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CYCLIC AMP INDUCIBLE EARLY REPRESSOR (ICER) MEDIATES THE TERMINATION OF CORTICOTROPIN RELEASING HORMONE TRANSCRIPTION IN HYPOTHALAMIC NEURONS

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Abstract

Elevations of inducible cAMP early repressor (ICER), the repressor isoform of the cAMP responsive element modulator (CREM), is associated with protein binding to the corticotrophin releasing hormone (CRH) promoter and termination of CRH transcriptional responses to stress. To determine whether endogenous ICER production represses CRH transcription, we examined the effect of CREM siRNA on forskolin-stimulated ICER formation and CRH transcription in the hypothalamic cell line, 4B, and in primary cultures of hypothalamic neurons. Co-transfection of 4B cells with CREM siRNA and a CRH promoter-driven luciferase reporter gene markedly reduced the induction of ICER by forskolin, and potentiated the stimulatory effect of forskolin on CRH promoter activity, compared with cells co-transfected with a non-specific oligonucleotide. The role of ICER on endogenous CRH expression was studied in primary cultures of hypothalamic neurons by examining the effect of CREM siRNA on forskolin-induced primary transcript (CRH hnRNA), using intronic real-time PCR. As observed during stress in vivo, forskolin-stimulated CRH hnRNA was transient, increasing up to 60 min and declining to near basal values by 3h. Transfection of CREM siRNA reduced forskolin-induced ICER by about 45% 48-h later, and partially reversed the declining phase of CRH hnRNA production at 3 h. The data provides evidence that endogenous ICER formation is required for termination of CRH transcription, and support the hypothesis that ICER is part of an intracellular feedback mechanism limiting the activation of CRH transcription during stress.

Keywords

Corticotropin releasing hormone; transcriptional regulation; Cyclic AMP-inducible early repressor; siRNA; hypothalamic neuronal cultures

INTRODUCTION

The neuropeptide corticotropin releasing hormone (CRH) regulates pituitary ACTH secretion, autonomic and behavioral responses to stress, being critical for maintaining circulating levels of glucocorticoids and homeostasis (Vale et al., 1983; Antoni, 1986). Appropriate regulation of CRH output requires rapid secretory responses as well as transcriptional activation of the gene in order to maintain mRNA levels required for translation of newly formed peptide (Aguilera et al., 2007). A number of pathological conditions, including psychiatric disorders such as depression are associated with alterations of CRH expression in the brain (Aguilera et

al., 2007; Holsboer and Ising, 2008; Bao et al., 2008). Therefore, elucidation of the mechanisms controlling CRH expression is essential to fully understand of the pathogenesis and therapeutic approaches to stress related disease.

While timely activation of CRH transcription is essential for stress adaptation, a prompt termination of the stress response is also critical to prevent deleterious effects of excessive CRH production (Munck and Naray-Fejes-Toth, 1994; Holsboer and Ising, 2008). A major mechanism for limiting the HPA axis stress response is the negative feedback by glucocorticoids. However, experiments in adrenalectomized rats have shown that transcriptional responses to prolonged stress are also transient in the absence of a glucocorticoid surge (Shepard et al., 2005), suggesting the presence of intracellular feedback mechanisms. We have previously demonstrated that the decline of CRH transcription during stress is associated with induction of inducible cAMP early repressor (ICER) in CRH neurons of the PVN (Shepard et al., 2005). ICER is a repressor isoform of the cAMP response element modulator (CREM), generated by cAMP-dependent activation of a promoter located in the intron of the CREM gene (Foulkes et al., 1991; Molina et al., 1993). ICER contains the DNA binding domain but not the transactivation domain of CREM and acts as competitive inhibitor of pCREB-dependent transcription (Foulkes et al., 1991). The CRH promoter contains a functional cyclic AMP response element (CRE) at -229 (Seasholtz et al., 1988), which is required for positive and negative regulation of the CRH promoter (Guardiola-Diaz et al., 1994; Guardiola-Diaz et al., 1996; Nicholson et al., 2004). ICER acts as a competitive inhibitor for phospho-CREB-dependent transcriptional activation (Sassone-Corsi, 1998), and is expressed in CRH neurons (Shepard et al., 2005). In addition, in vivo and in vitro studies have demonstrated binding of ICER to the CRH promoter, which parallels the decline of CRH transcription (Shepard et al., 2005; Liu et al., 2006; Aguilera et al., 2007). The above evidence strongly suggests that the induction of ICER is part of an intracellular feedback mechanism mediating the termination of CRH transcription.

In order to investigate the extent to which ICER is required for termination of CRH transcription, in this study we examined the effect of blocking endogenous ICER production using CREM siRNA in the hypothalamic cell line, 4B, and in primary cultures of hypothalamic neurons, on CRH transcription.

MATERIALS AND METHODS

The CRH promoter-driven luciferase reporter gene (pCRH-Luc) was created by two step cloning, using a 613-bp restriction fragment containing the CRH promoter (-498 to +115 bp relative to the proximal transcription start point), obtained by *XbaI/KpnI* digestion of the CRH gene clone prCRHBglII (provided by Dr. Audrey Seasholtz, Ann Arbor, MI), as previously described (Nikodemova et al., 2003). To knock out endogenous ICER production, siRNA duplexes, targeted against the sequence GATCCGAGCTCCTACTACT of the CREM gene and the control siRNA, silence inactive control #1 siRNA were obtained from Ambion, Austin, Texas.

Cell Cultures and transient transfections

The hypothalamic cell line, 4B, was provided by Dr John Kasckow, Cincinnati, OH. Cells were cultured in DMEM supplemented with 10% horse serum, 10% fetal bovine serum, and 100U penicillin and 100µg streptomycin/ml (culture media) in 150 cm² flasks. Cells were lifted with trypsin and aliquots of about 2 million cells co-transfected with 5µg of pCRH-luc or the empty reporter vector, pGL3 basic (Promega, Madison, WI), and 1.4 nmol of CREM siRNA or control oligonucleotide, by electroporation using an Amaxa Nucleofector (Lonza Walkersville Inc., Walkersville, MD) and the company protocol with solution number V. All cells were co-transfected with 60 ng of renilla luciferase plasmid in order to correct for transfection

efficiency. After transfection, cells were plated in 48-well plates at a density of 75,000 cells per well for luciferase activity assay, or in 60mm² plates at a density of 775,000 cells per well for Western analysis.

Primary cultures of hypothalamic cells were prepared from embryonic day 18 Sprague Dawley rats. Fetal rats were rapidly removed from 18-day pregnant rats after CO₂ sedation and decapitation. Fetuses were decapitated, and hypothalamic tissue dissected and collected in ice-cold buffer (pH 7.4, containing 137mM NaCl, 5mM KCl, 0.7mM Na₂HPO₄, 25mM HEPES buffer, and 100μg/ml gentamicin). Tissues were digested for 1h with collagenase type 2 (1mg/ml) (Worthington, Lakewood, NJ) and cells dispersed mechanically using a 10 ml pipette in the above buffer, supplemented with 1mg/ml glucose, 4mg/ml BSA, and 0.2mg/ml DNase,. Undigested tissue was incubated with collagenase for an additional 30 min before completing cell dispersion. The cell suspension was filtered through a 40μm cell strainer (BD Falcon) centrifuged at 200 × g for 10 minutes. Cells were washed in plating media (D-MEM/F12, 100μg/ml gentamicin, and 10% heat inactivated fetal bovine serum), before plating at a density of 3 × 10⁶ cells per well in 6-well plates coated with poly-L-lysine for RNA preparation, or 6.5 × 10⁶ cells per 10 cm culture dish for Western blot. Media was changed to Neurobasal Media (Gibco/Invitrogen) with B27 supplement (Invitrogen), 0.5mM L-glutamate, and 100μg/ml gentamicin on the second day to support selective neuron growth. From days 5 to 9 (i.e. for 5 days), cells were cultured in the above media containing 5μM cytosine arabinoside (Sigma), a selective inhibitor of DNA synthesis in order to prevent glial proliferation. On day 10, cells were changed into serum-free/B27 supplement-free Neurobasal medium containing 0.1% BSA for 2h before treatment in the conditions described in results and Figure legends.

CREM siRNA or non-specific oligonucleotides were transfected into primary hypothalamic neurons on day 10 of culture, using the GeneSilencer siRNA Transfection Reagent (Genlantis, San Diego, CA). Aliquots of 1.4 nmol siRNA (control or CREM) per 10cm dish, or 500 pmol per 60mm dish, and GeneSilencer reagent were diluted in serum-free medium (Opti-MEM, Invitrogen) according to the manufacturer's protocol, combined and incubated at room temperature for 15 min to allow the siRNA/lipid complex to form. Cells were changed to fresh culture medium (Neurobasal medium supplemented with B27) before addition of the transfection mix.

Luciferase Assay

After transfection, 4B cells were cultured for 18 h before changing medium to serum free DMEM containing 0.1% BSA. After 30 min preincubation, cells were incubated with 10 μM forskolin for an additional 6h, before washing in PBS and lysing in 100μl of passive lysis buffer. Luciferase activity in cell lysates was determined using reagents from Promega (Dual Luciferase Assay System) and a multiplate reader (Fluostar Optima (BMG LabTech, Durham, NC).

Protein extraction and Western analysis

Twenty four hours after transfection, nuclear extracts from 4B cells or primary hypothalamic neurons were prepared using NE-PERTM Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein concentration was quantified by spectrophotometry using the BCA Protein Assay (Pierce, Thermo Fisher Scientific Inc, Rockford, IL). For Western Blot, 50 μg of nuclear extract were loaded and separated in a 4–20% gradient SDS-PAGE. Proteins were transferred to a PDVF membrane (Perkin Elmer Life Sciences, Boston, MA), incubated with 5% blocking agent in 1xTBST (TBS plus 0.05% Tween-20) for one hour and incubated overnight with anti-CREM antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), which recognizes all CREM isoforms including ICER, at a 1:1000 dilution, or with anti-phospho-CERB antibody (Upstate Biotechnology, Lake Placid,

NY), at a 1:1000 dilution. After washing in 1xTBST, membranes were incubated for one hour at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG at a 1:20,000 dilution. Detection of immunoreactive CREM isoform bands was performed by using ECL Plus TM reagents (Amersham Pharmacia Biotech., Piscataway, NJ) followed by exposure to BioMax MR film (Eastman Kodak, Rochester, NY). After film exposure, blots were stripped and assayed for tubulin using a monoclonal antibody (Sigma-Aldrich, Inc., St. Louis, MO) to correct for protein loading in the gel. The intensity of the bands was quantified using a computer image analysis system (Imaging Research, Inc., Ontario, Canada) and the public domain NIH Image program (developed at the National Institutes of Health, and available at the Internet at: <http://rsb.info.nih.gov/nih-image>).

Total RNA isolation and Real Time PCR for CRH hnRNA

After 24h after transfection, primary neurons were harvested of TRIzol Reagent (Invitrogen) transferred to 1.5 ml microfuge tubes, and centrifuged at 11,000 g for 15 min after addition of chloroform. The RNA-containing upper aqueous phase was removed and RNA precipitated with an equal amount of 70% ethanol and transferred to an RNeasy spin column (QIAGEN). RNA was purified using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol using column DNase (QIAGEN) digestion to eliminate genomic contamination. Total RNA concentration was measured by NanoDrop spectrophotometry.

CRH primary transcript levels (CRH hnRNA) were measured by qRT-PCR, using the LUXTM gene expression system (Invitrogen). LUX fluorogenic and unlabelled primer pairs were designed using the D-LUX designer software (Invitrogen) by targeting the intron of the CRH gene. The sequence of FAM-labeled forward primer is CACAGGCGGCGAATAGCTTAAACCTG (FAM) G and the unlabeled reverse primer is CAGGTGACCCCTTCCTTGGAGA. Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used for the amplification mixture with each LUX primer at a final concentration of 200 nM and 2 µl of cDNA for a total reaction volume of 25 µl. PCR reactions were performed on spectrofluorometric thermal cycler (ABI PRISM 7300 real time PCR system) (Applied Biosystems). Samples were amplified by an initial denaturation at 50°C for 2 min, 95°C for 10 min and then cycled (45x) using 95 °C for 15 s, 57 °C for 30 s and 60°C for 45 s. Calibration curves to assess the efficiency of the PCR reaction were performed using a 5.5kb BglII rat genomic fragment containing the full rat CRH gene (−498 to 4,969) cloned into pUC18, kindly provided by Dr Audrey Seasholtz, Ann Arbor, Michigan.

For the experimental samples, 550ng of total RNA per sample were used for reverse transcription into cDNA. RNA was primed with oligo(dT)20, and cDNA was synthesized using SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions with CRH intronic primers were performed in parallel with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers used as control gene. Pre-designed Joe-labeled forward and unlabeled reverse GAPDH primers were obtained from Invitrogen. Relative quantification of CRH hnRNA levels was performed using the comparative threshold cycle method for CRH hnRNA and GAPDH, as described in the users manual (Livak and Schmittgen, 2001). The absence of RNA detection when the RT step was omitted indicated the lack of genomic DNA contamination in the RNA samples.

Statistical Analysis

Statistical significance of the differences between groups was calculated by two-way analysis of variance (ANOVA), followed by Fisher adjusted pairwise comparisons. Statistical significance was set at $p < 0.05$. Data are presented as means $\pm$ standard error of the mean (SEM) from the values in the number of observations indicated in results or legends to Figures.

RESULTS

Effect of ICER blockade on CRH promoter activity in 4B cells

The role of endogenous ICER on the time-dependent activation of the CRH promoter by forskolin was examined in 4B cells 18 h after co-transfection of CRHp-Luc and CREM siRNA. In cells transfected with a non-specific oligonucleotide, western blot analysis of ICER protein in cells incubated with forskolin for 3h revealed the expected induction of ICER from undetectable levels in basal conditions. Induction of ICER II was more marked than that for ICER I. Transfection of CREM siRNA oligonucleotides caused a significant decrease of 50 and 75% in ICER I and II, respectively compared to the levels observed after 3h forskolin stimulation in cells transfected with the non-specific oligonucleotide (Fig 1-A). In contrast, CREM siRNA has no effect on CREB content in the cells compared with the non-specific oligonucleotide (Fig 1-A). The consequence of blocking ICER production on CRH promoter activity is shown in Fig 1B. Co-transfection with ICER siRNA had no effect on basal CRH promoter activity but it significantly enhanced the effect of forskolin. Incubation with forskolin (0.3 μ M) for 6 h caused a 2-fold increase in CRH promoter activity in cells co-transfected with a non-specific oligonucleotide. When endogenous ICER production was blocked by co-transfection with CREM siRNA, forskolin stimulated CRH promoter activity by 3-fold the basal values ($p < 0.05$ vs forskolin-stimulated values in cells transfected with the non-specific oligonucleotide).

Effect of forskolin and CREM siRNA in primary cultures of hypothalamic cells

Consistent with previous observations in vivo and in the hypothalamic cell line 4B, incubation of hypothalamic neuronal cultures with forskolin resulted in a delayed increase in ICER protein, with values similar to basal at 30 min and 1h, and a marked increase by 3h (Figure 2-A). Stimulation of the cells with forskolin induced rapid increases in CRH hnRNA, reaching a maximum by 1 h and declining by 3h (Fig 2-B).

To determine whether the increase in ICER at 3 h mediated the declining phase of CRH transcription, cells were transfected with CREM siRNA for 48h before measuring the effect of forskolin. As shown in Figure 3-A, CREM siRNA caused a 42% decrease in ICER I, $p < 0.05$. ICER II levels showed a clear tendency to decrease but the changes were not statistically significant (Fig 3-A). Levels of CRH hnRNA were at threshold detection levels in basal conditions. Therefore, the effects were compared with forskolin-stimulated levels at 1h in neuronal cultures transfected with the non-specific oligonucleotide. Transfection of CREM siRNA caused a significant increase in CRH hnRNA levels at 3h incubation with forskolin compared with the levels in neuronal cultures transfected with the non-specific oligonucleotide. As shown in Fig 3-B, forskolin stimulated CRH hnRNA levels at 3h had declined by 60% compared with levels at 1h in neurons transfected with non-specific oligonucleotide. On the other hand, in neurons transfected with CREM siRNA, CRH hnRNA levels at 3h were not significantly different from CRH hnRNA levels at 1h forskolin in neurons transfected with the non-specific oligonucleotide. CRH hnRNA levels at 3 h were significantly higher in neurons transfected with CREM siRNA compared with those in neurons transfected with the non-specific oligonucleotide ($p < 0.05$).

DISCUSSION

ICER is an inducible member of the CREM family that functions as a repressor of CREB-dependent transcription (Foulkes et al., 1991). We have previously shown that there is a good correlation between induction of ICER in CRH neurons, ICER recruitment by the CRH promoter and the declining phase of CRH transcription in vivo (Shepard et al., 2005). This finding strongly suggested that ICER mediates the termination of CRH transcriptional

responses, thus explaining the transient nature of CRH transcription (Ma et al., 1997b; Shepard et al., 2005). The present studies, using siRNA to prevent ICER formation, extend these observations and provide evidence that endogenous ICER production during stimulation of CRH neurons indeed contributes to limiting activation of CRH transcription. The initial studies were performed in the hypothalamic cell line 4B, a cell line originally described as expressing endogenous CRH (Kasckow et al., 2003) but the extremely low CRH expression in current passages of the cells (Nikodemova et al., 2003; Liu et al., 2006; Liu et al., 2008), has impaired their use for studying endogenous CRH transcription. However, these cells have proven to be of use for studies on CRH transcription using reporter gene assays. The marked decreases in endogenous ICER (but not in expression of other genes such as CREB, beta-actin and GAPDH) in 4B cells or primary neuronal cultures, demonstrated the effectiveness of the siRNA to specifically silence the CREM gene. The fact that siRNA blockade of ICER production enhanced forskolin-stimulated CRH promoter activity in the hypothalamic cell line 4B, supports the view that ICER represses CRH transcription. This is consistent with previous observations indicating that over-expression of ICER in this cell line markedly inhibits the ability of forskolin to activate the CRH promoter (Liu et al., 2006).

An important question is whether ICER mediates the transient nature of CRH transcriptional responses, as suggested by the time correlation between the elevation in ICER and the decline in CRH transcription during stress (Shepard et al., 2005). However, reporter gene assays were found unsuitable for time dependence studies due to the long half life of firefly luciferase. Attempts at using destabilized luciferase vectors (pGL4-78, Promega, Madison WI) for these studies were also unsuccessful because the backbone of this vector showed promoter independent responses to forskolin and other stimulants (Liu and Aguilera, unpublished). In recent studies we have shown that primary cultures of fetal rat hypothalamic neurons provide an excellent system to measure endogenous CRH transcription by evaluating changes in primary transcript levels (hnRNA) using intronic qRT-PCR (Liu et al., 2008). Measurement of changes in CRH hnRNA by *in situ* hybridization has been extensively used as an index of changes in transcription (Herman et al., 1992; Ma et al., 1997a; Ma et al., 2001; Watts and Sanchez-Watts, 2002; Shepard et al., 2005). Consistent with previous *in vivo* and *in vitro* observations (Ma et al., 1997b; Shepard et al., 2005), the increases in CRH hnRNA levels during exposure to forskolin in hypothalamic neuronal cultures were transient. Thus, this system was found to be suitable to study the effect of suppressing ICER expression on the declining phase of CRH transcription. The present demonstration that blockade of endogenous ICER attenuates the decline of CRH transcription at 3h of incubation with forskolin (in the primary hypothalamic cultures), provides strong evidence that ICER indeed contributes to the late inhibition of transcription. The fact that CREM siRNA only partially prevented the decline in CRH transcription at 3h was probably due to the much lower efficiency of the CREM siRNA to suppress endogenous ICER production in the primary neuronal cultures as compared with the effect in 4B cells. While it is clear from the present experiments that ICER mediates repression of CRH transcription, it is not possible to rule out the participation of additional mechanisms in determining the duration and magnitude of the transcriptional response.

In conclusion, this study demonstrates that preventing ICER formation during stimulation of CRH transcription enhances the stimulatory action of forskolin on CRH promoter activity in a reporter gene assay, and attenuates the declining phase of endogenous CRH transcription in primary cultures of hypothalamic cells. The data provides evidence that endogenous ICER formation is required for termination of CRH transcription, and support the hypothesis that ICER is part of an intracellular feedback mechanism limiting the activation of CRH transcription during stress.

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Fig 1.

Effect of siRNA blockade of endogenous ICER on ICER protein levels (A) or CRH promoter activity (B). In A, 4B cells co-transfected with CRHp-luc and CREM siRNA were incubated for 3h with forskolin (Fsk) before preparation of protein extracts for western blot. Bars are the mean and SE of ICER and CREB levels, expressed as percent of forskolin-stimulated values in cells transfected with the non-specific oligonucleotide (mean and SE represented as the dotted lines and rectangles). In B, co-transfected cells were incubated for 6 h with or without forskolin before measurement of luciferase activity. Bars are the mean and SE of the fold-change from basal in cells transfected with the non-specific oligonucleotide. *, $p < 0.001$ vs non-specific oligonucleotide; &, $p < 0.01$ vs basal; &&, $p < 0.001$ vs basal; #, $p < 0.01$ vs forskolin, nonspecific oligonucleotide.

Fig 2.

Time course of the effects of forskolin on ICER I and II production (A) and CRH transcription (B). Cells were incubated for the time periods indicated before processing for western blot analysis or CRH hnRNA determination by intronic real time PCR (B). Data points are the mean and SE of the values in 3 experiments. *, $p < 0.001$ vs time 0; #, $p < 0.001$ vs 1h.

Fig 3.

Effect of siRNA blockade of endogenous ICER on ICER protein levels (A) or endogenous CRH transcription (B) in primary cultures of hypothalamic neurons. In A, cells were incubated for 3h with forskolin (Fsk) before preparation of protein extracts for western blot. Bars are the mean and SE of ICER and CREB levels, expressed as percent of forskolin-stimulated values in cells transfected with the non-specific oligonucleotide. *, $p < 0.05$.

In B, co-transfected cells were incubated for 6 h with or without forskolin before measurement of luciferase activity. Bars are the mean and SE of the fold-change from basal in cells transfected with the non-specific oligonucleotide.