Stimulation of extracellular signal-regulated kinase by pituitary adenylate cyclase-activating polypeptide in α T3-1 gonadotrophs

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Abstract

The putative hypophysiotropic factor pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates glycoprotein hormone α -subunit (α GSU) gene transcription and secretion in the clonal gonadotroph α T3-1 cell line. The specific signalling pathways regulating these actions of PACAP have not been clearly defined. We have examined the possibility that mitogen activated protein kinases (MAPKs) may play a role in mediating the effects of PACAP on α T3-1 gonadotrophs. Treatment of α T3-1 cells with PACAP (100 nM) or epidermal growth factor (EGF, 10 nM) for 5 min significantly stimulated extracellular signal-regulated kinase activity (ERK, a component of the MAPK pathway) as determined by an

immunocomplex assay. Pre-treatment of α T3-1 cells with the specific MAPK kinase (MEK) inhibitor, U0126, blocked PACAP and EGF-induced activation of ERK. Transcriptional stimulation of a human α GSU-luciferase reporter construct by PACAP was unaffected by U0126 treatment. However, pre-treatment with U0126 significantly inhibited PACAP stimulation of [3 H]-thymidine incorporation in α T3-1 cells. Thus our results suggest that PACAP stimulates ERK activation in α T3-1 cells, and that the functional effect of this ERK activation is increased DNA synthesis and cell proliferation rather than transcriptional activation of the α GSU gene.

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic peptide which has functional effects on pituitary gonadotrophs. These effects include stimulation of glycoprotein hormone α -subunit (α GSU) gene transcription (Burrin *et al.* 1998, Tsujii *et al.* 1994) and gonadotroph cell proliferation (Schomerus *et al.* 1994). PACAP receptors are members of the seven transmembrane G-protein-coupled receptor (GPCR) superfamily, but the intracellular signalling pathways that mediate the stimulatory and synergistic actions of PACAP on α GSU transcription or gonadotroph cell proliferation are poorly understood.

At least three subtypes of PACAP receptor exist (PAC₁-R, VPAC₁-R and VPAC₂-R). Activation of each of these receptors by PACAP stimulates adenylyl cyclase (AC) and there is also some suggestion that the phospholipase C (PLC) signalling pathway may be triggered to varying degrees (Rawlings and Hezerah 1996). Normal rat gonadotrophs express the PAC₁-R subtype, whereas the mouse gonadotroph derived α T3-1 cell line expresses PAC₁-R and VPAC₂-R. However, despite the suggestion that these receptors may be linked to the PLC system, previous studies have shown that the α GSU transcriptional response to PACAP in gonadotrophs is mediated via a

cAMP-dependent mechanism with the protein kinase C (PKC)/calcium pathway being unimportant (Burrin *et al.* 1998, Tsujii *et al.* 1995).

The downstream effectors that mediate the transcriptional effects of PACAP following AC activation have not been fully defined. The mitogen activated protein kinase (MAPK) family of enzymes are potential candidates and several studies have demonstrated that gonadotrophin releasing hormone (GnRH) can stimulate activation of p38, ERK and JNK in α T3-1 cells (Naor *et al.* 2000). Studies have also shown that ERK may play a role in mediating basal, and to a lesser extent, GnRH-stimulated expression of the α GSU gene (Sunderasan *et al.* 1996). The mechanism for activation of ERK by GnRH is thought to involve both Ca²⁺ and PKC with the influence of calcium being downstream of PKC (Mulvaney *et al.* 1999).

It is not yet determined whether PACAP can also activate the MAPK pathway in gonadotrophs, although it is reported to stimulate ERK in other cell types in a PKC-dependent manner (Barrie *et al.* 1997). The evidence for AC activation in gonadotrophs stimulating ERK is conflicting, with one study reporting an increase in ERK activity with forskolin (Reiss *et al.* 1997) while others failed to find a response (Johnson *et al.* 2000). In this study, we have examined whether PACAP stimulates the

activity of ERK in gonadotrophs and whether ERK activation is involved in mediating the stimulatory effects of PACAP on α GSU promoter activity or DNA synthesis in gonadotroph cells.

Materials and Methods

Materials

PACAP(1–38), Epidermal Growth Factor (EGF) and the protein kinase A inhibitor 14–22 amide (PKi), were purchased from CN Biosciences (Nottingham, UK). The specific MEK inhibitor, U0126, was obtained from Promega (Southampton, UK) and used at 1 μ M, a concentration previously reported to produce half-maximal ERK activity (Favata *et al.* 1998). All the above components were prepared as stock solutions in sterile water and applied to the cells in culture media. Nifedipine, GF109203X and thapsigargin were purchased from CN Biosciences, prepared as stock solutions in Me₂SO and applied to the cells in culture media. Cells were exposed to less than 0.01% Me₂SO and these concentrations of vehicle had no effect on responses of α T3–1 cells.

Cell Culture

α T3–1 cells were cultured in monolayer in Dulbecco's modified Eagle's medium supplemented with high glucose (4500 mg/L) containing 10% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μ g/ml) and fungizone (125 mg/L) (Life Technologies, Paisley, UK) (hereafter referred to as culture medium). Cells were plated at a density of 2×10^6 cells for ERK activity assays and 1×10^6 cells for transient transfection experiments. These cell densities were chosen to optimise protein requirements for the ERK activity assay and to yield sufficient light units for the luciferase assays. For kinase assays cells were serum-starved overnight before receiving treatments to reduce basal ERK activity as described previously (Wang & Maurer 1999).

Total protein extractions and measurement of ERK activity

Cells were treated with medium alone, 100 nM PACAP or 10 nM EGF for the indicated times, and total proteins extracted. The concentrations chosen were those shown previously to produce the maximal response for each agonist (Burrin *et al.* 1998, Roberson *et al.* 2000). ERK activity was measured using a commercially available kit (New England Biolabs, Hitchin, UK). After stimulation, the cells were washed briefly with ice-cold PBS, before treatment with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml (w/v)

leupeptin and 1 mM PMSF (added directly before use) and incubation on ice for 5 min. The protein concentration of the lysates were measured by the Bradford assay. The lysates were transferred to microcentrifuge tubes before immunoprecipitation with an immobilized phospho-p44/42 MAP kinase (ERK) (Thr202/Tyr204) monoclonal antibody. The immunoprecipitated protein was used in a kinase assay with 200 μ M ATP and 2 μ g Elk-1 fusion protein, before treatment with SDS sample buffer. 20 μ g of the samples were subjected to SDS-PAGE separation, protein transfer to nitrocellulose membrane and Western blotting (see above), using a phospho-Elk-1 antibody (1:1000) as the primary antibody and a combination of HRP-conjugated anti-rabbit secondary antibody (1:2000) with an HRP-conjugated anti-biotin antibody (1:1000) to detect the biotinylated protein markers. The proteins were visualized by enhanced chemiluminescence, using the supplied LumiGLO and peroxidase reagents. Autoradiographs were analysed by scanning densitometry using Gel Base/Gel Blot Pro software (Synoptics Ltd, Cambridge, UK). Peak height intensities were used to calculate ERK phosphorylation of Elk-1, and these values were expressed as fold values compared with the untreated controls.

Plasmids and transient transfection studies

The reporter constructs -517 α LUC, pA3 LUC and Bos β Gal, have been described previously (Holdstock *et al.* 1996). The plasmid containing the Elk-1 activation domain fused to the Gal-4 DNA-binding domain (Gal-4-Elk-1) and the Gal-4 promoter-luciferase (Gal-4-LUC) were obtained from Professor A. F. Rosso (University of Iowa, Iowa, USA) and have been described previously (Durham & Rosso 1998). Cells were transfected by the calcium phosphate technique without glycerol shock, with either 10 μ g of -517 α LUC, pA3 LUC, Gal-4-Elk-1 or Gal-4-LUC and with 5 μ g of Bos β Gal (used as an internal control for transfection efficiencies). The cells were stimulated for 8 h with culture medium, without or with 100 nM PACAP and in the presence or absence of U0126. The cells were harvested and cellular extracts were assayed for luciferase and β -Galactosidase activity as described previously (Burrin *et al.* 1998).

[³H]-thymidine incorporation

α T3–1 cells were plated at 5×10^4 cells/well in media containing 2% charcoal-stripped FCS and the indicated concentrations of PACAP and U0126 for 72 h before addition of 1 μ Ci/well of [³H]-thymidine (Amersham, Bucks., UK) for a further 6 h. Cells were trypsinised and harvested (Tomtec, UK) on to filters before counting in the presence of scintillation fluid using a MicroBeta 1450 β -counter (Wallac, UK).

Figure 1 Elk-1 phosphorylation in α T3-1 cells by PACAP (100 nM) and EGF (10 nM). α T3-1 cells were treated with PACAP (100 nM) or EGF (10 nM) for the indicated times (min). ERK activation was determined by an immunoprecipitation kinase assay and subsequent scanning densitometry (upper panel). An autoradiograph of pElk-1 is shown (lower panel) with accompanying scanning densitometry data (* $P < 0.05$, ** $P < 0.01$ compared with control) (upper panel, $n = 4$ experiments).

Data presentation and statistical analysis

All graphical data were prepared using GraphPad Prism 3.02 (GraphPad, San Diego, USA) and analyzed using pre-programmed analysis equations within Prism. Data are presented as results which are representative of several experiments (performed a minimum of three times), or as normalized data pooled from multiple experiments. 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.

Results

Preliminary studies using Western blotting revealed that both PACAP (100 nM) and EGF (10 nM) phosphorylated ERK without altering total ERK expression with activity being maximal at 5 min and decreasing to control levels by 15 min (data not shown). α T3-1 cells were treated with PACAP or EGF for 5 min and ERK activation was measured using an immunocomplex assay. Activation at 5 min was enhanced by PACAP (2.1 ± 0.4 -fold, $P < 0.05$) but the effect was less pronounced than with EGF (3.5 ± 0.8 -fold ($P < 0.01$)) (Fig. 1). PACAP and EGF-stimulated ERK activation was blocked by pre-treatment with a MAPK kinase (MEK) inhibitor, U0126 (1 μ M) (Fig. 2 upper panel). Pre-treatment of α T3-1 cells with inhibitors of the PKA (PKi), PKC (GF109203X), Ca^{2+} entry (nifedipine) and Ca^{2+} mobilisation (thapsigargin) pathways revealed that PACAP activation of ERK was PKC sensitive as GF109203X blocked pElk-1 phosphorylation, but no other inhibitor altered the effects of PACAP (Fig. 2 lower panel). U0126 also blocked PACAP

Figure 2 Inhibition of PACAP and EGF-stimulated ERK activation in α T3-1 cells. Upper panel, cells were pre-treated with 0 or 1 μ M U0126 for 30 min and then treated for 5 min with PACAP (100 nM), EGF (10 nM), or no further treatment (control). Lower panel, cells were pre-treated with 0 (lane 1,2), 1 μ M U0126 (lane 3), 1 μ M PKi (lane 4), 1 μ M GF109203X (lane 5), 1 μ M nifedipine (lane 6), 1 μ M thapsigargin (lane 7) and then treated for 5 min with PACAP (100 nM) in the continued presence of inhibitors (lanes 2-7) or no further treatment (lane 1). Each blot is representative of at least three separate experiments ($n = 3$).

activation of a transiently transfected Gal-4-Elk-1/Gal-4-LUC expression vector/reporter gene system (Fig. 3). PACAP stimulated Gal4-LUC activity by 2.0 ± 0.2 -fold above basal ($P < 0.01$), but U0126 significantly attenuated this effect to 1.2 ± 0.1 -fold respectively (* $P < 0.05$ respectively, compared with control). The PACAP effect on the human α GSU promoter was not affected by U0126 (Fig. 3).

PACAP can stimulate DNA synthesis in α T3-1 cells, as determined by [^3H]-thymidine incorporation (Schomerus

Figure 3 Effects of U0126 on PACAP-stimulated gene transcription in α T3-1 cells. Cells were transiently transfected with either -517 α LUC or Gal4-Elk-1 expression vector and Gal4-LUC, and subsequently pre-treated with 0 or 1 μ M U0126 for 30 min, then stimulated with PACAP (100 nM) in the continued absence (open bars) or presence (filled bars) of U0126, harvested after 8 h and assayed for LUC activity. Basal promoter activity was normalised to 1. The results are expressed as fold increase over basal and are mean $\pm$ S.E.M. of at least 6 separate transfections ($n = 6$, $P < 0.05$).

Figure 4 Effects of U0126 on PACAP-stimulated [3 H]-thymidine incorporation α T3-1 cells. Cells were pre-treated with 0 or 1 μ M U0126 for 30 min, then incubated in the absence (basal, dotted line) or presence of PACAP (1 nM or 100 nM) in the continued absence (open bars) or presence (filled bars) of U0126 for a further 72 h. 1 Ci/well [3 H]-thymidine was added for the last 6 h of culture. Basal thymidine incorporation was normalised to 1. Results are expressed as fold increase over basal incorporation, and show means $\pm$ S.E.M. of at least 10 individual incubations ($n=10$, ** $P<0.01$, *** $P<0.001$).

et al. 1994). To establish whether transient ERK activation by PACAP mediates these effects, α T3-1 cells were pre-treated without or with 1 μ M U0126 for 30 min before being cultured for 72 h in 2% (v/v) charcoal-stripped FCS-supplemented culture media without or with 1 or 100 nM PACAP in the continued absence or presence of U0126. 1 μ Ci/well of [3 H]-thymidine was added for the last 6 h of culture. U0126 alone reduced basal thymidine incorporation by approximately 25% (data not shown). Both 1 nM and 100 nM PACAP significantly stimulated thymidine incorporation (by 3.1 ± 0.5 -fold and 3.8 ± 0.7 -fold respectively, $P<0.001$) (Fig. 4). Treatment of α T3-1 cells with U0126 significantly attenuated the effects of both 1 nM and 100 nM PACAP, suggesting that ERK activation is involved in mediating the effects of PACAP on DNA synthesis in gonadotrophs.

Discussion

These studies provide novel evidence that PACAP stimulates ERK activation in α T3-1 gonadotrophs as revealed by phosphorylation of the MAPK regulated transcription factor, Elk-1. PACAP treatment also resulted in activation of the Gal4-Elk-1 fusion protein, providing further evidence for activation of the MAPK signalling cascade in α T3-1 cells. These findings are consistent with an earlier report in which activation of ERK was obtained in response to PACAP in PC12 (chromaffin) cells (Barrie *et al.* 1997) and studies of another G-protein coupled

receptor (the GnRH receptor) stimulating ERK activity in gonadotrophs (Sunderasan *et al.* 1996, Naor *et al.* 2000).

ERK activation by PACAP and EGF was rapid and transient in α T3-1 cells. A similar transient activation of ERK has been observed following EGF treatment of α T3-1 cells where again, activation returned to control levels by 20 min (Reiss *et al.* 1997). We used concentrations of EGF and PACAP that have been reported previously as those producing the maximal response in gonadotrophs (Sunderasan *et al.* 1996, Reiss *et al.* 1997, Burrin *et al.* 1998). Thus, the magnitude of PACAP-stimulated ERK activation appears less than that observed for EGF in α T3-1 cells.

In α T3-1 cells, mRNA for both the PAC $_1$ -R and the VPAC $_2$ -R has been demonstrated. The PAC $_1$ -R has been shown to activate adenyl cyclase through G $_s$ -coupled receptors and PLC is also activated to varying degrees, through G $_{q/11}$ (Rawlings and Hezarah 1996). The VPAC $_2$ -R is thought to activate only adenyl cyclase. Since PACAP has been shown to stimulate cAMP production and inositol phospholipid turnover and to increase cytosolic free Ca $^{2+}$ concentration in α T3-1 cells (Schomerus *et al.* 1994, Tsujii *et al.* 1995) it has been suggested that its actions on this cell type are mediated via the PAC $_1$ -R (Rawlings and Hezarah 1996). Both Ca $^{2+}$ influx and PKC activation have been shown to lead to MAPK activation in gonadotroph cells (Mulvaney *et al.* 1999, Sunderasan *et al.* 1996, Reiss *et al.* 1997) whereas cAMP is reported to have either no effect or in some instances to stimulate MAPK phosphorylation (Johnson *et al.* 2000). We therefore wished to determine which of the PACAP-activated intracellular signalling pathways were contributing to PACAP-induced ERK activation. Our results with cell permeable selective inhibitors of signal transduction pathways clearly demonstrate that the PKC inhibitor GF109203X was able to inhibit PACAP-induced activation of ERK and suggest that a PKC-dependent pathway contributes to PACAP effects on ERK activation as was also shown in PC12 cells (Barrie *et al.* 1997). GF109203X inhibits the activity of most PKC isoforms and avoids the complication of chronic phorbol ester treatment, which both activates and depletes PKC activity. This compound has previously been used to demonstrate the PKC-dependent activation of ERK by GnRH in α T3-1 cells (Reiss *et al.* 1997), and TRH in GH $_3$ cells (Wang and Maurer 1999). The lack of effect of other cell signalling inhibitors on PACAP induced ERK activation suggests that neither influx of extracellular Ca $^{2+}$, mobilization of intracellular Ca $^{2+}$, or PKA activation are involved in mediating the effects of PACAP on the MAPK signalling cascade.

In similar experiments (data not shown) we were also able to confirm previous reports from other groups that the phorbol ester, PMA, stimulated ERK activation and that this effect could also be blocked with GF109203X. Thus, as has been shown for GnRH, activation of ERK

by PACAP appears to be PKC dependent. Interestingly, for GnRH, influx of extracellular calcium through nifedipine-sensitive voltage-gated calcium channels is also required for GnRH-induced activation of ERK (Mulvaney *et al.* 1999) with the influence of calcium lying downstream of PKC. Nifedipine had no effect in our experiments and thus the effect of PACAP on ERK appears to differ from that of GnRH and be independent of calcium influx.

Activation of the MAPK pathway is sufficient to stimulate mouse α GSU gene transcription as demonstrated by experiments with a constitutively active form of Raf kinase (Roberson *et al.* 1995), which increased expression of mouse α GSU promoter reporter genes in a concentration-dependent manner. Our findings that PACAP, a known stimulator of α GSU gene transcription, also activated ERK led us to investigate whether the MAPK pathway mediated the effects of PACAP on α GSU gene expression. Treatment of α T3-1 cells with the specific MEK inhibitor, U0126, suggested a modest inhibition of basal expression of human α LUC (data not shown) but clearly failed to affect PACAP stimulation of the human α GSU promoter. U0126 is a selective inhibitor of the MAPK signalling cascade, which specifically inhibits MEK and prevents PMA-induction of gene transcription in several cell types (Favata *et al.* 1998). We found this inhibitor to be equally effective in blocking ERK activation by PACAP and EGF, as measured in the immunocomplex assay. At the same concentration, U0126 also inhibited PACAP activation of the Gal-4-Elk-1 fusion protein confirming its effective inhibition of an ERK-mediated transcriptional response. Our results support a role for the MAPK signalling pathway in basal expression of the human α GSU gene in α T3-1 cells, but ERK activation does not appear to mediate PACAP induced α GSU promoter activity.

In addition to its stimulatory action on α GSU transcription, PACAP also induces gonadotroph cell proliferation and DNA synthesis (Schomerus *et al.* 1994). We confirmed the stimulatory effects of PACAP on DNA synthesis in α T3-1 cells and showed that inhibition of MAPK activation by U0126 significantly attenuated this action. It is probably important that PACAP effects on MAPK are only transient since it has been previously reported that the kinetics of MAPK activation can have important effects on cellular responses. For example, in PC12 cells, sustained MAPK activation has been associated with neuronal differentiation (Traverse *et al.* 1992) whereas transient activation of MAPK by EGF results in cell proliferation rather than differentiation (Marshall 1995). Thus, the transient activation of ERK induced by PACAP in α T3-1 cells may represent the signalling mechanism mediating a DNA synthetic and cellular proliferative response in these cells. Interestingly, in PC12 cells, PACAP induces sustained ERK activation, which results in their differentiation rather than their proliferation (Barrie *et al.* 1997).

In summary, our results demonstrate for the first time that PACAP causes ERK activation in α T3-1 cells via a PKC-dependent mechanism. These effects are transient rather than sustained. It is unlikely that this activation of ERK mediates PACAP-induced stimulation of α GSU transcription, although the MAPK signalling pathway may be involved in basal expression of the α GSU gene. Although the role of the MAPK signalling pathway in gonadotrophs remains to be fully elucidated, our studies would suggest that the cellular response to PACAP mediated by transient activation of ERK in α T3-1 cells is DNA synthesis and cell proliferation.

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