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

Kazunori Kageyama, Komaki Hanada, Yasumasa Iwasaki¹, Satoru Sakihara, Takeshi Nigawara, John Kasckow² and Toshihiro Suda

Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

¹Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi University, Oko-cho, Kohasu, Nankoku 783-8505, Japan

²Department of Psychiatry, University of Cincinnati School of Medicine, 231 Bethesda Avenue (ML 559), Cincinnati, Ohio 45267, USA

(Correspondence should be addressed to K Kageyama; Email: kkageyama@hkg.odn.ne.jp)

Abstract

Corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) are the two major regulatory peptides in the hypothalamic–pituitary–adrenal 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 adrenocorticotrophic 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.

Journal of Endocrinology (2007) **195**, 199–211

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 adrenocorticotrophic 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-preferring 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, Shioda *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.* 1993, Takaki *et al.* 1994). IL-6 prompts lymphocytic 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 (Kasckow *et al.* 2003a). A fetal rat hypothalamic 4B cell line shows characteristics of the parvocellular neurons of the PVN (Kasckow *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, Hiroshima 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

(Roche Diagnostics). FuGENE 3 $\mu\text{l}/1\text{ }\mu\text{g}$ DNA was used. For each well, the total amount of DNA was 0.5 μg . The culture medium was then replaced with DMEM supplemented with 10% FBS. One day before each experiment, cells were washed and starved overnight in DMEM supplemented with 0.2% BSA.

Luciferase activity

The luciferase assay was performed according to the manufacturer's protocol. At the end of each experiment, cells were washed twice with PBS without Ca^{2+} and Mg^{2+} , harvested with PicaGene lysis buffer (Toyo Inki, Tokyo, Japan), and centrifuged at 12 000 r.p.m. for 2 min. For the luciferase assay, 20 μl of each supernatant was used. The reactions were started by the injection of 100 μl luciferin solution, PicaGene buffer. Light output was measured for 20 s at room temperature using a luminometer (Berthold Lumat LB9501, Postfach, Germany). Activity of β -galactosidase was used as an internal control. Cells were treated in triplicate in each experiment, with the average of three independent experiments shown in figures.

cAMP assay

Serum-starved 4B cells were pre-incubated for 20 min with 0.1 mM of 3-isobutyl-1-methylxanthine in assay medium and then treated at 37 °C for 20 min with the indicated concentrations of each peptide. The medium was aspirated and cells extracted with 1 ml of 95% ethanol containing 0.1 M HCl. cAMP content was measured in the supernatants using commercial cAMP EIA kits (Amersham Pharmacia Biotech). Cells were treated in triplicate in each experiment, with the average of three independent experiments shown in figures.

RNA extraction

Cellular total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Then, 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.

Reverse transcriptase-PCR (RT-PCR)

PCR was carried out in a programmable thermal controller (Bio-Rad) with the following oligonucleotide primers: CRF-F (5'-TGGATCTCACCTTCCACCTTCTG-3'), CRF-R (5'-CCGATAATCTCCATCAGTTTCCTG-3'); AVP-F (5'-ACATCCGACATGGAGCTG-3'), AVP-R (5'-GCGG-AAAAACCTCTCTCG-3') (Nagano *et al.* 1997); β 2-Microglobulin (B2MG)-F (5'-CGGTGACCG- TGATCTTT-CTGGT-3'), B2MG-R (5'-GGTGACGGTTTTGGGCTC-CTT-3') (Neumann *et al.* 1997); PAC1 receptor-F (5'-TTT-CATCGGCATCATCATCCTT-3'), PAC1 receptor-R (5'-CCTTCCAGCTCCTCCATTCCTCTT-3') (Ajpru

et al. 2002); VPAC1 receptor-F (5'-TGATTGAGATA-CAGCGTCAG-3'), VPAC1 receptor-R (5'-CCT-CAGAGCAGTGGTCTATC-3') (Chaudhary & Baumann 2002); VPAC2 receptor-F (5'-TTGGGCTACAGTG-TTTCTCT-3'), VPAC2 receptor-R (5'-ATTAGCAG-CAGTGTGGACTT-3'). Conditions for the CRF were 1 $\times$ (95 °C, 3 min), 40 $\times$ (95 °C, 20 s; 62 °C, 30 s; 72 °C, 30 s) and 1 $\times$ (95 °C, 1 min; 55 °C, 1 min). Conditions for the AVP were 45 $\times$ (94 °C, 1 min; 54 °C, 1 min; 72 °C, 2 min). Conditions for the B2MG were 1 $\times$ (95 °C, 3 min), 48 $\times$ (93 °C, 1 min; 65 °C, 1 min; 72 °C, 1 min) and 1 $\times$ (72 °C, 5 min). Conditions for the PAC1 receptor were 1 $\times$ (94 °C, 2 min), 40 $\times$ (94 °C, 1 min; 58 °C, 1 min; 72 °C, 2 min) and 1 $\times$ (75 °C, 5 min). Conditions for the VPAC1 and VPAC2 receptors were 1 $\times$ (94 °C, 5 min), 35 $\times$ (94 °C, 30 s; 55 °C, 40 s; 72 °C, 70 s), and 1 $\times$ (72 °C, 7 min). Products were separated by electrophoresis on a 1.2 or 4% (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 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 1 $\times$ 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 (C_T). Using diluted samples, the amplification efficacies 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 $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). In brief, for each sample assayed, the C_T for reactions amplifying a gene of

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 (ΔC_T). Untreated controls were chosen as reference samples, and the ΔC_T for all experimental samples was reduced by the average ΔC_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 $\pm$ 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 μ M 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 μ M PACAP.

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.

(ANOVA; $P < 0.0001$; Fig. 2A(i)). 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 ($P < 0.005$). PACAP stimulated CRF 5'-promoter activity in a dose-dependent manner with significant enhancing effects of PACAP observed at 1 nM to 1 μ M (ANOVA; $P < 0.0001$) (Fig. 2A(ii)).

Next, we examined the impact of PACAP on the time- and dose-dependent changes in AVP luciferase activity using 4B cells transfected with AVP-803luc (Fig. 2B). The time course study showed that PACAP significantly increased AVP

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 ($P < 0.005$). PACAP stimulated AVP luciferase activity in a dose-dependent manner, with significant stimulatory effects at 1 nM to 1 μ M (ANOVA; $P < 0.0001$; Fig. 2B(ii)).

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 μ M.

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 μ M 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 μ M PD98059, a selective mitogen-activated protein (MAP) kinase 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

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)). + $P < 0.05$, ++ $P < 0.005$ (when compared with 100 nM PACAP). ## $P < 0.005$ (when compared with 10 nM PACAP). Cells were pre-incubated with medium containing 1 μ M H89, 10 μ M PD98059, or vehicle for 30 min and then incubated for 2 h with medium containing 10 or 100 nM PACAP, or vehicle. Cells treated with PD98059 are indicated as PD.

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.

Figure 6 Effects of forskolin or PACAP on IL-6 mRNA levels 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 mRNA levels. (A(i)) Time-dependent changes in forskolin-induced IL-6 mRNA levels. Cells were incubated with medium containing 10 μM forskolin. (A(ii)) Dose-dependent changes in forskolin-induced IL-6 mRNA levels. Cells were incubated for 2 h with medium containing 0.1–10 μM forskolin (F). (B) Time- and dose-dependent changes in PACAP-induced IL-6 mRNA levels. (B(i)) Time-dependent changes in PACAP-induced IL-6 mRNA levels. Cells were incubated with medium containing 100 nM PACAP. (B(ii)) Dose-dependent changes in PACAP-induced IL-6 mRNA levels. Cells were incubated for 2 h with medium containing 1 nM to 1 μM PACAP.

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).

Figure 7 Effects of PACAP on IL-6 production in 4B cells. $**P < 0.005$ (when compared with control (C)). Cells were incubated for 48 h with medium alone (control), or with medium containing forskolin (F) or PACAP. The titers of IL-6 in the culture supernatants were measured by IL-6 ELISA.

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 μ M.

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 μ M 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-dependent 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

Figure 8 Effects of IL-6 on CRF or AVP 5'-promoter activity in 4B cells. * $P < 0.05$, ** $P < 0.005$ (when compared with control (C)). (A) Time- and dose-dependent changes in IL-6-induced CRF 5'-promoter activity. (A(i)) Time course of IL-6-induced CRF 5'-promoter activity. Cells were incubated with medium containing 5 nM IL-6. (A(ii)) Dose-dependent changes in IL-6-induced CRF luciferase activity. Cells were incubated for 24 h with medium containing 5 pM to 5 nM IL-6. (B) Time- and dose-dependent changes in IL-6-induced AVP 5'-promoter activity. (B(i)) Time course of IL-6-induced AVP 5'-promoter activity. Cells were incubated with medium containing 5 nM IL-6. (B(ii)) Dose-dependent changes in IL-6-induced AVP 5'-promoter activity. Cells were incubated for 24 h with medium containing 5 pM to 5 nM IL-6.

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

Figure 9 Effects of anti-IL-6 Ab on PACAP-induced CRF or AVP 5'-promoter activity in 4B cells. ** $P < 0.005$ (when compared with control (C)). + $P < 0.05$, ++ $P < 0.005$ (when compared with 100 nM PACAP). * $P < 0.05$ (when compared with 10 nM PACAP). (A) Effects of PACAP on IL-6 protein levels. The 4B cells were stimulated with 100 nM PACAP for duration shown. Expression of IL-6 protein was examined by western blot analysis. (B) Cells were pre-incubated with medium containing anti-IL-6 Ab or control IgG for 30 min and then incubated for 2 h with medium containing 10 or 100 nM PACAP or vehicle. Cells treated with control IgG are indicated as C.

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 could 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 corticotrophs.

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.

Acknowledgements

We thank Dr Ken Itoh's laboratory (Center for Advanced Medical Research) for technical assistance. K K is supported by a grant from the Funds for the Promotion of Aomori Medical Research. This work was also supported in part by Health and Labor Science Research Grants (Research on Measures for Intractable Diseases) from the Ministry of Health, Labor, and Welfare of Japan, and by a grant to T S from the Ministry of Education, Science and Culture of Japan (No. 18591014). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Agarwal A, Halvorson LM & Legradi G 2005 Pituitary adenylate cyclase-activating polypeptide (PACAP) mimics neuroendocrine and behavioral manifestations of stress: evidence for PKA-mediated expression of the corticotropin-releasing hormone (CRH) gene. *Brain Research. Molecular Brain Research* **138** 45–57.
- Ajpru S, McArthur AJ, Piggins HD & Sugden D 2002 Identification of PAC1 receptor isoform mRNAs by real-time PCR in rat suprachiasmatic nucleus. *Brain Research. Molecular Brain Research* **105** 29–37.
- Aoki Y, Iwasaki Y, Katahira M, Oiso Y & Saito H 1997 Regulation of the rat proopiomelanocortin gene expression in AtT-20 cells. II: Effects of the pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide. *Endocrinology* **138** 1930–1934.
- Boutillier AL, Monnier D, Koch B & Loeffler JP 1994 Pituitary adenyl cyclase-activating peptide: a hypophysiotropic factor that stimulates proopiomelanocortin gene transcription, and proopiomelanocortin-derived peptide secretion in corticotrophic cells. *Neuroendocrinology* **60** 493–502.
- Burbach JP, Luckman SM, Murphy D & Gainer H 2001 Gene regulation in the magnocellular hypothalamo-neurohypophyseal system. *Physiological Reviews* **81** 1197–1267.
- Castell JV, Gomez-Lechon MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R & Heinrich PC 1989 Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS Letters* **242** 237–239.
- Chaudhary P & Baumann TK 2002 Expression of VPAC2 receptor and PAC1 receptor splice variants in the trigeminal ganglion of the adult rat. *Brain Research. Molecular Brain Research* **104** 137–142.
- Dunn AJ 2004 Interleukin-6 access to the axis. *Endocrinology* **145** 3578–3579.
- Ghorbel MT, Sharman G, Leroux M, Barrett T, Donovan DM, Becker KG & Murphy D 2003 Microarray analysis reveals interleukin-6 as a novel secretory product of the hypothalamo-neurohypophyseal system. *Journal of Biological Chemistry* **278** 19280–19285.
- Gillies GE, Linton EA & Lowry PJ 1982 Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* **299** 355–357.
- Gonzalez-Hernandez T, Afonso-Oramas D, Cruz-Muros I, Barroso-Chinea P, Abreu P, del Mar Perez-Delgado M, Rancel-Torres N & del Carmen Gonzalez M 2006 Interleukin-6 and nitric oxide synthase expression in the vasopressin and corticotrophin-releasing factor systems of the rat hypothalamus. *Journal of Histochemistry and Cytochemistry* **54** 427–441.
- Grinevich V, Fournier A & Pelletier G 1997 Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on corticotropin-releasing hormone (CRH) gene expression in the rat hypothalamic paraventricular nucleus. *Brain Research* **773** 190–196.
- Hannibal J, Mikkelsen JD, Fahrenkrug J & Larsen PJ 1995 Pituitary adenylate cyclase-activating peptide gene expression in corticotropin-releasing factor-containing parvocellular neurons of the rat hypothalamic paraventricular nucleus is induced by colchicine, but not by adrenalectomy, acute osmotic, ether, or restraint stress. *Endocrinology* **136** 4116–4124.
- Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP *et al.* 1998 International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacological Reviews* **50** 265–270.
- Itoi K, Horiba N, Tozawa F, Sakai Y, Sakai K, Abe K, Demura H & Suda T 1996 Major role of 3',5'-cyclic adenosine monophosphate-dependent protein kinase A pathway in corticotropin-releasing factor gene expression in the rat hypothalamus *in vivo*. *Endocrinology* **137** 2389–2396.
- Kaskow JW, Aguilera G, Mulchahey JJ, Sherif S & Herman JP 2003a *In vitro* regulation of corticotropin-releasing hormone. *Life Sciences* **73** 769–781.
- Karalis K, Muglia LJ, Bae D & Hilderbrand JA 1997 CRH and the immune system. *Journal of Neuroimmunology* **72** 131–136.
- Kaskow J, Mulchahey JJ, Aguilera G, Pisarska M, Nikodemova M, Chen HC, Herman JP, Murphy EK, Liu Y, Rizvi TA *et al.* 2003b Corticotropin-releasing hormone (CRH) expression and protein kinase A mediated CRH receptor signalling in an immortalized hypothalamic cell line. *Journal of Neuroendocrinology* **15** 521–529.
- Komori T, Miyahara S, Yamamoto M, Matsumoto T, Zhang K, Nakagawa M, Nomura S, Motomura E, Shiroyama T & Okazaki Y 2003 Effects of odorants on the hypothalamic-pituitary-adrenal axis and interleukin-6 (IL-6) and IL-6 receptor mRNA expression in rat hypothalamus after restraint stress. *Chemical Senses* **28** 767–771.
- Kozicz T & Arimura A 2002 Dopamine- and cyclic AMP-regulated phosphoprotein-immunoreactive neurons activated by acute stress are innervated by fiber terminals immunopositive for pituitary adenylate cyclase-activating polypeptide in the extended amygdala in the rat. *Regulatory Peptides* **109** 63–70.
- Legradi G, Hannibal J & Lechan RM 1998 Pituitary adenylate cyclase-activating polypeptide-nerve terminals densely innervate corticotropin-releasing hormone-neurons in the hypothalamic paraventricular nucleus of the rat. *Neuroscience Letters* **246** 145–148.
- Liu Y, Kalintchenko N, Sassone-Corsi P & Aguilera G 2006 Inhibition of corticotropin-releasing hormone transcription by inducible cAMP-early repressor in the hypothalamic cell line, 4B. *Journal of Neuroendocrinology* **18** 42–49.
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-ΔΔC(T))} method. *Methods* **25** 402–408.
- Mastorakos G, Weber JS, Magiakou MA, Gunn H & Chrousos GP 1994 Hypothalamic-pituitary-adrenal axis activation and stimulation of systemic vasopressin secretion by recombinant interleukin-6 in humans: potential implications for the syndrome of inappropriate vasopressin secretion. *Journal of Clinical Endocrinology and Metabolism* **79** 934–939.

- Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD & Coy DH 1989 Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochemical and Biophysical Research Communications* **164** 567–574.
- Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N & Arimura A 1990 Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochemical and Biophysical Research Communications* **170** 643–648.
- Mouri T, Itoi K, Takahashi K, Suda T, Murakami O, Yoshinaga K, Andoh N, Ohtani H, Masuda T & Sasano N 1993 Colocalization of corticotropin-releasing factor and vasopressin in the paraventricular nucleus of the human hypothalamus. *Neuroendocrinology* **57** 34–39.
- Nagano K, Toba K, Akishita M, Kozaki K, Eto M, Hashimoto M, Sudoh N, Yoshimizu M & Ouchi Y 1997 Identification of arginine vasopressin mRNA in rat aortic smooth muscle cells. *Biochemical and Biophysical Research Communications* **231** 831–834.
- Navarra P, Tsagarakis S, Faria MS, Rees LH, Besser GM & Grossman AB 1991 Interleukins-1 and -6 stimulate the release of corticotropin-releasing hormone-41 from rat hypothalamus *in vitro* via the eicosanoid cyclo-oxygenase pathway. *Endocrinology* **128** 37–44.
- Neumann H, Schmidt H, Cavalie A, Jenne D & Wekerle H 1997 Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha. *Journal of Experimental Medicine* **185** 305–316.
- Nikodemova M, Kaskow J, Liu H, Manganiello V & Aguilera G 2003 Cyclic adenosine 3',5'-monophosphate regulation of corticotropin-releasing hormone promoter activity in AtT-20 cells and in a transformed hypothalamic cell line. *Endocrinology* **144** 1292–1300.
- Nomura M, Ueta Y, Serino R, Kabashima N, Shibuya I & Yamashita H 1996 PACAP type I receptor gene expression in the paraventricular and supraoptic nuclei of rats. *Neuroreport* **8** 67–70.
- Nomura M, Ueta Y, Serino R, Yamamoto Y, Shibuya I & Yamashita H 1999 Effects of centrally administered pituitary adenylate cyclase-activating polypeptide on *c-fos* gene expression and heteronuclear RNA for vasopressin in rat paraventricular and supraoptic nuclei. *Neuroendocrinology* **69** 167–180.
- Piggins HD, Stamp JA, Burns J, Rusak B & Semba K 1996 Distribution of pituitary adenylate cyclase activating polypeptide (PACAP) immunoreactivity in the hypothalamus and extended amygdala of the rat. *Journal of Comparative Neurology* **376** 278–294.
- Schimpl A & Wecker E 1972 Replacement of T-cell function by a T-cell product. *Nature: New Biology* **237** 15–17.
- Seasholtz AF, Thompson RC & Douglass JO 1988 Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. *Molecular Endocrinology* **2** 1311–1319.
- Shioda S, Shuto Y, Somogyvari-Vigh A, Legradi G, Onda H, Coy DH, Nakajo S & Arimura A 1997a Localization and gene expression of the receptor for pituitary adenylate cyclase-activating polypeptide in the rat brain. *Neuroscience Research* **28** 345–354.
- Shioda S, Yada T, Nakajo S, Nakaya K, Nakai Y & Arimura A 1997b Pituitary adenylate cyclase-activating polypeptide (PACAP): a novel regulator of vasopressin-containing neurons. *Brain Research* **765** 81–90.
- Silverman MN, Miller AH, Biron CA & Pearce BD 2004 Characterization of an interleukin-6- and adrenocorticotropin-dependent, immune-to-adrenal pathway during viral infection. *Endocrinology* **145** 3580–3589.
- Spengler D, Rupprecht R, Van LP & Holsboer F 1992 Identification and characterization of a 3',5'-cyclic adenosine monophosphate-responsive element in the human corticotropin-releasing hormone gene promoter. *Molecular Endocrinology* **6** 1931–1941.
- Suda T, Yajima F, Tomori N, Demura H & Shizume K 1985 *In vitro* study of immunoreactive corticotropin-releasing factor release from the rat hypothalamus. *Life Sciences* **37** 1499–1505.
- Takaki A, Huang QH, Somogyvari-Vigh A & Arimura A 1994 Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines. *Neuroimmunomodulation* **1** 335–342.
- Turnbull AV & Rivier CL 1999 Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiological Reviews* **79** 1–71.
- Vallieres L & Rivest S 1999 Interleukin-6 is a needed proinflammatory cytokine in the prolonged neural activity and transcriptional activation of corticotropin-releasing factor during endotoxemia. *Endocrinology* **140** 3890–3903.
- Vandesande F, Dierickx K & De Mey J 1977 The origin of the vasopressinergic and oxytocinergic fibres of the external region of the median eminence of the rat hypophysis. *Cell Tissue Research* **180** 443–452.
- Venihaki M, Dikkes P, Carrigan A & Karalis KP 2001 Corticotropin-releasing hormone regulates IL-6 expression during inflammation. *Journal of Clinical Investigation* **108** 1159–1166.
- Whitnall MH 1993 Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Progress in Neurobiology* **40** 573–629.
- Yoshida M, Iwasaki Y, Asai M, Takayasu S, Taguchi T, Itoi K, Hashimoto K & Oiso Y 2006 Identification of a functional AP1 element in the rat vasopressin gene promoter. *Endocrinology* **147** 2850–2863.
- Zhou D, Kusnecov AW, Shurin MR, DePaoli M & Rabin BS 1993 Exposure to physical and psychological stressors elevates plasma interleukin 6: relationship to the activation of hypothalamic-pituitary-adrenal axis. *Endocrinology* **133** 2523–2530.

Received in final form 31 July 2007

Accepted 23 August 2007

Made available online as an Accepted Preprint
23 August 2007