaries and testes—to regulate folliculogenesis, spermatogenesis, steroidogenesis, and ovulation (2).

Anterior Pituitary

The pituitary gland, or hypophysis, is an endocrine gland responsible for cellular homeostasis and is located below the hypothalamus, at the base of the brain (3). It is composed of two major components, the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis). The posterior pituitary is connected to the hypothalamus by the pituitary stalk, which carries the axons of the neurosecretory cells that release oxytocin and antidiuretic hormone. The posterior pituitary originates from the neuroectoderm while the anterior pituitary arises from the oral ectoderm. The anterior pituitary is initially composed of three different sections: the pars distalis, pars intermedia, and pars tuberalis. Upon adulthood, the pars intermedia regresses or disappears all together. The pars distalis contains five different cell types: somatotrophs, corticotrophs, thyrotrophs, lactotrophs, and gonadotrophs. Somatotrophs are responsible for secreting growth hormone that target adipose and liver tissue to promote growth. Corticotrophs produce and secrete adrenocorticotropic hormone, which targets the adrenal cortex to stimulate steroid biosynthesis. Thyrotrophs synthesize and secrete thyroid stimulating hormone (TSH) which targets the thyroid to produce thyroid hormone. Lactotrophs produce and secrete prolactin, which targets the mammary gland to create milk. Lastly are the gonadotrophs, which are responsible for synthesizing and releasing FSH and LH (3).

The gonadotrophs only make up about 10% of the anterior pituitary cells. Because of this small proportion as well as the heterogeneity of the cells, in vivo studies are difficult to conduct (4). Fortunately, the Mellon Laboratory created

immortalized gonadotrope cell lines by directing the expression of the simian virus (SV40) T-antigen oncogene linked to the promoter and enhancer region of the glycoprotein hormone LHβ promoter gene into gonadotrope cells of transgenic mice. The LβT2 cell line models a fully differentiated, mature gonadotrope cell able to express and secrete LH, FSH, and the hormones activin, follistatin, and inhibin (5).

Follicle Stimulating Hormone and Luteinizing Hormone

Both FSH and LH are heterodimeric glycoproteins composed of common α-subunit, called α-GSU (glycoprotein subunit), and a unique β-subunit, which confers biological specificity (6). The α-subunit is common to FSH, LH, TSH, and hCG (human chorionic gonadotropin) and is produced in excess. The β-subunits, on the other hand, are tightly regulated and thus the synthesis of these subunits is the rate-limiting step in mature hormone production (7).

FSH and LH are profoundly important in reproduction regulating development, growth, puberty, and the reproductive cycle. Specifically, FSH stimulates follicular growth and maturation as well as estradiol (E2) synthesis by the granulosa cell in women. In men, FSH stimulates the first division of meiosis to form secondary spermatocytes (2). LH is responsible for triggering ovulation and stimulating androgen production by theca cells in women. In men, LH triggers the Leydig cells to produce testosterone (8). Mutations in either of these hormones have significant effects on fertility and development. Women with mutations in FSHβ experience infertility and amenorrhea due to the absence of

follicular maturation. Men with FSHβ mutations are often azoospermic. Inactivating mutations in LH result in incomplete pubertal development and infertility in both men and women (9). Because defects in LH and FSH have substantial effects on reproductive fitness, it is important to understand the regulation and regulatory elements of these hormones.

GnRH signaling pathways

Not only is GnRH the main regulator of LH and FSH release from the anterior pituitary, it is also the primary regulator of LHβ and FSHβ subunit transcription. The GnRH receptor is a seven-transmembrane G-protein coupled receptor on the surface of the gonadotrope cells of the anterior pituitary (Figure 1). GnRH binding to its receptor activates heterotrimeric G proteins. GnRH primarily activates $G_{q/11}$ subfamily of G-proteins, however, recent evidence has shown that G_s subfamily of G-proteins are also activated (10, 11).

$G_{q/11}$ G-proteins activation triggers the activation of phospholipase C, which leads to phosphoinositide turnover. This causes an increase in intracellular diacylglycerol (DAG) and IP3. IP3 binds to the receptors on the endoplasmic reticulum and causes the release of calcium from intracellular stores (11). The increase in DAG stimulates PKC activity, which through activation of MAPK family proteins facilitates gonadotropin subunit transcription. Calcium activates a variety of calcium/calmodulin dependent kinases. Following activation, MAPK translocates to the nucleus and phosphorylates several transcription factors

leading to the increase in their transcriptional activity (12). MAPK activity is necessary for induction of immediate early genes.

G_s G-proteins signal an increase of intracellular cAMP. The primary target of cAMP is cAMP-dependent protein kinase, also known as Protein Kinase A (PKA). PKA is comprised of a regulatory subunit dimer and two catalytic subunits. Without cAMP, PKA remains in an auto-inhibited state. Once cAMP binds to the regulatory subunit, the catalytic subunits are released to phosphorylate targets (13).The major target for PKA is the transcription factor CREB (cAMP response-element binding protein), whose transcriptional activity is activated by phosphorylation (14). The function of other cAMP targets has yet to be discovered in the gonadotropes.

Recently, another class of cAMP sensors called Epac proteins (exchange proteins directly activated by cAMP) was discovered. These proteins serve as cAMP dependent guanine-nucleotide-exchange factors for the small GTPases Rap1 and Rap2. Epac proteins consist of an N-terminal regulatory region and a C-terminal catalytic region. The regulatory region auto-inhibits the catalytic region. Upon binding of cAMP, the catalytic unit can activate Rap proteins. Epac proteins are known to be involved in insulin secretion and neurotransmitter release (15). Their function in the gonadotropes or in GnRH signaling, though, has not yet been elucidated.

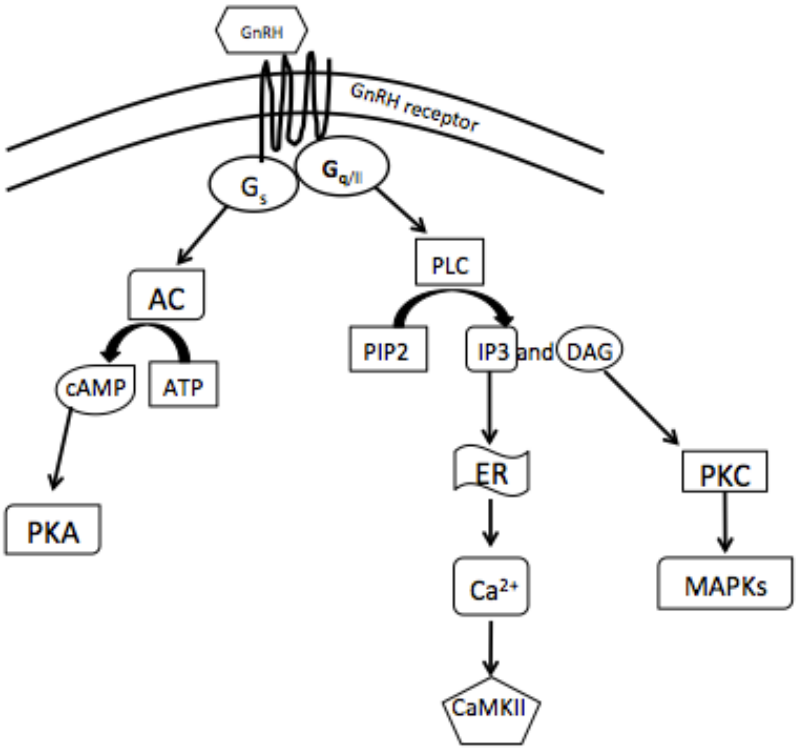


Figure 1: GnRH Signaling Pathways
Binding of GnRH to its 7 transmembrane, G-protein coupled receptor triggers activation of $G_{q/11}$ subfamily of G-proteins followed by the activation of phospholipase C which cleaves PIP2 to IP3 and DAG. DAG activates PKC which phosphorylates MAPKs. IP3 triggers the release of calcium from the endoplasmic reticulum. Calcium activates CaM Kinases II. G_s subfamily of G-proteins is also activated by GnRH binding, activating adenylate cyclase to increase cAMP. cAMP can then activate PKA.

Regulation of gonadotropin β-subunits by GnRH

Induction of LHβ by GnRH occurs through the upregulation of immediate early gene Egr1. Egr1 is induced through the MAPK pathway by a number of growth factors and cytokines, including GnRH. Its induction has been shown to involve CREB (16). Following upregulation, Egr1 interacts with basal factors to promote LHβ transcription. There are two Egr1 sites in the proximal promoter of

LHβ that are necessary for GnRH-induced transcription and are highly conserved (5). Mutations in these sites abrogate induction of LHβ by GnRH (17).

Induction of FSHβ by GnRH occurs through the upregulation of another set of immediately early genes that comprise activator protein-1 (AP-1). AP-1 is made up of various dimers consisting of Fos isoforms and Jun isoforms. Fos isoforms are both tightly regulated and induced by GnRH (18). c-Fos in particular is one of the most highly induced genes in the gonadotropes following GnRH stimulation (19). Fos members only form heterodimers with Jun members, however, Jun can form both homodimers and heterodimers to form AP-1. AP-1 binds to a heptanucleotide recognition sequence, TGA(C/G)TC (AP-1 site), and to a octanucleotide sequence, TGACGTCA (CRE site), with a lesser affinity (20).The induction of FSHβ requires the binding of AP-1 to the AP-1 half site in the proximal FSHβ promoter (21).

The gonadotropes are able to produce both FSH and LH in response the GnRH signal. However, these hormones are differentially regulated throughout the menstrual cycle, relying on similar pathways and immediate early genes. How this is achieved is not fully answered. However, studies have suggested that the frequency of GnRH pulses is partially responsible for the differential regulation of LH and FSH, with the transcription of LHβ favoring high frequency pulses and FSHβ favoring lower frequency pulses (7).

Recently, G_s and G_q sub-families of G-proteins and their respective secondary messengers, cAMP and DAG/Ca^{2+}, have been investigated as a possible model of how gonadotropes decode GnRH pulses. In response to a

tonic or constant GnRH signal, cAMP reporters show a rapid but transient increase in signal while DAG and Ca^{2+} reporters show rapid and prolonged signals. However, in response to pulsatile treatment of GnRH, cAMP reporters are activated with a prolonged signal while DAG and Ca^{2+} reporters show desensitization (22). Thus, it is possible that these secondary messengers decode the frequency of GnRH leading to the differential regulation of LHβ and FSHβ transcription.

PACAP

Another hypothalamic hormone that may play a role in gonadotropes is PACAP (Pituitary Adenylate Cyclase Activating Polypeptide). It is known to predominately stimulate cAMP production. The PAC1 receptor isoform is expressed in the gonadotropes (23). Additionally, previous papers have shown that PACAP can stimulate the release of LH, FSH, and α-subunit. The time course of gonadotropin release is much more modest than of cells treated with GnRH, however, they match that of cAMP agonist treated cells (24).

Summary

Proper functioning of the hypothalamic-pituitary-gonadal axis is required for reproductive success. GnRH serves as a trophic factor stimulating the gonadotropes to synthesize and release both LH and FSH.

Gonadotrope cells only comprise 10% of the cells in the anterior pituitary. For this reason, we use LβT2 cells as a model for mature gonadotropes in our studies.

LHβ and FSHβ confer biological specificity. They are regulated mainly by GnRH through the activation of $G_{q/11}$, which increases intracellular calcium, and G_s, to a lesser extent, which increases cAMP. Mutations in these genes can cause incomplete pubertal development and infertility. The regulation of LHβ and FSHβ subunits are governed mainly by GnRH. The upregulation of immediate early response gene Egr1 is required for LHβ induction by GnRH, mapping to Egr1 sites within the proximal promoter. AP-1 proteins consisting of c-Fos and c-Jun heterodimers mediate GnRH responsiveness of FSHβ at AP-1 sites.

The main question in gonadotrope physiology is how the two gonadotropin subunits, LHβ and FSHβ, are differentially regulated within the same gonadotrope cells. We hypothesize that differential regulation occurs through diverse activation and accumulation of secondary messengers, calcium and cAMP. Thus we started our studies by analysis of nuclear targets of these secondary messengers. To target exclusively cAMP signaling, we used PACAP, a hormone known to stimulate the cAMP/PKA pathway and compared its effects to GnRH, which activates both calcium and cAMP.

Our studies may lead to better understanding of molecular causes of pathophysiologic condition where differential regulation of LH and FSH is disregulated, such as premature ovarian failure characterized by the increase in only FSH or polycystic ovary syndrome, characterized by the increase in only LH.

A better understanding of hormone regulation may lead to new treatments in some cases of infertility.

II

Materials and Methods

Plasmid Constructs

The 1kb of the murine FSHβ proximal promoter and 1.8kb of the rat LHβ promoter (or regulatory region) linked to luciferase reporter in pGL3 plasmid were described previously (25) (21), (26). The 398 bp truncation of the murine FSHβ promoter and the 1000 kb murine c-Fos promoter linked to luciferase reporter in pGL3 plasmid were described previously (18). Truncations of FSHβ promoter linked to luciferase were described previously (26). Expression vectors PKI, PKA, and PKA K72H were graciously given by Dr. Susan Taylor (Howard Hughes Medical Institute, UCSD, San Diego, CA). CREB expression vectors were kindly given by Marc Montminy (Salk Institute, La Jolla, CA). EPAC expression vectors were obtained from Daniel Altschuler (University of Pittsburgh, Pittsburgh, PA). PACAP Receptor expression vector was kindly gifted by Dr. Norihito Sintani (Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan). SRF expression vector was obtained from Addgene (Cambridge, MA). ELK expression vector was kindly provided by Dr. Andrew Sharrocks (The University of Manchester, Manchester, UK). ERK2 expression vector was kindly provided by Dr. Peiquing Sun (Scripps Institute, La Jolla, CA).

Cell Culture

Immortalized LβT2 cells were cultured at 37°C in Dulbecco's Modification of Eagles Medium (DMEM, Mediatech, Manassas, VA) containing 10% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA) and penicillin-streptomycin antibiotics (Invitrogen / Life Technologies, Grand Island, NY). 1X Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) was used to re-suspend cells.

Hormones and Inhibitors

Cells were treated with 10nM Gonadotropin-releasing hormone (GnRH), which was purchased from Sigma-Aldrich (St. Louis, MO). Pituitary adenylate cyclase activating polypeptide (PACAP) was purchased from Calbiochem (La Jolla, CA); cells were treated using a final concentration of 100 nM. Inhibitors were purchased from Calbiochem (La Jolla, CA).

Transient Transfections

Cells were plated one day before transfection in 12-well plates in DMEM containing 10% FBS. Cells were transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. A mix of 0.5 μg of luciferase reporter plasmid and 0.1 μg of β-galactosidase, a reporter driven by a Herpes virus thymidine kinase (TK) promoter, to control for transfection efficiency, was transfected into the cells. Cells were starved in serum free DMEM containing 0.1% BSA and penicillin-streptomycin antibiotics the night before harvesting. 5 hours before harvesting, cells were treated with 10nM GnRH,

100nM PACAP, or vehicle. Inhibitors were applied 15 minutes prior to GnRH or PACAP treatment.

Luciferase and β-galactosidase Assay

Cells were washed with 1X PBS and lysed with a buffer containing 100nM K-PO_4 and 0.2% Triton X-100 48 hours after transfection. 20 µl of lysate was loaded into 96-well Costar plates. Both luciferase and β-galacatosidase activity were measured with a luminometer (Vertias Microplate luminometer from Turner Biosystems / Promega, Madison, WI). For luciferase assay, 100 µl of a luciferin buffer containing 25mM Tris pH 7.8, 15mM $MgSO_4$, 10mM ATP, and 65µM luciferin or 100 µl of Accelerator (Applied Biosystems, Foster City, CA) was injected into each well. Lysate for β-galactosidase assay was first incubated with Tropix Galact-light substrate (Applied Biosystems, Foster City, CA) according to manufacture's protocol. Then, 100 µl of catalyst, Accelerator, (Applied Biosystems, Foster City, CA) was injected by the luminometer before reading. Each transfection was performed in triplicate and repeated three times.

Whole Cell Extract

LβT2 cells were starved overnight in serum free DMEM containing 0.1% BSA. Some cells were treated with 10nM GnRH for 0.25, 0.5, 1, 2 or 4 hours. Cells were rinsed with cold 1x PBS then lysed with a buffer containing 20mM Tris pH 7.4, 140mM NaCl, protease inhibitors (Sigma-Aldrich), 1mM PMSF, 10mM NaF, 1% NP-40, 0.5mM EDTA, and 0.1mM EGTA. Protein concentration was

determined using Bio-rad Protein Assay (Bio-rad, Hercules, CA). A standard curve was generated to calculate the concentration.

Immunoprecipitation

LβT2 cells were plated in 10cm plates one day prior to transfection with expression vectors containing FLAG ERK or ELK coding sequence using FuGENE 6 according to manufacturer's instructions. After following the lysis and protein determination procedure for whole cell extract, equal amount of proteins were incubated with anti-FLAG antibody linked to agarose beads (Sigma-Aldrich, St. Louis, MO) for 3 hours. Beads were washed three times with 500µl of cold lysis buffer. 30µl of 2x Laemmli sample buffer was added to the beads.

Western Blot

Equal amounts of protein from whole cell extracts in 4x sample buffer or after immunoprecipitation were loaded into an SDS-PAGE gel consisting of 4% stacking gel and 10% or 12.5% separating gel. The proteins were separated by gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 hour at room temperature in 10% nonfat dry milk in TBST (20mM Tris 7.4, 0.1% Tween, 150mM NaCl, and 0.5% BSA). Membranes were then probed with primary antibodies to p-ERK, p-ELK, or FLAG. Anti-rabbit or mouse secondary antibodies linked to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect

bands. To detect complexes, Amersham (enhanced chemiluminescence) ECL reagent (GE Healthcare, Piscataway, NJ) was used.

Statistical Analysis

All experiments was performed at least three times. Transfections for luciferase assay were also performed in triplicate. To control for transfection efficiency, luciferase values were divided by β-galactosidase values. This ratio was then normalized to the empty vector luciferase/β-galactosidase ratios for each reporter. Normalized luciferase/β-galatosidase values were then averaged from three experiments. The JMP9 program was used to perform ANOVA, Tukey's posthoc, and Dunnett's test with a significance value set at $p \leq 0.05$. Bands from western blots were also quantified and normalized to a loading control protein: FLAG tag.

III

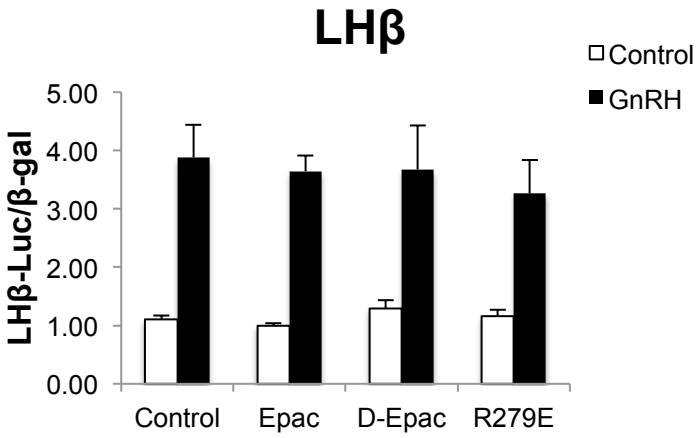
Results

Epac proteins may not play a role in the induction of LHβ and FSHβ by GnRH

Studies have shown that GnRH receptor-mediated signaling involves both $G_{q/11}$ and G_s proteins (11) (27). The signaling pathways downstream of $G_{q/11}$ activation have been studied extensively, while the G_s pathway and its downstream transcription factors are poorly understood. Following G_s activation, adenylate cyclase converts ATP to cAMP. Increase in intracellular cAMP levels are known to activate a myriad of cAMP binding effector proteins including PKA and Epac proteins (28). We began by examining the role of Epac proteins in GnRH induction of FSHβ and LHβ subunits. LβT2 cells were transfected with either the -1800 base pair rat LHβ promoter or the -1000 base pair murine FSHβ promoter fused to a pGL3 luciferase reporter, wildtype or mutated Epac expression vector, and Herpes Virus thymidine kinase β-galactosidase (β-gal). Epac expression vectors used were wildtype, dominant negative Epac (R279E), and mutated Epac. Dominant negative Epac (R279E) is deficient in cAMP binding. Delta Epac (D-Epac) is a mutant that has a deletion of a DEP domain that anchors it to the membrane. Cells were then treated for 5 hours with 10 nM GnRH. Analysis was performed on normalized luciferase expression numbers over β-gal to control for transfection efficiency. It was determined that wild type,

dominant negative EPAC deficient in cAMP binding (R279E), mutated EPAC, and empty vector showed no statistically significant difference in both basal and GnRH induced LHβ or FSHβ promoter expression (Figure 2A and 2B).

A)



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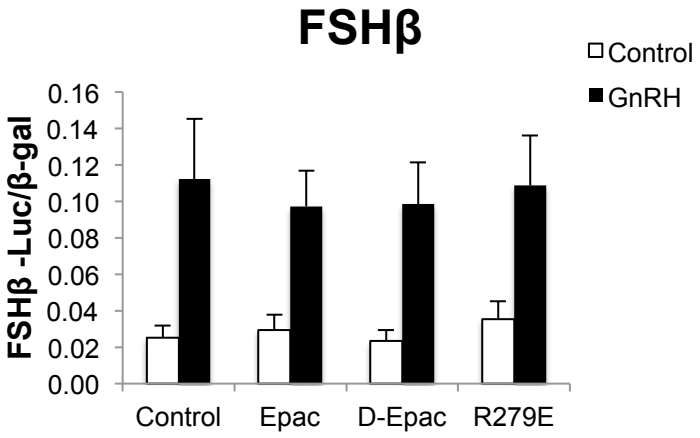


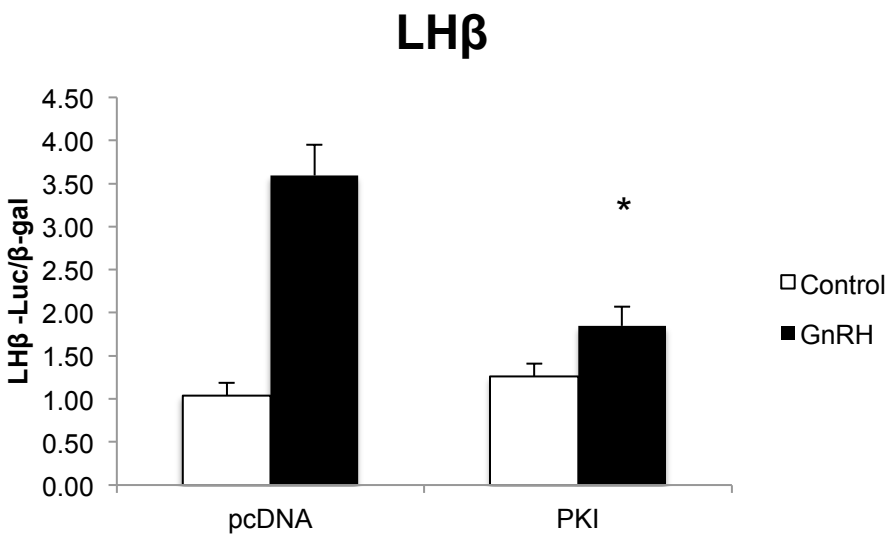
Figure 2: Epac proteins do not play a role in LHβ or FSHβ induction by GnRH

LβT2 cells were co-transfected with (A) -1800 base-pair (bp) rat LHβ promoter fused to a pGL3 luciferase reporter or (B) -1000 base-pair (bp) murine FSHβ promoter fused to a pGL3 luciferase reporter and either Epac (Wildtype), D-Epac (Mutant), or R279E (Dominant Negative) expression vector, then treated with vehicle or GnRH. There was no significant change in basal expression or induction by GnRH of either (A) LHβ or (B) FSHβ. Results are presented as the average of luciferase/β-gal ratios of three experiments performed in triplicates.

PKA is necessary for induction of LHβ and FSHβ by GnRH

Because EPAC proteins had no significant effect, we decided to investigate the role of PKA in LHβ and FSHβ induction. Previous data from our lab showed that induction of both LHβ and FSHβ by GnRH was strongly reduced by an inhibitor called H-89. H-89 inhibits PKA by competitively binding to its ATP pocket (29). The effectiveness of H-89 in reducing LHβ and FSHβ induction by GnRH indicates that PKA is important in GnRH signaling. However, inhibitors often act non-specifically by affecting other pathways, especially H-89 (30). Therefore to confirm that PKA is necessary for GnRH induction of the gonadotropins with another method, an inhibitory peptide of PKA called PKI was co-transfected with LHβ or FSHβ prior to treatment with GnRH. Addition of PKI significantly reduced the LHβ and FSHβ promoters' responsiveness to GnRH (Figure 3A and B). Thus, PKA is a necessary signaling intermediate for FSHβ and LHβ induction by GnRH.

A)



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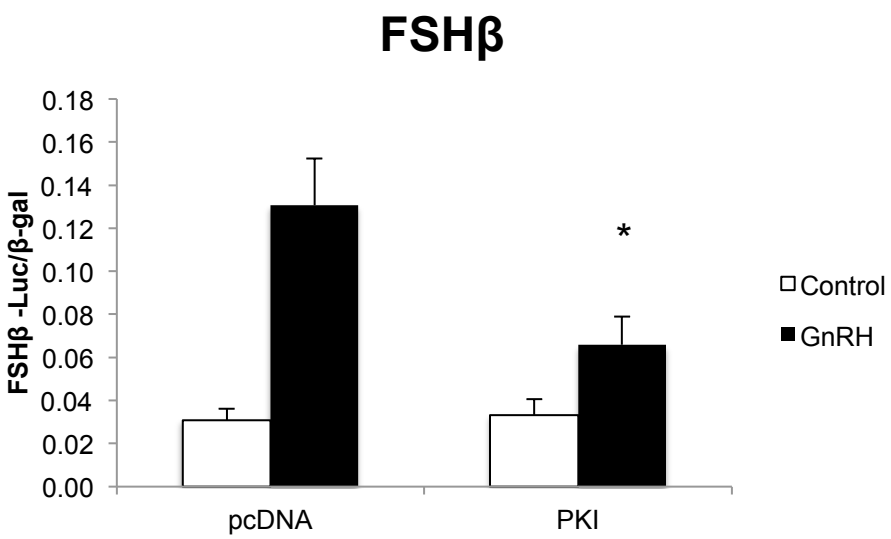
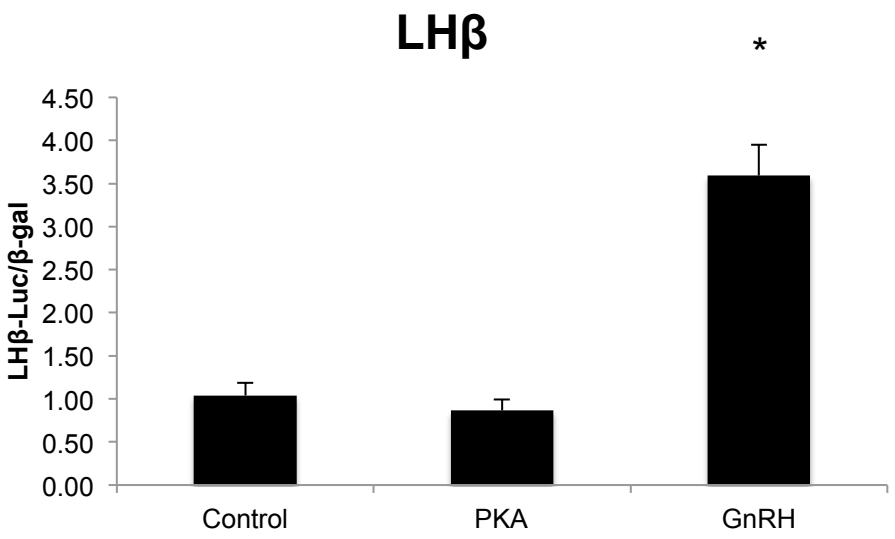


Figure 3: PKA is necessary for GnRH induction of LHβ and FSHβ
LβT2 cells were co-transfected with either (A) -1800 base-pair (bp) rat LHβ promoter fused to a pGL3 luciferase reporter or (B) -1000 base-pair (bp) murine FSHβ promoter fused to a pGL3 luciferase reporter and inhibitory peptide (PKI) expression vector. Results are presented as luciferase/β-gal ratio averaged over three experiments done in triplicate. (*) indicates a significant reduction in GnRH induction compared to empty vector.

PKA is sufficient for induction of FSHβ but not LHβ

Although PKA activity is necessary for maximal induction of gonadotropin β-subnits by GnRH, it remained unclear whether PKA was sufficient for induction. To answer this question, we used an expression vector coding for the catalytic subunit of PKA. Lacking the regulatory subunit, the catalytic subunit of PKA is constitutively active. Cells were co-transfected with the PKA expression vector and LHβ or FSHβ reporter. Surprisingly, there was no statistically significant change in expression levels of the LHβ promoter (Figure 4A). However, the introduction of PKA increased the basal expression of the FSHβ promoter 2-fold (Figure 4B). The results indicate that though PKA is necessary for GnRH induction, as shown by the reduced induction after transfecting inhibitor peptide PKI and treating with GnRH, PKA is not sufficient to induce LHβ expression. To examine why this might be, we examined the induction of Egr1 and c-Fos, key transcription factors in LHβ and FSHβ transcription, respectively. -1000 basepair (bp) promoter of murine Egr1 was co-transfected with PKA expression vector. There was no significant change in the induction of Egr1 with PKA overexpression (Figure 5A). Since GnRH normally stimulates Egr1 over 30 fold, we postulate this may be why LHβ is not induced by PKA. Since FSHβ was induced by overexpression of PKA and c-Fos is a key transcription factor in FSHβ induction by GnRH, we wanted to see if the presence of active PKA could also induce c-Fos. Cells were co-transfected using the -1000 promoter region of c-Fos fused to a pGL3 reporter and PKA. We observed that c-Fos is indeed significantly induced by constitutively active PKA (Figure 5B).

A)



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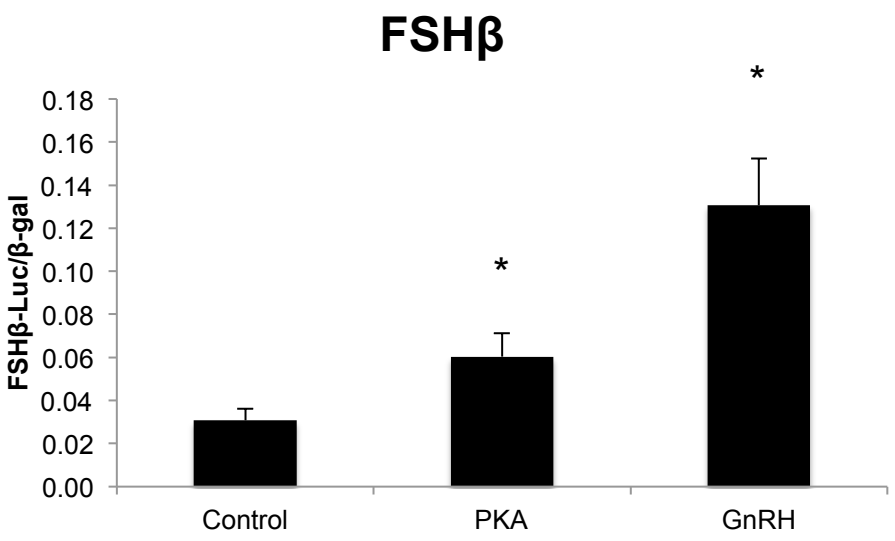
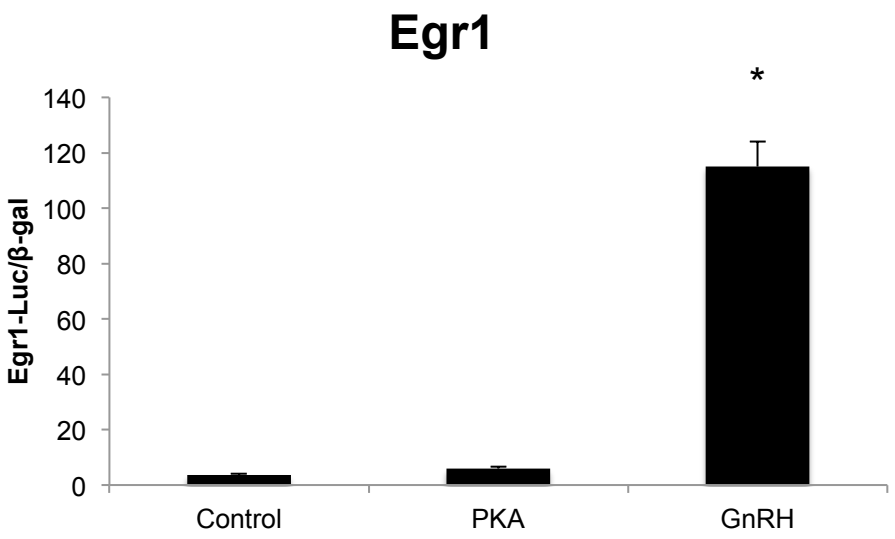


Figure 4: PKA is sufficient for FSHβ induction but not LHβ
LβT2 cells were co-transfected with either (A) -1800 base-pair (bp) rat LHβ promoter fused to a pGL3 luciferase reporter or (B) -1000 base-pair (bp) murine FSHβ promoter fused to a pGL3 luciferase reporter and the PKA catalytic expression vector. A set of cells were transfected with just the LHβ-luciferase reporter or FSHβ-luciferase reporter and then treated with GnRH. (*) indicates a value significantly greater than control.

A)



B)

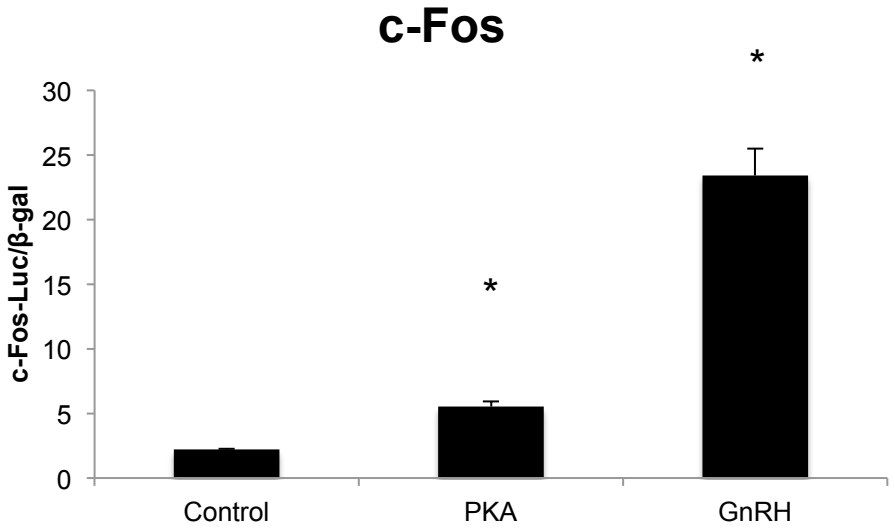
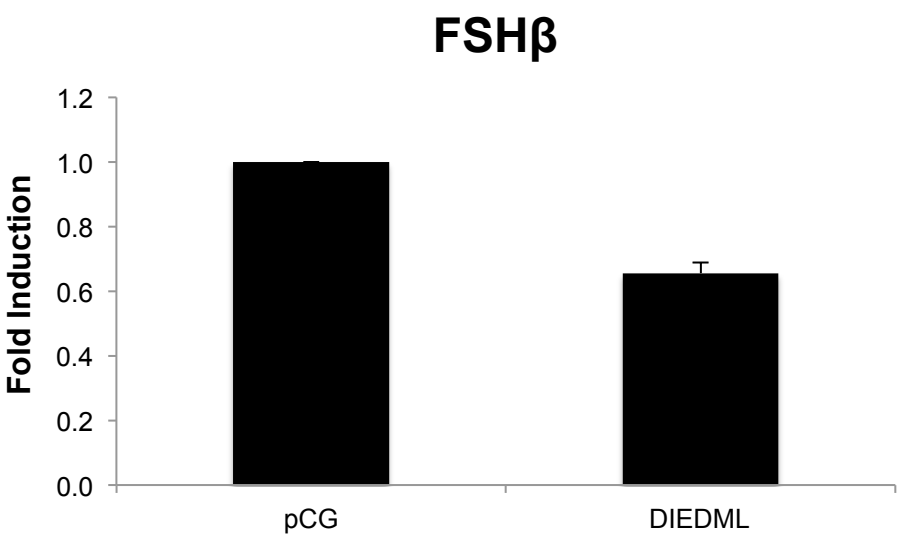


Figure 5: Overexpression of PKA induces c-Fos but not Egr1
LβT2 cells were co-transfected with either (A) -1000 base-pair (bp) murine Egr1 promoter fused to a pGL3 luciferase reporter or (B) -1000 base-pair (bp) murine c-Fos promoter fused to a pGL3 luciferase reporter and the PKA catalytic expression vector. A set of cells were transfected with just the c-Fos-luciferase reporter or Egr1-luciferase reporter and then treated with GnRH. (*) indicates a value significantly greater than control.

Constitutively active CREB does not activate FSHβ

A large body of evidence indicates that the primary target of PKA signaling in the nucleus is cyclic AMP response element (CRE)-binding protein (CREB), which is phosphorylated at Ser 133 by PKA following an increase in intracellular cAMP. CREB is known to induce gene expression in response to growth factors (31). To investigate if activated CREB has an effect on FSHβ transcription, we used a constitutively active CREB mutant. The DIEDML mutant contains a mutation of six amino acids in the kinase-inducible domain (KID), which allows it to constitutively interact with a coactivator CREB-binding protein to activate transcription (32). Constitutively active CREB activated transcription of the empty vector, however, after normalization to pGL3, it was determined FSHβ was not induced by constitutively active CREB (Figure 6A). Thus, CREB may not be the transcription factor mediating PKA induction of FSHβ. To verify if constitutively active CREB is functional and maps to its respective site, the expression vector was co-transfected with a multimer containing the CRE binding sites linked 4 times in tandem and to a pGL3 luciferase reporter. Constitutively active CREB significantly induced the CRE multimer and thus indeed acts as a constitutively active form of CREB (Figure 6B).

A)



B)

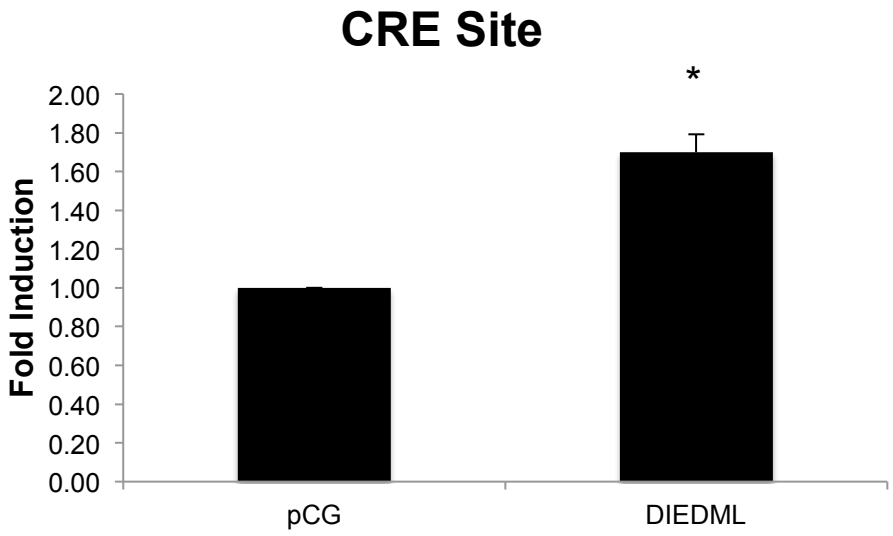


Figure 6: Overexpression of constitutively active CREB does not induce FSHβ

LβT2 cells were co-transfected with either (A) -1000 basepair (bp) murine FSHβ promoter fused to a pGL3 luciferase reporter or (B) 4x CRE site multimer fused to a pGL3 luciferase reporter and constitutively active CREB (DIEDML). Luciferase/β-gal ratios were normalized to pGL3 and then to empty vector and results are presented as the average fold induction of three experiments performed in triplicate.

PKA maps to the -30 region of the proximal promoter of FSHβ

To gain better insight into which regions are critical for the induction of FSHβ by PKA, we decided to perform transfection analysis using truncations of the FSHβ promoter. Induction by PKA was only reduced when the region between -95 and the transcription start site was eliminated. This is indicated by the statistically significant reduction between -95 and empty vector pGL3 (Figure 7). Thus, there are important elements in the proximal promoter region that enable responses to PKA.

To do finer mapping of this induction by PKA in this region, transfection analysis was performed using 10 base pair deletions within the -398 bp promoter region of FSHβ. These 10 base pair deletions ranged from -70/-61 to -20/-11. Deletion of the -30/-21 region showed significant reduction in fold induction by PKA (Figure 8A). To further map the response, 3 base pair mutations created within -30/-21 region were used as well as a 2 base pair mutation within -70/-69 that comprises the AP1 site. When three base pairs from -29/-27 that are within the TATA box were mutated, fold induction by PKA was significantly lowered (Figure 8B). Therefore, the region within -30/-21, particularly the TATA box is crucial for FSHβ induction by PKA. It was surprising that induction by PKA does not require the -70/-61 region, as it contains the AP1 site that is necessary for full FSHβ induction by GnRH. Therefore, PKA may only be a minor pathway in induction by GnRH.

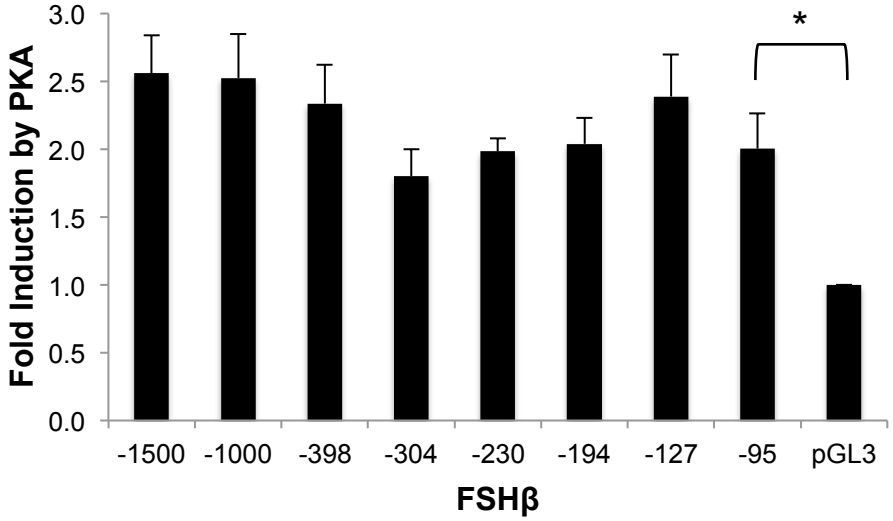
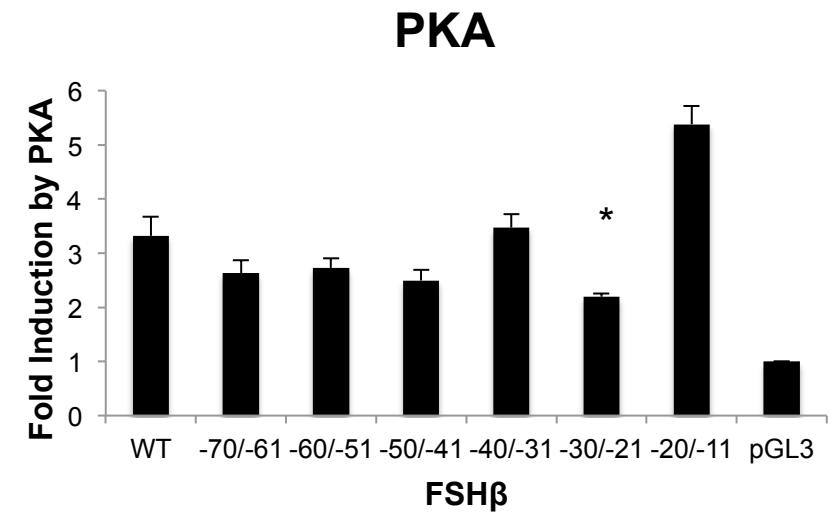


Figure 7: Induction of FSHβ by PKA maps to the proximal promoter region LβT2 cells were co-transfected with truncations of the murine FSHβ promoter and the PKA catalytic subunit expression vector. (*) indicates statistically significant reduction between consecutive truncations.

A)



B)

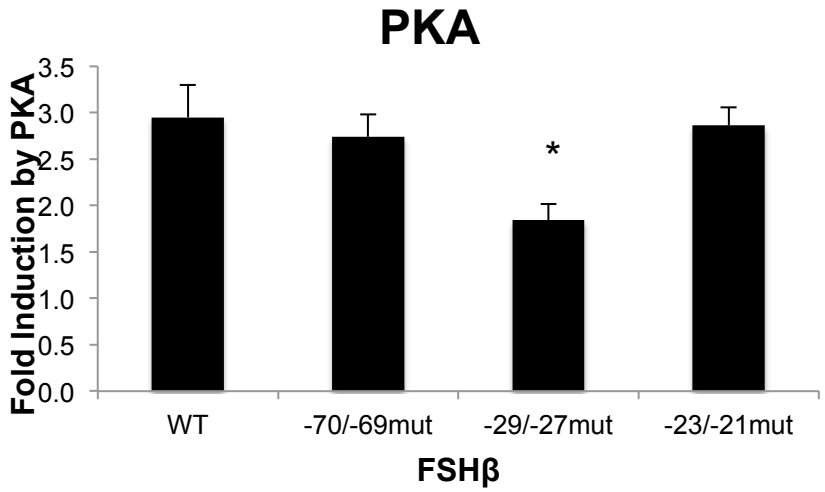


Figure 8: Induction by PKA maps to the -30/-21 region of the FSHβ proximal promoter

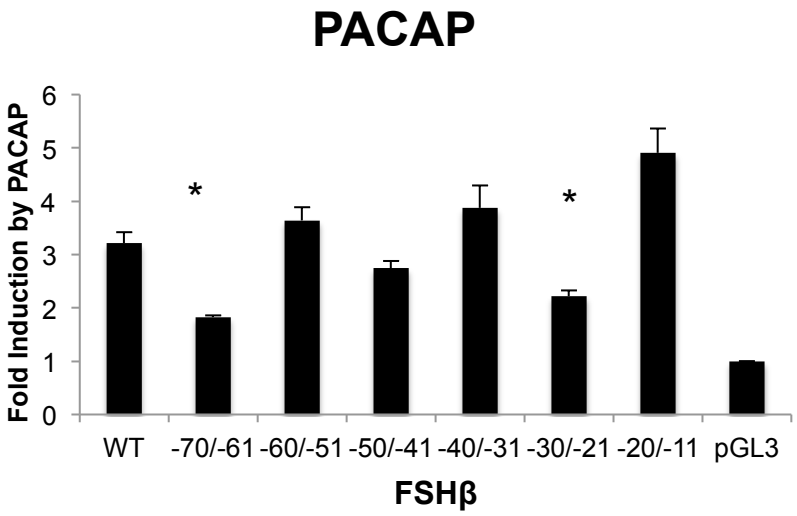
LβT2 cells were co-transfected with either (A) 10 basepair (bp) internal deletions in the -398 FSHβ promoter linked to a pGL3 luciferase reporter or (B) mutations in the FSHβ promoter linked to a pGL3 luciferase reporter and the PKA catalytic subunit expression vector. Results are presented as fold induction by PKA, normalized over basal. (*) indicates statistically significant decrease in induction as compared to wildtype.

PACAP maps to -70 region and -30 region, matching GnRH

To better match physiological conditions while still targeting cAMP signaling, we decided to treat cells with a hormone secreted by the hypothalamus called pituitary adenylate cyclase-activating polypeptide. PACAP is known to stimulate LH, FSH, and α-GSU secretion by the gonadotropes and to increase intracellular cAMP levels (23). However, there is an interesting caveat with LβT2 cells. They fail to express functional PACAP receptors, likely because these cells are a transformed cell line. However, gonadotropes in vivo and α-T3 cells express PACAP receptor (33). We could not reasonably use primary cells because they only comprise 10% of the pituitary cells. α-T3 cells are less differentiated and do not express gonadotropin β-subunits, thus we could not use them for our analysis. In order to observe induction of FSHβ by PACAP, we had to overexpress the PACAP receptor in LβT2 cells prior to treating with PACAP. Induction of FSHβ by PACAP was mapped using the same 10 base pair deletions within the proximal region of the -398 promoter of FSHβ as well as the TATA box and AP1 site mutations mentioned previously. Surprisingly, not only was the -30/-21 important for induction, the -70/-61 was as well (Figure 9A). Of the mutations, the 2 base pair mutation made at -70/-69 (AP1 site) and the 3 base pair mutation made at -23/-21 (adjacent to the TATA box) in the FSHβ promoter significantly lowered induction (Figure 9B). Induction by PACAP has been shown to occur predominantly through cAMP and PKA (23). Therefore, it was surprising to find that PACAP induction did not match PKA. Instead induction

by PACAP matched GnRH (Figure 10A and 10B), requiring both AP1 and the 3' TATA region for full induction.

A)



B)

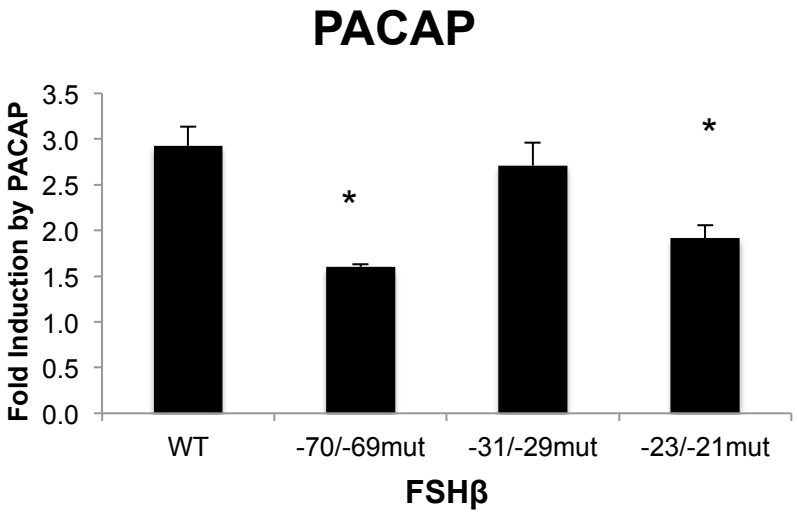
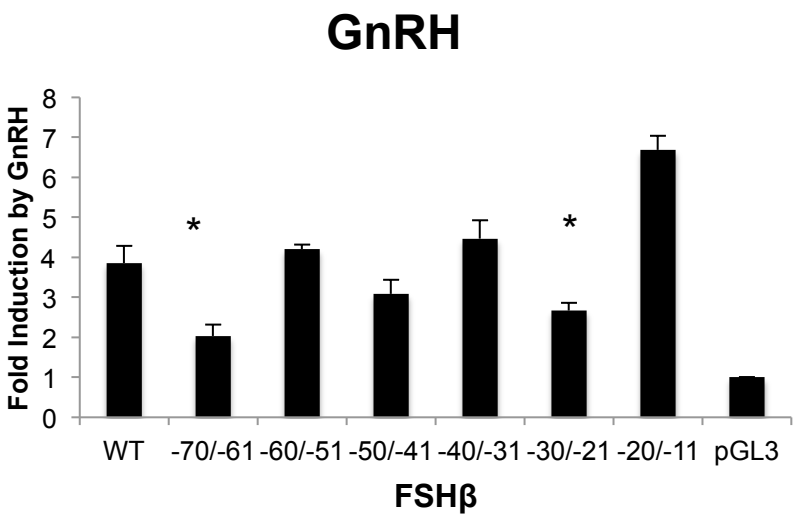


Figure 9: Induction by PACAP maps to both -30/-21 and -70/-61 region of the FSHβ proximal promoter, matching GnRH
(A) LβT2 cells were co-transfected with either (A) 10 basepair (bp) internal deletions in the -398 FSHβ promoter linked to a pGL3 luciferase reporter or (B) mutations in the -398 FSHβ promoter linked to a pGL3 luciferase reporter and PACAP receptor expression vector. Cells were then treated with PACAP. Results are presented as fold induction by PACAP, normalized over basal. (*) indicates statistically significant decrease in induction as compared to wildtype.

A)



B)

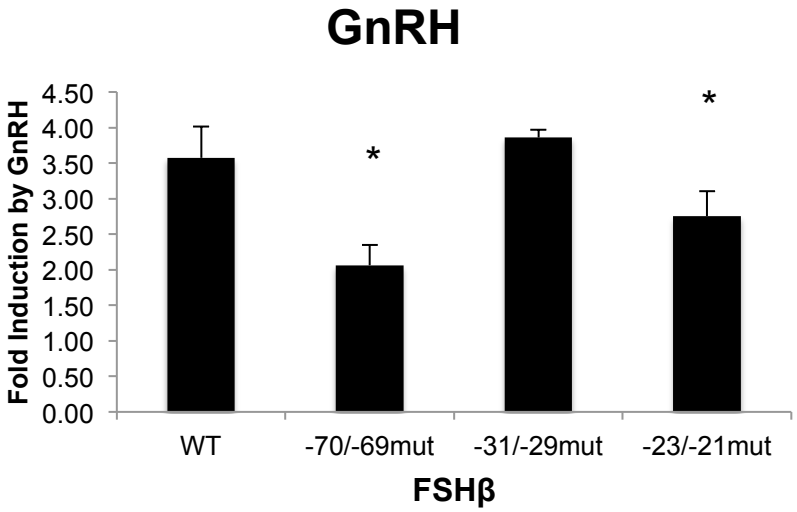


Figure 10: Induction of GnRH maps to the -30/-21 and -70/-61 region of the FSHβ proximal promoter

LβT2 cells were transfected with either (A) 10 basepair (bp) internal deletions in the -398 FSHβ promoter linked to a pGL3 luciferase reporter or (B) mutations in the -398 FSHβ promoter linked to a pGL3 luciferase reporter. Cells were then treated with GnRH. Results are presented as fold induction by GnRH, normalized over basal. (*) indicates statistically significant decrease in induction as compared to wildtype.

PACAP induces API isoforms requiring ERK1/2 and p38 MAPK pathways

After mapping the induction to specific regions, we wanted to know what transcription factors can be activated by PACAP and be recruited to the FSHβ promoter. Because PACAP maps to the AP1 site, we investigated whether the transcription factors that act on AP1, c-Fos and c-Jun, can also be induced by PACAP. Transient transfection assays were performed using luciferase reporters linked to c-Fos and c-Jun promoter regions. Both c-Fos and c-Jun reporters showed significant induction following PACAP treatment compared to vehicle control (Figure 11). GnRH also induces both c-Fos and c-Jun, which has already been reported in the literature and confirmed once again in the same assay with cells treated with GnRH. Thus, not only does PACAP map to the same site as GnRH on the FSHβ promoter, it also induces the same transcription factors, c-Jun and c-Fos, that are necessary for FSHβ induction.

Since the induction of c-Fos as compared to c-Jun was much higher, we used c-Fos-luciferase as a tool to analyze the mechanism of PACAP signaling in the gonadotrope. We began with the inhibitor approach combined with transient transfection to study the signaling pathways important for c-Fos induction. Inhibitors were applied 15 minutes prior to treating the cells with PACAP. The inhibitors used were H-89 (Protein Kinase A), KN-93 (Ca2+/calmodulin-dependent kinase II), BIM (Protein Kinase C), UO 126 (ERK1/2 branch of MAPK Kinase/MEK), SB (p38), and JNK (c-Jun N-terminal kinase). Induction of c-Fos by PACAP was significantly reduced by PKA, ERK1/2, and p38 inhibition (Figure 12a). Induction of c-Fos by GnRH was significantly reduced by ERK1/2, PKA and

Ca^{2+}/calmodulin-dependent kinase II inhibitors (Figure 12b). This data suggests that c-Fos induction by PACAP, similar to GnRH, requires the ERK1/2 pathway. PACAP also uses the p38 branch of MAPK. However, H-89 has been shown to have effects on different pathways outside of the PKA pathway (30). Therefore, we cannot conclude that induction of c-Fos by PACAP requires PKA. Our lab had previously reported that GnRH induction of c-Fos required CamKII and was confirmed in this experiment as CamKII inhibitor reduced induction.

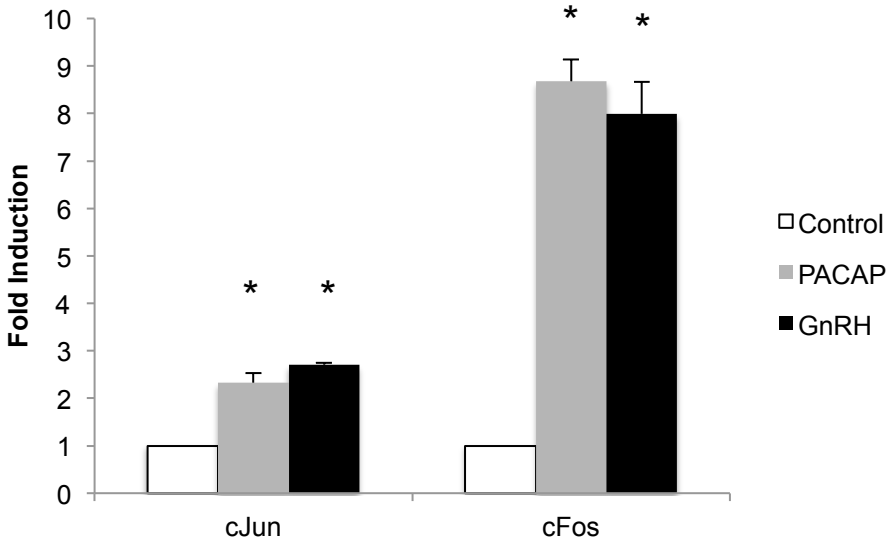
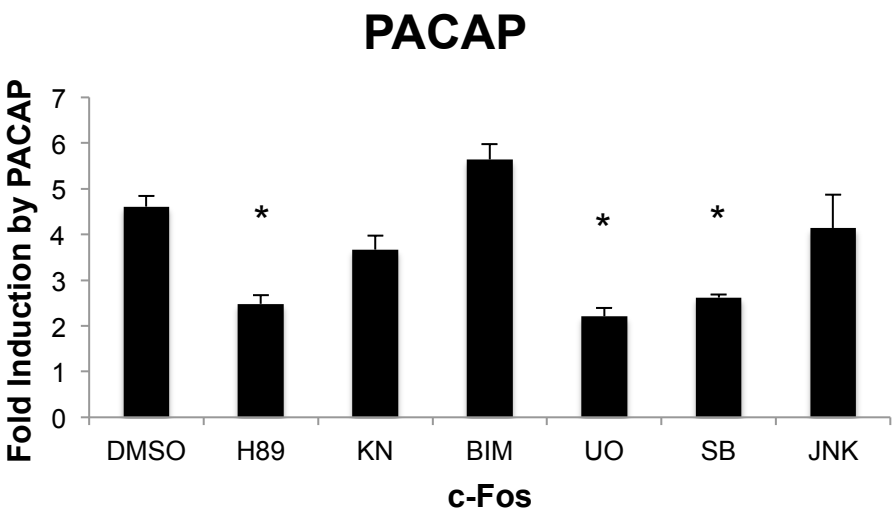


Figure 11: PACAP can induce both c-Jun and c-Fos, comparably to GnRH
LβT2 cells were co-transfected with -1000/+200 c-Jun promoter or -1000 c-Fos promoter linked to a pGL3 luciferase reporter and PACAP receptor expression vector. Results are presented as fold induction by PACAP or GnRH, normalized over basal. (*) indicates induction statistically larger than control.

A)



B)

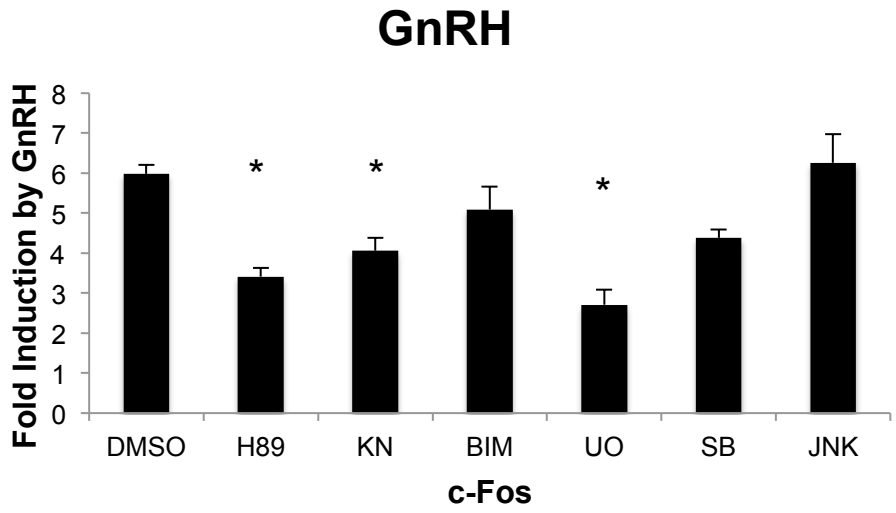


Figure 12: Induction of c-Fos by PACAP requires ERK1/2 and p38 MAPK pathways, while GnRH requires ERK1/2 and calmodulin-dependent kinase II pathways.
LβT2 cells were transfected with -1000 c-Fos promoter linked to a pGL3 luciferase reporter and for (A) PACAP receptor expression vector. The cells were then pre-treated with inhibitors before (A) PACAP or (B) GnRH treatment. (*) indicates a significant decrease in induction compared to control.

SRF site is necessary and sufficient for induction of c-Fos by PACAP

To map the site necessary for induction on the c-Fos promoter, we used c-Fos reporters containing mutations at sites known to be important for induction by growth factors. We included a mutation in the serum response factor (SRF) site, which has been shown to be important for c-Fos induction by GnRH and the Ets/ELK1 and STAT sites, which are important for induction by other growth factors (18). We found that induction of c-Fos by PACAP was significantly reduced by the SRF binding site mutant (Figure 13), indicating that the SRF site is crucial for induction by both PACAP and GnRH. Since both PACAP and GnRH map to the same site on the c-Fos promoter, they may share signaling pathways.

After establishing that induction of c-Fos by PACAP requires the SRF site and FSHβ requires the AP1 site, we wanted to see if the site alone was sufficient for induction. The lab previously created isolated response elements linked 4 times in tandem—4x multimers of the AP1 and SRF sites linked to a minimal thymidine kinase promoter in pGL3. Following analysis, we determined that both AP1 and SRF multimers were sufficient for PACAP induction. Thus, we can conclude that the SRF site in the c-Fos promoter is both necessary and sufficient for c-Fos induction (Figure 14). The AP1 site is also necessary and sufficient for FSHβ induction by PACAP.

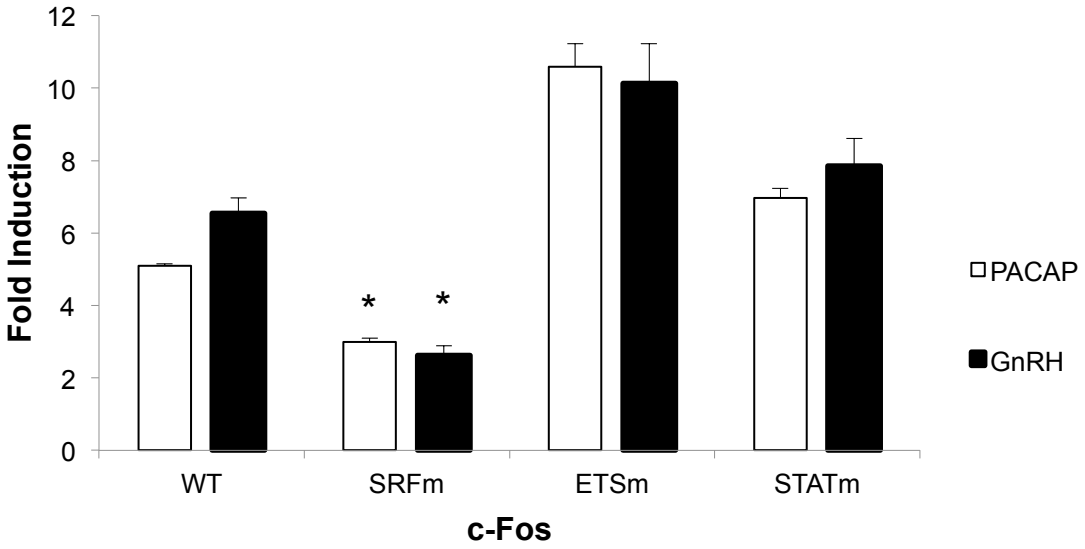


Figure 13: SRF site is necessary for c-Fos induction by PACAP
LβT2 cells were transfected with c-Fos mutants within the -1000 basepair (bp) c-Fos promoter linked to a pGL3 luciferase reporter . Cells were treated with PACAP or GnRH. Results are presented as fold induction, normalized over basal. (*) indicates a significant decrease compared to wildtype control.

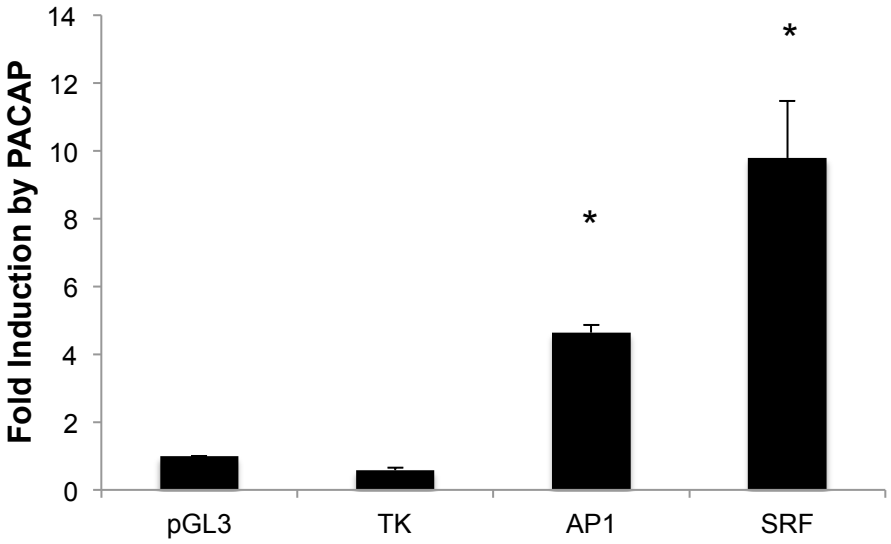


Figure 14: SRF site is sufficient for c-Fos induction by PACAP
LβT2 cells were transfected with 4x multimers linked to a pGL3 luciferase reporter then treated with PACAP. Results are presented as fold induction, normalized over basal. (*) indicates a significant increase compared to control.

Functional ELK and SRF proteins necessary for induction of c-Fos by PACAP

After identifying and confirming the SRF site as necessary and sufficient for PACAP induction of c-Fos, we wanted to know if the same transcription factors activated following GnRH treatment were also necessary for PACAP signaling. c-Fos transcription is thought to be activated by phosphorylation of ELK that then interacts with SRF at the SRF site to form a transcriptionally active complex (18).Thus, we wanted to see if overexpression of an ELK mutant, which is unable to be phosphorylated, or dominant negative SRF proteins would have any effect on induction of c-Fos by PACAP. We determined that c-Fos induction by PACAP was abrogated with the introduction of the mutated ELK protein. Overexpression of dominant negative SRF protein also muted induction. Interestingly, dominant negative SRF reduced basal expression of c-Fos luciferase as well. Thus, induction of c-Fos by PACAP works through both ELK and SRF at the SRF site and SRF may be important for basal expression of c-Fos (Figure 15).

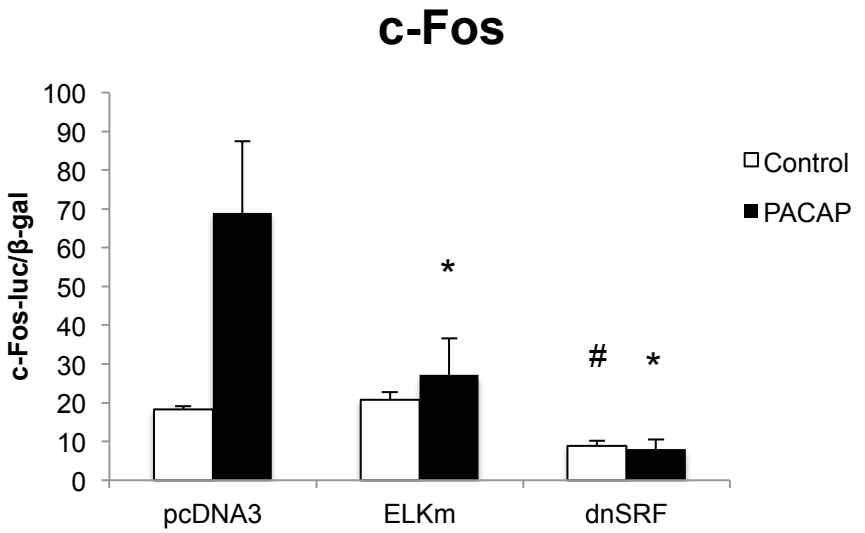


Figure 15: ELK and SRF are important for c-Fos induction by PACAP
LβT2 cells were co-transfected with -1000 murine c-Fos promoter linked to a pGL3 reporter and mutant ELK or dominant negative SRF expression vectors. Results are presented as c-Fos-luc/β-gal ratios normalized to pGL3. (*) indicates significantly lower induction compared to control. (#) indicates a significant decrease in basal expression.

PACAP activates ERK and ELK proteins

Given that MAPK inhibition and SRF site mutation decreased c-Fos induction by PACAP, we determined if PACAP does in fact activate key signaling molecules in the pathway. LβT2 cells have a very low transfection efficiency of 20% and only these cells would express the PACAP receptor. Therefore, we decided to perform immunoprecipitation by co-transfecting PACAP receptor and FLAG-tagged proteins of interest: ERK and ELK. Following treatment with vehicle, PACAP, or GnRH for two hours, FLAG-tagged proteins were immunoprecipitated and western blots were performed. Both ERK and ELK are clearly phosphorylated after PACAP treatment (Figure 16a and 16b). GnRH was included in the experiment as a control since previous studies have shown that

ERK and ELK are phosphorylated following GnRH stimulation (12). Indeed, ERK and ELK were phosphorylated following GnRH treatment (Figure 15a and Figure 15b). Thus, PACAP signaling causes the phosphorylation of important signaling intermediates, ERK and ELK, in the MAPK pathway.

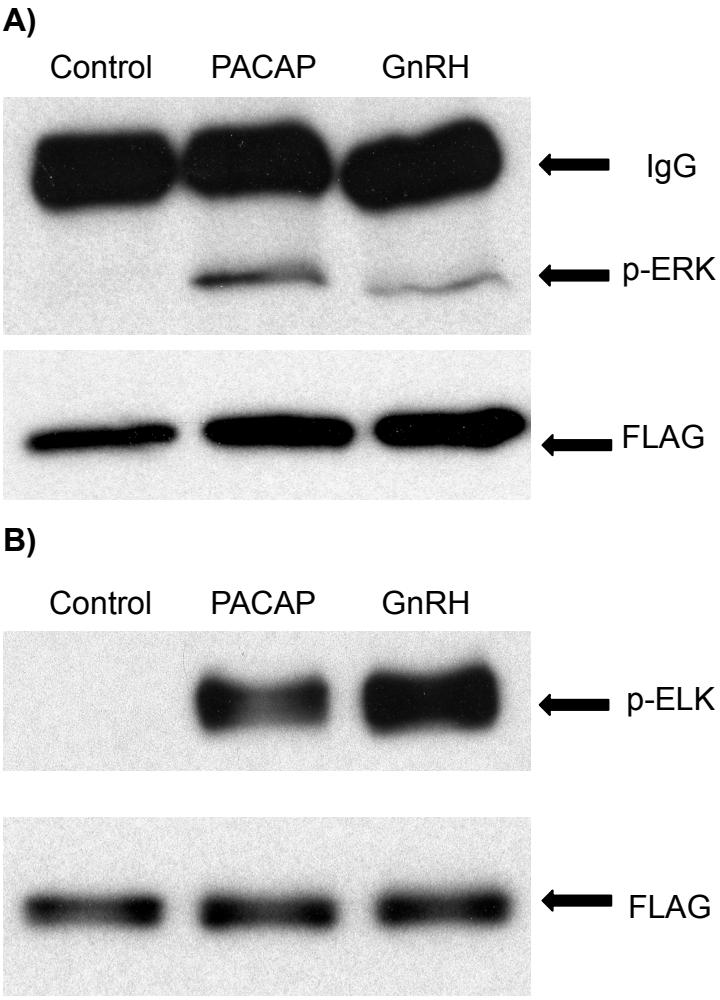
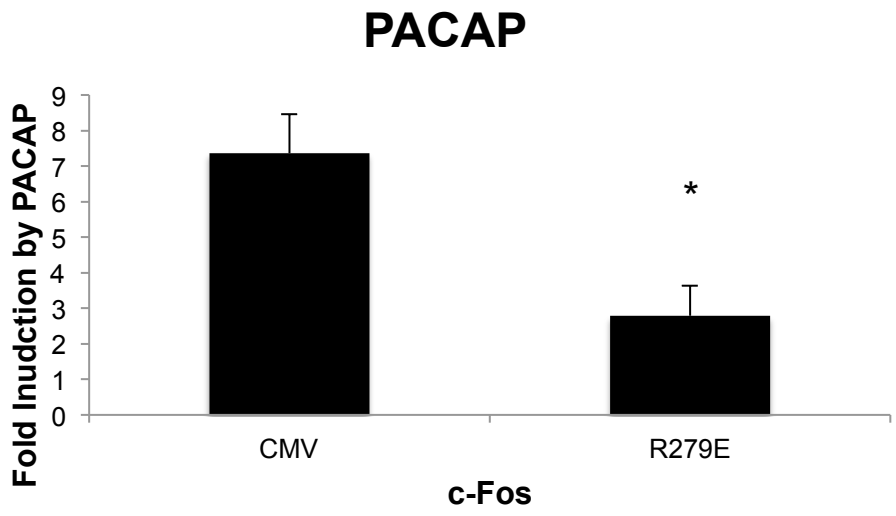


Figure 16: PACAP can activate ERK and ELK
Westerns were performed with immunoprecipitated proteins from LβT2 cells co-transfected with (A) FLAG-ERK or (B) FLAG-ELK expression vectors and PACAP receptor or empty vector. Cells were treated with vehicle, PACAP, or GnRH. FLAG proteins were immunoprecipitated using FLAG antibody linked to agarose beads. p-ERK, p-ELK, and FLAG antibodies were used to detect proteins of interest.

Epac is necessary for induction of c-Fos by PACAP but not GnRH

Studies have shown that PACAP predominantly stimulates the increase of intracellular cAMP and thus initiates the PKA pathway, but from our experiments, we determined that PACAP and PKA do not map to the same regions on FSHβ promoter (23). We decided, then, to investigate other possible factors activated by an increase in cAMP and identified novel signaling molecules called Epac. Epac proteins are cAMP-dependent guanine-nucleotide-exchange factors for the small GTPases Rap1 and Rap2. There is evidence that Rap GTPases, which are regulated by Epac, can activate PLC and calcium pathways in other cell types (34). Therefore, Epac proteins could potentially link PACAP-induced production of cAMP to the MAPK pathway. R279E dominant negative Epac was contransfected with the c-Fos reporter and cells were then treated with PACAP. Interestingly, dominant negative Epac significantly reduced PACAP induction of c-Fos (Figure 17a). When the cells were treated with GnRH, dominant negative Epac had no significant effect on induction of c-Fos (Figre 17b). This suggests that Epac may be the signaling intermediate linking the PACAP signaling pathways to MAPK, which is shared by GnRH.

A)



B)

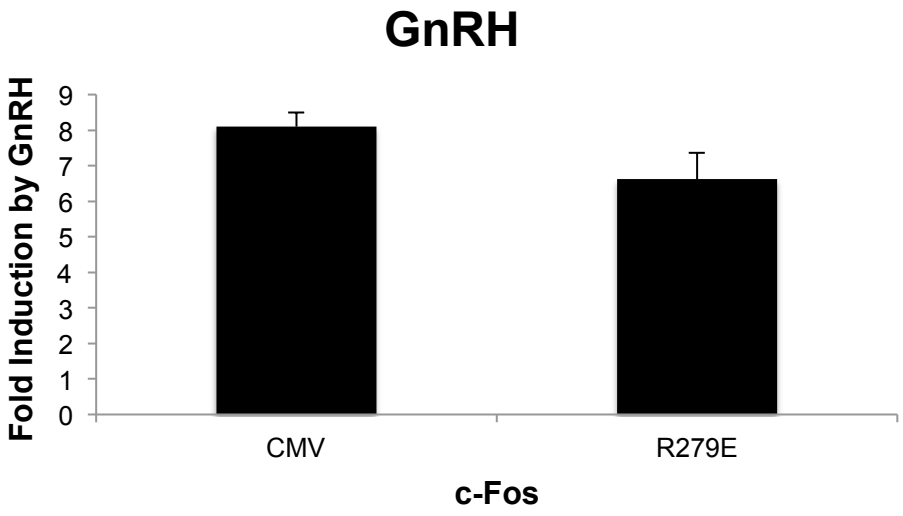


Figure 17: Dominant negative Epac reduces c-Fos induction by PACAP but not GnRH

LβT2 cells were co-transfected with -1000 basepair (bp) murine c-Fos promoter linked to a pGL3 luciferase reporter and a dominant negative Epac expression vector. A) Cells were also transfected with PACAP receptor expression vector prior to treatment with PACAP. B) Cells were treated with GnRH. Results are presented as fold induction by PACAP or GnRH, normalized over basal. (*) indicates a significantly reduced induction as compared to control.

IV

Discussion

Differential regulation of LH and FSH is critical for reproductive fitness. Our studies address the hypothesis that differential regulation by GnRH may stem from the divergent accumulation of intermediate, secondary messengers cAMP and calcium that activate downstream signaling pathways, PKA and Epac, or PKC, respectively. We find that cAMP and PKA may play a role in differential regulation since PKA activates FSH but not LH. However, we were unable to identify transcription factors or promoter elements that are the targets of PKA, nor was the PKA effect high enough to allow for a more careful analysis. Thus, we shifted our efforts to the PACAP pathway since it predominantly activates cAMP and compared its effect to that of GnRH, which activates both cAMP and calcium. We determined that PACAP signaling through Epac bridges to MAPK and does not work through PKA. Crosstalk at MAPK between PACAP and GnRH may be significant for incorporation of different hypothalamic signals in gonadotropin regulation.

cAMP and PKA have been shown to be crucial secondary messengers in the gonadotropin gene expression (11, 35). Using GnRH or forskolin, a selective pharmacological stimulator of cAMP and PKA, a study by Ferris et al showed that cAMP stimulates LHβ promoter activity. The induction by both GnRH and forskolin are abrogated when an inhibitory peptide of PKA called PKI is

overexpressed, suggesting that LHβ is at least in part dependent on PKA (35). In our studies, we showed that overexpression of the PKA catalytic subunit induced FSHβ-luciferase reporter by 2-fold but insufficient to induce LHβ-luciferase reporter. This is likely explained by the induction of c-Fos but not Egr-1 by PKA, essential transcription factors for induction of FSHβ and LHβ respectively. While FSHβ is induced by PKA activity, the induction is low and does not map to the AP-1 site, which is necessary for induction by GnRH.

Additionally, PKA has also been implicated in ERK activation and c-Fos induction, as inhibitory peptide, PKI, attenuates the phosphorylation of ERK and c-Fos expression by both GnRH and Forskolin stimulation (11). In our studies, we found that overexpression of PKA was insufficient to induce phosphorylation of ERK or c-Fos expression (data not shown). This data, together with low FSHβ induction and failure to map to the AP-1 site, leads us to conclude that while cAMP signaling is an important pathway, PKA may not be primary player for gonadotropin induction by GnRH.

Thus, this led us to investigate other potential targets of cAMP. In other studies using LβT2 cells, a specific increase in cAMP was achieved by treating cells with forskolin (11, 35). We were, however, unable to obtain significant induction. To better match physiological conditions, we selected PACAP, which is known for its predominant stimulation of cAMP and PKA (36, 23). Surprisingly, we observed that the induction by PKA and by PACAP do not map to the same regions on the FSHβ promoter. Instead, PACAP induction maps to the same AP-1 as GnRH. Furthermore, PACAP induces c-Fos. When we performed detailed

mapping studies on the c-Fos promoter, we determined that the response maps to the SRF/TCF site, which is the same site that GnRH maps to on the c-Fos promoter (18). The SRF site is a crucial site in the c-Fos promoter. c-Fos induction involves the phosphorylation of ERK by MAPK pathways and ELK's subsequent interaction with SRF at the SRF site, forming a transcriptionally active TCF-tertiary complex factor. We observed that signaling intermediates such as ERK and ELK that are activated upon GnRH stimulation were also activated with PACAP treatment, suggesting that cAMP somehow bridges to the MAPK pathway. This led us to investigate whether another cAMP sensor, Epac, played a role in PACAP induction and cAMP signaling in the gonadotropes. In other cell types, Epac has been linked to the activation of phospholipase C, which activates PKC and subsequently multiple MAPK pathways (34). Additionally, another study has linked Epac to Ca^{2+} mobilization and activation of p38 MAPK in cerebellar neurons (37). Interestingly, in our studies, we found that Epac does not play a role in the induction by GnRH of early immediate gene c-Fos. GnRH likely activates MAPK through direct interaction of the GnRH receptor with Raf (38) or through PKC, which does not require cAMP and Epac. However, Epac does play a role in the induction by PACAP, as we observed that dominant negative Epac abrogate the induction of c-Fos by PACAP. This discovery is particularly noteworthy as it suggests a possible mechanism for crosstalk between PACAP and GnRH signaling pathways.

Understanding the function of PACAP in gonadotropin regulation and reproduction is important. Mice deficient in either PACAP hormone or PAC1

receptor show reduced fertility (39, 40). Also, PACAP levels are highest in the median eminence during the proestrus stage, corresponding with an increase in GnRH during the preovulatory surge (41). GnRH has been shown to upregulate PAC1 receptor expression in the gonadotropes (42). Thus, PACAP may play some role in regulating the LH surge and increase in FSH right before ovulation.

We will continue to study the signaling mechanisms and physiological relevance of PACAP. However, we expect in vivo studies to be complicated as anterior pituitary cells are heterogeneous, with gonadotropes representing only 10% and the other cell types of the anterior pituitary also expressing PACAP receptor (43). To further explore the potentially crucial role of Epac signaling, we will first assess the known interacting molecules. Epac activates Rap, which activates various scaffold proteins. Epac1 has also been reported to activate R-Ras, a small Ras-like GTPase in HEK-293 cells (15). We will analyze these downstream targets and elucidate their roles in gonadotropin gene expression. Analysis of Epac in vivo will be difficult as currently there are no transgenic mice available. However, staining using immunohistochemistry to visualize co-localization of Epac and LH can be performed.

This study aimed to expand the knowledge of cAMP signaling in the gonadotropes. We discovered that PKA, a known target of cAMP, may contribute to gonadotropin gene expression, however, is not sufficient for full induction. We identified the regions on the FSHβ promoter that are critical for induction by the hypothalamic hormone PACAP, which predominantly stimulates an increase in cAMP. The downstream targets of PACAP, ERK1/2 and ELK, match those of

GnRH, which suggest that these two pathways crosstalk. Lastly, we uncovered a novel mechanism for this crosstalk through the cAMP sensor Epac, which has not been studied in the gonadotropes. Our findings shed light on the complexity of signaling within the gonadotropes and its ability to differentially regulate LHβ and FSHβ.

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