BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther, 593-598 (2017)

Sustained Intracellular Acidosis Triggers the Na^+/H^+ Exchager-1 Activation in Glutamate Excitotoxicity

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Abstract

The Na^+/H^+ exchanger-1 (NHE-1) is a ubiquitously expressed pH-regulatory membrane protein that functions in the brain, heart, and other organs. It is increased by intracellular acidosis through the interaction of intracellular H^+ with an allosteric modifier site in the transport domain. In the previous study, we reported that glutamate-induced NHE-1 phosphorylation mediated by activation of protein kinase C-β (PKC-β) in cultured neuron cells via extracellular signal-regulated kinases (ERK)/p90 ribosomal s6 kinases (p90RSK) pathway results in NHE-1 activation. However, whether glutamate stimulates NHE-1 activity solely by the allosteric mechanism remains elusive. Cultured primary cortical neuronal cells were subjected to intracellular acidosis by exposure to 100 μM glutamate or 20 mM NH_4Cl. After the desired duration of intracellular acidosis, the phosphorylation and activation of PKC-β, ERK1/2 and p90RSK were determined by Western blotting. We investigated whether the duration of intracellular acidosis is controlled by glutamate exposure time. The NHE-1 activation increased while intracellular acidosis sustained for >3 min. To determine if sustained intracellular acidosis induced NHE-1 phosphorylation, we examined phosphorylation of NHE-1 induced by intracellular acidosis by transient exposure to NH_4Cl. Sustained intracellular acidosis led to activation and phosphorylation of NHE-1. In addition, sustained intracellular acidosis also activated the PKC-β, ERK1/2, and p90RSK in neuronal cells. We conclude that glutamate stimulates NHE-1 activity through sustained intracellular acidosis, which mediates NHE-1 phosphorylation regulated by PKC-β/ERK1/2/p90RSK pathway in neuronal cells.

Key Words: Glutamate, Na^+/H^+ exchanger-1, Sustained acidosis, Cortical neurons, Protein kinase C-β, Extracellular signal-regulated kinases 1/2

INTRODUCTION

Neuronal excitability and neurotransmission are metabolically powerful activities that induce excessive changes in intracellular pH (Chesler and Kaila, 1992). These pH alterations further regulate electrical activity by regulating the conductance of various pH-sensitive neurotransmitters-, voltage-, and proton-gated ion channels (Jinadasa *et al*., 2014). The influence of intracellular and extracellular pH oscillation on neuronal excitability is well established and partly attributed to H^+ sensitivity of neurotransmitter receptors and voltage-gated ion channels (Tang *et al*., 1990; Rocha *et al*., 2008). These pH changes have to feedback mechanism associated with a physiological role for controlling of the neuronal functions (DeVries, 2001). On the contrary, excessive intracellular acidosis has been postulated to contribute to ischemic neuronal cell death (Hartley and Dubinsky, 1993). Previous studies suggested that ischemia-induced shifts in brain pH could be accounted for by the neurotransmitter-induced pH change within the neuronal cells. Moreover, physiological levels of glutamate have been reported to produce parallel increase in H^+ and Ca^{2+} concentrations; extended periods of elevated H^+ concentration results in *in vitro* neurotoxicity, contributing synergistic excitotoxic neuronal cell death (Hartley and Dubinsky, 1993). In a recent study, Rathje *et al*. (2013) reported that N-methyl-D-aspartate (NMDA) receptor activation-induced intracellular acidification modulates Na^+/H^+ exchanger-1 (NHE-1) activity through PDZ domain (presented in PSD-95, DlgA and ZO-1)-containing protein, which interacts with C kinase 1 (PICK-1) regulation in hippocampal cells. In-

Open Access **https://doi.org/10.4062/biomolther.2017.018**

Received Feb 2, 2017 **Revised** Mar 8, 2017 **Accepted** Mar 18, 2017
Published Online Jun 14, 2017

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deed, it has been reported that various NHE inhibitors such as cariporide, zoniporide, and SM20220 prevent glutamate-induced neuronal cell death (Matsumoto *et al*., 2004; Lee *et al*., 2009; Lee and Jung, 2012). Furthermore, we recently investigated that glutamate-induced Na^+/H^+ exchanger-1 (NHE-1) phosphorylation mediated by activation of protein kinase C-β (PKC-β) in cultured neuron cells via extracellular-signal-regulated kinase 1/2 (ERK1/2)/ribosomal s6 kinase (p90RSK) pathways result in NHE-1 activation in cortical neurons (Lee *et al*., 2014). However, the mechanism underlying mediation of regulatory function of glutamate in initiating the step of NHE-1 phosphorylation remains elusive. We therefore examined how glutamate leads to some aspects of intracellular acidosis and whether neuronal acidosis controls NHE-1 phosphorylation in cortical neuronal cells.

MATERIALS AND METHODS

Chemicals reagents

Cariporide was synthesized at the Bio-organic Division of the Korea Research Institute of Chemical Technology (Daejeon, Korea). Glutamate was purchased from Sigma (St. Louis, MO, USA). U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene) was from Tocris (Ballwin, MO, USA) and PKC-β inhibitor (3-(1-(3-Imidazol-1-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 (Darmstadt, Germany).

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 (Suwon, Korea). Primary mouse cortical neurons were cultured as described previously (Lee and Jung, 2012). 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, USA). 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, Welgene, Daegu, 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, Chemicon, Temecula, CA, USA) and GFAP (glial fibrillary acidic protein, glial cell markers, Sigma) staining (data not shown).

Measurements of pH_i and NHE activity

NHE activity was measured following a previously described method with a few modifications (Kim *et al*., 2007). 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, USA) and pH_i changes were measured. For primary cultured neuronal cells, cells grown on poly-D-lysine-coated glass cover slips 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 based on morphological examination. BCECF-AM fluorescence was recorded at excitation wavelengths of 440 and 490 nm using a recording setup (Delta Ram; PTI Inc., Brunswick, NJ, USA). 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.

Subcellular fractionation for the isolation of PKC and immunoblotting

Subcellular fractionation for PKC was performed as described previously (Jung *et al*., 2004). Briefly, cells were harvested in homogenization buffer (20 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycoltetraacetic acid (EGTA), 5 mM Dithiothreitol (DTT), 6 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (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 content was determined using the Bradford protein assay (Biorad, Hercules, CA, USA). The samples were resolved on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). 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, USA). The blots were then rinsed with Tris-buffered saline and incubated with horseradish peroxidase-conjugated secondary IgG (Cell Signaling Technologies, Beverly, MA, USA) for 1 h. Bound antibody was detected with an ECL kit (Intron) and bands analyzed using a LAS1000 (Fuji Photo Film, Tokyo, Japan).

Isolation of ERK1/2 and p90RSK from cell lysates

Isolation of ERK1/2 and p90RSK was performed as described previously (Lee *et al*., 2014). Briefly, 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 content was determined using the BCA™ protein assay (Pierce, Rockford, IL, USA). Protein samples were denatured in Laemli

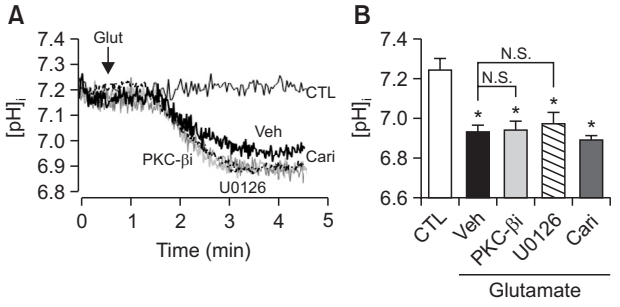

Fig. 1. The change in pH_i in neurons following glutamate exposure. (A) Representative recordings of pH_i in primary cortical neuron cultures in response to intracellular acidosis due to exposure to 100 μM glutamate for 1 min. The pH_i levels were measured in control (CTL, thin black), 100 μM glutamate alone (vehicle (Veh), thick black), glutamate+0.1 μM PKC-β inhibitor (PKC-βi, light gray), glutamate+0.1 μM cariporide (Cari, dark gray) and glutamate+10 μM U0126 (dot black). (B) The pH_i levels were calculated using the first 200 seconds of each curve. Cells were treated with or without glutamate in the presence or absence of 0.1 μM PKC-βi, 10 μM U0126, or 0.1 μM cariporide (n≥3, values are mean ± SEM, *p<0.05 vs. CTL).

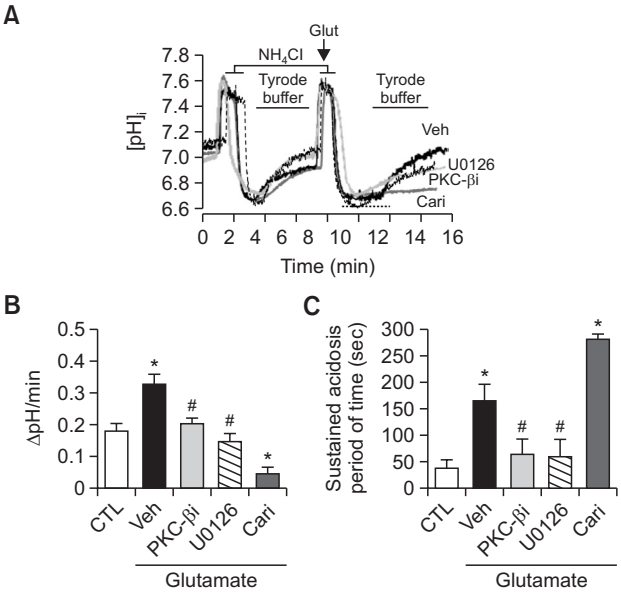

Fig. 2. The activation of NHE-1 in neurons following glutamate exposure. (A) Representative recordings of pH_i in response to intracellular acidosis by transient (1 min) exposure to 20 mM NH_4Cl. The pH_i levels were measured in control and 100 μM glutamate loaded conditions. The pH_i levels were measured in the presence of 100 μM glutamate alone (Veh, black), glutamate+0.1 μM PKC-βi (light gray), glutamate+0.1 μM Cari (dark gray) and glutamate+10 μM U0126 (dot black). (B) pH_i recovery rates were calculated using the first 60 s of each recovery curve. Cells were treated with or without glutamate in the presence or absence of 0.1 μM PKC-βi, 10 μM U0126, or 0.1 μM Cari (cariporide). (C) Sustained acidosis period of time was measured at below pH 6.8 after glutamate treatment. Cells were treated with or without glutamate in the presence or absence of 0.1 μM PKC-βi, 10 μM U0126, or 0.1 μM Cari (cariporide) (n=5, values are mean ± SEM, *p<0.05 vs. CTL, #p<0.05 vs. vehicle with glutamate alone (Veh)).

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.

Analysis of NHE-1 phosphorylation by immunoprecipitation

Phosphorylation level of NHE-1 was measured as described by Snabaitis *et al.* (2008). 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 NHE-1 antibody (BD Bioscience, San Jose, CA, USA) or rabbit monoclonal phospho-serine antibody (Invitrogen).

Statistical analysis

All data are presented as the means ± SEM of at least three separate determinations in each group. Numerical data were compared using Student's *t*-test or one-way ANOVA *post hoc* test for the unpaired observations between the two groups. A *p*-value <0.05 was considered statistically significant.

RESULTS

Changes in intracellular pH in neuronal cells following exposure to glutamate

There was a rapid and significant decrease in pH_i following the application of 100 μM glutamate. After about 4 min of glutamate treatment, pH_i decreased from 7.20 ± 0.05 to 6.95 ± 0.03 in neurons. PKC-βi (PKC-β1/2 inhibitor) and U0126 (MEK1/2 inhibitor) as inhibitors of NHE-1 phosphorylation (Lee *et al.*, 2014) and cariporide, a potent and selective pharmacological inhibitor of NHE-1 (Lee and Jung, 2012) did not affect intracellular acidic condition following glutamate exposure, suggesting that glutamate-induced intracellular acidosis is not mediated by activation or phosphorylation of NHE-1 in neuronal cells (Fig. 1). To elucidate the relationship between acidification and NHE-1 phosphorylation and activation in neurons following glutamate exposure, we measured the NHE activity. We previously investigated that intrinsic buffer capacity of neurons is altered by glutamate and confirmed it in present study. As shown in Fig. 2A, changes in pH_i were induced by applying progressively lower concentrations of NH_3/NH_4^+. In response to 100 μM glutamate, NHE-1 activity increased about 2-fold in neuronal cells (from 0.17 ± 0.03 to 0.32 ± 0.04 pH_i/min), and this was decreased by PKC-βi (to 0.19 ± 0.02 pH_i/min), U0126 (0.13 ± 0.04 pH_i/min) and by cariporide (to 0.04 ± 0.02 pH_i/min), respectively (Fig. 2B). Interestingly, neurons treated by glutamate sustained acidosis for about 3 min (Fig. 2A, dotted bar) and then increase in NHE-1 activity was noted. However, the glutamate-induced sustained acidosis (168.4 ± 36.2 sec) was significantly extinguished by PKC-βi (67.2 ± 38.6 sec) and

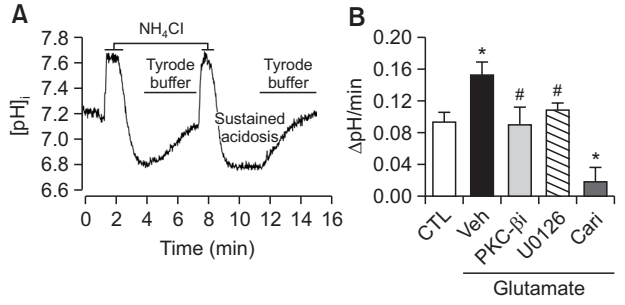

Fig. 3. NHE-1 activation was mediated by sustained acidosis in neurons treated with NH_4Cl. (A) In the sustained acidosis groups, the duration of intracellular acidosis during the second acid pulse was extended to 3 min by initial washout with NH_4Cl with Na^+-free solution, and normal extracellular $[Na^+]o$ was subsequently reintroduced. (B) The pH_i recovery rates were calculated using the first 60 seconds of each recovery curve. Cells were treated with or without NH_4Cl in the presence or absence of 0.1 μM PKC-βi, 10 μM U0126, or 0.1 μM cariporide (n=5, values are mean ± SEM, *p<0.05 vs. control (CTL), $^{\#}p$<0.05 vs. vehicle with NH_4Cl (Veh)).

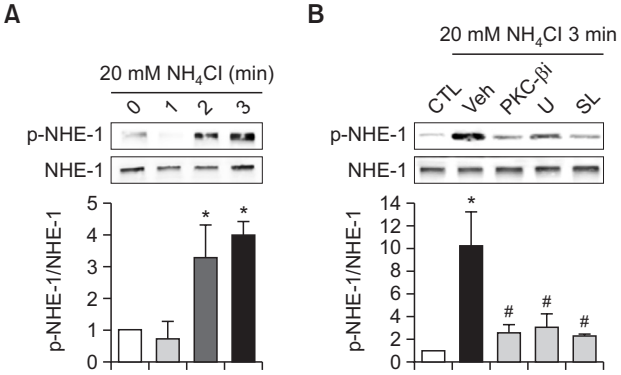

Fig. 4. The phosphorylation of NHE-1 in neurons treated with NH_4Cl. (A) Cells were treated with 20 mM NH_4Cl for ~ 3 min, which was washed out in the presence of 3 μM cariporide. Samples were lysed and immunoprecipitated with NHE-1 antibody and immunoblotted for phosphor-Ser (upper) or NHE-1 (lower). (B) Cells were treated with 20 mM NH_4Cl for 3 min in the presence or absence (Veh) of 0.1 μM PKC-βi, 10 μM U0126 (U), or 1 μM SL0101 (SL). Samples were lysed and immunoprecipitated with NHE-1 antibody and immunoblotted for phosphor-Ser (upper) or NHE-1 (lower) (n=3, values are mean ± SEM, *p<0.05 vs. CTL, $^{\#}p$<0.05 vs. Veh).

U0126 (to 62.3 ± 48.5 sec), respectively, to control level (46.0 ± 20.3 sec) (Fig. 2C). Cariporide led to extensive decrease in NHE-1 activity than control levels and maintained prolonged acidosis condition in glutamate-induced neuronal cells (Fig. 2B, 2C). These result suggests the possibility of occurrence of NHE-1 activation mediated by sustained acidosis in neurons induced by glutamate.

Changes in NHE-1 activity following sustained acidosis

To investigate NHE-1 activity stimulated by sustained intracellular acidosis, we established sustained acidosis condition by extending the duration of intracellular acidosis by 3 min through initial washout of NH_4Cl with Na^+-free solution and subsequent reintroduction of normal extracellular Na^+ (Haworth *et al*., 2003). As shown in Fig. 3, sustained intracellular acidosis increased NHE-1 activity (from 0.09 ± 0.015 to 0.15 ± 0.023 ΔpH_i/min) and the activity was decreased by PKC-βi (to 0.08 ± 0.03 ΔpH_i/min), U0126 (to 0.102 ± 0.01 ΔpH_i/min), and cariporide (to 0.015 ± 0.016 ΔpH_i/min), respectively.

Changes in NHE-1 phosphorylation following sustained acidosis

We subsequently investigated whether sustained acidosis leads to phosphorylation of NHE-1 in neurons. To determine the effect of the duration of intracellular acidosis on NHE-1 phosphorylation, neurons were exposed for ~3 min to 20 mM NH_4Cl, which was washed out in the presence of cariporide, thus lowering pH_i to ~6.6 (Haworth *et al*., 2006). Immunoprecipitation of NHE-1 followed by immunoblotting for phosphoserine (p-Ser) was performed to examine their interaction. NHE-1 binding to p-Ser was found to increase in neurons following NH_4Cl exposure. As illustrated in Fig. 4A, the intensity of the protein band representing the phosphorylation level of NHE-1 was significantly increased in neurons exposed to NH_4Cl (~3 min). A PKC-β/ERK1/2-p90RSK pathway has been shown to phosphorylate the regulatory domain of NHE-1 and possibly mediate the glutamate-induced stimulations of NHE-1 activity in neuronal cells (Lee *et al*., 2014). Therefore, we investigated whether PKC-β/ERK1/2-p90RSK pathways is also involved in the phosphorylation of NHE-1 in neuronal cells treated with NH_4Cl for 3 min. As shown in Fig. 4B, NH_4Cl-induced phosphorylation of NHE-1 was abolished by PKC-βi, U0126 or SL0101 (a p90RSK specific inhibitor) (from 10.2 ± 3.4 folds to 2.6 ± 1.0, 3.06 ± 1.2 or 2.3 ± 0.2 folds), respectively.

Sustained acidosis mediated the stimulation of NHE-1 activity through PKC-β/ERK1/2-p90RSK pathway in neurons treated with NH_4Cl

We investigated whether NH_4Cl-mediated sustained acidosis induces phosphorylation of NHE-1 through PKC-β/ERK1/2-p90RSK pathway, which was involved in glutamate-induced stimulation of NHE-1 activity in neuronal cells (Lee *et al*., 2014). As shown in Fig. 5, NH_4Cl treatment increased the activation of PKC-β1 and −β2 and phosphorylation of ERK1/2 and p90RSK. NH_4Cl-induced phosphorylation of ERK1/2 was significantly abolished by U0126 or PKC-βi, but U0126 did not inhibit the activation of PKC-β1 and −β2, whereas all inhibitors including PKC-β inhibitor, U0126 and SL0101, dramatically prevented NH_4Cl-induced phosphorylation of p90RSK. These finding suggests that the stimulation of NHE-1 activity after sustained acidosis exposure occurs via PKC-β-ERK1/2-p90RSK signaling pathway in neuronal cells.

DISCUSSION

In neuronal disease states, acidification is a phenomenon which forms part of attenuating excessive neuronal excitability (Chesler and Kaila, 1992). In addition, decrease in pH in the brain appears due to various problems that occur outside the brain such as metabolic acidosis, also several neurodegenerative disorders represent a close relationship between the pH and the nervous system (Ruffin *et al*., 2014). The regulation of intracellular pH in neuronal cells takes place through an active process, because H^+ ions do not passively pass through the cell membrane (Roos and Boron, 1981). Intracellular acidosis due to the activation of cell surface receptors that may be

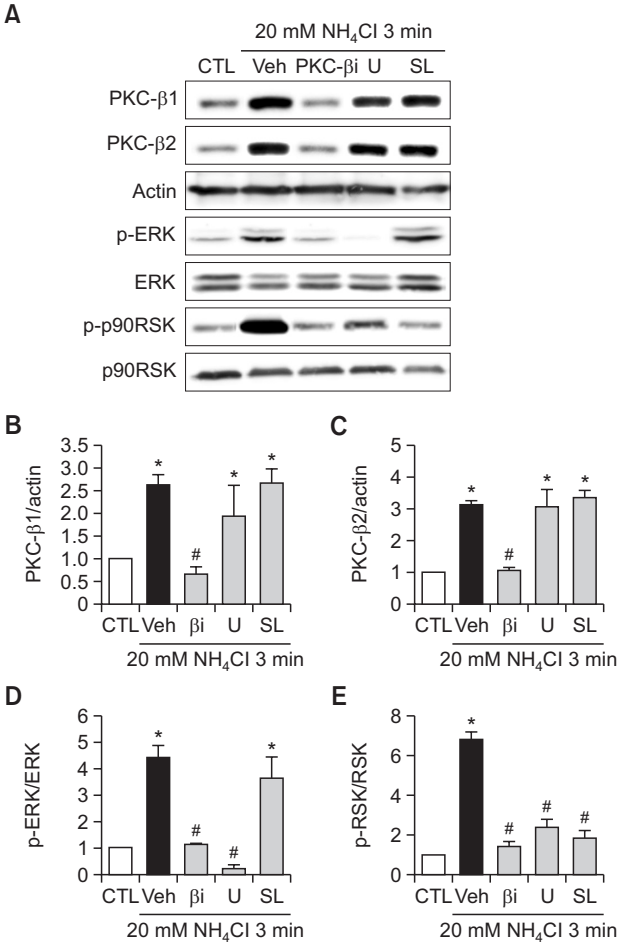

Fig. 5. Representative immunoblots for PKC-β1 and -β2 in the membrane fraction, p-ERK1/2, and p-p90RSK in whole fraction of cortical neurons. (A) Cells were treated with 20 mM NH_4Cl for 3 min in the presence or absence (Veh) of 0.1 μM PKC-βi, 10 μM U0126 (U), or 1 μM SL0101 (SL). PKC-β, p-ERK1/2, and p-p90RSK loadings were normalized versus actin, ERK1/2 and p90RSK, respectively. (B-E) Quantitative analysis for PKC-β, p-ERK1/2, and p-p90RSK (n=5, values are mean ± SEM, *p<0.05 vs. control (CTL), $^{\#}p$<0.05 vs. vehicle with NH_4Cl (Veh)).

generalized in the changes in intracellular and extracellular pH induced by glutamate have been described previously (Yamamoto *et al*., 2005). Although the source of increased intracellular H^+ ion concentration is not clear, acidification of the neuronal cells caused by glutamate has recently been proposed to be a Ca^{2+} dependent process (Dixon *et al*., 1993; Hartley and Dubinsky, 1993; Irwin *et al*., 1994). Glutamate-induced acidification can be displayed by increasing the Ca^{2+}-dependent intracellular H^+ concentration (Thomas, 1989). The reason for increase in H^+ concentration in a Ca^{2+}-dependent manner is diverse. Of the most convincing hypothesis is that the increase in intracellular Ca^{2+} concentration by glutamate displaces the H^+ from the acidic binding sites (Wang *et al*., 1994), suggesting that glutamate-induced neuronal acidification may appear with symptoms rather than the cause of the glutamate excitotoxicity. Acidification activates NHE in various cells including the neurons. This activation is due to the presence of an internal allosteric H^+ binding site in the transport domain of NHE that increases Na^+-dependent H^+-extrusion (Kersh *et al*., 2009). Additional regulation is achieved via kinase-mediated modulation of the NHE regulatory domain. For example, activation of NHE-1 is usually mediated by an elevation in intracellular Ca^{2+} concentration and/or by an increased activity of PKC isotypes involving phosphoinositide breakdown (Lipp and Reither, 2011). Despite the lack of evidence on direct phosphorylation, supportive evidence has been presented for a PKC-β-dependent up-regulation via various kinases binding to phosphorylation site on C-terminus of NHE (Takahashi *et al*., 1999; Itoh *et al*., 2005). In addition, we previously investigated that treatment of glutamate induces phosphorylation of NHE-1 with subsequent over-activation of NHE-1 leading to excitotoxicity via activation of PKC-β (Lee *et al*., 2014). In the present study, we hereby certify that glutamate induces phosphorylation and over-activation of NHE-1 via prolonged period of intracellular acidosis. To determine if glutamate-induced sustained acidosis stimulates NHE-1 activity solely by the allosteric mechanism, cultured cortical neuronal cells were subjected to intracellular acidosis by transient exposure to NH_4Cl. Apparently, NH_4Cl-induced intracellular acidosis increased phosphorylation and activation of NHE-1 through PKC-β, ERK1/2, and p90RSK pathways, which is a proven mechanism involving activation of NHE-1 activity by glutamate in neuronal cells (Lee *et al*., 2014). Furthermore, inhibition of PKC-β, ERK1/2, and p90RSK by pretreatment of neuronal cells with PKC-β inhibitor, U0126 and SL0101, respectively abolished the activation of NHE-1 by sustained NH_4Cl-induced intracellular acidosis.

During excitotoxic condition, over-release of glutamate that increase Ca^{2+} influx through their receptor and activates various protein kinases such as PKCs and mitogen-activated protein kinases (MAPKs). Synergistic effects of various kinase activation and increase in Ca^{2+} concentration following glutamate could lead to achievement of the mode of stimulation of NHE-1 activity by sustained intracellular acidosis in neuronal cells. Although future research may elucidate clear mechanism and possibly even different mechanisms for glutamate-induced intracellular acidosis in relation to intracellular Ca^{2+} level, existing data cannot support such conclusions. Therefore, future research is needed to assess accurately the role of increase in intracellular Ca^{2+} concentration in glutamate treatment that leads to acidification. However, it is apparent that the protein kinases may cause potential activation of NHE-1 in response to sustained intracellular acidosis. In addition, activation of PKC-β/ERK1/2/RSK pathway is necessary for the stimulation of NHE-1 activity by sustained intracellular acidosis in glutamate excitotoxicity.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A1A01059515), Republic of Korea.

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