IGF-I and vasoactive intestinal peptide (VIP) regulate cAMP-response element-binding protein (CREB)-dependent transcription via the mitogen-activated protein kinase (MAPK) pathway in pituitary cells: requirement of Rap1

M Fernández, F Sánchez-Franco, N Palacios, I Sánchez and L Cacicedo
Servicio de Endocrinología, Hospital Ramón y Cajal, Carretera de Colmenar, Km 9, 28034 Madrid, Spain
Servicio de Endocrinología, Hospital Carlos III-C.I.C., Instituto de Salud Carlos III, Sinesio Delgado, 10-12, 28029 Madrid, Spain.

Abstract

In previous studies we demonstrated that vasoactive intestinal peptide (VIP) mediation, and interactions between mitogen-activated protein kinase (MAPK) and cAMP/protein kinase A (PKA) signaling pathways are implicated in insulin-like growth factor I (IGF-I)- and VIP-induced lactotroph proliferation. These facts led us to investigate the intracellular mechanisms involved in IGF-I- and VIP-induced lactotroph proliferation. Exposure of cultured male rat pituitary cells to IGF-I (10^{-7} M) or VIP (10^{-7} M) stimulated the MAPK cascade. Studies in GH4C1 cells, with an expression vector for Rap1 GTPase-activating protein (Rap1 GAP1), demonstrated reduced VIP-induced MAPK activation, indicating that VIP-dependent activation of the extracellular signal-regulated kinase (ERK) pathway requires PKA-Rap1 signaling. IGF-I induced cAMP-response element (CRE)-binding protein (CREB) phosphorylation through the Ras-MAPK pathway, whereas VIP phosphorylated CREB directly via PKA. The mechanisms that regulate IGF-I- and VIP-CREB-dependent gene transcription were examined using GH4C1 cells transiently transfected with a CRE reporter gene. IGF-I and VIP stimulation of CRE-mediated transcription required activation of both Ras-MAPK and cAMP/PKA signaling. This activation was blocked in the presence of Rap1 GAP1. In summary, we showed that IGF-I and VIP induced lactotroph proliferation may involve Rap1.

Introduction

Insulin-like growth factor I (IGF-I) and vasoactive intestinal peptide (VIP) are two established mitogenic factors synthesized within the rat anterior pituitary gland (Arnaout et al. 1986, Segerson et al. 1989, Bach & Bondy 1992, Renner et al. 1996). IGF-I mRNA was found, by in situ hybridization, to be distributed throughout the pituitary (Bach & Bondy 1992). IGF-I receptor mRNA is abundantly and homogeneously distributed in the anterior pituitary and intermediate lobes (Rosenfeld et al. 1985, Fruchtman et al. 2002). Functional IGF-I receptors in lactotroph cells have been recently described in the fish pituitary gland (Fruchtman et al. 2002). Also, IGF-I receptors are expressed in pituitary cell lines, such as GH3 and GH4C1 cells (Rosenfeld et al. 1985, Castillo & Aranda 1997). The actions of IGF-I are mediated by activation of a tyrosine kinase receptor, which resembles the insulin receptor in both structural and functional aspects (Abbott et al. 1992, LeRoith et al. 1995). Binding of IGF-I to its receptor is followed by a rapid phosphorylation of several protein substrates, leading to the activation of phosphatidylinositol 3-kinase (PI 3'-kinase) and mitogen-activated protein kinase (MAPK; LeRoith et al. 2001). Previous studies have suggested the importance of the MAPK pathway in mediating signals leading to cellular proliferation (Coolican et al. 1997, Reiss et al. 1998). IGF-I has been reported to increase the proliferation of pituitary cells, such as corticotrophs and lactotrophs in mice (Oomizu et al. 1998). We have demonstrated previously that IGF-I induces proliferation of lactotrophs in rats (Fernández et al. 2003). IGF-I is also required for the growth of human pituitary adenoma cells (Renner et al. 1993) and rat GH3 cells (Hayashi 1984). In addition to its mitogenic properties, IGF-I has been implicated in the regulation of pituitary hormone synthesis and release (Yamashita & Melmed 1986, Lara et al. 1994, Fletcher et al. 1995). Moreover, IGF-I increases prolactin (PRL) mRNA levels in the GH4C1 rat pituitary cell line (Castillo & Aranda 1997).

VIP is a physiological PRL-releasing factor (Abbe et al. 1985, López et al. 1989) acting through neuroendocrine
mechanisms. It is also expressed in the anterior pituitary (Arnaout et al. 1986), where VIP mRNA and its receptor have been localized in lactotroph cells (Chew et al. 1996, Wanke & Rorstad 1990). VIP is regulated by thyroid hormones (Segerson et al. 1989), estrogens (Lam et al. 1990) and dopamine (Balsa et al. 1996). Pituitary VIP plays an important paracrine and/or autocrine role in the regulation of PRL secretion in both normal anterior pituitary and GH3 cells (Hagen et al. 1986, Nagy et al. 1988). Moreover, VIP increases PRL mRNA levels in GH3 and GH4C1 cells (Carrillo et al. 1985, Le Péchon-Vallée et al. 2000). In previous studies, we demonstrated that VIP stimulates the proliferation of lactotroph cells in rats (Fernández et al. 2003). The actions of VIP are mediated by activation of adenylate cyclase and the increase in intracellular cAMP levels (Robberecht et al. 1979, Wanke & Rorstad 1990). Previous studies have provided evidence of an important role for cAMP not only in the regulation of lactotroph functions, such as secretion, synthesis and transcriptional regulation of PRL (Maurer 1981, Swennen & Denef 1982, Liang et al. 1992, Romano et al. 2003), but also in the proliferation of lactotrophs (Suzuki et al. 1999). Moreover, VIP activates MAPK cascade through the cAMP/protein kinase A (PKA) pathway in the GH4C1 rat pituitary cell line (Le Péchon-Vallée et al. 2000). We have shown previously that VIP-induced lactotroph proliferation is mediated by cAMP/PKA and MAPK signaling pathways (Fernández et al. 2003). Recent studies have provided new insights into cAMP signal transduction and demonstrated that Rap1 can play a crucial role in mediating cAMP-induced MAPK activation in specific cell types (Vossler et al. 1997, Alleaume et al. 2003). These studies have shown that cAMP can stimulate Rap1 activation, leading to activation of B-Raf, MAPK/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) and MAPK.

We have demonstrated previously that IGF-I induces pituitary VIP gene expression and secretion (Lara et al. 1994). Moreover, IGF-I-induced lactotroph proliferation is mediated by VIP, and interactions between MAPK and cAMP/PKA signaling pathways are implicated in the lactotroph proliferation induced by IGF-I and VIP (Fernández et al. 2003). One well-studied target of both MAPK and cAMP/PKA signaling pathways is the transcription factor CREB. CREB-binding protein (CREB; Shaywitz & Greenberg 1999). CREB binds as a dimer to a conserved CRE found in the promoter regions of numerous eukaryotic genes (Montminy 1997) including c-fos (Monnier et al. 1994). Phosphorylation of serine 133 is a critical event in CREB activation (Yamamoto et al. 1988) and induces an increase in CREB trans-activation potential by allowing the recruitment and binding to coactivators such as CREB-binding protein (CBP; Chrivia et al. 1993, Kwok et al. 1994). It is increasingly evident that signaling events in addition to CREB phosphorylation are required for full CREB-dependent transcription (Shaywitz & Greenberg 1999). Thus, the activity of the transcriptional co-activator CBP has been reported to be regulated by a variety of kinases, including PKA and ERKs (Kwok et al. 1994, Chawla et al. 1998, Liu et al. 1998). It is likely that the multiple actions of different signaling pathways may contribute to the stimulation of full CREB-dependent transcription by both cAMP/PKA and MAPK.

The aim of this study was to investigate the intracellular mechanisms involved in the action of IGF-I and VIP on the proliferation of lactotrophs. The first aim was to examine the ability of IGF-I and VIP to stimulate MAPK activity and the phosphorylation of CREB in pituitary cells and to study whether IGF-I- and VIP-induced CREB activation occurs within the lactotroph cells. The second aim was to examine whether activation of Rap1 is necessary for VIP-induced MAPK activation. Finally, the contribution of the MAPK and PKA-Rap1 signaling pathways to the control of gene transcription by IGF-I and VIP were also examined using the GH4C1 rat pituitary cell line.

Materials and methods

Reagents

Recombinant human IGF-I (rhIGF-I) was purchased from PreproTech EC (London, UK). Porcine VIP(1–28) was purchased from Bachem (La Jolla, CA, USA). All antibodies were purchased from Dako (Glostrup, Denmark) unless otherwise specified. Poly-L-ornithine was purchased from Sigma (St Louis, MO, USA). The chemical inhibitors PD98059, Rp-Adenosine 3’5’-cyclic monophospho-orthioate (Rp-cAMPS) and LY294002 were from Alexis Corp. (San Diego, CA, USA).

Buffers and media

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), Hank’s balanced salt solution (HBSS), PBS, penicillin/streptomycin, gentamycin and L-glutamine were purchased from BioWhittaker (Walkersville, MD, USA). Pituitary cell cultures were maintained in defined medium and consisted of DMEM (1 g/l glucose) supplemented with 1% BSA, 15 mM Hepes, 0–1 µM hydrocortisone, 0–5 mM 3,3’,5-triiodothyronine (T3), 10 µM transferrin, 10 mM glucagon, 0–2 nM parathyroid hormone (Sigma), L-glutamine (4 mM) and penicillin/streptomycin (100 U/ml). GH4C1 cells were incubated in defined medium and consisted of DMEM (4–5 g/l glucose) supplemented with 20 mM Hepes, 4 mM L-glutamine and 60 µg/ml gentamycin.
Cell culture
Preparation of primary rat anterior pituitary cell cultures was done as described previously (Carcedo & Sánchez-Franco 1986). 2-month-old male Sprague-Dawley rats were supplied by Charles River (Barcelona, Spain). They were maintained in a temperature- and light-controlled room and given food and water ad libitum. Animal handling was conducted in accordance with the guidelines on protection of animals used in scientific research set by Real Decreto 223/1988 March 14th and Orden October 13th 1989. The animals were killed by decapitation and their pituitary glands were removed under sterile conditions. The neurohypophyses were discarded and the anterior pituitaries collected and mechanico-enzymatically dispersed with 0·1% papain, 0·1% neutral protease and 0·1% DNase for 1 h at 37 °C. The dispersed pituitary cells were resuspended in defined medium containing FCS (3%), plated on poly-L-ornithine-coated 35 mm tissue-culture dishes and seeded at a density of 5 × 10^5 cells per dish. For immunocytochemical studies, the cells were plated on poly-L-ornithine-coated glass coverslips at a density of 10^5 cells/coverslip in 24-well culture plates. Cultures were kept in a humidified atmosphere of 5% CO₂/95% air at 37 °C. After 72 h of incubation in serum-supplemented defined medium (3% FCS; preincubation), cells were maintained in defined medium (serum-free) for 24 h. For transfection, GH4C1 cells were grown in defined medium containing 10% FCS. They were seeded in 35 mm culture dishes at a density of about 8 × 10^5 cells per dish, 24 h before transfection.

Experimental design
Signaling pathway studies were determined in anterior pituitary cells by quantitation of phosphorylated forms of p44 (ERK1) and p42 (ERK2) MAPKs and CREB. Pituitary cells were plated on poly-L-ornithine-coated 35 mm tissue-culture dishes as described above. After 72 h of incubation in serum-supplemented medium, cells were cultured in defined medium (serum-free) for 24 h. Then the medium was replaced by defined medium containing IGF-I (10⁻⁷ M) and VIP (10⁻⁷ M) for the times indicated. The levels of phosphorylated ERK1, ERK2 and CREB were quantitated by Western immunoblotting as described below. To determine the intracellular mechanisms involved in the action of IGF-I and VIP on the MAPK and CREB activation, cells were pre-treated with the MEK inhibitor PD98059 (10 µM), the selective inhibitor of PKA Rp-cAMPS (10 µM) and the PI 3-kinase inhibitor LY294002 (20 µM) for 45 min prior to the addition of IGF-I or VIP. Cells were then incubated for the times indicated. PD98059 was dissolved with dimethyl sulfoxide at a concentration of 3·7 mM. Rp-cAMPS was initially dissolved in distilled water at a concentration of 2·2 mM. LY294002 was dissolved with methanol at 3·2 mM. These agents were diluted immediately before use. To analyze the effect of Rap1 on VIP-induced activation, GH4C1 cells were transfected with either Rap1 GTPase-activating protein (Rap1 GAP1; 8 µg) or empty vector (8 µg). After 24 h of incubation in defined medium (serum-free), VIP was added for the indicated times.

Activation of CREB in lactotroph cells was analyzed in anterior pituitary cells by double-labeling immunocytochemistry staining for phosphorylated CREB and PRL. Cells were plated on poly-L-ornithine-coated glass coverslips in 24-well culture plates and treated as described above. P-CREB/PRL labeling index was examined immunologically, and the percentage of labelled cells was determined as indicated below.

Western immunoblots
At the end of the experiments anterior pituitary cells, growing in 35 mm dishes, were lysed in a buffer containing 25 mM Tris/HCl, pH 7·4, 50 mM Hepes, 100 mM NaF, 2 mM EDTA, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin. Total protein extracts (40–50 µg) were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. After blocking the membranes, immunodetection was performed using an antisera specific for tyrosine- and threonine-phosphorylated forms of p44 (ERK1) and p42 (ERK2) MAPKs (1:10 000 dilution; Sigma), or an antibody that recognizes phospho-Ser133 CREB (Ser133; 1:1000 dilution; Upstate Biotechnology, Lake Placid, NY, USA), followed by incubation with a goat peroxidase-conjugated anti-rabbit secondary antibody (Dako). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system supplied by Amersham Biosciences (Little Chalfont, Bucks, UK). All nitrocellulose membranes were systematically treated with a blocking solution of 5% non-fat dry milk in Tyrode buffer and reprobed with phosphorylation state-independent antibodies, ERK1, ERK2 (Sigma) and CREB (Upstate Biotechnology).

Immunocytochemistry
Immunocytochemistry was performed with cells plated on poly-L-ornithine-coated glass coverslips in 24-well culture plates. CREB-labeling indices indicative of the activation rate of lactotrophs were determined by double-labeling immunocytochemistry staining for PRL and phospho-Ser133 CREB (Ser133). Cells were fixed in 4% paraformaldehyde in PBS for 10 min at 4 °C, washed in PBS three times for 5 min each at room temperature, and permeabilized with 0·1% Triton X-100 for 10 min at room temperature.
temperature, and blocked with normal goat serum for 15 min. Cells were incubated with rabbit anti-CREB (Ser\textsuperscript{133}; 1:100 dilution) in blocking solution overnight at 4 °C. Cells were washed extensively with Tris-buffered saline, pH 7.4, after primary antibody incubation, and incubated in anti-rabbit IgG biotinylated secondary antibody (Zymed Laboratories, San Francisco, CA, USA) for 1 h. The cells were washed extensively with Tris-buffered saline three times for 5 min each and then the third reagent (avidin–biotin–peroxidase complex) was applied for 1 h at room temperature. The immunoreactivity of CREB was detected using 0-05% DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma) and 0-005% H\textsubscript{2}O\textsubscript{2} solution for 1-5 min at room temperature. The CREB-labeled nuclei were stained brown. After extensive washes in PBS and incubation with rabbit anti-PRL (NIDDK IC-5) at 1:4000 dilution overnight at 4 °C, cells were incubated with goat anti-rabbit rhodamine-conjugated secondary antisera (1:250 dilution). The plastic coverslips were mounted on glass slides with Mowiol (Sigma) and cells were observed with a fluorescence microscope. Immunostaining was abolished by omission of each primary antibody. No nuclear staining was observed using preimmune antisera instead of primary antibodies. Cells immunoreactive for PRL were counted with a 40× objective lens to determine the P-CREB/PRL labeling index, which expresses the percentage of pituitary cells immunoreactive for both PRL and phospho-Ser\textsuperscript{133} CREB (P-CREB) from all PRL-immunoreactive cells counted. Three slides were analyzed for each treatment group derived from the same cell preparations. Each experiment was repeated three times.

**Plasmids and transfections**

Dr P Stork kindly provided the FLAG-Rap1 GAP1 expression vector. pGL3-Control and pGL3-CRE expression vectors (Promega, Madison, WI, USA) were modified by Dr M.J. Lorenzo. GH4C1 cells were cultured in defined medium supplemented with 10% FCS. For transfection, GH4C1 cells were seeded into 35 mm culture dishes at an approximate density of 8×10\textsuperscript{3} cells per dish 24 h before transfection. DNA (4–8 µg total) was transfected using the Lipofectamine reagent (Invitrogen, Life Technologies, Paisley, Scotland, UK) according to the manufacturer’s protocol. Following overnight incubation, the cells were treated with IGF-I and VIP for 8 h, after which they were washed, lysed and analyzed for luciferase activity. Cells were transfected with pRSV-β-galactosidase expression vector to monitor transfection efficiency. Luciferase activity was normalized to galactosidase activity, and results are expressed as a percentage of the control group.

**Statistical analysis**

All data are expressed as means ± s.e.m. Tests for significance between sample groups were performed with a two-tailed t-test. For multiple comparisons, ANOVA was used with Fisher’s test for post-hoc comparisons. Differences were considered statistically significant if \( P < 0\text{.}005 \).

**Results**

**IGF-I and VIP stimulate MAPK activity in pituitary cells**

In previous studies we demonstrated that the MAPK signaling pathway is implicated in lactotroph proliferation induced by IGF-I and VIP (Fernández et al. 2003). To further investigate the role of the MAPK pathway on IGF-I and/or VIP action on lactotrophs, dissociated rat anterior pituitary cells were exposed to IGF-I (10\textsuperscript{-7} M) or VIP (10\textsuperscript{-7} M) for varying times and the levels of MAPK isoforms ERK1 and ERK2 were quantitated by Western immunoblotting.

As shown in Fig. 1A, treatment of pituitary cells with IGF-I (10\textsuperscript{-7} M) resulted in the rapid stimulation of the phosphorylation of ERK1 and ERK2. Maximum increase (5-fold) was observed after 2 min of IGF-I treatment. Phosphorylation declined to basal levels after 10 min, even under the continuous presence of the growth factor. There was no change in the levels of total immunoreactive ERK1 and ERK2, suggesting that the increased phosphorylation was not due to a change in the total amount of these kinases.

Treatment of pituitary cells with VIP (10\textsuperscript{-7} M; Fig. 1B) resulted in a rapid and more sustained stimulation of the phosphorylation of ERK1 and ERK2. The highest activation occurred at 2 min and persisted after 30 min. These results indicate that IGF-I-induced MAPK activation declined more rapidly than the VIP effect.

**MEK1/2 activation is required for IGF-I-induced ERK1 and ERK2 activation**

To determine the intracellular mechanisms involved in the action of IGF-I on ERK1 and ERK2 activation, cells were pre-treated with the MEK inhibitor PD98059 (10 µM), the PI 3′-kinase inhibitor LY294002 (20 µM) and the selective inhibitor of PKA Rp-cAMPS (10 µM). In previous studies we showed that at these concentrations PD98059 and Rp-cAMPS inhibited IGF-I- and VIP-induced lactotroph proliferation (Fernández et al. 2003). As shown in Fig. 2A, the effect of IGF-I on ERK1 and ERK2 activation was completely abolished by PD98059 (\( P < 0\text{.}001 \)). The blockade of the PI 3′-kinase pathway with LY294002 did not modify this activation.

Journal of Molecular Endocrinology (2005) 34, 699–712
We have demonstrated previously that IGF-I-induced lactotroph proliferation is mediated by VIP; thus it was important to determine whether the inactivation of PKA with Rp-cAMPS modified IGF-I-induced MAPK activation. IGF-I-induced ERK 1/2 activation was not affected by exposure to the PKA inhibitor (Fig. 2A). Pre-treatment with inhibitors had no effect on basal levels of MAPK activation, indicating that, at the concentrations used in the study, they caused no appreciable toxicity. These results indicate that IGF-I regulates MAPK phosphorylation via activation of the Ras-Raf-MEK signaling cascade.

Involvement of the cAMP-dependent protein kinase on VIP-induced ERK1 and ERK2 activation

The major signaling pathway that mediates responses to cAMP involves activation of the cAMP-dependent protein kinase (PKA). Previous studies have demonstrated that VIP activates the MAPK cascade through the cAMP/PKA pathway in the GH4C1 rat pituitary cell line (Le Péchon-Vallée et al. 2000). The inhibitor Rp-cAMPS (10 µM) was used to determine whether inhibition of PKA affected VIP-induced MAPK activation. The results, shown in Fig. 2B, indicate that the ability of VIP to activate ERK1/2 was completely abolished by Rp-cAMPS (P<0.01). These data indicate that PKA is required for VIP-induced stimulation of MAPK activity.

Involvement of Rap1 on VIP-induced ERK1 and ERK2 activation

As expected, the blockade of the MAPK cascade with PD98059 (10 µM) completely abolished VIP-induced ERK1/2 activation. Recent studies have demonstrated that Rap1 can play a crucial role in mediating cAMP-inducing MAPK activation (Vossler et al. 1997). To determine whether the activation of Rap1 is necessary for VIP-induced MAPK activation an expression vector for Rap1 GAP1 was used (Jordan et al. 1999). Forced expression of Rap1 GAP1 should maintain Rap1 in the inactive, GDP-bound state, and thus act as an antagonist to this signaling pathway. As shown in Fig. 3, Rap1 GAP1 reduced VIP-induced levels of phosphorylated ERK 1/2 at both time points tested. The ability of Rap1 GAP1 to reduce MAPK activation offers evidence that Rap1 may contribute to VIP-induced MAPK activation in GH4C1 cells.

IGF-I and VIP stimulate CREB activation in pituitary cells

CREB, one of the best-characterized stimulus-induced transcription factors, is a target of both MAPK and...
cAMP (Shaywitz & Greenberg 1999). All signaling pathways that activate CREB lead to phosphorylation of a particular residue, Ser133 (Montminy 1997). This phosphorylation is required for CREB-induced gene transcription. To investigate the role of IGF-I and VIP on CREB activation, dissociated rat anterior pituitary cells were incubated with IGF-I (10^{-7} M) or VIP (10^{-7} M) for varying times. The level of phosphorylated CREB was examined using an antibody that recognizes phospho-Ser133 CREB.

As shown in Fig. 4A, treatment of pituitary cells with IGF-I (10^{-7} M) resulted in the rapid stimulation of the phosphorylation of CREB. Maximum activation (3-fold) was observed after 5 and 10 min of IGF-I treatment. CREB activation declined to basal levels after 60 min. As shown in Fig. 4B, VIP (10^{-7} M) induced a rapid and sustained phosphorylation of CREB that started at 5 min, remained elevated for 60 min and declined to basal levels after 3 h.

**Inhibitory effect of MEK inhibitor on IGF-I-induced CREB activation**

To determine whether the effect of IGF-I on phosphorylation of CREB involved the MAPK pathway, cells were pretreated with the MEK inhibitor, PD98059 (10 µM). Furthermore, using the selective inhibitor of PKA, Rp-cAMPS (10 µM), we analyzed whether the effect of IGF-I on CREB phosphorylation involved the VIP-induced PKA activation. As shown in Fig. 5A, the effect of IGF-I on CREB activation was completely abolished by PD98059 (P<0.01). Rp-cAMPS did not modify IGF-I-induced CREB activation. These results indicate that IGF-I induces CREB phosphorylation via activation of the MAPK signaling pathway.

**Inhibitory effect of PKA inhibitor on VIP-induced CREB activation**

The data shown above suggested that VIP stimulated MAPK activation in pituitary cells. To determine...
whether the effect of VIP on CREB phosphorylation involved the MAPK and/or PKA pathways, cells were pre-treated with the MEK inhibitor, PD98059 (10 µM), or the PKA inhibitor, Rp-cAMPS (10 µM). The results, shown in Fig. 5B, indicate that PD98059 did not modify VIP-induced CREB activation, whereas the effect of VIP on CREB activation was completely abolished by Rp-cAMPS (P < 0.01). These results indicate that VIP stimulates CREB phosphorylation via the cAMP/PKA pathway.

**IGF-I and VIP stimulate CREB activation in lactotroph cells**

We have previously demonstrated that IGF-I and VIP induce lactotroph proliferation (Fernández et al. 2003). The data shown above indicate that both IGF-I and VIP stimulated CREB phosphorylation. Consequently, we analyzed whether IGF-I- and VIP-induced CREB activation occurs within the lactotroph cells. CREB phosphorylation was examined in anterior pituitary cells by double-labeling immunocytchemistry staining for phosphorylated CREB and PRL. As shown in Fig. 6, IGF-I (10⁻⁷ M) and VIP (10⁻⁷ M) induced a significant increase in phospho-CREB immunoreactivity in lactotroph cells (control, 22 ± 1.3%; IGF-I, 79 ± 2.3%; VIP, 68 ± 1.9%). Thus, these results indicate that CREB activation takes place in the lactotroph cells.

**IGF-I and VIP stimulate CRE-dependent transcription in GH4C1 cells**

We have demonstrated previously that IGF-I and VIP induce lactotroph proliferation through interactions between the MAPK and cAMP/PKA signaling pathways (Fernández et al. 2003). Also, we have confirmed that IGF-I and VIP increase the activation of CREB in lactotroph cells. Therefore, we next investigated the effect of IGF-I and VIP in the regulation of CRE-dependent transcription in GH4C1 cells. Following transfection of GH4C1 cells with a CRE-luciferase reporter, IGF-I and VIP stimulated CRE-dependent transcription (P < 0.01) as shown in Fig. 7A.

**Inhibitory effects of MAPK and PKA inhibitors on IGF-I- and VIP-induced CRE-dependent transcription**

Previous studies have demonstrated that both MAPK and PKA regulate CRE-dependent transcription (Grewal et al. 2000). To determine whether the effect of IGF-I and VIP on CRE-dependent transcription involved the MAPK and/or PKA signaling, GH4 cells were pre-treated with the MEK inhibitor PD98059 (10 µM) and the PKA inhibitor Rp-cAMPS (10 µM). The results, shown in Fig. 7B and C, indicate that the stimulatory effect of IGF-I and VIP on CRE-dependent transcription was abolished (P < 0.05) by PD98059 and Rp-cAMPS. These data suggest that in GH4C1 cells both MAPK and cAMP/PKA signaling pathways are required for IGF-I and VIP to induce CREB-dependent gene expression activation. This mechanism might be involved in IGF-I- and VIP-induced proliferation in pituitary lactotrophs.

**Rap1 is required for IGF-I- and VIP-induced CRE-dependent transcription**

To determine whether Rap1 contributes to IGF-I- and VIP-mediated gene expression, GH4C1 cells were transfected with the Rap1 GAP1 expression vector and a CRE-luciferase reporter gene. As shown in Fig. 8, at the higher concentrations tested Rap1 GAP1 was capable of reducing both IGF-I- and VIP-induced CRE-dependent transcription. These findings provide evidence that

---

**Figure 3** Effect of Rap1 GAP1 on VIP-induced MAPK activation. GH4C1 cells were transfected in defined medium without serum with empty expression vector (pcDNA3; 8 µg) or Rap1 GAP1 (8 µg) as indicated. After serum deprivation, VIP (10⁻⁷ M) was added for the indicated times. The upper panel shows a representative immunoblot using antibodies against total and phosphorylated (P-) ERK1 and ERK2. The lower panel shows the densitometric values (arbitrary densitometric units, a.d.u.) gathered from three independent experiments. Values represent means ± s.e.m. (n = 2). ***P < 0.01.

Discussion

This study demonstrates that IGF-I and VIP activates the MAPK signaling pathway in rat pituitary and GH4C1 cells. VIP-dependent activation of the ERK signaling pathway in GH4C1 cells requires the PKA-Rap1 pathway. The Rap1-ERK signaling pathway participates in the regulation of CREB-dependent transcription by both VIP and IGF-I. Phosphorylation of CREB by VIP and IGF-I occurs through distinct mechanisms. Thus, stimulation of Rap1-ERK pathway appears to contribute to IGF-I and VIP-mediated CREB-dependent transcription at a step downstream of CREB phosphorylation.

In previous studies we demonstrated that inhibition of the activation of the MAPK cascade blocked both IGF-I- and VIP-induced lactotroph proliferation (Fernández et al. 2003). These findings suggested that IGF-I and VIP may stimulate MAPK activity and that interactions between MAPK and cAMP/PKA signaling pathways were implicated in IGF-I- and VIP-dependent lactotroph proliferation. To further investigate the intracellular mechanism involved in the action of IGF-I and VIP we used primary cultures of rat pituitaries and the GH4C1 pituitary cell line. Our results indicate that IGF-I and VIP stimulate MAPK activity as assessed by the analysis of ERKs phosphorylation in rat pituitary and GH4C1 cells (data not shown).

These findings are in agreement with previous studies which demonstrated that IGF-I (Castillo & Aranda 1997) and VIP (Le Péchon-Vallée et al. 2000) stimulated MAPK activity in GH4C1 cells. We found that IGF-I- and VIP-induced activation of ERKs have somewhat

Figure 4 Time course of IGF-I- and VIP-induced CREB activation. Rat anterior pituitary cells were incubated in the presence or not of IGF-I (10^-7 M; A) and VIP (10^-7 M; B) for the times indicated. The upper panels show a representative immunoblot using antibodies against total and phospho-Ser^133 CREB. The lower panels show the densitometric values (arbitrary densitometric units, a.d.u.) gathered from three independent experiments. Values represent means±S.E.M. (n=3). ***P<0.001, **P<0.01 versus time 0.
different time courses. ERKs peaked quickly under both IGF-I and VIP; however, the levels declined faster under IGF-I than under VIP. Studies in other systems have provided evidence that the kinetics of MAPK induction may have an important effect on the induced response (Marshall 1995). In PC12 cells sustained activation is associated with translocation of ERKs to the nucleus and the induction of new gene expression whereas transient activation does not lead to nuclear translocation (Nguyen et al. 1993). In earlier studies, we demonstrated that IGF-I stimulates pituitary VIP gene expression and secretion (Lara et al. 1994), which is suggestive that IGF-I-induced MAPK activation could be mediated by VIP or another local factor. However, the rapid kinetics of MAPK activation induced by IGF-I and VIP indicate that these factors directly stimulate MAPK activity.

We next investigated the intracellular mechanism involved in the action of IGF-I and VIP on the ERKs activation. As expected we found that PD98059, a specific inhibitor of MEK (Alessi et al. 1995), was effective in totally suppressing MAPK activation in response to IGF-I. The presence of an antagonist for the activation of PKA (Pereira et al. 1987), Rp-cAMPS, did not modify IGF-I-induced MAPK activation. Inactivation of the PI 3′-kinase did not affect this activation either, although we had observed that LY294002 at the same doses markedly inhibited IGF-I-induced anti-apoptotic protein (Fernández et al. 2004). These results indicate that IGF-I induced ERK1 and ERK2 phosphorylation through the Ras-Raf-MEK signaling cascade in pituitary cells. Similar doses of the inhibitor Rp-cAMPS completely abolished VIP-induced MAPK activation, confirming PKA involvement in VIP-mediated stimulation of MAPK activity (Kievit et al. 2001) and in the somatolactotroph GH4C1 cell line (Romano et al. 2003). The inhibitor of MEK, as expected, was effective in totally suppressing ERKs phosphorylation in response to VIP. Our results confirm previous studies which have demonstrated that VIP activates the MAPK cascade through the cAMP/PKA pathway.
Figure 6 Effect of IGF-I and VIP treatment on CREB activation in lactotroph cells. Anterior pituitary cells were incubated in defined medium without serum for 24 h. Then the medium was replaced by defined medium and cells were cultured in the presence or absence of IGF-I (10⁻⁷ M) or VIP (10⁻⁷ M) for 10 min. (A) Effect of IGF-I and VIP treatment on CREB activation in lactotroph cells. The panel shows the double immunocytochemistry of phospho-CREB and PRL in cultured pituitary cells. PRL-immunoreactive cells (A, left). Phospho-CREB immunoreactivity in the nucleus (A, right). Scale bar, 25µm. (B) Quantitation of the P-CREB/PRL-labeling index. The panel shows the quantitative analysis of the percentage of pituitary cells immunoreactive for both PRL and P-CREB from all PRL immunoreactive cells counted. 20 fields of vision per dish were examined in three different dishes from three independent experiments. Values represent means±S.E.M. (n=3). *P<0.05.

Figure 7 Effect of IGF-I and VIP treatment on CRE-dependent transcription. GH4C1 cells were transfected in defined medium without serum with a CRE-luciferase reporter gene (see the Materials and methods section). 24 h after transfection, the cells were incubated in the presence or not of IGF-I (10⁻⁷ M) or VIP (10⁻⁷ M) for 8 h and collected for analysis of luciferase activity. Reporter gene activity is reported as luciferase activity from three independent transfections normalized to β-galactosidase activity. (A) Effect of IGF-I and VIP treatment on CRE-dependent transcription. (B) Effects of MEK and PKA inhibitors on IGF-I-induced CRE-dependent transcription. (C) Effects of MEK and PKA inhibitors on VIP-induced CRE-dependent transcription. Values represent means±S.E.M. (n=3). *P<0.05; **P<0.01.
pathway in the pituitary cell line GH4C1 (Le Péchon-Vallée et al. 2000). Recently, it has been reported that Rap1, a Ras homologue, can play a crucial role in mediating cAMP-induced MAPK activation in neurons (Vossler et al. 1997, Grewal et al. 2000), HT29 human colonic cells (Alleaume et al. 2003) and in GH3 pituitary cells (Kievit et al. 2001). These studies have shown that cAMP can stimulate Rap1 activation, leading to activation of B-Raf, MEK and ERK. Our study shows for the first time that VIP, a factor that increases intracellular cAMP levels, induces the rapid stimulation of MAPK activity through the PKA-ERK pathway in normal pituitary cells. Additionally, our studies in GH4C1 cells using an expression vector for Rap1 GAP1, which should inactivate Rap1, showed a drastic reduction on VIP-induced MAPK activation, thus confirming a PKA-dependent signalling pathway that may link VIP to ERKs via the small GTP-binding protein, Rap1. Recently, Romano et al. (2003) also reported that in GH4C1 pituitary cells Rap1 is involved in ERK activation by VIP. However, ERK1/2 can be activated or inhibited by cAMP in a cell-specific manner. The activation of Rap1 by PKA has been demonstrated in a wide variety of cells including GH4C1 (Romano et al. 2003) and GH3 pituitary cells (Yonehara et al. 2001). For Rap1 to activate ERKs, the Rafl isoform B-Raf must be expressed. B-Raf is the major Rafl isoform in the brain (Morice et al. 1999) and is also expressed in endocrine cells (Kievit et al. 2001). The results obtained from experiments conducted in GH4C1 pituitary cells suggest that VIP activation of ERKs requires Rap1 in primary cultures of pituitary cells. In previous studies we demonstrated that inhibition of the MAPK cascade blocked the ability of IGF-I and VIP to stimulate lactotroph proliferation (Fernández et al. 2003). The present findings together with previous work using GH3 and GH4C1 (Castillo & Aranda 1997, Le Péchon-Vallée et al. 2000, Kievit et al. 2001) suggest that VIP induces MAPK activation in lactotroph cells probably through the PKA-Rap1 pathway. In contrast to our results, a previous study (Wang et al. 2001) demonstrated that cAMP inhibits MAPK by inhibiting Rap1 in rat C6 glioma cells.

The data shown indicate that both IGF-I and VIP stimulate CREB activation in pituitary cells. The finding that CREB is phosphorylated and activated not only by PKA (Montminy 1997, Shaywitz & Greenberg 1999) but also by MAPK-dependent mechanisms (Xing et al. 1996, Pearson et al. 2001) is consistent with possible CREB involvement in the interactions of IGF-I and VIP on lactotroph proliferation. CREB is a regulator of transcription of early genes that are critical for cell proliferation and differentiation (Segal & Greenberg 1996). There is a precedent for CREB functioning as a factor critical for proliferation of pituitary somatotrophs. Transgenic mice expressing a transcriptionally inactive mutant of CREB exhibited a dwarf phenotype with atrophied pituitary glands and lactotroph cell densities that were slightly reduced (Struthers et al. 1991). Our previous observations that IGF-I and VIP induced lactotroph proliferation (Fernández et al. 2003) led us to analyze whether IGF-I and VIP stimulate CREB activation within lactotroph cells. Our data indicate that IGF-I and VIP stimulated CREB activation in lactotroph cells, which may contribute to IGF-I- and VIP-induced lactotroph proliferation.

We next analyzed the mechanism used by IGF-I and VIP to phosphorylate CREB. Our results indicate that IGF-I induced CREB activation via the MAPK signaling pathway. This is in agreement with previous studies which reported that growth factor-mediated phosphorylation of CREB occurred via the ERK dependent activation of the CREB kinase, p90 ribosomal S6 kinase (RSK2) (Xing et al. 1996). VIP phosphorylates CREB directly via PKA. Indeed, phosphorylation of CREB is often considered to be a measure of transcriptional activation by cAMP. Unlike IGF-I, the requirement for ERK activation does not seem to involve its ability to mediate CREB phosphorylation. Stimulation of intracellular cAMP levels by VIP can lead to CREB activation via PKA, presumably through a direct action on Ser133. Such an action has classically been thought to account for the ability of cAMP to stimulate CRE-dependent transcription (Montminy 1997, Grewal et al. 2000).
we demonstrated that both MAPK and PKA activation are required for IGF-I- and VIP-induced lactotroph proliferation (Fernández et al. 2003). The present study also examined the contribution of the MAPK and cAMP/PKA signaling pathways to the control of gene transcription by IGF-I and VIP using the GH4C1 cells. Our findings indicate that IGF-I and VIP stimulated CREB-dependent transcription, but this regulation occurred through distinct mechanisms (Fig. 9). IGF-I-mediated phosphorylation of CREB occurred through the Ras-Raf1-MEK pathway, whereas IGF-I-stimulated, CRE-mediated transcription also required activation of the MAPK/ERK pathway, possibly by locally IGF-I-induced VIP, in the regulation of CREB-dependent gene expression. p90 RSK, p90 ribosomal S6 kinase.

Figure 9 A model of regulation of CREB-dependent gene expression by VIP and IGF-I in lactotroph cells. Elevation of intracellular cAMP levels by VIP (left) phosphorylates CREB directly via PKA and possibly utilizes the Rap1-ERK pathway to stimulate CRE-mediated transcription. IGF-I-mediated phosphorylation of CREB (right) occurs through the Ras-Raf1-ERK pathway, but IGF-I requires activation of the MAPK/ERK pathway, possibly by locally IGF-I-induced VIP, in the regulation of CREB-dependent gene expression. p90 RSK, p90 ribosomal S6 kinase.

In summary, these results support a model (Fig. 9) in which VIP-dependent activation of the PKA-Rap1-ERK signaling pathway mediates VIP and IGF-I effects on CREB-dependent transcription in GH4C1 cells. Furthermore, we have shown that IGF-I and VIP play a stimulatory role in the MAPK activity and the phosphorylation of CREB in pituitary cells. In view of these results it is possible that the signaling pathway that mediates VIP- and IGF-I-induced lactotroph proliferation may involve the small GTP-binding protein, Rap1.

Acknowledgements

The authors are grateful to Dr Philip J S Stork and Dr Maria Jesús Lorenzo for helpful advice and generous gifts of a number of reagents. We also thank Constanza Navarro for technical assistance and Mary Harper for the preparation of the manuscript. This work was supported by grants PB98–1629–002–01 and SAF 2001–0016 from the Ministerio de Ciencia y Tecnología and FIS P1020720 from the Fondo de Investigaciones Sanitarias. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Fletcher TP, Thomas GB, Dunsmear FR, Moore LG & Clarke JJ 1995 IGF feedback effects on growth hormone secretion in ewes: evidence for action at the pituitary but not the hypothalamic level. *Journal of Endocrinology* 144 323–331.


Kievit P, Lauten JD & Maurer RA 2001 Analysis of the role of the mitogenic-activated protein kinase in mediating cyclic-adenosine 3′, 5′-monophosphate effects on prolactin promoter activity. *Molecular Endocrinology* 15 614–624.


LeRoith D, Werner H, Beiter-Johnson D & Roberts CT 1995 Molecular and cellular aspects of insulin-like growth factor I receptor. *Endocrine Reviews* 16 143–163.


Müller RA 2001 Analysis of the role of the mitogenic-activated protein kinase in mediating cyclic-adenosine 3′, 5′-monophosphate effects on prolactin promoter activity. *Molecular Endocrinology* 15 614–624.


Received 22 December 2004

Accepted 21 December 2004