Calcium/calmodulin kinase IV pathway is involved in the transcriptional regulation of the corticotropin-releasing hormone gene promoter in neuronal cells

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Abstract

Although corticotropin-releasing hormone (CRH) plays a pivotal role in the regulation of the hypothalamo-pituitary-adrenal axis, the mechanism of CRH gene expression in the neuronal cell is not completely understood. In this study, we examined the transcriptional regulation of human CRH gene 5'-promoter, using a human BE(2)C neuroblastoma cell line expressing intrinsic CRH. In particular, we focused on the involvement of calmodulin kinases (CaMKs), which are known to play an important role in excitation-induced gene expression through the rise in intracellular calcium in the central nervous system. RT-PCR analysis confirmed the expression of CaMK as well as CRH mRNA in BE(2)C cells. When we introduced ≈ 1.1 kb of the 5'-promoter region of the human CRH fused with luciferase reporter gene into the cells, a substantial transcriptional activity was observed, and this was further increased by the activation of the cAMP/PKA pathway. We then examined the effect of activation of CaMKs by introducing the expression vectors of each kinase, revealing a potent stimulatory effect of CaMKIV, but no effect of CaMKII. Depolarization of the cells caused an increase in CRH promoter activity, which was completely abolished by the treatment with the CaMK antagonist K252a. Interestingly, KCREB, a dominant negative form of CREB, antagonized the effect of the CaMKIV-mediated effect. Altogether, we conclude that not only the cAMP/PKA but also the calcium/CaMKIV signaling pathway is involved in the regulation of CRH gene expression. Furthermore, CREB is thought to be involved in CaMK- as well as cAMP/PKA-mediated CRH gene expression. Since the CRH gene is expressed in the neuronal cells of the hypothalamus, the calcium/CaMKIV signaling pathway may play an important role in the excitation-mediated regulation of CRH synthesis.

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Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is a major antistress system in mammalian species (Stratakis & Chrousos 1995). Corticotropin-releasing hormone (CRH), a neuropeptide hormone expressed mainly in the hypothalamus, is known to play a pivotal role in the regulation of the HPA axis because it serves as a major regulator of the synthesis and secretion of adrenocorticotropin by the pituitary (Vale *et al.* 1981). In fact, genetic

ablation of the CRH gene causes hypoadrenocorticism with marked attenuation of corticosterone responses to stressful stimuli (Muglia *et al.* 1995, Venihaki & Majzoub 1999). Therefore, it is important to clarify the transcriptional regulation of the CRH gene for a better understanding of the regulatory mechanism of the HPA axis at the molecular level.

Previous studies in the field of CRH gene regulation have focused on the role of the cAMP-protein kinase A (PKA) pathway, because

the canonical cAMP-responsive element (CRE) is located in the promoter region of the CRH gene (Seasholtz *et al.* 1988, Spengler *et al.* 1992). In addition, CRE-binding protein (CREB) has been shown to mediate the positive effects of the cAMP/PKA signaling pathway (Seasholtz *et al.* 1988, Spengler *et al.* 1992, Itoi *et al.* 1996, Wolff *et al.* 1999). Thus, it is well established that the cAMP/PKA/CREB pathway is one of the major regulatory systems for the positive regulation of the CRH gene.

In addition, since CRH is expressed in the neuronal cells of the hypothalamus, a rise in intracellular calcium, another major signaling event, occurs when they are activated, followed by the release of CRH. Therefore, it is tempting to speculate that activation of the calcium signaling system influences CRH gene expression as well. Indeed, it has been shown that there is a mutual link between depolarization of neuronal cells and the increase in gene expression (Schmidt 1996), and that the rise in intracellular calcium is associated with the transcriptional activation of many genes (Bading *et al.* 1995, Finkbeiner & Greenberg 1998). In this sense, Seasholtz and colleagues have already reported the increased CRH gene transcription following depolarization in PC12 adrenal chromaffin cells (Guardiola-Diaz *et al.* 1994).

Elevation of intracellular calcium causes various biologic responses, among which activation of the calcium/calmodulin system is one of the major signaling events in neuronal cells (Hook & Means 2001). Calmodulin is a calcium-binding protein, which activates calmodulin kinases (CaMKs) when the intracellular calcium level is increased. CaMKs, in turn, activate multiple downstream signaling pathways by phosphorylating their target proteins. CaMKs have been shown to be expressed abundantly in the brain, and to play important roles in the transcriptional regulation of many kinds of genes (Hook & Means 2001). Accordingly, it is possible that depolarization of the CRH neuron with subsequent CRH release simultaneously causes increased CRH gene expression involving activation of the calcium/calmodulin/CaMK cascade.

In this study, we explored the possible involvement of CaMKs in the regulation of CRH gene expression. For this purpose, we chose a human neuroblastoma cell line BE(2)C that intrinsically

expresses the CRH gene and also retains the properties of excitable neuronal cells (Kasckow *et al.* 1994, 1995). We also adopted the expression system of a constitutively active form of CaMKII and IV, to clarify the role of each kinase. Using this homologous cell line, we provided evidence indicating that CaMKIV is involved in the transcriptional regulation of the CRH gene. We also showed that CREB is responsible for the activation by CaMKIV as well as by cAMP/PKA.

Materials and methods

Reagents

Forskolin, 8Br-cAMP and nystatin were purchased from Sigma; KN62 and K252a from Calbiochem (San Diego, CA, USA) and fura-2 from Molecular Probe (Eugene, OR, USA).

Cell culture

BE(2)C, a human neuroblastoma cell line, was grown in DMEM/F12 culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% nonessential amino acid (Invitrogen) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Invitrogen) under 5% CO₂/95% atmosphere at 37 °C. Culture medium was changed twice a week, and cells were subcultured once a week.

RT-PCR

Total RNA was isolated from BE(2)C cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and RT-PCR analyses were carried out with a one-step RT-PCR kit (Toyobo, Tokyo, Japan). The specific primers for each amplification were as follows: human CRH forward, 5'-AAGCGCCTGGGAA GCGAGTG-3', reverse, 5'-GCGGCTGGAAGA AATCCAAG-3'; human CaMKIIα forward, 5'-TGACAGAGCAGCTGATTGAAG-3', reverse, 5'-GTGGACGATCTGCCATTTGC-3'; human CaMKIV forward, 5'-AGAAGCTCCAAGAATT CAATGC-3', reverse, 5'-GATCAGATCTTGCT GTGGAAC-3'. In all cases, the forward and reverse primers were designed to be located in

different exons, so that the PCR products from cDNA and genomic DNA could be distinguished.

Plasmid constructions

About 1.1 kb of the human CRH gene 5'-promoter region containing CRE (−907 to +171 bp, +1 designates the transcription start site) was amplified by PCR, and, after its sequence was confirmed, was fused with luciferase reporter gene (pA3Luc), obtaining a CRH-luciferase construct (CRH-Luc). Expression vectors containing the cDNAs for CaMKs (CaMKII_{WT}, CaMKII₂₉₀, CaMKIV_{WT} and CaMKIV₃₁₃) were constructed by subcloning each cDNA (kindly provided by Dr McKnight) (Matthews *et al.* 1994) into pRC/CMV (Invitrogen). An expression vector for dominant negative CREB (KCREB) has been described previously (Walton *et al.* 1992) and was kindly provided by Dr Goodman.

Transfection

BE(2)C cells were plated in 35-mm culture dishes with 60–70% confluency. On the next day, cells were incubated with serum-free medium containing 3 µg plasmid DNAs and 6 µl TransIT polyamine transfection reagent (Pan Vera, Madison, IL, USA) per dish for 8 h. The culture medium was then replaced with medium containing 10% FBS, and each experiment was carried out 24–48 h thereafter. β-Galactosidase (β-gal) or secreted placental alkaline phosphatase (SEAP) expression vector was used as an internal control. When a different amount of an expression vector for each treatment group was used in an experiment, a complementary amount of the same vector without cDNA was used, so that the net amount of the plasmid was equal among the groups. When cells were treated with high potassium, an adequate amount of potassium chloride solution was added to the culture medium, so that the final concentration of potassium was 60 mM.

Reporter assays

Luciferase assay was performed as previously described (Aoki *et al.* 1997, Iwasaki *et al.* 1997). β-gal and SEAP activities were determined

by Galacto-Light Plus assay kit (Tropix, Bedford, MA, USA) and SEAP reporter assay kit (Toyobo) respectively, according to the manufacturers' instructions.

Electrophysiology and [Ca²⁺]_i measurement

For [Ca²⁺]_i measurements, cells were seeded on 22-mm, round cover glasses and placed in 35-mm culture dishes. For electrophysiological experiments, they were seeded on 35-mm culture dishes. Electrophysiological studies and [Ca²⁺]_i measurements were performed on the third day after plating the cells. The perforated whole-cell clamp technique (Horn & Marty 1988) was used. The standard patch electrode solution contained (in mmol/l): 95 K aspartate, 47.5 KCl, 1 MgCl₂, 0.1 ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (tetramethylammonium (TMA) salt) and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (TMA salt, pH 7.2). The standard external solution was (in mmol/l): 128 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂ and 10 HEPES (Na salt, pH 7.4). High potassium solution was made by increasing the potassium of the extracellular solution to 60 mmol/l and decreasing extracellular Na⁺ iso-osmotically. During the experiments, the extracellular solution was continuously perfused by a peristaltic pump. The time needed to change the entire extracellular solution in our peristaltic pump system was about 2 min. Agents were applied by changing the perfusing solution. The liquid junction potential between the standard extracellular solution and other solutions used (internal and external) was measured with a 3 mol/l KCl electrode as a reference, and all the data were corrected for the liquid junctional potential. A List EPC-7 amplifier was used to record the membrane current and potential. All experiments were performed at room temperature (22–25 °C). Glass capillaries of 1.5 mm diameter with a filament were used to make patch electrodes. The resistance of the patch electrodes was 5–8 MΩ. For the perforated whole-cell clamp experiments, a fresh stock solution of nystatin was made in dimethylsulfoxide (50 mg/ml) daily. Shortly before recording, the stock solution was diluted with the patch electrode solution (final nystatin concentration, 200 µg/ml). Details of the perforated whole-cell clamp technique have been reported elsewhere (Horn & Marty 1988, Yamashita &

Hagiwara 1990). Current clamp recordings were started after the series resistance fell below 50 M Ω . Voltage clamp recordings were made after the series resistance fell below 20 M Ω . Because the amplitude of the current was less than 150 pA, the errors caused by the series resistance were ignored.

For $[Ca^{2+}]_i$ measurement, cells were loaded with fura-2 by incubating with 2 μ mol/l fura-2/AM in Hanks' balanced salt solution containing 0.1% BSA for 40 min at room temperature. $[Ca^{2+}]_i$ measurements were performed on a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Cells were excited at 340 and 380 nm alternately at a frequency of 100 Hz with CAM220 (Nihonbunko, Tokyo, Japan). Fluorescent light was collected from single cells. A band filter was used to monitor the fluorescent emission at 510 nm. The cytosolic free Ca^{2+} concentration was determined by the equation $[Ca^{2+}]_i = K(R - R_{min}) / (R_{max} - R)$ (Yamashita & Hagiwara 1990). In this equation, K represents $K_d (F_{min}/F_{max})$, where K_d is the dissociation constant of fura-2 (130 nmol/l at 25 °C), and F_{max}/F_{min} is the ratio of Ca^{2+} -free to Ca^{2+} -bound fura-2 fluorescence at 380 nm. R_{min} is the 340/380 fluorescence ratio of Ca^{2+} -free fura-2, and R_{max} is the 340/380 ratio of Ca^{2+} -bound fura-2. Calibration was performed by permeabilizing the cell to Ca^{2+} with 2 μ mol/l digitonin. Cells were first permeabilized in Ca^{2+} -free saline (5 mmol/l EGTA, 150 mmol/l KCl and 10 mmol/l HEPES, pH=7.2), for determination of R_{min} and F_{min} , and then in high Ca^{2+} saline (2.5 mmol/l $CaCl_2$, 150 mmol/l KCl and 10 mmol/l HEPES, pH=7.4) for determination of R_{max} and F_{max} . The $[Ca^{2+}]_i$ traces shown in the figures were filtered with a bandwidth of 1 Hz in order to reduce the noise. Agonists were applied by changing the bath solution with a peristaltic pump. In the $[Ca^{2+}]_i$ measurement experiment, it took about 30 s to change the bath solution in this peristaltic pump system.

Data analysis

Samples in each group of the experiments were in triplicate. All data were expressed as mean $\pm$ S.E. When statistical analyses were performed, data were compared by one-way ANOVA with Fisher's protected least-significant difference test, and P values below 0.05 were considered significant.

Figure 1 Expression of CRH mRNA in BE(2)C cells analyzed by reverse transcriptase (RT)-PCR. The photograph shows the amplified DNA fragments with the predicted lengths (176 and 463 bp for human CRH and CaMKIV mRNAs) derived from BE(2)C cells. M refers to DNA markers. RT-minus control reaction showed no amplification (data not shown).

Results

BE(2)C cells express endogenous CRH and CaMKIV

We first investigated whether BE(2)C expresses endogenous CRH and CaMKs by the RT-PCR technique. The result showed that bands with expected lengths for human CRH and CaMKIV were amplified (Fig. 1). On the other hand, the band for CaMKII was not, or was barely, detected.

Activation of cAMP/PKA pathway stimulates CRH 5'-promoter activity in BE(2)C cells

CRH gene promoter is known to be activated by the cAMP/PKA pathway (Seasholtz *et al.* 1988, Spengler *et al.* 1992). To confirm the positive signaling in BE(2)C cells, we transiently introduced the human CRH gene 5'-promoter (≈ 1.1 kb)-luciferase fusion gene (CRH-Luc) into the cells. When stimulated by forskolin, an activator of adenylate cyclase, a 4–6-fold increase in the promoter activity was observed (Figs 2A and B). Stimulation by cAMP analog 8Br-cAMP caused similar or even more pronounced effect (Fig. 2C). These results support the previous notion that the cAMP/PKA pathway is one of the major positive regulatory pathways for CRH gene expression.

Figure 2 Effects of the activation of cAMP/PKA pathway on CRH gene expression in BE(2)C cells. (A and B) the time course and dose-response effects of forskolin on the promoter activity of the CRH gene. BE(2)C cells transiently transfected with the full-length human CRH gene 5'-promoter-luciferase construct (CRH₉₀₇-Luc) were treated with forskolin (10 μM) for 0–12 h (A), or with various doses of forskolin (0–10 μM) for 5 h (B). (C) BE(2)C cells transfected as above were treated with 8Br-cAMP (5 mM) for 5 h.

* $P < 0.05$ vs control value.

Expression of activated CaMKIV stimulates CRH 5'-promoter activity in BE(2)C cells

CRH release is stimulated by depolarization of the CRH neuron with an increase in intracellular calcium, which simultaneously activates calmodulin/CaMKs. Therefore, we next investigated whether activation of CaMKs increases CRH gene expression. For this purpose, we coexpressed wild-type or constitutively active forms of CaMKII/IV along with CRH-Luc in BE(2)C cells (Matthews *et al.* 1994). We found a potent stimulatory effect of CaMKIV, but no effect of CaMKII expression, on CRH 5'-promoter activity (Figs 3A and B). In the latter case, only the constitutively active form of CaMKIV (CaMKIV₃₁₃) caused a significant effect. The enhancing effect of CaMKIV₃₁₃ was dose-dependent (Fig. 3C). Together, these results suggest that CaMKIV, but not CaMKII, is involved in the transcriptional regulation of the CRH gene.

The effects of high potassium and 8Br-cAMP on membrane potential and intracellular calcium

To determine whether depolarization of BE(2)C cells actually causes the rise in intracellular calcium, we examined the effect of high-potassium extracellular solution and 8Br-cAMP on the membrane

potential under the current clamp. In the standard extracellular solution containing 5 mM potassium, the resting potential was -55 ± 3 mV ($n=5$). When the extracellular solution was changed to high (60 mM) potassium solution, the membrane depolarized to -22 ± 4 mV ($n=5$). When the standard extracellular solution was replaced with that containing 8Br-cAMP (100 μM), the membrane potential changed from -53 ± 2 ($n=10$) to -42 ± 2 ($n=10$) mV. In these experimental conditions, we determined the intracellular calcium concentration by use of fura-2 (Tsien 1980). With the standard extracellular solution containing 5 mM potassium, the intracellular calcium concentration was 63 ± 10 nmol/l ($n=20$). When the extracellular solution was changed to high (60 mM) potassium solution, the intracellular calcium concentration increased in 7 out of the 20 cells. The average calcium concentration of the 20 cells significantly increased from 63 ± 10 to 138 ± 24 nmol/l ($P < 0.05$). Fig. 4A–C show representative positive responses. Addition of nitrendipine (5 μM) and ω-conotoxin GV1A (1 μM) to the extracellular solution abolished the increase (Fig. 4D). The same results were obtained in four other clusters of cells. When the standard extracellular solution was replaced with that containing 8Br-cAMP (100 μM), the intracellular calcium concentration increased in

Figure 3 Effects of CaMKII/IV coexpression on the CRH gene expression in BE(2)C cells. (A) BE(2)C cells were cotransfected transiently with CRH₉₀₇-Luc construct and either control (pRC/RSV without cDNA), activated form (CaMKII_{T290}, T290) or wild-type CaMKII (CaMKII_{WT}, WT). (B) BE(2)C cells were cotransfected transiently with CRH₉₀₇-Luc construct and either control (pRC/RSV without cDNA), activated form (CaMKIV_{T313}, T313) or wild-type CaMKIV (CaMKIV_{WT}, WT). (C) BE(2)C cells were cotransfected transiently with CRH₉₀₇-Luc construct and increasing doses of the activated form of CaMKIV expression vector (CaMKIV_{T313}). In all cases, cells were harvested at the end of the experiment, and the promoter activities were determined by luciferase assay. * $P < 0.05$ vs control value.

8 out of the 20 cells. The average calcium concentration of the 20 cells significantly increased from 64 ± 13 to 122 ± 27 nmol/l ($P < 0.05$). Fig. 4E and F show representative positive responses.

CaMKIV mediates the depolarization-induced increase in CRH 5'-promoter activity

We then studied the effect of high potassium-induced cellular depolarization on CRH gene expression. As shown in Fig. 5, treatment of the cells with high (60 mM) potassium solution increased the CRH 5'-promoter activity. To see the involvement of CaMKs in this response, we tested the effects of two different CaMK inhibitors (Yoshida *et al.* 2000). K252a, an inhibitor of both CaMKII and IV, completely abolished the positive effect of potassium-induced depolarization on CRH gene expression (Fig. 5B). In contrast, KN93, a specific inhibitor of CaMKII, showed no effect (Fig. 5A). These results show that neuronal excitation indeed increases the transcriptional activity of the CRH gene, and that endogenous CaMKs, probably CaMKIV, mediate the effect.

CREB mediates the CaMKIV-induced as well as PKA-induced activation of CRH 5'-promoter activity

Finally, we examined the transcriptional factor(s) which mediates the positive effect of CaMKIV activation. It has been shown that PKA promotes the transcriptional activity of CRH through phosphorylation of CREB. To determine whether this is also the case with CaMKIV, we used KCREB, the dominant negative form of CREB (Walton *et al.* 1992). KCREB significantly inhibited the PKA-stimulated transcription of CRH, as expected (Fig. 6A). Interestingly, KCREB similarly interfered with CaMKIV-stimulated CRH gene expression (Fig. 6B). The data strongly suggest that the transcription factor CREB mediates both PKA- and CaMKIV-mediated transcriptional activation of the CRH gene 5'-promoter.

Discussion

In this study, we examined the transcriptional regulation of the CRH gene, using the homologous

Figure 4 (A–C) Effect of high extracellular K^+ on $[Ca^{2+}]_i$. 'High K^+ ' indicates application of extracellular solution containing 60 mmol/l K^+ . (D) Effect of high extracellular K^+ on $[Ca^{2+}]_i$ under the treatments with nitrendipine (5 μ M) and ω -conotoxin GV1A (1 μ M). (E and F) Effect of 8Br-cAMP on $[Ca^{2+}]_i$. '8Br-cAMP' indicates application of 8Br-cAMP (100 μ M).

BE(2)C neuronal cell line, which expresses endogenous CRH. The data obtained indicate the involvement of the calcium/CaMKIV and the cAMP/PKA systems, both signaling pathways being converged into the activation of CRE/CREB of the CRH 5'-promoter. Since the CRH gene in the HPA axis is expressed in the hypothalamic neuronal cells, it is likely that excitation of the CRH neuron with the increase in intracellular calcium not only stimulates CRH release but also induces its gene expression through the calcium/calmodulin/CaMKIV pathway.

Regarding the molecular mechanism of CRH gene expression, most of the previous *in vitro* studies

were carried out with placental or heterologous neuronal cell lines (Seasholtz *et al.* 1988, Spengler *et al.* 1992, Guardiola-Diaz *et al.* 1994, Scatena & Adler 1998, Majzoub & Karalis 1999), because neuronal cell lines which expressed CRH were unavailable. Furthermore, these studies stressed the importance of the cAMP/PKA/CREB signaling pathway, because the canonical CRE (TGACGTCA) is located in its 5'-promoter region (Seasholtz *et al.* 1988, Vamvakopoulos *et al.* 1990, Spengler *et al.* 1992). Indeed, phosphorylated CREB is shown to bind the CRE, which elicits the transcription of CRH gene (Spengler *et al.* 1992, Wolf *et al.* 1999). In this study,

Figure 5 Effects of CaMKs inhibition on the CRH gene expression in BE(2)C cells. BE(2)C cells transfected with CRH₉₀₇-Luc construct were pretreated for 1 h with vehicle, or either KN93 (20 μM) (A) or K252a (1 μM) (B), and then treated with high potassium (60 mM). Cells were harvested at the end of the experiment, and the promoter activities were determined by luciferase assay. Open bars represent control groups; closed bars represent potassium-treated groups. Each value is shown as a percentage of the control value. * $P < 0.05$ vs control value.

treatment of the transfected cells with forskolin and the cAMP analog 8Br-cAMP, and coexpression of PKA catalytic subunit, all potently stimulated the 5'-promoter activity of the CRH gene, confirming the positive role of the signaling pathway.

Neuroendocrine cells, including CRH neurons, however, are excitable cells and release neuropeptide when cells are depolarized. Excitation of neuronal cells is usually accompanied by a rise in intracellular calcium, which is known to elicit the expression of various genes (Bading *et al.* 1995, Finkbeiner & Greenberg 1998), including CRH (Guardiola-Diaz *et al.* 1994, Ma *et al.* 1997). Therefore, it is tempting to speculate that a depolarization-induced rise in intracellular calcium is another positive regulator of CRH gene expression as well as secretion (Tsagarakis *et al.* 1991, Guardiola-Diaz *et al.* 1994). When treated with high extracellular potassium, BE(2)C cells depolarized at above the threshold of the excitation of the voltage-gated calcium channels, which increased the intracellular calcium concentration by increasing the calcium influx through the channels, as previously reported (Yamashita & Hagiwara 1990, Takano *et al.* 1996). In this

condition, a significant increase in the transcriptional activation of the CRH gene was observed, supporting the previous work by Seasholtz (Guardiola-Diaz *et al.* 1994). Furthermore, CaMKIV, one of the major signaling molecules following the rise in intracellular calcium in neuronal cells (Anderson & Kane 1998), was intrinsically expressed in BE(2)C cells, and the excitation-induced CRH gene expression was completely eliminated by treatment with K252a, an inhibitor of CaMKII/IV (Yoshida *et al.* 2000), but not with KN62, a selective inhibitor of CaMKII. Thus, our data strongly suggest that CaMKIV is involved in the depolarization-induced CRH gene expression. Interestingly, the effect of potassium-induced depolarization on the CRH gene promoter was weaker than those of forskolin or 8Br-cAMP, probably due to the decreased excitability of this cell line (at most, 200 nmol/l intracellular calcium). Furthermore, forskolin/8Br-cAMP is supposed to activate both calcium-independent (direct activation of CREB through PKA) and -dependent (through CaMKIV) pathways, possibly causing a more potent effect than potassium-induced depolarization alone.

Figure 6 Effects of CREB inhibition on the CRH gene expression in BE(2)C cells. BE(2)C cells were cotransfected transiently with CRH₉₀₇-Luc construct, PKAcet expression vector (PKA), and either CREB or KCREB (dominant negative form of CREB) expression vector (A). Alternatively, cells were cotransfected transiently with CRH₉₀₇-Luc construct, the active form of CaMKIV (CaMKIV_{T313}), and either CREB or KCREB expression vector (B). Cells were harvested at the end of the experiment, and the promoter activities were determined by luciferase assay. Each value is shown as a percentage of the value in CREB group. * $P < 0.05$ vs CREB group.

CaMKs are multifunctional serine/threonine kinases, among which CaMKIV is mainly expressed in the brain and some other limited tissues. It is known to be localized predominantly in the nucleus, and to become activated rapidly after the elevation of intracellular calcium (Sun *et al.* 1994). To confirm the role of CaMKIV in the regulation of CRH gene transcription, we coexpressed CaMK expression vectors, the results showing a potent stimulatory effect of CaMKIV, but not CaMKII. The lack of a CaMKII effect may be due to its cytosol-dominant location and/or to the phosphorylation of CREB at Ser142, which is known to suppress transcriptional activity. CaMKIV has a self-inhibitory domain in its molecule, and removal of the domain renders it constitutively active (Sun *et al.* 1994, Chatila *et al.* 1996, Anderson & Kane 1998). Our data show that the active form (CaMKIV₃₁₃) exerts the most potent stimulatory effect on CRH 5'-promoter activity, further supporting strongly the role of the kinase in the regulation of the CRH gene. This work is also in agreement with a previous *in vivo* work suggesting the involvement of CaMKs in the regulation of CRH in the hypothalamus, although the specific

involvement of CaMKIV has not been identified in the study (Tsagarakis *et al.* 1991).

The mechanism(s) whereby activation of CaMKs induces the expression of target genes in neuronal cells is not completely understood. In this study, we obtained results showing that the positive effect of the active form of CaMKIV was antagonized by KCREB, a dominant negative inhibitor of CREB, in a similar manner to the PKA-stimulated condition (Walton *et al.* 1992). This is not surprising, because the effect of CaMKIV is known to be mediated by CREB; indeed, CaMKIV has been shown to phosphorylate CREB at the Ser133 residue (Matthews *et al.* 1994, Shaywitz & Greenberg 1999, Soderling 1999). Therefore, it is likely that both PKA and CaMKIV share CREB as a common target protein. This notion coincides well with previous experimental data by Seasholtz (Guardiola-Diaz *et al.* 1994) suggesting that depolarization-induced CRH gene expression is also mediated by the CRE of the CRH gene 5'-promoter.

Finally, based on the present and previous works, the possible signaling pathways regulating the expression of the CRH gene in neuronal cells are

Figure 7 Schematic representation of the possible intracellular signaling pathways involved in the neurotransmitter-mediated transcriptional regulation of the CRH gene. The neurotransmitter(s) which stimulates adenylate cyclase via Gs protein increases intracellular cAMP, activates PKA and then activates CRH gene expression through phosphorylation of CREB. Other neurotransmitters which directly open the Ca channel and/or depolarize cells increase intracellular calcium, activate calmodulin and CaMKIV, and then increase CRH gene transcription through phosphorylation of CREB. Thus, in both cases, CREB seems to be the key transcriptional mediator of CRH gene expression in neuronal cells. In addition, immediate early gene-derived transcription factors, such as Nurr1/Nur77, might be involved in the positive regulation of the CRH gene as well.

schematically shown in Fig. 7. Some of the neurotransmitters stimulate CRH synthesis and secretion through G-protein-coupled adenylate cyclase activity, which activates the cAMP/PKA pathway. The activated PKA phosphorylates CREB directly, or indirectly through membrane depolarization with the resultant calcium/CaMK pathway. Alternatively, other neurotransmitters or excitatory amino acids that elicit membrane depolarization without cAMP production also enhance the expression of CRH, by increasing the intracellular calcium and subsequent activation of the calmodulin/CaMKIV pathway. In both cases, CREB is phosphorylated and acts as a final common transcription factor mediating the positive

regulation of the CRH gene. The immediate early genes Nurr1/Nur77 might also be involved in the regulation of the CRH gene (Murphy & Conneely 1997). Because most of the findings including the present data were obtained from *in vitro* experiments using cell lines, further studies using primary hypothalamic neuronal cell culture and/or animal studies will be necessary to confirm the *in vivo* regulation of the CRH gene.

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