GATA augments GNRH-mediated increases in *Adcyap1* gene expression in pituitary gonadotrope cells

Robin L Thomas, Natalie M Crawford, Constance M Grafer, Weiming Zheng and Lisa M Halvorson

Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9032, USA

Correspondence should be addressed to L M Halvorson
Email
lisa.halvorson@utsouthwestern.edu

Abstract

Pituitary adenylate cyclase-activating polypeptide 1 (PACAP or ADCYAP1) regulates gonadotropin biosynthesis and secretion, both alone and in conjunction with GNRH. Initially identified as a hypothalamic-releasing factor, ADCYAP1 subsequently has been identified in pituitary gonadotropes, suggesting it may act as an autocrine–paracrine factor in this tissue. GNRH has been shown to increase pituitary *Adcyap1* gene expression through the interaction of CREB and jun/fos with CRE/AP1 *cis*-elements in the proximal promoter. In these studies, we were interested in identifying additional transcription factors and cognate *cis*-elements which regulate *Adcyap1* gene promoter activity and chose to focus on the GATA family of transcription factors known to be critical for both pituitary cell differentiation and gonadotropin subunit expression. By transient transfection and electrophoretic mobility shift assay analysis, we demonstrate that GATA2 and GATA4 stimulate *Adcyap1* promoter activity via a GATA *cis*-element located at position –191 in the rat *Adcyap1* gene promoter. Furthermore, we show that addition of GATA2 or GATA4 significantly augments GNRH-mediated stimulation of *Adcyap1* gene promoter activity in the gonadotrope LβT2 cell line. Conversely, blunting GATA expression with specific siRNA inhibits the ability of GNRH to stimulate ADCYAP1 mRNA levels in these cells. These data demonstrate a complex interaction between GNRH and GATA on ADCYAP1 expression, providing important new insights into the regulation of gonadotrope function.

Key Words

- ▶ adenylate cyclase-activating polypeptide 1
- ▶ GATA
- ▶ gonadotropes
- ▶ PACAP
- ▶ pituitary

Journal of Molecular Endocrinology
(2013) 51, 313–324

Introduction

Proper functioning of the hypothalamic–pituitary–gonadal axis requires the complex interplay of gonadotropin-releasing hormone (GNRH), steroids, and other regulatory factors. In addition to the well-characterized effects of hypothalamic GNRH, the neuropeptide adenylate cyclase-activating polypeptide 1 (ADCYAP1; also known as PACAP) also regulates gonadotropin gene expression.

ADCYAP1 is secreted by hypothalamic neurons into the pituitary portal vasculature, binding to specific G protein-coupled receptors on pituitary cell membranes and activating the cAMP/protein kinase A signaling pathway (Sherwood *et al.* 2000, Vaudry *et al.* 2000). ADCYAP1 increases gonadotropin biosynthesis and secretion, alone and in synergy with GNRH (Culler & Paschall 1991,

Schomerus *et al.* 1994, Tsujii & Winters 1995, Winters *et al.* 1996, Purwana *et al.* 2010, Kanasaki *et al.* 2013). ADCYAP1 has been shown to increase α -subunit, *Lhb*, *Fshb*, and *Gnrhr* gene promoter activity and mRNA levels in gonadotropes (Schomerus *et al.* 1994, Tsujii & Winters 1995, Winters *et al.* 1996, Burrin *et al.* 1998, Katayama *et al.* 2000, Cheng & Leung 2001, Ngan *et al.* 2001, Pincas *et al.* 2001b, Fujii *et al.* 2002, Ferris *et al.* 2007, Purwana *et al.* 2010). Although less potent than GNRH, ADCYAP1 also stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion by primary pituitary cells and the gonadotrope cell line, α T3-1 (Culler & Paschall 1991, Hart *et al.* 1992, Perrin *et al.* 1993, Schomerus *et al.* 1994, Tsujii & Winters 1995, Winters *et al.* 1996, Koves *et al.* 2003). These stimulatory effects of ADCYAP1 on LH secretion in rats have been confirmed *in vivo* (Osuga *et al.* 1992, Radleff-Schlimme *et al.* 1998). GNRH and ADCYAP1 exert cooperative effects on gonadotrope function via multiple mechanisms including alterations in receptor expression. As shown by Kanasaki *et al.* (2009), treatment of gonadotrope cells with either pulsatile GNRH or ADCYAP1 stimulates both GNRH receptor (*GNRHR*) and PACAP receptor type 1 (*Adcyap1r1*) gene expression.

Although, originally identified as a hypothalamic-releasing factor, ADCYAP1 subsequently has been determined to have widespread distribution and function, including expression in the CNS and peripheral nervous system, smooth muscle, adrenal gland, placenta, anterior and posterior pituitary, ovaries, and testes (Arimura & Shioda 1995). Within the anterior pituitary gland, both gonadotropes and the folliculostellate cells have been shown to synthesize and secrete ADCYAP1 peptide (Koves *et al.* 1998, Jin *et al.* 2001, Szabo *et al.* 2002, Heinzlmann *et al.* 2008). Therefore, ADCYAP1 both acts on and is secreted by pituitary cells, forming a functional autocrine-paracrine loop in this tissue.

A limited number of studies have begun to elucidate the hormonal factors which regulate *Adcyap1* gene expression. Estradiol and progesterone have been suggested to stimulate *Adcyap1* expression in the hypothalamus and ovary (Ha *et al.* 2000, Park *et al.* 2000, Apostolakis *et al.* 2004, Moore *et al.* 2005). Additional studies have shown the ability of LH and FSH to promote *Adcyap1* expression in human granulosa-luteal cells obtained from patients undergoing IVF (Morelli *et al.* 2008). LH, FSH, human chorionic gonadotropin, and GNRH have also been shown to increase *Adcyap1* mRNA levels in the ovary (Ko *et al.* 1999, Lee *et al.* 1999, Park *et al.* 2001).

GNRH has also been demonstrated to increase ADCYAP1 biosynthesis in the pituitary. As shown by our

group and others, *Adcyap1* promoter activity and mRNA levels are markedly increased by static or pulsatile GNRH treatment in an immortalized gonadotrope cell line (Grafer *et al.* 2009, Purwana *et al.* 2010, Kanasaki *et al.* 2011). This stimulatory effect has been confirmed in rat primary pituitary cell cultures in which a threefold increase in *Adcyap1* mRNA levels has been observed in response to GNRH treatment (C M Grafer and L M Halvorson, 2009, unpublished observations). We have reported that the GNRH response is mediated via the PKA, PKC, and MAPK intracellular signaling pathways acting via three DNA-regulatory elements, a CRE site located at position -205 and two AP1 sites located at positions -275 and -448, in the proximal *Adcyap1* promoter (Grafer *et al.* 2009). Furthermore, these studies demonstrated a role for CREB and the AP1 proteins, jun and fos, in mediating this response. This work was the first to identify specific transcription factors and associated *cis*-elements with the importance for pituitary *Adcyap1* gene expression. We, therefore, were interested in identifying additional transcription factors with the importance for expression of this gene.

The GATA family of transcription factors has been shown to regulate gene expression at all levels of the reproductive axis. Although originally characterized in the hematopoietic and cardiac systems, GATA factors are now known to be expressed in hypothalamic GNRH neurons, the pituitary, the ovary, and the testes (LaVoie 2003, Viger *et al.* 2008). Vertebrates express six different GATA family members. The amino acid sequence of these factors is highly conserved with ~80% homology in the zinc finger DNA-binding domains. As indicated by their name, the GATA factors bind to the consensus motif GATA, although they have also been reported to bind nucleotide sequences containing core GATC or GATT elements (Merika & Orkin 1993, Nakagawa *et al.* 2001).

Within the pituitary gland, GATA transcription factors have been shown to be critical for both pituitary cell differentiation and function. During development, GATA2 directs cells toward the gonadotrope lineage and away from the thyrotrope lineage through reciprocal interactions with Pit1 (Dasen *et al.* 1999). Functional GATA *cis*-elements have also been identified in the promoter regions of the common α - and *Fshb*-subunit genes, as well as the *Gnrh* gene (Steger *et al.* 1994, Pincas *et al.* 2001a, Lo *et al.* 2011). Our laboratory has recently demonstrated that the adult mouse pituitary expresses GATA4 in addition to GATA2 and that both of these factors stimulate gonadotropin *Lhb* gene expression in the L β T2 gonadotrope cell line (Lo *et al.* 2011).

For the studies reported here, we hypothesized that GATA transcription factors play a role in the regulation of *Adcyap1* gene expression in pituitary gonadotropes. Our results demonstrate the ability of both GATA2 and GATA4 to stimulate basal and GNRH-induced *Adcyap1* gene expression via a GATA response element located at position –191 relative to the transcriptional start site in the proximal rat *Adcyap1* gene promoter.

Subjects and methods

RNA extraction and RT

Total RNA was prepared from cultured gonadotrope LβT2 cells using TRI Reagent (Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions. Total RNA samples were DNase-treated using the Turbo DNA-free Kit (Ambion, Inc.) and reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Inc.) primed with random hexamer. A parallel reaction lacking RT was used as an additional negative control. LβT2 cells were generously provided by Dr P L Mellon (University of California, San Diego, CA, USA).

Quantitative real-time PCR

cDNA (10–100 ng/reaction) was amplified in triplicate in a 384-well plate on a 7900HT Sequence Detection System (Applied Biosystems) using Applied Biosystems TaqMan Universal PCR Master Mix and gene-specific TaqMan Gene Expression Assay primer/probe sets with universal cycling conditions (Table 1). Each reaction was run in 15 µl total volume. The expression of each target gene was normalized to *18S* transcript expression in each sample. The relative target gene expression levels among treatments were calculated using the comparative C_T method as described in Applied Biosystems User Bulletin No. 2.

Plasmids

The rat *Adcyap1* gene promoter spanning region –1916 to +906 relative to the transcription start site exon 1A (gene ID: 24166) was subcloned into the pGL3-Basic luciferase reporter vector (Promega) (cDNA was kindly provided by Drs S L White and K M Braas (University of Vermont College of Medicine, Burlington, VT)). A CRE site mutant (mutCRE(–205)) was also provided containing an AC to TG mutations present at position –203. Additional deletion constructs and site-directed mutations were generated by the PCR or the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The fidelity of all constructs was verified by nucleotide sequencing. The cDNAs encoding mouse GATA2 and GATA4 were provided by D F Gordon and S Hammes, respectively, and were subcloned into pcDNA3.1(+) (Invitrogen, Inc.).

Transient transfection of cell lines

Green monkey kidney fibroblast cells (CV1) were maintained in monolayer culture in low glucose DMEM supplemented with 10% fetal bovine serum (FBS) (v/v) and 1% penicillin/streptomycin (v/v) at 37 °C in humidified 5% CO₂/95% air. CV1 cells (6–7 × 10⁴ cells/well) were cultured overnight in 12-well plates. Using the calcium phosphate precipitation method, each well was transfected with 400 ng/well of ADCYAP1 reporter vector and 0–30 ng/well of GATA2 or GATA4 expression vectors as indicated in the dose–response experiment (Fig. 1). For subsequent CV1 experiments, cells received 30 ng of GATA2 and 10 ng of GATA4 expression vector. The GATA response of the empty luciferase reporter vector was less than threefold (data not shown).

For LβT2 transfection experiments, cells were maintained in high glucose DMEM supplemented with

Table 1 PCR primers used in this study

Name of oligonucleotides	Sequences (mutated residues underlined)
EMSA	
WT (–209/–167)	5'-ATGCTGACGTCCTTTTACTGATACCGGATCATTACGTGACTGGG-3'
Mutant –191 GATA	5'-ATGCTGACGTCCTTTTACTGCCACCGGATCATTACGTGACTGGG-3'
Mutant –205 CRE	5'-ATGCTGTGGTCTTTTACTGATACCGGATCATTACGTGACTGGG-3'
Mutant –179 CRE	5'-ATGCTGACGTCCTTTTACTGATACCGGATCCTTTGGTGACTGGG-3'
Mutant –205/–179 CRE	5'-ATGCTGTGGTCTTTTACTGATACCGGATCCTTTGGTGACTGGG-3'
Mutagenesis	
Mutant –191 GATA	5'-GATGCTGACGTCCTTTTACTGCCACCGGATCATTACGTGACT-3'
Mutant –191 GATA/–205 CRE	5'-GATGCTGTGGTCTTTTACTGCCACCGGATCATTACGTGACT-3'
Mutant –191 GATA/–179 CRE	5'-GGATGCTGACGTCCTTTTACTGCCACCGGATCCTTTGGTGAC-3'
Mutant –191 GATA/–205 CRE/–179 CRE	5'-GGATGCTGTGGTCTTTTACTGCCACCGGATCCTTTGGTGAC-3'

Figure 1

GATA2 and GATA4 stimulate *Adcyap1* promoter activity in a dose-dependent fashion. Fibroblast CV1 cells were transiently transfected with a reporter construct containing region –1916/+906 of the rat *Adcyap1* gene promoter fused to a luciferase reporter, pGL3. Cells were cotransfected with the indicated amount of expression vector for either GATA2 or GATA4 and with the pSV-β-galactosidase vector to control for differences in transfection efficiency. Luciferase activity was normalized to β-galactosidase activity and expressed as fold-change relative to cells receiving the same amount of empty expression vector. All experiments were performed a minimum of three times with data expressed as the mean $\pm$ S.E.M.

10% FBS (v/v), 1% penicillin/streptomycin (v/v), and 1 mM sodium pyruvate. Cells ($2.5\text{--}3 \times 10^5$ cells/well) were grown overnight in antibiotic-free media in 12-well plates. The cells were then transfected with 400 ng/well of ADCYAP1 reporter vector and 100 ng/well of GATA2 or GATA4 expression vectors or empty-vector control using the FuGENE 6 Transfection Reagent (Roche Applied Sciences) and OptiMEM-1 Reduced Serum Medium (Invitrogen, Inc.) according to the manufacturer's instructions. The day following transfection, cells received fresh antibiotic-free DMEM. Cells were treated for 6 h with the GNRH analog [des-Gly¹⁰, D-Ala⁶]-LH-RH ethylamide acetate hydrate (10 nM or as indicated; Sigma-Aldrich) or vehicle before harvest. Cotransfection of LβT2 and CV1 cells with 48 ng/well of a pSV-β-galactosidase plasmid (Promega) allowed correction for differences in transfection efficiency within the experiments. Cells were harvested at ~48 h following transfection and the cell extracts were analyzed for luciferase activity and for β-galactosidase activity using the Galacto-Light Plus assay system (Tropix, Applied Biosystems) and a luminometer from Berthold Detection Systems (Pforzheim, Germany). Luciferase activity was normalized to β-galactosidase activity and results were calculated as fold-changes relative to expression in the control wells.

Data are shown as the mean $\pm$ S.E.M. from at least three experiments with three replicates performed within each experiment.

siRNA interference

For siRNA experiments, LβT2 cells were seeded onto 12-well plates at 2.5×10^5 cells/well in DMEM containing 10% FBS (without antibiotics) one day before siRNA transfection. Dharmacon ON-TARGET plus SMARTpool siRNAs directed toward mouse *Gata2* (L-062114-00-0005) or *Gata4* (L-040759-01-0005) or a nontargeting negative control (D-001810-10-05) was transfected into LβT2 cells using DharmaFECT-1 siRNA Transfection Reagent (Thermo Fisher Scientific, Lafayette, CO, USA). Each well was transfected with 1 ml of 100 nM siRNA diluted in OptiMEM-1 Reduced Serum Medium (Invitrogen, Inc.), complexed with 3 μl DharmaFECT-1. The cells were cultured overnight and the transfection mixture was replaced the next day with DMEM containing 10% FBS. After treatment for the final 6 h with 100 nM GNRH or vehicle control, the cells were harvested 48 h after transfection for total RNA isolation for quantitative real-time PCR (qPCR) analysis. Each combination of siRNA and hormonal treatment was tested in duplicate in three experiments.

For each experiment, qPCR data was calculated using the comparative C_T method and calibrator values were chosen to set the averages of replicate vehicle-treated or GNRH-treated, non-targeting (NTA) siRNA samples equal to 100. The various experimental vehicle-treated or GNRH-treated samples were then expressed relative to their appropriately treated NTA controls.

Statistical analysis

Statistical analysis was performed using the SigmaStat Software Package (SPSS Science). Data were analyzed for normality followed by ANOVA. The Tukey's test was used for *post hoc* comparisons. The *t*-test was used where only two groups were involved. Statistical significance was set at $P < 0.05$.

Results

GATA2 and GATA4 stimulate *Adcyap1* gene promoter activity in CV1 cells

In order to study the effects of GATA transcription factors on *Adcyap1* promoter activity, transient transfection of the full-length rat *Adcyap1* (–1916/+906) gene promoter

fused to the pGL3 luciferase reporter was conducted in CV1 cells. These cells are an immortalized green monkey kidney fibroblast cell line essentially devoid of any endogenous GATA (Robert *et al.* 2006). Dose–response experiments demonstrated maximal GATA2 ($26 \pm$ two-fold) stimulation of full-length rat *Adcyap1* gene promoter activity at 30 ng/well and maximal GATA4 ($42 \pm$ nine-fold) stimulation of full-length rat *Adcyap1* gene promoter activity at 10 ng/well (Fig. 1). These maximal doses were chosen for subsequent transfection experiments in the CV1 cell line.

The GATA response maps to positions –242 to –172 of the rat *Adcyap1* gene promoter

We next wished to determine the region of the *Adcyap1* gene promoter that confers GATA responsiveness. Transient transfection of serial 5' deletions of the rat *Adcyap1* gene demonstrated a dramatic loss of GATA2 and GATA4 responsiveness between nucleotide positions –402 and –77 (79 ± 2 and $89 \pm 2\%$, respectively, compared with the –1916/+906 *Adcyap1* promoter construct; Fig. 2A). A significant loss of GATA4 responsiveness was also observed with deletion between positions –915 and –402 (Fig. 2A). We chose to focus on the downstream region as it conferred both GATA2 and GATA4 effects.

As shown in Fig. 2B, a series of intermediate 5'-truncations between region –402 and –77 were tested to further localize the GATA-responsive region. Both the GATA2 and GATA4 responses were significantly decreased with deletion between positions –242 and –172 (80 ± 9 and $72 \pm 6\%$, respectively, compared with the –402/+906 *Adcyap1* promoter construct).

Identification of a putative GATA binding site in the rat *Adcyap1* gene promoter

By sequence homology, three GATA-like elements, located at positions –210, –191, and –184, were identified within region –242 to –172. By electrophoretic mobility shift assay (EMSA), *in vitro* translated and endogenous L β T2, GATA2, and GATA4 proteins did not bind to a nucleotide probe spanning either position –210 or –184 (data not shown). However, as shown in Fig. 3B, *in vitro* translated GATA2 and GATA4 (lanes 2 and 4 respectively) bound to the WT probe spanning position –191. The presence of GATA2 or GATA4 in the identified complex was confirmed by the addition of specific antibodies for the corresponding protein (lanes 3 and 5). Endogenous GATA2 and GATA4 present in L β T2 nuclear extracts

Figure 2

Mapping regions of GATA responsiveness in the *Adcyap1* gene promoter. CV1 cells were transiently transfected with various lengths of the rat *Adcyap1* promoter–luciferase reporter construct and expression vectors for GATA2 or GATA4. (A) Serial 5'-truncations across region –1916 to +906 relative to the transcriptional start site. Vector only refers to the empty luciferase reporter construct, pGL3. (B) More detailed serial 5'-truncations between positions –402 and –77 relative to the transcriptional start site. Bars with different letters vary significantly within GATA2 or GATA4 results ($P < 0.05$). All experiments were performed a minimum of three times with data expressed as the mean $\pm$ S.E.M.

were also found to interact with this region of the *Adcyap1* gene promoter (Fig. 3C). The loss of intensity of this complex with addition of the GATA2 antibody (lane 2) or GATA4 antibody (lane 3) verified the presence of GATA in this complex.

Interestingly, the –191 GATA site is flanked by two sites located at positions –205 and –179 with homology to the consensus CRE *cis*-element (Fig. 3A). Our laboratory has previously demonstrated CREB binding to the –205 CRE site, with mutation of this site blunting the ability of

GNRH to stimulate rat *Adcyap1* gene promoter activity (Grafer *et al.* 2009). An antibody directed against a number of CREB family members supershifted the upper bands, but did not appear to alter the ability of GATA to bind (lane 4).

GATA proteins bind to CRE sites which flank the GATA *cis*-element

Cold competition EMSA experiments were then performed in which LβT2 nuclear extract was incubated with the -209/-167 oligonucleotide probe and excess unlabeled oligonucleotides were added to determine their ability to compete for GATA binding (Fig. 3D). The oligonucleotides which were used contain the CRE and GATA sites present as WT or with the indicated mutations in the putative *cis*-elements. Mutation in the putative -191 GATA site eliminated the ability of the oligonucleotide to compete for GATA binding, but did not substantially alter the ability of CREB proteins to bind. Conversely, the ability of GATA to interact with the promoter region was not impacted by mutations in the CRE sites. Interestingly, mutations in both CRE sites were required to prevent CREB binding in this region.

Mutations in the -205 CRE, -191 GATA, and -179 CRE sites, singly or in combination, decrease GATA2 and GATA4 effect

We next wished to study the functional significance of mutation in the putative GATA and CRE sites on *Adcyap1* gene promoter activity. Transient transfection of GATA2 or GATA4 expression vectors and reporter constructs containing the WT full-length (-1916/+906) rat *Adcyap1* gene promoter or constructs containing mutations in these sites were conducted in CV1 fibroblast cells (Fig. 4).

Mutation at the putative -191 GATA *cis*-element decreased GATA2 and GATA4 stimulated *Adcyap1* promoter activity by 50 ± 5 and $53 \pm 6\%$, respectively, compared with WT. Mutation of the -205 CRE decreased GATA2 and GATA4 stimulated *Adcyap1* promoter activity by 41 ± 3 and $41 \pm 8\%$, respectively, while mutation at the -179 CRE decreased GATA2 and GATA4 stimulated

Figure 3

GATA2 and GATA4 bind to the proximal rat *Adcyap1* gene promoter on electrophoretic mobility shift assay (EMSA). (A) Depiction of the putative GATA *cis*-element and flanking CRE sites in the rat *Adcyap1* gene promoter. (B) Binding by *in vitro* translated GATA2, GATA4, or unprogrammed reticulocyte lysate (RL) to a 32 P-labeled oligonucleotide probe spanning positions -209 to -167 in the rat *Adcyap1* gene promoter. (C) Interaction of GATA2 and GATA4 present in LβT2 gonadotrope nuclear extracts with the -209/-167 oligonucleotide probe. Antibodies directed against GATA2, GATA4, or CREB family members were added to the incubation mixture as indicated. (D) LβT2 nuclear extract was incubated with the WT -209/-167 *Adcyap1* oligonucleotide probe and unlabeled oligonucleotide containing the WT sequence or with mutations in the CRE or GATA sites. Oligonucleotide competitors were added at 200-, 40-, and 8-fold molar excess. Shown are representative EMSA which have been performed a minimum of three times.

Figure 4

Site-directed mutations in the -191 GATA, -205 CRE, or -179 CRE sites, either singly or in combination, blunt GATA-stimulated *Adcyap1* promoter activity. CV1 cells were transiently transfected with GATA2 or GATA4 expression vectors and the full-length ($-1916/+906$) rat *Adcyap1* gene promoter (WT) or constructs containing mutations in the -191 GATA, -205 CRE, and/or -179 CRE sites fused to a luciferase reporter vector. Vector only refers to the empty luciferase reporter construct, pGL3. The response in all mutated constructs was significantly decreased compared with the full-length construct ($P < 0.001$). All experiments were performed a minimum of three times with data expressed as the mean $\pm$ S.E.M.

Adcyap1 promoter activity by 48 ± 4 and $36 \pm 6\%$ respectively. Combinatorial mutations of the GATA and CRE *cis*-elements further decreased GATA-stimulated *Adcyap1* promoter activity. Compared with the WT, all constructs containing mutations significantly decreased the effects of both GATA2 and GATA4 on *Adcyap1* promoter activity ($P < 0.001$). These data demonstrate that the -191 GATA, -205 CRE, and -179 CRE sites all play an important role in GATA-mediated stimulation of the rat *Adcyap1* promoter.

GNRH and GATA act cooperatively to stimulate *Adcyap1* promoter activity in LβT2 gonadotrope cells

Having demonstrated the ability of GATA to increase *Adcyap1* promoter activity in the CV1 fibroblast cell line, we now wanted to confirm this observation in gonadotrope LβT2 cells. Furthermore, as we have previously shown an important role for GNRH in mediating *Adcyap1* promoter activity, the GATA response was tested following treatment with GNRH or vehicle for 6 h. Overexpression of GATA2 (Fig. 5A) or GATA4 (Fig. 5B) significantly increased the stimulatory effect of GNRH on *Adcyap1*

promoter activity. Note that the GATA response is lower in the gonadotrope cell line as these cells express high levels of endogenous GATA protein (Lo *et al.* 2011).

As observed in the CV1 cells, a dramatic loss of *Adcyap1* promoter activity occurred with truncation from position -402 to -77 . The simultaneous loss of the GNRH response was expected as this region is known to contain the -205 CRE site as well as a -275 AP1 site, both of which have been shown to be critical for mediating the GNRH effect (Grafer *et al.* 2009). Additional transfection experiments compared the WT full-length rat *Adcyap1* gene promoter to a reporter construct containing a mutation in the -191 GATA site. The presence of this mutation eliminated the ability of both GATA2 and GATA4 to stimulate the *Adcyap1* promoter either alone or in the presence of GNRH, while the GNRH response was maintained (Fig. 5C and D). These data demonstrate that an intact -191 GATA site is required for cooperative activation of the rat *Adcyap1* promoter by GATA and GNRH treatment.

siRNA knockdown of endogenous GATA2 or GATA4 blunts GNRH-stimulated *Adcyap1* gene expression

As GATA2 and GATA4 significantly increase the ability of GNRH to stimulate *Adcyap1* promoter activity, we reasoned that decreasing intracellular expression of these factors using a siRNA approach should blunt the ability of GNRH to stimulate endogenous *Adcyap1* gene expression in LβT2 cells. As shown in Fig. 6, GNRH-induced *Adcyap1* mRNA levels were significantly decreased in the presence of siRNAs directed against *Gata2* or *Gata4* (59 ± 4 and $52 \pm 3\%$, respectively, relative to the NTA control siRNA). These data strongly support a requirement for GATA transcription factors in achieving maximal GNRH-stimulated *Adcyap1* gene expression. No effect was observed on vehicle-treated *Adcyap1* expression, likely due to the fact that *Adcyap1* mRNA levels are very low in the basal state, preventing the ability to detect a further decrease (data not shown).

The average decrease in *Gata2* mRNA levels using siRNAs directed against *Gata2* was 51 ± 4 and $55 \pm 4\%$ in the absence and presence of GNRH respectively. The average decrease in *Gata4* mRNA levels using siRNAs directed against *Gata4* was 67 ± 2 and $70 \pm 2\%$ in absence and presence of GNRH respectively (data not shown). We have previously demonstrated a 60–70% decrease in GATA protein expression using an identical protocol (Lo *et al.* 2011).

Figure 5

GATA significantly increases GNRH-mediated stimulation of *Adcyap1* promoter activity via the -191 GATA *cis*-element. Gonadotrope L β T2 cells were transiently transfected with GATA2 (A) or GATA4 (B) expression vectors and sequential 5'-truncations of the rat *Adcyap1* gene promoter fused to a luciferase reporter followed by treatment with 100 nM GNRH or vehicle for 6 h. L β T2 cells were transfected with GATA2 (C) or GATA4 (D) and either the WT -1916/+906 PACAP reporter vector or a construct containing a mutation in the -191 GATA site followed by treatment with 100 nM GNRH or vehicle for 6 h. * $P < 0.001$ vs constructs treated with GNRH alone. All experiments were performed a minimum of three times with data expressed as the mean $\pm$ S.E.M.

Discussion

Initially described as a hypothalamic neuropeptide, ADCYAP1 is now known to be expressed in a wide range of tissues in which it exerts multiple physiologic effects. Despite growing interest in this peptide, surprisingly little is known about the transcription factors and associated DNA-regulatory regions which mediate expression of the *Adcyap1* gene. We have previously reported that GNRH stimulates pituitary *Adcyap1* promoter activity via CREB and the AP1 proteins (Grafer *et al.* 2009). We now add GATA to the list of transcription factors known to play a critical role in basal and GNRH-stimulated expression of the *Adcyap1* gene.

Although highly homologous, GATA family members have been observed to vary in their effects on specific gene promoters as well as their activity within individual cell types and across physiologic states. This functional specificity has been attributed to differences in expression levels of the individual GATA proteins, as well as differences in the expression of cofactors and other transcription factors with which GATA factors are known to interact. In line with these prior observations, our results demonstrate that while GATA2 and GATA4 both markedly increase rat *Adcyap1* promoter activity, subtle differences may exist in regards to the importance of specific nucleotide regions for achieving maximal stimulation. For example, deletion from nucleotide position -915 to -402 substantially decreased GATA4-mediated transactivation, but not GATA2, suggesting the presence of a *cis*-element in this region with specificity for GATA4 (Fig. 2). Characterization of this region will be of interest in future studies.

Our studies demonstrate that the CRE elements which flank the -191 GATA *cis*-element contribute to GATA-mediated stimulation of the *Adcyap1* gene promoter, suggesting the presence of complex interactions between CREB and GATA factors on this promoter. Hong *et al.* (2006) have reported that GATA3 can form protein-protein bonds with CREB and a similar physical interaction may reasonably be postulated to occur with GATA2 and GATA4. In addition, they demonstrated that mutation of the CRE site markedly blunted the ability of GATA to stimulate expression of the tyrosine hydroxylase gene. Similarly, mutation of the CRE sites in the *Adcyap1* promoter sequence blunts GATA-mediated stimulation. The proximity of the CRE and GATA sites suggests that the CREB and GATA proteins may bind to their respective DNA elements and to each other simultaneously to generate a maximal response. Of note, the EMSA

Figure 6

Effect of siRNA knockdown of GATA2 or GATA4 on GNRH-induced *Adcyap1* mRNA expression. LβT2 cells were transfected with 100 nM of a non-targeting siRNA (NTA) or with 100 nM of siRNA directed against GATA2 or GATA4 followed by treatment with 100 nM GNRH for 6 h. Mouse *Adcyap1* or siRNA target gene mRNA levels were quantified by qPCR. Results are expressed as percent relative to *Adcyap1* expression in the GNRH-treated NTA transfected cells and represent the results from three experiments. * $P < 0.001$ vs non-targeting control siRNA.

competition experiments did not suggest that binding by one factor was dependent on binding by the other, but subtle effects may be missed by this approach. Further studies with DNA-binding mutants and protein-dimerization mutants will be required to characterize the mechanism by which the CRE sites contribute to the GATA response.

Perhaps surprisingly, endogenous *Adcyap1* mRNA levels are very low in the LβT2 cell line despite the presence of significant amounts of GATA protein. This observation may be a reflection of the balance between stimulatory and inhibitory factors which regulate *Adcyap1* promoter activity. At this time, a limited number of transcription factors have been identified which act on this promoter and it is quite possible that these cells also express high levels of a yet unidentified inhibitory factor. Of interest, *Adcyap1* mRNA levels are much more readily detected in the αT3-1 gonadotrope cell line which is generally considered to be less differentiated than the LβT2 cell line, suggesting gain of an inhibitory factor with pituitary development (Grafer *et al.* 2009).

As shown in Fig. 5, GNRH augments GATA-mediated stimulation of *Adcyap1* gene promoter activity. Multiple mechanisms can be postulated for this interaction of which the simplest would be a GNRH-mediated increase in GATA protein expression. We have performed preliminary experiments which suggest that 6 h of GNRH treatment does not alter GATA4 protein expression and may actually decrease GATA2 protein levels in LβT2 cells (data not

shown). Kamesaki *et al.* (1996) observed a decrease in GATA1 protein and simultaneous increase in GATA2 protein expression in an erythroid cell line following activation of the PKC system with phorbol ester treatment. As increase in PKC activity results in an arrest in erythroid cell differentiation, they postulated that this quantitative switch in the ratio of GATA proteins was critical for the regulation of genes which mediate differentiation. Our dose-response data suggest that GATA4 may be more active than GATA2 at lower concentrations, albeit with caveats regarding possible differences in protein expression between constructs. Therefore, it is tempting to speculate that a decrease in GATA2 would allow for greater access of the more transcriptionally active GATA4 to the -191 GATA site.

GATA proteins also undergo hormonally mediated post-translational modifications, including acetylation and phosphorylation, which most commonly increase their functional activity (Towatari *et al.* 1995, Tremblay & Viger 2003, Hayakawa *et al.* 2004, Wang *et al.* 2005). It has been reported that GATA proteins can be phosphorylated following the activation of the PKA, PKC, or MAPK intracellular signaling systems, all pathways known to be activated by GNRH treatment (Liu *et al.* 2002, Fowkes *et al.* 2003, Haisenleder *et al.* 2003, Coss *et al.* 2007). Using two gonadotrope cell lines, αT3-1 and LβT2, Fowkes *et al.* (2002) demonstrated an increase in GATA binding to a consensus GATA nucleotide probe following activation of the MAPKK (MEK) signaling system; however, it was not determined whether this was due to an increase in GATA protein levels or alteration in DNA-binding affinity. It is therefore possible that GNRH may increase transcriptional activity of one or both of the GATA proteins via post-transcriptional modifications rather than substantial changes in protein expression.

We have previously demonstrated that GNRH-mediated increases in rat *Adcyap1* gene promoter activity depend, in large part, on the presence of an intact AP1-like site at position -275 as well as a CRE-like *cis*-element at position -205 (Grafer *et al.* 2009). As a general rule, the AP1 family of jun and fos proteins is expressed at low levels before hormonal stimulation, while transcriptional activity of the constitutively expressed CREB family members is modulated by phosphorylation state. Interestingly, GNRH treatment leads to the recruitment of the AP1 proteins, jun and fos, to both of these sites without detectable increases in phospho-CREB binding. These sites may be better described as composite AP1/CRE sites. The ability of the -179 CRE site to bind AP1 proteins was not investigated as the mutation only modestly blunted the

GNRH response. In any case, GATA family members have been shown to functionally interact with both AP1 family members and CREB proteins (Kawana *et al.* 1995, Hong *et al.* 2006). We, therefore, postulate a complex interaction between GATA proteins and both AP1 and CREB proteins acting at multiple sites in the proximal *Adcyap1* gene promoter.

In summary, the data presented here demonstrate a central role for both GATA2 and GATA4 in mediating *Adcyap1* gene expression in pituitary gonadotropes. Although we focused on a pituitary cell model, our finding may also be relevant to other cell types. For example, expressions of both ADCYAP1 (Gras *et al.* 1996, Scaldaferrri *et al.* 1996, Shioda *et al.* 1996, Lee *et al.* 1999) and GATA transcription factors (LaVoie 2003) have also been demonstrated in ovarian granulosa cells and theca-interstitial cells. We propose that GATA transcription factors play an instrumental role in the regulation of *Adcyap1* gene expression, and therefore, normal reproductive function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Institutes of Health Grant R01 HD054782 (to L M H).

Acknowledgements

We thank Drs Karen Braas and Victor May, who were instrumental in the development of the rat *Adcyap1* promoter constructs that launched these studies.

References

- Apostolakis EM, Lanz R & O'Malley BW 2004 Pituitary adenylate cyclase-activating peptide: a pivotal modulator of steroid-induced reproductive behavior in female rodents. *Molecular Endocrinology* **18** 173–183. (doi:10.1210/me.2002-0386)
- Arimura A & Shioda S 1995 Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Frontiers in Neuroendocrinology* **16** 53–88. (doi:10.1006/frne.1995.1003)
- Burrin JM, Aylwin SJ, Holdstock JG & Sahye U 1998 Mechanism of action of pituitary adenylate cyclase-activating polypeptide on human glycoprotein hormone α -subunit transcription in α T3-1 gonadotropes. *Endocrinology* **139** 1731–1737. (doi:10.1210/en.139.4.1731)
- Cheng KW & Leung PC 2001 Human gonadotropin-releasing hormone receptor gene transcription: up-regulation by 3',5'-cyclic adenosine monophosphate/protein kinase A pathway. *Molecular and Cellular Endocrinology* **181** 15–26. (doi:10.1016/S0303-7207(01)00480-4)
- Coss D, Hand CM, Yaphockun KK, Ely HA & Mellon PL 2007 p38 mitogen-activated protein kinase is critical for synergistic induction of the FSH(β) gene by gonadotropin-releasing hormone and activin through augmentation of c-Fos induction and Smad phosphorylation. *Molecular Endocrinology* **21** 3071–3086. (doi:10.1210/me.2007-0247)
- Culler MD & Paschall CS 1991 Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. *Endocrinology* **129** 2260–2262. (doi:10.1210/endo-129-4-2260)
- Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK & Rosenfeld MG 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. *Cell* **97** 587–598. (doi:10.1016/S0092-8674(00)80770-9)
- Ferris HA, Walsh HE, Stevens J, Fallest PC & Shupnik MA 2007 Luteinizing hormone β promoter stimulation by adenylyl cyclase and cooperation with gonadotropin-releasing hormone 1 in transgenic mice and L β T2 Cells. *Biology of Reproduction* **77** 1073–1080. (doi:10.1095/biolreprod.107.064139)
- Fowkes RC, King P & Burrin JM 2002 Regulation of human glycoprotein hormone α -subunit gene transcription in L β T2 gonadotropes by protein kinase C and extracellular signal-regulated kinase 1/2. *Biology of Reproduction* **67** 725–734. (doi:10.1095/biolreprod67.3.725)
- Fowkes RC, Sidhu KK, Sosabowski JK, King P & Burrin JM 2003 Absence of pituitary adenylate cyclase-activating polypeptide-stimulated transcription of the human glycoprotein α -subunit gene in L β T2 gonadotropes reveals disrupted cAMP-mediated gene transcription. *Journal of Molecular Endocrinology* **31** 263–278. (doi:10.1677/jme.0.0310263)
- Fujii Y, Okada Y, Moore JP Jr, Dalkin AC & Winters SJ 2002 Evidence that PACAP and GnRH down-regulate follicle-stimulating hormone- β mRNA levels by stimulating follistatin gene expression: effects on folliculostellate cells, gonadotropes and L β T2 gonadotroph cells. *Molecular and Cellular Endocrinology* **192** 55–64. (doi:10.1016/S0303-7207(02)00109-0)
- Grafer CM, Thomas R, Lambrakos L, Montoya I, White S & Halvorson LM 2009 GnRH stimulates expression of PACAP in the pituitary gonadotropes via both the PKA and PKC signaling systems. *Molecular Endocrinology* **23** 1022–1032. (doi:10.1210/me.2008-0477)
- Gras S, Hannibal J, Georg B & Fahrenkrug J 1996 Transient periovulatory expression of pituitary adenylate cyclase activating peptide in rat ovarian cells. *Endocrinology* **137** 4779–4785. (doi:10.1210/en.137.11.4779)
- Ha CM, Kang JH, Choi EJ, Kim MS, Park JW, Kim Y, Choi WS, Chun SY, Kwon HB & Lee BJ 2000 Progesterone increases mRNA levels of pituitary adenylate cyclase-activating polypeptide (PACAP) and type I PACAP receptor (PAC1) in the rat hypothalamus. *Brain Research. Molecular Brain Research* **78** 59–68. (doi:10.1016/S0169-328X(00)00070-X)
- Haisenleder DJ, Ferris HA & Shupnik MA 2003 The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is mediated by calcium/calmodulin-dependent protein kinase type II. *Endocrinology* **144** 2409–2416. (doi:10.1210/en.2002-0013)
- Hart GR, Gowing H & Burrin JM 1992 Effects of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, on pituitary hormone release in rats. *Journal of Endocrinology* **134** 33–41. (doi:10.1677/joe.0.1340033)
- Hayakawa F, Towatari M, Ozawa Y, Tomita A, Privalsky ML & Saito H 2004 Functional regulation of GATA-2 by acetylation. *Journal of Leukocyte Biology* **75** 529–540. (doi:10.1189/jlb.0603289)
- Heinzlmann A, Kirilly E, Meltzer K, Szabo E, Baba A, Hashimoto H & Kovacs K 2008 PACAP is transiently expressed in anterior pituitary gland of rats: *in situ* hybridization and cell immunoblot assay studies. *Peptides* **29** 571–577. (doi:10.1016/j.peptides.2007.12.009)

- Hong SJ, Huh Y, Chae H, Hong S, Lardaro T & Kim KS 2006 GATA-3 regulates the transcriptional activity of tyrosine hydroxylase by interacting with CREB. *Journal of Neurochemistry* **98** 773–781. (doi:10.1111/j.1471-4159.2006.03924.x)
- Jin L, Tsumanuma I, Ruebel KH, Bayliss JM & Lloyd RV 2001 Analysis of homogeneous populations of anterior pituitary folliculostellate cells by laser capture microdissection and reverse transcription-polymerase chain reaction. *Endocrinology* **142** 1703–1709. (doi:10.1210/en.142.5.1703)
- Kamesaki H, Michaud GY, Irving SG, Suwabe N, Kamesaki S, Okuma M & Cossman J 1996 TPA-induced arrest of erythroid differentiation is coupled with downregulation of GATA-1 and upregulation of GATA-2 in an erythroid cell line SAM-1. *Blood* **87** 999–1005.
- Kanasaki H, Mutiara S, Oride A, Purwana IN & Miyazaki K 2009 Pulse frequency-dependent gonadotropin gene expression by adenylate cyclase-activating polypeptide 1 in perfused mouse pituitary gonadotroph L β T2 cells. *Biology of Reproduction* **81** 465–472. (doi:10.1095/biolreprod.108.074765)
- Kanasaki H, Purwana IN, Mijiddorj T, Oride A & Miyazaki K 2011 Possible involvement of PACAP and PACAP type 1 receptor in GnRH-induced FSH β -subunit gene expression. *Regulatory Peptides* **167** 227–232. (doi:10.1016/j.regpep.2011.02.010)
- Kanasaki H, Purwana IN & Miyazaki K 2013 Possible role of PACAP and its PAC1 receptor in the differential regulation of pituitary LH β - and FSH β -subunit gene expression by pulsatile GnRH stimulation. *Biology of Reproduction* **88** 35. (doi:10.1095/biolreprod.112.105601)
- Katayama T, Nakashima M, Kyan H, Murakami N & Kuroda H 2000 A role of pituitary adenylate cyclase activating polypeptide (PACAP) as a regulator of paracrine interactions between folliculo-stellate cells and gonadotropes through the control of activin–folliculin interactions. *Journal of Veterinary Medical Science* **62** 731–736. (doi:10.1292/jvms.62.731)
- Kawana M, Lee ME, Quertermous EE & Quertermous T 1995 Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. *Molecular and Cellular Biology* **15** 4225–4231.
- Ko C, In YH & Park-Sarge OK 1999 Role of progesterone receptor activation in pituitary adenylate cyclase activating polypeptide gene expression in rat ovary. *Endocrinology* **140** 5185–5194. (doi:10.1210/en.140.11.5185)
- Koves K, Kantor O, Scammell JG & Arimura A 1998 PACAP colocalizes with luteinizing and follicle-stimulating hormone immunoreactivities in the anterior lobe of the pituitary gland. *Peptides* **19** 1069–1072. (doi:10.1016/S0196-9781(98)00049-7)
- Koves K, Kantor O, Molnar J, Heinzlmann A, Szabo E, Szabo F, Nemeskeri A, Horvath J & Arimura A 2003 The role of PACAP in gonadotropic hormone secretion at hypothalamic and pituitary levels. *Journal of Molecular Neuroscience* **20** 141–152. (doi:10.1385/JMN:20:2:141)
- LaVoie HA 2003 The role of GATA in mammalian reproduction. *Experimental Biology and Medicine* **228** 1282–1290.
- Lee J, Park HJ, Choi HS, Kwon HB, Arimura A, Lee BJ, Choi WS & Chun SY 1999 Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (PACAP) messenger ribonucleic acid in the rat ovary and the role of PACAP as a follicle survival factor. *Endocrinology* **140** 818–826. (doi:10.1210/en.140.2.818)
- Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM & Webster NJ 2002 Involvement of both Gq(11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L β T2 cells. *Journal of Biological Chemistry* **277** 32099–32108. (doi:10.1074/jbc.M203639200)
- Lo A, Zheng W, Gong Y, Crochet JR & Halvorson LM 2011 GATA transcription factors regulate LH β gene expression. *Journal of Molecular Endocrinology* **47** 45–58. (doi:10.1530/JME-10-0137)
- Merika M & Orkin SH 1993 DNA-binding specificity of GATA family transcription factors. *Molecular and Cellular Biology* **13** 3999–4010.
- Moore JP Jr, Burger LL, Dalkin AC & Winters SJ 2005 Pituitary adenylate cyclase activating polypeptide messenger RNA in the paraventricular nucleus and anterior pituitary during the rat estrous cycle. *Biology of Reproduction* **73** 491–499. (doi:10.1095/biolreprod.105.041624)
- Morelli MB, Barberi M, Gambardella A, Borini A, Cecconi S, Cotichio G & Canipari R 2008 Characterization, expression, and functional activity of pituitary adenylate cyclase-activating polypeptide and its receptors in human granulosa–luteal cells. *Journal of Clinical Endocrinology and Metabolism* **93** 4924–4932. (doi:10.1210/jc.2007-2621)
- Nakagawa R, Sakai Y, Takashima A, Terada T, Kobayashi A & Maeda M 2001 GATADNA-binding protein expressed in mouse I-10 Leydig testicular tumor cells. *Biochemical and Biophysical Research Communications* **283** 412–416. (doi:10.1006/bbrc.2001.4802)
- Ngan ES, Leung PC & Chow BK 2001 Interplay of pituitary adenylate cyclase-activating polypeptide with a silencer element to regulate the upstream promoter of the human gonadotropin-releasing hormone receptor gene. *Molecular and Cellular Endocrinology* **176** 135–144. (doi:10.1016/S0303-7207(01)00402-6)
- Osuga Y, Mitsushashi N & Mizuno M 1992 *In vivo* effect of pituitary adenylate cyclase activating polypeptide 38 (PACAP 38) on the secretion of luteinizing hormone (LH) in male rats. *Endocrinologia Japonica* **39** 153–156. (doi:10.1507/endocrj1954.39.153)
- Park JI, Kim WJ, Wang L, Park HJ, Lee J, Park JH, Kwon HB, Tsafiri A & Chun SY 2000 Involvement of progesterone in gonadotrophin-induced pituitary adenylate cyclase-activating polypeptide gene expression in pre-ovulatory follicles of rat ovary. *Molecular Human Reproduction* **6** 238–245. (doi:10.1093/molehr/6.3.238)
- Park JY, Park JH, Park HJ, Lee JY, Lee YI, Lee K & Chun SY 2001 Stage-dependent regulation of ovarian pituitary adenylate cyclase-activating polypeptide mRNA levels by GnRH in cultured rat granulosa cells. *Endocrinology* **142** 3828–3835. (doi:10.1210/en.142.9.3828)
- Perrin D, Soling HD, Wuttke W & Jarry H 1993 The stimulatory effect of pituitary adenylate cyclase activating polypeptide (PACAP) on LH release from rat pituitary cells *in vitro* does not involve calcium mobilization. *Experimental and Clinical Endocrinology* **101** 290–296. (doi:10.1055/s-0029-1211246)
- Pincas H, Amoyel K, Counis R & Laverriere JN 2001a Proximal cis-acting elements, including steroidogenic factor 1, mediate the efficiency of a distal enhancer in the promoter of the rat gonadotropin-releasing hormone receptor gene. *Molecular Endocrinology* **15** 319–337. (doi:10.1210/me.15.2.319)
- Pincas H, Laverriere JN & Counis R 2001b Pituitary adenylate cyclase-activating polypeptide and cyclic adenosine 3',5'-monophosphate stimulate the promoter activity of the rat gonadotropin-releasing hormone receptor gene via a bipartite response element in gonadotrope-derived cells. *Journal of Biological Chemistry* **276** 23562–23571. (doi:10.1074/jbc.M100563200)
- Purwana IN, Kanasaki H, Oride A, Mijiddorj T, Shintani N, Hashimoto H, Baba A & Miyazaki K 2010 GnRH-induced PACAP and PAC1 receptor expression in pituitary gonadotrophs: a possible role in the regulation of gonadotropin subunit gene expression. *Peptides* **31** 1748–1755. (doi:10.1016/j.peptides.2010.05.012)
- Radleff-Schlimme A, Leonhardt S, Wuttke W & Jarry H 1998 Evidence for PACAP to be an autocrine factor on gonadotrope cells. *Annals of the New York Academy of Sciences* **865** 486–491. (doi:10.1111/j.1749-6632.1998.tb11222.x)
- Robert NM, Miyamoto Y, Taniguchi H & Viger RS 2006 LRH-1/NR5A2 cooperates with GATA factors to regulate inhibin α -subunit promoter activity. *Molecular and Cellular Endocrinology* **257–258** 65–74. (doi:10.1016/j.mce.2006.06.011)
- Scaldeferri L, Arora K, Lee SH, Catt KJ & Moretti C 1996 Expression of PACAP and its type-I receptor isoforms in the rat ovary. *Molecular and Cellular Endocrinology* **117** 227–232. (doi:10.1016/0303-7207(95)03752-7)
- Schomerus E, Poch A, Bunting R, Mason WT & McArdle CA 1994 Effects of pituitary adenylate cyclase-activating polypeptide in the pituitary: activation of two signal transduction pathways in the gonadotrope-derived α T3-1 cell line. *Endocrinology* **134** 315–323. (doi:10.1210/en.134.1.315)

- Sherwood NM, Krueckl SL & McRory JE 2000 The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocrine Reviews* **21** 619–670. (doi:10.1210/er.21.6.619)
- Shioda S, Nakai Y, Nakajo S, Nakaya K & Arimura A 1996 Localization of pituitary adenylate cyclase-activating polypeptide and its type I receptors in the rat ovary: immunohistochemistry and *in situ* hybridization. *Annals of the New York Academy of Sciences* **805** 677–683. (doi:10.1111/j.1749-6632.1996.tb17540.x)
- Steger DJ, Hecht JH & Mellon PL 1994 GATA-binding proteins regulate the human gonadotropin α -subunit gene in the placenta and pituitary gland. *Molecular and Cellular Biology* **14** 5592–5602.
- Szabo E, Nemeskeri A, Heinzlmann A, Suzuki N, Arimura A & Kovacs K 2002 Cell immunoblot assay study demonstrating the release of PACAP from individual anterior pituitary cells of rats and the effect of PACAP on LH release. *Regulatory Peptides* **109** 75–81. (doi:10.1016/S0167-0115(02)00186-6)
- Towatari M, May GE, Marais R, Perkins GR, Marshall CJ, Cowley S & Enver T 1995 Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. *Journal of Biological Chemistry* **270** 4101–4107. (doi:10.1074/jbc.270.8.4101)
- Tremblay JJ & Viger RS 2003 Transcription factor GATA-4 is activated by phosphorylation of serine 261 via the cAMP/protein kinase a signaling pathway in gonadal cells. *Journal of Biological Chemistry* **278** 22128–22135. (doi:10.1074/jbc.M213149200)
- Tsujii T & Winters SJ 1995 Effects of pulsatile pituitary adenylate cyclase activating polypeptide (PACAP) on gonadotropin secretion and subunit mRNA levels in perfused rat pituitary cells. *Life Sciences* **56** 1103–1111. (doi:10.1016/0024-3205(95)00047-A)
- Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A & Vaudry H 2000 Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological Reviews* **52** 269–324.
- Viger RS, Guittot SM, Anttonen M, Wilson DB & Heikinheimo M 2008 Role of the GATA family of transcription factors in endocrine development, function, and disease. *Molecular Endocrinology* **22** 781–798. (doi:10.1210/me.2007-0513)
- Wang J, Paradis P, Aries A, Komati H, Lefebvre C, Wang H & Nemer M 2005 Convergence of protein kinase C and JAK–STAT signaling on transcription factor GATA-4. *Molecular and Cellular Biology* **25** 9829–9844. (doi:10.1128/MCB.25.22.9829-9844.2005)
- Winters SJ, Tsujii T & Attardi B 1996 Effects of GnRH and PACAP on gonadotropin secretion and subunit messenger RNAs. *Annals of the New York Academy of Sciences* **805** 343–352 (discussion 352–354). (doi:10.1111/j.1749-6632.1996.tb17495.x)

Received in final form 15 August 2013

Accepted 6 September 2013

Accepted Preprint published online 9 September 2013