Corticotropin-releasing hormone stimulates SGK-1 kinase expression in cultured hippocampal neurons via CRH-R1

Hui Sheng, Tingting Sun, Binhai Cong, Ping He, Yanmin Zhang, Jin Yan, Changlin Lu, and Xin Ni

Department of Physiology, Neurobiology, and Psychology, Second Military Medical University; and Key Laboratory of Molecular Neurobiology, Ministry of Education, Shanghai, China

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Corticotropin-releasing hormone (CRH), a 41-amino acid polypeptide originally isolated from ovine hypothalamus, is the primary factor driving stress-induced adrenocorticotropin secretion from the anterior pituitary and capable of integrating the neuroendocrine, behavioral, autonomic, and immune response to stress (35, 47). The anatomic distribution of CRH and its receptors in brain suggests that this peptide also may modulate the neuronal function of hippocampus (2, 13, 39, 44). Previous studies have shown that CRH produces a long-lasting enhancement of synaptic efficacy in the rat hippocampus in vivo (50, 51) and facilitates the induction and stability of long-term potentiation in hippocampus (5). Injection of CRH into hippocampus enhances classical fear conditioning in mice (40) and improves the retention of one-way inhibitory avoidance learning in rats (27). In addition, it has also been demonstrated that CRH has neuroprotective activity against amyloid β-peptide- or glutamate-induced cell death (3, 14, 37). However, the molecular basis responsible for CRH actions in hippocampus is not fully understood.

Serum/glucocorticoid-inducible kinase-1 (SGK-1), a novel protein kinase, was originally identified as transcriptionally induced by serum and glucocorticoids (52). SGK-1 is a member of the PKA, PKG, and PKC (“AGC”) family of serine/threonine protein kinases. The catalytic domain of SGK-1 is 54% homologous to the catalytic domain of PKB (22, 52). It has been demonstrated that SGK-1 is expressed in all tissues that have been studied, including the pancreas, liver, heart, lung, muscle, intestine, ovary, and brain (25, 49). SGK-1 is involved in regulation of ion channel activity and abundance in the lung, kidney, and heart (20, 48, 53) as well as cell survival and proliferative responses (8, 42). Some studies have shown that SGK-1 is involved in the control of synaptic efficacy and plasticity (12, 30) and facilitates memory consolidation of spatial learning in rats (46).

It is known that most members of AGC family members are regulated predominantly at the enzymatic level by posttranslational phosphorylation and dephosphorylation (18, 19). However, SGK-1 is transcriptionally induced by a diverse set of stimuli including hormones, heat shock, ultraviolet radiation, and oxidative stress (1, 2). Moreover, it has been shown that SGK-1 protein is ubiquitinated (7), which would allow quick removal of SGK-1 gene products upon termination of the stimulus. Thus, the mechanisms by which SGK-1 expression is regulated have become of interest. In addition to glucocorticoids, various endogenous factors such as gonadotropins, insulin, and transforming growth factor are shown to induce SGK-1 transcript expression (1, 22, 34, 38). Signaling molecules involved in the transcriptional regulation of SGK-1 include cyclic AMP (cAMP), PKC, and MAPK (26, 31, 36, 38).

SGK-1 has been found to be expressed in many brain areas, including hippocampus and cortex, and induced by various stress stimuli (23, 32, 34). However, the bioactive factors that mediate SGK-1 expression in the nervous system are not fully characterized. In hippocampus, CRH receptors (CRH-Rs) are expressed in neurons (2, 39, 43) and are able to activate more than one signaling cascade, including the PLC/PKC and adenylate cyclase (AC)/PKA pathways (6, 43). Therefore, we hypothesized that CRH could regulate SGK-1 expression to exert its profound action on hippocampal neurons. In the present study, we determined the effects of CRH on SGK mRNA and protein expression in cultured hippocampal neurons and then investigated whether these effects could

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have occurred through an AC/PKA- or PLC/PKC-dependent pathway.

MATERIALS AND METHODS

Preparation of hippocampal neuron cultures. All animal procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University. Primary hippocampal neurons were cultured according to modified Nelson’s method, as described previously (33, 43). Briefly, the hippocampi were dissected from neonatal (P1) Sprague-Dawley rats in ice-cold dissection solution containing sucrose-glucose-HEPES (136 mM NaCl, 5.4 mM KCl, 0.2 mM Na2HPO4, 2 mM KH2PO4, 16.7 mM glucose, 20.8 mM sucrose, 0.0012% phenol red, and 10 mM HEPES, pH 7.4) and then incubated with 0.125% trypsin at 37°C for 20–25 min. Single-cell suspension was obtained by mechanical dissociation using a Pasteur pipette with a fire-narrowed tip in DMEM containing 10% heat-inactivated fetal calf serum (FCS) and 10% horse serum.

Cells were then plated at a density of 1 × 10⁵ cells/cm² on poly-l-lysine-coated culture plates or glass coverslips and maintained in 5% CO2 at 37°C in DMEM containing 10% FCS and 10% horse serum overnight. The culture medium was then changed to serum-free B27/neurobasal medium (Life Technologies, Grand Island, NY). One-half of the medium was replaced with fresh medium every 3 days. B27/neurobasal medium (Life Technologies, Grand Island, NY). One-half of the medium was replaced with fresh medium every 3 days. B27/neurobasal medium (Life Technologies, Grand Island, NY). One-half of the medium was replaced with fresh medium every 3 days.

On the 8th day after plating, cells were changed to fresh medium and contained one of following treatments: human/rat CRH (0 –100 nmol/l; Sigma-Aldrich), antalarmin (0 –100 nmol/l; Sigma-Aldrich), astressin 2B (0 –100 nmol/l; Sigma-Aldrich), SQ22536 (10 μmol/l; Sigma-Aldrich), H89 (10 μmol/l; Calbiochem, La Jolla, CA), forskolin (1 μmol/l; Sigma-Aldrich), U73122 (1 μmol/l; Sigma-Aldrich), Go6976 (0.1 μmol/l; Calbiochem), and phorbol 12-myristate 13-acetate (0–1 μmol/l; Calbiochem). Antalarmin, H89, forskolin, U73122, Go6976, and phorbol 12-myristate 13-acetate (PMA) were first dissolved in dimethyl sulfoxide (DMSO) and then diluted by DME to achieve the final concentration of DMSO less than 0.01%. Control cultures were maintained without additives or contained the same final solvent concentration, and each treatment was performed in triplicate for each preparation of cells. After 1–24 h, the cells were collected for total RNA and protein extraction or fixed in 4% paraformaldehyde for 1 h.

Immunofluorescence analysis. Fixed cells were washed with PBS and incubated with 10% BSA for 1 h. To ensure achieving specific staining of SGK-1, two antibodies from different sources were used: mouse monoantibodies against SGK-1 (sc-28338, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antibodies against SGK-1 (Cell Signaling Technology, Danvers, MA). Both SGK-1 antibodies were raised against a short peptide mapping near the COOH terminus of SGK-1. The cells were incubated with one of two antibodies at a dilution of 1:500 overnight at 4°C. For double staining, some slices were incubated with glial fibrillary acidic protein (GFAP) antibody (Santa Cruz Biotechnology) at a dilution of 1:500. All dilutions were made in 1% BSA in PBS. Subsequently, the specimens were washed with PBS three times and then incubated with fluorescein isothiocyanate/rhodamine-conjugated anti-mouse IgG (1:100) or fluorescein isothiocyanate/rhodamine-conjugated anti-rabbit at 37°C for 1 h in the dark. Thoroughly rinsed with PBS, the cell nuclei were visualized by applying the DNA-specific dye hoechst at a final concentration of 5 μg/ml. For negative controls, these two primary antibodies were either substituted with a normal IgG in same dilution or preabsorbed with blocking peptide (supplied with antibodies by Santa Cruz Biotechnology). Peptide-antibody ratio was 5:1 (wt/wt). CRH- and dexamethasone-treated cell slides were used for negative control slides.

Results were viewed under fluorescent microscope with the use of appropriate filters.

To determine the frequency histograms of SGK-1 neurons in cultured hippocampal neurons, a threshold of average cytoplasmic density level of immunoreactive product was set, determined using an image of negative control neurons (normal IgG control). The optical density threshold was then applied to all other CRH-treated neurons. The areas of all measured cells were obtained at the same time, and for each cell the optical density of immunoreactive product and the cell areas were plotted. At least 500 cells from the CRH-treated neurons or vehicle were measured for each slide.

Western blotting analysis. Western blot was conducted as described previously (43). Briefly, cells were scraped off the plate in the presence of lysis buffer containing the following chemicals: 60 mM Tris·HCl, 2% sodium dodecyl sulfate (SDS), 10% sucrose, 2 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany), 1 mM sodium orthovanadate (Sigma-Aldrich), and 10 μg/ml aprotinin (Bayer, Leverkusen, Germany). The lysates were then quickly sonicated and centrifuged at 4°C. The supernatant was harvested and diluted in sample buffer (250 mM Tris·HCl, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.002% bromophenol blue). Aliquots of proteins were separated by SDS-PAGE (10%) and subsequently transferred to nitrocellulose membranes by electoblotting. The membrane was blocked in 0.1% Tris-buffered saline-Tween-20 (TBST) containing 5% skim milk powder for 2 h and then incubated SGK-1 antibodies (Santa Cruz Biotechnology) at a dilution of 1:1,000 or antibodies against phosphorylated PLC-β3 (Cell Signaling Technology) at a dilution of 1:1,000 overnight at 4°C. After three washes with TBST, the membrane was incubated with the secondary antibodies of horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immuno-reactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Santa Cruz Biotechnology). The light-emitting bands were detected with X-ray film.

Total RNA extraction, RT-PCR, and quantitative real-time RT-PCR. Total RNA was prepared using Trizol reagent (Life Technologies). Two micrograms RNA was reverse transcribed with oligo(dT)₁₂₋₁₈ primer using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and then quickly sonicated and centrifuged at 4°C. The primers for CRH-R1 were sense 5'-CCC TGC CCT ATC ATT GTC G-3' and antisense 5'-CCG AATGAGGGAATGGTGACG3' (accession no. NM_019232). The primers for CRH-R2 were sense 5'-CCC TGC CCT ATC ATT GTC G-3' and antisense 5'-CCG AATGAGGGAATGGTGACG3' (accession no. NM_019232). The primers for CRH-R1 were sense 5'-CCC TGC CCT ATC ATT GTC G-3' and antisense 5'-CCG AATGAGGGAATGGTGACG3' (accession no. NM_019232). The primers for CRH-R2 were sense 5'-CCC TGC CCT ATC ATT GTC G-3' and antisense 5'-CCG AATGAGGGAATGGTGACG3' (accession no. NM_019232). The primers for CRH-R1 were sense 5'-CCC TGC CCT ATC ATT GTC G-3' and antisense 5'-CCG AATGAGGGAATGGTGACG3' (accession no. NM_019232).

Quantitative real-time PCR was carried out using Rotor-Gene 3000 (Corbett Research). The reaction solution consisted of 2.0 μl of diluted cDNA product, 0.1 μM of each paired primer, 100 nM deoxynucleotide triphosphates, 1 U Taq DNA polymerase (Promega), and 1X PCR buffer. SYBR Green (BMA, Rockland, ME) was used as detection dye. Quantitative real-time PCR conditions were optimized according to preliminary experiments to achieve linear relationship between initial RNA concentration and PCR product. The temperature range to detect the melting temperature of the PCR product was set from 60 to 95°C. Amplification of two housekeeping genes, β-actin and GAPDH, was measured for each sample as an internal PCR control for sample loading and normalization. The specificity of the primers was verified by using the melting curve as well as subsequent sequencing of the quantitative real-time PCR products. The size of CRH-R1 or CRH-R2 PCR product was 267 and 230 bp, respectively. The size of amplicon for SGK-1 was 190 bp. To determine the relative quantitation of gene expression for both target and housekeeping genes, the comparative Cₜ (threshold cycle) method with arithmetic formulae was used (29). Subtracting the Cₜ of the target gene yields the ΔCₜ in each group (control and experimental groups), which was entered into the equation 2^-ΔΔCₜ and calculated for the exponential amplification of
PCR. For each experiment, the amount of SGK-1 mRNA under various treatment conditions is expressed relative to the amount of transcript present in the untreated control. Because very similar data were obtained by using either β-actin or GAPDH as an internal control, β-actin was used for calculation of ΔCt in presentation of results.

RIA of cAMP. After 8 days of plating, neurons were treated with increasing concentrations of CRH for 3, 5, and 30 min and then terminated by the addition of 0.1 ml of 0.3 mol/l HCl. Cells were frozen overnight, followed by heating of the tubes in boiling water for 5 min. The supernatants were collected by centrifuge and stored at −20°C for later assay for cAMP.

cAMP was assayed using commercially available RIA kits (Shanghai Institute of Biological Product, Shanghai, China). The sensitivity was 0.1 pmol/l. The mean intra- and interassay coefficients of variation were 4.24 and 5.57%, respectively (manufacturer’s data).

Statistical analyses. The values are expressed as means ± SE. The data were analyzed by Student’s t-test, paired t-test, and one-way ANOVA followed by least significant difference t-test. Significance was set at P < 0.05.

RESULTS

Expression of SGK-1 in cultured hippocampal neurons. As shown in Fig. 1A, immunofluorescence analysis revealed that immunoreactive staining of SGK-1 was localized mainly in the somata and nucleus of neurons. In some cells, the processes were positively labeled by SGK-1.

Our previous study showed that >95% of cells prepared by modified Nelson’s method are neurons (43). To test whether astrocytes express SGK-1 in the absence or presence of dexamethasone, cells were double-stained with antibodies of SGK-1 and GFAP, the marker for astrocytes. As shown in Fig. 1, SGK-1-positive cells were not stained by GFAP but by MAP-2, the marker of neuron.

CRH stimulates SGK-1 expression in hippocampal neurons. As shown in Fig. 2A, CRH significantly increased SGK-1 mRNA levels in a dose-dependent manner over the concentration range of 1 pmol/l to 10 nmol/l. Maximal effect was obtained at a concentration of 10 nmol/l, which caused an ~2.5-fold increase in SGK-1 mRNA level after a 24-h incubation period. CRH (10 nmol/l) treatment also resulted in a time-dependent increase in SGK-1 mRNA expression, with a significant increase by 30 min and the maximal increase by 3 h (Fig. 2B).

Fig. 1. Immunofluorescence analysis of serum/glucocorticoid-inducible kinase-1 (SGK-1) expression in cultured hippocampal neurons. A: immunostaining with antibody against SGK-1. B: cells were stained with the primary antibodies of SGK-1 that were preabsorbed with blocking peptide. C: negative control: primary antibodies were substituted with normal mouse IgG. Cells were treated with dexamethasone (1 μmol/l) for 24 h and were then immunostained for SGK-1 (D) and microtubule-associated protein-2 (MAP-2; E) or SGK-1 (G) and glial fibrillary acidic protein (GFAP; H). F: SGK-1 and MAP-2 overlite. I: SGK-1 and GFAP overlite. Original magnifications, ×200 (A–C) or ×100 (D–I).

Fig. 2. Concentration and time-dependent effect of corticotropin-releasing hormone (CRH) on SGK-1 transcript levels in cultured hippocampal neurons. A: hippocampal neurons were incubated with indicated concentrations of CRH for 24 h. B: cells were incubated for indicated time with CRH (10 nmol/l). SGK-1 mRNA levels were determined by real-time quantitative RT-PCR. Data are normalized to the control and presented as means ± SE (n = 5). *P < 0.05, **P < 0.01 vs. control.
To elucidate whether CRH subsequently influences SGK-1 protein expression, Western blot and immunofluorescence analysis were applied to real protein intensity and the frequency of SGK-1-positive neurons, respectively. Western blot analysis showed that CRH (1 pmol/l to 10 nmol/l) enhanced SGK-1 protein level in a dose-dependent manner over a 24-h treatment period (Fig. 3A). The frequency of SGK-1-positive neurons was significantly increased after 3 h of treatment, and the maximal effect was obtained by a 24-h treatment period (Fig. 3B). The frequency of SGK-1-positive cells was 22 ± 1.7% in absence of CRH. Cells were treated with increasing concentrations of CRH (1 pmol/l to 10 nmol/l) for 24 h, and the frequency of SGK-1-positive cells was significantly increased to 55 ± 3.3 and 66 ± 2.9% at doses of 1 pmol/l and 10 nmol/l, respectively (Fig. 3, C and D). To test whether astrocytes express SGK-1 in response to CRH, cells were double-stained with antibodies of SGK-1 and GFAP. It was found that the SGK-1-positive neurons were not stained by GFAP (data not shown).

Fig. 3. Effects of CRH on SGK-1 protein levels in cultured hippocampal neurons. A: neurons were treated with increasing concentrations of CRH for 24 h. The protein of cells was extracted for Western blot analysis. Representative protein bands are presented at top of histogram. Data of SGK-1 protein levels are normalized to the control and presented as means ± SE (n = 3). **P < 0.01 vs. control. B: neurons were incubated with 10 nmol/l CRH for indicated time. Cells were then fixed in 4% paraformaldehyde for immunofluorescence analysis. Cumulative data showing the rates of SGK-1-positive neurons (n = 5). Data are presented as means ± SE. **P < 0.01 vs. control. C: neurons were incubated with 1 pmol/l (b), 100 pmol/l (c), 10 nmol/l (d), and vehicle (a) for 24 h. Then, cells were fixed in 4% paraformaldehyde for immunofluorescence analysis. Original magnifications, ×100. D: cumulative data showing the rates of SGK-1-positive neurons in absence and presence of CRH (n = 4). Data are presented as means ± SE. **P < 0.01 vs. control.

Fig. 4. CRH receptor (CRH-R1) mediated CRH-induced SGK-1 expression. A: real-time quantitative RT-PCR analysis CRH-R1 and CRH-R2 mRNA levels in hippocampal neurons. Data are presented as means ± SE (n = 7), **P < 0.01 vs. CRH-R1. B: real-time quantitative RT-PCR analysis of SGK-1 mRNA levels. Cells were treated with CRH (10 nmol/l), antalarmin (Anta; 100 nmol/l), CRH (10 nmol/l) in combination with antalarmin (CRH + Anta; 100 nmol/l), astressin 2b (Astr; 100 nmol/l), or CRH (10 nmol/l) in combination with astressin 2b (CRH + Astr; 100 nmol/l) for 24 h (n = 5). Data are normalized to the control and presented as means ± SE. Ctl, control. **P < 0.01 vs. control. C: Western blot analysis of SGK-1 protein levels in neurons that were treated with CRH (1 pmol/l), CRH (1 pmol/l) + Anta (100 pmol/l), and CRH (1 pmol/l) + Astr (100 pmol/l) (n = 3). Representative protein bands are presented at top of histogram. Data are normalized to the control and presented as means ± SE. **P < 0.01 vs. control. D: immunofluorescence analysis of SGK-1-positive neurons. Cells were treated with CRH (10 nmol/l), Anta (100 nmol/l), CRH (10 nmol/l) + Anta (100 nmol/l), Astr (100 nmol/l), or CRH (10 nmol/l) + Astr (100 pmol/l) for 24 h. Data are presented as means ± SE (n = 5). **P < 0.01 vs. control. E: SGK-1 mRNA level in cultured neurons after a 24-h treatment with indicated concentrations of urocortin II (Uro II). Data are normalized to the control and presented as means ± SE (n = 4). F: cumulative data showing the rates of SGK-1-positive neurons after a 24-h treatment with indicated concentrations of Uro II. Data are presented as means ± SE (n = 4).
CRH receptor type 1 but not type 2 mediates CRH-induced SGK-1 expression. Our previous study had shown that cultured hippocampal neurons express both CRH-R1 and CRH-R2 (43). Quantitative RT-PCR analysis showed that the mRNA level of CRH-R1 was higher than that of CRH-R2 in cultured hippocampal neurons (Fig. 4A). To determine the subtype of CRH receptor-mediated CRH effect, the specific antagonist of CRH-R1 or CRH-R2 was used. As shown in Fig. 4, the CRH-R1 antagonist antalarmin (0.1 μmol/l) did not affect SGK-1 mRNA expression but completely blocked the CRH (10 nmol/l)-induced SGK-1 mRNA expression. Antalamin (0.1 μmol/l) blocked CRH (10 nmol/l)-induced increase in frequency of SGK-1-positive neurons. Cells treated with CRH-R2 antagonist astressin 2B (0.1 μmol/l) and CRH (10 nmol/l) had SGK-1 mRNA levels that were not significantly different from those of cells treated with CRH (10 nmol/l) alone. There was no significant difference in SGK-1 protein levels between the cells treated with CRH (1 pmol/l) alone and the cells treated with astressin 2B (100 pmol/l) and CRH (1 pmol/l).

To further clarify whether CRH-R2 is involved in the CRH-induced SGK-1 expression, we examined the effect of the CRH-R2 exclusive agonist urocortin II on SGK-1 expression. Application of urocortin II (0.1–10 nmol/l) did not affect SGK-1 expression at mRNA and protein levels (Fig. 4A, E and F).

CRH-R1 is involved in CRH stimulation of cAMP production and phosphorylated PLC-β3 expression. It is known that CRH receptors could couple to multiple G proteins and induce at least activation of AC/PKA and PLC/PKC signaling pathways. Our previous study demonstrated that CRH dose-dependently stimulated cAMP production and expression of phosphorylated PLC-β3 in cultured hippocampal neurons over a concentration range of 1 pmol/l to 10 nmol/l (43). In the present study, we determined whether CRH-R1 was involved in CRH-induced cAMP production and phosphorylated PLC-β3 expression. As shown Fig. 5A, enhancement of cAMP production induced by CRH (10 nmol/l) could be blocked by antalamin (0.1 μmol/l). CRH (10 nmol/l)-induced expression of phosphorylated PLC-β3 was also reversed by antalamin (0.1 μmol/l) (Fig. 5B).

The role of AC/PKA signaling pathway in CRH-induced SGK-1 expression. We further observed the effects of blocking AC and PKA on CRH-induced SGK-1 expression. SQ22356 (10 μmol/l) itself did not affect SGK-1 mRNA and protein expression, but it was able to block CRH-induced increase in SGK-1 mRNA and protein expression. Treatment of cells with H89 (10 μmol/l) exhibited a similar effect as SQ22356, completely blocking CRH-induced SGK-1 expression at mRNA and protein levels (Fig. 6).

To confirm the role of the AC/PKA signaling pathway in the regulation of SGK-1 expression, we observed the effect of AC activator forskolin on SGK-1 expression. The results showed that forskolin (0.1 μmol/l) significantly increased SGK-1 mRNA and protein expression (Fig. 6).

The effect of PLC and PKC inhibitors on CRH-induced SGK-1 expression. Because CRH significantly induced phosphorylated PLC-β3 expression in cultured hippocampal neurons, we tested the effect of PLC inhibitor U73122 on CRH-induced SGK-1 expression. Treatment of cells with U73122 (1 μmol/l) alone significantly increased SGK-1 mRNA and protein expression (Fig. 7). Cells that were treated with U73122 (1 μmol/l) and CRH (10 nmol/l) had SGK-1 mRNA and protein levels that were not significantly different from those of cells treated with CRH (10 nmol/l) alone (Fig. 7).

PKC is a major downstream signaling molecule that can be activated by the PLC signaling pathway. Thus, we tested whether PKC signaling pathway is involved in CRH regulation of SGK-1 expression. Blocking PKC activity with Go6976 (0.1 μmol/l) did not block CRH-induced SGK-1 expression at mRNA and protein levels (Fig. 7). Moreover, treatment of neurons with Go6976 alone could significantly increase the frequency of SGK-1-positive neurons as well as protein intensity and mRNA level of SGK-1 (Fig. 7).

In addition, we also observed the effect of PKC activator PMA (0.01–1 μmol/l) on SGK-1 mRNA and protein expression. PMA treatment did not result in significant changes in mRNA level, protein intensity of SGK-1, or the frequency of SGK-1-positive neurons (data not shown).

DISCUSSION

In this study, we have demonstrated that CRH dose-dependently stimulated SGK-1 mRNA and protein expression in cultured hippocampal neurons. The stimulatory effect of CRH on SGK-1 expression was completely reversed by CRH-R1 antagonist. However, CRH-R2 antagonist did not block CRH-induced SGK-1 expression. Urocortin II, the exclusive CRH-R2 agonist, did not affect SGK-1 expression. We also
found that CRH-R1 mediated CRH-induced increase in cAMP production in these cells and that blocking PKA and AC prevented CRH-induced SGK-1 expression. These findings suggest that CRH may be one of bioactive factors that induce SGK-1 expression in hippocampal neurons.

Both CRH-R1 and -R2 are identified in hippocampus and primary hippocampal neurons (2, 39, 43). Moreover, both CRH-R1 and -R2 have been implicated in the modulation of hippocampal functions in vivo and in vitro. More recently, we reported that CRH-R1 mediates CRH regulation of N-methyl-D-aspartate (NMDA) receptor activity in hippocampal neurons (43). Some studies have shown that CRH-R1 mediates the anxiogenic-like effect (9) and neuroprotective activity of CRH (15, 40). Sananbenesi et al. (41) have demonstrated that CRH-R2 in hippocampus is a link to stress-enhanced memory consolidation. The present study showed that activation of CRH-R1 could induce expression of SGK-1, a novel protein kinase, in cultured hippocampal neurons, suggesting that CRH-R1 may exert its function through the SGK-1 signaling pathway in hippocampal neurons.

It has been found that CRH receptors activate multiple G proteins and subsequently lead to activation of multiple signaling pathways, including the AC/PKA and PLC/PKC pathways (6, 16, 43). Blank et al. (6) found that CRH receptors couple to Gs, Gi, and Gq to evoke hippocampal neuronal excitability via PKA- or PKC-dependent signaling pathways in mice. Elliott-Hunt et al. reported that, in rat hippocampus, CRH activates PKA and MAPK signaling pathways to exert neuroprotective effects (14). In the present study, we showed that CRH acts on CRH-R1 to stimulate cAMP production in primary hippocampal neurons. It is known that PKA is activated by cAMP, which is produced by AC; thus, AC and PKA inhibitors were applied to elucidate the role of PKA in CRH stimulation of SGK-1 expression. We found that blocking either PKA or AC completely prevented CRH-induced SGK-1 expression. These data suggested that the AC/PKA signaling pathway is involved in CRH induction of SGK-1 expression. Sequence analysis revealed that there are three cAMP-responsive element-binding protein sites in the SGK-1 promoter region (45), providing the molecular basis that activation of PKA signaling pathway upregulates SGK-1 expression. Studies by Alliston et al. (1) and Gonzalez-Robayna et al. (17) demonstrated that, in rat ovarian granulose cells, activation of the cAMP pathway induces SGK-1 expression. We also found that activation of CRH-R1 resulted in not only activation of cAMP/PKA but also PLC-β3 signaling pathways in hippocampal neurons. In this study, we also examined the effect of PLC and PKC inhibitors on CRH-induced SGK-1 expression. It was found that blockage of PLC and PKC cannot reverse CRH effect. Moreover, PLC as well as PKC inhibitors significantly induced SGK-1 expression. Although we did not show the effect of PLC activator on SGK-1 expression, we demonstrated that PKC activator did not influence SGK-1 mRNA and protein levels. Thus, it could be suggested that CRH stimulation of SGK-1 expression may be independent of the PLC/PKC signaling.
pathway. Interestingly, our previous study showed that the PLC/PKC but not AC/PKA signaling pathway is involved in CRH regulation of NMDA activity in cultured hippocampal neurons (43). Together, this suggests that CRH may exhibit multiple effects in hippocampal neurons through different signaling pathways.

Although activation of PKC by PMA did not affect SGK-1 expression, inhibition of PLC and PKC resulted in an increase in SGK-1 mRNA and protein expression, which may suggest that maintenance of SGK-1 expression at a relative low level in cultured hippocampal neurons is due to the tonic inhibitory effect of the intrinsic PLC/PKC signaling pathway. A study by Lang et al. (24) showed that SGK-1 transcript could be induced by activation of PKC in fibroblasts. Thus, it is suggested that regulation of SGK-1 expression by PKC signaling pathway may be dependent on cell context. However, the role of PLC/PKC signaling pathway in the modulation of SGK-1 expression remains to be further elucidated.

It has been known that SGK-1 transcript expression can be acutely regulated by various stimuli (34, 38). We also observed, in this study, that SGK-1 mRNA expression was significantly enhanced after cells were exposed to CRH for 30 min. A number of stimuli, such as stress, hyper- or hypo-osmotic stress, and transient ischemia, have been shown to induce SGK-1 transcript expression (4, 28, 34). It is found that global ischemia could induce CRH release in hippocampus (21). During exposure to stress, CRH can be secreted directly from the nerve terminals located in the hippocampus (10). Thus, CRH might be one of the mediators that control SGK-1 expression in response to various stimuli, such as ischemia. Nevertheless, the specific link between stress and SGK-1 expression needs to be further elucidated.

SGK-1 has been implicated in modulation of various physiological functions such as activity of ion channels, cell survival, and proliferation (8, 20, 42, 48, 53). In the nervous system, SGK-1 has been shown to be involved in learning and memory, neuroprotection, and dendrite growth (12, 30, 42, 46). Interestingly, CRH has been demonstrated to be involved in learning, dendrite growth, and neuroprotection (3, 11, 14, 27, 37, 40). The results of the present study indicate that there may be a functional link between CRH and SGK-1 in hippocampal neurons. Thus, it would be of interest to further study the functions controlled by the CRH-SGK-1 system in the future.

In summary, CRH acts on CRH-R1 to stimulate SGK-1 mRNA and protein expression in cultured hippocampal neurons via a mechanism that is involved in AC/PKA signaling pathways. Our results suggest that CRH may exert its function through the SGK-1 signaling pathway in hippocampus.

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