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INVOLVEMENT OF A NEGATIVE GLUCOCORTICOID RESPONSE ELEMENT ON CORTICOTROPIN-RELEASING FACTOR GENE PROMOTER IN HYPOTHALAMIC CELLS

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Abstract Corticotropin-releasing factor (CRF) plays a central role in controlling the hypothalamic-pituitary-adrenal axis during stressful periods. CRF, produced in the hypothalamic paraventricular nucleus (PVN) in response to stress, stimulates adrenocorticotropic hormone (ACTH) release in the anterior pituitary. ACTH then stimulates glucocorticoid release from the adrenal glands. Glucocorticoid in turn inhibits hypothalamic PVN production of CRF and pituitary production of ACTH. We previously demonstrated that protein kinase A (PKA) pathway takes a main part in producing CRF in the hypothalamus. A functional cAMP-response element (CRE) on the 5'-promoter region is important to increase CRF gene expression. Glucocorticoids inhibit CRF promoter activity in the pituitary cells, while in the placenta and the bed nucleus of the stria terminalis, glucocorticoids stimulate it. The effect of glucocorticoids on CRF gene, therefore, would be tissue specific. Although to study the mechanism regulating CRF transcription in the PVN has been the lack of availability of a representative cell line, a rat fetal hypothalamic cell line has been recently available. We found that forskolin-stimulated CRF gene transcription is mediated by CRE on the CRF 5'-promoter region in the hypothalamic cells. Both PKA and, in part, p38 mitogen-activated protein kinase pathways contribute to the forskolin-induced CRF promoter activity. Glucocorticoid-dependent repression of forskolin-stimulated CRF promoter activity was localized to promoter sequences between −278 and −249 bp. Taken together, these findings indicate that a negative glucocorticoid regulatory element is critical for the repression of CRF gene in the hypothalamic cells.

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Key words: cyclic AMP; corticotropin-releasing factor receptor; glucocorticoids; hypothalamus; promoter

Introduction

Corticotropin-releasing factor (CRF) plays a central role in controlling the hypothalamic-pituitary-adrenal axis during stressful periods (Figure 1)[1,2)].

CRF, produced in the hypothalamic paraventricular nucleus (PVN) in the brain in response to stress [1)], is secreted into the pituitary portal circulation, resulting in the release of adrenocorticotropic hormone (ACTH) via CRF receptor type 1 (CRF_1 receptor) from the anterior pituitary (AP). ACTH then stimulates glucocorticoid release from the adrenal glands[3)]. Glucocorticoid in turn inhibits hypothalamic PVN production of CRF and pituitary production of ACTH, hence ensuring that serum glucocorticoid levels are appropriate to the stress experienced. In addition to well-established inhibitory effects on proopiomelanocortin and CRF gene expression[4,5)], glucocorticoids are known to be potent anti-inflammatory agents, and to modulate production of inflammatory factors such as cytokines[6)].

We previously demonstrated that protein kinase A (PKA) pathway takes a main part

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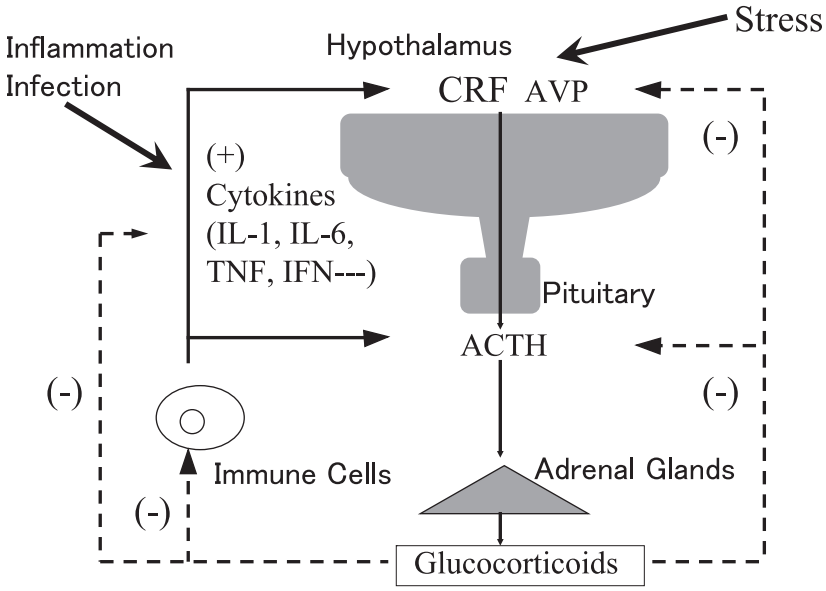

Figure 1 Hypothalamic-pituitary-adrenal axis in stress.

in CRF release in hypothalamic explants[7]. 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. A functional cAMP-response element (CRE) in the 5'-promoter region is important to increase CRF gene expression[8,9]. CRE activation of the PKA pathway leads to binding of CRE-binding protein to the CRE on the CRF promoter in human placental cells[10].

Glucocorticoid suppresses CRF gene expression levels in the PVN. In a subclone of AtT-20 cells permanently transfected with the CRF gene, dexamethasone also suppressed CRF gene expression levels[11]. Glucocorticoids would regulate CRF through inhibition of gene transcription in these cells. The CRF promoter does not contain a classical consensus glucocorticoid regulatory element (GRE), but there are a number of regions in the sequence where glucocorticoid receptors (GRs) are able to bind[11]. Malkoski et al. demonstrated glucocorticoid regulatory regions in the CRF promoter[12]. They demonstrated that using a series of 5'-nested deletions, dexamethasone-dependent repression of cAMP-stimulated CRF promoter activity was localized to promoter sequences between −278 and −249 bp. High-affinity binding of GR DNA-binding domain to this promoter region was observed using an electrophoretic mobility shift assay. Therefore, a negative GRE (nGRE) mediates the inhibition of CRF promoter activity by glucocorticoids.

Little is known about the signaling pathways involved in the control of CRF transcription in parvocellular PVN neurons. A major impediment to studying the mechanism regulating CRF transcription in parvocellular neurons has been the lack of availability of a representative cell line[13]. Recently, a rat fetal hypothalamic cell line has been available[14]. The hypothalamic cells, 4B cells, express CRF mRNA and CRF-like immunoreactivity. These cells also express vasopressin, CRF_1 receptor, and GR. In these cells transfected with CRF puromoter-luciferase construct, forskolin increased the CRF luciferase activity in parallel with increases in intracellular cAMP[15].

In the present study, we examined whether CRE is involved in the responses of CRF

promoter activity after PKA activation. We further explored the involvement of nGRE on CRF gene promoter in the 4B hypothalamic cells.

Materials and Methods

Constructs and transfection

A 1077-base pair (bp) restriction fragment containing the human CRF promoter (-907 to +170 relative to the proximal transfection start point) was obtained by PCR. A series of deletion mutant constructs of the CRF gene promoter (CRF-220luc, CRF-233luc, CRF-248luc, and CRF-278luc) were constructed using the specific primers. A mutant construct (CRF-233Mluc) in which CRE element (TGACGTCA) was mutated (TGGATCCA) was made from the by a site-directed mutagenesis technique following the manufacturer's instructions (Stratagene, La Jolla, CA, USA).

CRF luciferase activity

Luciferase assay was performed according to the manufacturer's protocol. At the end of each experiment, cells were washed two times with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, harvested with PicaGene lysis buffer (Toyo Inki, Tokyo, Japan), and centrifuged at 12,000 rpm for 2 min. For the luciferase assay, 20 μL of each supernatant was used. The reactions were started by the injection of 100 μL of luciferin solution, PicaGene buffer. Light output was measured for 20 sec at room temperature using a luminometer (Berthold Lumat LB9501, Postfach, Germany). Activity of β-galactosidase was used as an internal control.

Results

Effects of CRE deletion on forskolin-induced CRF gene expression in 4B cells.

To determine whether CRE is involved in regulation of CRF gene expression by forskolin, 4B cells transfected with a series of deletion mutant constructs of the CRF gene promoter, such as CRF-233luc, CRF-233Mluc, or CRF-220luc, were stimulated with10 μM forskolin. 4B cells transfected with CRF-233luc showed a similar potent CRF promoter activity to the cells transfected with CRF-907luc, while neither 4B cells transfected with CRF-233Mluc nor CRF-220luc showed such an activity.

Effects of dexamethasone on forskolin-induced CRF gene expression in 4B cells.

We next examined the dose-dependency of the glucocorticoid effect. Incubation with 10 or 100 nM dexamethasone decreased the CRF luciferase activity. RU486 (100 nM) recovered the inhibitory effects of dexamethasone (100 nM) on forskolin-induced CRF luciferase activity.

Effects of nGRE deletion on the dexamethasone suppression of CRF gene expression in 4B cells.

4B cells transfected with CRF-907luc or CRF-278luc showed the inhibitory effect of dexamethasone on the forskolin-induced CRF promoter activity, while the cells transfected with CRF-248luc or CRF-233luc showed no inhibitory effect of dexamethasone. Therefore, glucocorticoid-dependent repression of cAMP-stimulated CRF promoter activity was localized to promoter sequences between −278 and −248 bp.

Discussion

We found that forskolin increased CRF 5' -promoter activity in 4B hypothalamic cells, in agreement with those of previous studies using other cells[8,9]. Further, we found that neither 4B cells transfected with CRF-233Mluc nor CRF-220luc showed such an activity. Together, forskolin-stimulated CRF gene transcription is mediated by CRE, which includes −220 to −233 bp, on the CRF 5'-promoter region in hypothalamic 4B cells (Figure 2).

Glucocorticoids regulate CRF directly through inhibition of gene transcription in a subclone of AtT-20 cells[11]. However, CRF promoter does not contain a classical consensus GRE.

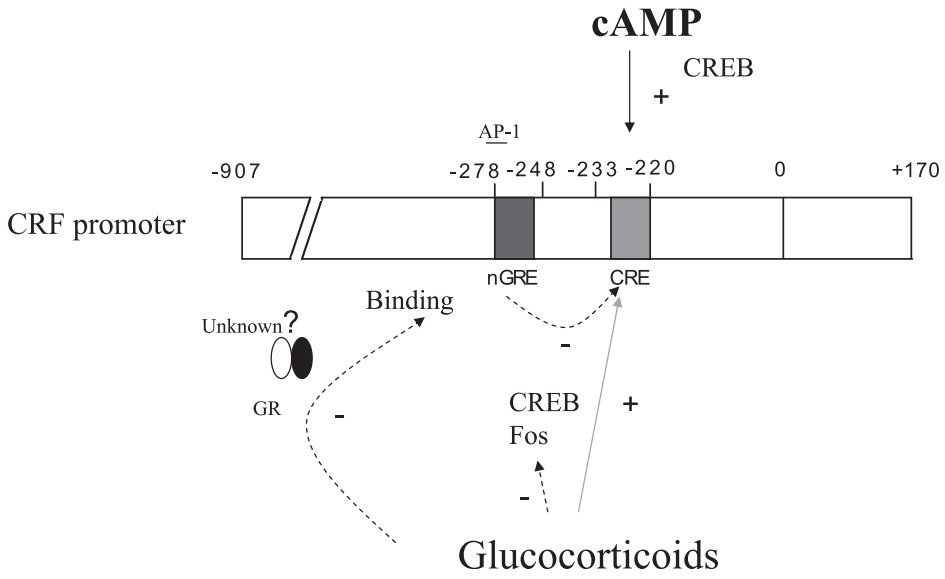

Figure 2 A schematic model of the CRF promoter regulation in the hypothalamic cells.

We demonstrated that a promoter region is involved in the negative regulation of CRF gene expression in hypothalamic cells. We further demonstrated that the glucocorticoids suppression of cAMP-stimulated CRF promoter activity was involved in the promoter sequences between −278 and −248 bp of CRF gene promoter in hypothalamic cells. Our results consistent with Malkoski's hypothesis that glucocorticoid-dependent repression of cAMP-stimulated CRF promoter activity was caused by the nGRE[16].

However, outside the PVN, in the placenta and the bed nucleus of the stria terminalis, glucocorticoids stimulate CRF gene expression[13]. Therefore, the effects of glucocorticoids on tissue specificity would be suggested. Malkoski et al. showed that high-affinity binding sites for both GR and activator protein 1 (AP-1) nucleoproteins were identified at adjacent elements within the nGRE[12]. Mutations that disrupted either GR or AP-1 binding activity were associated with loss of glucocorticoid-dependent repression. These results suggest that the nGRE works as a composite regulatory element, involving direct DNA binding of GR and AP-1 nucleoproteins. King et al. proposed[17] that transcription factor differences among tissues cause the negative or positive regulation, because CREB and Fos were detected in AtT-20 while CREB and cJun in placental cels. Further, glucocorticoids can inhibit CREB and cFos in the PVN[18,19]. The modified expression of the transcription factors may change the interaction between the nGRE and CRE, resulting in suppression of CRF gene transcription. Although such factors to inhibit CRF gene are undetermined in the hypothalamus, it is clear that nGRE is critical for the repression of CRF gene in hypothalamic cells (Figure 2).

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