Erratum

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In the version of this article published in the June issue, 2008, the authors misspelled the last name of the third author as Makoto Kanazaki. The correct name is Makoto Kanzaki (M. Kanzaki). The error was corrected in the online version (PDF), which now differs from the printed version.
Electric Pulse Stimulation Induces NMDA Glutamate Receptor mRNA in NIH3T3 Mouse Fibroblasts

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Excess glutamate and Ca\(^{2+}\) influx into neurons exacerbate brain damage such as ischemia. Astrocytes at the site of damage proliferate and attenuate the glutamate- and Ca\(^{2+}\)-induced neuronal damage by removing excess glutamate and Ca\(^{2+}\) through the N-methyl-D-aspartate (NMDA) glutamate receptor and the L-type Ca\(^{2+}\) channel, respectively. Fibroblasts are commonly mobilized to the site of damage, probably supporting the restoration process. Notably, fibroblasts express the L-type voltage-sensitive Ca\(^{2+}\) channel, but not central nervous system-specific NMDA glutamate receptor. We examined if electric pulse stimulation (EPS) was capable of inducing NMDA receptor on fibroblasts by way of Ca\(^{2+}\) channel activation, so that they could potentially have a neuroprotective role. To activate L-type Ca\(^{2+}\) channel, we delivered electric pulse to cultured NIH3T3 mouse fibroblasts. EPS of 20 V with a pulse duration of 2 msec at a frequency of 1 Hz for more than 1 h up to 24 h successfully introduced Ca\(^{2+}\) into NIH3T3 fibroblasts as detected by Fluo-4AM calcium imaging, which was totally inhibited by a L-type Ca\(^{2+}\) channel inhibitor, verapamil. Remarkable expression of NMDA receptor mRNA in the fibroblasts after 24-h EPS was demonstrated by RT-PCR. Verapamil treatment during EPS totally abrogated the EPS-induced NMDA receptor mRNA expression. To the best of our knowledge, this is the first report showing that electric pulse is able to induce sustained Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channel in a non-excitatory fibroblast, which leads to the expression CNS-specific NMDA receptor mRNA. Neuroprotective role of NMDA receptor induced in fibroblasts needs to be further examined.

NIH3T3; fibroblasts; electric pulse stimulation (EPS); Ca\(^{2+}\) influx; NMDA receptor.

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During brain injury such as stroke or several chronic neurodegenerative diseases, glutamate is released from damaged neurons and accumulates in the extracellular space. Glutamate, the major excitatory neurotransmitter in the mammalian brain, leads to the death of neurons whenever its concentration increases and its receptors are active for prolonged periods. Stimulation of
N-methyl-D-aspartate (NMDA) glutamate receptors by increased glutamate triggers neuronal death by a process known as excitotoxicity (Olney et al. 1971; Choi et al. 1988; Koh et al. 1990). Entry of calcium through the NMDA receptor subtype is considered to contribute to the induction of excitotoxic neuronal death. Increases in intracellular calcium level induce the activity of several intracellular enzymatic processes involved in the degradation of cell components, such as endonucleases, phospholipases, and proteases (Lafon-Cazal et al. 1993; Mattson 2007). Calpains are calcium-activated cysteine proteases involved in physiological and pathological processes (Oliver et al. 1989; del Cerro et al. 1990; Denny et al. 1990; Vanderklish et al. 1996) and are involved in necrotic cell death through the degradation of several substrates essential for cell survival, including enzymes, transcription factors, receptors, transporters, channels, and cytoskeletal proteins such as spectrin (Wang 2000; Goll et al. 2003). Calpain activation has been implicated in excitotoxicity triggered under pathological conditions such as ischemia (Seubert et al. 1989; Lee et al. 1991; Rami and Krieglstein 1993).

During brain or central nervous system (CNS) injury, neuroprotective processes are known to be triggered, although their effects are generally not sufficient. CNS or brain injury facilitates astrogial proliferation a process well known as gliosis (Kajihara et al. 2001). These injury-reactive astrocytes may play a major role in neuroprotection. Accumulated glutamate may be taken up by astrogial NMDA receptors and then converted into non-toxic glutamine by glia-specific glutamine synthetase (GS) attenuating the excitotoxicity of extracellular glutamate (Rothstein et al. 1996). Moreover, the up-regulated L-type Ca channels in reactive astrocytes after brain injury in mice were suggested to maintain ionic homeostasis in injured brain regions (Westenbroek et al. 1998).

Fibroblast infiltration after CNS injury such as ischemia or trauma is generally considered as to compromise neuronal regeneration and restoration (Fernandez and Pallini 1985). Interestingly, however, fibroblasts express L-type Ca channel (Davidson and Guo 2000; Westenbroek et al. 2004), the role of which has yet to be documented. Therefore, we have postulated that the L-type Ca channel may play a protective role in the CNS after injury as well as those on astrocytes. As L-type Ca channels are voltage-dependent, we delivered electric pulse stimulation (EPS) to cultured NIH3T3 mouse fibroblasts and examined its potential neuroprotective role. Assuming similarity between fibroblasts and astrocytes, we examined the expression of putatively protective NMDA glutamate receptor mRNA after Ca channel activation by electric pulse stimulation.

Materials and Methods

Animals

This study was performed according to the Guidelines and Regulations for Laboratory Animal Care of Tohoku University Graduate School of Medicine. Normal male mice (C57BL/6CR, 6 weeks old) were housed in a cage at a controlled temperature of 20-23°C under a 12:12-h light-dark cycle. The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium at 50 mg/kg body mass, and then the whole brain was rapidly isolated.

Cell line and culture conditions

The NIH3T3 (TKG0297) mouse fibroblast cell line was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan). NIH3T3 cells were grown on 8-well plates (BD Biosciences, San Jose, CA, USA) for RT-PCR and western blotting or on 35-mm glass-bottomed dishes (Matsunami glass ind., Osaka) for calcium imaging in 10% FBS (Gibco, Carlsbad, CA, USA) in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO). Mouse neuro2a (N2A) cells were obtained from Health Science Research Resources Bank (HSRRB, Osaka).

Electrical cell stimulation

Twenty-four-hour EPS was applied to the cells in the C-Dishes (IonOptix, Milton, MA, USA) using a C-Pace pulse generator (C-Pace 100; IonOptix). The stimulator was set at a pulse voltage of 20 V, pulse frequency of 1 Hz, pulse duration of 2 ms, pulse train of 115 min, and 5-min of rest, as described previously (Fujita et al. 2007). The cells in C-Dishes were maintained in a 5% CO2 incubator at 37°C. During intracellular Ca2+...
visualization, electric pulses (1 Hz, 2 msec pulse duration) were applied using a MyoPacer (Field Stimulator; IonOptix).

Reagents

NIH3T3 cells were treated with the phenylalkylamines calcium channel blocker verapamil (concentration, 10^{-5} M; Molecular Probes, Eugene, OR, USA), which binds specifically to the α1-subunit of the L-type calcium channel. The intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM; 10^{-3} M; Sigma St. Louis, MO, USA) was applied to the cells after electric pulse stimulation to determine the viability of the cells.

RNA preparation and semi-quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted from cells using Trizol Reagent (Molecular Probes), in accordance with the manufacturer’s instructions. Extracted total RNA (1 μg in each 20 μl sample) was subjected to reverse transcription reaction to obtain cDNA using an RT Kit Rever Tra Dash (TOYOBO, Osaka). A cDNA template, 1 or 2 μl in a total volume of 24 or 25 μl containing Platinum Taq DNA polymerase (Invitrogen, Frederick, MD, USA) was amplified by PCR under the following condition: 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. PCR amplicons were selected at different cycles and compared to ensure that the amplifications was within the linear range. The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV transilluminator. The primer sequences used are shown in Table 1.

Calcium imaging

Cells were incubated at 37°C in the dark for 20 min in the presence of 2.5 μM fluo4-AM (Dojindo Laboratories, Kumamoto) in 0.1% dimethylsulfoxide in DMEM. Excitation at 488 nm and image acquisition were performed with a confocal microscope (Fluoview1000; Olympus, Tokyo). Experiments were performed at room temperature.

RESULTS

EPS induces an increase in intracellular Ca^{2+} concentration via L-type voltage dependent calcium channels

We detected the expression of L,P,T-type voltage-dependent calcium channel mRNAs in NIH3T3 by RT-PCR analysis (Fig. 1), and L-type calcium channel mRNA was the most abundant species.

Using cells loaded with the Ca^{2+} indicator Fluo-4 AM, we found that EPS induced an increase in intracellular Ca^{2+} concentration, as indicated by the increase in Fluo-4 AM intensity in NIH3T3 cells (Fig. 2A). One hour of EPS did not induce morphological alterations (Fig. 2A). Continuation of EPS up to 24 h induced cellular swelling as well as an increase in the Ca concentration. The calcium channel blocker verapamil, which binds specifically to the α1-subunit of the L-type calcium channel, completely disrupted both EPS-induced increase in intracellular Ca^{2+} and swelling (Fig. 2B). We also confirmed that NIH3T3 cells treated with BAPTA after 24-h EPS showed extensive reduction of intracellular Fluo-4 AM intensity (data not shown), indicating that

<table>
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<tr>
<th>Target</th>
<th>Forward primer sequence (5'-3')</th>
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<td>Cav3.3</td>
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24-h EPS did not induce cell death.

**Expression of CNS-specific NMDA receptor mRNA was detected by semi-quantitative RT-PCR**

We found that EPS induced the expression of CNS-specific NMDA receptor NR1 subunit mRNA in NIH3T3 fibroblasts. The NMDA receptor is a complex consisting of two obligatory NR1 subunits, which have eight splice variants, and up to two of four NR2 subunits (NR2A–NR2D). According to the development and activity, the component of NMDA receptor complexes changes but NR1 subunit is expressed invariably (van Zundert et al. 2004). To examine whether the expression NMDA receptor mRNA is dependent on calcium signaling, we examined the effects of treatment with verapamil. Treatment with 10^{-5} M verapamil under conditions of 24-h EPS blocked the expression of NMDA receptor mRNA (Fig. 3). These results suggest that the expression of NMDA receptor mRNA is induced by Ca^{2+} influx via L-type voltage-sensitive calcium channels.

**DISCUSSION**

To the best of our knowledge, this is the first report showing that electric pulse is capable of inducing sustained Ca^{2+} influx via L-type calcium-sensitive channel in a non-excitatory fibroblast. Moreover, no study has ever demonstrated that electric stimulation lead to the expression of CNS-specific NMDA receptor mRNA in a non-excitatory fibroblast. As the expression of NMDA receptor mRNA was blocked by L-type calcium-sensitive channel antagonist, NMDA receptor transcription was suggested to be dependent on increases in the calcium concentration through voltage-dependent Ca influx through L-type calcium channels.

The NMDA receptor has a dual nature as a mediator of excitotoxic cell death and a mediator of activity-dependent cell survival, which likely results from divergent patterns of kinase activation, transcription factor activation, and gene expression in neurons (Lee et al. 2005). Interestingly, the activation of NMDA receptors causes depolarization, which in turn causes swelling in neurons (Isokawa 2005). Glutamate, at similar concentrations required to induce neuronal cell death, has also been shown to increase cell volume in cultured astrocytes (Han et al. 2004). As NMDA-antagonists significantly decrease seizure-related astrocyte swelling in the cerebral cortex (Szakacs et al. 2003), it is likely that cellular swelling represents the activity of NMDA glutamate receptors. In this study, we did not directly examine the function of NMDA glutamate receptors, but as we observed cellular swelling of NIH3T3 after 24-h EPS when NMDA mRNA was clearly detected, we assumed that EPS had not only induced transcription of NMDA receptor mRNA, but also expression of functional receptors on NIH3T3 cells. It should be noted that no cellular swelling was detectable at 1 h of EPS.

Astrocytes have neuroprotective effects through glia-specific enzyme glutamine synthases (Muscoli et al. 2005; Pertusa et al. 2007). In this study, we did not demonstrate neuroprotective function of NIH3T3 cells exposed to EPS, but the similarity with astrocytes in which EPS resulted in fibroblast-induced functional NMDA receptor expression by activation of L-type Ca channels may represent their potential neuroprotective
Fig. 2. \( \text{Ca}^{2+} \) influx via L-type calcium-sensitive channel in NIH3T3 fibroblasts. (A) Cells were loaded with the calcium indicator dye Fluo-4 AM. Confocal image of the same field of view of NIH3T3 cells during the experiment for 1 h at room temperature with EPS. (B) L-type calcium channel blocker verapamil disrupted EPS-induced increases in intracellular \( \text{Ca}^{2+} \) and swelling. The images shown are from representative fields of each sample. (C) The number of nuclei with fluorescence intensity over 2,000 were counted. The means(± s.d.) of at least three independent experiments are shown. * Significantly different \( (p < 0.01) \) from the non-EPS and EPS + verapamil.
function in the wounds. Further studies are required to confirm these findings.

In summary, EPS to NIH3T3 fibroblasts induced Ca\(^{2+}\) influx via L-type calcium-sensitive channels, which induced NMDA receptor mRNA and cellular swelling, suggesting the expression of functional NMDA glutamate receptors represents a potential neuroprotective role of fibroblasts in wounds.

Acknowledgments

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References


