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PACAP induces FSHβ gene expression via EPAC

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Abstract

Gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are heterodimers of a common α subunit and unique β subunits. Regulation of their levels, primarily by GnRH, is critical for re-productive function. Several other hormones modulate gonadotropin expression, either independently or by modifying the responsiveness to GnRH. Pituitary adenylate cyclase activating peptide (PACAP) is one such hormone. Four-hour treatment of female mouse primary pituitary cells by either GnRH or PACAP induced FSHβ expression, while 24-h treatment repressed FSHβ. Both PACAP and GnRH caused FSH secretion into the medium. In the gonadotropes, PACAP activates primarily Gαs and increases concentration of cAMP, while GnRH primarily functions via Gαq and increases calcium concentration. Herein, we compared PACAP and GnRH signaling pathways that lead to the induction of FSHβ expression. Interestingly, constitutively active Gαs represses LHβ and induces FSHβ expression, while Gαq induces both β-subunits. We determined that FSHβ induction by PACAP requires functional EPAC, a cAMP sensor protein that serves as a guanine exchange factors for small G proteins that then bridges cAMP signaling to MAPK pathway. We further demonstrate that in addition to the prototypical small G protein Ras, two members of the Rho subfamily, Rac and CDC42 are also necessary for PACAP induction of FSHβ, likely via activation of p38 MAPK that leads to induction of cFOS, a critical transcription factor that is necessary and sufficient for FSHβ induction. Therefore, PACAP-induced cAMP pathway leads to MAPK activation that stimulates cFOS induction, to induce the expression of FSHβ subunit and increase FSH concentration.

Keywords

PACAP; GnRH; EPAC; Gonadotropin; FSH

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Declarations of interest

None.

Disclosure statement

The authors have no conflicts of interest to declare.

Statement of ethics

All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines.

1. Introduction

Regulation of gonadotropin levels and therefore reproduction depends on the integration of the endocrine, paracrine and autocrine signals in the gonadotrope cells of the anterior pituitary (Coss, 2018). Gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are heterodimers of a common alpha subunit (α-GSU, *Cga*) that dimerizes with unique beta subunits (LHβ, *Lhb* and FSHβ, *Fshb*) (Pierce and Parsons, 1981). β subunits confer the biological specificity of LH and FSH and their synthesis is the rate-limiting step for production of the mature hormones (Papavasiliou et al., 1986; Kaiser et al., 1997). Synthesis and secretion of LH and FSH are primarily regulated by the gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH binding to the GnRH receptor, expressed specifically in the gonadotrope, causes activation of Gαq and Gα11 types of G-proteins (Stanislaus et al., 1997), with the subsequent stimulation of phospholipase Cβ, increase in the intracellular calcium concentration, and activation of protein kinase C (PKC) and calcium/calmodulin kinase II (CamKII), (Liu et al., 2002b; Haisenleder et al., 2003). GnRH receptor signaling also leads to activation of the mitogen-activated protein kinase (MAPK) pathways: ERK 1/2, JNK, and p38 (Roberson et al., 1995; Sundaresan et al., 1996; Liu et al., 2002b), via PKC (Naor, 2009; Ely et al., 2011) or via calcium influx through voltage gated calcium channel (Mulvaney et al., 1999).

Pituitary adenylate cyclase activating peptide (PACAP) is another hypothalamic neuropeptide that regulates gonadotropin expression (Counis et al., 2007; Halvorson, 2014). It was initially identified as a novel hypothalamic factor that increases adenylate cyclase (AC) signaling in the pituitary cultures, as its name suggests (Miyata et al., 1989). PACAP belongs to the vasoactive intestinal polypeptide (VIP)-secretin-glucagon superfamily and is widely distributed in the brain. It is also expressed in peripheral organs, primarily in the endocrine pancreas, gonads, adrenal, respiratory and urogenital tracts, where it exerts pleiotropic effects (Vaudry et al., 2009). PACAP exists as either a 38-amino acid or 27-amino acid form, both derived from the same precursor protein encoded by the *Adcyap1* gene. Vast majority of PACAP protein in most tissues is PACAP38, while PACAP27 represents less than 10% of total PACAP. PACAP binds to three receptor subtypes, the PACAP type 1 receptor (PAC1R), the vasoactive intestinal polypeptide type 1 receptor (VPAC1R), and the VPAC2R, but the binding affinity is much higher for PAC1R than for others (Pisegna and Wank, 1993). All receptors belong to the family of G protein-coupled receptors and are expressed in a variety of tissues. The predominant receptor for PACAP, PAC1R, couples mainly to Gαs protein and induces rapid cAMP production, which ultimately activates PKA (Vaudry et al., 2009). cAMP concentration is significantly increased following PACAP stimulation of gonadotrope cells as well (Harada et al., 2007).

PACAP is also secreted by gonadotropes and folliculostelate cells in the pituitary and functions in the autocrine or paracrine manner to modulate response to GnRH (Counis et al., 2007). GnRH increases the expression of PACAP receptor in the gonadotrope and in turn, PACAP potentiates the effect of GnRH (Culler and Paschall, 1991; Purwana et al., 2010). While GnRH regulates expression of both β subunits via intermediates, immediate early genes, mechanisms of PACAP regulation of gonadotropin β subunits was not thoroughly analyzed. GnRH induces LHβ expression through induction of the early growth response-1

(Egr-1) transcription factor (Kaiser et al., 2000; Weck et al., 2000). Upon induction, Egr-1 interacts with tissue specific and basal factors to regulate the LHβ promoter (Jorgensen et al., 2004; Coss, 2018). PACAP stimulates the release of LH (Culler and Paschall, 1991; Hart et al., 1992) and LHβ expression when administered in a pulsatile manner in rat primary cells (Tsujii and Winters, 1995) and continuously in LβT2 cells model (Ferris et al., 2007; Harada et al., 2007). However, the promoter elements and transcription factors mediating PACAP effects on LHβ levels are not known.

FSHβ induction by GnRH occurs via activator protein-1 (AP-1) transcription factor, cFOS (Coss et al., 2004; Wang et al., 2008). AP-1 also interacts with basal and hormonally-regulated transcription factors to integrate the responses at the level of the promoter (Coss et al., 2007; Lamba et al., 2008; Bernard et al., 2010; Ely et al., 2011; Reddy et al., 2013; Roybal et al., 2014). Role of PACAP in regulation of FSHβ transcription is not clear. PACAP increased FSHβ expression in LβT2 go-nadotrope cell model when administered in either pulsatile manner (Kanasaki et al., 2009) or continuously (Harada et al., 2007) and in monkey primary pituitary cells (Kawakami et al., 2002). In other studies, however, PACAP dowregulated FSHβ expression in rat primary pituitary cells (Tsujii et al., 1994; Tsujii and Winters, 1995) This repression may occur via follistatin induction (Winters et al., 1997). Since PACAP expression in the pituitary decreases after birth, concomitant with an increase in FSHβ levels, PACAP downregulation of FSHβ may be critical during development (Winters and Moore, 2011). Some inconsistencies that exist regarding a role of PACAP in the gonadotrope and gonadotropin expression, may be attributed to the level of PACAP receptor expression in different models (Fowkes et al., 2003a). Alternatively, the inconsistencies may be explained by crosstalk of pathways, since with cotreatment, a signaling pathway may be augmented, while pretreatment may lead to downregulation and inability to respond to a subsequent signal. The fact that pretreatment with either PACAP or GnRH diminishes the effect of the other can likely be explained by downregulation (Fowkes et al., 2003b; Larivière et al., 2008). This hypothesis is strengthened with findings from transgenic male mice with permanently elevated PACAP levels that have delayed puberty and decreased gonadotropin levels (Moore et al., 2012). However, the signaling pathways and transcription factors involved in PACAP regulation of FSHβ expression have not been determined (Halvorson, 2014). In this manuscript, we sought to determine mechanisms of PACAP regulation of FSHβ expression and identify signaling pathways of this regulation and compare these with pathways activated by GnRH.

2. Materials and methods

2.1. Animals

C57BL/6 J mice were maintained under a 12-h light, 12-h dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines. Primary pituitary cells in culture were obtained as described before (Coss et al., 2005) and treated with 10 nM GnRH or 100 nM PACAP for 4 h or 24 h, after overnight in the serum free medium. Secretion of gonadotropin hormones to the medium was measured using Luminex pituitary panel

(Millipore) and analyzed using MagPix Luminex instrument (Lainez et al., 2018; Lainez and Coss, 2019), according to manufacturers' instructions.

2.2. Materials

Immortalized gonadotrope cells line, LβT2 was kindly provided by Pamela Mellon (UCSD, California) and cultured as described previously. The 1 kb of the murine FSHβ proximal promoter and 1.8 kb of the rat LHβ regulatory region linked to luciferase reporter in pGL3 plasmid were described previously (Coss et al., 2004, 2005; Roybal et al., 2014; Jonak et al., 2017, 2018). Truncations of FSHβ promoter linked to luciferase were described previously (Coss et al., 2004). cFOS expression vector and 1 kb cFOS luciferase reporter were also described before (Ely et al., 2011; Reddy et al., 2013; Jonak et al., 2018). Gαs and Gαq expression vectors and small G proteins expression vectors were purchased from cDNA Resource Center. CREB expression vectors were obtained from 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). ELK expression vector was kindly provided by Dr. Andrew Sharrocks (The University of Manchester, Manchester, UK). ERK2 and p38 expression vectors were kindly provided by Dr. Peiquig Sun (Scripps Institute, La Jolla, CA). Cells were treated with 10 nM Gonadotropin-releasing hormone (GnRH, Sigma-Aldrich, St. Louis, MO) or 100 nM Pituitary adenylate cyclase activating polypeptide (PACAP 38 Calbiochem, La Jolla, CA). Inhibitors were purchased from Calbiochem (La Jolla, CA) and cells pretreated for 30 min with: 1 μM BIM (to inhibit PKC); 10 μM H89 (to inhibit PKA); 10 μM KN-93 (KN; to inhibit CamKII); 5 μM UO126 (UO; to inhibit MEK1 and activation of ERK1/2), 20 μM SB 202190 (SB; to inhibit p38), or 10 μM SP 600125 (JNK; to inhibit JNK), prior to hormone treatment.

2.3. 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 with 0.5 μg of luciferase reporter plasmid and 0.1 μg of β-galactosidase, driven by a Herpes virus thymidine kinase (TK) promoter, to control for transfection efficiency. Different batches of LβT2 cells express variable levels of PACAP receptors and the expression is diminished with passaging, as described before (Fowkes et al., 2003a; Purwana et al., 2010; Winters and Moore, 2011; Halvorson, 2014). To normalize the response to PACAP, we overexpressed PACAP receptor in LβT2 cells by adding 0.1 μg of the expression vector in the transfection mix. Cells were starved in serum free DMEM containing 0.1% BSA and penicillin-streptomycin antibiotics the night before harvesting. 4 h before harvesting, cells were treated with 10 nM GnRH, 100 nM PACAP, or vehicle. Inhibitors (concentration detailed above) were applied 30 min prior to GnRH or PACAP treatment. Cells were washed with 1X PBS and lysed with a buffer containing 100 nM K-PO4 and 0.2% Triton X-100 48 h after transfection. 20 μl of lysate was loaded into 96-well plates. Both luciferase and β-galacatosidase activity were measured with a luminometer (Vertias Microplate luminometer from Turner Biosystems/Promega, Madison, WI). 100 μl of a luciferin buffer containing 25 mM Tris pH 7.8, 15 mM MgSO4, 10 mM ATP, and 65 μM

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 Galacto-light substrate (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Then, 100 μl of catalyst, Accelerator, (Applied Biosystems, Foster City, CA) was injected before reading. Each transfection was performed in triplicate and repeated three times. Luciferase values in each sample were normalized to β-galactosidase values. Results are represented as mean of 3 experiments. ANOVA and Tukey's posthoc test was used to determine statistical significance, $p < 0.05$.

2.4. Whole cell extract, immunoprecipitation and Western blot

LβT2 cells were starved overnight in serum free DMEM containing 0.1% BSA. Cells were rinsed with cold 1x PBS then lysed with a buffer containing 20 mM Tris pH 7.4, 140 mM NaCl, protease inhibitors (Sigma-Aldrich), 1 mM PMSF, 10 mM NaF, 1% NP-40, 0.5 mM EDTA, and 0.1 mM EGTA. Protein concentration was determined using Bio-rad Protein Assay (Bio-rad, Hercules, CA) and a standard curve was generated to calculate the concentration. Equal amount of proteins were incubated with anti-FLAG or anti-HA antibody linked to agarose beads (Sigma-Aldrich, St. Louis, MO) for 3 h. Beads were washed three times with 500 μl of cold lysis buffer. 30 μl of 2x Laemmli sample buffer was added to the beads.

Equal amounts of protein from whole cell extracts in 4x sample buffer or after immunoprecipitation were loaded into an SDS-PAGE gel and proteins were separated by gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 h at room temperature in 10% nonfat milk in TBST (20 mM Tris 7.4, 0.1% Tween, 150 mM NaCl, and 0.5% BSA). Membranes were then probed with primary antibodies to p-ERK, p-38, p-ELK, or FLAG and HA. After anti-rabbit or mouse secondary antibodies linked to horseradish peroxidase (Amersham), enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, NJ) was used to detect complexes. Experiments were repeated three times and representative images shown.

2.5. RT-qPCR

RNA was obtained with MicroRNA kit (Ambion, Austin TX) according to the manufacturer's instructions. Contaminating DNA was removed with DNA-free reagent (Ambion, Austin, TX) and RNA was reverse transcribed using Superscript IV (Invitrogen, Carlsbad, CA). Quantitative Real-Time PCR was performed in iCycler from Bio-Rad (Hercules, CA), using SYBR Green (Bio-Rad, Hercules, CA) and the following primers: LHb forward: CTGTCAACGCAACTCTGG; LHb reverse: ACAGGAGGCAAAGCAGC; GAPDH forward: TGCACCACCAACT GCTTAG; GAPDH reverse: GGATGCAGGGATGATGTTC; FSHb forward: GCCGTTTCTGCATAAGC; FSHb reverse: CAATCTTACGGTCTCGTAT ACC; under the following conditions: 95 C for 15 min, followed by 40 cycles at 95 C for 15 s, 54 C for 30 s and 72 C for 30 s. Each sample was assayed in triplicate and the experiment was repeated 4 times. A standard curve with dilutions of 10 pg/well, 1 pg/well, 100 fg/well and 10 fg/well of a plasmid containing LHβ cDNA was generated in each run with the samples. In each experiment, the amount of LHβ was calculated by comparing threshold cycle obtained for each sample with the standard

curve generated in the same run. Replicates were averaged and divided by the mean value of GAPDH in the same sample. Preliminary analysis determined that GAPDH does not change with treatment. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction. ANOVA and Tukey's posthoc test was used to determine statistical significance $p < 0.05$.

3. Results

3.1. PACAP induces FSHβ expression

In order to assess if PACAP regulates gonadotropin β subunits expression, we dispersed primary pituitary cells in culture from 4-month old C57Bl/6 J female mice and treated them with 10 nM GnRH, which serves as a positive control, and with 100 nM PACAP for 4 h. PACAP induced LHβ (*Lhb*) expression 2.8 fold and FSHβ (*Fshb*) expression 5.2-fold, while GnRH induces *Lhb* 4.6 fold, and *Fshb* 3.0 fold (Fig. 1A). Thus, induction by PACAP is comparable to the induction by GnRH. We then treated the primary pituitary cells for 24 h to analyze the effects of longer treatment. 24-hour treatment with PACAP induced LHβ (*Lhb*) expression 1.58 fold, while *Lhb* expression was reduced to 83% after 24-h GnRH treatment (Fig. 1B). PACAP treatment for 24 h reduced *Fshb* mRNA to 55% of vehicle treated cells, while GnRH treatment diminished *Fshb* to 52%. As indicated in the introduction, it is possible that long-term treatment downregulates the receptors or components of the intracellular signaling pathway, which caused a switch from induction to repression of *Fshb* with both PACAP and GnRH treatments. On the other hand, follistatin is induced by PACAP, which abrogates *Fshb* induction by intra-pituitary activin (Winters et al., 1997; Winters and Moore, 2011). We analyzed expression of follistatin (*Fst*) following 4-h treatment (Fig. 1C) and following 24-h treatment (Fig. 1D) by PACAP or GnRH. 4-hour treatment by PACAP induced *Fst* 1.3 fold, while 4-h GnRH treatment reduced *Fst* mRNA to 80% (Fig. 1C). PACAP and GnRH treatment for 24 h induced *Fst* 1.5 fold and 1.3 fold, respectively. Therefore, follistatin is induced by PACAP at both 4 h and 24 h to a similar level, while FSHβ is induced by both GnRH and PACAP after 4-h treatment and repressed after 24-h treatment in adult female mouse pituitary cells.

We investigated secretion of LH and FSH from female mouse primary pituitary cells following 24-h PACAP or GnRH treatment. LH concentration in the medium increased from 20 ng/ml to 94.8 ng/ml, a 4.7 fold increase, with PACAP treatment; and to 180 ng/ml, a 9 fold increase, with GnRH treatment (Fig. 1E). PACAP increased FSH concentration from 9.9 ng/ml to 25.5 ng/ml, a 2.6 fold increase, while GnRH increased FSH in the medium to 41.6 ng/ml, a 4.2 fold increase (Fig. 1E). Therefore, PACAP, as well as GnRH, caused secretion of both LH and FSH.

To analyze molecular mechanisms of this induction and compare GnRH and PACAP signaling pathways, we employed LβT2 cells, a model of mature gonadotrope that endogenously expresses gonadotropin β subunits and responds to hormonal stimuli (Alarid et al., 1996). We transiently transfected LHβ-luciferase and FSHβ-luciferase reporters that were used previously to analyze GnRH regulation of gonadotropin expression (Coss et al., 2005; Jonak et al., 2017, 2018) and treated cells with PACAP and GnRH for 4 h. PACAP

induced LHβ reporter 1.6 fold and FSHβ reporter 2.8 fold. This induction is also comparable to induction by GnRH of 2.4 fold for LHβ reporter and 2.9 fold for FSHβ reporter (Fig. 2A).

Both PACAP receptor (PAC1R) and GnRH receptor (GnRHR) belong to the G-coupled 7-transmembrane receptor family and activate G-proteins following ligand binding (Halvorson, 2014; Oride et al., 2018). PACAP, however, primarily activates Gαs which leads to an increase in cAMP, while GnRH primarily activates Gαq/11 which leads to an increase in calcium. To ascertain if these G proteins can activate transcription of gonadotropin β subunits, we co-transfected constitutively active forms of Gαs and Gαq with gonadotropin β reporters. Surprisingly, Gαs suppressed LHβ reporter by 50%, and activated FSHβ by 2.2 fold. As expected, constitutively active Gαq induced LHβ 2 fold and FSHβ 3.7 fold. Therefore, PACAP and GnRH increase the expression of both gonadotropin β subunits in primary pituitary gonadotropes and in LβT2 gonadotrope cell model.

3.2. PACAP activates cFOS to induce FSHβ expression

Mechanism of FSHβ mRNA regulation by PACAP has not been explored. Thus, we initiated these studies using LβT2 cells to examine a role of CREB as a prototypical nuclear target of Gαs-cAMP-PKA signaling pathway, potentially activated by PACAP. We used multimerized cAMP response elements (CRE-luciferase), a consensus binding site for CREB (Mayr and Montminy, 2001), as a positive control. We co-transfected non-phosphorylatable, mutant CREB protein (CREBm, (Struthers et al., 1991)) that serves as a dominant negative, or an empty vector control, with CRE-multimer and treated cells with vehicle or PACAP. PACAP induced CRE-luciferase 4 fold and CREBm abrogated this induction (Fig. 3A). On the other hand, CREBm did not affect induction of FSHβ-luciferase by PACAP (Fig. 3B). We then co-transfected constitutively active form of CREB (CA CREB), which induced CRE-luciferase 2.4 fold (Fig. 3C). CA CREB did not induce FSHβ-luciferase (Fig. 3D). These results indicate that CREB is unlikely to regulate of FSHβ expression.

To determine the elements in the FSHβ promoter that PACAP may employ for induction, we used truncation analysis, and determined that PACAP induction of FSHβ is abrogated with a truncation from −95 to −60 base pairs from the transcriptional start side (Fig. 4A). This is the same region necessary for the FSHβ responsiveness to GnRH, which induces FSHβ via induction of cFOS that binds AP-1 site at −76 (Coss et al., 2004; Wang et al., 2008). Therefore, we transfected a reporter with the mutation in the −76 AP-1 site and compared its induction to the induction of the wild type reporter. −76 site mutation diminished induction of the FSHβ reporter by PACAP (Fig. 4B). Then, we analyzed if PACAP induces cFOS in the gonadotrope cells, since PACAP increases cFOS expression in the brain (Meloni et al., 2016). We determined that cFOS is induced at the mRNA (not shown) and protein level (Fig. 4C) following PACAP treatment. PACAP also induces cFOS reporter 9.7 fold (Fig. 4D). Since previously we have shown that overexpression of cFOS is sufficient to induce FSHβ in the LβT2 cells (Ely et al., 2011; Jonak et al., 2018), we postulate that PACAP induces FSHβ via cFOS as well.

3.3. PACAP uses EPAC to activate MAPK pathway

Since GnRH and PACAP may activate FSHβ transcription via the cFOS intermediate gene, but they bind receptors that preferentially activate different G proteins, we wanted to analyze signaling pathways activated by PACAP and compare them to GnRH-activated signaling pathways. Our previous studies determined that GnRH signals primarily via CamKII activation following intracellular calcium increase, with some contribution of ERK 1/2 and p38 pathways, to activate FSHβ and cFOS transcription (Ely et al., 2011). To analyze PACAP signaling pathways necessary for induction of cFOS and FSHβ we used inhibitors and determined that PACAP does not utilize CamKII (Cam), but that the activity of PKA, ERK 1/2 and p38 is necessary for maximal induction of FSHβ (Fig. 5A) and cFOS (Fig. 5B) by PACAP.

To determine if PACAP activates ERK 1/2 and p38, we analyzed their phosphorylation with western blots following vehicle (C), PACAP (P) or GnRH (G) treatments. These experiments determined that PACAP treatment, as well as GnRH, leads to phosphorylation and activation of ERK 1/2 (Fig. 6), which is in agreement with previous reports (Fowkes et al., 2003b; Purwana et al., 2010). We further determined that PACAP activates p38 as well (Fig. 6). Transcription factor ELK is a nuclear target of ERK 1/2 and p38 kinases, which when phosphorylated forms an active complex with SRF to activate transcription of cFOS (Sharrocks, 2002). ELK is also phosphorylated following PACAP and GnRH (Fig. 6). Therefore, PACAP uses classical growth factor pathway, via MAPK phosphorylation of ELK to activate expression of cFOS, and subsequently FSHβ.

To determine how PACAP activates MAPK pathway, we postulated that it may use exchange protein directly activated by cAMP (EPAC). EPAC is a guanine nucleotide exchange factor that is activated by cAMP and in turn activates Ras superfamily of small GTPases, which then leads to MAPK activation (Lewis et al., 2016; Robichaux and Cheng, 2018). We obtained two different forms of dominant negative EPAC: dn1, created by mutation of the arginine residue at the position 279 to glutamate which prevents cAMP binding, and dn2, that lacks Ras association domain (Hochbaum et al., 2008); and co-transfected them with FSHβ (Fig. 7A) and cFOS (Fig. 7B) reporters. Cells were treated with PACAP (black bars) or GnRH (gray bars). Dominant negative EPACs diminished induction of FSHβ and cFOS by PACAP, but had no effect on the induction by GnRH. We previously determined that CamKII is necessary for GnRH induction of FSHβ and cFOS (Ely et al., 2011), and here we transfected dominant negative CamKII to assess its necessity for PACAP induction of FSHβ and cFOS. As shown before, dominant negative CamKII is necessary for induction of FSHβ (Fig. 7C) and cFOS (Fig. 7D) by GnRH, but not by PACAP. Taken together our results indicate that while GnRH activates FSHβ and cFOS transcription via CamKII and MAPK, PACAP induces FSHβ and cFOS via MAPK pathways that are activated by cAMP-mediated EPAC activation.

As stated above, EPAC molecules activate small GTPases by exchanging GDP for GTP (Robichaux and Cheng, 2018). Thus, we assessed several small G proteins for their roles in FSHβ and cFOS induction. First, we transfected constitutively active forms to determine which small G proteins can activate transcription of FSHβ and cFOS reporters. Constitutively active Ras, Rac and CDC42 activate transcription of FSHβ (Fig. 8A) and

cFOS (Fig. 8B). We also analyzed which of these small G proteins can phosphorylate ERK1/2 and p38 by western blots. ERK2 isoform is more abundant than ERK1 (Shin et al., 2013), and out of four p38 isoforms, p38β plays a role in normal physiological processes while p38α is mostly involved in cell responses to stress, and γ and δ have low abundance (Askari et al., 2007). Thus, we co-transfected FLAG-tagged ERK2 and HA-tagged p38β with constitutively active forms of small G proteins. ERK2 is activated by Ras, while p38β is activated by Ras, Rac and CDC42 in the gonadotrope cell model (Fig. 8C).

We then determined which of these small G proteins are necessary for PACAP and GnRH induction of FSHβ and cFOS by transfecting dominant negative mutants. Consistent with our previous results that GnRH stimulation leads to CamKII activation that is necessary for FSHβ induction, small G proteins are not necessary for FSHβ induction by GnRH (Fig. 9A). For PACAP induction of FSHβ, however, Ras, Rac and CDC42 are necessary (Fig. 9A). Rho, Rac and CDC42 are necessary for maximal induction of cFOS by either GnRH or PACAP (Fig. 9B). Our results demonstrate the necessity of several small G proteins, which may be targets of EPAC signaling following cAMP increase, for induction of FSHβ by PACAP. Taken together, our results indicate that GnRH activates CamKII, via increase in intracellular calcium by Gαq-PLCβ, to induce cFOS and FSHβ, while PACAP activates EPAC via increase in cAMP by Gαs-adenylate cyclase, that leads to activation of MAPK through several small G proteins, that in turn induces cFOS and FSHβ (Fig. 10).

4. Discussion

Concentrations of pituitary gonadotropins, LH and FSH, are regulated primarily by the hypothalamic peptide GnRH, which stimulates their synthesis and secretion. Other factors, such as gonadal steroids and gonadal peptides such as inhibin, or pituitary-expressed peptides that function in autocrine or paracrine manner, such as activin and follistatin, influence gonadotropin levels, directly or by modulating the responsiveness to GnRH (Coss, 2018). In addition, PACAP, a product of the *Adcyap1* gene expressed in the hypothalamus and the pituitary, also regulates gonadotropin synthesis and release, both alone and in cooperation with GnRH (Winters and Moore, 2011; Halvorson, 2014). In this manuscript, we address the mechanisms of this regulation by analyzing signaling pathways and promoter elements activated by PACAP and compare them to GnRH. Although PACAP regulates expression of all three gonadotropin subunits, promoter elements responsive to PACAP were only analyzed for the common α-subunit (Attardi and Winters, 1998; Burrin et al., 1998), and in this manuscript we analyzed the induction of FSHβ and promoter elements therein. We determine that both PACAP and GnRH stimulate FSHβ expression after 4-h treatment of primary pituitary cells from the female mouse, but repress FSHβ after 24 h. We then analyzed pathways that lead to induction of FSHβ and future studies will analyze mechanisms of repression following 24-h treatment. Similar to GnRH, PACAP induces FSHβ via induction of cFOS. We further investigate roles of small G proteins and EPAC molecules in both GnRH and PACAP signaling in order to explain the mechanisms of cAMP-activated pathways stimulated by PACAP receptor activation, bridging to MAPK pathways and cFOS and FSHβ promoters.

PACAP, expressed in the hypothalamus, may be secreted from the nerve terminals within the median eminence and carried by the portal circulation to stimulate the pituitary, and act as a hypophysiotropic neurohormone. Alternatively, in the pituitary PACAP is expressed by the gonadotrope and may function in an autocrine manner, and by the folliculostellate cells and may function in the paracrine manner. Either way, PACAP regulates gonadotropin hormone synthesis and gonadotrope responsiveness to GnRH. Some studies determined that PACAP stimulates all three gonadotropin subunits expression, including FSHβ (Kawakami et al., 2002; Harada et al., 2007; Kanasaki et al., 2009; Purwana et al., 2010). Others, on the other hand reported that while PACAP induces α-GSU and LHβ, it represses FSHβ in rat primary pituitary culture (Tsujii and Winters, 1995; Winters et al., 1996). Repression of FSHβ by PACAP is indirect via induction of follistatin and may be critical during development (Winters and Moore, 2011). In agreement, we also determined that PACAP induces follistatin expression, albeit to a relatively low level. We found that PACAP induces both LHβ and FSHβ in primary pituitary cells from female mice after 4 h of treatment, but represses FSHβ after 24 h treatment although LHβ mRNA remains elevated compared to controls. Several possibilities can be proposed to explain the differences in our study and studies that demonstrated repression of FSHβ. First, the difference may be due to the physiological state of the animals from which the pituitaries were extracted or to the stress level the animals experienced during transport or procedure. For example, different levels of steroid hormones can contribute to the differences in results, since several studies that reported repression of FSHβ used gonadectomized animals that lack sex steroids. Second, the duration of the treatment is different. As discussed in the introduction, duration of treatment may augment or dowregulate the pathway involved in crosstalk (McArdle and Counis, 1996; McArdle et al., 1996). We used acute, 4-h treatment to prevent downregulation of signaling pathways. Indeed, in our studies as well, 24-h treatment by either PACAP or GnRH repressed FSHβ. Third, the studies that observed repression of FSHβ likely had a higher level of folliculostellate cells due to longer culture prior to the hormone treatment. These studies reported that the cells were cultured for 3 days prior to the 9-h treatment. However, in primary pituitary cultures, secretory cells are rapidly overgrown by folliculostellate cells, which secrete follistatin, a potent FSHβ repressor (Kawakami et al., 2002). Our studies were performed in freshly isolated cells to minimize changes during culture. Fourth, there are significant species differences in regulation of gonadotropin hormones (Bernard et al., 2010). Finally, sex differences may play a critical role since we used primary cells from adult female mice, while previous studies used male rat pituitary cells. Gonadotropin β subunits in particular exhibit substantial sex differences in their levels of expression (Bjelobaba et al., 2015; Kreisman et al., 2017).

PACAP signaling in the gonadotrope cells causes an increase in cAMP via Gαs activation of adenylate cyclase (Harada et al., 2007; Winters and Moore, 2007). Although some studies have shown increase in calcium, calcium does not play a role in PACAP induction of gonadotropin subunits (Attardi and Winters, 1998; Burrin et al., 1998), indicating that PACAP functions primarily via cAMP, as expected from the Gαs activation by PACAP receptor. We investigated roles of Gαs, activated by PACAP and Gαq, activated by GnRH, by transfecting constitutively active forms of these G proteins. As expected Gαq induced both LHβ and FSHβ, while surprisingly, Gαs repressed LHβ and induced FSHβ. This was

unexpected, since PACAP induces LHβ, and will require further investigations which are beyond the scope of this report that focuses on FSHβ induction. The most common nuclear target of the cAMP pathway is CREB transcription factor, but we could not find any evidence of CREB involvement in the FSHβ expression. Our results concur with previous findings that PACAP activates MAPK pathways (Fowkes et al., 2001; Harada et al., 2007), which lead to FSHβ induction. This poses a question of how does cAMP activate MAPK pathways in the gonadotrope. One potential mediator is EPAC that we addressed here. We show that functional EPAC is necessary for FSHβ induction by PACAP, but not by GnRH.

Several guanine exchange factors (GEF) interact with trimeric G proteins and activate small G proteins that in turn activate MAPK cascade (Goldsmith and Dhanasekaran, 2007). EPAC (exchange protein directly activated by cAMP) is a cAMP sensor protein that serves as a GEF for small G proteins (Quilliam et al., 2002). Gαs activation and subsequent generation of cAMP activates EPAC, which elicits GDP–GTP exchange in Rap-1 small G protein. The activated GTP-bound Rap-1 activates B-Raf and the downstream MAPK (Goldsmith and Dhanasekaran, 2007). This may play a role in Gαs induction of FSHβ. Gαs-mediated cAMP–PKA signaling pathway can also inhibit MAPK pathways, which entails serine phosphorylation of another Raf isoform, C-Raf, at a residue that prevents Ras-mediated phosphorylation and activation (Goldsmith and Dhanasekaran, 2007). This may play a role in the repression of LHβ by Gαs that we observed and will be explored further. EPAC proteins bind to cAMP with high affinity and activate the Ras superfamily of small GTPases (Bos, 2006; Robichaux and Cheng, 2018). EPAC was originally identified as a GEF for Rap-1 GTPases, which are activated by cAMP independent of PKA (Kawasaki et al., 1998). Later studies determined that EPAC can activate other branches of Ras superfamily as well (Shariati et al., 2016). There are two isoforms of EPAC, EPAC1 and EPAC2, products of independent genes in mammals. While EPAC1 is ubiquitously expressed in all tissues, EPAC2 is more limited in its tissue distribution (Lewis et al., 2016) and we concentrated our studies on EPAC1. We determined that EPAC1 is necessary for PACAP induction of FSHβ.

There are five main braches of the small G protein superfamily: Ras, Rho, Ran, Arf and Rab and each has its cohort of regulatory proteins, such as GEFs, which promote small G protein activation by exchanging GDP for GTP (Loirand et al., 2013). The Ran, Arf, Rab family are localized in the specific subcellular compartments and regulate nuclear, Golgi and ER trafficking, and thus, were not investigated in our studies. Because Ras and Rho families are involved in receptor signaling, we focused on the members of these groups. The Ras family contains several Ras and Rap isoforms, while Rho contains Rho, Rac and CDC42 isoforms (Rossman et al., 2005; Hall, 2012). They each can interact with 20–30 downstream proteins, regulating numerous signaling pathways as of yet unclear specificity (Hall, 2012). They can also activate each other, increasing complexity further. We demonstrate that Rac and CDC42 activate p38 in the gonadotrope cells, consistent with previous reports using Cos-1 cells (Gallo and Johnson, 2002). We also show that they are involved in the cFOS and FSHβ induction by PACAP likely via p38 or through activation of SRF (Miralles et al., 2003), since we have shown previously that SRF is sufficient and necessary for cFOS induction by GnRH (Ely et al., 2011). Therefore, Rac and CDC42 may be the targets of cAMP-EPAC pathway activated by PACAP that leads to FSHβ induction.

Herein, we compared GnRH and PACAP signaling pathways that lead to induction of FSHβ. Future studies will analyze whether down-regulation of FSHβ by 24-h treatment is due to the follistatin induction, or due to down-regulation of secondary messengers and signaling pathways. In our studies, follistatin was induced at both 4-h treatment and 24-h treatment, while FSHβ is induced after 4 h and repressed after 24 h. Our previous investigations of GnRH signaling pathways that activate gonadotropin gene expression, and PACAP signaling pathways in this report, reveal critical signaling molecules necessary for β subunit induction. GnRHR activates downstream signaling pathways through activation of trimeric Gαq/11 proteins. The Gαq pathway leads to gonadotropin expression via CamKII activation of SRF transcription factor that induces both Egr-1 (Witham et al., 2013) and cFOS (Ely et al., 2011) and through them gonadotropin β subunits (Coss, 2018). CamKII activation of SRF had been reported in neuronal cells, and was also sufficient for cFOS induction. In neurons, this pathway is activated by depolarization mediated-calcium influx, while growth factor induction of cFOS in the same cells requires the MAPK pathway. In the gonadotrope cells, GnRH causes calcium influx that is necessary for cFOS induction (Mulvaney et al., 1999; Liu et al., 2002a). Thus, GnRH induction of cFOS is similar to cell depolarization rather than to hormone/ growth factor signaling. GnRH activation of the MAPK pathway augments this activation via phosphorylation of ELK-1 that interacts with SRF. PACAP activation of Gαs, a trimeric G protein that leads to activation of adenylate cyclase and cAMP formation, activates EPAC that links Gαs-adenylate cyclase-cAMP pathway to MAPK signaling via small G proteins, in particular Rac and CDC42. MAPK, ERK1/2 and p38, activate cFOS and FSHβ transcription via ELK-1 phosphorylation.

5. Conclusion

Both GnRH and PACAP induce FSHβ following 4-h treatment and both repress FSHβ after 24-h treatment of female mouse primary pituitary cells. FSH is secreted from these cells in response to either GnRH or PACAP. The repression after 24 h may occur due to the induction of follistatin or stem from the downregulation of receptors and second messenger depletion. In this manuscript, we further analyzed that mechanism of induction. Signaling pathways activated by PACAP and GnRH converge on the expression of cFOS transcription factor that is necessary for FSHβ induction (Fig. 10). While GnRH activates Gαq and causes an increase of intracellular calcium that activates CamKII and MAPK, PACAP activates Gαs that increases cAMP, which is turn activates MAPK via EPAC and small G proteins.

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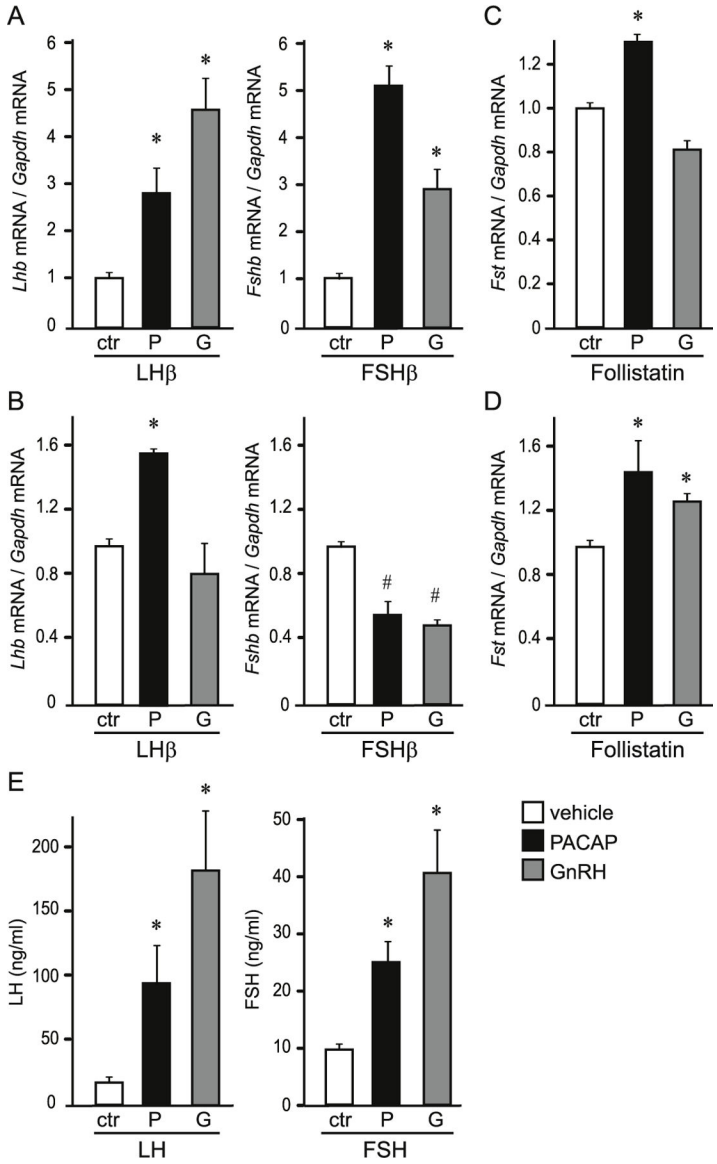

Fig. 1. PACAP induces LHβ and FSHβ in mouse pituitary cells.

Primary pituitary cells from 16-week old female mice were dispersed by enzymatic dissociation and treated with GnRH (G) or PACAP (P) for 4 h in serum free medium (A and C) or for 24 h in serum free medium (B and D). Total mRNA was reverse transcribed and qPCR performed to assess levels of *Lhb* (LHβ) and *Fshb* (FSHβ) mRNAs (A, B) and *Fst* (Follistatin) mRNA (C, D). E, Concentration of LH and FSH in the medium after 24-h treatment with vehicle (ctr), 100 nM PACAP (P) or 10 nM GnRH (G) was analyzed using Luminex assay. Experiments were performed three times. * indicates significant induction by hormones compared to vehicle treated cells, while # indicates significant repression ($p < 0.05$, ANOVA and Tukey's posthoc test).

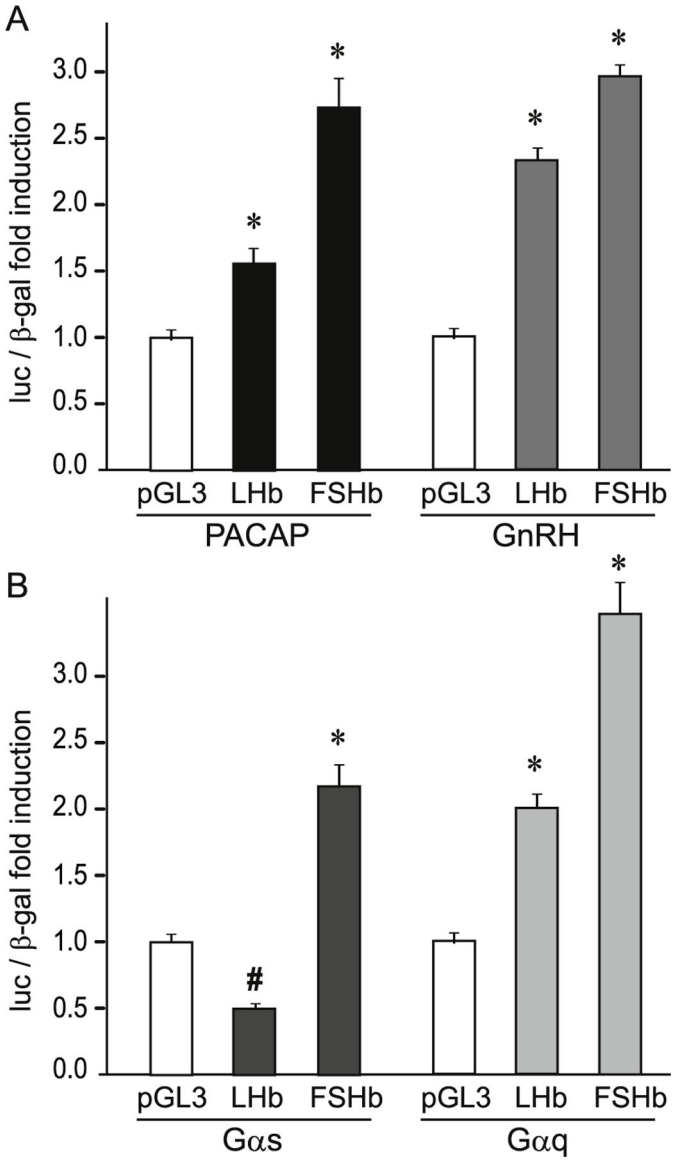

Fig. 2. PACAP induces LHβ and FSHβ in LβT2 cells. A, LβT2 cells.

A, LβT2 cells were transfected with 1.8 kb LHβ-luciferase reporter and 1 kb FSHβ-luciferase reporter and treated with GnRH or PACAP for 4 h. Luciferase values were normalized to β-galactosidase and results presented as fold induction from vehicle treated cells. Significant induction by hormones compare to vehicle treated cells ($p < 0.05$, ANOVA and Tukey's posthoc test) is indicated with *. B, Cells were co-transfected with constitutively active Gαs or Gαq expression vectors and after 48 h, expression of the reporters in cells with Gα was compared to the expression in cells co-transfected with empty vector control. Significant induction of the reporter expression in Gα-expressing cells compared to empty vector transfected control cells is indicated with *, while significant repression is indicated with # ($p < 0.05$, ANOVA and Tukey's test).

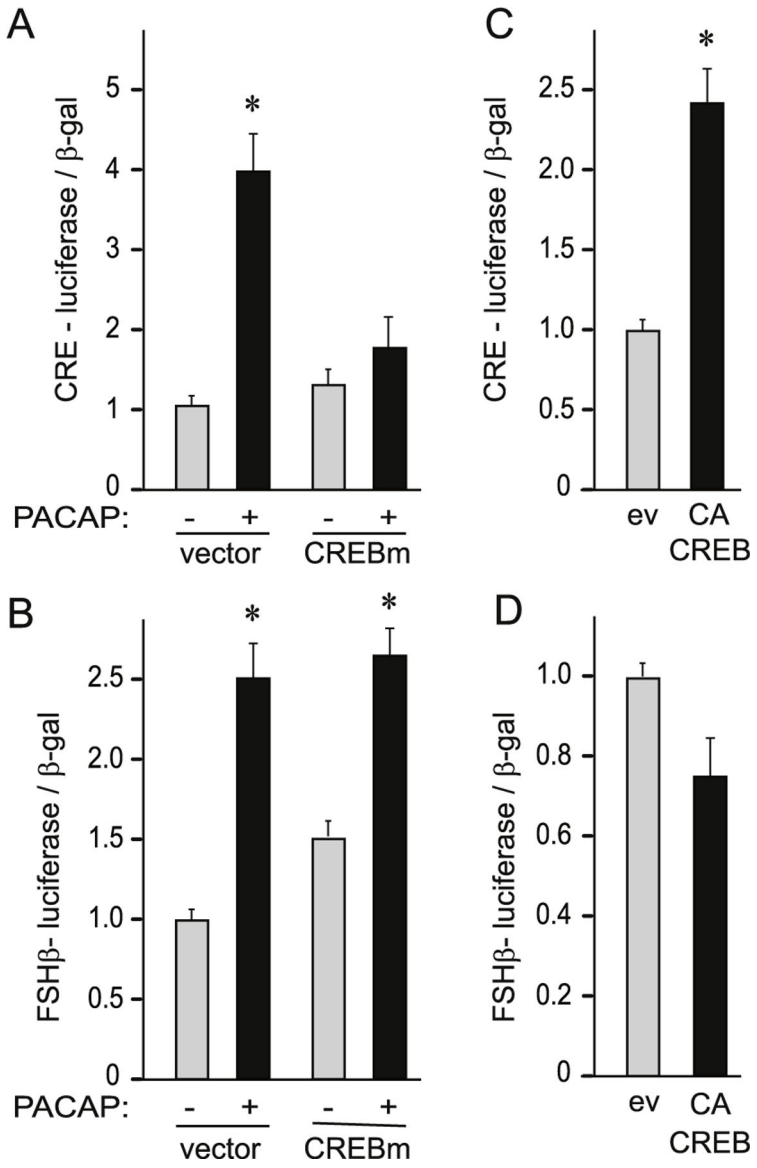

Fig. 3. CREB induces CRE element but not FSHβ reporter.

Reporter containing 4 copies of the cAMP-Response element (CRE), a consensus binding site for CREB (A, C) fused to luciferase gene, or a reporter containing 1 kb FSHβ regulatory region, FSHβ-luciferase (B, D) was co-transfected with the dominant negative CREB mutant (CREBm; A, B) or with the constitutively active (CA) CREB mutant (C, D) in LβT2 cells. Cells were treated with vehicle or PACAP for 4 h to assess a role for CREB in reporter expression. * indicates significant reporter induction with PACAP (A, B) or with CA CREB (C, D).

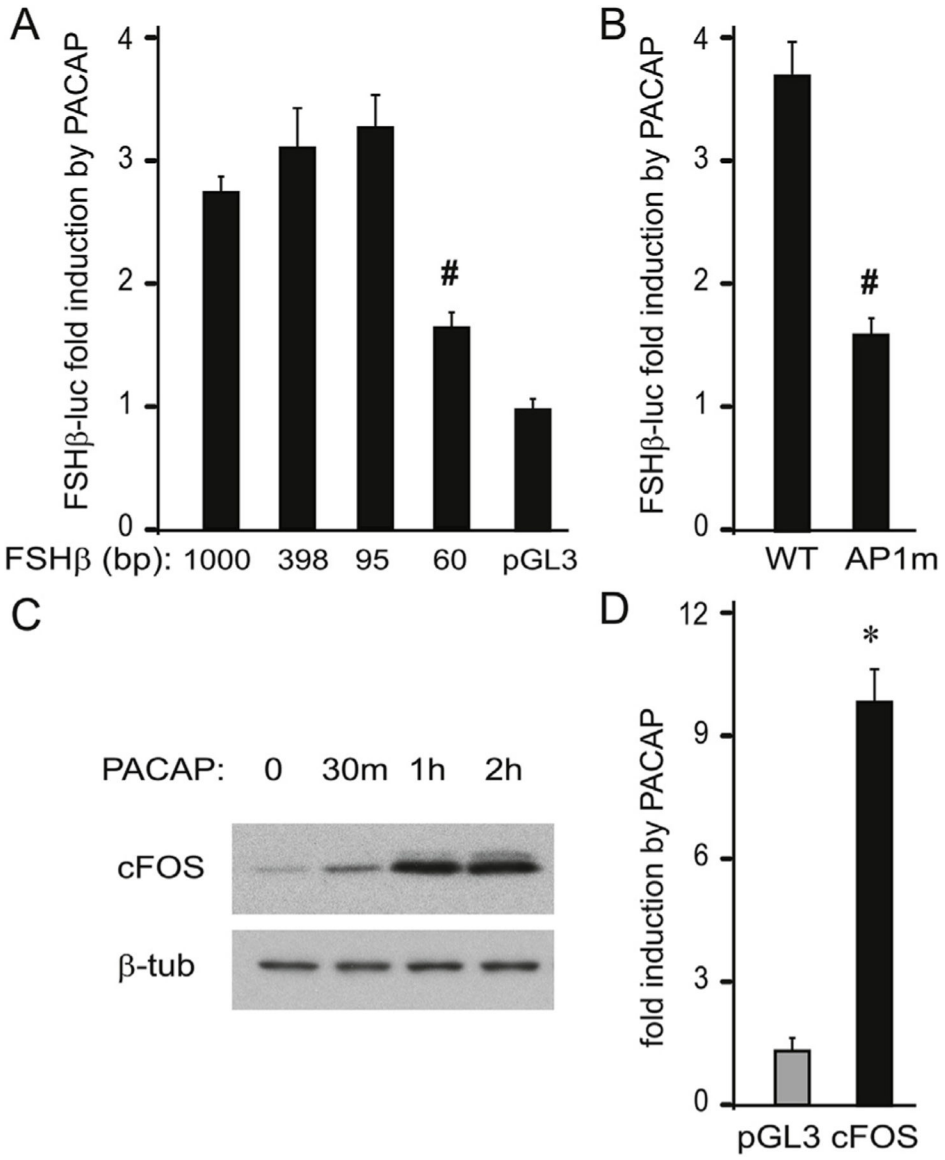

Fig. 4. PACAP induction maps to the AP1 site in the FSHβ promoter.

A, To map the region necessary for PACAP induction, different lengths of the FSHβ reporter were transfected and cell treated with PACAP for 4 h after overnight starvation. The results are represented as fold induction by PACAP from vehicle-treated cells for each truncation. # indicates significantly lower fold induction of the truncation compared to the previous longer truncation. Results represent the mean ± SEM of 3 independent experiments each performed in triplicate. B, Two bp mutation in the −76 AP1 response element was introduced into the reporter and its induction by PACAP compared to the induction of the wild-type reporter. Results are presented as fold induction. Significantly different fold induction in the cells with AP1 mutation versus the cells transfected with wild-type reporter is marked with #. C, Western blots demonstrate induction of cFOS protein following PACAP treatment of LβT2 cells for times indicated above each lane. D, 1 kb cFOS-luciferase

reporter was transfected in LβT2 cell and * indicates significant induction by PACAP treatment compared to vehicle treated cells.

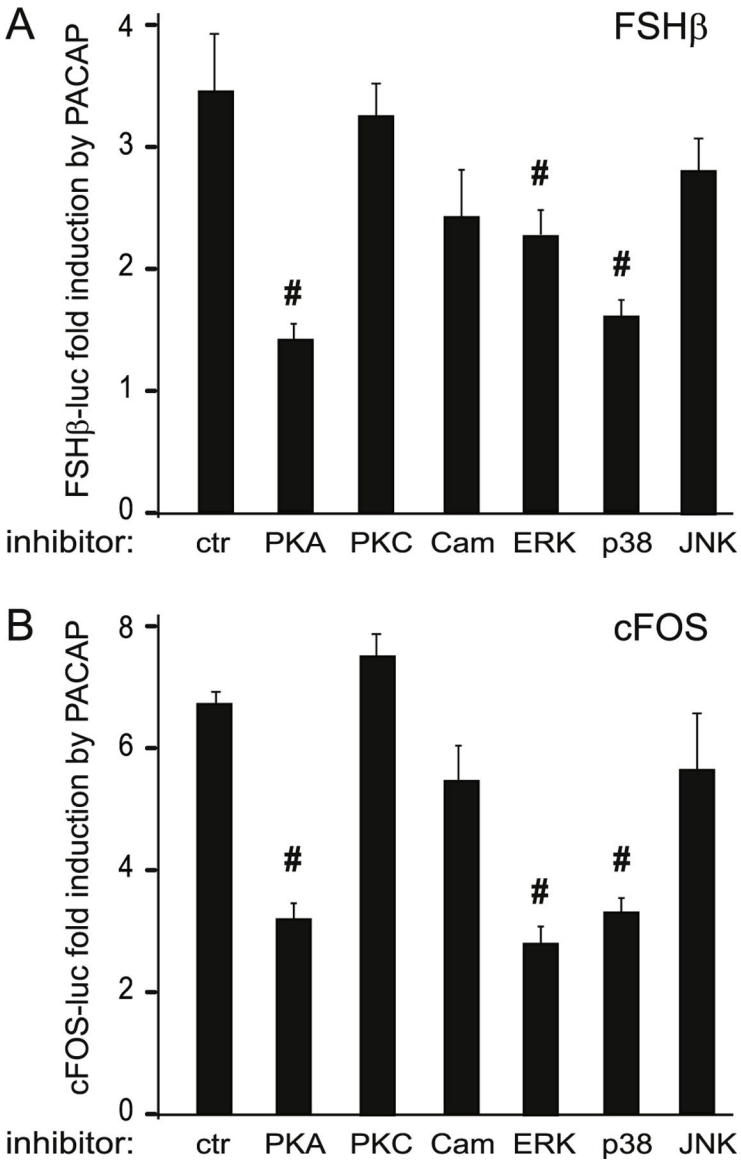

Fig. 5. Inhibition of MAPK diminishes PACAP induction of FSHβ.

LβT2 cells were transfected with 1 kb FSHβ-luciferase (A) or 1 kb cFOS luciferase (B) and were pretreated for 30 min with 10 μM H89 (to inhibit PKA); 1 μM BIM (to inhibit PKC); 10 μM KN-93 (to inhibit CamKII, Cam); 5 μM UO126 (to inhibit MEK1 and activation of ERK1/2), 20 μM SB 202190 (to inhibit p38), or 10 μM SP 600125 (to inhibit JNK). Cells were treated with 100 nM PACAP for 4 h. Luciferase values in each sample were normalized to β-galactosidase values. Experiment was repeated 3 times in triplicate and mean ± standard error is presented. Results were represented as fold induction by PACAP from the vehicle control treated cells with the same inhibitor. # represents significant decrease in fold induction from PACAP treated cells without inhibitor, $p < 0.05$ determined by ANOVA and Tukey's posthoc test.

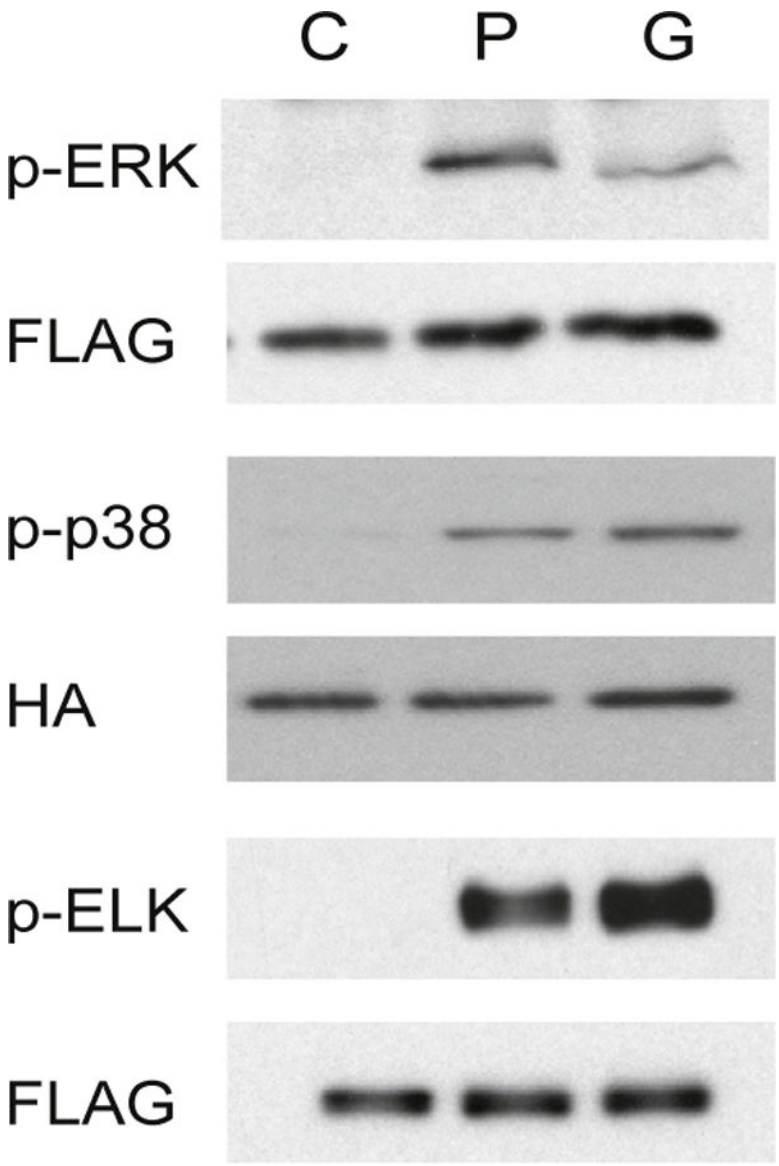

Fig. 6. PACAP activates p38, ERK1/2 and ELK.

LβT2 cells were transfected with Flag-tagged ERK2 or ELK, or HA-tagged p38β. After treatment with GnRH or PACAP, lysates were precipitated with anti-Flag or anti-HA beads and western for phosphorylated forms of ERK2, p38β and ELK performed. Experiment was repeated 3 times and representative images shown.

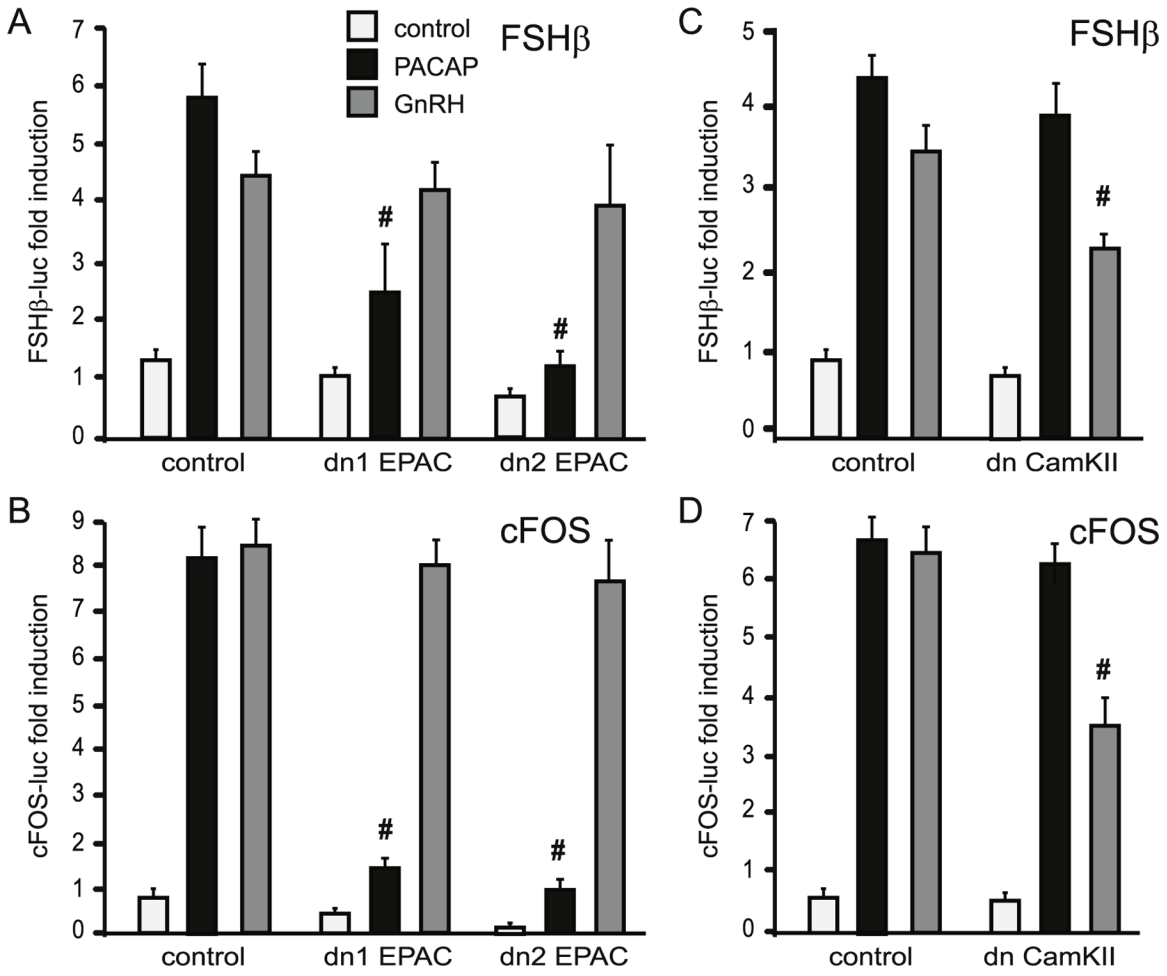

Fig. 7. EPAC plays a role in PACAP induction of FSHβ.

LβT2 cells were co-transfected with FSHβ reporter (A, C) or cFOS reporter (B, D) and empty vector control (control), or dominant negative EPAC mutants (dn EPAC; A, B)) or dominant negative CamKII mutant, used previously (C, D). Cells were treated with vehicle (white bars), PACAP (black bars) or GnRH (grey bars) to assess a role for EPAC or CamKII in reporter induction by PACAP or GnRH. Luciferase levels in each well were normalized to β-galactosidase values. Significantly lower expression in the reporter induction by PACAP or GnRH due to the presence of the dominant negative mutant compared to the empty vector control is indicated with #, determined by ANOVA and Tukey's posthoc test.

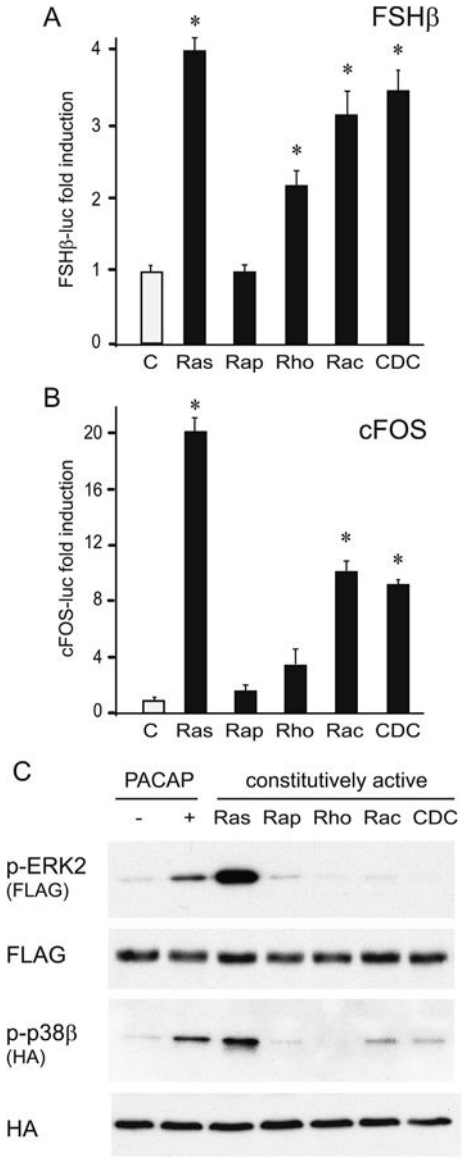

Fig. 8. Several small G proteins induce FSHβ.

LβT2 cells were co-transfected with FSHβ reporter (A) or cFOS reporter (B) and constitutively active mutations of small G proteins. Reporter expression was analyzed 48 h later. Luciferase values in each sample were normalized to β-galactosidase values. Results are presented as mean of three independent experiments performed in triplicate and * indicates significant induction of the reporter by a G protein compared to expression with empty vector control, determined by ANOVA and Tukey's posthoc test. C, Flag-tagged ERK2 or HA-tagged p38β were co-transfected with constitutively active small G proteins. ERK2 or p38β were precipitated with anti-Flag or anti-HA beads respectively. Anti-phospho-ERK1/2 and antiphospho-p38 antibodies were used to assess activation of these proteins. Experiment was repeated three times and representative images shown.

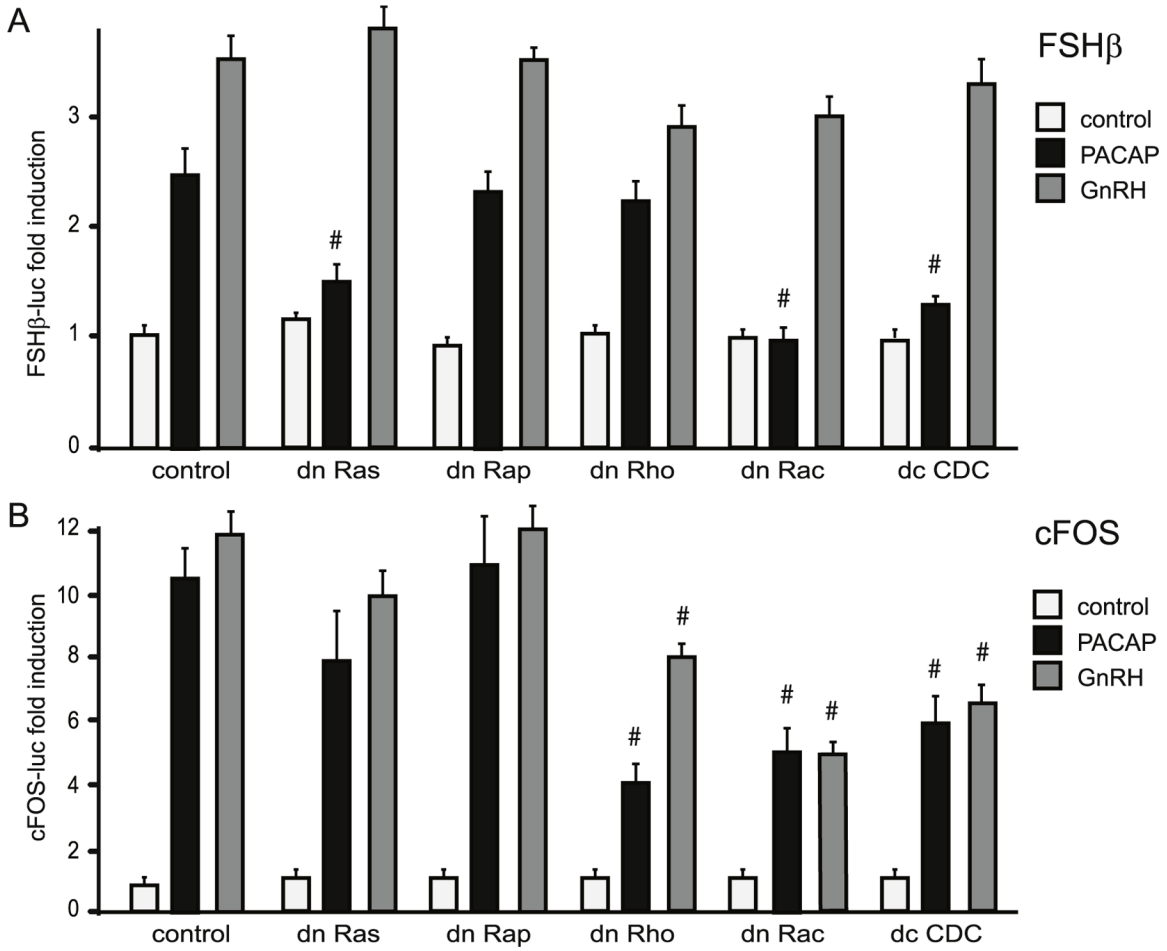

Fig. 9. Ras, Rac and Cdc42 are involved in PACAP induction of FSHβ.

FSHβ reporter (A) or cFOS reporter (B) was co-transfected with empty vector control (control) or dominant negative mutants (dn) of small G proteins. LβT2 cells were then treated with vehicle (white bars), PACAP (black bars) or GnRH (grey bars) to assess small G protein role in reporter induction. Luciferase values in each sample were normalized to β-galactosidase values. Significantly lower expression of the reporter induction by PACAP or GnRH due to the presence of the dominant negative mutant, compared to the empty vector control (control) is indicated with #, determined by ANOVA and Tukey's posthoc test.

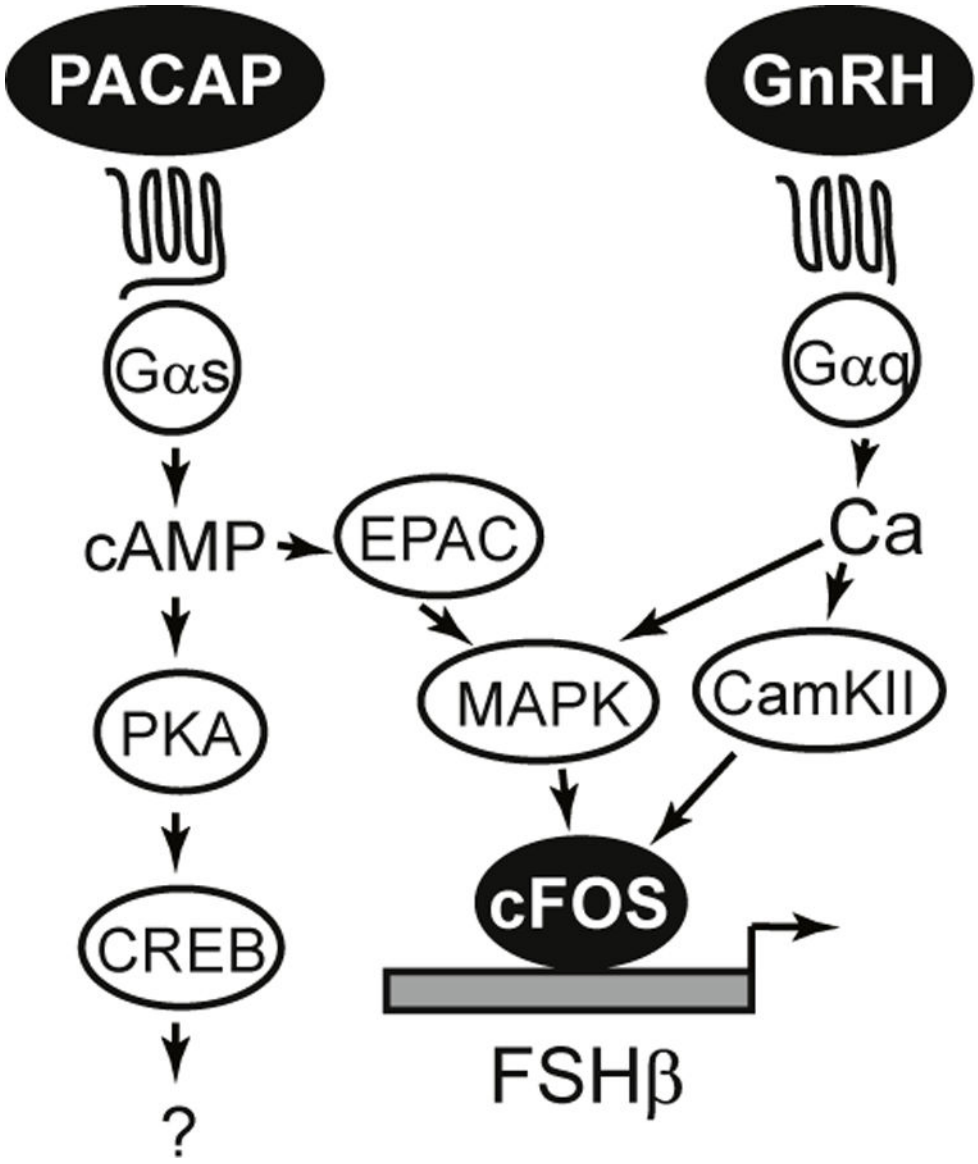

Fig. 10. Signaling pathways involved in FSHβ induction by GnRH and PACAP.

GnRH signals via Gαq that causes an increase in the intracellular calcium. This increase activates CamKII that is sufficient and necessary for FSHβ induction. MAPK is also activated, and contributes to the induction of FSHβ by GnRH maybe by extending the cFOS half-life, as we have shown before, or by potentiating induction by GnRH via ELK1 phosphorylation. PACAP activates Gαs which leads to an increase in cAMP concentration that activates EPAC. EPAC, in turn, leads to activation of several small G proteins that activate MAPK pathways, in particular ERK1/2 and p38 that via cFOS induction induce FSHβ.