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Signaling Pathways and Promoter Regions that Mediate Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) Self-Regulation in Gonadotrophs

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is thought to play a role in the development and regulation of gonadotrophs. PACAP levels are very high in the rodent fetal pituitary, and decline substantially and rapidly at birth, followed by a significant rise in FSHβ and GnRH-R expression. Because there is evidence that PACAP stimulates its own transcription, we propose that this self-regulation is interrupted around the time of birth. To begin to examine the mechanisms for PACAP self-regulation, we used two well-established gonadotroph cell lines, αT3–1 cells and the more mature LβT2 cells which were transfected with a PACAP promoter-reporter construct As *in vivo*, the basal PACAP transcription level is significantly lower in the more mature LβT2 cells in which basal cAMP signaling is also much reduced. The PACAP promoter was stimulated by PACAP in both cell lines. Treatment with inhibitors of second messenger pathways implicated PKA, PKC and MAPK in PACAP transcription. Three regions of the PACAP promoter were found to confer inhibition or stimulation of PACAP transcription. By inhibiting cAMP response element binding (CREB) activity and mutating a proximal CREB binding site, we found that CREB is essential for promoter activation. Finally, overexpression of PACAP receptor HOP1 isoform, to increase the level in LβT2 cells to that of αT3–1 cells and simulate the E19 pituitary, increased PACAP- stimulated sensitivity and significantly altered downstream gene transcription. These results provide novel insight into the feed-forward regulation of PACAP expression that may help initiate gonadotroph function at birth.

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Credit Author Statement

Dr. Rongquiang Yang performed the majority of the experiments as part of his Ph.D. dissertation. Dr. Yang wrote the initial draft of the manuscript.

Drs. Moore and Winters were responsible for the design and oversight of the experiments and for final editing of the manuscript. Dr. Moore also performed some of the experiments presented in this manuscript.

Keywords

Pituitary adenylate cyclase-activating polypeptide; receptor; neuropeptide; gonadotrophs; promoter; pathway

1. Introduction

Gonadotropin synthesis is regulated by signals originating from the hypothalamus and gonads as well as the pituitary. Pituitary adenylate cyclase activating polypeptide (PACAP) is both a hypophysiotropic and a paracrine regulator of gonadotrophs. PACAP binds to G protein-coupled receptors, and activates calcium mobilization and stimulates the cAMP/protein kinase A (PKA) second messenger system in gonadotrophs (1–3). PACAP has been demonstrated to modify gonadotroph responsiveness to GnRH (3–8) and to regulate each of the gonadotropin subunit genes directly (9–12). The effect of PACAP to inhibit the synthesis of FSHβ and decrease GnRH-R is partly by stimulating follistatin transcription in gonadotrophs and folliculostellate cells (4,8,13) which blocks up-regulation by activin (14,15). PACAP and its receptors are expressed within gonadotrophs in the rat pituitary and by immortalized gonadotroph cell lines. Therefore, self-regulation of pituitary PACAP production may play an important role in gonadotroph function.

At the time of birth there is a dramatic rise in GnRH-R and FSHβ gene expression (15–19). We reported previously that PACAP and follistatin expression levels are high in the embryonic pituitary, and decline significantly and in parallel at birth (15). From those results, we hypothesize that an interruption in PACAP self-regulation from the embryonic to newborn anterior pituitary partly initiates the transition from fetal to newborn gonadotroph function by its effect on follistatin production.

Little is known however, about the mechanisms that regulate pituitary PACAP gene expression. Both GnRH and androgens have been shown to increase PACAP expression in gonadotroph cell lines (20,21). In addition, treatment of adult rats with PACAP increases pituitary PACAP mRNA levels (22) and PACAP mRNA levels in primary pituitary cell cultures decline following treatment with the PACAP6–38 antagonist (23). Furthermore, PACAP mRNA levels are increased in neuroblastoma cells (24) and in PC12 pheochromocytoma cells (25) by forskolin and by exogenous PACAP. These findings suggest a potential cAMP dependent, feed-forward auto-regulatory mechanism for PACAP expression.

The immortalized gonadotroph cell lines, αT3–1 and LβT2, have been utilized extensively to investigate the influence of PACAP on the regulation of each of the gonadotropins and the GnRH receptor (4,9,10,26,27). The αT3–1 cell line represents developmentally immature gonadotrophs as it expresses the common gonadotropin α-subunit (*Cga*) and GnRH receptor (GnRH-R) but does not express LHβ or FSHβ subunits (28). The more mature LβT2 gonadotroph cells express *cga* and GnRH-R and also express high levels of LHβ as well as low, but stimulus- responsive levels of FSHβ (29,30). Both cell lines express the PACAP specific receptor, PAC_1R, at variable levels. In addition, PACAP mRNA is readily detectable in untreated αT3–1 cells while detection in LβT2 cells requires exogenous stimulation

(7,21,22). Therefore, the combined use of αT3–1 and LβT2 cells represents an excellent model to evaluate differential expression levels of PACAP in gonadotrophs and mimics its developmental pattern within the perinatal pituitary (15).

In this series of experiments, we used αT3–1 and LβT2 cells to investigate the mechanisms by which PACAP self-regulation occurs as an initial step toward understanding the sequence of events that leads to the dramatic and rapid perinatal decline in pituitary PACAP expression. Mouse PACAP promoter-reporter constructs (1.2 kb) were transfected into αT3–1 and LβT2 gonadotroph cells, as models of immature and mature gonadotrophs, respectively (28). We measured basal and stimulated promoter activity in the two cell lines in the absence or presence of PACAP, and demonstrate the involvement of the PKA, PKC and MEKI signaling pathways in the activity of the PACAP promoter. Furthermore, we identified potential promoter regions, which mediate the regulation of PACAP. We also utilized gene-array analysis to determine which signaling pathways are modulated in gonadotrophs following PACAP exposure.

2. Materials and Methods

2.1. Animals

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals according to a protocol approved by the Animal Care and Use Committee of the University of Louisville. Timed-pregnant Sprague-Dawley rats were delivered from the supplier (Charles Rivers Laboratories) and housed for one week with free access to rat chow and water. Pregnant females were sacrificed on gestational day 19, and the pituitaries from the fetuses were collected using a stereoscopic microscope. Pituitaries were also collected from male rat pups sacrificed on postnatal days 10 and 30.

2.2. Cell Culture

αT3–1 and LβT2 immortalized mouse pituitary gonadotroph cells were kindly provided by Dr. Pamela Mellon (University of California, San Diego, CA). αT3–1 cells were grown in Eagle's Minimal Essential Medium (MEM) containing glucose (4.5 g/L), NaHCO3 (2.2 g/L) and HEPES (5.96 g/L), with penicillin, streptomycin, and fluconazole, and supplemented with 10% fetal bovine serum (FBS). LβT2 cells were grown in Dulbecco's MEM (DMEM) containing HEPES (22.5 mM), $NaHCO_3$ (40 mM), 10% charcoal-stripped FBS, penicillin, streptomycin, and fluconazole.

2.3. Mouse PACpro-luc reporter constructs

A PCR generated cDNA fragment, including the promoter (−1218 − + 36) region of mouse PACAP, was cloned into the pSTBlue-1 vector by TA cloning. The cDNA was sequenced, and found to be identical to published sequences. The cDNA was excised with restriction enzymes, and cloned into the pGL3-Basic vector (Promega, Madison, WI). Truncated promoter sequences were produced utilizing the restriction enzyme *Kpn I* paired with one of four other enzymes (−1018 by *Spe I*, −700 by *Bts I*, −541 by *Nde I*, −200 by *Aat II*) followed by re-ligation. A CRE site mutation in the full- length construct was constructed utilizing the restriction enzyme *Aat II*, which disrupts the CRE site sequence.

2.4. Expression vectors

A dominant-negative inhibitor protein expression vector of CREB (31), A-CREB, and an empty vector (filler) plasmid, pRc/CMV500 were obtained from Dr. C. Vinson (Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA). A rat PAC_1R-Short expression vector was obtained from Dr. Laurent Journot (National Center of Scientific Research, Montpellier, France). A human PAC_1R-Hop1 expression vector was obtained from Dr. Eve Lutz (Royal College, Glasgow, UK)

2.5. Transfections

For transfections, αT3–1 and LβT2 cells were plated in 6-well plates at $1–2\times10^6$ cells/well, and used within 1–2 days at 50–80% confluency. Approximately 3 h prior to transfection, the media were replaced with fresh media (MEM+10% dextran-coated-charcoal-stripped FBS). Cells were transfected with Fugene-6 or GeneJammer Transfection Reagent (Stratagene, La Jolla, CA) according to the manufacturers' protocols. For each plasmid, phRL-TK vector (Promega, Madison, WI) was co-transfected into the cells (0.2 mM) to use Renilla luciferase expression to monitor transfection efficiency. After 24 h, cells were washed and incubated with fresh media. After 48 h, cells were treated with test substances for six hours; cells were then lysed and assayed for luciferase activity.

2.6. RNA extraction and reverse transcription

Total RNA was prepared from cultured αT3–1 and LβT2 cells and rat pituitaries using the QIAGEN RNAeasy Kit (Qiagen, Valencia, CA) following the instructions of the manufacturer. 1μg total RNA from each sample was reverse- transcribed using Superscript III reverse transcriptase (Invitrogen, Grand Island, NY) primed with *oligo(dT)* following the instructions from Invitrogen. Semi-quantitative PCR analyses were normalized with parallel PCR amplification of mouse GAPDH mRNA utilizing specific PCR primers (forward, 5′-GGCATTGCTCTCAATGACAA-3′; reverse, 5′-TGTGAGGGAGATGCTCAGTG-3′), 19 cycles by denaturing for 30 s at 94 °C, annealing for 30 s at 58 °C, extension for 45 s at 72 °C.

2.7. Restriction enzyme analysis of the various PAC_1R subtypes

PCR is first performed to amplify PAC_1R mRNA. To specifically identify the PAC_1R isoforms, the PCR band corresponding to the insert form (HIP, HOP1 or HOP2) of the receptor is cut from an agarose gel and the products are purified by extraction and precipitation. These products are then digested with one of three cassette-specific restriction enzymes and subsequently rerun on an agarose gel. The restriction enzymes used are *Ava II* (which cuts HIP, but not HOP forms), *Blp I* (which cuts both HOP forms, but not HIP), and *Pvu II* (which cuts HOPl, but not HOP2 nor HIP).

2.8. Microarray analysis

Microarray analysis was performed at the University of Louisville Microarray Core Facility according to instructions from Affymetrix (Santa Clara, CA). mRNA was converted into double stranded cDNA using a *T7-oligo (dT)* promoter primer sequence. The double-stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription

reactions which were carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for cRNA amplification. The biotinylated cRNA was purified, fragmented, and used in the hybridization cocktail containing control oligonucleotide B2 and four controls bacterial and phage cDNA (BioB, BioC, BioD, cre). The labeled cRNA was hybridized to the Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), using the protocol provided by Affymetrix. The Mouse Genome 430 2.0 Array is comprised of over 45,000 probe sets representing over 21,000 well-substantiated mouse genes. The sequence clusters were created from the UniGene database (Build 107, June 2002) and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute for Genome Research (MGSC, April 2002). Alterations in RNA transcript levels were analyzed using Partek Genomics Suite 6.2 (Partek Inc., St. Louis, MO). Data analysis was performed using Partek Genomics Suite 6.2 (Partek Inc., St. Louis, MO). The Affymetrix probe level signal values were summarized using the RMA algorithm. Significantly up- or down-regulated genes were identified by analysis of variance (ANOVA) with FDR-corrected p-values < 0.05. The genes that showed 2-fold induction or 2-fold suppression were transferred to separate up and down lists, respectively. The gene sets with an FDR corrected p-value of less than 0.05 were identified in these lists, and Ingenuity Pathway Analysis software (Ingenuity Systems Inc., Redwood City, CA) was used to interpret the interactive pathway networks between the selected genes from the microarray data.

2.8. Statistical Analyses

All luciferase assays were performed with triplicate samples, and all experiments were repeated at least three times. Values were expressed as mean ±SEM. Statistical analysis was performed using ANOVA with post hoc Tukey's test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Comparison of PACAP and basal PACAP promoter activity in αT3–1 and LβT2 cells and PAC_1R mRNA expression in αT3–1 and LβT2 cells and pre and postnatal rat pituitaries.

PACAP is biosynthesized in and secreted by αT3–1 cells as well as primary pituitary cells (22). However, PACAP mRNA is nearly undetectable in LβT2 cells (21) (Fig.1A). Similarly, the PACAP promoter construct is substantially more active under basal culture conditions in αT3–1 cells when compared to LβT2 cells (Fig. 1B). Thus, the level of expression in these immature and mature cell lines, respectively, parallels the dramatic decrease in PACAP expression that occurs at or about the time of birth (15). Analysis of PAC_1R mRNA confirmed that both cell lines express the long (single cassette) and short (null cassette) forms of PAC_1R (Fig. 1C). In αT3–1 cells, the predominant form of PAC_1R is the long form, and the level is substantially higher than in LβT2 cells. Both isoforms are expressed at near equal levels in LβT2 cells. Restriction enzyme analysis of the long form in both cell lines revealed a composition that includes a mixture of the HOP1 and HOP2 cassettes and an absence of the HIP cassette (Fig. 1D). The level of PAC_1R mRNA expression in pre- and postnatal rats (1E and F) reveals that HOP1 isoform predominates in the fetal pituitary, and

that HOP1 and short isoforms are present in nearly equal levels postnatally, which is recapitulated in the immature and mature cell lines, respectively.

3.2. Effects of PACAP on mouse PACAP promoter activity in gonadotroph cells

To examine how PACAP affects activation of the mouse PACAP promoter in gonadotrophs, we utilized a luciferase-reporter construct containing the mouse PACAP promoter (−1218 −+36). Figure 2 shows that PACAP promoter activity is stimulated by PACAP in both αT3–1 and LβT2 cells but is more sensitive to stimulation by PACAP in LβT2 cells possibly because of the difference in basal activity (Fig. 2A and B). In addition, PACAP-stimulated PACAP promoter activity in both cell lines is blocked by pretreatment with a PACAP antagonist (PACAP 6–38, 1 μM) while basal PACAP promoter activity is decreased by the PACAP antagonist only in the basally active αT3–1 cells (Fig 2C and D).

3.3. Effects of PACAP on mouse PACAP promoter activity when PAC_1R receptors are overexpressed in gonadotroph cell lines

The PAC_1R specific receptor short and Hop1 forms are expressed in the rat pituitary (32) and in αT3–1 cells (2). To study their role in PACAP feed-forward regulation individually, we overexpressed the PAC_1R-Short or -Hop1 in αT3–1 and LβT2 cells (Fig. 3A). When these cell lines were also transfected with the mPACpro-luc reporter construct, 10 nM PACAP significantly increased PACAP- stimulated promoter activation more strongly in PAC_1R-Short and PAC_1-R-Hop1 transfected cells than in wild-type (WT) αT3–1 (Fig. 3B) or LβT2 cells (Fig. 3C). Compared to WT LβT2 cells, PAC_1R-short overexpression induced a 3-fold increase in PACAP stimulated PACAP promoter activity while PAC_1R-Hop1 overexpression resulted in a 4-fold increase in PACAP activation of the PACAP promoter (Fig. 3C). Based on the level of expression following transfection (Fig. 3A), the Hop1 form appears to be more effective than the short form in increasing PACAP-stimulated PACAP promoter activity in LβT2 cells. In αT3–1 cells, PAC_1R-Hop1 is expressed endogenously at a high level, and therefore only PAC_1R-short overexpression significantly increased PACAP stimulation of the PACAP promoter. Thus, both the short and the Hop1 PAC_1R effectively transduce the PACAP signal to the PACAP promoter.

3.4. Evaluation of second messenger cascades involved in stimulation of the PACAP promoter

PACAP was named because it increases cAMP production, but it also increases PKC activity and Ca^{2+} mobilization (1,33–35). However, little is known about the signaling pathways by which PACAP stimulates the PACAP promoter. To determine the important pathways that regulate PACAP promoter activity, we used the pharmacological inhibitors BIM (PKC inhibitor), H-89 (PKA inhibitor) and PD98059 (MAPK inhibitor) in cultures of αT3–1 and LβT2 cells transfected with the mPACpro-luc reporter construct. Basal promoter activity in αT3–1 cells was decreased when the PKA (50%) or MAPK (30%) pathways (Fig. 4A) were inhibited but not when PKC signaling was blocked with BIM at doses previously shown to inhibit forskolin-stimulated follistatin promoter activity (36). In contrast, basal promoter activity was reduced by H89 but was unaffected by PKC or MAPK inhibitors in LβT2 cells (Fig. 4B). In αT3–1 cells stimulated with PACAP, H-89 and PD98059 markedly decreased PACAP promoter activity while BIM was less effective (Fig. 4C). The MAPK inhibitor was

substantially less effective in blocking PACAP-stimulated PACAP promoter activity in LβT2 cells. These results demonstrate that the PKC, PKA and MAPK pathways are all involved in PACAP induced PACAP promoter activity and suggest that the PKA and MAPK pathways are more critical mediators of PACAP promoter activity in αT3–1 cells.

3.5. Regional analysis of basal and stimulated mouse PACAP promoter activity in αT3–1 and LβT2 cells

By analysis of previously reported gene sequences, we identified a series of putative binding sites for transcription factors on the PACAP promoter that share at least 80% homology to their consensus sequences, and are present in pituitary (Fig. 5) (37,38). Based on these findings, we constructed five mouse PACAP promoter reporter constructs, each with a sequential truncation of approximately 200 bp, and treated αT3–1 and LβT2 cells for 6 hours with 10 nM PACAP or control media.

Fig. 5 (A, B) shows changes in basal promoter activity after sequential promoter truncation. We found two regions that may mediate an inhibitory signal to the PACAP promoter. One element is located between position −700 and −541 and is effective both in αT3–1 and LβT2 cells. The second region, between −1218 and −1018, appears to mediate inhibition only in αT3–1 cells. In figure 5C and D, in which cells were treated with PACAP, the most distal region of the mouse PACAP promoter (−1218 to −1018) was essential for maximum PACAP induced activity in αT3–1 but not in LβT2 cells. The promoter region from −541 to −200 contains elements that mediate the effects of PACAP in both cell lines evidenced by a pronounced loss of promoter activation following deletion of this region (Fig. 5C and D). The middle region of the PACAP promoter (−700 to −541) does not influence gonadotroph responsiveness to PACAP induced promoter activation.

3.6. PACAP promoter activity requires CREB activation.

PKA and MAPK signaling mediate PACAP expression in gonadotrophs, and serial deletions of the PACAP promoter identified the −541 to −200 region to be critical for promoter activity. This region of the mouse PACAP promoter contains sequences with homology to the consensus CRE (−205 and −179) element, a well-described mediator of PKA signaling as well as two putative AP-1 sites (−448 and −275). Furthermore, we showed that PACAP induces CRE activities utilizing a luciferase reporter construct with a CRE element in both cell lines (23). To evaluate the role of CREB signaling, αT3–1 and LβT2 cells were transfected with the mPACpro-luc construct along with increasing concentrations of an expression plasmid encoding a dominant negative inhibitor of CREB (A-CREB) and a filler plasmid (31). A-CREB decreased both basal (Fig. 6A and B) and PACAP-induced (Fig. 6C and D) promoter activity dose-dependently to a similar extent in αT3–1 and LβT2 cells.

We also constructed a reporter construct containing a mutation of the −205 CRE sequence in the −1218/+36 mPACpro-luc construct. In both cell lines, −205 CRE site mutation significantly attenuated PACAP- induced promoter activity compared to the WT reporter construct (Fig. 7A and B). Furthermore, neither overexpression of the PAC_1R-Short nor Hop1 PACAP receptor isoforms in LβT2 cells rescued the loss of stimulation caused by the

CRE mutation although there were marginally significant increases compared to LβT2 cells with native PACAP receptor expression (Fig. 7C).

3.7. Gene-array analysis of canonical signaling cascades regulated by PACAP signaling in LβT2 cells

To evaluate novel genes that could be either up- or down-regulated by PACAP signaling, we performed gene chip microarray analysis comparing untreated or 10 nM PACAP- treated LβT2 cells and PACAP- treated LβT2 cells with PAC_1R-Hop1 overexpression, the major form in αT3–1 cells, and in the E19 mouse pituitary. As depicted in Table 1, PACAP treatment for 6 h of LβT2 cells transfected with control vector resulted in significant ($p<0.01$, change > 20%) alterations in 223 genes (106 increased, 117 decreased). PACAP treatment of HOP1 transfected LβT2 cells resulted in significant alterations of 393 genes (171 increased, 222 decreased), of which, 113 (44 increased, 69 decreased) were common to PACAP- treated control cells. Ingenuity Systems Pathway Analysis revealed significant changes in gene expression of putative molecules related to specific canonical pathways (Table 1). PACAP treatment of control- and Hop1-transfected LβT2 cells resulted in changes in combinations of molecules that indicate an overall increase in PKA, PI3K/AKT, ERK/MAPK and NGF signaling. PACAP treatment resulted in significant changes in specific molecules known to regulate gonadotroph function such as increases in the PKA (PRKACB, CREM) and PI3K/AKT (cRAF, Bcl-XL, p21cip1, eNOS) signaling pathways while decreasing molecules in the ERK/MAPK (Myc, PIK3R1, TLN2) and BMP (BMPR2, Runx2) pathways. Overexpression of HOP1 PAC_1R lead to additional changes in gonadotroph-influencing pathways including significant increases in molecules in the JAK/STAT (Bcl-XL, p21cip1, RAF1, CDKN1A), TGF-β (INHα, RAF1, HNF [acvr1 and acvrIIa were decreased]) and Interleukin (BAX1, RAF1, GNB3, CDH1, CXCL 10/11, I-TAC) signaling pathways and significant decreases in molecules in the GnRH (EGR1, GnRHr), insulin and IGF-1 (FOX01, SOCS2), EGF (NRAS, PIK3R1, MAP3K), Wnt/β-catenin (β-catenin, ACVR1, ACVR2A, Frizzled family receptor), gonadal hormone (HSP90, SRY) signaling pathways. Many other molecules related to signaling pathways not previously associated with gonadotroph function were also significantly altered.

4. Discussion

Pituitary PACAP expression is high in the fetus, and dramatically decreases at or around the time of birth, initiating a series of events that result in sexual maturation (15,39) which we have proposed reflects a feed-forward mechanism for the paracrine regulation of PACAP expression. Because few reports have examined the auto-regulation of PACAP expression, we designed experiments to study PACAP-evoked activation of the mouse PACAP promoter in gonadotrophs. We first examined basal PACAP transcription in two gonadotroph cell lines, αT3–1 and LβT2 cells. αT3–1 cells were again found to have a much higher level of basal PACAP mRNA (7,21,22) as well as higher PACAP promoter activity when compared to LβT2 cells, and to also have much higher levels of cAMP signaling (36). LβT2 cells are viewed as more mature because they express LHβ and FSHβ as well as the common a subunit mRNA. Thus, the relative levels of PACAP expression in immature αT3–1 cells and

mature LβT2 cells recapitulate the expression levels found in the fetal and postnatal pituitary (15), and provide a model to study gonadotrophs with varying levels of PACAP expression.

The −1218 to +36 region of the mouse PACAP promoter was cloned into a luciferase-reporter plasmid. The promoter was constitutively active in both gonadotroph cell lines. Higher PACAP expression in αT3–1 cells was recapitulated by higher basal levels of the PACAP promoter construct, and explain why the PACAP antagonist suppresses basal promoter activity only in the αT3–1 cell line. PACAP promoter activity was significantly stimulated by PACAP in both cell lines even though the level of expression of PAC_1R is much lower in LβT2 cells. Furthermore, dose- response curves revealed that LβT2 cells are more sensitive to PACAP treatment than are αT3–1 cells. Decreased responsiveness in αT3–1 cells could potentially be due to the already high basal cAMP levels (36), higher endogenous PACAP levels, or by receptor desensitization due to the high endogenous PACAP levels.

Alternate splicing of PAC_1R has been extensively studied but the impact on various signaling pathways in gonadotrophs is not well understood. The short and HOP 1/2 isoforms have been shown to have equal effects on cAMP and IP_3 production (40) and signaling through each is PKA and PLC dependent and can stimulate ERK signaling through a ras-independent mechanism that relies on influx of extracellular calcium (3,41,42). Both the short and the hop-1 receptor isoforms are expressed in normal gonadotrophs (43). Single cell PCR analysis revealed that an individual gonadotroph can express either or both receptor isoforms with no difference in the characteristic calcium response to PACAP stimulation (43). Overexpression of either the PAC_1R-Short or Hop1 forms substantially increased PACAP- stimulated PACAP promoter activity in LβT2 cells with much smaller increases in αT3–1 cells. These differences may be due to the much higher level of endogenous PAC_1R expression in αT3–1 cells (Fig 1C). The higher level of endogenous PAC_1R-Hop than PAC_1R short in αT3–1 cells may explain why over-expression of PAC_1-R-short more effectively increased PACAP transcription in these cells. The relative level of PAC_1R expression in the gonadotroph cell lines also mimics the observed changes in rat pituitaries between pre- and postnatal development (Fig 1E) with the Hop1 form predominating on E19 and a substantial increase in the short form postnatally. Moreover, the Hop-1 form was more effective than the Short form as an activator of PACAP transcription in LβT2 cells even though the level of receptor expression, both endogenously and with overexpression (Fig. 3A), was similar, implying variable activation of different signaling pathways.

PAC_1R is known to activate several signaling pathways including the PKA, PKC and MAPK pathways (44–46), and while all isoforms stimulate cAMP production, the Hop1 isoform also stimulates PLD (47). BIM, H-89 and PD98059 were used to disrupt the PKC, PKA, and MAPK pathways, respectively, in un-stimulated and PACAP-stimulated αT3–1 and LβT2 cells. The results using H-89 imply that the PKA pathway plays a key role in PACAP promoter activity both in αT3–1 and LβT2 cells. Blocking the MAPK pathway also markedly inhibited basal and PACAP -stimulated PACAP expression in Hop-1 rich αT3–1 cells. On the other hand, MAPK signaling appears to play a lesser role in PACAP self-regulation in the more mature LβT2 cell line in which MAPK was demonstrated to have a regulatory role in GnRH-stimulated PACAP expression (21).

In a regional analysis of the mouse PACAP promoter, we found three potential regions which could play a role in gonadotroph PACAP expression. The region between −1218 and −1018 appears to inhibit basal activity in αT3–1 cells whereas −700 to −541 is inhibitory for basal activity in both cell lines. Sequence analysis of −1218 and −1018 revealed a putative PR/GR binding site. The region from −700 to −541 may mediate an inhibitory signal for basal PACAP regulation, especially in LβT2 cells, but is without effect when either cell line is stimulated by PACAP. In this region, there is a consensus binding site for SMAD3, a transcription factor which, after its phosphorylated form complexes with the common SMAD4, mediates effects of multiple regulatory proteins in the pituitary including activin, bone morphogenic proteins BMP 6 and 7, growth differentiation factor 9 (GDF9) and GnRH (48,49). SMAD3 was previously shown to be essential for FSH synthesis and FSHβ transcription (50,51).

Region −541 to −200 of the mouse PACAP promoter was determined to be critical for promoter activity in both cell lines. A similar region was previously found to be essential for GnRH stimulation of the rat PACAP promoter in LβT2 cells (21). Sequence analysis revealed a putative CRE binding site located at −205. Its binding protein, the CREB-family, is dependent on the PKA pathway (52). Over-expression of a CREB dominant-negative expression vector in αT3–1 and LβT2 cells revealed that CRE activation is essential for basal and stimulated PACAP promoter activity. The same result was obtained with a CRE-site mutant reporter construct. Furthermore, overexpression of PAC_1R did not reverse these results. Therefore, stimulation of PACAP promoter requires participation of the CRE-family. These results imply that PKA signaling induces CRE-family transcript factor binding to the PACAP promoter which may be the most important regulatory pathway for PACAP expression. PACAP has also been shown to stimulate the MAPK pathway leading to cFOS induction (12). Both the mouse and rat PACAP promoters contain AP1 binding sequences at −448 and −274, and mutation of these sequences significantly decreases GnRH-stimulation of the rat promoter (21). However, mutation of the −205 CRE alone is sufficient to completely block PACAP- stimulated PACAP transcription in αT3–1 cells (Fig. 7). These results suggest that interruption of cAMP signaling may be the mechanism for the developmental decline in pituitary PACAP expression in vivo (15) and for the lower level of expression in mature LβT-2 than in immature αT3–1 immortalized gonadotroph cells.

Despite nearly undetectable levels of PACAP mRNA with LβT2 cells, basal activity of the PACAP reporter construct was observable and suppressed by pharmacological inhibitors and dominant-negative A-CREB expression. However basal expression was not suppressed by removal of the −205 CRE sequence that is essential for PACAP and GnRH stimulation (21) of the PACAP promoter. This suggests that the proximal PACAP promoter, which includes a CRE at −179, is all that is required for basal PACAP transcription in the αT3–1 and LβT2 cell lines. The endogenous factor which permits basal activity of the PACAP promoter is unknown but is likely not specific to PACAP promoter activity. Similar to this investigation of PACAP promoter activity, disruption of the cAMP-PKA-CREB pathway also has been demonstrated to reduce basal promoter activity of reporter constructs for follistatin, *cga*, and CRE-responsive reporters in LβT2 cells (36,53–55).

Additional analysis revealed some minor differences between the mouse and rat PACAP promoters regarding responsiveness and promoter sequences. Both the rat and mouse PACAP promoters were initially tested in luciferase reporter constructs in αT3–1 cells. The mouse PACAP promoter displayed higher basal and forskolin-stimulated activity when compared to the rat promoter (data not shown). For this reason, and the fact that the αT3–1 and LβT2 cells are immortalized mouse gonadotroph lines, we elected to utilize the mouse PACAP promoter in our further experiments. Analysis of the mouse and rat PACAP promoter sequences revealed an additional AP-1 site at the −948 position of the mouse promoter that is absent in the rat promoter. A potential TATA box, located at position −605 of the mouse promoter is also absent in the rat PACAP promoter as well as a 29 bp sequence between −844 and −815 of the mouse promoter.

Because LβT2 cells were found to be more responsive to PACAP exposure, and overexpression of the PAC_1R-HOP resulted in the highest observed responsiveness, these experimental models were utilized to evaluate downstream targets of PACAP signaling. Global analysis of alterations in gene expression in LβT2 cells in response to PACAP stimulation confirmed known changes in gene expression and revealed changes in molecules not previously associated with PACAP signaling. PACAP receptors are known to stimulate the PKA and PI3K pathways, and molecules associated with these pathways were increased significantly in response to 6h PACAP exposure. Ingenuity Pathway Analysis predicted a net increase in ERK/MAPK signaling (Table 1) in response to PACAP, and MEK1 and MEK2 were both increased by PACAP. Confirmation of the importance of MAPK signaling was demonstrated by a significant decrease in PACAP promoter activity in the presence of a MEK1 inhibitor (Fig 4). The predicted decrease in BMP signaling was strongly influenced by the significant decrease in BMP receptor type II expression. BMPs have been demonstrated to selectively increase synthesis and secretion of FSH while PACAP has the opposite effect (56–59) so that the decreases in BMP receptor expression and signaling may contribute to the selective down regulation of FSH in response to PACAP.

Of particular interest were the changes in gene expression following overexpression of the Hop1 isoform. PAC_1R-HOP is present at a high level in the pituitary of E19 rats and in immature αT3–1 gonadotroph cells that also express high levels of PACAP and cAMP. Increased PACAP signaling through the Hop1 receptor led to increases in molecules related to interleukin signaling including molecules in the JAK/STAT signaling cascade. The interleukins have been shown to inhibit gonadotropin release, and stimulation of various interleukin signaling pathways may have a role in the suppressive effect of PACAP on FSH production (60–64). The predicted decrease in TGFβ signaling was strongly influenced by the observed decreased expression of the activin receptors I and IIa which may contribute to the low level of expression of FSH in fetal gonadotrophs in which PACAP expression is high. The gene array results also suggest that PACAP may influence EGF, IGF-1, insulin and Wnt/β-catenin as each of these pathways was predicted to be reduced following PACAP exposure of cells with PAC_1R-Hop1 overexpression (65–71). Of particular interest were the effects of PACAP on GnRH signaling. PACAP exposure for 6h significantly decreased GnRH receptor expression and signaling, as well as in EGR1 expression in LβT2 cells with PAC_1R-Hop1 overexpression. A decrease in these molecules was also observed in transgenic mice that overexpress PACAP in the pituitary (72). Pituitary PACAP transgenic mice have

lifelong suppression of gonadotropin secretion due in part to increased follistatin and decreased EGR1 and GnRH receptor expression (72). The global gene expression analysis reveals that PACAP may interact with multiple extracellular and intracellular signaling pathways to regulate its level of transcription and thereby gonadotroph function.

5. Conclusions

In summary, we investigated auto-regulation of the PACAP promoter in gonadotrophs. Using several complimentary approaches, we demonstrate that much of the up-regulation of PACAP expression in gonadotroph cells is through the PKA pathway. MAPK signaling is also important in the immature αT3–1 cell line but much less so in mature LβT2 gonadotroph cells. Thus PKA and MAPK signaling contribute to the high level of PACAP expression and cAMP production that typifies immature gonadotrophs. DNA regulatory elements in the proximal region (−541 to −200) of the PACAP promoter are essential for up-regulation of PACAP, and one distal region may be important for down-regulation. Furthermore, we found that the −205 CRE-site is the most important in these regions for PACAP auto-regulation. We proposed previously that an increase in hypothalamic dopamine at birth, with subsequent suppression of cAMP signaling in gonadotrophs, may initiate the sequence of events that results in less PACAP and follistatin in neonates (15,72). This is the first report to identify specific regions of the mouse PACAP promoter that contribute to PACAP auto-regulation in gonadotrophs. The results suggest that experiments utilizing immortalized gonadotroph cell lines of varying stages of maturation have the potential to identify factors that regulate gonadotroph PACAP expression during development.

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Highlights

- PKA and MAPK signaling contribute to PACAP auto-regulation
- A proximal CRE in the PACAP promoter is essential for PACAP auto-regulation
- Gonadotroph cell lines mimic PACAP and PAC_1R expression in the perinatal pituitary
- Suppression of cAMP signaling may initiate the decline in pituitary PACAP in neonates

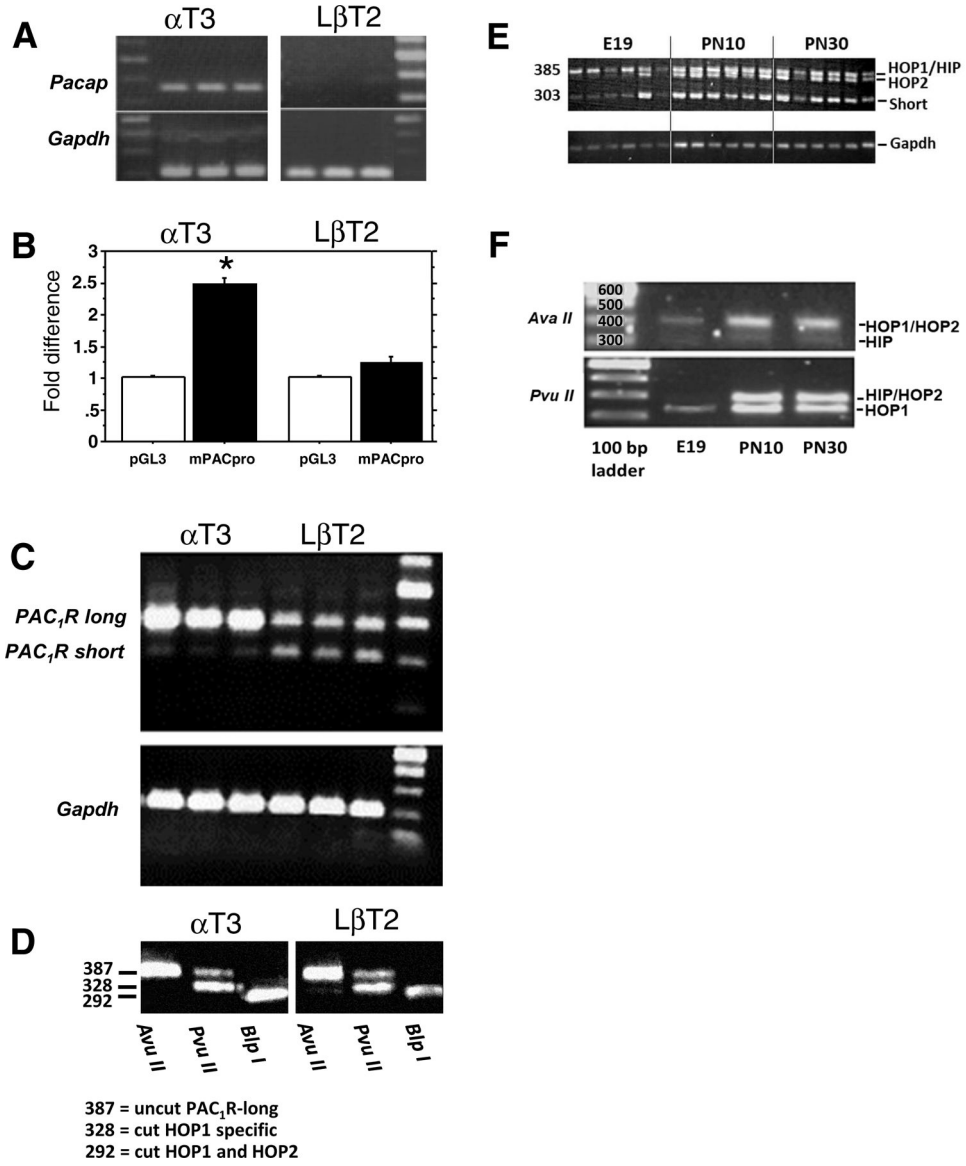


Figure 1.

Comparison of PACAP and basal PACAP promoter activity in αT3–1 and LβT2 cells and PAC_1R mRNA expression in αT3–1 and LβT2 cells and pre and postnatal rat pituitaries. Semi-quantitative PCR analysis (A) of PACAP mRNA expression levels in αT3–1 and LβT2 cells. (B) αT3 and LβT2 cells were transfected with the pGL3 vector alone or the mPACpro-luc construct and cultured for six hours. Results were from three experiments with triplicate wells. Data are expressed as fold difference normalized to pGL3 vector alone.* Significantly different than control vector ($P<0.05$) by ANOVA. (C) Semi-quantitative PCR analysis of PAC_1R and Gapdh mRNA levels in αT3–1 and LβT2 cells. (D) Restriction enzyme analysis of HIP (*Ava II*), HOP 1 (*Pvu II*), and HOP 1/2 (*Blp I*) PAC_1R isoforms. Semi-quantitative PCR (E) and restriction enzyme analysis (F) of PAC_1R and Gapdh mRNA levels in pituitaries from male rats at embryonic day 19 (E19) and postnatal days (PN) 10 and 30. (E)

The PCR products were run through 2.5% agarose to separate the 387 and 384 base pair bands.

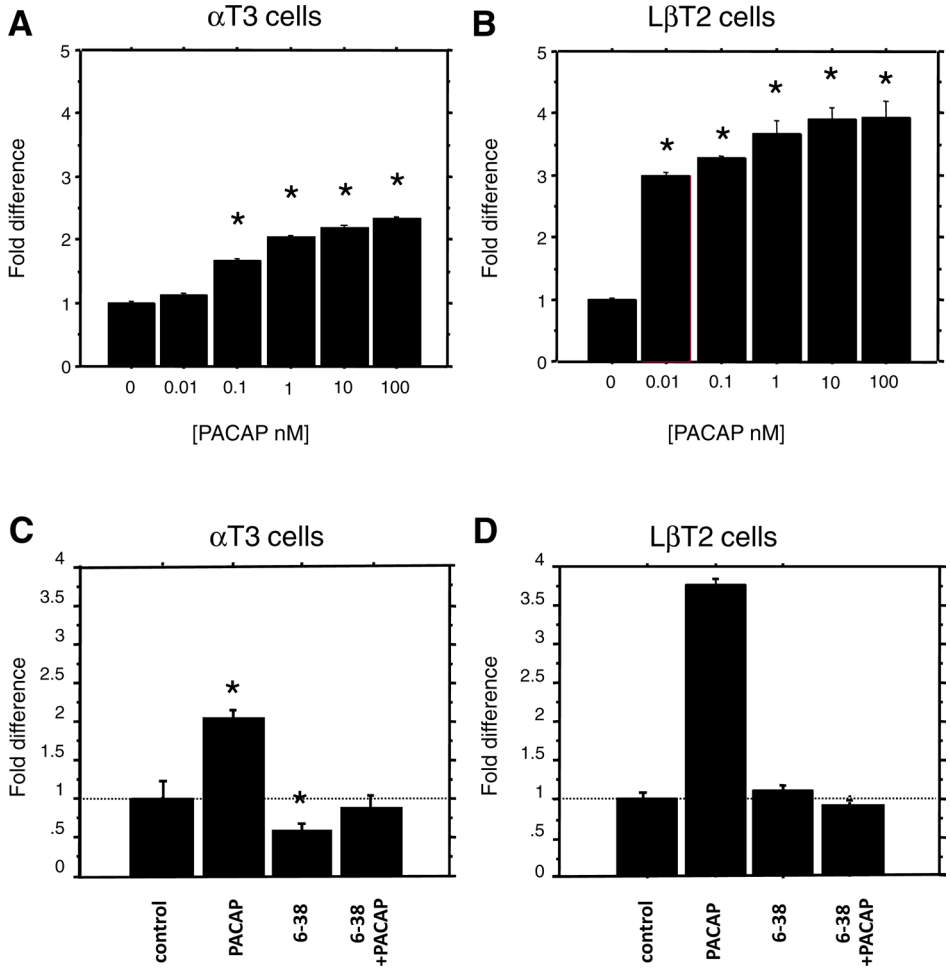


Figure 2.

αT3–1 (A) and LβT2 (B) cells were transfected with the mPACpro-luc construct and treated for six hours with increasing concentrations (0–100 nM) of PACAP. In addition, αT3–1 (C) and LβT2 (D) cells were transfected with the mPACpro-luc construct and treated for six hours with medium alone (control) or media containing 10 nM PACAP, 1 mM PACAP antagonist (6–38), or pretreatment with PACAP (6–38) for 30 min followed by 10 nM PACAP. Data are expressed as fold difference normalized to media alone. * Significantly different than media alone ($P<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

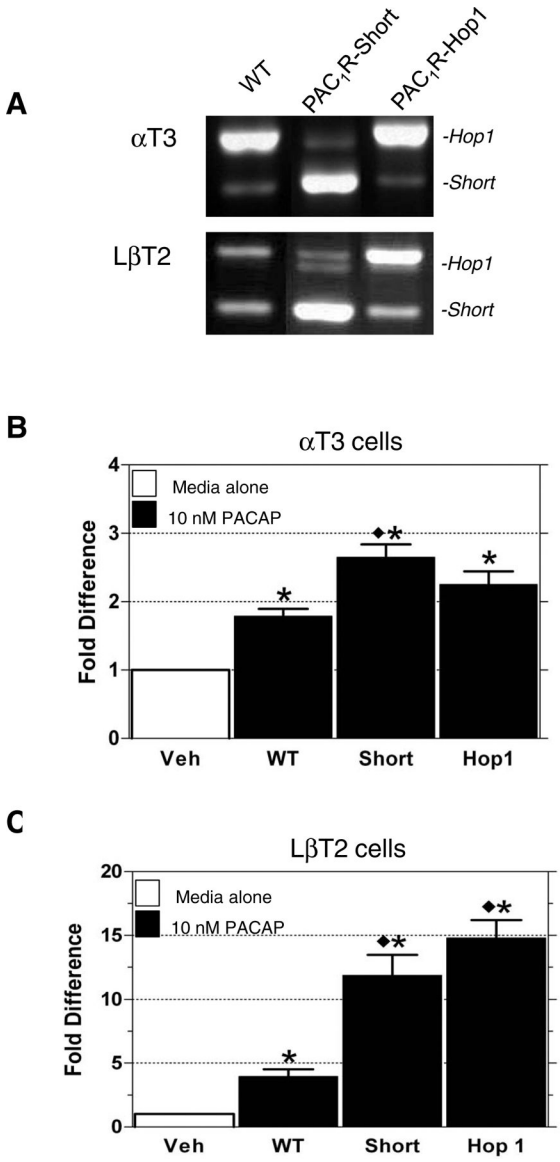


Figure 3.

PAC_1R mRNA isoform expression in wild type αT3–1 and LβT2 cells, and cells transfected with PAC_1R-Short or PAC_1R-Hop1 expression vector (A). αT3–1 cells (B) and LβT2 (C) were transfected with the mPACpro-luc construct (2.5 μg) and PAC_1R expression vector (50 ng) and treated for six hours with 10 nM PACAP. Data are expressed as fold difference normalized to media alone. * Significantly different than media alone ($P<0.05$) by ANOVA. ◆ Significantly different than wild-type cells with 10 nM PACAP ($P<0.05$) by Tukey's test. Results were from three experiments with triplicate wells.

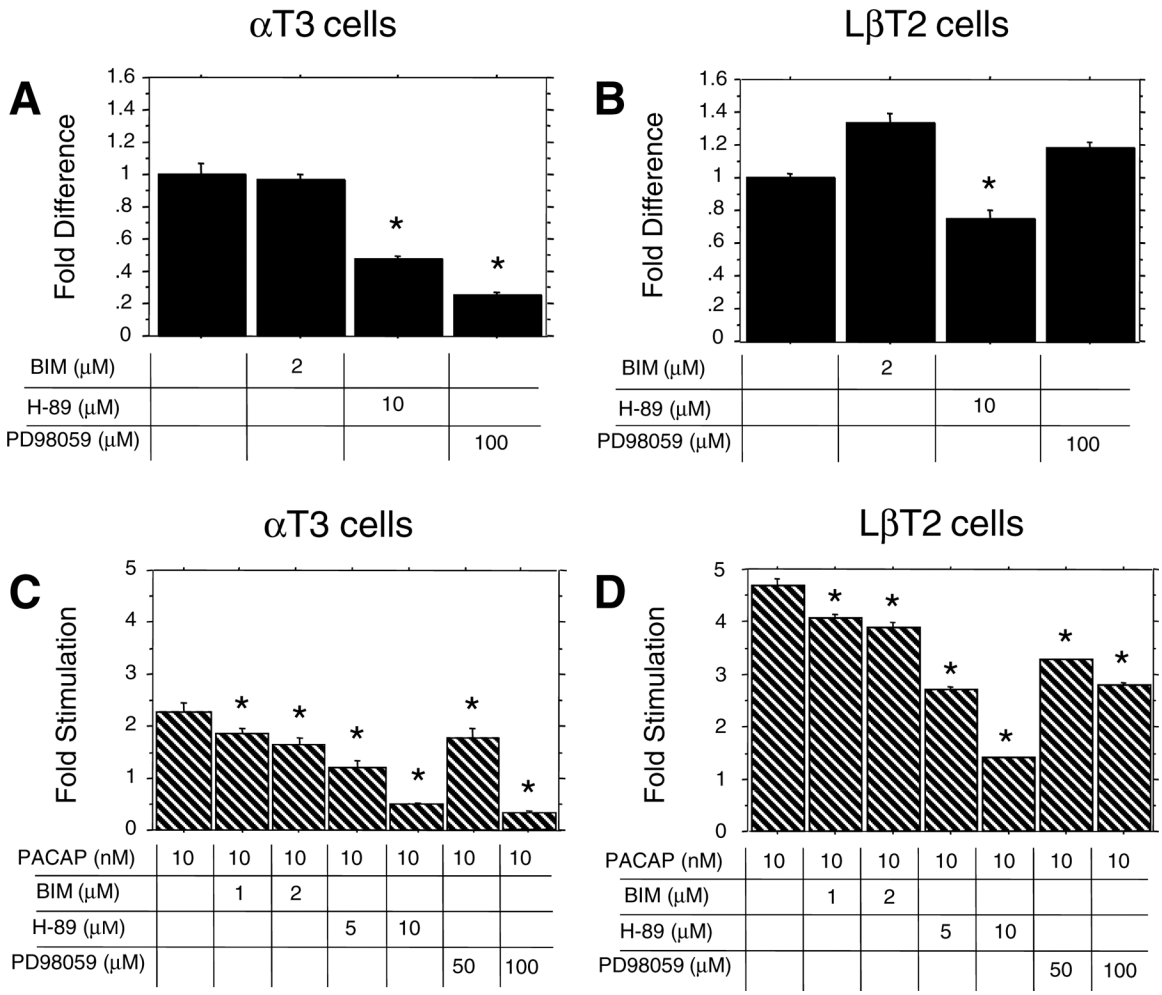


Figure 4.

αT3–1 (A, C) and LβT2 (B, D) cells were transfected with the mPACpro-luc construct and treated for six hours with media alone (A, B) or 10 nM PACAP (C, D) with or without the indicated concentrations of the PKC inhibitor, bisndolylmaleimide (BIM), the PKA inhibitor, H89, or the MEK1 inhibitor, PD98059. Data are expressed as fold difference or stimulation normalized to media alone. * Significantly different than media alone ($p<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

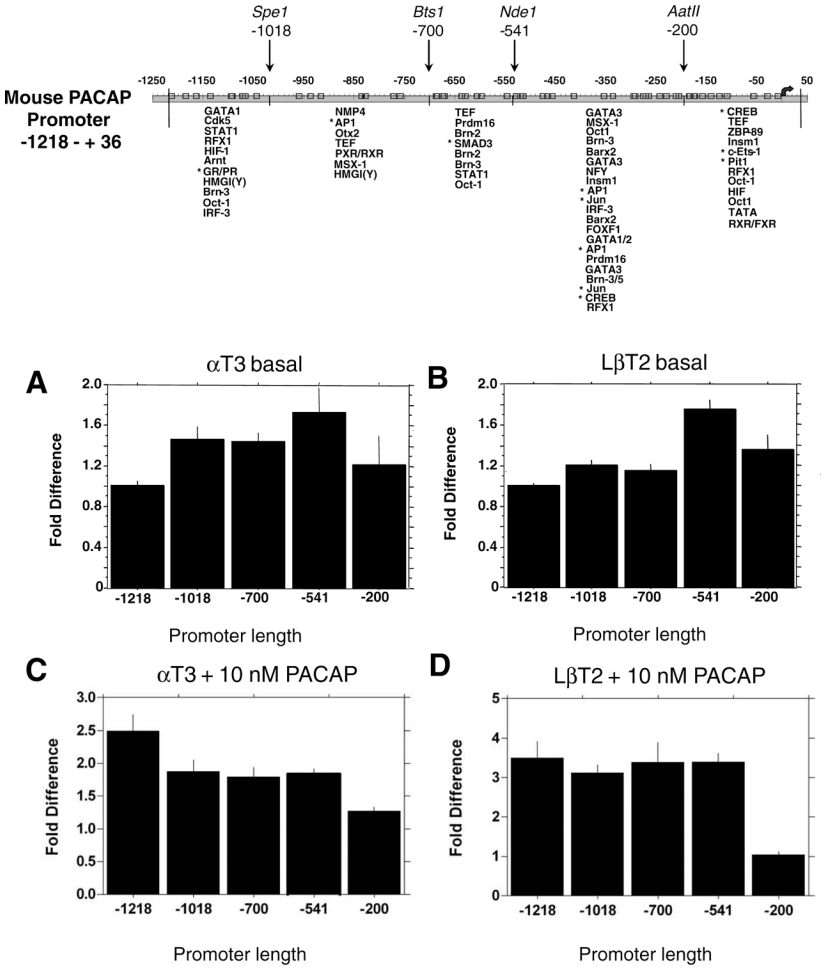


Figure 5.

Above is a schematic diagram depicting the regions of the mouse PACAP promoter altered in this investigation. The transcription factors demarked by asterisks are of particular interest as they have been previously demonstrated to be involved in regulation of one or more of the gonadotropin subunit genes. αT3–1 (A, C) and LβT2 (B, D) cells were transfected with DNA constructs containing various lengths of the mouse PACAP promoter reporter construct (mPACpro-luc) and treated for six hours with media alone (A, B) or media containing 10 nM PACAP (C, D). Data are expressed as fold difference normalized to media alone treatment of mPACpro-luc transfected cells. * Significantly different than −1218 promoter activity. Results are from three experiments with triplicate wells.

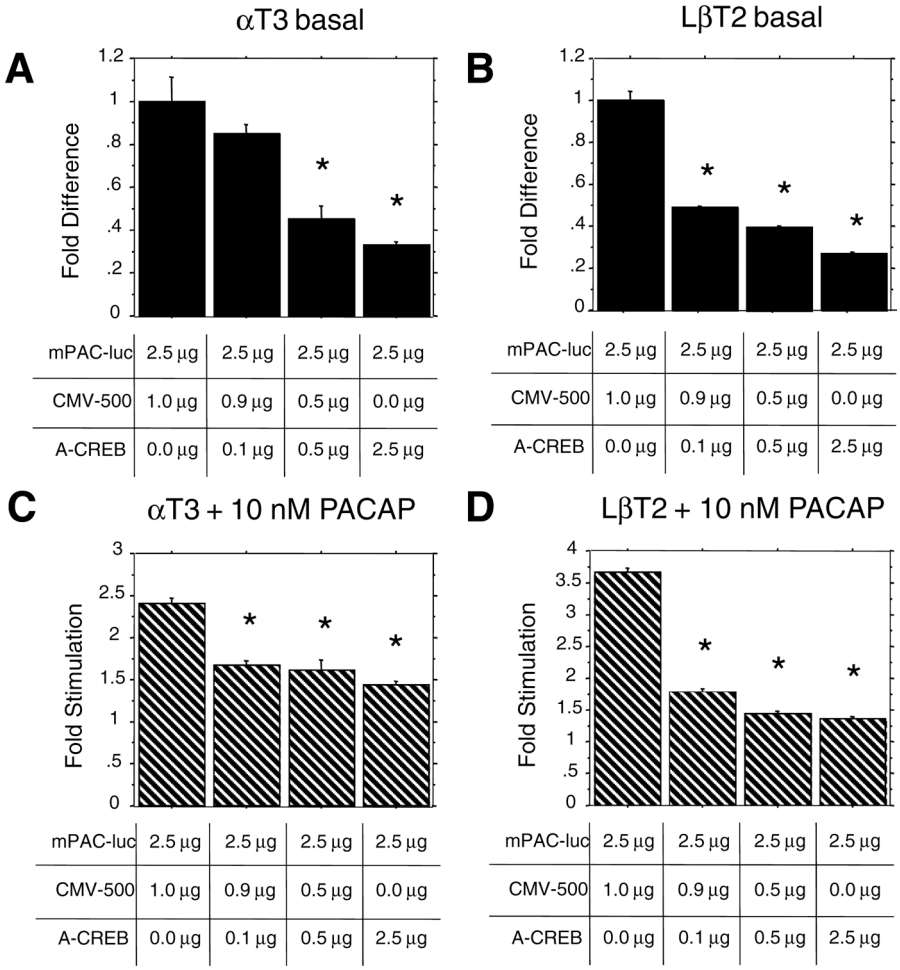


Figure 6.

αT3–1 (A, C) and LβT2 (B, D) cells were transfected with the mPACpro-luc construct and with the indicated concentrations of expression plasmid coding for the dominant negative inhibitor of CREB (A-CREB), and a filler plasmid (CMV-500) to control for total transfected DNA amount. Transfected cells were treated for six hours with media alone (A, B) or 10 nM PACAP (C, D) and the cell lysates were collected for luciferase activity determination. Data are expressed as fold difference or stimulation normalized to media alone. * Significantly different than media exposure alone ($p<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

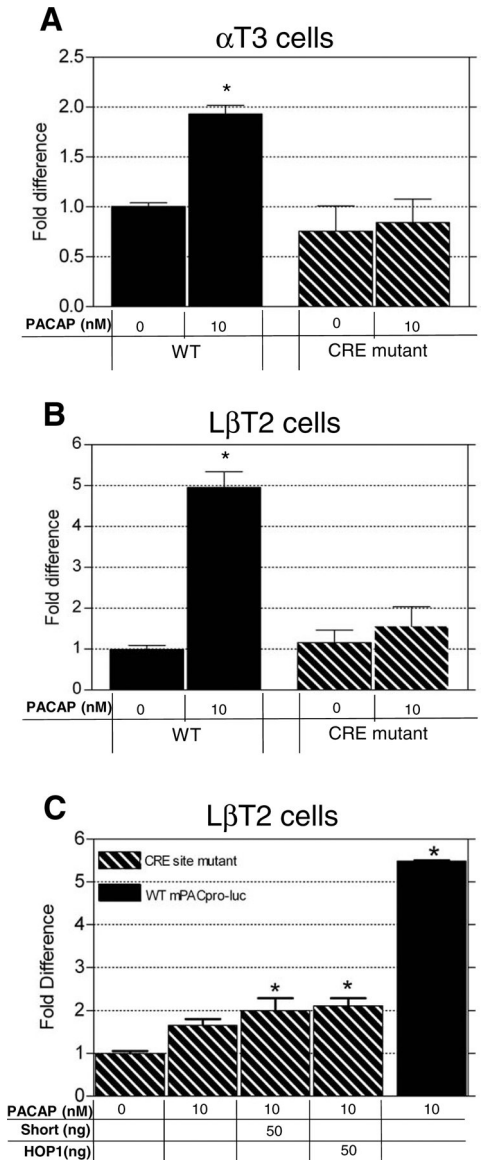


Figure 7.

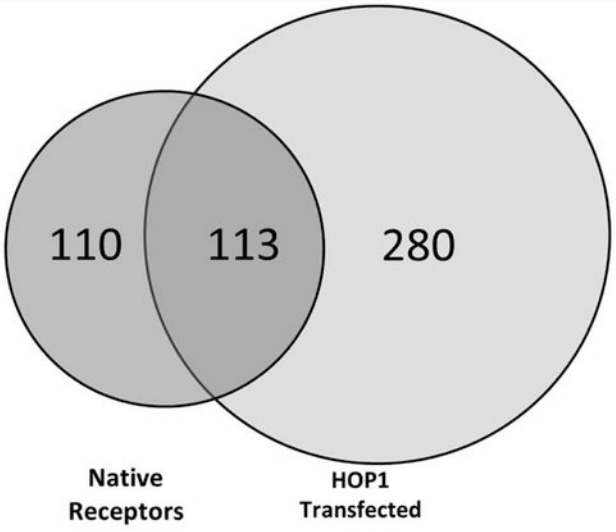
αT3–1 (A) and LβT2 (B, C) cells were transfected with the CRE-mutant mPACpro-luc (A, B) construct and with the PAC_1R expression plasmid. (C) Transfected cells were treated for six hours with media alone and 10 nM PACAP and the cell lysates were collected for luciferase activity determination. Data are expressed as fold difference to media alone. * Significantly different than media alone with WT construct ($P<0.05$) by Tukey's test. Results were from three experiments with triplicate wells.

Table 1.

Canonical pathways in which gene expression is affected significantly by PACAP signaling. ★ = significantly greater in HOP1 transfected cells.

Altered by PACAP in both	Specific to native receptors	Specific to HOP1 transfected
Up Regulated	**Up Regulated**	**Up Regulated**
PKA Signaling ★	Glutamate Metabolism	Ceremide Signaling
PI3K/AKT Signaling ★	**Down Regulated**	Inositol Metabolism
ERK/MAPK Signaling ★	Mismatch Repair in Eukaryotes	Inositol Phosphate Metabolism
NGF Signaling ★	Agrin Interactions at NMJ	IL-2 Signaling
Neurotrophin/TRK Signaling	RAN Signaling	IL-8 Signaling
DHA Signaling	Human Stem Cell Pluripotency	IL-17 Signaling
ALS Signaling	ER Stress Pathway	VEGF Signaling
P53 Signaling		EGF Signaling
Down Regulated		Glucocorticoid Anti-inflamatory
EIF2 Signaling ★		Insulin Receptor Signaling
ATM Signaling ★		IGF-1 Signaling
BMP Signaling ★		Apoptosis Signaling
mTOR Signaling		Cyclins and Cell Cycle Regulation
HGF Signaling		JAK/STAT Signaling
Role of CHKs in Cell Cycle Control		PTEN Signaling
		Fc Epsilon RI Signaling
		Down Regulated
		GnRH Signaling
		Prolactin Signaling
		Androgen Signaling
		TGF-β Signaling
		Estrogen Receptor Signaling
		PDGF Signaling
		G2/M DNA Damage Regulation
		LPS-stimulated MAPK Signaling
		SAPK/JNK Signaling
		Gα 12/13 Signaling
		Melatonin Signaling
		PPARα/RxRα Activation
		AMPK Signaling
		CNTF Signaling
		Wnt/β-catenin
		Sonic Hedgehog Signaling
		PAK Signaling
		Cell Cycle Regulation by BTGs

Distribution of genes significantly altered > 20%



	Altered in Both	Native Only	HOP1 Only
Up-Regulated	44	62	127
Down-Regulated	69	48	153