Absence of pituitary adenylate cyclase-activating polypeptide-stimulated transcription of the human glycoprotein α-subunit gene in LβT2 gonadotrophs reveals disrupted cAMP-mediated gene transcription

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Abstract

Hormone regulation of anterior pituitary expression of the common glycoprotein hormone α-subunit (αGSU) is mediated by multiple response elements residing in the first −435 bp of the human promoter. In rat pituitary cells and mouse αT3–1 precursor gonadotrophs, the human αGSU promoter is strongly responsive to activators of the adenylyl cyclase/cAMP pathway, such as the hypothalamic releasing hormone, pituitary adenylate cyclase-activating polypeptide (PACAP) and forskolin (an adenylyl cyclase activator). However, the role of PACAP and cAMP in regulating αGSU transcription in the more differentiated LβT2 gonadotroph is unclear. Here, we investigate the regulation of the human αGSU promoter by PACAP and forskolin in LβT2 and αT3-1 gonadotrophs. PACAP failed to stimulate αGSU promoter activity or cAMP production in LβT2 cells, in marked contrast to αT3–1 cells. LβT2 gonadotrophs expressed extremely low levels of any PACAP type 1 receptors (PAC1-R) isoform by RT-PCR and lacked PAC1-R by radioligand binding. Forskolin stimulated the αGSU promoter in LβT2 cells, but by less than 30% of the response seen in αT3-1 gonadotrophs. This blunted cAMP transcriptional effect was not due to different levels of cAMP generation, or altered expression of the cAMP target proteins CREB, Akt, CBP or ICER. However, only LβT2 cells showed detectable expression of the protein kinase A type 1α regulatory subunit. Binding of activating transcription factor-2 and phosphorylated CREB to the consensus CRE was observed in both LβT2 and αT3-1 gonadotrophs, yet forskolin failed to stimulate either CRE- or CREB-mediated transcription in LβT2 cells. Collectively, these data demonstrate the lack of functional PACAP receptors in LβT2 gonadotrophs, and a pronounced attenuation in the responsiveness of this differentiated gonadotroph cell line to cAMP stimulus.

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Introduction

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are heterodimeric glycoproteins, which comprise a common α-subunit (αGSU) and distinct β-subunits that confer specificity (Gharib et al. 1990). αGSU expression is restricted to gonadotroph and thyrotroph cells in the anterior pituitary but is also found in placental tissue where chorionic gonadotrophin is expressed. The expression of the αGSU gene in pituitary development is one of the first indicators of cell lineage differentiation (Dasen & Rosenfeld 1999), and the regulation of αGSU transcription involves multiple transcriptional elements, which reside within the first −435 bp of the proximal human promoter (Heckert et al. 1996). Common and distinct transcription factors are involved in regulating αGSU transcription in pituitary and chorionic tissues. Tandem cAMP response elements (CREs) and an upstream regulatory element are important for αGSU transcription in the placenta (Heckert...
et al. 1996), whereas multiple elements contribute to gonadotroph αGSU expression, including the pituitary glycoprotein hormone basal element, the CREs, a consensus GATA site and a steroidogenic factor-1 (SF-1) site (the gonadotroph-specific element) (Barnhart & Mellon 1994, Steger et al. 1994, Heckert et al. 1996). These sites have been implicated in basal and hormone-stimulated αGSU promoter activity.

Gonadotroph cells are subject to hormonal regulation from hypothalamic releasing factors, such as gonadotrophin-releasing hormone (GnRH) and pituitary adenylate cyclase-activating polypeptide (PACAP), as well as the gonadal steroids. GnRH and PACAP both act via their specific seven transmembrane G-protein-coupled receptors (GPCRs) in gonadotrophs and gonadotroph cell lines such as αT3-1 and LβT2 cells. The GnRH receptor couples to Goq/Gαq1 to stimulate the diacylglycerol (DAG)/inositol phosphate (IP)/Ca2+/protein kinase C (PKC) pathway, as well as all four members of the mitogen-activated protein kinase (MAPK) family, and these pathways contribute to the transcriptional effects of GnRH on the αGSU, LHβ and FSHβ promoters (Holdstock et al. 1996, Saunders et al. 1998, Naor et al. 2000). PACAP acts predominantly via splice variants of the PACAP type 1 receptor (PAC1-R) isoform in gonadotrophs, which is a member of the secretin/glucagon family of GPCRs (Schomerus et al. 1994, Rawlings & Hezareh 1999). As such, PAC1-R activation results in coupling to both Goq and Gαi to stimulate the DAG/IP/Ca2+/PKC pathway and also to adenylyl cyclase, resulting in cAMP production. Most recently, we have shown PACAP to stimulate the activity of a member of the MAPK family, extracellular signal-regulated kinase 1/2 (ERK1/2), in αT3-1 cells in a PKC-dependent manner (Fowkes et al. 2001). This appears to regulate the proliferative effects of PACAP in these cells. We, and others, have previously shown the transcriptional effects of PACAP on the αGSU promoter to be mimicked by cAMP analogues and to be unaffected by pharmacological blockade of Ca2+ entry or PKC activity (Tsujii et al. 1994, Burrin et al. 1998). Furthermore, the increase in αGSU mRNA transcripts seen in response to PACAP is not altered following PKC depletion (Tsujii et al. 1995). Many of these studies have used the murine gonadotroph progenitor cell line, αT3-1, which is representative of gonadotroph precursor cells of embryonic day e13-5, expressing SF-1 and the GnRH receptor (Windle et al. 1990, Horn et al. 1991). Recently, investigations of gonadotrophin gene expression have employed the more differentiated LβT2 cell line (Weck et al. 1998, Liu et al. 2002), representative of murine gonadotrophs at e17.5–18.5. Unlike αT3-1 cells, LβT2 cells express the mature gonadotrophins (LHβ and FSHβ) (Turgeon et al. 1996), and have thus far proved useful tools for investigating the hormonal regulation of LH and FSH transcription. We have recently demonstrated a role for PKC and ERK1/2 in regulating αGSU transcription in LβT2 cells (Fowkes et al. 2002). However, the cAMP pathway in this cell line remains to be elucidated.

The classic mechanism for cAMP-mediated gene transcription has been elucidated for many genes, and involves a pathway from adenylyl cyclase activation, cAMP generation and protein kinase A (PKA) activation to phosphorylation of downstream molecules, including transcription factors such as CRE-binding protein (CREB) and other members of the activating transcription factor (ATF) family (McArdle & Counis 1996). More recently, the cAMP signalling pathway has been expanded following evidence that the phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B (PKB) growth factor signalling pathway can mediate effects of cAMP. These signalling molecules have been implicated in mediating cAMP effects on another pituitary gene, prolactin (Hayakawa et al. 2002). The expression and action of PI3K/Akt in mediating effects of gonadotroph signalling has not been investigated.

In the current study we have examined the role of cAMP/PKA in regulating αGSU gene transcription in LβT2 gonadotrophs. We show that PACAP fails to stimulate αGSU promoter activity or cAMP generation in LβT2 cells, and that forskolin-stimulated αGSU transcription is significantly attenuated compared with similar observations in αT3-1 cells. The relative contribution of known and novel cAMP target proteins/genes underlying these reduced responses has also been investigated.

**Materials and methods**

**Materials**

All chemicals were purchased from Sigma (Poole, Dorset, UK) or BDH-Merck (Poole, Dorset, UK).
unless otherwise stated. PACAP (residues 1–38) was obtained from CN Biosciences (Nottingham, Notts, UK) in lyophilized form and stored at −20 °C as a 1 mM stock solutions in sterile water. Forskolin was obtained from Sigma and stored as a 100 mM stock solution in DMSO at 4 °C. All stimulants were diluted directly into culture medium (see below) before each experiment.

Cell culture

LβT2 and αT3-1 cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with high glucose (4500 mg/l) containing 10% (v/v) fetal calf serum, penicillin (100 IU/ml), streptomycin (100 mg/l) and Fungizone (125 mg/l) (Life Technologies, Paisley, Strathclyde, UK) (hereafter referred to as culture medium). Cells were passaged twice weekly in culture medium). Cells were plated twice weekly and incubated at 37 °C in a humidified 5% (v/v) CO₂/95% (v/v) air incubator. Cells were plated in six-well plates at a density of 1 × 10⁶ cells/well for transient transfection experiments and Western blotting/nuclear protein preparation.

Plasmids and transient transfection studies

The reporter construct −517αLUC contains 517 bp of the 5’ flanking sequence and 44 bp of exon 1 of the human αGSU gene, linked to the luciferase (LUC) reporter gene in the plasmid pA3 LUC, and has been previously described (Maxwell et al. 1989, Holdstock et al. 1996). The internal control plasmid BosβGal contains the promoter of the human elongation factor 1 gene driving expression of β-galactosidase (Mizushima & Nagata 1990). The promoterless LUC expression vector pA3 LUC was used as a control plasmid for basal LUC expression, and the expression vector BosβGal was used as an internal control to normalize transfection efficiencies. The plasmids containing the CREB activation domain fused to the Gal-4 DNA-binding domain (Gal-4-CREB) and the Gal-4 promoter-LUC (Gal-4-LUC) were obtained from Dr H Takemori (University of Osaka, Japan) and have been described previously (Doi et al. 2002). The heterologous CRE reporter gene, TKCRE, was obtained from Dr S Goodbourn (St Georges Medical School, London, UK) and consisted of the consensus CRE from the somatostatin promoter fused to the minimal thymidine kinase promoter. All constructs were verified for orientation and correct sequence by restriction endonuclease digests and the dideoxy-DNA sequencing method. Large-scale preparation and purification of plasmids was performed by alkaline lysis and resin purification (Qiagen Ltd, Dorking, Surrey, UK). Cells at 1 × 10⁶/well were transfected by the calcium phosphate method as described previously without glycerol shock (Graham & van der Eb 1973). Cells were transfected for 4 h with 5 µg −517αLUC, pA3 LUC, TKCRE, Gal4-CREB or Gal4-LUC and co-transfected with 5 µg BosβGal. The cells were subsequently stimulated for 8 h in culture medium without (control) or with 100 nM PACAP or 10 µM forskolin. The cells were harvested and cellular extracts were assayed for LUC and β-galactosidase activity as described previously. LUC data from separate experiments were pooled by normalizing to the level of β-galactosidase activity. Each treatment group contained triplicate cultures and experiments were repeated at least twice.

PCR for PAC₁-R receptor, cAMP response element modulator protein (CREM) and ICER expression in LβT2 and αT3–1 cells

mRNA was isolated from LβT2 and αT3-1 cells in culture (1 × 10⁶ cells/well) using the QuickPrep micro mRNA purification kit (Pharmacia Biotech, St Albans, Herts, UK), which utilizes oligo (dT) cellulose to extract poly (A) mRNA. After washing in low- and high-salt buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA and NaCl (0.1 M or 0.5 M respectively)), the mRNA was eluted in 10 mM Tris–HCl and 1 mM EDTA and precipitated with glycogen (0.25 g/l), 0.25 M potassium acetate and 95% ethanol. After centrifugation, the RNA pellet was washed with 75% ethanol and allowed to dry before being re-suspended in diethyl pyrocarbonate (DEPC)-treated water. Reverse transcription was performed using the First Strand cDNA synthesis kit (Pharmacia Biotech), which utilizes Moloney murine leukaemia virus reverse transcriptase and a NotI-d(T)₁₈ bifunctional primer. Reverse transcription was also performed on DEPC-treated water, for use as a negative control in subsequent PCR reactions. Specific intron-spanning primers directed to the rodent PAC₁-R receptor were used to amplify up the PAC₁-R hip, hop or hiphop splice variants, which were as follows: forward
5′-CATCATCGGGGTTGGGACAC-3′, reverse 5′-CTACTCGGCTCTGGGACTTTC-3′ (Bresson-Bépoldin et al. 1998). Rodent GAPDH was co-amplified to serve as a qualitative control to show equal loading of cDNA (Perkin-Elmer, Cheshire, UK), which were as follows: forward 5′-TGCACCACCAACTGCTTAG-3′, reverse 5′-GGATGCAAGGATGATGTT-3′. The PCR was run for 35 cycles under the following conditions: 95°C for 30 s, 60°C for 30 s, 72°C for 45 s, followed by a final extension step at 72°C for 10 min. For the CREM and inducible cAMP early repressor (ICER) PCR, a common reverse primer was used for both reactions, encoding the leucine zipper region of the gene (LZIP reverse 5′-CCTCCAGCTCCTCATTTGCTTT-3′) with specific forward primers for CREM (5′-TGGAAA CAGTTGAATCACAG-3′) or ICER (5′-ATGGC TGTAACTGGAGATGAAACT-3′) (Thonberg et al. 2001). The PCR was run for 35 cycles under the following conditions: 95°C for 1 min, 58°C for 2 min, 72°C for 2 min, followed by a final extension step at 72°C for 10 min. Ten microlitres of the PCR products were mixed with loading buffer and run on a 1·6%-agarose gel stained with ethidium bromide.

Iodination and purification of PACAP

PACAP-27 was radiolabelled with 125I using an adaptation of the method of Christophe et al. (1976). Briefly, to a siliconized tube were added 5 µl 1 mg/ml 10% acetonitrile/water solution of PACAP-27 (CN Biosciences), 10 µl sodium phosphate buffer (0·3 M, pH 7·4), 3·7–7·4 MBq 125I (ICN Pharmaceuticals, Inc., CA, USA) and 5 µl chloramine-T (1 mg/ml in 0·3 M sodium phosphate buffer, pH 7·4). The mixture was reacted for 5 min and then diluted to 50 µl with 25% acetonitrile/water. The 125I-PACAP-27 solution was immediately purified from unlabelled PACAP and free 125I via RP-HPLC.

Radioligand binding assays of PAC1-R

Radioligand binding for PACAP was performed as described previously (Schomerus et al. 1994). Cells were removed from T75 culture flasks in a physiological saline solution (PSS: 127 mM NaCl, 1·8 mM CaCl2, 5 mM KCl, 2 mM MgCl2, 0·5 mM NaH2PO4, 5 mM NaHCO3, 10 mM glucose, 0·1% BSA, and 10 mM Hepes, pH 7·4) by scraping and collected by centrifugation and re-suspension in PSS containing 1 mg/ml bacitracin (Sigma). The binding incubation was for 30 min at RT in 100 µl PSS with 1 mg/ml bacitracin, 0·2–6 nM 125I-PACAP, 0 or 100 nM PACAP and cells. The cell suspensions were then layered over 150 µl of an inert mineral oil mixture in Eppendorf tubes. The oil mixture was a 3:2 ratio of silicone oil to di-isononyl phthalate from Fluka Biochemicals (Poole, Dorset, UK). Cells were separated from the incubation medium by centrifugation (2 min at 12 000 g). The microfuge tubes were frozen and the tips were cut off and collected for determination of the radioactivity in the cell pellets by gamma counting. Non-specific binding was defined as that seen with 10 µM PACAP. In some experiments iodinated buserilin was used instead of PACAP to assess GnRH receptor binding (obtained from Prof. Sandow, Aventis Pharma GmbH, Frankfurt, Germany), and cold GnRH was used to compete 125I-buserilin binding.

Measurement of total cAMP by enzyme immunoassay (EIA)

cAMP levels were determined according to the manufacturer’s instructions using the Biotrak cAMP EIA kit (Amersham-Pharmacia). LBT2 and αT3-1 cells were stimulated with 0 or 100 nM PACAP or 10 µM forskolin for 1 h, before adding 500 µl 100% ethanol (−20°C) and freezing the plates at −20°C for at least 2 h. Prior to assay, the plates were thawed before centrifugation at 500 g for 5 min at 4°C. The supernatant was removed and evaporated to dryness in a heated rotary vacuum centrifuge. The pellets were re-suspended in 500 µl assay buffer and stored at −20°C to await assay.

Nuclear protein extraction and Western blotting

Nuclear protein extracts were prepared from LBT2 and αT3-1 cells using a modification of a method described previously (Schreiber et al. 1989). Briefly, 1 × 10⁶ cells were cultured in six-well plates overnight in serum-free DMEM, before replacement of media with 0 or 10 µM forskolin for indicated time-points. Media were removed and the cells washed with PBS and scraped into 2 ml ice-cold PBS. Following centrifugation (5 min,
1500 g, 4 °C), the cells were re-suspended in 400 µl ice-cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)) and transferred to a cold microfuge tube. Having left the cells to swell on ice for 15 min, 25 µl 10% Nonidet P-40 (made in buffer A) were added to each sample followed by vortexing for 10 s. Following microcentrifugation (10 000 g, 30 s), the pellets were re-suspended in 300 µl buffer B (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) before vigorous rocking on a shaking platform at 4 °C for 15 min. The nuclear extract was centrifuged (10 000 g, 5 min), and stored at −70 °C prior to protein determination by the Bradford assay. Normalized concentrations of nuclear extract (typically between 10 and 15 µg) were loaded onto a 10% SDS-PAGE stacking gel and electrophoresed at 200 V. Briefly, samples were boiled in an equal volume of 2× sample buffer to cell lysate, electrophoresed and transferred to Hybond-ECL membranes (Amersham). The membrane was blocked with 5% non-fat milk and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-cJun (Santa Cruz Biotechnology), rabbit anti-ATF-2 or rabbit anti-ATF-1 (Upstate Biotechnology), rabbit anti-phosphoCREB, rabbit anti-CREB (Cell Signalling Technology, NE, USA; anti-phosphoCREB recognizes phosphoCREB and phosphoATF-1), rabbit anti-phosphoAkt, rabbit anti-Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; both antibodies recognize phosphorylated and total Akt1, 2 and 3), rabbit anti-PKA-Cα (partially cross-reactive with β and γ isoforms), rabbit anti-PKA-R-Iα (Santa Cruz Biotechnology; partially cross-reactive with RIβ isoform), mouse anti-PKA-R-Iα (BD Transduction Laboratories, UK), or 1:5000 dilution of mouse anti-β-actin (AbCam, Cambridge, Cambridge, UK).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using 1–3 µg nuclear extract per reaction. Probes were created by filling in the 5′ AGCT overhangs of the annealed CRE oligonucleotides with Klenow polymerase using a mixture of dATP, dGTP, dTTP and [α-32P]dCTP (ICN, Hants, UK) (CRE: forward 5′-AGCT-AAA-TTG-ACG-TCA-TGG-TAA-AAA-TTG-ACG-TCA-TGG-TA-3′, reverse 5′-AGCT-TAC-CAT-GTC-AAT-TTT-TAC-CAT-GAC-GTC-AAT-TT-3′). Three microlitres of nuclear extracts were incubated at room temperature for 5 min in 20 µl 20 mM Tris (pH 8.0), 60 mM KCl, 2 mM MgCl2, 1.2 mM DTT, 12% glycerol, 2.5 µg [poly(dL.dC)].poly(dL.dC)] (Amersham-Pharmacia). For gel shifts, 2 µl rabbit anti-phosphoCREB (Upstate Biotechnology), rabbit anti-AKT-2 or rabbit anti-cJun (Santa Cruz Biotechnology) were added to the reaction to identify components of the specific protein complexes, and these samples were incubated on ice for 60 min. The reactions were then incubated for 15 min at 30 °C in the presence of 1 ng probe. Complexes were electrophoresed on a 5% native acrylamide gel, dried and visualized by autoradiography.

Data presentation and statistical analysis

All graphical data were prepared using GraphPad Prism 3·0 (GraphPad, San Diego, CA, USA) and analysed using pre-programmed analysis equations within Prism. Transfection data are presented as normalized data pooled from multiple experiments (each in triplicate, and performed at least three
times). Where appropriate, an ANOVA was performed on data followed by Student’s t-test or Tukey’s multiple comparisons test, accepting $P<0.05$ as significant. Multiple passages of LβT2 and αT3-1 cells were used during the course of this study, ranging from early (passage ~5) to late (passage ~50), without detected differences in responsiveness.

**Results**

Our previous studies comparing the activity of the human αGSU promoter in LβT2 and αT3-1 cells had revealed that basal promoter activity was significantly reduced in LβT2 cells compared with αT3-1 cells, but the response to GnRH was similar (Fowkes et al. 2002). To investigate the effect of PACAP or the adenylyl cyclase activator, forskolin, on the αGSU promoter, LβT2 and αT3-1 cells were transfected with 5 µg/well of the −517αLUC promoter and 5 µg BosβGal and stimulated with 0 or 100 nM PACAP or 10 µM forskolin for 8 h post-transfection. As expected, αT3-1 cells responded well to PACAP and forskolin stimulation (Fig. 1A), where basal LUC activity was increased to 20.3 ± 4.9-fold and 27.5 ± 4.9-fold ($P<0.05$ and $P<0.001$ for PACAP and forskolin respectively). In contrast, PACAP-stimulation of transfected LβT2 cells only resulted in a 1.7 ± 0.3-fold increase over basal ($P>0.05$, ns). However, forskolin significantly stimulated −517αLUC activity in LβT2 cells 5.4 ± 0.3-fold ($P<0.001$), but this response was also significantly lower than that seen in αT3-1 cells ($P<0.001$). To determine whether the absence of a PACAP response and the reduced forskolin response was due to a lack of cAMP production in LβT2 cells, total cAMP production was measured from LβT2 and αT3-1 cells treated with 0 or 100 nM PACAP or 10 µM forskolin for up to 60 min. As shown (Fig. 1B), both cell lines responded to forskolin (60 min) to a similar extent (by 30.7 ± 4.2-fold and 31.2 ± 24.5-fold over basal, for LβT2 and αT3-1 cells respectively, $P<0.001$). PACAP significantly increased cAMP production in αT3-1 cells within 10 min and peaked at 7.5 ± 1.4-fold over basal at 1 h ($P<0.01$). However, no increase was seen in cAMP levels following PACAP stimulation in LβT2 cells at any time-point. Taken together, these data reveal that LβT2 cells fail to respond transcriptionally to PACAP, but do exhibit forskolin-stimulated αGSU transcription, albeit at levels significantly lower than those seen in αT3-1 gonadotrophs.

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**Figure 1** Comparison of PACAP and forskolin effects in LβT2 and αT3-1 gonadotrophs. (A) LβT2 and αT3-1 cells were transiently transfected with 5 µg −517αLUC and 5 µg BosβGal, before stimulation with either 0 (basal), 100 nM PACAP or 10 µM forskolin for 8 h. The data are means±S.E.M., expressed as fold increase over basal promoter activity, with values indicated over the relevant bars, and are normalized from at least three individual experiments each performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly different from basal, or as indicated by brackets. (B) LβT2 and αT3-1 cells were stimulated for up to 1 h with either 0 (basal), 100 nM PACAP or 10 µM forskolin. Total cAMP (intra- and extracellular) cAMP was ethanol extracted and measured by cAMP EIA. The data shown are means±S.E.M., pooled from at least eight individual stimulations and expressed as fold increase over basal. *$P<0.05$, **$P<0.01$ and ***$P<0.001$, significantly different from basal.
The lack of cAMP production following PACAP stimulation in LßT2 cells suggested an apparent lack of functional PACAP receptors in these cells. To confirm the presence or absence of the PAC1-R receptor in these gonadotroph cell lines, radioligand binding using \(^{125}\text{I}-\text{PACAP}\) was performed on whole cells in suspension. As shown (Fig. 2A), specific PACAP binding was observed in \(\alpha\text{T3-1}\) cells, which showed displacement of tracer with 100 pM to 100 nM unlabelled PACAP, but no displacement was seen in LßT2 cells. To verify the radioligand binding assay, similar studies were performed with iodinated buserilin, a potent GnRH agonist. As shown (Fig. 2A, right), specific GnRH binding was observed in both LßT2 and \(\alpha\text{T3-1}\) cells. In order to identify the presence of PAC1-R in LßT2 cells, PCR was performed from LßT2 and \(\alpha\text{T3-1}\) cDNA using specific intron-spanning primers designed to amplify multiple splice variants of the PAC1-R. \(\text{hiphop}\) and \(\text{hip or hop}\) variants were detected in \(\alpha\text{T3-1}\) cells, as had been reported previously (Rawlings & Hezareh 1996), but only a weak band for either \(\text{hip}\) or \(\text{hop}\) in LßT2 cells. Taken together, these data suggest that the lack of a transcriptional response to PACAP in LßT2 cells is probably due to an absence of functional PAC1-R in these cells.
The regulation of cAMP effects involves many downstream signalling molecules, which may have positive or negative effects on cAMP-mediated gene transcription. To establish whether there were differences in the downstream cAMP signalling components, Western blotting was performed. LβT2 and αT3-1 cells were serum-starved for 2 h prior to stimulation with 10 µM forskolin for 15 min. Total proteins were extracted and resolved by SDS-PAGE electrophoresis, followed by Western blotting for Ser133-phosphoCREB and total CREB. Forskolin up-regulated CREB phosphorylation in both LβT2 and αT3-1 cells (Fig. 3A). The level of the CREB accessory protein, CBP, was examined in nuclear extracts of LβT2 and αT3-1 cells. As shown, similar levels of the 265 kDa CBP protein were observed in both cell lines (Fig. 3B). As recent publications had suggested that novel mediators of cAMP signalling can regulate the effects of cAMP on pituitary gene transcription, we examined whether forskolin could activate the PKB/Akt pathway. Following treatment with 10 µM forskolin for up to 60 min, total protein extracts were prepared from LβT2 and αT3-1 cells and Western blotting for phosphoAkt and total Akt were performed. PhosphoAkt was detected in all samples, and forskolin failed to alter this phosphorylation status in either cell line (Fig. 3C). cAMP signalling conventionally leads to the activation of PKA, a holoenzyme that consists of catalytic and regulatory subunits. The expression of different isoforms of these subunits can dictate the activity of PKA in any given cell type (Brandon et al. 1997). We therefore sought to establish the expression profile of PKA subunits in LβT2 and αT3-1 cells. Following treatment with 10 µM forskolin for up to 60 min, total protein extracts were prepared from LβT2 and αT3-1 cells and Western blotting for PKA-CαII, PKA-RIIα and PKA-RIα was performed. Similar levels of PKA-CαII and PKA-RIα were observed in both cell lines, with an apparent increase in PKA-RIα following forskolin stimulation in αT3-1 cells (Fig. 3D). However, only LβT2 cells expressed detectable levels of PKA-RIIα (Fig. 3D), which also tended to increase with forskolin stimulation. Protein loading was confirmed by blotting for β-actin levels. Collectively, these data suggest that the levels of several putative cAMP signalling components are similar in LβT2 and αT3-1 cells and respond to forskolin appropriately. The major difference is that PKA-RIIα is only expressed in LβT2 cells.

cAMP regulation of gene transcription is classically regulated via CRE sequences in the proximal promoter region of specific genes, and these CREs can bind a number of transcription factors. To establish whether nuclear proteins from LβT2 cells exhibit altered DNA-binding characteristics, we performed EMSAs using the tandem CRE from the human αGSU as the probe. Inclusion of anti-ATF-2, anti-cJun or anti-phosphoCREB antisera in the reaction mixture was used to identify the protein complexes. The two largest complexes contained ATF-2, whereas the smaller two complexes contained phosphoCREB in extracts from both LβT2 and αT3-1 cells (Fig. 4). These data suggest proteins that bind the CRE in LβT2 and αT3-1 cells are identical.

The CREB/ATF family of transcription factors are known to regulate cAMP-mediated gene transcription in many cells. In addition to CREB, several homologous proteins have also been shown to bind CRE sites in promoters, namely CREM and its associated gene product ICER. We examined whether LβT2 and αT3-1 cells express CREM or ICER. cDNA was made from LβT2 and αT3-1 cells treated with 10 µM forskolin for 6 h, and PCR was performed for ICER and CREM expression. No transcripts were detected for CREM in either LβT2 or αT3-1 cells, but specific products were seen with the positive control cDNA from rat testis (Fig. 5, top). In contrast, no ICER transcripts were detected in the testis cDNA, but both LβT2 and αT3-1 cells expressed similar levels of ICER-II and ICER-IIγ when stimulated with forskolin (Fig. 5, middle). These findings suggest that changes in the level of ICER are not responsible for the differential transcriptional response to forskolin observed in LβT2 cells.

As we had observed that forskolin could stimulate the αGSU promoter, we wanted to establish whether a CRE sequence could act as a transcriptional responsive cassette in LβT2 cells. We transiently transfected LβT2 and αT3-1 cells with 5 µg of a heterologous reporter construct encoding the perfect palindromic CRE sequence from somatostatin promoter upstream of the minimal thymidine kinase promoter. Following transfection, the cells were stimulated with 0 or 10 µM forskolin for 8 h. In αT3-1 cells, promoter activity was significantly enhanced to 3·3 ± 0·2-fold.
Figure 3 Expression of cAMP-stimulated signalling molecules in LβT2 and αT3-1 cells. (A) LβT2 and αT3-1 cells were stimulated with 0 or 10 µM forskolin for 15 min prior to extraction of total proteins and Western blotting for Ser133-phosphoCREB or total CREB. (B) Western blotting for CBP was performed on nuclear proteins from LβT2 and αT3-1 cells. HeLa cell nuclear extracts were used as a positive control. (C and D) Total proteins were extracted from LβT2 and αT3-1 cells stimulated with 0 or 10 µM forskolin for up to 60 min, before Western blotting for phosphoAkt and total Akt1/2 (C) or PKA-Cα, PKA-RIIα or PKA-RIα (D). β-actin was used as a measure of loading efficiency (D). In all cases, the autoradiographs are representative of at least two individual experiments.
over basal ($P<0.001$), but no response was seen in LβT2 cells (Fig. 6A). This suggests that the CRE sequence alone is incapable of acting as a cAMP-response cassette in LβT2 cells.

Having established that forskolin could enhance CREB phosphorylation in both LβT2 cells and αT3-1 cells, we investigated whether the transcriptional activity of CREB could be similarly induced. LβT2 and αT3-1 cells were co-transfected with 5 µg Gal-4-CREB expression vector, 5 µg Gal-4-LUC reporter gene and 5 µg BosβGal, before stimulation for 8 h with 0 or 10 µM forskolin. Surprisingly, forskolin only modestly increased CREB transcriptional activity in LβT2 cells under control conditions (to $2.8 \pm 0.9$-fold, Fig. 6B). In contrast, forskolin potently enhanced CREB transcriptional activity (to $32.7 \pm 4.7$-fold, $P<0.001$) in αT3-1 cells. Taken together, these data reveal marked differences in cAMP-mediated gene transcription in LβT2 and αT3-1 gonadotrophs.

**Discussion**

In the present study we have examined the regulation of human αGSU promoter activity by cAMP, using transient transfection of LβT2 cells, a mouse pituitary clonal gonadotroph cell line which is reported to exhibit functional characteristics consistent with those of mature, differentiated gonadotrophs (Turgeon et al. 1996). Our results suggest that, unlike the less-differentiated αT3-1 clonal gonadotroph, these cells lack functional PACAP receptors and mount a poor transcriptional response to cAMP.

It is well established that cAMP is an intracellular mediator of PACAP action (Schomerus et al. 1994, Tsujii et al. 1995, Rawlings & Hezareh 1996) and in gonadotrophs, PACAP is a more potent stimulator than GnRH of the cAMP/PKA pathway (McArdle & Counis 1996). PACAP has been shown to increase αGSU mRNA concentrations and αGSU gene transcription in primary rat pituitary cell
cultures and αT3-1 gonadotrophs, an effect that was markedly reduced when αGSU promoter constructs containing a mutant CRE site were used (Burrin et al. 1998). In the αT3-1 cell line, PACAP continues to stimulate αGSU mRNA levels in the presence of PKC depletion (Tsujii et al. 1995), pharmacological blockade of calcium entry (Burrin et al. 1998) and inhibition of MAPK signalling (Fowkes et al. 2001), all evidence that supports a role for the cAMP pathway in mediating the stimulatory transcriptional effects of PACAP on the αGSU promoter. In addition, previous studies have shown that the PKA inhibitor, H-89, can attenuate the effect of PACAP on the mouse αGSU promoter, albeit when used at concentrations in excess of the \( K_i \) for this compound (Attardi & Winters 1998). Our current studies confirm a potent 20-fold stimulation of αGSU transcription in this cell line, similar to the response seen with the adenyl cyclase activator, forskolin. In view of these data, which clearly implicate the cAMP signalling pathway in mediating the actions of PACAP on αGSU gene transcription in gonadotrophs, it was of considerable interest to observe the lack of αGSU transcriptional response to PACAP in the LβT2 cell line.

To further investigate this observation, we performed receptor-binding studies and RT-PCR to look for PAC1-R mRNA. We were unable to demonstrate the presence of PACAP receptors by ligand binding, and found no measurable cAMP production in these cells following stimulation with PACAP, leading us to conclude that LβT2 cells lacked functional PACAP receptors activating the cAMP pathway. However, we were able to detect some PAC1-R-specific transcripts by PCR, albeit at extremely low copy numbers. This is in agreement with recent reports of PAC1-R expression in LβT2 cells (Agarwal et al. 2002, Fujii et al. 2002), although Fujii et al. required amplification for 40 cycles in order to detect the receptor. Neither of these studies has performed ligand-binding experiments to confirm specific PACAP binding in these cells, nor have the downstream signalling pathways activated by PACAP been elucidated. Therefore, it is likely that although LβT2 cells may express various isoforms of the PAC1-R, they do not express PAC1-R protein as determined by radioligand-binding assays.

Several recent studies have investigated the effects of PACAP in LβT2 cells. Although PACAP has been reported to stimulate LHβ, FSHβ and PACAP expression, and CRE activity, the magnitude of these effects in all examples is consistently no more than 2-fold over basal (Horton & Halvorson 2000, Agarwal et al. 2002, Fujii et al.
Indeed, our current observations report a 1.7-fold increase in αGSU promoter activity following PACAP stimulation of transfected LβT2 cells, an effect that in the context of our experiments was not statistically significant. Garrel et al. (2002) also report that PACAP failed to stimulate the neuronal nitric oxide synthase promoter in LβT2 cells, in contrast to their observations in αT3-1 cells and primary pituitary cells. They were surprised by the modest effect of PACAP on a heterologous CRE reporter gene, and suggest that PACAP might be acting via a non-cAMP-mediated pathway in LβT2 cells. There are no reports of a non-cAMP-coupled PACAP receptor being expressed in pituitary cells. However, a splice variant of the PAC1-R is expressed in rat cerebellum and β-islets (known as PAC1-R-TM4) which solely activates L-type Ca2+ channels (Chatterjee et al. 1996). We have previously shown that the response of the human αGSU promoter to elevated extracellular Ca2+ is modest, eliciting a 2-fold increase in promoter activity at best (Holdstock et al. 1996). This effect is similar to the PACAP response seen in LβT2 cells (Fujii et al. 2002, Garrel et al. 2002, data herein), but it is unclear whether the TM4 variant is expressed in these cells. The lack of specific PACAP binding demonstrated in this study suggests that even if TM4 variant receptors are expressed in LβT2 cells, they are present in extremely low numbers. Another possible explanation for the modest effects of PACAP on pituitary gene transcription in LβT2 cells is that PACAP may act via non-classic receptors, i.e. non-PACAP receptors. Such cross-reactivity by PACAP has been previously observed in smooth muscle cells where PACAP and VIP bind specifically to the natriuretic peptide clearance receptor (NPR-C) (Murthy et al. 1998). This receptor couples to Gqi in smooth muscle cells, and leads to an increase in calcium influx (Murthy et al. 1998). However, it is not clear whether LβT2 cells express NPR-C. Finally, cell lines are known to mutate over time and the absence of functional PAC1-R receptors might reflect receptor loss due to mutation. We have used LβT2 cells at early, mid and late passage number (from 5 to 50), without observing any response to PACAP.

Activation of the cAMP/PKA pathway via a GPCR has been reported in LβT2 cells. Recently, GnRH was shown to stimulate cAMP accumulation via Gαi activity (Liu et al. 2002), and we have observed a potent effect of glucagon-like peptide 1 (GLP-1) on αGSU transcription in LβT2 cells following transient expression of the GLP-1 receptor (R C Fowkes, K K Sidhu & J M Burrin, 2002, Garrel et al. 2002). Indeed, our current observations report a 1.7-fold increase in αGSU promoter activity following PACAP stimulation of transfected LβT2 cells, an effect that in the context of our experiments was not statistically significant. Garrel et al. (2002) also report that PACAP failed to stimulate the neuronal nitric oxide synthase promoter in LβT2 cells, in contrast to their observations in αT3-1 cells and primary pituitary cells. They were surprised by the modest effect of PACAP on a heterologous CRE reporter gene, and suggest that PACAP might be acting via a non-cAMP-mediated pathway in LβT2 cells. There are no reports of a non-cAMP-coupled PACAP receptor being expressed in pituitary cells. However, a splice variant of the PAC1-R is expressed in rat cerebellum and β-islets (known as PAC1-R-TM4) which solely activates L-type Ca2+ channels (Chatterjee et al. 1996). We have previously shown that the response of the human αGSU promoter to elevated extracellular Ca2+ is modest, eliciting a 2-fold increase in promoter activity at best (Holdstock et al. 1996). This effect is similar to the PACAP response seen in LβT2 cells (Fujii et al. 2002, Garrel et al. 2002, data herein), but it is unclear whether the TM4 variant is expressed in these cells. The lack of specific PACAP binding demonstrated in this study suggests that even if TM4 variant receptors are expressed in LβT2 cells, they are present in extremely low numbers. Another possible explanation for the modest effects of PACAP on pituitary gene transcription in LβT2 cells is that PACAP may act via non-classic receptors, i.e. non-PACAP receptors. Such cross-reactivity by PACAP has been previously observed in smooth muscle cells where PACAP and VIP bind specifically to the natriuretic peptide clearance receptor (NPR-C) (Murthy et al. 1998). This receptor couples to Gqi in smooth muscle cells, and leads to an increase in calcium influx (Murthy et al. 1998). However, it is not clear whether LβT2 cells express NPR-C. Finally, cell lines are known to mutate over time and the absence of functional PAC1-R receptors might reflect receptor loss due to mutation. We have used LβT2 cells at early, mid and late passage number (from 5 to 50), without observing any response to PACAP.

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Disrupted cAMP-mediated αGSU transcription in LβT2 cells  ·  R C Fowkes and others

unpublished observations). Furthermore, GnRH stimulation of the PACAP promoter in LβT2 cells was shown to be both PKC- and PKA-sensitive (Agarwal et al. 2002). In our current study, we observed a significant effect of forskolin on the αGSU promoter in LβT2 cells. However, the magnitude of this response was significantly less than that observed in parallel observations in αT3-1 cells, despite the fact that similar levels of cAMP were measured following forskolin stimulation. This difference in responsiveness appears to be specific to the cAMP/PKA pathway, as we have previously observed that GnRH and the PKC activator, PMA, activate the αGSU promoter by the same magnitude in both LβT2 and αT3-1 cells (Fowkes et al. 2002). In addition, differences in transfection efficiency do not underlie the current observations, because the activity of the promoter-less control, pA3 LUC, or the minimal thymidine kinase promoter, TKLUC, is not significantly different between LβT2 and αT3-1 cells (Fowkes et al. 2002), and all transfections included the β-galactosidase plasmid to correct for differences in efficiency. Furthermore, the differences between promoter activity in these two gonadotroph-derived cell lines are maintained when lipofection-based transfection reagents are used instead of calcium phosphate (RC Fowkes & JM Burrin, unpublished observations).

We attempted to establish why the forskolin effect on αGSU transcription was less potent in LβT2 cells. Previous investigations of immortalized cell lines have revealed differences in the levels of signalling proteins, which might reflect changes which occur during cellular transformation and oncogenesis. For example, the ubiquitous signalling protein CREB is not expressed in the human adrenocortical cell line H295R, but this is compensated for by overexpression of CREM isoforms (Groussin et al. 2000). We therefore examined whether similar differences could be found between LβT2 and αT3-1 gonadotrophs. Forskolin significantly enhanced CREB phosphorylation at Ser133 to a similar extent in both cell lines, and the basal levels of CREB and its accessory protein CBP were also identical in LβT2 and αT3-1 cells. As recent reports have detailed cAMP activation of the PKB/Akt pathway in pituitary cells (Hayakawa et al. 2002), we investigated Akt phosphorylation in both gonadotroph cell lines. Forskolin treatment failed to alter the basal level of Akt phosphorylation in either cell line. The differences in cAMP responsiveness between LβT2 and αT3-1 cells could also be due to expression of inhibitors of the cAMP/PKA pathway. Thus we examined the levels of CREM and ICER by RT-PCR, as these CREBs have been reported to regulate cAMP-mediated transcription in the pituitary and gonads (De Cesare & Sassone-Corsi 2000). Following forskolin stimulation, both LβT2 and αT3-1 cells expressed two specific ICER transcripts corresponding to ICER II and ICER IIγ, but CREM was not detected. Therefore, changes in the levels of cAMP/PKA inhibitors do not underlie the differences in cAMP responsiveness in these two gonadotroph cell lines.

Generation of cAMP following adenyl cyclase activation leads to the activation of PKA. PKA consists of catalytic (C) and regulatory (R) subunits, which are associated when the enzyme is in the resting state (Brandon et al. 1997). Following the binding of cAMP, the catalytic and regulatory subunits dissociate and the catalytic subunit is freed to phosphorylate target proteins. Previous studies have shown that αT3-1 gonadotrophs and rat anterior pituitary cells express PKA-C and PKA-RII and II (Garrel et al. 1995, 1997), and that the level of these subunits changes with cAMP treatment. PKA-C declines in the presence of 8-Br-cAMP whereas PKA-R increases during the same treatment paradigm (Garrel et al. 1995). The recent availability of PKA-C and PKA-R subunit-specific antibodies has allowed identification of these subtypes in LβT2 and αT3-1 cells. Our results demonstrate that PKA-Cα was found at similar levels in both gonadotroph cell lines and was not affected by forskolin treatment. PKA-RIα was also expressed in both cell lines, and appeared to be up-regulated in response to forskolin in αT3-1 cells but less so in LβT2 cells. However, the major difference in subunit expression was observed in PKA-RIIα, which was only detected in LβT2 cells. Therefore, the possibility exists that LβT2 cells express more PKA regulatory subunits than αT3-1 cells, and therefore may require higher levels of cAMP to dissociate the PKA-C than would be the case in αT3-1 cells. Further studies using PKA isoform-specific agonists/antagonists (e.g. Rp-cAMPs, 8-Br-cAMP, dibutylryl cAMP) are required to establish the exact mechanism behind this apparent discrepancy in LβT2 and αT3-1 cells. Furthermore, the A-kinase anchoring peptides are
important regulators of PKA activity (Colledge & Scott 1999), although it is as yet unclear as to their expression or role in signal transduction in gonadotrophs.

cAMP-mediated activation of the human αGSU is directed partially via the tandem CREs as determined by mutational analysis (Burrin et al. 1998). The CREs of the human αGSU bind similar proteins in trophoblast and gonadotroph cells (Heckert et al. 1996). As we had failed to observe any differences in the levels of CREBs in LβT2 and αT3-1 cells, we examined whether the DNA-binding characteristics were altered, using EMSA analysis. The expected four complex pattern was observed using nuclear extracts of LβT2 and αT3-1 cells, and the tandem αGSU CRE as a probe. The largest of these complexes contained ATF-2, whereas the smaller two complexes contained phosphoCREB, as determined by supershifts. However, the relative contribution of these two proteins to CRE complex formation was not significantly different between the two gonadotroph cell lines. The CRE is a powerful regulator of gene transcription, and has previously been shown to be sufficient to mediate basal and cAMP-stimulated αGSU gene transcription in placental cells (Jameson et al. 1989). To establish whether a CRE could act as a transcriptionally responsive cassette, we examined the ability of forskolin to stimulate the ability of the classic CRE from the somatostatin promoter. Forskolin stimulated CRE-mediated promoter activity in αT3-1 cells but not at all in LβT2 cells. To establish whether transcription driven via a Gal-4-CREB fusion protein was similarly affected, we co-transfected a full-length Gal-4-CREB with the Gal-4-LUC reporter gene in to both gonadotroph cell lines. Forskolin again potently stimulated promoter activity in αT3-1, but LβT2 cells responded very poorly by comparison. The apparent discrepancy between CREB phosphorylation (as determined by Western blotting) and CREB transcriptional activity (as determined by Gal-4-CREB transfections) in LβT2 cells is difficult to reconcile. However, the antibody used to detect CREB phosphorylation is specific for the Ser133 residue, the major target site for protein kinase-induced phosphorylation in CREB. Proximally located to this Ser residue is another Ser at 142, which is specifically a calcium/calmodulin-dependent protein kinase II site and phosphorylation at this residue is thought to inhibit CREB activity (Sun et al. 1994). Therefore, it is possible that in LβT2 cells, CREB is phosphorylated at both of these residues, which would reduce its ability to mediate gene transcription and to recruit co-activators.

The understanding of gonadotrophin gene transcription has been considerably enhanced by the availability of gonadotroph-derived cell lines, with clear advances being made in the understanding of peptide hormone regulation of the αGSU, LHβ and FSHβ genes. However, we have clearly shown that the LβT2 and αT3-1 gonadotroph models have significant discrepancies in their responses to cAMP that may or may not be reflective of changes occurring during gonadotroph development. These important differences must be taken into account when examining gene expression in these developmentally distinct cell lines.

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