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Interaction of Gonadal Steroids and Gonadotropin-Releasing Hormone on Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and PACAP Receptor Expression in Cultured Rat Anterior Pituitary Cells

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors are expressed in the hypothalamus, the gonadotrope cells of the anterior pituitary gland, and the gonads, forming an autocrine–paracrine system in these tissues. Within the pituitary, PACAP functions either alone or synergistically with gonadotropin-releasing hormone (GnRH) to stimulate gonadotropin gene expression and secretion. Our goal was to define the hormonal regulation of pituitary PACAP and PACAP receptor (PAC1) gene expression by dihydrotestosterone (DHT), estradiol, and progesterone alone or in conjunction with GnRH. Treatment of adult male rat pituitary cell cultures with DHT or progesterone augmented GnRH-mediated increase in PACAP messenger RNA (mRNA) levels, but neither had an effect when present alone. Conversely, estradiol treatment blunted PACAP gene expression but did not alter GnRH effects on PACAP expression. Expression of PACAP receptor mRNA was decreased by GnRH treatment, minimally increased by DHT treatment, but not altered by the addition of estradiol or progesterone. DHT and GnRH together blunted PACAP receptor gene expression. Taken together, these results suggest that the activity of the intrapituitary PACAP-PAC1 system is regulated via the complex interaction of gonadal steroids and hypothalamic GnRH.

Keywords

anterior pituitary, PACAP, dihydrotestosterone, estradiol, progesterone, GnRH

Introduction

Sexual maturation and reproduction in mammals depend on the normal development and function of the hypothalamic–pituitary–gonadal axis. The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), stimulates the gonadotrope subpopulation of the anterior pituitary gland to synthesize and secrete the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These gonadotropins then regulate the activity of the gonads to produce mature gametes and gonadal steroids, namely, estrogen, progesterone, and androgens. The gonadal steroids modulate gonadotropin gene expression by feedback actions at both the anterior pituitary and the hypothalamus.

Although gonadotropin biosynthesis and secretion are strongly regulated by GnRH,[1] accumulating evidence indicates that the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) also modulates gonadotrope function, acting either alone or in conjunction with GnRH. The PACAP has been demonstrated to increase the α-subunit messenger RNA (mRNA) levels and to increase the length of *LH*β transcript poly(A) tails and LH secretion in vivo in rats.[2-5] In cultured primary rat pituitary cells, PACAP stimulates the transcription of follistatin by the folliculostellate cells, which in turn inhibits activin signaling leading to the repression of *FSH*β gene expression.[2] Pituitary *PACAP* transcript number has been shown to vary across the rat estrus cycle with peak levels occurring at 24 hours on proestrus, supporting the notion that locally derived PACAP may play an important role in modulating gonadotrope function.[6,7]

The PACAP was first isolated from sheep hypothalamic extracts and named for its ability to enhance cyclic adenosine

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monophosphate production by rat anterior pituitary cells.[8] Since its discovery, numerous studies have investigated the structure, distribution, and developmental expression of PACAP and its receptors in a wide array of cell types and body systems, including the reproductive, neural, and gastrointestinal systems.[7,9–11] Although initially isolated as a hypothalamic-releasing factor, PACAP and its receptors have been found more recently at all the levels of the hypothalamic–pituitary–gonadal axis as well as in the endometrium and placenta.[12-14] The PACAP mRNA and protein have been detected in the germ cells of the rat and human testis as well as in the ovarian granulosa, theca-interstitial, and luteal cells.[12,15] As PACAP receptors are also present in the gonads, PACAP appears to play a local role in regulating gametogenesis and steroidogenesis. In the anterior pituitary gland, PACAP expression has been localized to the gonadotropes and folliculostellate cells.[16,17] The importance of PACAP to reproductive function is highlighted by reports that both PACAP and PACAP receptor (PAC1) null female mice have decreased fertility.[18-20]

Three distinct subtypes of PACAP receptors have been cloned. The PACAP receptors are G protein-coupled receptors that activate a number of signaling pathways, most importantly the cyclic AMP/protein kinase A signaling pathway.[9] The PAC1 receptor binds PACAP with high affinity and the closely related peptide vasoactive intestinal peptide (VIP) with a much lower affinity. The vasoactive intestinal polypeptide receptor 1 (VPAC1) and receptor 2 (VPAC2) receptors bind PACAP and VIP with equal affinity. All anterior pituitary cells express at least one of the PACAP receptor subtypes, with PAC1 the most prevalent. Therefore, PACAP both acts on and is secreted by the pituitary cells, forming a functional autocrine–paracrine loop within this tissue.[21,22]

Prior studies have clearly demonstrated that gonadotropin gene expression is modulated by gonadal steroids in addition to the neuropeptides GnRH and PACAP. For example, androgens specifically increase *FSH*β but not α-subunit or *LH*β mRNA levels in both male and female rat pituitary cell cultures.[23] Progesterone and estrogen have been shown to potentiate GnRH-induced increase in *LH*β gene expression.[24,25]

The GnRH and gonadal steroids act via binding to unique receptors at the target cell. The GnRH binds to a specific G-protein-coupled membrane receptor, GnRH receptor (GnRHR), to activate the protein kinase C, protein kinase A, and calcium intracellular signaling pathways and thereby increase the gonadotropin gene expression.[26-28] In contrast, gonadal steroids exert their effects primarily via association with corresponding nuclear receptors, the estrogen receptors (ERα or ERβ), progesterone receptors (PRA or PRB), and androgen receptor (AR).[29,30] These activated nuclear receptors then bind to the promoter elements of their target genes directly or indirectly via tethering through other DNA-bound transcription factors.[31] Although less well understood, estrogen and progesterone can also bind with high affinity to other cell components, including the cell membrane, to produce rapid effects on target cell activity.[32,33]

A limited number of studies have investigated the hormonal regulation of *PACAP* and *PAC1* receptor gene expression. Outside the pituitary, estradiol has been reported to stimulate *PACAP* mRNA levels in the hypothalamic ventromedial and arcuate nuclei, and progesterone has been shown to increase *PACAP* and *PAC1* mRNA levels in the rat hypothalamus and ovary.[34-36] Gonadectomy leads to decreased PACAP levels in both brain and pituitary tissues of male and female Wistar rats.[37] In addition, LH and FSH have been shown to induce *PACAP* transcript number in rat preovulatory follicles and in human granulosa–luteal cells obtained from patients undergoing in vitro fertilization.[38,39]

The regulation of pituitary *PACAP* and *PAC1* expression by GnRH has been studied in the LβT2 gonadotrope cell line. Work in our laboratory demonstrated the ability of GnRH to increase *PACAP* promoter activity and mRNA expression via the PKC, PKA, and MAPK pathways.[26] Purwana and colleagues subsequently confirmed the stimulatory effect of GnRH on *PACAP* transcript levels.[40] This group also reported a GnRH-mediated increase in PACAP receptor mRNA levels, which we had not observed in our prior study. To our knowledge, the effect of GnRH on *PACAP* and *PAC1* expression in primary pituitary cells has not been reported. Furthermore, nothing is known about the effects of gonadal steroids on pituitary *PACAP* expression, despite the fact that these steroids are known to play a pivotal role in feedback regulation of anterior pituitary function.

The overall goal of the studies reported here was to further define the hormonal factors that regulate *PACAP* and *PAC1* receptor expression in the anterior pituitary gland. Specifically, we characterized the effects of dihydrotestosterone (DHT), estradiol, and progesterone alone or in combination with GnRH on *PACAP* and *PAC1* mRNA levels in dispersed, cultured adult male rat anterior pituitary cells. Our findings suggest that, in addition to direct actions on the gonadotropin genes, GnRH and gonadal steroids may regulate gonadotropin expression indirectly via alterations in the local PACAP-PAC1 system.

Materials and Methods

Animals and Pituitary Tissue Collection

Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts) at 57 to 70 days of age. Rats were housed in the University of Texas Southwestern Medical Center Animal Resource Center on a 14 light–10 dark cycle. Food and water were available ad libitum. After brief CO_2 exposure, the animals were decapitated, and the anterior pituitaries were used for dispersion and culture as described below. All animal procedures were performed in accordance with the guidelines established by the UT Southwestern Institutional Animal Care and Use Committee.

Pituitary Dispersion and Culture

The anterior pituitaries were washed twice in Hanks balanced salts solution (HBSS) supplemented with 0.1% fetal bovine serum (FBS) and 15 mmol/L 4-(2-hydroxyethyl)-1-piper

azineethanesulfonic acid (HEPES). The gland was cut into 1 to 2 mm fragments inside a Petri dish using a sterile blade while immersing in HBSS washing medium. The tissue fragments were collected into a conical tube, washed in 10 mL HBSS washing medium, and gently centrifuged. The tissue pellet was suspended in a 50-mL flask containing 5 mL HBSS dissociation medium consisting of 0.3% trypsin, 2 μg/mL deoxyribonuclease (DNase), 15 mmol/L HEPES, 1.2 mmol/L EDTA, and 0.3% bovine serum albumin. The tissue and enzymatic mixture was incubated in a water bath at 30°C with gently shaking for 20 minutes. The tissue blocks were gently triturated approximately 20 times with a disposable Pasteur pipette. The cell suspension was transferred to a new conical tube containing an equal volume of Dulbecco-modified Eagle medium (DMEM; Invitrogen, Carlsbad, California), with high glucose and 10% FBS, 23 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 1× penicillin/streptomycin (DMEM culture medium). The undissociated tissue was allowed to settle at the bottom of the flask, followed by further enzymatic dissociation and trituration as described above. The gland was fully dispersed after 3 dissociation periods. The cell suspensions obtained from each dissociation were combined and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in DMEM culture medium.

The dispersed primary cells were counted and plated in 48-well tissue culture plates at a density of 2 50 000 cells/well. After overnight culture in DMEM culture medium, the cells were gently washed and cultured with phenol red-free Opti-MEM I (Invitrogen) for 2 hours. Next, the cells were treated with DHT (5α-androstan-17β-ol-3-one, A8380; Sigma-Aldrich Inc, Saint Louis, MO), estradiol (17β-estradiol, 3301; Calbiochem, EMD Millipore, Billerica, MA), or progesterone (P6149; Sigma-Aldrich), with or without GnRH analog (L4897; Sigma-Aldrich). The concentration and duration of each hormone treatment are indicated in the corresponding figure legend. Control cell cultures were treated with equal amount of vehicles (ethanol for steroids and H_2O for GnRH). Duplicate cell wells were used for every treatment in each experiment, which was conducted a minimum of 3 times.

RNA Extraction and Reverse Transcription

Total RNA was prepared from primary pituitary cells using TRI Reagent (Ambion, Austin, TX), according to the manufacturer's instructions. Total RNA samples were DNase treated using the Turbo DNA-free kit (Ambion). The DNase-treated total RNA was reverse transcribed at 50°C for 50 minutes in the presence of 250 ng of random hexamers and 200 units Superscript III reverse transcriptase in 1× first strand buffer containing 0.5 mmol/L deoxyribonucleotide triphosphate and 40 units RNase Out (Invitrogen). A parallel reaction lacking the reverse transcriptase was prepared as a negative control.

Polymerase Chain Reactions

Quantitative real-time polymerase chain reaction (qPCR) was performed with the above reverse transcribed complementary DNA in a 384-well plate on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) using Taqman Universal PCR Master Mix and gene-specific Taqman Gene Expression Assays (Table 1) with universal cycling conditions. Each reaction was run in 15 μL total volume, and each sample was run in triplicates. The expression of each target gene was normalized to 18S transcript expression in the sample. The relative target gene expression levels were calculated using the Comparative C_T method as described in Applied Biosystems User Bulletin No. 2.

Table 1. Quantitative PCR Primer Sequences.

Genes	Assay ID/Primer Sequences
Tagman assays	
AR	Rn00560747_m1
FSHβ	Rn01484594_m1
GnRHR	Rn00578981_m1
PACAP	Rn00566438_m1
PAC1	Rn00591653_m1
SYBR green method	
PRB	Forward: 5′-CCAATACCGATCTCCCTGGAC-3′
	Reverse: 5′-CTTCCACTCCAGAGAAAGCTCC-3′

Abbreviations: AR, androgen receptor; GnRHR, GnRH receptor; FSHβ, follicle-stimulating hormone β; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1, PACAP receptor; PCR, polymerase chain reaction; PRB, progesterone receptor.

Statistical Analysis

For each experiment, the result from one of the control wells was arbitrarily set at 1, and the remainder of the results corrected against that value. The experimental results were averaged, and the standard error of the mean was calculated counting each independent experiment as a single N. Statistical analysis was performed using the SigmaStat Software package (SPSS Science, Chicago, Illinois). Data were analyzed for normality followed by analysis of variance. The Tukey test was used for post hoc comparison. The student *t* test was used, where only 2 groups were involved. Statistical significance was set at $P < .05$.

Results

The GnRH Alters PACAP and PAC1 mRNA Levels in Rat Primary Pituitary Cells

The GnRH treatment of dispersed adult male rat pituitary cells for 6 hours increased PACAP mRNA expression by approximately 5-fold ($P < .05$; Figure 1A). The GnRH treatment for 24 hours also significantly increased PACAP mRNA expression but to a lesser degree. This temporal response is in accordance with observation in the LβT2 gonadotrope cell line.[26] PAC1 mRNA level was not affected by GnRH treatment for 6 hours, but it was significantly decreased after 24 hours of GnRH treatment (Figure 1B).

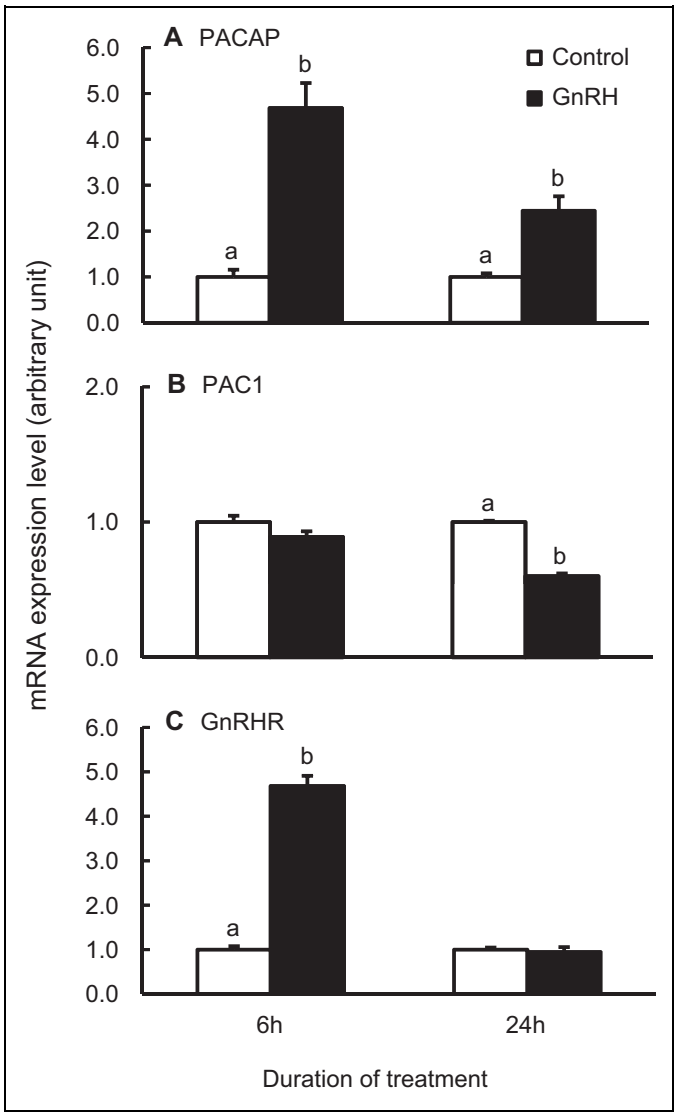

Figure 1. The GnRH regulates the mRNA levels of PACAP (A), PACAP receptor (PAC1; B), and GnRHR (C) in dispersed adult male rat pituitary cells. Cells were treated with 100 nmol/L GnRH analog for 6 or 24 hours and harvested for total RNA isolation. The mRNA expression levels were determined by real-time PCR using Taqman gene expression assays with those of the control samples set as 1.0 at each time point, respectively. Bars represent the mean ± standard error of the mean of each treatment group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$). GnRH indicates gonadotropin-releasing hormone; mRNA, messenger RNA; PACAP, pituitary adenylate cyclase-activating polypeptide.

As it is known that GnRH induces GnRHR expression, GnRHR mRNA levels were determined as an internal control for the experimental protocol.[41,42] Consistent with the previous reports, GnRHR mRNA expression increased approximately 5-fold following 6 hours of GnRH treatment with a return to control levels following 24 hours treatment (Figure 1C). Based on these results, in subsequent experiments the cells were treated with GnRH for only the last 6 hours before sample collection, as detailed in each figure legend.

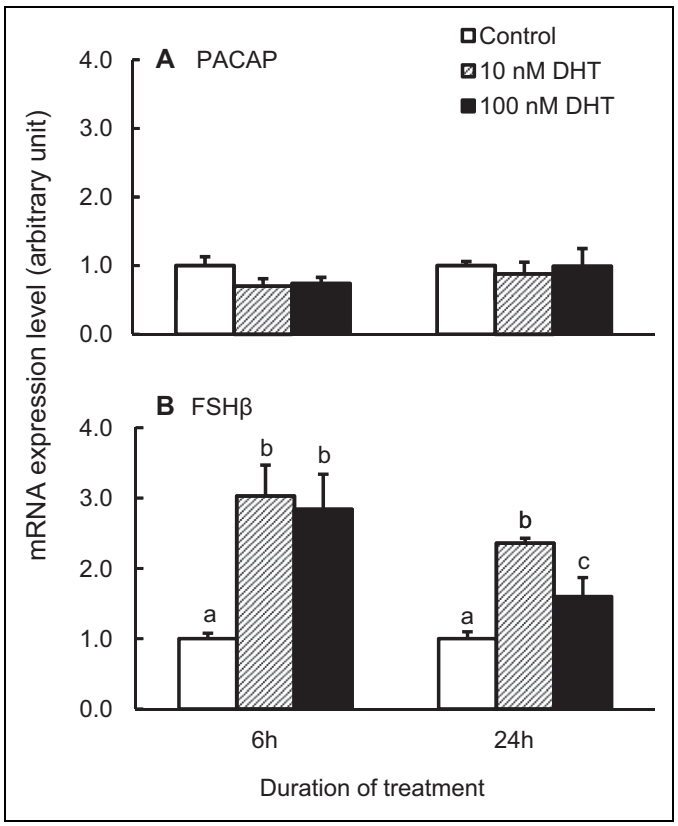

Figure 2. Pituitary adenylate cyclase-activating polypeptide (PACAP; A) and follicle-stimulating hormone β (FSHβ; B) messenger RNA (mRNA) expression in dispersed adult male rat pituitary cells in response to treatment with vehicle, 10, or 100 nmol/L dihydrotestosterone (DHT) for 6 or 24 hours. The samples were processed and analyzed as described in Figure 1. Bars represent the mean ± standard error of the mean of each group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$).

Dihydrotestosterone Augments GnRH-Mediated Increases in PACAP mRNA Levels

We next investigated the effect of DHT alone on PACAP mRNA expression. As presented in Figure 2A, while 6 hours DHT treatment decreased PACAP mRNA expression by about 25%, this response was not statistically significant. To confirm our experimental procedure, we also measured the expression of *FSH*β mRNA, because it has been reported previously that DHT increases pituitary *FSH*β mRNA and serum FSH levels in rats.[23,43,44] As expected, DHT significantly increased *FSH*β mRNA in dispersed pituitary cells at both the 6- and the 24-hourtime points (Figure 2B).

As both DHT and GnRH are present simultaneously in normal physiology, we chose to define the interaction of these 2 hormones on pituitary PACAP mRNA expression. The dispersed pituitary cells were treated with 100 nmol/L DHT for 6 or 24 hours, and GnRH was included in the culture medium during the last 6 hours of treatment. Once again, there was a nonsignificant decrease in PACAP mRNA levels in the presence of DHT with a significant increase in response to GnRH treatment at both time

points (Figure 3A). When both DHT and GnRH were included in the culture medium, the expression of PACAP mRNA was significantly greater than that with GnRH treatment alone.

We hypothesized that DHT and GnRH could exert cooperative effects via hormone-mediated stimulation of either AR or GnRHR expression. Arguing against this mechanism, AR expression was unchanged by DHT and/or GnRH treatment (data not shown), and DHT actually blunted the ability of GnRH to stimulate expression of its receptor (Figure 3C).

The PAC1 mRNA expression was also analyzed. The DHT treatment caused a biphasic change in PAC1 mRNA expression, with a significant, albeit small, increase at 6 hours but no change by 24 hours of treatment (Figure 3B). As observed previously, GnRH alone did not affect PAC1 expression, because it was only given for 6 hours. Interestingly, when DHT and GnRH were used together, they tended to suppress PAC1 mRNA expression below the other treatment group (Figure 3B).

Similarly, as an internal control, FSHβ mRNA expression was measured. The DHT alone caused a significant increase in FSHβ mRNA expression, and GnRH alone decreased the expression but not to a significant level when compared with the vehicle control (Figure 3D). When both DHT and GnRH were present, the stimulatory effect of DHT on FSHβ mRNA expression was totally suppressed by GnRH.

Estradiol Blunts PACAP Gene Expression When Present Alone

Estradiol controls pituitary function through its feedback directly to the pituitary or indirectly to the hypothalamus. Treatment of dispersed pituitary cells with 10 or 100 nmol/L estradiol for 6 hours decreased PACAP transcript number significantly, a response lost with prolonged treatment of 24 hours (Figure 4A). In contrast, estradiol treatment for 6 or 24 hours did not alter the expression of the PAC1 or GnRHR genes (Figure 4B and C).

To monitor the effectiveness of the estradiol treatment, mRNA levels of the PRB was measured. As presented in Figure 4D, estradiol significantly increased PRB mRNA levels at both 6 and 24 hours, consistent with its reported ability to enhance the PRB expression in gonadotropes.[45]

We next investigated the responses to treatment with both estradiol and GnRH. As demonstrated in Figure 5, GnRH-mediated increases in PACAP gene expression were not altered by the presence of 10 or 100 nmol/L E2 (Figure 5A). Estradiol did not augment the ability of GnRH to increase the GnRHR transcripts, and, conversely, GnRH treatment did not change the ability of estradiol to stimulate the PRB mRNA levels (Figure 5C and D). The PAC1 gene expression did not change in response to 6 hours of GnRH treatment in the presence of estradiol (Figure 5B).

Progesterone Potentiates GnRH Effects on PACAP mRNA Levels

Finally, the effect of progesterone on PACAP mRNA expression was studied. In order to induce PR expression, dispersed

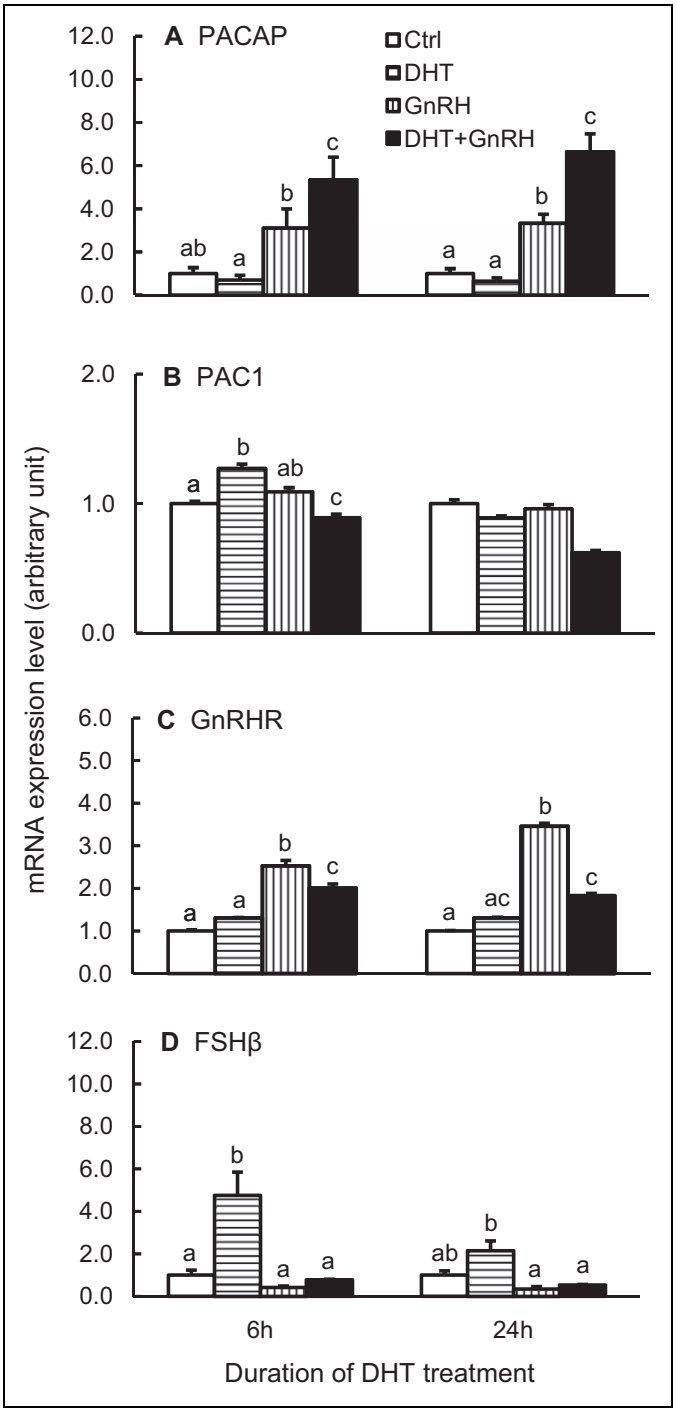

Figure 3. Interaction between DHT and GnRH on PACAP (A) and PAC1 (B) gene expression. The GnRHR (C) and FSHβ (D) mRNA levels were also analyzed. Dispersed cells were treated with vehicle or 100 nmol/L DHT for 6 or 24 hours, and 100 nmol/L GnRH analog (or equal volume of H_2O as control) was included for the last 6 hours of incubation. The samples were then processed and measured as described in Figure 1. Bars represent the mean ± standard error of the mean of each group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$). DHT indicates dihydrotestosterone; FSHβ, follicle-stimulating hormone β; GnRH, gonadotropin-releasing hormone; mRNA, messenger RNA; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1, PACAP receptor.

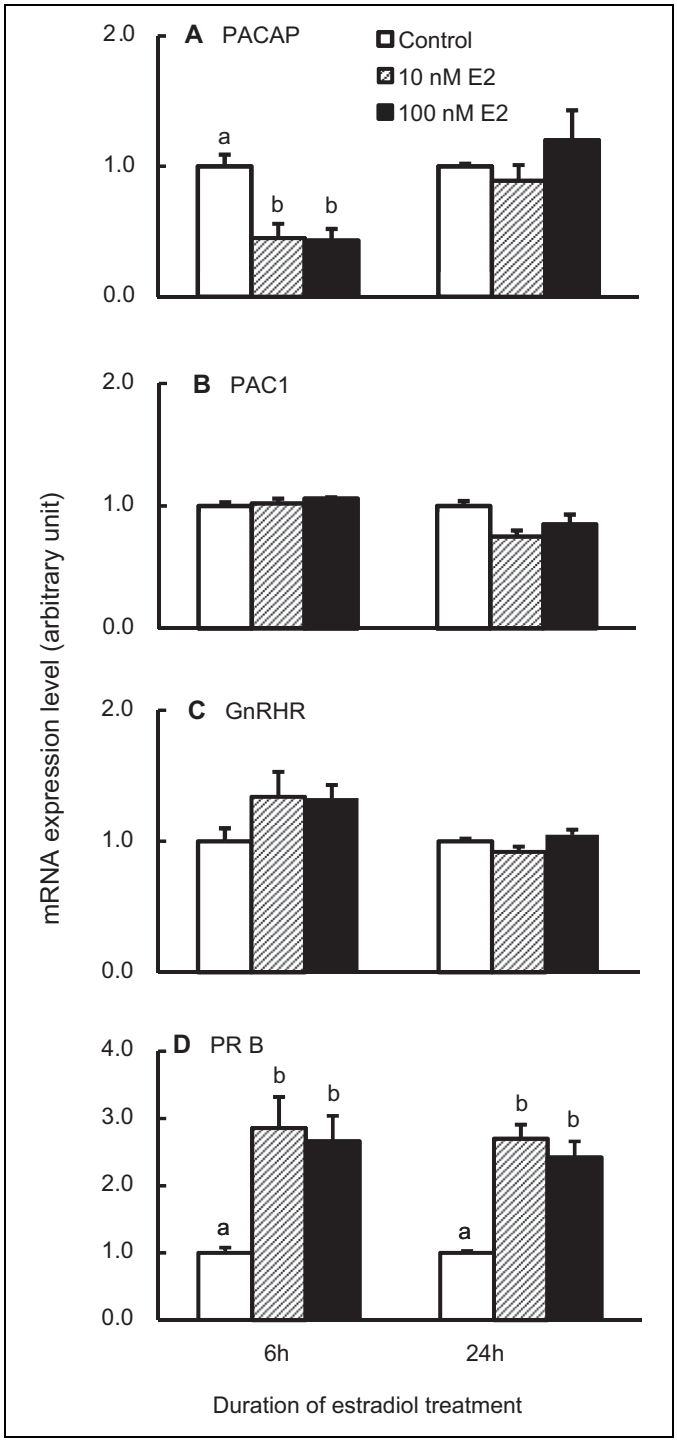

Figure 4. Expression of pituitary adenylate cyclase-activating polypeptide (PACAP; A), PACAP receptor (PAC1; B), gonadotropin-releasing hormone receptor (GnRHR; C), and progesterone receptors (PRB; D) in rat pituitary cells treated with estradiol (E_2). PRB messenger RNA (mRNA) expression was used as positive controls for E_2 treatment. The cells were treated with vehicle, 10, or 100 nmol/L E_2 for 6 or 24 hours. Samples were analyzed as described in Figure 1. Bars represent the mean $\pm$ standard error of the mean of each group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$).

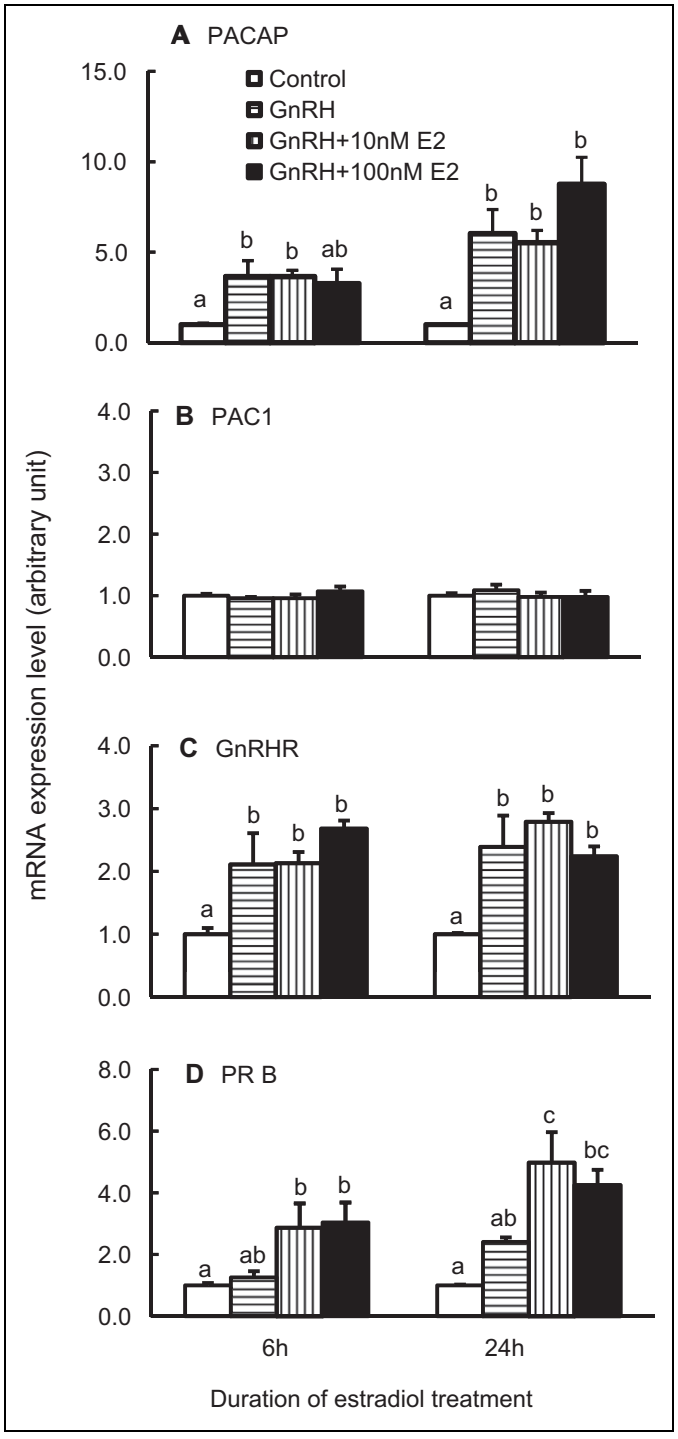

Figure 5. Expression of pituitary adenylate cyclase-activating polypeptide (PACAP; A), PACAP receptor (PAC1; B), GnRHR (C), and progesterone receptor (PRB; D) in rat pituitary cells treated with vehicle, 10, or 100 nmol/L E_2 for 6 or 24 hours, and 100 nmol/L gonadotropin-releasing hormone (GnRH) analog (or equal volume of H_2O as control) was included for the last 6 hours of incubation. The samples were measured as described in Figure 1. Bars represent the mean $\pm$ standard error of the mean of each group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$).

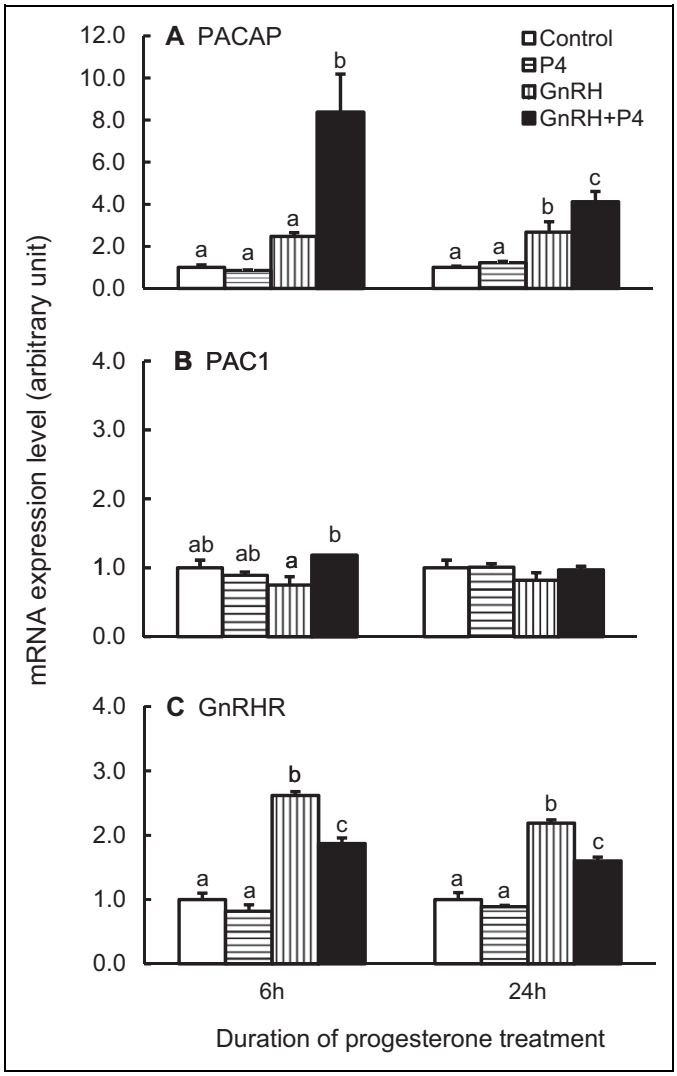

Figure 6. Expression of pituitary adenylate cyclase-activating polypeptide (PACAP; A), PACAP receptor (PAC1; B), and GnRHR (C) in rat pituitary cells. The cells were first primed with 1 nmol/L E_2 for 24 hours, then with constant E_2 priming treated with 100 nmol/L progesterone (P4) for 6 or 24 hours, and 100 nmol/L gonadotropin-releasing hormone (GnRH) analog (or equal volume of H_2O as control) was added at 6 hours before sample collection. The samples were processed and measured as described in Figure 1. Bars represent the mean $\pm$ standard error of the mean of each group ($n \geq 3$). Bars at the same time point with different letters vary significantly ($P < .05$).

anterior pituitary cells were primed with 1 nmol/L estradiol 24 hours prior to and during the progesterone and/or GnRH treatments.

Progesterone treatment alone did not affect the expression of PACAP mRNA, and GnRH increased the expression as expected (Figure 6A). When both progesterone and GnRH were included in the culture medium, PACAP mRNA expression was increased over 3-fold higher than that of GnRH alone at 6 hours treatment. Progesterone alone did not affect PACAP receptor and GnRHR mRNA expression (Figure 6B and C), but it inhibited the stimulatory effects of GnRH on GnRHR expression (Figure 6C). The GnRH, as observed previously (Figure 1), stimulated GnRHR mRNA expression but did not alter PAC1 expression even in the presence of progesterone (Figure 6B and C).

Discussion

Gonadotropin biosynthesis and secretion are tightly regulated through the interaction of hypothalamic-releasing factors, most notably GnRH, gonadal steroid feedback, and intrapituitary regulatory pathways including the activin-follistatin system. More recent investigations have begun to elucidate an important role for both hypothalamic and locally produced PACAP in the regulation of pituitary cell function.

In the experiments reported here, we tested the effects of various hormonal treatments on male rat anterior pituitary cells, which had been dispersed and plated in static culture. Male rats were utilized in order to eliminate any potential confounding effects of estrous cycle stage. As primary end points, we analyzed the expression levels of the transcripts, which encode PACAP, whose expression is limited to the gonadotropes and folliculostellate cells, and the PACAP receptor, PAC1, which is expressed more widely in the pituitary. Gonadotrope cells are unique within this mixed cell population, as they express the GnRHR as well as androgen, estrogen, and progesterone nuclear hormone receptors.[46-48] To our knowledge, our data are the first to characterize regulation of PACAP and PAC1 gene expression by GnRH and gonadal steroids in primary pituitary cells. As PACAP itself regulates gonadotropin expression, these data suggest that, in addition to direct action via their receptors, GnRH and gonadal steroids may also modulate gonadotrope function through alterations in a pituitary PACAP autocrine–paracrine system. It will be of interest in the future to define changes in pituitary PACAP protein levels; however, the amount of PACAP present in the anterior pituitary is below the level of detection by standard Western blot in rodents and will likely require the development of a sensitive RIA protocol.[49]

We have previously reported the ability of GnRH to increase PACAP mRNA expression by over 100-fold in the LβT2 gonadotrope cell line.[26] We now confirm a significant GnRH response in primary gonadotropes of 2- to 6-fold. The smaller response in the mixed cell preparation is likely due to the fact that PACAP mRNA produced by the folliculostellate cells is unchanged in response to GnRH.[7,50,51] Variations in the fold change across experiments may be the result of minor differences in the treatment protocols, such as differences in the vehicle or the length of incubation times that would have led to differences in paracrine factor involvement.

Although Purwana and colleagues reported a GnRH-mediated increase in PAC1 mRNA levels in the LβT2 cell line after 12 or 24 hours treatment, in our studies of primary pituitary cells, PAC1 expression was unchanged following 6 hours of GnRH treatment and actually decreased with 24 hours of treatment.[52] Whether this discrepancy is due to intrinsic differences in primary versus immortalized gonadotrope cells or to other factors will require further investigation. Also

of interest, although only the level of transcript expression is analyzed, the fact that GnRH increases PACAP mRNA levels at an early time point and subsequently blunts receptor expression may suggest that the subsequent PACAP effects are temporally self-limited (Figure 1). Of note, the degree and timing of the GnRH effect varied depending on the presence or absence of steroids, adding further complexity to the response.

Although treatment with DHT alone did not alter PACAP expression in primary pituitary cells, our results clearly indicate that GnRH-induced PACAP expression is significantly enhanced in the presence of DHT. The DHT treatment resulted in minimal albeit statistically significant changes in PAC1 transcript number which, as observed in response to GnRH, tended to be the converse of the PACAP response. The functional interaction between DHT and GnRH can be postulated to occur through multiple mechanisms. One of these possibilities, steroid-mediated changes in GnRHR expression, does not appear to be a contributing factor as GnRHR expression drops rather than increases at least at the mRNA level.

In the classical steroid hormone receptor genomic pathway, steroids exert their effects via interactions with cognate receptors that bind to DNA directly. Both AR and PR binding sites have in fact been identified in the rat PACAP gene promoter (Grafer et al, unpublished data). Arguing against this mechanism, however, AR mRNA expression was unchanged in our experiments. Steroid receptors and their cofactors have also been shown to be phosphorylated and thereby activated by intracellular signaling pathways such as the MAPK pathways,[53] which are known to be the downstream effectors of GnRH action. It is therefore possible that ligand-independent activation of AR subsequently allows for ligand-dependent effects, which are not detectable in the presence of steroid ligand alone.[54-58]

It has been reported that progesterone induces PACAP and PAC1 mRNA expression in the rat hypothalamus and ovary.[35,36] In contrast, progesterone did not affect the expression of either of these genes in primary pituitary cell cultures, demonstrating tissue-specific differences in hormonal responsiveness. Progesterone did significantly augment GnRH induction of PACAP mRNA expression in dispersed pituitary cells. Although the mechanism for this interaction remains unknown, it is interesting to note that progesterone also enhances the ability of GnRH to stimulate the expression of the gonadotropin genes, LHβ, and FSHβ.[25,59]

The response to estradiol differed from the response to the other 2 gonadal steroids in that PACAP gene expression was altered in the presence of estradiol alone, but there was no apparent functional interaction with GnRH. Of note, estradiol-mediated suppression was observed only at the 6 hours time point and lost by 24 hours of treatment. Tissue-dependent differences in steroid response were again noted as estradiol decreased the PACAP mRNA levels in pituitary cell cultures, although it has been reported to stimulate PACAP gene expression in the hypothalamic ventromedial and arcuate nuclei.[34]

The effects of estradiol and progesterone treatment on PACAP mRNA levels, which we observed in vitro, are in accordance with changes described previously in pituitary PACAP gene expression across the estrous cycle. Moore et al reported that pituitary expression of PACAP mRNA levels decline moderately on the afternoon of proestrus with a significant increase at 24 hours as determined by qPCR.[6] A similar pattern was observed by Heinzlmann et al using in situ hybridization.[60] Our data suggest that the late evening peak in pituitary PACAP mRNA levels may be attributable to the loss of inhibitory estradiol input, which occurs during proestrus as well as the stimulatory effects of increasing progesterone levels acting in concert with GnRH.[61]

The combinatorial effects of GnRH and DHT are gene specific within the gonadotrope. Although GnRH and DHT synergistically increased PACAP mRNA expression (Figure 3A), DHT blocked the increase in GnRHR mRNA induced by GnRH (Figure 3C), and GnRH blocked the DHT-mediated stimulation of FSHβ transcript number (Figure 3D). The PACAP and FSHβ expression are known to be regulated by locally produced activin and its inhibitor follistatin.[2] Furthermore, a number of studies have demonstrated the ability of DHT and GnRH to regulate the pituitary follistatin expression, thereby altering the ability of local activin to stimulate FSHβ gene expression.[62,63] In fact, it has been reported that androgen-mediated regulation of the FSHβ gene requires protein–protein interactions between the AR and the Smad family of transcription factors, which convey activin signaling.[64,65] In addition, androgens have been shown to be correlated with an increase in Smad expression and phosphorylation as well as a decrease in follistatin mRNA levels in primary rat pituitary cells.[62] In contrast, GnRH has been shown to increase follistatin promoter activity and mRNA expression in primary pituitary and LβT2 gonadotrope cells. It can be hypothesized that this GnRH-mediated increase in follistatin could blunt the ability of androgens to stimulate the FSHβ mRNA expression. Adding further complexity, GnRH and DHT synergize to stimulate the FSHβ promoter activity in contrast to the inhibition observed at the mRNA level in our studies, perhaps suggesting differential effects on mRNA transcription and stability.[65]

In summary, the results reported here suggest that the activity of the intrapituitary PACAP-PAC1 system is regulated via the complex interaction of gonadal steroids and hypothalamic GnRH. As PACAP contributes to the regulation of gonadotropin biosynthesis and secretion, these results establish a novel mechanism for the modulation of gonadotrope gene expression and, thereby, normal reproductive function.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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