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Differential regulation of corticotropin-releasing factor receptor type 1 (CRF_1 receptor) mRNA via protein kinase A and mitogen-activated protein kinase pathways in rat anterior pituitary cells

Takako Moriyama [a], Kazunori Kageyama [a,*], Yoko Kasagi [a,1], Yasumasa Iwasaki [b], Takeshi Nigawara [a], Satoru Sakihara [a], Toshihiro Suda [a]

[a] *The Third Department of Internal Medicine, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan*
[b] *Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi 783-8505, Japan*

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Abstract

Corticotropin-releasing factor (CRF) receptor type 1 (CRF_1 receptor) mRNA levels are down-regulated by CRF via the cyclic AMP-protein kinase A (PKA) pathway. In this study, we focused on the involvement of both the mitogen-activated protein (MAP) kinase pathway and PKA in this regulation. Real-time PCR (RT-PCR) revealed that a MAP kinase, extracellular signal-regulated kinases 1/2, pathway was also involved in the down-regulation of CRF_1 receptor mRNA levels by CRF in the rat anterior pituitary (AP). Down-regulation of CRF_1 receptor mRNA levels was caused by a post-transcriptional system such as mRNA degradation, as incubation with CRF significantly decreased the half-life of CRF_1 receptor mRNA. Furthermore, pre-treatment with a PKA inhibitor completely blocked CRF-induced CRF_1 receptor mRNA destabilization, while pre-treatment with an extracellular signal-regulated kinases 1/2 inhibitor had no inhibitory effect. These results suggested that in the rat AP, down-regulation of CRF_1 receptor mRNA levels is caused by mRNA degradation via PKA, but not by the MAP kinase pathway.

Keywords: Corticotropin-releasing factor; Protein kinase A; Mitogen-activated protein kinase; Corticotropin-releasing factor receptor; Anterior pituitary; mRNA stability

1. Introduction

Corticotropin-releasing factor (CRF) acts as the primary mediator of the hypothalamic–pituitary–adrenal (HPA) axis under stress conditions (Vale et al., 1981; Suda et al., 2004). CRF is produced in the hypothalamic paraventricular nucleus (PVN) in the brain in response to stress (Vale et al., 1981; Whitnall, 1993) and secreted into the pituitary portal circulation, resulting in the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (AP) (Whitnall, 1993). CRF exerts its biological actions by binding to CRF receptors (Vale et al., 1997) that belong to the seven-transmembrane-domain G-protein-coupled receptor superfamily. CRF receptor type 1 (CRF_1 receptor) mRNA is expressed abundantly in the AP and the brain with major expression sites in the cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulbs (Perrin and Vale, 1999). In the pituitary corticotroph, CRF_1 receptor is the major subtype and is responsible for regulating the synthesis and secretion of ACTH, which in turn stimulates glucocorticoid release from the adrenal glands. Therefore, in order to understand the functional regulation of the HPA axis, it is necessary to elucidate the regulation of CRF_1 receptor on the corticotroph.

Alterations of the HPA axis cause dynamic changes in CRF_1 receptor levels in the AP (Nikodemova et al., 2002; Aguilera et al., 2004). CRF_1 receptor mRNA and protein levels are influenced by multiple factors such as CRF, arginine vasopressin (AVP), glucocorticoids and cytokines, which are released during stress to coordinate the HPA axis (Nikodemova et al., 2002; Aguilera et al., 2004; Nigawara et al., 2003). In the corticotroph, it is well known that CRF induces proopiomelanocortin transcription and ACTH secretion through the cAMP-protein kinase A (PKA) pathway. In addition, CRF down-regulates

* Corresponding author. Tel.: +81 172 39 5062; fax: +81 172 39 5063.
E-mail address: kkageyama@hkg.odn.ne.jp (K. Kageyama).
[1] Present address: Yokohama Medical Center, National Hospital Organization, 3-60-2 Harajuku, Totsuka-ku, Yokohama, Kanagawa 233-8503, Japan.

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CRF_1 receptor mRNA levels in the rat AP (Sakai et al., 1996; Pozzoli et al., 1996). We reported recently that down-regulation of CRF_1 receptor mRNA by CRF was induced via a cAMP-PKA and possibly by the cAMP-responsible element binding protein (CREB) pathway (Kasagi et al., 2002), while CRF_2 receptor is down-regulated via cAMP-PKA and p38 MAP kinase pathways (Kageyama et al., 2003; Kageyama et al., 2005). In addition to the signal transduction pathways discussed above there is accumulating evidence that CRF modulates mitogen-activated protein (MAP) kinase. For example, CRF increases MAP kinase activity in ovine AP cells (Li et al., 1998) and also triggers neurite outgrowth of catecholaminergic immortalized neuron cells via extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Cibelli et al., 2001).

On the basis of this evidence, this study examined the hypothesis that MAP kinase pathways may be involved in the down-regulation of CRF_1 receptor mRNA levels by CRF in rat AP cells. We also explored the effect of CRF on CRF_1 receptor mRNA stability and the pathway involved in this process, for the reason that down-regulation of CRF_1 receptor mRNA levels may be caused by a post-transcriptional system such as mRNA degradation.

2. Materials and methods

2.1. Animals

Adult male Wistar rats were obtained from CLEA Japan (Tokyo, Japan). The rats were housed in an air-conditioned room with a controlled cycle (light on at 08:00 h, off at 20:00 h) and provided rat chow and water ad libitum. All animal experiments in this paper were carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

2.2. Materials

CRF was purchased from Peptide Institute (Osaka, Japan) and PD98059, SB203580, actinomycin D (Act D) and protein kinase A inhibitor 14–22 amide (PKAi) from Calbiochem (San Diego, CA, USA). Act D was dissolved in 2.5% diluted dimethylsulfoxide (DMSO) and the other compounds in 0.2% bovine serum albumin (BSA) solution.

2.3. Cell cultures

AP cells obtained from adult male Wistar rats weighing 200–250 g were dispersed as described previously (Kasagi et al., 2002). In brief, anterior pituitaries were cut into small pieces and incubated for 30 min at 37 °C in 5 ml of sterile HEPES-buffered saline containing 0.4% collagenase (Gibco-BRL, Gaithersberg, MD, USA), 0.002% deoxyribonuclease (Sigma Chemical Co., St. Louis, MO, USA), 0.04% dispase (Godo Shusei Co., Tokyo, Japan) and 2% BSA (Nakarai Tesque, Kyoto, Japan). The dispersed cells were then washed and suspended in HEPES-buffered DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco-BRL). Aliquots of 1.2×10^6 cells were placed in six-well (35 mm diameter) culture dishes (Iwaki Glass, Funabashi, Japan) and cultured at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. On the fourth day, the cells were washed and starved overnight by incubation in HEPES-buffered DMEM supplemented with 0.2% BSA, 100 μg/ml streptomycin and 100 U/ml penicillin. On the fifth day, the cells were pre-incubated for 30 min with or without medium containing various inhibitors and then incubated in medium containing CRF. At the end of incubation, the medium and total cellular RNA were collected and stored at −80 °C. All treatments were performed in triplicate and repeated three times.

2.4. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Cellular total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNAs were synthesized from total RNA (0.5 μg) with random hexamer as the primer using the SuperScript First-Strand Synthesis System for RT-PCR Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions.

The resulting cDNAs were then subjected to real-time PCR as follows. The expression level of rat CRFR1 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). Beta 2 microglobulin (B2M) was used as a housekeeping gene to standardize the values, because the B2M mRNA levels were not changed in any treatments of these studies. Each reaction consisted of 1×TaqMan universal PCR Master Mix (Applied Biosystems), 1×Assays-on-Demand Gene Expression Products (Rn00678611_m1 for rat CRF_1 receptor and Rn00560865_m1 for rat B2M) and 2 μl cDNA in a total volume of 50 μl using the following parameters on the 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 involve specific sets of primers and TaqMan probe spanning exon/exon junction and should not therefore 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 the diluted samples, the amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical.

2.5. Relative quantitative gene expression

Relative quantitative gene expression was calculated with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). In brief, for each sample assayed, the C_T for reactions amplifying a gene of interest (rat CRF_1 receptor) and the housekeeping gene (rat B2M) were determined. The C_T of the gene of interest for each sample was corrected by subtracting the C_T of the housekeeping gene (ΔC_T). Untreated controls were chosen as reference samples with mean ΔC_T for the control samples being subtracted from the ΔC_T for all the experimental samples ($\Delta\,\Delta C_T$). Finally, experimental mRNA abundance relative to control mRNA abundance was calculated by the formula $2^{-\Delta\Delta C_T}$.

2.6. ACTH assay

The culture medium was collected from the dishes and stored at −80 °C for measurement of ACTH levels using a radio immunometric assay (ACTH IRMA kit, Mitsubishi Kagaku Iatron, Tokyo, Japan).

2.7. mRNA stability study

AP cells were incubated for 2 h with either medium alone or 100 nM CRF pre-treated for 30 min with or without medium containing either 10 μM PKAi or 10 μM PD98059. At the end of stimulation, Act D was added to a final concentration of 5 μg/ml. The cells were then harvested using the RNeasy Mini Kit and the RNA extracted immediately ($t = 0$) or at hourly intervals up to 180 min ($t = 180$) after the addition of Act D. CRF_1 receptor mRNA stability was assessed using real-time RT-PCR in order to quantify the amount of CRF_1 receptor mRNA at each time point relative to the amount of CRF_1 receptor mRNA in $t = 0$ in each treatment group. The half-lives of CRF_1 receptor mRNA were then calculated as described in literature (Geng and Strobel, 1998).

2.8. Statistical analysis

All values are expressed as the mean ± S.E.M. of three independent experiments. Statistical analysis of the data was performed using one-way ANOVA, followed by Scheffe's F post hoc test. The level of statistical significance was set at $P < 0.05$.