Pituitary adenylate cyclase-activating polypeptide stimulates corticotropin-releasing factor, vasopressin and interleukin-6 gene transcription in hypothalamic 4B cells

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Abstract

Corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) are the two major regulatory peptides in the hypothalamic–pituitary–adrenal (HPA) axis. CRF, produced in the hypothalamic paraventricular nucleus (PVN) in response to stress, is secreted into the pituitary portal circulation, resulting in the release of adrenocorticotropic hormone from the anterior pituitary. AVP is synthesized in the PVN and supraoptic nucleus by various stressors. Hypothalamic 4B cells coexpress CRF and AVP. In 4B cells transfected with either a CRF or an AVP promoter-luciferase construct, forskolin increased the transcriptional activity of CRF or AVP. In the present study, we tried to determine whether pituitary adenylate cyclase-activating polypeptide (PACAP) regulates both CRF and AVP genes in the hypothalamic cells, because receptors for PACAP were expressed in the hypothalamic cells. PACAP stimulated activity of both CRF and AVP promoter via protein kinase A pathway. PACAP stimulated interleukin (IL)-6 promoter activity and the levels of IL-6 mRNA and protein. IL-6 stimulated activity of both CRF and AVP promoter in a dose-dependent manner. Finally, we found that the stimulatory effects of PACAP on both activities were significantly inhibited by treatment with anti-IL-6 monoclonal antibody. These data suggest that PACAP is involved in regulating the synthesis of IL-6 mRNA and IL-6 protein, and that the increase in endogenous IL-6 also contributes to stimulate the expression of both CRF and AVP genes. Taken together, these findings indicate that PACAP stimulates the transcription of CRF, AVP, and IL-6 genes in hypothalamic 4B cells.


Introduction

Corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) are the two major regulatory peptides in the hypothalamic–pituitary–adrenal (HPA) axis. CRF, produced in the hypothalamic paraventricular nucleus (PVN) in response to stress (Whitnall 1993), is secreted into the pituitary portal circulation, resulting in the release of adrenocorticotropic hormone (ACTH) via the CRF receptor type 1 (CRF1 receptor) from the anterior pituitary (AP). ACTH then stimulates glucocorticoid release from the adrenal glands (Whitnall 1993). Glucocorticoid in turn inhibits hypothalamic PVN production of CRF and pituitary production of ACTH. We previously demonstrated that the protein kinase A (PKA) pathway plays a major role in releasing CRF in hypothalamic tissues (Suda et al. 1985). Other studies also support the hypothesis that stimulation of the PKA pathway in CRF neurons increases CRF expression, and indicate that cyclic AMP (cAMP)-dependent signaling activates CRF gene expression (Seasholtz et al. 1988, Spengler et al. 1992). A functional cAMP-response element (CRE) in the 5′-promoter region of CRF gene takes a part in regulating its gene expression (Seasholtz et al. 1988, Spengler et al. 1992).

AVP is synthesized in both magnocellular and parvocellular regions of the PVN, and in the supraoptic nucleus (SON). Expression of the AVP gene in the SON and PVN is regulated by inflammation, cytokines, and an acute stress (Burbach et al. 2001). Magnocellular AVP neurons in the PVN and SON project to the neural lobe of the pituitary, whereas AVP neurons in the parvocellular region of the PVN project to the external zone of the median eminence (ME) to stimulate ACTH production in the corticotrophs (Vandesande et al. 1977).

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family, is found in two forms in the
mammalian central nervous system (CN): a 38 amino acid peptide (PACAP38) and an N-terminally truncated 27 amino acid peptide (PACAP27; Miyata et al. 1989, 1990). PACAP acts by binding to PACAP receptor 1 (PAC1 receptor) and two VIP-prefering receptors (VPAC1 and VPAC2 receptors). These receptors have different affinities for PACAP and VIP (Harmar et al. 1998). PACAP and the PACAP-selective PAC1 receptors are known to be highly expressed in the hypothalamus, including the parvocellular and magnocellular subdivisions of the PVN, and the SON (Nomura et al. 1996, Shiода et al. 1997a).

PACAP has shown to stimulate cAMP production in the AP (Miyata et al. 1989). PACAP also increases CRF mRNA levels in the parvocellular region of the PVN, suggesting that PACAP is involved in the positive regulation of CRF gene expression (Grinevich et al. 1997). Therefore, it is suggested that PACAP might regulate the HPA axis by stimulation of CRF gene expression in the hypothalamus and through direct effects on pituitary corticotrophs (Boutillier et al. 1994, Aoki et al. 1997, Agarwal et al. 2005). PACAP also activates AVP neurons of the PVN or SON via PAC1 receptor (Shioda et al. 1997b, Nomura et al. 1999).

Interleukin (IL)-6 also shows a variety of biological activities. Plasma IL-6 levels rise in response to both immune activation and non-immune stress (Zhou et al. 1997). IL-6 promotes lymphpoctic proliferation and differentiation (Schimpl & Wecker. 1972) and induces production of acute-phase proteins in the liver (Castell et al. 1989). In addition, IL-6 stimulates the HPA axis (Venihaki et al. 2001), increasing glucocorticoid levels, which in turn suppress IL-6 production (Karalis et al. 1997, Turnbull & Rivier 1999). Therefore, IL-6 is an important mediator of the interaction between the neuroendocrine and immune systems. IL-6 also plays a role in the regulation of CRF and AVP. For example, IL-6 increases CRF gene expression and secretion in the hypothalamus (Navarra et al. 1991, Vallieres & Rivest 1999). IL-6 is coexpressed with AVP in the SON and PVN neurons (Ghorbel et al. 2003) and also induces AVP secretion in the hypothalamus (Mastorakos et al. 1994).

Little is known about the signaling pathways involved in the control of CRF and AVP transcription in parvocellular PVN neurons due to the lack of availability of homologous hypothalamic cells (Kascikow et al. 2003a). A fetal rat hypothalamic 4B cell line shows characteristics of the parvocellular neurons of the PVN (Kascikow et al. 2003b) and expresses CRF, AVP, and CRF₁ and glucocorticoid receptors. In the cells transfected with either a CRF or AVP promoter-luciferase construct, forskolin increased CRF or AVP transcriptional activity in parallel with increases in intracellular cAMP (Nikodemova et al. 2003). In the present study, we asked whether PACAP is involved in the responses of CRF and AVP promoter activities in the hypothalamic 4B cells. We further explored the involvement of PACAP in the regulation of IL-6 gene expression in the hypothalamic cells.

### Materials and Methods

#### Materials

Forskolin was purchased from Calbiochem (San Diego, CA, USA). PACAP38 was purchased from the Peptide Institute (Osaka, Japan). H89 was purchased from Seikagaku Corporation (Tokyo, Japan). PD98059 and SQ22536 were purchased from Calbiochem. Rat IL-6 was purchased from PeproTech (London, UK). Anti-rat IL-6 MAB was purchased from Biosource (Camarillo, CA, USA).

#### Animals

Adult male Wistar rats (body weight, BW 280–320 g) were housed in a temperature-controlled room with controlled lighting (light 0600–1800 h) and were given free access to laboratory chow and tap water. The rats were decapitated for tissue collection (hypothalamic tissue and anterior pituitary). This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

#### Cell culture

Rat hypothalamic 4B cells were incubated in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were plated at 10⁴ cells/cm² 4 days before each experiment, with the medium being changed for every 48 h. On day 3, cells were washed and starved overnight with DMEM supplemented with 0.2% BSA. On day 4, cells were incubated in medium with added vehicle, forskolin, with or without medium containing one of various inhibitors or an antagonist.

#### Constructs and transfection

A 1077 bp restriction fragment containing the CRF promoter (−907 to +170 relative to the proximal transcription start point) and an 829 bp restriction fragment containing the AVP promoter (−803 to +26) were obtained by PCR. The PCR products were then confirmed by sequences. The DNA fragments were used to produce the CRF and AVP promoter-driven luciferase reporter constructs, CRF−907luc and AVP−803luc respectively by a two-step cloning method. First, the DNA fragment was cloned into pGEM-T Easy vector (Promega Corp.), then digested with Kpn I and Hind III, and subcloned into Kpn I and Hind III cloning sites of the pA3–Luc plasmid. A 543 bp restriction fragment containing the IL−6 promoter (−522 to +21) was obtained from rat genomic DNA. This DNA fragment was used to produce the IL−6 promoter-driven luciferase reporter construct, IL−6luc, by the two-step cloning method.

For the luciferase activity assay, cells were placed in 12-well (22 mm diameter) culture trays at 60% confluency. The next day, cells were transfected following the manufacturer’s instructions using the FuGENE 6 Transfection Reagent Kit.
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In the present study, we investigated the role of PACAP in the hypothalamus, a central organ involved in the control of energy metabolism and feeding behavior. The hypothalamus contains numerous PACAP-immunoreactive neurons, and PACAP is co-localized with other neuronal peptides such as cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY). These findings suggest that PACAP may play a role in the regulation of energy metabolism and feeding behavior.

Materials and methods

Cell culture and treatment

PC12 cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen). PC12 cells were plated onto 24-well plates at a density of 2 × 10^5 cells per well. After 24 h, the medium was replaced with fresh DMEM containing 0.2% BSA. Cells were treated with PACAP (100 nM) for 20 min, followed by the injection of 100 μl luciferin solution, PicaGene buffer, and the luciferase assay was performed according to the manufacturer's protocol. At the end of each experiment, cells were washed and starved overnight in DMEM supplemented with 0.2% BSA.

Reverse transcriptase-PCR (RT-PCR)

cDNAs were synthesized from total RNA (0.5 μg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for reverse transcriptase-PCR (RT-PCR) Kit (Invitrogen Corp.) according to the manufacturer's instructions. PCR was carried out in a programmable thermal controller using the following oligonucleotide primers: CRF-F (5′-ACATCCGACATGGAGCTG-3′), CRF-R (5′-CCCCATAATCTCCATCAGTTTCCCTG-3′); AVP-F (5′-ACATCCGACATGGAGCTG-3′), AVP-R (5′-GCGGAAAACCTCTCG-3′) (Nagano et al. 1997); β2-Microglobulin (B2MG)-F (5′-CCGTGAGCC-3′), B2MG-R (5′-GGTGACGTTCCTATCAGTTTCCCTG-3′) (Neumann et al. 1997); PAC1 receptor-F (5′-TTTCTCAGCAGCCATCATCAGCTCTTTCCCTG-3′), PAC1 receptor-R (5′-CCTCCAGCAGCCATCATCAGCTCTTTCCCTG-3′) (Ajpru et al. 2002); VPAC1 receptor-F (5′-TGATTGAGATA-CAGCCTCAGC-3′), VPAC1 receptor-R (5′-CCTCAGGACATGTTCTAC-3′) (Chaudhry & Baumann 2002); VPAC2 receptor-F (5′-TTGGGCTACAGTTTCTCTTCTT-3′), VPAC2 receptor-R (5′-ATTAGCAGCAGTTGAACTTTC-3′). Conditions for the CRF were 1× (95 °C, 3 min), 40× (95 °C, 20 s; 62 °C, 30 s; 72 °C, 30 s) and 1× (95 °C, 1 min; 55 °C, 1 min). Conditions for the AVP were 45× (94 °C, 1 min; 54 °C, 1 min; 72 °C, 2 min). Conditions for the B2MG were 1× (95 °C, 3 min), 48× (93 °C, 1 min; 65 °C, 1 min; 72 °C, 1 min) and 1× (72 °C, 5 min). Conditions for the PAC1 receptor were 1× (94 °C, 2 min), 40× (94 °C, 1 min; 58 °C, 1 min; 72 °C, 2 min) and 1× (75 °C, 5 min). Conditions for the VPAC1 and VPAC2 receptors were 1× (94 °C, 5 min), 35× (94 °C, 30 s; 55 °C, 40 s; 72 °C, 70 s), and 1× (72 °C, 7 min). Products were separated by electrophoresis on a 1–2% (for CRF) agarose gel containing ethidium bromide, followed by quantitative analysis using the NIH image software 1.61 (Bethesda, MD, USA). The results are expressed as corrected arbitrary units. The expected sizes of PCR products for CRF and AVP were 101 and 270 bp respectively. The expected size of PCR products for B2MG was 332 bp. The expected sizes of PCR products for PAC1 receptor were 374 bp (a variant with a single 84 bp insert) and 290 bp (short isoform). The expected sizes of PCR products for VPAC1 and VPAC2 receptors were 509 and 607 bp respectively.

Real-time RT-PCR

Resulting cDNAs were subjected to real-time PCR as follows. The expression level of rat IL-6 mRNA was evaluated using quantitative real-time PCR based on specific sets of primers and probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA, USA). B2M was used as a housekeeping gene to normalize values. Each reaction consisted of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× Assays-on-Demand Gene Expression Products (Rn00561420 m1 for rat IL-6; Rn00560865 m1 for rat B2MG), and 2 μl cDNA in a total volume of 50 μl using the following parameters with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The above assays involved specific sets of primers and TaqMan probe spanning exon/exon junctions and, therefore, should not have been influenced by DNA contamination. Data were collected and recorded by ABI PRISM 7000 SDS Software (Applied Biosystems) and expressed as a function of threshold cycle (Ct). Using diluted samples, the amplification efficiencies for each gene of interest and the housekeeping gene amplimers were found to be identical.

Relative quantitative gene expression

Relative quantitative gene expression was calculated with the 2−ΔΔCt method (Livak & Schmittgen 2001). In brief, for each sample assayed, the Ct for reactions amplifying a gene of

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interest (rat IL-6) and a housekeeping gene (rat B2MG) was determined. The gene of interest $C_T$ for each sample was corrected by subtracting the $C_T$ for the housekeeping gene ($\Delta C_T$). Untreated controls were chosen as reference samples, and the $\Delta C_T$ for all experimental samples was reduced by the average $\Delta C_T$ for the control samples ($\Delta \Delta C_T$). Finally, experimental mRNA abundance relative to control mRNA abundance was calculated by the formula $2^{-\Delta \Delta C_T}$.

**Western blot analysis**

After treatment with PACAP, cells were washed twice with PBS and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. Samples were boiled and subjected to electrophoresis on a gradient (4–20%) polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking by Detector Block blocking buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), the membrane was incubated for 1 h with a rabbit anti-CRE-binding protein (CREB) and anti-phosphorylated (p)-CREB antibodies (Cell Signaling Technology, Beverly, MA, USA), or anti-monoclonal IL-6 (BioSource International Inc.), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Daiichi Kagaku). Detection was performed using a chemiluminescent substrate Super-signal WestPico (Pierce Chemical Co., Rockford, IL, USA), and the membrane was exposed to BioMax film (Eastman Kodak Co.) followed by quantitative analysis using the NIH image software 1.61. The results are expressed as corrected arbitrary units.

**IL-6 assay**

Serum-starved 4B cells were incubated at 37 °C for 48 h with the indicated concentrations of each peptide. The medium was then aspirated and IL-6 levels in the supernatants were measured using commercial IL-6 ELISA kits (Biosource International). All samples from each experiment were determined in the same assay.

**Statistical analysis**

All values are expressed as the mean ± S.E.M. Statistical analyses of data were performed using one-way ANOVA, followed by Bonferroni/Dunn post hoc test. The level of statistical significance was set at $P<0.05$.

**Figure 1**  Expression of CRF, AVP, and receptors for PACAP. Cells were treated in triplicate in each experiment, with the average of three independent experiments shown in figures. Statistical analyses were performed using ANOVA, followed by post hoc test. *$P<0.05$, **$P<0.005$ (when compared with control). (A) Expression of PAC1, VPAC1, and VPAC2 receptors in 4B cells. Short isoform (290 bp) and a variant with a single 84 bp insert (374 bp) of PAC1 receptor were detected by RT-PCR. 4B, 4B cells; Hypo, hypothalamus; AP, anterior pituitary; C1, negative control for 4B; C2, negative control for hypo; C3, negative control for AP. (B) Effects of PACAP on CRF and AVP mRNA levels. The 4B cells were stimulated with 100 nM PACAP for 2, 6, and 24 h. Expression of CRF and AVP mRNA was examined by RT-PCR. (C) Effects of PACAP on CREB phosphorylation. The 4B cells were stimulated with 100 nM PACAP for the duration shown. CREB phosphorylation was examined by western blot analysis.
Results

Expression of CRF, AVP, and receptors for PACAP mRNA in 4B cells

PAC1 receptor gene expression was detected in 4B cells, hypothalamus, and anterior pituitary tissues (Fig. 1A). PAC1 receptor has a short isoform (290 bp) and a variant with a single 84 bp insert (374 bp). Expression levels of VPAC1 receptor were very low in 4B cells, while VPAC2 was detected in 4B cells and rat hypothalamus.

Expression of both CRF and AVP mRNA was examined by RT-PCR. Basal levels of both mRNA were very low or undetectable, while PACAP significantly increased both CRF and AVP mRNA levels from 2 to 24 h after experiment in 4B cells (Fig. 1B). The maximal effect of PACAP was observed at 2 h after experiment.

To determine whether PACAP affected CREB phosphorylation, 4B cells were incubated with PACAP. As shown in Fig. 1C, incubation with 100 nM PACAP significantly increased CREB phosphorylation from 10 to 30 min after the addition.

Effects of PACAP on CRF or AVP 5'-promoter activity in 4B cells

We examined the impact of PACAP on the time- and dose-dependent changes in CRF 5'-promoter activity using 4B cells transfected with CRF-907luc. The time course study showed that PACAP significantly increased CRF 5'-promoter activity.

Figure 2 Effects of PACAP on CRF or AVP 5'-promoter activity in 4B cells. Cells treated with medium alone are indicated as C. Statistical analyses were performed using one-way ANOVA, followed by post hoc test. *P<0.05, **P<0.005 (when compared with control (C)). (A) Time- and dose-dependent changes in PACAP-induced CRF 5'-promoter activity. (A(i)) Time-dependent changes in PACAP-induced CRF 5'-promoter activity. Cells were incubated with medium containing 100 nM PACAP. (A(ii)) Dose-dependent changes in PACAP-induced CRF 5'-promoter activity. Cells were incubated for 2 h with medium containing 1 nM to 1 μM PACAP. (B) Time- and dose-dependent changes in PACAP-induced AVP 5'-promoter activity. (B(i)) Time-dependent changes in PACAP-induced AVP 5'-promoter activity. Cells were incubated with medium containing 100 nM PACAP. (B(ii)) Dose-dependent changes in PACAP-induced AVP 5'-promoter activity. Cells were incubated for 2 h with medium containing 1 nM to 1 μM PACAP.
The maximal effect of PACAP occurred at 2 h after experiment, with an approximate fivefold increase in CRF 5′-promoter activity when compared with the basal level in CRF-907luc (P<0.0001; Fig. 2A(i)). PACAP stimulated CRF 5′-promoter activity in a dose-dependent manner, with significant stimulatory effects at 1 nM to 1 μM (ANOVA; P<0.0001; Fig. 2B(i)).

**Effects of PACAP on cAMP accumulation in 4B cells**

Figure 3 shows the levels of cAMP production following PACAP stimulation of 4B cells. Both forskolin and PACAP induced intracellular cAMP production in a dose-dependent manner. Significant stimulatory effects of PACAP were observed at 10 nM to 1 μM.

**Effects of a PKA or extracellular signal-related kinases inhibitor on PACAP-induced CRF or AVP 5′-promoter activity in 4B cells**

To determine whether PKA pathway is involved in the PACAP-induced CRF or AVP 5′-promoter activity, 4B cells were incubated with H89, a potent PKA inhibitor. As shown in Fig. 4, the stimulatory effects of 100 nM PACAP on both activities were partially inhibited by treatment with 1 μM H89, while these of a smaller dose, 10 nM PACAP were completely done. The effects of 10 nM PACAP on both CRF and AVP promoter activities were not inhibited by 10 μM PD98059, a selective mitogen-activated protein (MAP) kinase extracellular signal-related kinases inhibitor.

**Effects of forskolin or PACAP on IL-6 5′-promoter activity in 4B cells**

Forskolin or PACAP may stimulate IL-6 gene expression in 4B cells. Therefore, we examined the time- and dose-dependency of forskolin and PACAP on IL-6 5′-promoter activity (ANOVA; P<0.0001) (Fig. 2B(i)). The maximal effect of PACAP was observed 2 h after the experiment, with an approximate fourfold increase when compared with the basal level in AVP-803luc (P<0.005).

Figure 4 Effects of a PKA or ERK inhibitor on PACAP-induced CRF or AVP 5′-promoter activity in 4B cells.

*P<0.05, **P<0.005 (when compared with control (C)). Cells were pre-incubated for 20 min with medium containing 0.1 mM 3-isobutyl-1-methylxanthine, followed by the addition of forskolin (F) or PACAP. Cells were then incubated for 20 min with medium alone (control), or with medium containing F or PACAP. The level of intracellular cAMP was measured by cAMP EIA.

**Figure 3** Effects of PACAP on cAMP accumulation in 4B cells. **P<0.005 (when compared with control (C)).** Cells were pre-incubated for 20 min with medium containing 0.1 mM 3-isobutyl-1-methylxanthine, followed by the addition of forskolin (F) or PACAP. Cells were then incubated for 20 min with medium alone (control), or with medium containing F or PACAP. The level of intracellular cAMP was measured by cAMP EIA.

Involvement of PACAP in hypothalamic cells


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Figure 5 Effects of forskolin or PACAP on IL-6 5'-promoter activity in 4B cells. *P<0.05, **P<0.005 (when compared with control (C)). (A) Time- and dose-dependent changes in forskolin-induced IL-6 5'-promoter activity. (A(i)) Time-dependent changes in forskolin-induced IL-6 5'-promoter activity. Cells were incubated with medium containing 10 μM forskolin. (A(ii)) Dose-dependent changes in forskolin-induced IL-6 5'-promoter activity. Cells were incubated for 24 h with medium containing 0.01 to 100 μM forskolin (F). (B) Time- and dose-dependent changes in PACAP-induced IL-6 5'-promoter activity. (B(i)) Time-dependent changes in PACAP-induced IL-6 5'-promoter activity. Cells were incubated with medium containing 100 nM PACAP. (B(ii)) Dose-dependent changes in PACAP-induced IL-6 5'-promoter activity. Cells were incubated for 2 h with medium containing 0.1 nM to 1 μM PACAP. (C) Effect of PACAP on forskolin-induced IL-6 5'-promoter activity. Cells were incubated for 6 h with medium alone (C) or medium containing 100 μM forskolin and/or 1 μM PACAP. (D) Effects of an adenylate cyclase inhibitor on PACAP-induced IL-6 5'-promoter activity. Cells were pre-incubated with medium containing 100 μM SQ22536, or vehicle for 30 min and then incubated for 2 h with medium containing 100 nM PACAP, or vehicle. Cells treated with SQ22536 are indicated as SQ.
activity (Fig. 5A). The time course study revealed gradual increases in IL-6 5′-promoter activity in the presence of 10 μM forskolin. Significant stimulatory effects of forskolin were observed at 0.1–100 μM.

We next examined the effect of PACAP on the time- and dose-dependent changes in IL-6 5′-promoter activity, using 4B cells transfected with IL-6luc (Fig. 5B). The maximal effects of PACAP were observed at 2 and 6 h after experiment, with an approximate threefold increase when compared with the basal level. PACAP stimulated IL-6 5′-promoter activity in a dose-dependent manner with significant stimulatory effects observed at 10 nM to 1 μM.

When PACAP was simultaneously used with forskolin, the combination showed no additive effect on IL-6 5′-promoter activity (Fig. 5C).

To determine whether an adenylate cyclase pathway was involved in PACAP-induced IL-6 5′-promoter activity, 4B cells were pre-incubated for 30 min with 100 μM SQ22536, an adenylate cyclase inhibitor. As shown in Fig. 5D, treatment with SQ22536 significantly inhibited IL-6 5′-promoter activity.

**Effects of forskolin or PACAP on IL-6 mRNA levels in 4B cells**

Next, we investigated the modulation of IL-6 mRNA levels by forskolin and PACAP. The maximal effect of forskolin on IL-6 mRNA levels occurred at 2 h after experiment, with an approximate tenfold increase when compared with the basal level (Fig. 6A). Significant stimulatory effects of forskolin were observed at 1 and 10 μM.

We also examined time- and dose-dependent changes in IL-6 mRNA levels in the presence of PACAP (Fig. 6B).
Maximal stimulation by PACAP was observed at 2 h after experiment, with an approximate ninefold increase of IL-6 mRNA levels. PACAP stimulated IL-6 mRNA levels in a dose-dependent manner with significant stimulation at 10 nM to 1 μM.

Effects of PACAP on IL-6 production in 4B cells

To examine whether PACAP induced a functional response in the 4B cells, we examined the effects of PACAP on IL-6 output levels or production (Fig. 7). Incubation with 0.01 nM to 10 μM forskolin or 0.1 to 100 nM PACAP increased IL-6 output levels in a dose-dependent manner (ANOVA; *P* < 0.0001). PACAP stimulation was significant at 100 nM.

Effects of IL-6 on CRF and AVP 5'-promoter activities in 4B cells

IL-6 is a candidate modulator of CRF and AVP gene expression. Therefore, we examined the time- and dose-dependency of the IL-6 effects on both CRF and AVP 5'-promoter activities. To examine CRF 5'-promoter activity, 4B cells transfected with CRF-907luc were incubated with IL-6 (Fig. 8A). The time course revealed a gradual increase in CRF 5'-promoter following exposure to 5 nM IL-6. Maximal stimulation by IL-6 occurred after 24 h, with an approximate twofold increase when compared with the basal level. IL-6 stimulation of CRF 5'-promoter was dose dependent with significant stimulatory effects observed at 500 pM.

Next, 4B cells transfected with AVP-803luc were incubated with IL-6 to examine changes in AVP 5'-promoter activity (Fig. 8B). There were gradual increases in AVP 5'-promoter activity in the presence of 5 nM IL-6. The maximal effect of IL-6 was observed after 24 h, with an approximate twofold increase when compared with the basal level. IL-6 stimulated AVP 5'-promoter activity in a dose-dependent manner, with 500 pM inducing significant stimulation.

Effects of anti-IL-6 Ab on PACAP-induced CRF or AVP 5'-promoter activity in 4B cells

PACAP (100 nM) stimulated IL-6 protein expression from 2 to 24 h after experiment in 4B cells (Fig. 9A).

Finally, to determine whether endogenous IL-6 production is involved in the PACAP-induced CRF or AVP 5'-promoter activity, 4B cells were pre-incubated for 30 min with anti-IL-6 MAB, and then incubated for 2 h with 10 or 100 nM PACAP. As shown in Fig. 9B, the stimulatory effects of 100 nM or a smaller dose, 10 nM, PACAP on both activities were significantly, but not completely, inhibited by treatment with the anti-IL-6 MAB.

Discussion

In this study, we first found that PACAP stimulated activity of both CRF and AVP promoter in hypothalamic 4B cells. Although PACAP has been shown to modulate hypothalamic CRF gene expression in vivo (Grinevich et al. 1997), it was difficult to study the mechanism regulating CRF transcription in parvocellular neurons because of the lack of availability of a representative cell line. We used a homologous hypothalamic 4B cell, which shows the characteristics of the parvocellular cells of the PVN, because the cells were shown to express CRF, AVP, and CRF1 and glucocorticoid receptors (Kasckow et al. 2003b). In this and other recent studies (Liu et al. 2006), basal levels of both CRF and AVP mRNA were very low or undetectable in the 4B cells, presumably caused by changes in the cell condition through current passages of the cells, while PACAP stimulated both CRF and AVP mRNA levels in 4B cells. PACAP induced intracellular cAMP production in hypothalamic 4B cells and stimulated CRF gene transcription via the cAMP–PKA pathway. Indeed, nerve fibers containing PACAP connect to CRF neurons (Hannibal et al. 1995, Legradi et al. 1998). Other studies also suggest that PACAP stimulates CRF gene via the cAMP/PKA signaling pathway (Agarwal et al. 2005). In hypothalamic 4B cells, the cAMP–PKA pathway was involved in PACAP-induced CRF stimulation of gene transcription. Activation of the PKA pathway, causing phosphorylation of CREB, acts on the CRF promoter (Seasholtz et al. 1988, Spengler et al. 1992, Itoi et al. 1996). A functional CRE in the 5'-promoter region takes a part in regulating CRF gene expression (Seasholtz et al. 1988, Spengler et al. 1992). H89, a PKA inhibitor, completely
blocked a smaller dose of PACAP-induced CRF stimulation of gene transcription in hypothalamic 4B cells. However, the inhibitor did not completely block a larger dose of PACAP-induced CRF stimulation, suggesting the limitation of the inhibitor. Otherwise, it is possible that an additional pathway may be involved in this regulation. The difference between CRF/AVP promoter activity and cAMP response to PACAP also may suggest that the effect of PACAP is mediated by other signals in addition to cAMP.

PACAP also stimulated both AVP gene transcription and mRNA expression via the cAMP–PKA pathway in hypothalamic 4B cells. Gene transcription of AVP is stimulated by Fos/Jun family member proteins through an activation protein 1 (AP1) site in neuroblastoma cells (Yoshida et al. 2006). This effect is achieved via both PKA and protein kinase C signaling pathways. Therefore, our results are consistent with those of the prior study showing that intracellular cAMP production is involved in the stimulation of AVP gene transcription via the PKA pathway in hypothalamic 4B cells. PACAP is detected in nerve terminals that innervate AVP-containing neurons in the rat hypothalamus (Shioda et al. 1997b), and its receptor mRNA is highly expressed in AVP-containing neurons (Shioda et al. 1997b). Another report demonstrated that PAC1 receptors were expressed in the parvocellular and magnocellular subdivisions of the PVN (Nomura et al. 1996). The 4B cells also expressed PAC1 and VPAC receptors. Therefore, activation of PAC1 or VPAC receptors by PACAP induces production of intracellular cAMP, activating the transcription of AVP gene in hypothalamic cells. Taken together, we propose that
PACAP-induced intracellular cAMP production is involved in stimulating the transcription of both CRF and AVP genes at least via the PKA pathway in hypothalamic 4B cells. The role of PACAP in the PVN of the hypothalamus has not been clearly determined, although PACAP might contribute to stress responses through CRF and AVP stimulation. The extended amygdala and the bed nuclei of the stria terminalis are identified as innervation sites of PACAP neurons, suggesting an important role in stress responses (Piggins et al. 1996, Kozicz & Arimura 2002).

PACAP also stimulates cAMP production in the AP. PACAP, therefore, could regulate pituitary corticotrophs directly (Boutillier et al. 1994). CRF and AVP neurons in the parvocellular region of PVN project to the external zone of the ME (Seasholtz et al. 1988, Gonzalez-Hernandez et al. 2006). Furthermore, AVP and CRF in parvocellular PVN neurons exert synergistic effects on ACTH secretion from the AP (Gillies et al. 1982, Mouri et al. 1993). Therefore, PACAP may contribute to stress responses through stimulation of CRF and AVP neurons of the hypothalamus, causing ACTH secretion.

The treatment with both PACAP and forskolin showed no additive effect on IL-6 5'-promoter activity. An adenylate cyclase inhibitor significantly inhibited PACAP-induced IL-6 5'-promoter activity. Therefore, PACAP may stimulate IL-6 production through intracellular cAMP pathway. On the other hand, it is possible that the differences in the activation of IL-6 promoter between PACAP and forskolin may suggest the differences in signal pathway, in addition to cAMP, in the IL-6 regulation.

Cytokines affect the HPA axis at multiple levels (Dunn 2004). Silverman et al. (2004), demonstrated that IL-6 was able to activate the HPA axis directly by actions on the hypothalamus, the pituitary, and the adrenal cortex. IL-6 and its receptor mRNA levels in the hypothalamus are modulated by an acute stress or changes in the HPA axis (Komori et al. 2003). Therefore, IL-6 in the hypothalamus is also a candidate for stimulation of CRF and AVP gene expression. PACAP may participate in the regulation of CRF and AVP genes via IL-6 production. For example, both forskolin and PACAP stimulated IL-6 promoter activity, IL-6 mRNA, and protein release. Therefore, PACAP stimulates IL-6 synthesis and secretion. PACAP increased IL-6 output levels to 70 pg/ml which would correspond to about 3.5 pM in the medium, while significant stimulatory effects on both CRF and AVP promoter activities required the treatment with at least 500 pM IL-6 in our study. This result suggests that IL-6 may not be the only stimulus in response to PACAP. However, we found that the stimulatory effects of PACAP on both activities were significantly inhibited by treatment with the
anti-IL-6 MAB. Therefore, we propose that endogenous IL-6 production is involved in the PACAP-induced CRF and AVP 5′-promoter activities, and IL-6 stimulates transcription of both genes in an autocrine manner in hypothalamic 4B cells. Considering the delayed response to IL-6, and the partial inhibition of PACAP effects by IL-6 Ab, IL-6 may be important to sustain the activity of CRF and AVP promoter. Finally, note that subpopulations of parvocellular cells coexpress CRF, AVP, and IL-6, and project to the external layer of the ME (Gonzalez-Hernandez et al. 2006). Thus, it is possible that IL-6, produced in the hypothalamus, both directly and indirectly through CRF and AVP, stimulates ACTH secretion in pituitary corticortrophs.

In conclusion, this study demonstrated that PACAP stimulates transcription of CRF and AVP genes via a cAMP-dependent PKA pathway in hypothalamic 4B cells. The PACAP-induced intracellular cAMP pathway was also involved in the regulation of IL-6 gene transcription and protein production in hypothalamic 4B cells.

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