Absence of pituitary adenylate cyclase-activating polypeptide-stimulated transcription of the human glycoprotein α -subunit gene in L β T2 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 (α GSU) is mediated by multiple response elements residing in the first -435 bp of the human promoter. In rat pituitary cells and mouse α T3-1 precursor gonadotrophs, the human α GSU 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 α GSU transcription in the more differentiated L β T2 gonadotroph is unclear. Here, we investigate the regulation of the human α GSU promoter by PACAP and forskolin in L β T2 and α T3-1 gonadotrophs. PACAP failed to stimulate α GSU promoter activity or cAMP production in L β T2 cells, in marked contrast to α T3-1 cells. L β T2 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 α GSU promoter in L β T2 cells, but by less than 30% of the response seen in α T3-1 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 L β T2 cells showed detectable expression of the protein kinase A type II α regulatory subunit. Binding of activating transcription factor-2 and phosphorylated CREB to the consensus CRE was observed in both L β T2 and α T3-1 gonadotrophs, yet forskolin failed to stimulate either CRE- or CREB-mediated transcription in L β T2 cells. Collectively, these data demonstrate the lack of functional PACAP receptors in L β T2 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 (α GSU) and distinct β -subunits that confer specificity (Gharib *et al.* 1990). α GSU 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 α GSU gene in pituitary development

is one of the first indicators of cell lineage differentiation (Dasen & Rosenfeld 1999), and the regulation of α GSU 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 α GSU transcription in pituitary and chorionic tissues. Tandem cAMP response elements (CREs) and an upstream regulatory element are important for α GSU transcription in the placenta (Heckert

et al. 1996), whereas multiple elements contribute to gonadotroph α GSU 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 α GSU 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 α T3-1 and L β T2 cells. The GnRH receptor couples to $G_{\alpha_q}/G_{\alpha_{11}}$ to stimulate the diacylglycerol (DAG)/inositol phosphate (IP)/ Ca^{2+} /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 α GSU, LH β and FSH β 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 (PAC $_1$ -R) isoform in gonadotrophs, which is a member of the secretin/glucagon family of GPCRs (Schomerus *et al.* 1994, Rawlings & Hezareh 1999). As such, PAC $_1$ -R activation results in coupling to both G_{α_q} and G_{α_s} to stimulate the DAG/IP/ Ca^{2+} /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 α T3-1 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 α GSU promoter to be mimicked by cAMP analogues and to be unaffected by pharmacological blockade of Ca^{2+} entry or PKC activity (Tsujii *et al.* 1994, Burrin *et al.* 1998). Furthermore, the increase in α GSU 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, α T3-1, 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 L β T2 cell line (Weck *et al.* 1998, Liu *et al.* 2002), representative of murine gonadotrophs at e17.5–18.5. Unlike α T3-1 cells, L β T2 cells express the mature gonadotrophins (LH β and FSH β) (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 α GSU transcription in L β T2 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 α GSU gene transcription in L β T2 gonadotrophs. We show that PACAP fails to stimulate α GSU promoter activity or cAMP generation in L β T2 cells, and that forskolin-stimulated α GSU transcription is significantly attenuated compared with similar observations in α T3-1 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

L β T2 and α T3-1 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 and incubated at 37°C in a humidified 5% (v/v) $\text{CO}_2/95\%$ (v/v) air incubator. Cells were plated in six-well plates at a density of 1×10^6 cells/well for transient transfection experiments and Western blotting/nuclear protein preparation.

Plasmids and transient transfection studies

The reporter construct -517α LUC contains 517 bp of the 5' flanking sequence and 44 bp of exon 1 of the human α GSU 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 Bos β Gal 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 Bos β Gal 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 1×10^6 /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 -517α LUC, pA3 LUC, TKCRE, Gal4-CREB or Gal4-LUC and co-transfected with 5 μg Bos β Gal. 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 PAC $_1$ -R receptor, cAMP response element modulator protein (CREM) and ICER expression in L β T2 and α T3-1 cells

mRNA was isolated from L β T2 and α T3-1 cells in culture (1×10^6 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 Not1-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 PAC $_1$ -R receptor were used to amplify up the PAC $_1$ -R hip, hop or hip-hop splice variants, which were as follows: forward

5'-CATCATCGGGTGGGGGACAC-3', reverse 5'-CCTTCCAGCTCCTCCATTTCTCTT-3' (Bresson-Bépolin *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'-GGATGCAGGGATGATGTT-3'. The PCR was run for 35 cycles under the following conditions: 94 °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'-CTAATCTGTTTTGGGAGAGCAAAT-3') with specific forward primers for CREM (5'-TGGAAA CAGTTGAATCACAG-3') or ICER (5'-ATGGC TGTAACCTGGAGATGAAACT-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 ^{125}I 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 ^{125}I (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 ^{125}I -PACAP-27 solution was immediately purified from unlabelled PACAP and free ^{125}I via RP-HPLC.

Radioligand binding assays of PAC $_1$ -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 CaCl $_2$, 5 mM KCl, 2 mM MgCl $_2$, 0.5 mM NaH $_2$ PO $_4$, 5 mM NaHCO $_3$, 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 ^{125}I -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 ^{125}I -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). L β T2 and α T3-1 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 L β T2 and α T3-1 cells using a modification of a method described previously (Schreiber *et al.* 1989). Briefly, 1×10^6 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 μ l 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 μ l 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 μ l 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 μ g) were loaded onto a 10% SDS-PAGE stacking gel and electrophoresed at 200 V. Briefly, samples were boiled in an equal volume of 2 $\times$ sample buffer to cell lysate, electrophoresed and transferred to Hybond-ECL membranes (Amersham-Pharmacia). The membrane was blocked with 5% non-fat milk and incubated overnight at 4 °C with agitation, using 1 μ g/ml rabbit anti-CBP (CREB accessory protein) (specific for 165–265 kDa isoforms of CBP; Upstate Biotechnology, Lake Placid, NY, USA). The membrane was then washed three times with PBS containing 0.05% Tween and subsequently incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (Dako, Glostrup, Denmark) for 2 h at room temperature, again with agitation. The membrane was washed as before and bound antibody detected using enhanced chemiluminescence (Amersham-Pharmacia).

Total protein preparation

Total proteins were extracted from L β T2 and α T3-1 cells using the commercially available CytoBuster reagent (CN Biosciences) with added protein phosphatase inhibitor cocktails I and II (Sigma) (referred to as extraction buffer). Briefly, 1×10^6 L β T2 or α T3-1 cells/well were plated in six-well plates and left to adhere overnight. Following a 2 h serum-starvation, the cells were incubated with indicated stimuli and inhibitors for various time-points. The cells were washed briefly with ice-cold PBS before adding 150 μ l/well of extraction buffer, and sonicated for 5 s before

storage at –70 °C to await analyses by Western blotting (as above) using 1:1000 dilution of 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-C α (partially cross-reactive with β and γ isoforms), rabbit anti-PKA-RII α (Santa Cruz Biotechnology; partially cross-reactive with RII β isoform), mouse anti-PKA-R-I α (BD Transduction Laboratories, UK), or 1:5000 dilution of mouse anti- β -actin (AbCam, Cambridge, Cambs, UK).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using 1–3 μ g 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 - 32 P]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-GAC-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 μ l 20 mM Tris (pH 8.0), 60 mM KCl, 2 mM MgCl₂, 1.2 mM DTT, 12% glycerol, 2.5 μ g [poly(dI.dC).poly(dI.dC)] (Amersham-Pharmacia). For gel shifts, 2 μ l rabbit anti-phosphoCREB (Upstate Biotechnology), rabbit anti-ATF-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 L β T2 and α T3-1 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 α GSU promoter in L β T2 and α T3-1 cells had revealed that basal promoter activity was significantly reduced in L β T2 cells compared with α T3-1 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 α GSU promoter, L β T2 and α T3-1 cells were transfected with 5 μ g/well of the -517α LUC promoter and 5 μ g Bos β Gal and stimulated with 0 or 100 nM PACAP or 10 μ M forskolin for 8 h post-transfection. As expected, α T3-1 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 L β T2 cells only resulted in a 1.7 ± 0.3 -fold increase over basal ($P > 0.05$, ns). However, forskolin significantly stimulated -517α LUC activity in L β T2 cells 5.4 ± 0.3 -fold ($P < 0.001$), but this response was also significantly lower than that seen in α T3-1 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 L β T2 cells, total cAMP production was measured from L β T2 and α T3-1 cells treated with 0 or 100 nM PACAP or 10 μ M forskolin for up to 60 min. As shown (Fig. 1B), both cell lines responded to forskolin (60 min) to a similar extent (by 307.4 ± 4.2 -fold and 312.3 ± 24.5 -fold over basal, for L β T2 and α T3-1 cells respectively, $P < 0.001$). PACAP significantly increased cAMP production in α T3-1 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 L β T2 cells at any time-point. Taken together, these data reveal that L β T2 cells fail to respond transcriptionally to PACAP, but do exhibit

Figure 1 Comparison of PACAP and forskolin effects in L β T2 and α T3-1 gonadotrophs. (A) L β T2 and α T3-1 cells were transiently transfected with 5 μ g -517α LUC and 5 μ g Bos β Gal, before stimulation with either 0 (basal), 100 nM PACAP or 10 μ M forskolin for 8 h. The data are means $\pm$ 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. * $P < 0.05$, *** $P < 0.001$, significantly different from basal, or as indicated by brackets. (B) L β T2 and α T3-1 cells were stimulated for up to 1 h with either 0 (basal), 100 nM PACAP or 10 μ M forskolin. Total cAMP (intra- and extracellular) cAMP was ethanol extracted and measured by cAMP EIA. The data shown are means $\pm$ 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.

forskolin-stimulated α GSU transcription, albeit at levels significantly lower than those seen in α T3-1 gonadotrophs.

Figure 2 Comparison of PAC₁-R expression in L β T2 and α T3-1 gonadotrophs. (A) radioligand-binding assays for PACAP (left panel) and GnRH (right panel) were performed on whole L β T2 and α T3-1 cells in suspension. The data shown are means $\pm$ S.E.M., expressed as % of total binding, from three independent experiments performed in triplicate. (B) RT-PCR analysis of PAC₁-R expression in L β T2 and α T3-1 cells. Separate reactions were performed with specific primers for PAC₁-R (upper) or GAPDH (lower), and each gel is representative of at least three separate experiments performed on different passages of cells.

The lack of cAMP production following PACAP stimulation in L β T2 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}I -PACAP was performed on whole cells in suspension. As shown (Fig. 2A), specific PACAP binding was observed in α T3-1 cells, which showed displacement of tracer with 100 pM to 100 nM unlabelled PACAP, but no displacement was seen in L β T2 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 L β T2 and α T3-1 cells. In order to identify the presence of PAC₁-R in L β T2 cells, PCR was performed from L β T2 and α T3-1 cDNA using specific intron-spanning primers designed to amplify multiple splice variants of the PAC₁-R. *hiphop* and *hip* or *hop* variants were detected in α T3-1 cells, as had been reported previously (Rawlings & Hezareh 1996), but only a weak band for either *hip* or *hop* in L β T2 cells. Taken together, these data suggest that the lack of a transcriptional response to PACAP in L β T2 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. L β T2 and α T3-1 cells were serum-starved for 2 h prior to stimulation with 10 μ M 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 L β T2 and α T3-1 cells (Fig. 3A). The level of the CREB accessory protein, CBP, was examined in nuclear extracts of L β T2 and α T3-1 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 μ M forskolin for up to 60 min, total protein extracts were prepared from L β T2 and α T3-1 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 L β T2 and α T3-1 cells. Following treatment with 10 μ M forskolin for up to 60 min, total protein extracts were prepared from L β T2 and α T3-1 cells and Western blotting for PKA-C α II, PKA-RII α and PKA-RI α was performed. Similar levels of PKA-C α II and PKA-RI α were observed in both cell lines, with an apparent increase in PKA-RI α following forskolin stimulation in α T3-1 cells (Fig. 3D). However, only L β T2 cells expressed detectable levels of PKA-RII α (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 L β T2 and α T3-1 cells and respond to forskolin appropriately. The major

difference is that PKA-RII α is only expressed in L β T2 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 L β T2 cells exhibit altered DNA-binding characteristics, we performed EMSAs using the tandem CRE from the human α GSU 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 L β T2 and α T3-1 cells (Fig. 4). These data suggest proteins that bind the CRE in L β T2 and α T3-1 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 L β T2 and α T3-1 cells express CREM or ICER. cDNA was made from L β T2 and α T3-1 cells treated with 10 μ M forskolin for 6 h, and PCR was performed for ICER and CREM expression. No transcripts were detected for CREM in either L β T2 or α T3-1 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 L β T2 and α T3-1 cells expressed similar levels of ICER-II and ICER-II γ 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 L β T2 cells.

As we had observed that forskolin could stimulate the α GSU promoter, we wanted to establish whether a CRE sequence could act as a transcriptional responsive cassette in L β T2 cells. We transiently transfected L β T2 and α T3-1 cells with 5 μ g 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 μ M forskolin for 8 h. In α T3-1 cells, promoter activity was significantly enhanced to 3.3 ± 0.2 -fold

Figure 3 Expression of cAMP-stimulated signalling molecules in L β T2 and α T3-1 cells. (A) L β T2 and α T3-1 cells were stimulated with 0 or 10 μ M 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 L β T2 and α T3-1 cells. HeLa cell nuclear extracts were used as a positive control. (C and D) Total proteins were extracted from L β T2 and α T3-1 cells stimulated with 0 or 10 μ M forskolin for up to 60 min, before Western blotting for phosphoAkt and total Akt1/2 (C) or PKA-C α , PKA-RII α or PKA-RI α (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.

Figure 4 Analysis of CRE-binding proteins in L β T2 and α T3-1 cells. EMSA analysis of nuclear extracts of L β T2 and α T3-1 cells was performed using the consensus tandem CRE sequence from the wild-type human α GSU promoter as the 32 P-labelled probe. In some instances, the proteins were pre-incubated with either anti-ATF-2, anti-cJun or anti-phosphoCREB antibodies to identify the complexes formed. The autoradiograph is representative of at least three separate experiments.

over basal ($P < 0.001$), but no response was seen in L β T2 cells (Fig. 6A). This suggests that the CRE sequence alone is incapable of acting as a cAMP-response cassette in L β T2 cells.

Having established that forskolin could enhance CREB phosphorylation in both L β T2 cells and α T3-1 cells, we investigated whether the transcriptional activity of CREB could be similarly induced. L β T2 and α T3-1 cells were co-transfected with 5 μ g Gal-4-CREB expression vector, 5 μ g Gal-4-LUC reporter gene and 5 μ g Bos β Gal, before stimulation for 8 h with 0 or 10 μ M forskolin. Surprisingly, forskolin only modestly increased CREB transcriptional activity in L β T2 cells under control conditions (to 2.8 ± 0.9 -fold, Fig. 6B). In contrast, forskolin potently enhanced CREB transcriptional activity (to 32.7 ± 4.7 -fold, $P < 0.001$) in α T3-1 cells. Taken together, these data reveal marked differences in cAMP-mediated gene transcription in L β T2 and α T3-1 gonadotrophs.

Discussion

In the present study we have examined the regulation of human α GSU promoter activity by cAMP, using transient transfection of L β T2 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 α T3-1 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 α GSU mRNA concentrations and α GSU gene transcription in primary rat pituitary cell

Figure 5 Expression of CREM and ICER in L β T2 and α T3-1 cells. RT-PCR for CREM and ICER was performed on cDNA extracted from L β T2 and α T3-1 cells treated with 0 or 10 μ M forskolin for 8 h. Rat testis cDNA was used as a control for CREM (upper panel), where no specific transcripts were detected in either L β T2 or α T3-1 cells. Two ICER-specific transcripts were detected in both L β T2 and α T3-1 cells following forskolin stimulation. GAPDH was used to monitor the integrity of the cDNA. The autoradiograph is representative of at least three separate experiments.

cultures and α T3-1 gonadotrophs, an effect that was markedly reduced when α GSU promoter constructs containing a mutant CRE site were used (Burrin *et al.* 1998). In the α T3-1 cell line, PACAP continues to stimulate α GSU 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 α GSU promoter. In addition, previous studies have shown that the PKA inhibitor, H-89, can attenuate the effect of PACAP on the mouse α GSU 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 α GSU transcription in this cell line, similar to the response seen with the adenylyl cyclase activator, forskolin. In view of these data, which clearly implicate the cAMP signalling pathway in mediating the actions of PACAP on α GSU gene transcription in gonadotrophs, it was of considerable interest to observe the lack of α GSU transcriptional response to PACAP in the L β T2 cell line.

To further investigate this observation, we performed receptor-binding studies and RT-PCR

to look for PAC $_1$ -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 L β T2 cells lacked functional PACAP receptors activating the cAMP pathway. However, we were able to detect some PAC $_1$ -R-specific transcripts by PCR, albeit at extremely low copy numbers. This is in agreement with recent reports of PAC $_1$ -R expression in L β T2 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 L β T2 cells may express various isoforms of the PAC $_1$ -R, they do not express PAC $_1$ -R protein as determined by radioligand-binding assays.

Several recent studies have investigated the effects of PACAP in L β T2 cells. Although PACAP has been reported to stimulate LH β , FSH β 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.*

Figure 6 Comparison of CRE- and CREB-mediated transcription in L β T2 and α T3-1 cells. (A) L β T2 and α T3-1 cells were transfected with 5 μ g CRE-TKLUC and 5 μ g Bos β Gal, before stimulation with 0 or 10 μ M forskolin for 8 h. The data shown are means $\pm$ S.E.M., expressed as fold increase over basal, pooled from at least three individual experiments each performed in triplicate. *** P < 0.001, significantly different from basal. (B) L β T2 and α T3-1 cells were transfected with 5 μ g GAL-4-CREB, 5 μ g GAL-4-LUC and 5 μ g Bos β Gal, before stimulation with 0 or 10 μ M forskolin for 8 h. The data shown are means $\pm$ S.E.M., expressed as fold increase over basal, pooled from at least three individual experiments each performed in triplicate. *** P < 0.001, significantly different from basal.

2002, Garrel *et al.* 2002). Indeed, our current observations report a 1.7-fold increase in α GSU promoter activity following PACAP stimulation of transfected L β T2 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 L β T2 cells, in contrast to their observations in α T3-1 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 L β T2 cells. There are no reports of a non-cAMP-coupled PACAP receptor being expressed in pituitary cells. However, a splice variant of the PAC $_1$ -R is expressed in rat cerebellum and β -islets (known as PAC $_1$ -R-TM4) which solely activates L-type Ca $^{2+}$ channels (Chatterjee *et al.* 1996). We have previously shown that the response of the human α GSU promoter to elevated extracellular Ca $^{2+}$ 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 L β T2 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 L β T2 cells, they are present in extremely low numbers. Another possible explanation for the modest effects of PACAP on pituitary gene transcription in L β T2 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 G α_i in smooth muscle cells, and leads to an increase in calcium influx (Murthy *et al.* 1998). However, it is not clear whether L β T2 cells express NPR-C. Finally, cell lines are known to mutate over time and the absence of functional PAC $_1$ -R receptors might reflect receptor loss due to mutation. We have used L β T2 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 L β T2 cells. Recently, GnRH was shown to stimulate cAMP accumulation via G α_s activity (Liu *et al.* 2002), and we have observed a potent effect of glucagon-like peptide 1 (GLP-1) on α GSU transcription in L β T2 cells following transient expression of the GLP-1 receptor (R C Fowkes, K K Sidhu & J M Burrin,

unpublished observations). Furthermore, GnRH stimulation of the PACAP promoter in L β T2 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 α GSU promoter in L β T2 cells. However, the magnitude of this response was significantly less than that observed in parallel observations in α T3-1 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 α GSU promoter by the same magnitude in both L β T2 and α T3-1 cells (Fowkes *et al.* 2002). In addition, differences in transfection efficiency do not underlie the current observations, because the activity of the promoterless control, pA3 LUC, or the minimal thymidine kinase promoter, TKLUC, is not significantly different between L β T2 and α T3-1 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 α GSU transcription was less potent in L β T2 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 L β T2 and α T3-1 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 L β T2 and α T3-1 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 L β T2 and α T3-1 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 L β T2 and α T3-1 cells expressed two specific ICER transcripts corresponding to ICER II and ICER II γ , 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 adenylyl 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 α T3-1 gonadotrophs and rat anterior pituitary cells express PKA-C and PKA-RI 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 L β T2 and α T3-1 cells. Our results demonstrate that PKA-C α was found at similar levels in both gonadotroph cell lines and was not affected by forskolin treatment. PKA-RI α was also expressed in both cell lines, and appeared to be up-regulated in response to forskolin in α T3-1 cells but less so in L β T2 cells. However, the major difference in subunit expression was observed in PKA-RII α , which was only detected in L β T2 cells. Therefore, the possibility exists that L β T2 cells express more PKA regulatory subunits than α T3-1 cells, and therefore may require higher levels of cAMP to dissociate the PKA-C than would be the case in α T3-1 cells. Further studies using PKA isoform-specific agonists/antagonists (e.g. Rp-cAMPs, 8-Br-cAMP, dibutyryl cAMP) are required to establish the exact mechanism behind this apparent discrepancy in L β T2 and α T3-1 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 α GSU is directed partially via the tandem CREs as determined by mutational analysis (Burrin *et al.* 1998). The CREs of the human α GSU 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 L β T2 and α T3-1 cells, we examined whether the DNA-binding characteristics were altered, using EMSA analysis. The expected four complex pattern was observed using nuclear extracts of L β T2 and α T3-1 cells, and the tandem α GSU 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 α GSU 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 α T3-1 cells but not at all in L β T2 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 α T3-1, but L β T2 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 L β T2 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 L β T2 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 α GSU, LH β and FSH β genes. However, we have clearly shown that the L β T2 and α T3-1 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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