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Protein kinase C-β mediates neuronal activation of Na^+/H^+ exchanger-1 during glutamate excitotoxicity

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ABSTRACT

Na^+/H^+ exchanger-1 (NHE-1) activity is known to play a critical role in the neuronal injury caused by glutamate. However, the underlying mechanism is not clear. This study shows that NHE-1 activation and its phosphorylation during glutamate exposure were attenuated by the inhibition of protein kinase C (PKC)-βI and -βII, leading to reduced neuronal death. In addition, activations of PKC-βI and -βII by PKC-βI and -βII CAT plasmid or by PMA, PKC-β pharmacological activator have stimulated the activity and phosphorylation of NHE-1, which were abolished by inhibition of PKC-β in neuronal cells. Furthermore, the inhibition of PKC-β has mediated neuroprotective effect on glutamate-induced cells, which is similar to neuroprotective efficacy of siRNA NHE-1 transfection. Taken together, these results suggest that activation of the PKC-βI and -βII pathway by glutamate increases the activity and phosphorylation of NHE-1, and that these increases contribute to neuronal cell death. In this study, we demonstrate that PKC-βI and -βII are involved in the regulation of NHE-1 activation following glutamate exposure in neuron.

1. Introduction

The Na^+/H^+ exchanger-1 (NHE-1) is a pH-regulatory protein that is distributed almost universally in mammalian tissues. NHE-1 extrudes one H^+ and takes up one Na^+ ion when a decrease in intracellular pH occurs [1], and has been implicated in a number of cellular functions, which include cell volume regulation, cell migration, and cell proliferation. Increased NHE-1 activity during ischemic conditions is a key step in cell volume regulation and acid–base homeostasis in neuronal cells [2,3]. During brain ischemia, increases in intracellular Na^+ concentration due to NHE-1 activation result in an intracellular Ca^{2+} overload by Na^+/Ca^{2+} exchanger and subsequent neuronal damage [4]. These inter-relationships underlie the importance of understanding the regulatory mechanisms that control NHE-1 during ischemic injury. A great deal of effort has gone into determining the regulatory mechanisms of NHE-1 [5], though NHE-1 has generally been studied by observing its direct phosphorylation. It was recently found that the activations of serine/threonine kinases, such as, extracellular signal-related kinase1/2 (ERK1/2) and 90-kDa ribosomal S6 kinase (p90RSK), are required for the phosphorylation of NHE-1 induced by ischemia [6].

Protein kinase C (PKC) has been reported to be able to regulate NHE-1 in various cells, such as, cardiomyocytes and fibroblasts [7,8], but no study has been undertaken to investigate the relationship between NHE-1 regulation and PKC in neuronal cells. Furthermore, nothing is known regarding the possible roles of major PKC isoforms in the regulation of NHE-1 activation. In the present study, we investigated whether the activation of PKC affects the stimulation of NHE-1 activity by glutamate excitotoxicity, and in addition, we sought to identify the PKC isoforms responsible for the regulation of NHE-1 in neuronal cells.

2. Methods

2.1. Chemicals reagents

Cariporide was synthesized at the Bio-organic Division of the Korea Research Institute of Chemical Technology (Daejon, Korea). Glutamate and 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Gö6976 was purchased from Biomol Research Labs Inc. (Plymouth Meeting, PA). MK-801 ((+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene) were from Tocris (Ballwin, MO), and PKC-β inhibitor (3-(1-(3-Imidazol-1-

Abbreviations: NHE-1, Na^+/H^+ exchanger-1; PKC, protein kinase C; PMA, 12-myristate 13-acetate; ERK1/2, extracellular signal-related kinase1/2; p90RSK, 90-kDa ribosomal S6 kinase; Gö6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene; SL0101, Kaempferol-3-O–(3′,4′-di-O–acetyl-α-L-rhamnopyranoside); SK-N-MC, human neuroblastoma cell line.

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ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione) and SL0101 (Kaempferol-3-O-(3′,4′-di-O-acetyl-α-L-rhamnopyrano-side)) were purchased from Calbiochem (Germany).

2.2. Primary cultures of cortical neurons

All experimental procedures were performed in accordance with the guidelines on the use and care of laboratory animals issued by the Animal Care Committee at Ajou University. Primary mouse cortical neurons were cultured as described previously [9]. Briefly, cerebral cortices were removed from the brains of fetal ICR mice on gestation day 14, gently titrated 3–4 times using a large-bore Pasteur pipette, dissociated into individual cells using a small-bore Pasteur pipette, and plated on 6- or 24-well plates precoated with 100 μg/ml poly-D-lysine (Sigma) and 4 μg/ml natural mouse laminin (Gibco-BRL, Gaithersburg, MD). Cells (approximately 2.5×10^5 cells/10 ml) were maintained in culture media, consisting of Eagle's minimum essential medium (MEM) (Earle's salts, JBI, Korea) supplemented with 21 mM glucose, 5% fetal bovine serum (Gibco-BRL), 5% horse serum (Gibco-BRL), and 2 mM L-glutamine. Cytosine arabinofuranoside (10 μM Ara-C, Sigma) was added to cultures on culture days 3–4 *in vitro* (DIV 3–4) to prevent glial cell overgrowth. Cells were maintained in 5% CO_2 atmosphere at 37 °C for 7–8 days, and then used for experiments. More than 80% of the cell population at this stage was neuronal cells, as determined by NeuN (neuronal nuclei, specific neuronal markers) and GFAP (glial fibrillary acidic protein, glial cell markers) staining (data not shown).

2.3. SK-N-MC cell cultures

SK-N-MC neuroblastoma cells were purchased from the ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) containing 10% fetal bovine serum and 20 mM glucose, and maintained in a humidified 5% CO_2 incubator at 37 °C. The cells were used for experiments after reaching ~80% confluence.

2.4. RNA preparation and RT-PCR

Cultured cells were washed with ice-cold PBS and total RNA was extracted using the easy-blue® kit (Intron, Korea). Reverse transcription (RT) was carried out using AMV reverse transcriptase (Takara, Japan) according to the manufacturer's instructions. The primer sequences used in this study for NHE-1 were; 5′-TCTGCCGTCTCAACTG TCTTA-3′ (forward) and 5′-CCCTTCAACTCC TCATTCACCA-3′ (reverse). PCR amplification was performed over 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 35 s. PCR products were separated by electrophoresis on 1% agarose gels, which were then stained with ethidium bromide and photographed. The optical densities of NHE-1 bands were measured using a Gel doc system (GEL DOC 2000, Bio-Rad, Hercules, CA). Optical density measurements were normalized versus glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5. Measurements of pH_i and NHE activity

NHE activity was measured using as previously described method with a few modifications [10]. Briefly, cells were loaded with a pH-sensitive fluorescent dye BCECF-AM (acetoxymethyl esters of 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein, Invitrogen, Carlsbad, CA) and pH_i changes were measured. For primary cultured neuronal cells, cells grown on poly-D-lysine-coated glass coverslips were loaded with 5 μM BCECF-AM by incubation for 15 min at room temperature in standard HEPES-buffered solution. The standard HEPES-buffered solution contained in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). Cells were then washed with standard HEPES-buffered solution, and assembled in the bottom of a perfusion chamber. The chamber was placed on an inverted microscope and intralobular ducts were identified by morphology. BCECF-AM fluorescence was recorded at excitation wavelengths of 440 and 490 nm using a recording setup (Delta Ram; PTI Inc., Brunswick, NJ). NHE activities were measured by estimating Na^+-dependent pH_i recovery in acidified cells as follows. Cells were first acidified by a NH_4^+ (20 mM) pulse, and then perfused with a Na^+-free solution prepared by replacing Na^+ in the standard HEPES-buffered solution. Maximal Na^+-dependent pH_i recovery was measured in cells acidified to a pH of 6.3–6.4. Buffer capacity was calculated by measuring pH_i in response to 5–20 mM NH_4Cl pulses. During the experiment, the intrinsic buffer capacity was found to show a negative linear relationship with pH_i between pH values of 6.2 and 7.6.

2.6. Subcellular fractionation for the isolation of PKC and Immunoblotting

Subcellular fractionation for PKC was performed as described previously [11]. Briefly, cells were harvested in homogenization buffer (20 mM Tris–HCl, 2 mM EDTA, 5 mM EGTA, 5 mM DTT, 6 mM β-mercaptoethanol, 1 mM PMSF, 20 μM leupeptin, and 10 μg/ml aprotinin, pH 7.4) and centrifuged at 100,000 *g* for 1 h at 4 °C. Supernatants were retained as cytosolic fractions. Pellets were resuspended in 1% Triton X-100-containing homogenization buffer, and centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatants are referred to as membrane fractions. Protein contents were determined using the Bradford protein assay (Biorad, Hercules, CA). The samples were resolved on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Blots were incubated in 5% non-fat dry milk for 1 h at room temperature, and then incubated overnight at 4 °C with a polyclonal antibody against PKC isoform (Santa Cruz, CA). The blots were then rinsed with Tris-buffered saline and incubated with horse-radish peroxidase-conjugated secondary IgG (Cell Signaling Technologies, Beverly, MA) for 1 h. Bound antibody was detected with an ECL kit (Intron), and bands analyzed using a LAS1000 (Fuji Photo Film, Japan).

2.7. Isolation of ERK1/2 and p90RSK from cell lysates

Cells were harvested in RIPA buffer (150 mM NaCl, 20 mM Tris–HCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, and protease inhibitors at pH 7.4), homogenized, and nuclei and cell debris were removed by centrifugation at 10,000 *g* for 15 min at 4 °C. Supernatants were collected for immunoblotting. Protein contents were determined using the BCA™ protein assay (Pierce Rockford, IL). Protein samples were denatured in Laemli buffer (1:4 by volume), and total ERK1/2 and p-ERK1/2 levels were quantified by immunoblotting using polyclonal antibody against ERK1/2 and monoclonal antibody against p-ERK1/2, respectively (both from Cell Signaling Technologies). Polyclonal antibodies against RSK and phosphorylated p90RSK (both from Cell Signaling Technologies) were used to detect total p90RSK and p-p90RSK, respectively.

2.8. Analysis of NHE-1 phosphorylation by immunoprecipitation

Phosphorylation levels of NHE-1 were measure as described by Snabaitis et al. [12]. Cells were lysed in ice-cold RIPA buffer as described above and centrifuged at 10,000 *g* for 15 min at 4 °C. Supernatants containing proteins were collected and incubated overnight at 4 °C with mouse monoclonal antibody against the phosphor-Ser 14-3-3β protein binding motif (Cell Signaling Technologies) or with goat monoclonal NHE-1 antibody (Santa Cruz). The immunocomplexes obtained were mixed with protein A and G (Merck, Germany) for 4 h at 4 °C and then washed three times with ice-cold modified RIPA buffer. Immunocomplexes were dissociated from beads by heating at 100 °C for 5 min. Protein samples from immunocomplexes were resolved on 8% SDS-PAGE and analyzed by immunoblotting using goat polyclonal