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NMDA and non-NMDA receptor-mediated differential Ca^{2+} load and greater vulnerability of motor neurons in spinal cord cultures

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Abstract

Glutamate receptor activated neuronal cell death has been implicated in the pathogenesis of motor neuron disease but the molecular mechanism responsible for neuronal dysfunction needs to be elucidated. In the present study, we examined the contribution of NMDA and non-NMDA sub-types of glutamate receptors in selective vulnerability of motor neurons. Glutamate receptor activated Ca^{2+} signaling, mitochondrial functions and neurotoxicity in motor neurons and other spinal neurons were studied in mixed spinal cord primary cultures. Exposure of cells to glutamate receptor agonists glutamate, NMDA and AMPA elevated the intracellular Ca^{2+}, mitochondrial Ca^{2+} and caused mitochondrial depolarization and cytotoxicity in both motor neurons and other spinal neurons but a striking difference was observed in the magnitude and temporal patterns of the $[Ca^{2+}]_i$ responses between the two neuronal cell types. The motor neurons elicited higher Ca^{2+} load than the other spinal neurons and the $[Ca^{2+}]_i$ levels were elevated for a longer duration in motor neurons. AMPA receptor stimulation was more effective than NMDA. Both the NMDA and non-NMDA receptor antagonists APV and NBQX inhibited the Ca^{2+} entry and decreased the cell death significantly; however, NBQX was more potent than APV. Our results demonstrate that both NMDA and non-NMDA sub-types of glutamate receptors contribute to glutamate-mediated motor neuron damage but AMPA receptors play the major role. AMPA receptor-mediated excessive Ca^{2+} load and differential handling/regulation of Ca^{2+} buffering by mitochondria in motor neurons could be central in their selective vulnerability to excitotoxicity.
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1. Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system and plays an important role in learning and memory and execution of motor acts (Kubo and Ito, 2004). However, excessive release and inadequate uptake of synaptic glutamate results in excitotoxicity that is implicated in variety of acute and chronic neurological disorders, viz. ischemia, stroke, epilepsy, Huntington disease, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Choi and Rothman, 1990; Rothstein et al., 1992; Harris et al., 1995; Heath and Shaw, 2002). Glutamate can induce neuronal cell death through activation of three classes of ionotropic glutamate receptors (iGluRs), viz. *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors. There are compelling evidences suggesting that glutamate-mediated neurotoxicity is triggered primarily due to large influx of Ca^{2+} on activation of iGluRs but the immediate calcium dependent events responsible for cell death remain to be elucidated. Earlier reports have suggested that glutamate receptor stimulated rise in intracellular Ca^{2+} may be crucial in activating the calcium dependent cytoplasmic enzymes and mitochondrial Ca^{2+} overload resulting in ROS production and subsequent neuronal death (Nicholls and Budd, 1998; Schinder et al., 1996).

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that is manifested clinically by

Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA); APV, α-2-amino-5-phosphonopentanoic acid; NBQX, 2-3 dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; EMEM, Eagle's minimal essential medium; TMRM, tetramethyl rhodamine methylester; HEPES, *N*-2 hydroxyethyl piperazine-*n*-2 ethanesulphonic acid; PBS, phosphate buffered saline

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muscle weakness and pathologically by selective degeneration of motor neurons in spinal cord, brainstem and cerebral cortex. An increase in glutamate levels in plasma and cerebrospinal fluid (CSF) of patients suffering from ALS has been reported and initiation of neuronal injury by excitatory amino acid glutamate has been proposed as one of the possible cause of this disease. However, the contribution of NMDA and non-NMDA sub-types of glutamate receptors in selective vulnerability of motor neurons is not clearly known. Some reports have suggested that NMDA receptors mediate the motor neuron degeneration (Urushitani et al., 2001; Van Den Bosch and Robberecht, 2000; Van Westerlaak et al., 2001) while other studies have proposed that motor neurons are highly vulnerable to AMPA/Kainate receptor-mediated injury (Carriedo et al., 1996, 2000; Van Damme et al., 2003). The spinal neurons express high levels of NMDA (Tolle et al., 1993) and Ca^{2+} permeable AMPA receptors (Williams et al., 1997) but the role played by the iGluRs in selective injury to motor neurons in preference to other spinal neurons particularly the cell selectivity and receptor specificity during the same excitotoxic insult are not well understood. Moreover, most of the previous studies have been carried out on the effect of either NMDA or AMPA/Kainate receptor agonists on cultured spinal cord or cortical neurons separately but did not provide a comparative study of both kinds of receptor agonists in one model system under similar experimental conditions. We have previously reported that the CSF from ALS patients induces higher $[Ca^{2+}]_i$ and toxicity in motor neurons than in other spinal neurons (Sen et al., 2005). We found that AMPA receptor antagonist NBQX was more potent than NMDA receptor antagonist APV in inhibiting the CSF induced Ca^{2+} influx and toxicity.

The present study was undertaken to understand the relative contribution of NMDA and AMPA receptors in selective vulnerability of motor neurons. Our results demonstrate that in mixed spinal cord cultures motor neurons exhibit significantly greater sensitivity to NMDA as well as AMPA receptor activation as compared to the other spinal neurons. Motor neurons exhibited dramatically higher and prolonged increase in cytosolic and mitochondrial Ca^{2+} irrespective of the glutamate receptor agonist though AMPA was more potent than NMDA. We propose that this differential handling/regulation of glutamate induced Ca^{2+} load could be central in the selective vulnerability of motor neurons to excitotoxicity.

2. Experimental procedure

2.1. Materials

Glutamate, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), 2-amino-5-phosphonopentanoic acid (APV), 7-sulfamoyl-benzo(f)quinoxaline (NBQX), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), oligomycin and Eagle's minimal essential medium (EMEM) were purchased from Sigma–Aldrich, India. Horse serum and fetal bovine serum was obtained from Life Technologies (Grand Island, NY, USA). SMI-32 antibody was from Sternberger Monoclonal Inc., USA, monoclonal anti MAP-2 antibody was from BD Pharmingen, Singapore. The Zenon Alexafluor 488 and Alexafluor 633 antibody labeling kits, Fura-2 acetoxymethyl ester (AM), tetramethylrhodamine methyl ester (TMRM), calcein acetoxymethyl ester (AM), ethidium homodimer and pluronic were purchased from Molecular Probes, Invitogen, USA. All other chemicals were analytical grade and were obtained from commercial sources.

2.2. Spinal cord cultures

Studies were conducted using primary spinal cord cultures from 13- to 15-day-old embryonic Sprague Dawley rats. Animal experiments were carried out in accordance with the guidelines laid down by the institute ethics committee for the care and use of animal for experimental work. Cultures were prepared as described earlier with minor modifications (Sen et al., 2005). The spinal cords were dissected in a sterile laminar flow cabinet under a dissection microscope and meninges were removed. Spinal cord suspensions were then plated at a density of 2.5×10^5 cells/ml (approximately four spinal cords per six Petri dishes). Cells were plated in 35 mm Petri dishes containing 24 mm poly-L-lysine coated coverslips. Plating medium consisted of Eagle's minimal essential medium with 2 mM glutamine, supplemented with 10% horse serum, 10% fetal bovine serum, 25 mM glucose and muscle extract from 2-day-old rat. Cultures were maintained at 37 °C in 5% CO_2 atmosphere. After 4–6 days *in vitro*, cultures were treated with 10 μM cytosine arabinoside for 1–3 days. Subsequently, the cultures were maintained in a medium similar to the plating medium but do not contain fetal bovine serum. Medium was changed twice a week and experiments were performed after 13–15 days *in vitro* cultures.

2.3. Identification of motor neurons in culture

Motor neurons and other spinal neurons in spinal cord culture were identified by immunostaining with monoclonal antibody SMI-32 that labels non-phosphorylated neurofilaments in motor neurons (Gotow and Tanaka, 1994; Carriedo et al., 1996) and antimicrotubule associated protein-2 (MAP-2) antibody which stains all types of neurons. After 13–15 days *in vitro*, the cultures were fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, followed by permeabilization with 0.1% triton X-100 in PBS for 10 min. Cells were then washed three times with PBS, incubated with blocking solution (10% bovine serum albumin in PBS) for 30 min and washed with PBS. Subsequently cells were incubated with prelabeled SMI-32 (Alexafluor 488) and MAP-2 (Alexafluor 633) antibodies (1:500 dilution each) for 30 min. The primary antibodies were labeled with fluorophores using Zenon antibody labeling kits (Molecular Probes, USA). The immunostained cells were visualized by Olympus FV-1000 confocal microscope. For Ca^{2+} imaging experiments, the motor neurons and other spinal neurons were identified by morphological criteria as described by (Schaffner et al., 1987). Cells with somal diameter >20 μm and extensive arborization were considered as motor neurons. At the end of some randomly selected Ca^{2+} imaging experiments cultures were fixed and the identification of motor neurons was confirmed by SMI-32 and MAP-2 staining.

2.4. Imaging of intracellular calcium $[Ca^{2+}]_i$

Intracellular calcium was monitored using the fluorescent indicator Fura-2AM as described earlier (Sen et al., 2005). Cultures were loaded with 5 μM Fura-2AM in HEPES-buffered salt solution (HBSS) containing 0.16% pluronic acid for 45 min at 37 °C, washed three times with HBSS and kept in the dark for an additional 30 min to allow for complete dye desertification. Cultures were then maintained at 25 °C in HBSS containing (in mM): Na^+ 130, K^+ 5.4, Mg^{2+} 0.8, Ca^{2+} 1.8, Cl^- 130.6, HEPES 20 and glucose 15, pH 7.4 and the cover slip was mounted on a perfusion chamber on the stage of an inverted microscope (Olympus IX70, Japan). The bath volume of the chamber was 0.5 ml. Fluorescence images were acquired using an apo 20× or 40× objective (Olympus). Glutamate/NMDA/AMPA (50 μM) was added to the static bath during recording.

Ratiometric fluorescence imaging was performed with a fluorescence imaging system (TILL Photonics, Germany). Fura-2 loaded cells were alternately excited at 340 and 380 nm with polychrome IV, a compact xenon arc lamp source coupled to a high-speed monochromator. The emitted fluorescence was selected with filter. The fluorescence images were acquired with a 12-bit Peltier cooled CCD camera. The data acquisition and analysis was done with TILL Vision software. Fields containing at least one morphologically identified motor neuron and three to five other spinal neurons were selected for imaging. Recording was performed in HBSS buffer without Mg^{2+} to prevent the inactivation of NMDA receptors. $[Ca^{2+}]_i$

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