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Hypoxic injury of isolated axons is independent of ionotropic glutamate receptors

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Axonal injury in white matter is an important consequence of many acute neurological diseases including ischemia. A role for glutamate-mediated excitotoxicity is suggested by observations from *in vitro* and *in situ* models that AMPA/kainate blockers can reduce axonal injury. We assessed axonal vulnerability in primary murine neuronal cultures, with axons isolated from their cell bodies using a compartmented chamber design. Transient removal of oxygen and glucose in the axon compartment resulted in irreversible loss of axon length and neurofilament labeling. This injury was not prevented by addition of ionotropic glutamate receptor blockers and could not be reproduced by glutamate receptor agonists. However, hypoxic injury was prevented by blockade of voltage-gated sodium channels, inhibition of calpain and removal of extracellular calcium. These results suggest that isolated, unmyelinated axons are vulnerable to hypoxic injury which is mediated by influx of sodium and calcium but is independent of glutamate receptor activation.

Keywords: Glutamate; AMPA/kainate; NMDA; Calpain; Axon; Excitotoxicity; Ischemia; Oxygen–glucose deprivation; White matter

Introduction

Ischemic damage to axons in cerebral white matter contributes to neurological dysfunction after stroke, cardiac arrest, and perinatal encephalopathy (Dewar et al., 1999; Volpe, 2001). Axonal injury is also important in brain and spinal cord trauma, multiple sclerosis, and neurodegenerative diseases (Bjartmar and Trapp, 2001; Stys, 2005). Many of the initial ionic events leading to hypoxia-induced loss of axon conduction have been well characterized, largely in isolated preparations of rodent optic nerve (Waxman et al., 1991). Energy failure causes depletion of ATP and axon depolarization. This is followed by accumulation of axoplasmic Na^+ via non-inactivating voltage-gated Na channels (Stys and Lopachin, 1998) and of intra-axonal free Ca^{2+} by activation of voltage-sensitive Ca^{2+} channels (Fern et al., 1995), reversal of Na^+/Ca^{2+} exchange (Li et al., 2000; Brown et al., 2001; Ouardouz et al., 2005), and release from intracellular stores (Ouardouz et al., 2003).

Recent evidence suggests that excessive glutamate receptor activation (or excitotoxicity) may also contribute to white matter injury in several conditions. The immediate target of this injury may be white matter glial cells. In particular, the central nervous system myelin-forming cells, oligodendrocytes, express functional glutamate receptors (Gallo et al., 1994) and can be injured *in vitro* by overactivation of ionotropic AMPA/kainate receptors (Yoshioka et al., 1995; McDonald et al., 1998; Fern and Moller, 2000). New studies suggest that distal oligodendrocyte processes express NMDA receptors which may also contribute to their injury (Salter and Fern, 2005; Karadottir et al., 2005; Micu et al., 2006). Supporting the role of glial excitotoxicity *in vitro*, AMPA/kainate antagonists, such as NBQX, have been shown to reduce oligodendrocyte or myelin loss in white matter in slice and animal models of brain and spinal cord injury (Rosenberg et al., 1999; Li et al., 2000; Follett et al., 2000; Wilke et al., 2004).

In these models, AMPA/kainate blockade prevents injury of white matter axons as well as oligodendrocytes. For example, axonal protection has been observed in cortical brain slice (Tekkok and Goldberg, 2001), isolated spinal cord (Agrawal and Fehlings, 1997; Li et al., 2000), and *in vitro* rodent models including spinal cord trauma (Wrathall et al., 1994), spinal ischemia (Kanellopoulos et al., 2000), stroke (McCracken et al., 2002), and experimental allergic encephalomyelitis (Pitt et al., 2000). Glutamate receptor subunits have been observed in axons (Li et al., 2000), but it is not known whether functional receptors are expressed on axolemma. Therefore, these observations raise the following question: is AMPA/kainate receptor-dependent injury mediated by glutamate action directly on axons, or indirectly, through receptors located on neuronal cell bodies or white matter glial cells? The issue cannot be resolved using standard pharmacological approaches in intact models nor in

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conventional dissociated cell culture systems. Therefore, we examined the vulnerability of isolated axons using a compartmented chamber system (Ivins et al., 1998). Primary murine cortical cultures were plated on one side of a glass coverslip, and axons allowed to project under the coverslip to a different chamber. We examined the vulnerability of isolated cortical axons to oxygen–glucose deprivation (OGD) and assessed the roles of extracellular cations and glutamate. Axon injury was assessed by neurofilament immunocytochemistry and by fluorescence microscopy using neurons derived from mice expressing the green fluorescent protein derivative YFP.

Methods

Cell culture

Cortical neurons were cultured using a two-compartment chamber design which was previously shown to promote neurite outgrowth from the side containing neuronal cell bodies, without chemical exchange between chambers (Ivins et al., 1998). Chambers were constructed of a 5 mm hemisected cylinder of Teflon tubing attached with sterile silicon grease to a glass coverslip barrier; the bottom of each chamber assembly was placed with silicon grease on a 35 mm polystyrene tissue culture dish previously treated with poly-D-lysine and laminin.

Cortices were isolated from E15 Swiss Webster (Charles Rivers, Wilmington, Maryland) or transgenic C57Bl/6 mice expressing YFP under the neuronal Thy1.1 promoter (Feng et al., 2000). The thy1-YFP-16 transgenic line provided high YFP expression in cortical neurons at early developmental ages. This tissue was briefly triturated yielding microexplants that were plated in the inner chamber of the culture array. Both the inner and the outer chamber were supplied with 10% serum containing minimal essential media (MEM)-based media (Gibco, Grand Island, New York) with 20 mM D-glucose, 2 mM glutamine for 14 days. Before experiments were performed, the external media was replaced with 0.04% mM trypan blue for 2 h to establish the chambers' integrity. Those with visible leaks were discarded.

Cell culture toxicity paradigms

Drugs and OGD treatments were applied to the outer, axonal compartment only. Neuronal cell bodies in the inner compartment were maintained in 10% serum-containing media (also containing oxygen and glucose) during axonal toxicity paradigms. Cultures were rinsed three times with serum-free MEM buffer containing 20 mM D-glucose warmed to 37°C and then exposed to drugs diluted in the same media. For experiments with recovery periods, cultures were washed three times with regular media and returned to 37°C. OGD experiments were performed in an anaerobic chamber as described previously (Forma; Goldberg and Choi, 1993).

All drugs were purchased from Sigma (St. Louis, Missouri) except for (s)-AMPA (Tocris Cookson, Ballwin, Missouri) and ALLM (Calbiochem, La Jolla, California). Reagents were dissolved in water or DMSO as specified by the vendor and stored in stock solutions protected from light at −20°C for no more than 3 months until use.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde and 0.025% glutaraldehyde in PBS for 30 min. Cultures were permeabilized with 0.125% Triton X-100 and blocked in 5% normal goat serum (NGS) in PBS for 30 min. Primary and secondary antibodies were diluted in 5% NGS and applied sequentially for 4 h at room temperature or overnight at 4°C.

Primary antibodies used in this study included mouse monoclonal neurofilament antibodies SMI31 and SMI32 (Sternberger Monoclonals, Baltimore, Maryland; 1:10,000 and 1:1000, respectively), and rabbit polyclonal antibodies anti-MAP2 (Boehringer Mannheim; 1:1000), and anti-tau (ICN Biochemicals, Costa Mesa, California; 1:1000). Secondary antibodies included Alexa-488 conjugated goat anti-mouse and goat anti-rabbit (Molecular Probes Eugene, Oregon; 1:1,000) and CY3 conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, Pennsylvania; 1:1,000). For double labeling, cultures were re-blocked and the second set of antibodies was applied similarly. Control experiments for single and double labeling demonstrated no cross-reactivity.

Microscopy

Cells were examined under epifluorescence illumination on a Nikon Eclipse TE300 (Nikon Inc., Melville, New York) inverted microscope using either a 10×, N.A. 0.30 or 20×, N.A. 0.45 objective. Digital images were acquired using an RT Color Spot Camera (Diagnostic Instruments, Hitschfel Instruments, Inc.).

Measurement of axon lengths

Axon integrity was assessed with immunofluorescence for axon cytoskeletal components including phosphorylated neurofilaments H and M (SMI31) and tau. We quantified the health of the axons by measuring their total length extended from the edge of the coverslip as the most consistent measure among control cultures. Fluorescent images at 100× were taken at 2–3 consecutive sites of axonal crossing in each dish and analyzed using image processing software (Metamorph, Universal Imaging, West Chester, Pennsylvania). Ten axons were randomly identified at the site where they protruded underneath the coverslip barrier, and the lengths were traced from the edge of the coverslip to their ends. Calibration from pixels to microns was accomplished by reference to a hemocytometer image. At least three dishes from two different plating dates were used for all experiments. For YFP experiments, axon integrity was assessed over time. Images of the same sites were taken before (T0) and 24 h after the paradigm (T24). Axon lengths were measured as described above. The average length was calculated as T24/T0. Four different cultures from three different plating dates were used for YFP experiments. Average values from each dish were identified as a single observation for statistical analysis.

Results

Hypoxic injury in isolated cortical axons

To assess vulnerability of isolated axons, independent of effects upon neuronal cells bodies and dendrites, we made use of a chamber model (Ivins et al., 1998) modified for cortical neurons. Each chamber was constructed with a semicircular wall of Teflon tubing, and a glass coverslip divider fixed to the Teflon tubing and culture surface with sterile silicon grease (Fig. 1). Cortical microexplants plated in the internal compartment projected neurites under the coverslip and into external compartment (Fig. 1). The