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Ifenprodil, a NR2B-selective antagonist of NMDA receptor, inhibits reverse Na^+/Ca^{2+} exchanger in neurons

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ABSTRACT

Glutamate-induced delayed calcium dysregulation (DCD) is causally linked to excitotoxic neuronal death. The mechanisms of DCD are not completely understood, but it has been proposed that the excessive influx of external Ca^{2+} is essential for DCD. The NMDA-subtype of glutamate receptor (NMDAR) and the plasmalemmal Na^+/Ca^{2+} exchanger operating in the reverse mode (NCX_{rev}) have been implicated in DCD. In experiments with "younger" neurons, 6–8 days in vitro (6–8 DIV), in which the NR2A-containing NMDAR expression is low, ifenprodil, an inhibitor of NR2B-containing NMDAR, completely prevented DCD whereas PEAQX, another NMDAR antagonist that preferentially interacts with NR2A-NMDAR, was without effect. With "older" neurons (13–16 DIV), in which NR2A- and NR2B-NMDARs are expressed to a greater extent, both ifenprodil and PEAQX applied separately failed to prevent DCD. However, combined application of ifenprodil and PEAQX completely averted DCD. Ifenprodil and ifenprodil-like NR2B-NMDAR antagonists Ro 25-6981 and Co 101244 but not PEAQX or AP-5 inhibited gramicidin- and Na^+/NMDG-replacement-induced increases in cytosolic Ca^{2+} mediated predominantly by NCX_{rev}. This suggests that ifenprodil, Ro 25-6981, and Co 101244 inhibit NCX_{rev}. The ability of ifenprodil to inhibit NCX_{rev} correlates with its efficacy in preventing DCD and emphasizes an important role of NCX_{rev} in DCD. Overall our data suggest that both NR2A- and NR2B-NMDARs are involved in DCD in "older" neurons, and it is necessary to inhibit both NMDARs and NCX_{rev} to prevent glutamate-induced DCD.

1. Introduction

Glutamate excitotoxicity is a key component in a variety of neuropathologies including stroke, traumatic brain injury, and age-related neurodegenerations (Bramlett and Dietrich, 2004; Hazell, 2007; Salinska et al., 2005). A sustained increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), or delayed Ca^{2+} dysregulation (DCD), represents a major detrimental factor in glutamate excitotoxicity (Nicholls and Budd, 1998; Tymianski et al., 1993a). Two major hypotheses concerning the mechanism of DCD postulate that in neurons exposed to glutamate an increase in $[Ca^{2+}]_c$ occurs predominantly due to influx of external Ca^{2+} via activated N-methyl-D-aspartate (NMDA)-subtype of glutamate receptors (NMDAR) (Tymianski et al., 1993b) or via the reverse Na^+/Ca^{2+} exchanger (NCX_{rev}) (Hoyt et al., 1998; Kiedrowski, 1999). Significant efforts were aimed at developing neuroprotection based on NMDAR and NCX_{rev} antagonists (Scatton, 1994). However, earlier studies with high-affinity NMDAR antagonists such as MK801 revealed serious problems associated with strong inhibition of vitally important glutamate neurotransmission leading to increased likelihood of neurodegenerations (Ikonomidou et al., 1999; Lipton, 2004). In contrast, the lack of selective and efficacious inhibitors of neuronal NCX_{rev} significantly hindered development of neuroprotective strategies based solely on NCX_{rev} inhibition (Jeffs et al., 2007).

NMDARs are formed by heteromeric complexes consisting of NR1 and NR2 subunits (McBain and Mayer, 1994). The NR1 subunit is abundantly expressed in CNS (Janssens and Lesage, 2001). The NR2 subunit that contains the glutamate binding site has four different isoforms, encoded by different genes *NR2A–NR2D*

Abbreviations: DCD, delayed Ca^{2+} dysregulation; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; NCX, Na^+/Ca^{2+} exchanger; NCX_{rev}, reverse NCX; PEAQX, ({[(1S)-1-(4-bromophenyl)ethyl]amino}-(2,3-dioxo-1,4-dihydroquinoxalin-5-yl) methyl)phosphonic acid; NMDG, N-methyl-D-glucamine; GPT, glutamate pyruvate transaminase; AP-5, D-(-)-2-amino-5-phosphonopentanoic acid; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid); TTX, tetrodotoxin.

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(Cull-Candy et al., 2001). Ifenprodil was found to be the first neuroprotective agent selective for NR2B-containing NMDARs (NR2B-NMDARs) (Carter et al., 1988, 1989; Williams, 1993). Importantly, ifenprodil increases the potency of protons to block the NMDAR (Mott et al., 1998) and protects neurons against glutamate excitotoxicity in an activity-dependent manner (Kew et al., 1996). This mechanism was proposed to significantly contribute to ifenprodil efficacy and the lack of unwanted side effects of this drug (Scatton, 1994).

In our previous study, we found that both NMDAR and NCX_{rev} contribute to DCD in neurons exposed to glutamate and, consequently, both Ca^{2+} influx mechanisms have to be inhibited to prevent DCD (Brittain et al., 2012). Ifenprodil inhibits DCD in younger neurons exposed to glutamate (Stanika et al., 2009). This effect was attributed to ifenprodil-mediated inhibition of NR2B-NMDAR. However, whether ifenprodil inhibits NCX_{rev} is unknown. In the present study, we hypothesized that ifenprodil as well as ifenprodil-like NR2B-selective NMDAR antagonists Ro 25-6981 and Co 101244, in addition to antagonizing NR2B-NMDAR, also inhibit NCX_{rev}. The obtained results support this hypothesis and suggest that ifenprodil, Ro 25-6981, and Co 101244 suppress NCX_{rev} activity.

2. Materials and methods

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.1. Materials

Glutamate, glycine, and gramicidin were purchased from Sigma (St. Louis, MO). Fura-2FF-AM and Fura-2-AM were from Teflabs (Austin, TX). Fluo-4FF-AM and SBFI-AM were from Invitrogen (Carlsbad, CA). Ifenprodil and PEAQX were from Sigma. Ro 25-6981 and Co 101244 were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culturing

Primary cultures of hippocampal neurons were prepared from postnatal day 1 rat pups, according to Institutional Animal Care and Use Committee (IACUC) approved protocol. For fluorescence measurements, neurons were plated on glass-bottomed Petri dishes without preplated glia as previously described (Dubinsky, 1993). For all platings, 35 μg/ml uridine plus 15 μg/ml 5-fluoro-2′-deoxyuridine were added 24 h after plating to inhibit proliferation of non-neuronal cells. Neuronal cultures were maintained in a 5% CO_2 atmosphere at 37 °C in Earl's MEM supplemented with 10% NuSerum (BD Bioscience, Bedford, MA), 27 mM glucose, and 26 mM $NaHCO_3$ (Dubinsky et al., 1995).

2.3. Fluorescence imaging

In our experiments, we used "younger" hippocampal neurons grown for 6–8 days in vitro (6–8 DIV) and "older" neurons grown for 13–16 DIV. The standard bath solution contained 139 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM NaHEPES, pH 7.4, 5 mM glucose, and 65 mM sucrose. Sucrose was used to maintain osmolarity similar to that in the growth medium (340 mosm) (Wang and Thayer, 1996; White and Reynolds, 1996). Fluorescence imaging was performed with a Nikon Eclipse TE2000-U inverted microscope using a Nikon objectives Plan Fluor 20 × 0.45 NA or Super Fluor 40 × 1.3 NA and an EM-CCD Hamamatsu C9100-12 camera (Hamamatsu Photonic Systems, Bridgewater, NJ) controlled by Simple PCI software 6.1 (Compix Inc., Sewickley, PA) or Photometrics Cool $SNAP_{HQ}$ camera (Roper Scientific, Tucson, AZ) controlled by MetaFluor software 6.3 (Molecular Devices, Downingtown, PA). The excitation light was delivered by a Lambda-LS system (Sutter Instruments, Novato, CA). To minimize photobleaching and phototoxicity, the images were taken every 15 s during the time-course of the experiment.

For fluorescence microscopy experiments, neurons were loaded with either 2.6 μM Fura-2AM (Figs. 1 and 2 and Suppl. Figs. 1 and 2) or 2.6 μM Fura-2FF-AM (Figs. 4–7 and Suppl. Fig. 5) for 60 min at 37 °C in the presence of 0.015% Pluronic F-127. The excitation filters (340 ± 5 and 380 ± 7 nm) were controlled by a Lambda 10-2 optical filter changer (Sutter Instruments, Novato, CA). Fluorescence was recorded from individual neurons through a 505 nm dichroic mirror at 535 ± 25 nm. The changes in $[Ca^{2+}]_c$ were monitored by following Fura-2 or Fura-2FF F_{340}/F_{380} ratio. Alternatively, the changes in $[Ca^{2+}]_c$ were monitored simultaneously with changes in $[Na^+]_c$ using a Ca^{2+}-sensitive fluorescent dye Fluo-4FF-AM and a Na^+-sensitive dye SBFI-AM (Suppl. Fig. 4). Neurons were loaded simultaneously with 2.5 μM Fluo-4FF-AM for 30 min and 9 μM SBFI-AM for 1 h at 37 °C. The excitation wavelengths were 340 ± 5 and 380 ± 7 nm for SBFI and 480 ± 20 nm for Fluo-4FF. Fluorescence was recorded from individual neurons through a 505 nm dichroic mirror at 535 ± 25 nm. The changes in $[Na^+]_c$ were monitored by following SBFI F_{340}/F_{380} ratio. The changes in $[Ca^{2+}]_c$ were monitored by following Fluo-4FF F_{480} and normalized as F/F_0. Quantification of Fluo-4FF signals was carried out following manufacturer instructions (http://probes.invitrogen.com/media/pis/mp01240.pdf). $[Ca^{2+}]_c$ and $[Na^+]_c$ were calculated using Grynkiewicz method (Grynkiewicz et al., 1985) assuming K_d for Fura-2 is 0.225 μm, for Fura-2FF is 5.5 μM, for Fluo-4FF is 9.7 μM, and for SBFI is 11.3 mM. In all experiments, the background was subtracted from fluorescence signals. Since Ca^{2+} binding and spectroscopic properties of fluorescent dyes can vary significantly in intracellular environment, the presented cytosolic Ca^{2+} concentrations should be considered estimates as stated previously by other investigators (Dietz et al., 2007; Stanika et al., 2009).

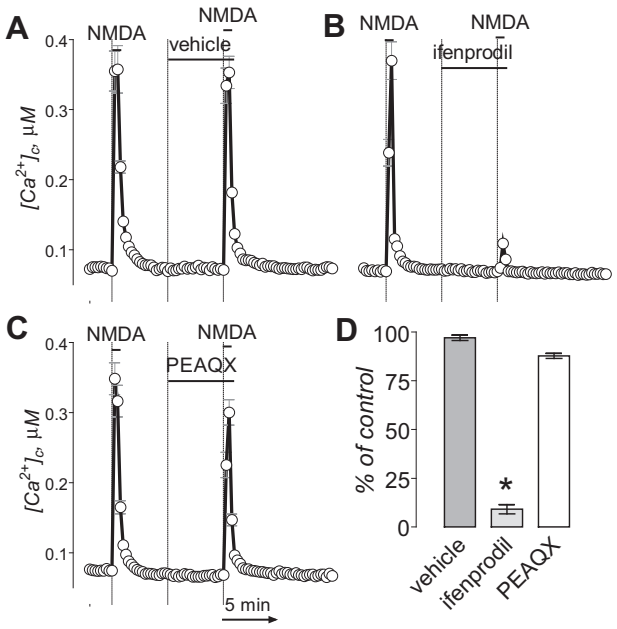

Fig. 1. In "younger" neurons (6–8 DIV), ifenprodil completely inhibited Ca^{2+} influx induced by NMDA. The bath solution was supplemented with 1 μM tetrodotoxin and 5 μM nifedipine. Neurons were loaded with 2.6 μM Fura-2AM. In these experiments, we used two 30-s NMDA (30 μM, plus 10 μM glycine) pulses. The inhibitors or vehicle were applied 5 min before the second NMDA pulse and amplitude of $[Ca^{2+}]_c$ increase was compared to $[Ca^{2+}]_c$ increase in response to the first NMDA pulse. In A-C, where indicated, vehicle (0.2% DMSO), ifenprodil (1 μM) or PEAQX (5 μM) were applied. NMDA (30 μM, plus 10 μM glycine) was applied twice for 30 s as indicated. The Ca^{2+} influx into neurons was evaluated by measuring amplitude of the increases in $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ was calculated using the Grynkiewicz method (Grynkiewicz et al., 1985). The time scale shown in panel C is applicable to traces in A and B. In D, statistical analysis of the Ca^{2+} influx inhibition. Data are mean ± SEM, $^*p < 0.01$ compared to vehicle, $n = 3$.

2.4. Western blot

Cultured hippocampal neurons were washed with PBS and lysed with a solution containing 50 mM Tris–HCl, pH 7.35, 2 mM EDTA, 5 mM dithiothreitol, and 1% Nonidet P-40, and supplemented with a Proteinase Inhibitor Cocktail (Roche, Indianapolis, IN). The lysate was centrifuged in Eppendorf microcentrifuge 5415D at 13,000 rpm for 10 min and total protein was determined in the supernatant using Bradford assay (Bio-Red Laboratories, Hercules, CA). Aliquots of this solution 30 μg/line were loaded onto 3–8% Tris-Acetate gel (Invitrogen, Carlsbad, CA). Electrophoresis and protein transfer onto Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) was performed as described for the NuPage electrophoresis system (Invitrogen). The membranes were blocked with 5% BSA and 0.15% Triton X-100 in phosphate-buffered saline (PBS), pH 7.0, incubated for an hour at room temperature with one of the following primary antibodies: anti-NR2B rabbit polyclonal antibody or anti-NR2A rabbit polyclonal antibody (Millipore, Temecula, CA) at 1:2000 dilution. Blots were developed using goat anti-rabbit or goat anti-mouse IgG (1:20000) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and Supersignal West Pico chemiluminescent reagent (Pierce, Rockford, IL). Molecular weight marker HiMark™ Pre-Stained Standards (15 μl, Invitrogen) were used to determine molecular weights of the