The cAMP signaling system regulates LHβ gene expression: roles of early growth response protein-1, SP1 and steroidogenic factor-1

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Abstract

Expression of the gonadotropin genes has been shown to be modulated by pharmacological or physiological activators of both the protein kinase C (PKC) and the cAMP second messenger signaling pathways. Over the past few years, a substantial amount of progress has been made in the identification and characterization of the transcription factors and cognate cis-elements which mediate the PKC response in the LH β-subunit (LHβ) gene. In contrast, little is known regarding the molecular mechanisms which mediate cAMP-mediated regulation of this gene. Using pituitary cell lines, we now demonstrate that rat LHβ gene promoter activity is stimulated following activation of the cAMP system by the adenylate cyclase activating agent, forskolin, or by the peptide, pituitary adenylate cyclase-activating peptide. The forskolin response was eliminated with mutation of a previously identified 3′ cis-acting element for the early growth response protein-1 (Egr-1) when evaluated in the context of region −207/+5 of the LHβ gene. Activation of the cAMP system increased Egr-1 gene promoter activity, Egr-1 protein levels and Egr-1 binding to the LHβ gene promoter, supporting the role of this transcription factor in mediating the cAMP response. Analysis of a longer LHβ promoter construct (−797/+5) revealed additional contribution by upstream Sp1 DNA-regulatory regions. Of interest, forskolin-induced stimulation of LHβ gene promoter activity was observed to increase synergistically with introduction of the transcription factor, steroidogenic factor-1 (SF-1). Although SF-1 is a critical mediator of the cAMP response in other genes, mutation of the SF-1 DNA-binding sites in the rat LHβ gene did not alter the forskolin response nor did forskolin increase SF-1 protein levels in a gonadotrope cell line. In a further set of experiments, it was determined that forskolin-responsiveness was maintained following mutation of the previously defined homeobox-binding element at position −100. We conclude that both Egr-1 and Sp1 contribute to cAMP-dependent transcription of the rat LHβ gene promoter. While SF-1 does not act independently to mediate the cAMP/PKA response, SF-1 is important for magnification of this response.


Introduction

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are critical mediators of sexual development and adult reproductive function. LH and FSH consist of a common α-subunit linked non-covalently to one of two unique β-subunits (LHβ and FSHβ respectively). As would be predicted by their central role in reproductive physiology, expression of the gonadotropin genes is tightly regulated at both the biosynthetic and secretory levels through the complex interaction of hypothalamic, pituitary and gonadally derived factors. These factors, in turn, activate an array of intracellular signaling systems. The hypothalamic factor gonadotropin-releasing hormone (GnRH) has been widely recognized to alter gonadotropin biosynthesis via activation of both the protein kinase C (PKC) and calcium pathways (Stojilkovic et al. 1994, Garrel et al. 1997, Saunders et al. 1998, Weck et al. 1998). Recent evidence suggests that GnRH may also stimulate
PKA regulation of LHβ gene promoter

the cAMP/protein kinase A (PKA) signaling system (Stanislaus et al. 1994, Liu et al. 2002). Gonadotropin gene expression is further modulated by additional hypothalamic peptides, including pituitary adenylate-cyclase activating peptide (PACAP). PACAP is a member of the vasoactive intestinal peptide/secretin/glucagon family of peptides. As its name suggests, PACAP activates the cAMP/PKA system by binding to specific cell-surface G-protein coupled receptors (Miyata et al. 1989, Schomerus et al. 1994, Tsuji et al. 1994).

A number of studies have pointed towards a role for the cAMP/PKA system in modulating expression of the LHβ gene. In early investigations, pharmacological activation of the adenylate cyclase system was shown to increase steady-state LHβ mRNA levels (Starzec et al. 1986, 1989). This cAMP-induced increase was blocked by the transcriptional inhibitor, actinomycin D, consistent with a role for Egr-1 in mediating this site(s) (Kaiser et al. 1998). Egr-1 gene expression is markedly increased by GnRH or phorbol ester treatment of pituitary cell lines, which form DNA-regulatory pairs with the SF-1 cis-elements, also known as the gonadotrope-specific elements (GSEs) (Lee et al. 1996, Halvorson et al. 1998, Wolfe & Call 1999). Egr-1 gene expression may also be increased by activation of the cAMP/PKA signaling system as suggested by studies in the ovary, as well as non-reproductive systems (Bernal-Mizrachi et al. 2000, Espey et al. 2000, Tai et al. 2001).

The transcription factor, Sp1, likewise has been implicated in both basal and GnRH-stimulated expression of the LHβ gene through action at its two binding sites located 5′ to the SF-1 and Egr-1 cis-acting elements (Kaiser et al. 1998a, Weck et al. 2000). Sp1 binds to GC-rich sequences, which are similar to, but distinct from, the consensus Egr-1 cis-element (Berg 1992). Constitutively expressed in a wide range of cell types, Sp1 is best known as a

Figure 1 Schematic of identified and putative DNA-regulatory elements in the rat LHβ gene promoter.

regulator of basal gene transcription; however, cAMP-induced transcription of the CYP11A gene (P450 scc) has been attributed to the presence of an Sp1 binding site in addition to a GSE (Begeot et al. 1993, Venepally & Waterman 1995, Liu & Simpson 1997). It is not known if Sp1 plays a similar role in mediating the cAMP/PKA response in the LHβ gene.

SF-1, also known as NR5A1, also has been shown to be critical for gonadotropin gene expression. An orphan member of the nuclear hormone receptor superfamily, SF-1/NR5A1 is expressed in the gonadotrope subpopulation of the anterior pituitary gland as well as in the gonads and adrenal gland (Ingraham et al. 1994, Luo et al. 1994). The SF-1 DNA-regulatory region, or GSE, resembles a nuclear hormone receptor half-site and is present as two copies in the LHβ gene promoter (Barnhart & Mellon 1994, Halvorson et al. 1998, Wolfe 1999). SF-1 has been implicated in mediating cAMP/PKA responses in a wide variety of gonadal and adrenal genes, including the CYP19 (aromatase), CYP11A (P450 scc), CYP17 and StAR genes (Lynch et al. 1993, Clemens et al. 1994, Michael et al. 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Chau et al. 1997, Liu & Simpson 1997, Jacob & Lund 1998, Sandhoff et al. 1998). Thus, SF-1, Egr-1 and Sp1 potentially may all play a role in mediating cAMP-induced expression of the LHβ gene.

The homeobox cis-element located at position −100 in the LHβ gene promoter also deserves consideration as a potential modulator of cAMP-induced LHβ gene transcription. This DNA-regulatory region has been shown to bind members of the Ptx (pituitary homeobox) and Otx (orthodenticle-related homeobox) families of homeobox proteins (Tremblay et al. 1998, Rosenberg & Mellon 2002). Ptx1 is required for normal anterior pituitary development as well as for the expression of a broad array of pituitary-specific genes (Lamonerie et al. 1996, Lanctot et al. 1997, Tremblay & Drouin 1999, Quirk et al. 2001). Ptx1 acts in synergy with Egr-1 and SF-1 to generate GnRH-induced transcriptional activity of the LHβ gene; however, this effect is thought to be the result of Ptx1 interaction with modified transcription factors, particularly Egr-1, rather than through alteration of Ptx1 gene expression itself (Tremblay & Drouin 1999). LHβ gene expression also has been demonstrated to be regulated by Otx1 and an Otx-related factor may be required for gonadotrope maturation (Acampora et al. 1998, Tremblay et al. 1998, 1999, Rosenberg & Mellon 2002). Thus, while clearly critical for gonadotrope function, it is unknown whether these homeobox proteins are able to directly mediate hormonal responsiveness.

In summary, substantial progress has been made in the identification of basal and GnRH/PKCl/α-adrenergic-responsive elements in the LHβ gene promoter. Limited data from prior studies have suggested that the cAMP/PKA system may also mediate LHβ gene expression; however, this observation has not been evaluated at the molecular level. In the present study, we demonstrate that activation of the cAMP/PKA system through various methods does, in fact, increase rat LHβ gene promoter activity in a gonadotrope-derived cell line. We then investigate the transcriptional mechanisms which mediate this response with a focus on the previously identified LHβ DNA-regulatory elements for Egr-1, Sp1, SF-1 and Ptx1/Otx.

Materials and methods

Electrophoretic mobility shift assay (EMSA) analysis

The nucleotide sequence of the rat LHβ gene promoter is based on sequencing data available at GenBank accession number AF020505, which differs slightly from that of Jameson et al. (1984). Probes were created by T4 polynucleotide kinase end-labeling with [γ-32P]ATP followed by purification over a NICK column (Pharmacia Biotech, Uppsala, Sweden). A double-stranded oligonucleotide corresponding to region −67/−35 of the rat LHβ gene promoter was used to detect Egr-1 DNA-binding (Halvorson et al. 1998, 1999).

In vitro-translated Egr-1 was generated from 3·2 kb of the mouse Egr-1 cDNA (provided by D Nathans, Johns Hopkins University, Baltimore, MD) using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). The resultant product was determined to be of appropriate size by comparison with [35S]methionine-labeled protein markers using SDS-PAGE. The method of Andrews & Faller (1991) was used to prepare crude nuclear extracts from the mouse gonadotrope-derived cell line.
αT3–1, or the rat somatolactotrope cell line, GH₃, following treatment with vehicle or forskolin for 1 h. Protein samples were incubated with 50 000 c.p.m. of oligonucleotide probe in DNA-binding buffer (20 mM Hepes (pH 7-9), 60 mM KCl, 5 mM MgCl₂, 10 mM phenylmethylsulfonyl-fluoride, 10 mM dithiothreitol, 1 mg/ml BSA and 5% (v/v) glycerol) for 30 min on ice. Where indicated, 1 ml Egr-1 polyclonal antisera (sc-110; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added 30 min following the addition of probe and the incubation continued for 2 h. Protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0-5 × Tris–borate–EDTA buffer and subjected to autoradiography.

Plasmids used in transfection studies

The largest LHβ reporter construct used for these studies contained 794 bp of the 5′-flanking sequence of the rat LHβ gene and the first 5 bp of the 5′-untranslated region fused to a luciferase reporter gene, pXP2 (Nordeen 1988). Deletions in this construct were created by subcloning PCR products containing the LHβ promoter sequences into the pXP2 vector using BamHI/HindIII sites which were introduced by the primers (Kaiser et al. 1998b). Mutations were introduced into the LHβ promoter region using the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The SF-1, Egr-1 and Sp1, and Otx/Ptx mutations have been described previously and are known to eliminate DNA binding on EMSA (Kaiser et al. 1998)

To create GSE4-GH50, an oligonucleotide was designed containing four copies of the 5′-GSE site flanked by BamHI/BglII restriction sites (sense strand: 5′-GATCCCTTTCTGACCTTGTCTGTCTCGCCTCTGACCTTGTCTGT-3′ present as a tandem repeat). This oligonucleotide was inserted upstream of the minimal growth hormone promoter, GH50, in the pXP1 luciferase reporter plasmid (Nordeen 1988, Suen et al. 1994). All reporter constructs were confirmed by dideoxy-sequencing.

The SF-1 expression vector contains 2·1 kb of the mouse SF-1 cDNA driven by cytomegalovirus (CMV) promoter sequences in the vector, pCMV5 (provided by K L Parker, Southwestern University School of Medicine, Dallas, TX) (Lala et al. 1992). The ΔLBD-SF-1 expression vector lacks the ligand-binding domain of SF-1 and was created by excising the SalI-SalI region of the pCMV5-SF-1 vector. The CMV expression vectors encoding the wild-type and constitutively active G-protein α-subunit (GaS) protein were provided by R Iyengar, Mount Sinai School of Medicine, New York, NY (Chen & Iyengar 1994). The Egr-1 expression vector was created by cloning 3·2 kb of the mouse Egr-1 cDNA into pCMV5 at BamHI and HindIII restriction sites (Egr-1 cDNA provided by D Nathans, Johns Hopkins University, Baltimore, MD) (Christy et al. 1988). The Egr-luc reporter construct contains 1·2 kb of the mouse Egr-1 gene promoter sequence cloned into the SalI site of pXP2 (Egr-1 5′-flanking sequence provided by V Sukhatme, Harvard Medical School, Boston, MA) (Sukhatme et al. 1987). The SF-1-luc expression vector contains 1885 bp upstream of the mouse SF-1 transcriptional start site in the vector pLKSβ-LUC and was kindly supplied by Y Sadovsky, Washington University, St Louis, MO (Woodson et al. 1997).

Transfection experiments

Rat somatolactotrope GH₃ cells or mouse gonadotrope-derived LBT2 cells were cultured to 50–70% confluence in low glucose (GH₃) or high glucose (LBT2) Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). The LBT2 cell line was generously provided by P Mellon (University of California, San Diego, CA, USA) (Turgeon et al. 1996). For the GH₃ cell line, approximately 5 × 10⁶ cells were suspended in 0·4 ml Dulbecco’s PBS plus 5 mM glucose with the DNA to be transfected. The cells received a single electrical pulse of 240 V at a total capacitance of 1000 mF using an Invitrogen Electroporator II apparatus (Invitrogen). GH₃ cells received 1·5 µg/well of the reporter constructs. As indicated for the GH₃ cell line, cells also received 1 µg/well of the SF-1 expression vector or an equivalent amount of the appropriate ‘empty’ expression vector. For the LBT2 cell line, cells growing in 3·5 cm tissue culture wells were transfected with reporter plasmids (1·5 µg/well) using the calcium phosphate precipitation method. Co-transfection with an RSV-β-galactosidase plasmid (1 µg/well) allowed
correction for differences in transfection efficiency between wells in all experiments.

Cells were treated with vehicle, forskolin (2.5 µM) (Sigma, St Louis, MO, USA), or PACAP-38 (10 nM) (Calbiochem, La Jolla, CA, USA), for 4–6 h starting approximately 40 h following transfection. In a subset of experiments, cells were treated with the selective PKA inhibitor, H-89 (10 µM), or with the PKC inhibitor, GF 109203X (5 µM), starting 1 h prior to DMSO or forskolin treatment (inhibitors obtained through LC Laboratories, Woburn, MA, USA). Cells were then harvested and the cell extracts analyzed for luciferase and β-galactosidase activity (Edlund et al. 1985, deWet et al. 1987). Luciferase activity was normalized to the level of β-galactosidase activity and results calculated as fold-change relative to expression in the control wells. Data are shown as the means ± S.E.M. from three to ten independent experiments.

**Western blot analysis**

Nuclear extracts were obtained from LβT2 cells as described by Tremblay et al. (1998). Protein concentration were determined using the BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA). In vitro-translated Egr-1 and SF-1 were generated using the TNT Coupled Reticulocyte Lysate System (Promega) as described for EMSA. Nuclear proteins (5 µg) were resolved on a 10% SDS-PAGE in 25 mM Tris/250 mM glycine (TG) buffer. The protein was subsequently transferred onto 0.2 µM Protran nitrocellulose (Schleicher & Schuell, Keene, NH, USA) in TG buffer containing 20% methanol. The membrane was blocked overnight at 4 °C in 5% Carnation non-fat dry milk in TBS (10 mM Tris–HCl, 0.9% NaCl, pH 7.5) and then washed with TBS-T (TBS, 0.05% Tween-20). The blot was incubated overnight at 4 °C in 5% milk-TBS with a polyclonal antibody directed against SF-1 (Upstate Biotechnology, Inc., Lake Placid, NY, USA) or Egr-1 (Santa Cruz Biotechnology), diluted to 1:1000 or 1:8000 respectively. After washing with TBS-T, the blot was then incubated at room temperature for 1 h in 5% milk in TBS-T containing donkey anti-rabbit IgG-horseradish peroxidase diluted 1:1000 (Santa Cruz Biotechnology). The blot was washed with TBS-T and then TBS and developed using Western Blotting Luminol Reagent according to the manufacturer’s instructions (Santa Cruz Biotechnology).

**Statistical analysis**

ANOVA followed by comparisons with Student’s t-test was used to assess whether promoter activity was statistically different between the indicated groups. Statistical significance was set at the P<0.05 level.

**Results**

**Activation of the cAMP/PKA signaling system stimulates LHβ gene expression in two pituitary cell lines**

Transfection experiments were performed in two pituitary cell lines, a gonadotrope-derived cell line (LβT2) and a somatolactotropin cell line (GH3). LβT2 cells were transiently transfected with a reporter construct containing region −794/+5 of the rat LHβ gene promoter. Cells were treated with PACAP, a known physiological activator of the cAMP/PKA system in gonadotrope cells. As shown in Fig. 2A, PACAP treatment modestly, but significantly, increased LHβ gene promoter activity relative to control wells (2-fold). Although PACAP is best known for activation of the cAMP/PKA system, it also has been shown to stimulate the phospholipase C (PLC) and calcium pathways (Schomerus et al. 1994, Hezareh et al. 1996, Piseigna & Wank 1996, Bresson-Bepoldin et al. 1998). Therefore, in order to more specifically investigate the cAMP system, cells were treated with forskolin, a pharmacological activator of adenylate cyclase. As observed with PACAP treatment, luciferase activity was stimulated with the addition of forskolin (1.8-fold).

The ability of forskolin to augment LHβ gene promoter activity was also investigated in GH3 cells. GH3 cells have been used extensively to study gonadotropin gene regulation and have been found to provide qualitatively accurate observations with regards to both basal and hormonally regulated LHβ gene expression (Kim et al. 1990, Clayton et al. 1991, Kuphal et al. 1994, Stanislaus et al. 1994, Saunders et al. 1998, Pinter et al. 1999). This cell line lacks endogenous SF-1, thereby permitting independent analysis of the effects of signaling pathways and SF-1 (Kaiser et al. 1994, Halvorson et al. 1998).
As shown in Fig. 2B, LHβ gene promoter activity was significantly increased with forskolin treatment or in the presence of a constitutively active mutant form of the GαS protein (3- and 2-fold respectively). Co-transfection with the CMV-driven SF-1 expression vector resulted in a 15-fold increase in luciferase activity, consistent with previously published results (Halvorson et al. 1998). Of interest, LHβ promoter activity increased by nearly 50-fold in the presence of SF-1 and either forskolin or the mutant GαS, demonstrating synergy between SF-1 and cAMP signaling.

We next used pharmacological inhibitors in order to formally test the specificity of forskolin in our system. As shown in Fig. 2C, neither the PKA blocking agent, H-89, nor the PKC inhibitor, GF 109203X, altered basal LHβ-driven luciferase activity. However, the forskolin-stimulated increase in LHβ promoter activity was significantly blunted by H-89, but not by GF 109203X, consistent with a specific effect of forskolin on the cAMP/PKA activity.

The LHβ gene promoter contains two regions which contribute to the cAMP/PKA response

Sequential 5′-deletion constructs of the rat LHβ gene promoter were tested for forskolin-responsiveness. This analysis identified two regions which contribute to the forskolin response, the first

Figure 2 The cAMP/PKA system acts both alone and in synergy with SF-1 to increase LHβ gene promoter activity. (A) LβT2 gonadotrope cells were transiently transfected with −794/+5 rat LHβ gene promoter linked to a luciferase reporter construct, pXP2. Cells were treated for 4–6 h with PACAP-38 (10 nM), forskolin (2.5 μM) or the appropriate vehicle and harvested 48 h following transfection. Results are shown as the means±S.E.M. of eight experiments. *P<0.01 vs control. (B) GH3 cells were transiently transfected with region −207/+5 of the rat LHβ gene promoter linked to a luciferase reporter construct, pXP2. A subset of cells also received CMV-driven expression vectors encoding SF-1 and/or a constitutively active GαS protein. Control wells received equivalent amounts of ‘empty’ expression vector. All cells were co-transfected with an RSV-β-galactosidase vector. Approximately 40 h after transfection, cells were treated for 4–6 h with vehicle or with the adenylate cyclase activating agent, forskolin. Luciferase activity was normalized to β-galactosidase activity and promoter activity was calculated as fold-change over expression in the control wells. Results are shown as the means±S.E.M. of four independent experiments. *P<0.01 vs control; **P<0.05 vs SF-1 alone. (C) A luciferase reporter construct containing region −797/+5 of the rat LHβ gene promoter was transiently transfected into GH3 cells, followed by treatment with vehicle or 2.5 μM forskolin for 4–6 h starting 40 h after transfection. Where indicated, cells received the PKA inhibitor, H-89, or the PKC blocking agent GF 109203X, beginning 1 h prior to the addition of forskolin. Results are shown as the means±S.E.M. of three independent experiments. *P<0.001 vs forskolin; NS=not significant, P>0.05 vs control.
between positions −797 and −207 and the second between position −134 and the transcriptional start site (Fig. 3).

**Loss of the cAMP/PKA response with mutation of the Egr-1 binding sites**

Pharmacological or hormonal activation of the cAMP/PKA signaling system has been reported to increase Egr-1 gene expression (mRNA and/or protein levels) in the ovary and in pheochromocytoma and insulinoma cell lines (Bernal-Mizrachi et al. 2000, Espey et al. 2000, Tai et al. 2001). We hypothesized that cAMP-responsiveness of the LHβ gene may be mediated likewise by Egr-1 acting through previously identified Egr-1 cis-elements in this gene. As shown in Fig. 4, GH3 cells were transfected with a −207/+5 rat LHβ gene reporter construct present as the wild-type construct or with mutations in one or both of the previously characterized Egr-1 DNA cis-elements. Cells were treated as in Fig. 2. The results are presented relative to the forskolin response of the wild-type construct, which has been set at 100%. Data given as the means±S.E.M. of three independent experiments. *P<0.01 vs wild-type.

The forskolin response is not attributable to the presence of SF-1 or its cognate cis-elements (GSEs) in the LHβ gene promoter

The proximal forskolin-responsive region identified in Fig. 3 was also noted to contain previously characterized DNA-regulatory elements for the orphan nuclear receptor, SF-1. It is clear that SF-1 cannot be implicated in the ability of forskolin treatment alone to increase LHβ gene promoter activity in the SF-1-deficient GH3 cell line. Nevertheless, as ultimate interest is in the physiological regulation of gonadotrope function, it is critical to determine whether SF-1 and its associated cis-elements are involved in the observed cAMP-dependent transcription of the LHβ gene. SF-1 has been demonstrated to be crucial for mediating cAMP/PKA-induced increases in the

We therefore investigated whether the interaction between forskolin and SF-1 was altered with deletion of the SF-1 ligand-binding domain containing the AF-2 domain. As shown in Fig. 5A, the presence of the carboxy-truncated SF-1 construct (ΔLBD) markedly increased LHβ promoter activity, although to a lesser extent than observed with the native SF-1 (4- vs 9-fold respectively). The addition of forskolin further stimulated LHβ gene expression in the presence of either SF-1 construct (upper panel). The data were recalculated to evaluate whether the magnitude of the forskolin response differed depending on which SF-1 construct was present. As shown in the lower panel, the fold-change in LHβ promoter activity was not significantly different between constructs.

**Figure 5** Role of SF-1 and associated DNA-regulatory elements (GSEs) in mediating cAMP/PKA-responsiveness of the LHβ gene promoter. GH3 cells were transiently transfected with various rat LHβ gene promoter reporter constructs. Cells were co-transfected with CMV-driven SF-1 expression vectors or control ‘empty’ vectors as well as the RSV-β-galactosidase vector. Prior to harvesting, cells received 2-5 μM forskolin or vehicle for 4–6 h starting 40 h after transfection. Results are shown as the means±S.E.M. of three to eight experiments with each data point tested in triplicate. *P<0.001; NS=not significant, P>0.05. (A) Comparison of the forskolin (F) effect in the presence of CMV-driven expression vectors encoding the full-length vs a carboxy-truncated SF-1 mutant lacking the ligand binding domain (ΔLBD). Promoter activity was calculated as fold-change relative to control wells (upper panel). Alternatively, data was expressed as the response to forskolin treatment in the presence of the intact or the truncated SF-1 vs the response to forskolin treatment alone (lower panel). (B) PKA effects in a heterologous LHβ-GSE-promoter construct. GH3 cells were transiently transfected with a construct containing the growth hormone minimal promoter, GH50, linked to a luciferase reporter construct, pXP1, with or without the addition of four copies of the 5′-GSE site. (C) Maintenance of the forskolin response following mutation of the SF-1 binding sites (GSEs). GH3 cells were transfected with region −207/+5 of the rat LHβ gene promoter present as either the wild-type sequence or with mutations in both GSE sites.
(approximately 3-fold with either construct), suggesting that the observed synergy between SF-1 and the cAMP system is not dependent on alterations at the carboxy-terminus of the SF-1 molecule.

As an alternative approach, we reasoned that a forskolin response should be observed in the presence of SF-1 DNA-regulatory elements if, in fact, activation of the cAMP/PKA system is directly altering SF-1 functional activity. Four copies of the rat LHβ 5′-GSE sequence were inserted upstream of the growth hormone minimal promoter, GH50, in the pXP1 reporter vector. As seen in Fig. 5B, these sequences conferred an SF-1 response, but failed to confer a response to forskolin (10.5- and 11.8-fold for SF-1 alone and SF-1 plus forskolin respectively).

These results suggest that the cAMP/PKA effect is unlikely to be due to direct effects on the transcription factor, SF-1. This conclusion was confirmed by transfecting cells with a construct containing point mutations in both GSE sites within the context of the native rat LHβ gene promoter. These mutations have been shown to eliminate SF-1 DNA-binding and SF-1-induced transactivation (Halvorson et al. 1998). In contrast to the loss of the response to SF-1, forskolin-induced stimulation was conserved in the mutated construct (1.9- vs 1.8-fold in the wild-type construct) (Fig. 5C). These data strongly suggest that SF-1 and its cognate cis-element are not required for mediating the cAMP response in the rat LHβ gene promoter.

**Figure 6** Activation of the cAMP/PKA system induces Egr-1 gene expression. (A) Cells from the gonadotrope-derived cell line, LβT2, were treated with a vehicle control (lane C), 2.5 μM forskolin for 1 h (lane F1) or 4 h (lane F4), or with 100 ng/ml PMA for 1 h (lane P1). Nuclear proteins (5 μg) or in vitro-translated Egr-1 or SF-1 were then separated on 10% SDS-PAGE gels followed by chemiluminescent Western blot analysis. I.V., in vitro translated Egr-1. (B) Band intensity from (A) was quantified using a Kodak EDAS 290 Image Analysis station and calculated as fold-change relative to vehicle-treated cell extracts. (C) LβT2 or GH3 cells were transiently transfected with a luciferase reporter construct containing 1.2 kb of the 5′-flanking region of the Egr-1 gene. An RSV-β-galactosidase vector was co-transfected to control for transfection efficiency. Cells were treated with vehicle or 2.5 μM forskolin for 4 h prior to harvesting at 48 h post-transfection. Following normalization to β-galactosidase activity, promoter activity was calculated as fold-change in luciferase activity in treated vs untreated wells. Results are shown as the means±S.E.M. of five independent experiments. *P<0.001.
Activation of the cAMP/PKA signaling system induces Egr-1, but not SF-1, gene expression

Results from experiments presented in Figs 4 and 5 suggest that the cAMP/PKA signaling system acts via Egr-1, but not SF-1, to stimulate LHβ gene expression. In order to further evaluate these observations, we asked whether activation of the cAMP system altered Egr-1 and/or SF-1 protein expression in gonadotrope cells. As shown by Western blot analysis in Fig. 6A and B, treatment of gonadotrope LβT2 cells with 2.5 µM forskolin markedly increased Egr-1 protein levels, but did not alter SF-1 protein levels in this protocol. The observed induction of Egr-1 protein expression by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), is consistent with previous reports (Dorn et al. 1999).

The observed increase in Egr-1 protein levels may be due to increased Egr-1 biosynthesis and/or post-translational modification, resulting in increased protein stability. In order to determine whether activation of the cAMP pathway system altered Egr-1 biosynthesis, pituitary cell lines were transiently transfected with a luciferase reporter construct containing 1.2 kb of the Egr-1 gene promoter. Forskolin treatment increased Egr-1 promoter activity nearly 3-fold in the LβT2 cell line, with a 4-fold increase in the line GH3 cells (Fig. 6C). In contrast, SF-1 promoter activity was not regulated by forskolin in the GH3 cell line (data not shown).

We next used EMSA to determine whether increased Egr1 gene expression results in altered Egr-1 DNA-binding to the rat LHβ gene promoter. Nuclear extracts from untreated GH3 or gonadotrope αT3–1 cells formed a single dominant band with an oligonucleotide probe spanning the 3′′-Egr-1 site (Fig. 7B, lanes 2 and 7). Prior studies utilizing specific antibodies have demonstrated the presence of the transcription factor, Sp1, in this complex (Halvorson et al. 1999). The addition of forskolin (2.5 or 25 µM for 1 h) induced a faster migrating complex (lanes 3, 5, 8 and 10) that contained Egr-1 as shown by the addition of an Egr-1-specific antibody (lanes 4, 6, 9 and 11). The similar mobility of in vitro-translated Egr-1 (lane 1) was consistent with the presence of Egr-1 in the treated extracts.

Taken together, these results suggest that the cAMP signaling system induces Egr-1 transactivation capability, at least in part, via increased biosynthesis of the Egr-1 gene.

Sp1 DNA-binding sites contribute to the full forskolin response of the −797/+5LHβ gene promoter region

We then tested the importance of the Egr-1 cis-elements for cAMP/PKA-responsiveness in the context of a longer region of the rat LHβ gene 5′-flanking sequence (−797/+5). As shown in Fig. 8, the forskolin effect was significantly blunted with the introduction of point mutations into the Egr-1 sites. Nevertheless, a substantial response remained in the mutant promoter constructs suggesting the presence of a non-Egr-1, PKA-responsive regulatory region in the more distal LHβ gene promoter.

Figure 7 Forskolin treatment induces Egr-1 binding to rat LHβ gene promoter sequences. Activation of the cAMP/PKA system induces Egr-1 gene expression. EMSA was performed using in vitro-translated Egr-1 (lane 1) or nuclear extracts from GH3 cells (left panel) or from the gonadotrope-derived αT3–1 cell line (right panel). Nuclear extracts were derived from cells maintained in the absence or presence of forskolin (2.5 or 25 µM for 1 h). Region −67/−35 of the rat LHβ gene promoter containing the 3′′-Egr-1-binding site was 32P labeled and used as a probe. Where indicated, antiserum specific for Egr-1 was added 2 h prior to electrophoresis. NE=nuclear extract; * indicates Egr-1-containing complex.
Region −451/−386 of the rat LHβ gene promoter has been shown to contain two binding sites for the ubiquitous transcription factor, Sp1. These sites have been shown to contribute to both basal and GnRH-mediated LHβ gene expression (Kaiser et al. 1998a). Mutation in the LHβ-Sp1 sites significantly decreased the forskolin response, with further blunting in a construct containing mutations in both the Sp1 and Egr-1 cis-elements. These data suggest that both Sp1 and Egr-1 contribute to the full cAMP response of the −797/+5 rat LHβ gene promoter.

Mutation of the homeodomain cis-element does not alter cAMP-responsiveness in the rat LHβ gene

As shown in Fig. 8, a small residual cAMP-response was noted in the −797/+5 rat LHβ gene promoter construct despite mutation of both the Sp1 and Egr-1 cis-elements. These results suggested a contribution by a third DNA-regulatory region. In an attempt to identify this site, we analyzed a previously identified homeodomain cis-element located at position −100 in the rat LHβ gene promoter. Mutation of this site did not alter the magnitude of the forskolin response, arguing against a role for Ptx/Otx-related proteins in mediating this response (data not shown).

Discussion

LHβ gene expression is regulated by the complex interaction of hypothalamic, pituitary and gonadal factors, which in turn activate a variety of intracellular signaling systems. GnRH-induced stimulation of the PKC system has been widely recognized and intensively investigated (Kaiser et al. 1998b, Halvorson et al. 1999, Wolfe & Call 1999, Weck et al. 2000, Vasilyev et al. 2002). Studies have suggested that gonadotrope function is also mediated by physiological modulators of the cAMP/PKA system. The hypothalamic peptide, PACAP, is perhaps the best studied of these factors. PACAP has been shown to increase cAMP levels in both primary pituitary cells as well as in gonadotrope cell lines (Miyata et al. 1989, Schomerus et al. 1994). Interestingly, GnRH has recently been demonstrated to activate Gα protein and increase intracellular cAMP levels in the gonadotrope LβT2 cell line, confirming prior studies in GH3 cells stably expressing the GnRH receptor (Stanislaus et al. 1994, Liu et al. 2002). As reported by Ortmann et al. (1999) the gonadal steroid, estradiol, may also modulate adenylate cyclase activity. Furthermore, it is increasingly clear that crosstalk occurs between the PKC and PKA systems in the gonadotrope (McArdle et al. 1994, Garrel et al. 1997). Collectively, these data strongly support the physiological relevance of the cAMP/PKA pathway in regulating gene expression in gonadotrope cells.

In prior studies, PACAP has been shown to increase gonadotropin secretion and to modulate steady-state α-, LHβ- and FSHβ-subunit mRNA levels (McArdle et al. 1994, Tsujii & Winters 1995, Winters et al. 1997, Attardi & Winters 1998, Burrin et al. 1998, Fujii et al. 2002). We now demonstrate PACAP increases LHβ gene promoter activity in the gonadotrope-derived cell line, LβT2 (Fig. 2). These results suggest that increased transcriptional activity contributes to the observed PACAP-induced increase in LHβ mRNA levels. As previously noted, PACAP stimulates the PLC and calcium pathways in addition to increasing

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Figure 8 Both Egr-1 and Sp1 DNA-binding sites contribute to the full cAMP response of region −797/+5 of the LHβ gene promoter. GH3 cells were transiently transfected with region −797/+5 of the rat LHβ gene promoter present as the wild-type sequence or with mutations in the previously described Egr-1 and Sp1 cis-elements. Forskolin treatment and data calculation were as described in Fig. 5B and represent seven independent experiments. The results are presented relative to the forskolin-response of the wild-type construct, which has been set at 100%. * P<0.05 vs wild-type.
intracellular cAMP levels (Schomerus et al. 1994, Hezareh et al. 1996, Pisegna & Wank 1996, Bresson-Bepoldin et al. 1998). In order to focus our studies on the effects of the cAMP/PKA signaling system, we chose to utilize the specific adenylate cyclase activator, forskolin, for the remainder of our experiments.

Forskolin treatment stimulated LHβ gene promoter activity in both LβT2 and GH3 cells (Fig. 2). Forskolin increased Egr-1 promoter activity, protein levels, and Egr-1 binding to the LHβ gene promoter (Figs 6 and 7). Furthermore, mutation of the 3′-Egr-1 cis-element in the proximal LHβ gene promoter was observed to blunt the forskolin response (Fig. 4). Based on these data, we propose that cAMP/PKA-induced stimulation of LHβ promoter activity is achieved, in part, through induction of Egr-1 gene expression.

It should be noted that two other groups did not observe a forskolin-mediated increase in steady-state Egr-1 mRNA levels or Egr-1 gene promoter activity using the gonadotrope-derived αT3–1 cell line (Tremblay & Drouin 1999, Duan et al. 2002). Interestingly, one of these groups did observe that GnRH-induced increases in Egr-1 transcription and protein levels could be partially abrogated by the PKA blocking agent, H-89, results which support a role for cAMP/PKA in modulating Egr-1 gene function (Duan et al. 2002).

Egr-1 is a member of the immediate early gene family whose members are markedly regulated at the biosynthetic and post-translational level by a wide variety of growth and differentiation factors (Christy et al. 1988, Cao et al. 1990). Forskolin treatment has been shown to increase Egr-1 promoter and protein expression in insulinoma and pheochromocytoma cell lines respectively (Bernal-Mizrachi et al. 2000, Tai et al. 2001). Richards and co-workers (Espey et al. 2000) have shown that Egr-1 mRNA and protein levels are increased by human chorionic gonadotropin (hCG) treatment in ovarian granulosa cells, presumably due to LH receptor-mediated activation of the cAMP pathway. More recently, Yoshino et al. (2002) demonstrated that both hCG and 8-Br-cAMP increase Egr-1 mRNA levels in luteinized granulosa cells. Thus, current data now exist to suggest that cAMP modulates Egr-1 gene expression at multiple levels of the reproductive axis.

Mutation of the Sp1 DNA-binding sites further blunted the cAMP response in the context of the longer region of the rat LHβ gene promoter (Fig. 8). Constitutively expressed, Sp1 classically has been thought to act as a basal transcription factor. Nevertheless, recent studies have implicated Sp1 in the hormonal regulation of a variety of genes, including cAMP-induced transcription of the CYP17 and CYP11A (P450 scc) steroidalogenic genes (Begeot et al. 1993, Venepally & Waterman 1995, Borroni et al. 1997, Ahlgren et al. 1999). The precise mechanism by which Sp1 mediates the cAMP effect in the rat LHβ gene remains to be determined. In preliminary studies, forskolin treatment did not alter the intensity of endogenous Sp1 binding to LHβ-Sp1 sites, implying that Sp1 protein levels are not altered (data not shown). Sp1 is known to be modified by N- and O-linked glycosylation as well as by phosphorylation (Jackson et al. 1990). It has been proposed that these post-translational modifications may alter Sp1 interactions with other transcription factors, such as SF-1, and/or with specific co-activators including the cAMP-responsive co-factor, CBP/p300 (Liu & Simpson 1999).

Unlike the highly conserved Egr-1 and SF-1 sites, the Sp1 cis-elements are not conserved across species, suggesting that each species may utilize different combinations of transcription factors to achieve cAMP-dependent stimulation of the LHβ gene. Of note, a DNA-regulatory site for the CCAAT-binding factor NF-Y has been characterized in the bovine LHβ gene promoter in the region of the Sp1 sites of the rat gene (Keri et al. 2000). NF-Y has been shown to contribute to cAMP-dependent regulation of both the rat fatty acid synthase and TIMP-2 genes (Roder et al. 1997, Zhong et al. 2000). It would be of interest to evaluate whether the NF-Y cis-acting elements mediate cAMP-responsiveness in the bovine LHβ gene.

The orphan nuclear receptor SF-1 is known to alter LHβ gene expression independently, as well as through interactions with other transcription factors and with the PKC signaling system (Halvorson et al. 1996, 1998, 1999, Keri & Nilson 1996, Dorn et al. 1999, Wolfe 1999). We now demonstrate that the presence of SF-1 augments cAMP-induced stimulation of the rat LHβ gene promoter activity when tested in SF-1-deficient GH3 cells. A similar observation has been reported for the equine LHβ gene in placental JEG3 cells (Heckert 2001).
SF-1 is believed to be directly responsible for conferring cAMP-dependent transcription in a number of genes, although the mechanism by which this effect is achieved remains unclear (Lynch et al. 1993, Clemens et al. 1994, Michael et al. 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Chau et al. 1997, Liu & Simpson 1997, Jacob & Lund 1998, Sandhoff et al. 1998, Aesoy et al. 2002). In a variety of studies in non-pituitary cells, neither hormonal nor pharmacological stimulation of the cAMP/PKA pathway increased steady-state SF-1 mRNA levels (Zhang & Mellon 1996, Chau et al. 1997, Aesoy et al. 2002). Aesoy et al. (2002) have shown that prolonged forskolin treatment (10 µM × 24 h) increases SF-1 protein levels by decreasing SF-1 degradation in adrenocortical cells. We did not observe a forskolin-mediated increase in gonadotrope-derived SF-1 protein (Fig. 7); however, we cannot rule out the possibility that a higher dose and/or longer treatment regimen would increase pituitary SF-1. Nevertheless, this response would not explain the ability of forskolin to induce LHβ gene transcription in the 4h time course demonstrated in our studies. Furthermore, the persistent cAMP response with the 4 h time course demonstrated in our studies. We, therefore, propose that Sp1, Egr-1 and SF1 play critical roles in integrating the transcriptional response of the LHβ-subunit gene to a variety of hormonal stimuli.

In conclusion, our results demonstrate that activation of the cAMP system by PACAP or forskolin increases LHβ gene promoter activity. The forskolin response is mediated via Egr-1 and Sp1 acting at their cognate DNA-binding sites in the rat LHβ gene. Although not required for cAMP-responsiveness, SF-1 markedly augments cAMP-induced LHβ gene transcription. Of note, this mechanism is similar to a model proposed for GnRH/PKC-induced activation of the rat LHβ gene (Kaiser et al. 2000). We, therefore, propose that Sp1, Egr-1 and SF1 play critical roles in integrating the transcriptional response of the LHβ-subunit gene to a variety of hormonal stimuli.

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