Neuroscience 210 (2012) 38–46

SNARE-DEPENDENT UPREGULATION OF POTASSIUM CHLORIDE CO-TRANSPORTER 2 ACTIVITY AFTER METABOTROPIC ZINC RECEPTOR ACTIVATION IN RAT CORTICAL NEURONS *IN VITRO*

R. A. SAADI,[a] K. HE,[a] K. A. HARTNETT,[a] K. KANDLER,[a,b] M. HERSHFINKEL[c] AND E. AIZENMAN[a,c*]

[a]*Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*

[b]*Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*

[c]*Department of Morphology, Ben-Gurion University, Faculty of Health Sciences, Beer-Sheva 84105, Israel*

Abstract—The major outward chloride transporter in neurons is the potassium chloride co-transporter 2 (KCC2), critical for maintaining an inhibitory reversal potential for $GABA_A$ receptor channels. In a recent study, we showed that Zn^{2+} regulates $GABA_A$ reversal potentials in the hippocampus by enhancing the activity of KCC2 through an increase in its surface expression. Zn^{2+} initiates this process by activating the Gq-coupled metabotropic Zn^{2+} receptor/G protein-linked receptor 39 (mZnR/GPR39). Here, we first demonstrated that mZnR/GPR39 is functional in cortical neurons in culture, and then tested the hypothesis that the increase in KCC2 activity is mediated through a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process. We established the presence of functional mZnR in rat cultured cortical neurons by loading cells with a Ca^{2+} indicator and exposing cells to Zn^{2+}, which triggered consistent Ca^{2+} responses that were blocked by the Gq antagonist YM-254890, but not by the metabotropic glutamate receptor antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG). Importantly, Zn^{2+} treatment under these conditions did not increase the intracellular concentrations of Zn^{2+} itself. We then measured KCC2 activity by monitoring both the rate and relative amount of furosemide-sensitive NH_4^+ influx through the co-transporter using an intracellular pH-sensitive fluorescent indicator. We observed that Zn^{2+} pretreatment induced a Ca^{2+}-dependent increase in KCC2 activity. The effects of Zn^{2+} on KCC2 activity were also observed in wild-type mouse cortical neurons in culture, but not in neurons obtained from mZnR/GPR39$^{-/-}$ mice, suggesting that Zn^{2+} acts through mZnR/GPR39 activation to upregulate KCC2 activity. We next transfected rat cortical neurons with a plasmid encoding botulinum toxin C1 (Botox C1), which cleaves the SNARE proteins syntaxin 1 and synaptosomal-associated protein 25 (SNAP-25). Basal KCC2 activity was similar in both transfected and non-transfected neurons. Non-transfected cells, or cells transfected with marker vector alone, showed a Zn^{2+}-dependent increase in KCC2 activity. In contrast, KCC2 activity in neurons expressing Botox C1 was unchanged by Zn^{2+}. These results suggest that SNARE proteins are necessary for the increased activity of KCC2 after Zn^{2+} stimulation of mZnR/GPR39.

Key words: zinc, metabotropic zinc receptor, GPR39, KCC2, botulinum toxin, SNARE.

Zn^{2+} is found in glutamate-containing synaptic vesicles in neurons that are abundant in the cerebral cortex, limbic structures and auditory brainstem, among other regions (Frederickson et al., 2005; Sensi et al., 2009). Vesicular Zn^{2+} has been shown to be released in a calcium- and activity-dependent manner (Qian and Noebels, 2005, 2006) and can reduce postsynaptic neuronal excitability (Vogt et al., 2000; Smart et al., 2004; Sensi et al., 2009). As such, possible anticonvulsant actions of the metal have been noted (Fukahori and Itoh, 1990; Elsas et al., 2009). Vesicular Zn^{2+} has also been shown to regulate the Ca^{2+} sensitivity of release at high firing frequencies (Lavoie et al., 2011). Zinc can modify excitability by allosterically modulating both excitatory and inhibitory neurotransmitter receptors (Hosie et al., 2003; Rachline et al., 2005; Paoletti et al., 2009; Sensi et al., 2009). Recent studies have demonstrated that synaptically released zinc can also directly act on a postsynaptic metabotropic zinc receptor/G protein-linked receptor 39 (mZnR/GPR39) (Besser et al., 2009; Chorin et al., 2011). Activation of this receptor initiates innositol triphosphate signaling via a Gq protein, resulting in intracellular calcium release (Hershfinkel et al., 2001). In hippocampal slices, mZnR activation and subsequent calcium liberation leads to upregulation of potassium/chloride co-transporter 2 (KCC2) activity, inducing a hyperpolarizing shift in the $GABA_A$ receptor channel reversal potential (Chorin et al., 2011).

KCC2 is the major outward transporter of chloride in neurons, necessary and sufficient for creating a chloride equilibrium potential negative to the resting membrane voltage (Lu et al., 1999; Lee et al., 2005), thereby rendering $GABA_A$-mediated synaptic potentials inhibitory (Farrant and Kaila, 2007; Viitanen et al., 2010). Increases in KCC2 activity can enhance the inhibitory actions of GABA,

*Correspondence to: E. Aizenman, Department of Neurobiology, University of Pittsburgh School of Medicine, E1456 BST, Pittsburgh, PA 15261, USA. Tel: +1-412-648-9434; fax: +1-412-648-1441.
E-mail address: redox@pitt.edu (E. Aizenman).

Abbreviations: a.u., arbitrary units; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; Botox C1, botulinum toxin C1; DIV, days *in vitro*; DMEM, Dulbecco's modified minimal essential medium; GPR39, G protein-linked receptor 39; HBSS, HEPES-buffered physiological salt solution; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); KCC2, potassium chloride co-transporter 2; KO, knockout; MCPG, (RS)-α-methyl-4-carboxyphenylglycine; mZnR, metabotropic zinc receptor; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TPEN, *N*,*N*,*N'*,*N'*-tetrakis-(2-pyridalmethyl)-ethylenediamine; WT, wild-type.

0306-4522/12 $36.00
10.1016/j.neuroscience.2012.03.001

which may provide high excitatory or epileptic activity with a self-regulating dampening drive (Huberfeld et al., 2007; Zhu et al., 2008; Khirug et al., 2010). In the present study we tested the hypothesis that a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process mediates the enhanced activity of KCC2 after mZnR/GPR39 activation.

EXPERIMENTAL PROCEDURES

Cell culture

Primary cortical cultures obtained from embryonic day 16 Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were prepared as described by Hartnett et al. (1997) in accordance with approved protocols. Embryonic cortices were dissociated with trypsin and, subsequently, the cell suspension was adjusted to 670,000 cells per 35-mm well in six-well tissue culture plates, each containing five 12-mm poly-L-ornithine-treated glass coverslips. Cultures were maintained at 37 °C in 5% CO_2, in a growth medium composed of a volume-to-volume mixture of 80% Dulbecco's modified minimal essential medium (DMEM+ GlutaMAX-1; Sigma-Aldrich, St Louis, MO, USA), 10% Ham's F12-nutrients (*F*-12+GlutaMAX-1; Sigma-Aldrich) and 10% heat-inactivated and iron-supplemented bovine calf serum with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 24 U/ml penicillin and 24 μg/ml streptomycin. Nonneuronal cell proliferation was inhibited after 2 weeks in a culture with 1–2 μm cytosine arabinoside, after which the cultures were maintained in growth medium containing 2% serum without F12-nutrients. Rat cultures were utilized at 18–25 days *in vitro* (DIV).

Mouse cortical neurons were prepared in a similar manner utilizing E15–E17 GPR39$^{-/-}$ or wild-type mice from the same genetic background (Moechars et al., 2006). The resulting cell suspension was prepared in growth medium and was adjusted to 1.2×10^6 cells per 35-mm well (5 poly-L-ornithine-treated glass coverslips each). At 3 DIV the growth medium was changed to one devoid of L-glutamine and with the addition of 25% Neurobasal medium+2.5% B27 Supplements. Mouse cortical neurons were utilized at 7–12 DIV. The presence of KCC2 at this developmental stage was confirmed by immunoblots (see later in the text) and with functional assays (Titz et al., 2006; Hershfinkel et al., 2009; Chorin et al., 2011).

Fluorescence imaging

Cells were visualized using an epifluorescence microscope and perfused with a HEPES-buffered physiological salt solution (HBSS: 115 mM NaCl, 2.5 mM KCl, 2.0 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, 1 mM $CaCl_2$; pH 7.2). The response time course to the agents added via the perfusion were somewhat slower than those reported earlier (Besser et al., 2009; Chorin et al., 2011). This is caused by differences in the speed of perfusion and larger recording chamber volume used in the present work. Images were acquired by exciting fluorescent dyes every 10 s using a computer-controlled monochromator (Polychrome II; TILL Photonics, Martinsried, Germany) and CCD camera (IMAGO; TILL Photonics). All measurements of relative fluorescence units were background corrected. Each field under observation (20×) contained approximately 15–30 neurons, which were selected as regions of interest (ROI) to form individual fluorescence traces. Traces obtained from all neurons in a single coverslip were averaged and considered a single data point, except during transfection studies, where transfected (DsRed+, see later in the text) and non-transfected cells from the same coverslip were evaluated independently. All results are expressed as the mean±SEM.

To measure intracellular free Zn^{2+}, neurons were loaded with FluoZin-3 AM (5 μM for 30 min; prepared in HBSS), a non-ratiometric (excitation: 485 nm, emission: 520 nm), highly Zn^{2+}-selective fluorescent indicator. After acquisition of baseline fluorescence (~5 min), a treatment of 200 μM $ZnCl_2$, a concentration sufficient to activate the mZnR/GPR39 (Hershfinkel et al., 2001; Besser et al., 2009), was perfused into the chamber for 2.5 min and subsequently monitored for 10 min in Zn^{2+}-free HBSS. Cells were also treated with 200 μM $ZnCl_2$ plus 5 μM pyrithione, a zinc ionophore, for 2.5 min. Neuronal free Zn^{+2} was then chelated by 20 μM *N,N,N',N'*-tetrakis-(2-pyridalmethyl)-ethylenediamine (TPEN), a membrane-permeant Zn^{+2} chelator. Δ*F* values were measured as the difference between the average baseline fluorescence before the treatment and the average of data points encompassing the peak fluorescence after each treatment.

The ratiometric, calcium sensitive, fluorescent dye Fura-2 AM (5 μM for 30 min; prepared in HBSS) was used to measure intracellular Ca^{2+} in cortical neurons after activation of the mZnR/GPR39. Fluorescent measurements at 510 nm emission were taken as a ratio of the signals obtained upon excitation by 340 nm/380 nm. Baseline fluorescence was measured for 5 min and cells were subsequently treated with 200 μM $ZnCl_2$ and then 300 μM glutamate (2.5 min each). The Δ*F* for each treatment was measured as the difference between the average baseline fluorescence just before the treatment and at peak after the treatment was administered. A separate group of coverslips was pretreated with 10 μM YM-254890, a Gq antagonist (Takasaki et al., 2004), for 15 min, directly before being placed into the perfusion chamber.

KCC2 activity was measured using the ratiometric, pH-sensitive fluorescent dye BCECF-AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester) during exposure to 5 mM NH_4Cl (Titz et al., 2006; Hershfinkel et al., 2009; Chorin et al., 2011). NH_4^+, as a surrogate for K^+, can be transported into neurons by KCC2 and induce a decrease in intracellular pH. Cells were loaded with 1.5 μM BCECF-AM (prepared in HBSS containing 1% BSA) for 15 min and rinsed for 20 min. Fluorescent measurements were taken as a ratio of the signals obtained upon excitation by 440 nm/485 nm using a 510 nm emission filter. Baseline fluorescence was monitored for 5 min, and then cells were treated with NH_4Cl (5 mM, 5 min). To monitor the effects of Zn^{2+} on KCC2 activity $ZnCl_2$ (200 μM, 2 min) was added, followed by a 1 min rinse, before addition of NH_4Cl. Some cells were treated with bumetanide (1 μM), furosemide (100 μM) or BAPTA-AM (13 mM). The activity of KCC2 was measured as both the rate of change in BCECF fluorescence (slope; a.u./min, where a.u. are arbitrary units of fluorescence) and the total change in intracellular BCECF fluorescence (Δ*F* a.u.; see Fig. 1). Note that results are presented as positive numbers for clarity, although acidification causes decreases in BCECF fluorescence.

Neuronal transfection

Rat cortical neurons at DIV19-23 were transfected in a 24-well plate with 1.5 μg of plasmid DNA (1 part DsRed-expressing plasmid: 1 part botulinum toxin C1-expressing plasmid or empty parent vector) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). DNA-lipid complexes were allowed to form for 30 min at room temperature before addition to the cortical cultures for approximately 3.5–4 h during which time cells were maintained at 37 °C, 5% CO_2. Twenty-four hours after the transfection, cells were utilized for imaging studies as described previously in the text.

Genotyping and immunoblotting

GPR39$^{-/-}$ (Moechars et al., 2006) were kindly provided by Diederik Moechars (Janssen Pharmaceutical Companies of Johnson & Johnson, Beerse, Belgium). DNA was isolated from mouse-tail biopsy samples utilizing the Genetra Puregene Mouse Tail Kit (Qiagen Inc., Valencia, CA, USA). Wild-type (WT) selection primers, 5′ACCCTCATCTTGGTGTACCT3′ and 5′ATGTAG-CGCT-CAAAGCTGAG3′, and knockout (KO) selection primers, 5′