Depolarization-Induced Ca\textsuperscript{2+} Release in Ischemic Spinal Cord White Matter Involves L-type Ca\textsuperscript{2+} Channel Activation of Ryanodine Receptors

Mohamed Ouardouz,\textsuperscript{1} Maria A. Nikolaeva,\textsuperscript{1}
Elaine Codere\textsuperscript{1}, Gerald W. Zamponi,\textsuperscript{2}
John E. McRory,\textsuperscript{2} Bruce D. Trapp,\textsuperscript{2}
Xinghua Yin,\textsuperscript{3} Weili Wang,\textsuperscript{1} John Wouffe,\textsuperscript{1}
and Peter K. Stys\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1}Ottawa Health Research Institute
Division of Neuroscience
University of Ottawa
Ottawa, Ontario K1Y 4K9

\textsuperscript{2}Cellular and Molecular Neurobiology Research Group
Department of Physiology and Biophysics
University of Calgary
Calgary, Alberta T2N 4N1
Canada

\textsuperscript{3}Department of Neurosciences
Cleveland Clinic Foundation
Cleveland, Ohio 44195

Summary

The mechanisms of Ca\textsuperscript{2+} release from intracellular stores in CNS white matter remain undefined. In rat dorsal columns, electrophysiological recordings showed that in vitro ischemia caused severe injury, which persisted after removal of extracellular Ca\textsuperscript{2+}; Ca\textsuperscript{2+} imaging confirmed that an axoplasmic Ca\textsuperscript{2+} rise persisted in Ca\textsuperscript{2+}-free perfusate. However, depletion of Ca\textsuperscript{2+} stores or reduction of ischemic depolarization (low Na\textsuperscript{+}, TTX) were protective, but only in Ca\textsuperscript{2+}-free bath. Ryanodine or blockers of L-type Ca\textsuperscript{2+} channel voltage sensors (nimodipine, diltiazem, but not Cd\textsuperscript{2+}) were also protective in zero Ca\textsuperscript{2+}, but their effects were not additive with ryanodine. Immunoprecipitation revealed an association between L-type Ca\textsuperscript{2+} channels and RyRs, and immunohistochemistry confirmed colocalization of Ca\textsuperscript{2+} channels and RyR clusters on axons. Similar to “excitation-contraction coupling” in skeletal muscle, these results indicate a functional coupling whereby depolarization sensed by L-type Ca\textsuperscript{2+} channels activates RyRs, thus releasing damaging amounts of Ca\textsuperscript{2+} under pathological conditions in white matter.

Introduction

In the CNS, myelinated axons are critically important for transmitting information between neurons. A variety of disorders, such as ischemia during stroke, brain and spinal cord trauma, and demyelination seen in multiple sclerosis, are characterized by abnormal or failed transmission of action potentials along axons, often resulting in serious clinical disability. Although the precise mechanisms of axonal injury are not completely understood, intracellular Ca\textsuperscript{2+} overload is generally thought to play a major role (Stys, 2004). Indeed, removal of extracellular Ca\textsuperscript{2+} during injury protects axons in many in vitro models of white matter anoxia and trauma (Stys et al., 1990; Tekkoc and Goldberg, 2001; Waxman et al., 1993). During in vitro anoxia, the increase in intraxonal Ca\textsuperscript{2+} concentration is mainly due to influx of extracellular Ca\textsuperscript{2+} across the axolemma through reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (Imaizumi et al., 1997; Stys et al., 1992, 1997). In addition, inhibitors of voltage-sensitive Ca\textsuperscript{2+} channels are also protective during white matter anoxia and trauma, implicating these channels in Ca\textsuperscript{2+}-dependent injury (Agrawal et al., 2000; Brown et al., 2001). However, an increase in cytosolic Ca\textsuperscript{2+} can occur not only from influx across the plasmalemma, but also through release of Ca\textsuperscript{2+} from intracellular stores. Given the substantial quantities of total axoplasmic Ca\textsuperscript{2+} in central axons (approaching 1 mM; LoPachin and Stys, 1995; Stys et al., 1997), a role for stores in the damaging intracellular Ca\textsuperscript{2+} increase in white matter is possible (Stys, 2004; Thorell et al., 2002).

Intracellular Ca\textsuperscript{2+} release channels can be divided into two major categories: inositol-1,4,5-triphosphate receptors (IP3R) and ryanodine receptors (RyR), both of which are present on the endoplasmic reticulum (ER), releasing Ca\textsuperscript{2+} from this organelle in response to a variety of stimuli. Three different ryanodine receptor isoforms have been identified in mammalian tissues: RyR1, RyR2, and RyR3 (McPherson and Campbell, 1993). RyR1 is expressed preferentially in skeletal muscle, while RyR2 is mainly cardiac (Otsu et al., 1990). However, all three are present in brain (Furuichi et al., 1994; Giannini et al., 1995; Kuwajima et al., 1992; Lai et al., 1992; McPherson and Campbell, 1993). In skeletal muscle fibers, depolarization of the sarclemma induces release of Ca\textsuperscript{2+} from ER, which does not require influx of extracellular Ca\textsuperscript{2+}, but depends instead on sarcosomal depolarization. In this arrangement, the L-type Ca\textsuperscript{2+} channel located on the sarclemma functions as a voltage sensor, transducing depolarization to activation of RyR1 and release of Ca\textsuperscript{2+} from the ER. Influx of Ca\textsuperscript{2+} through the L-type Ca\textsuperscript{2+} channel is not required for activation of RyR1 receptors in these cells (Franzini-Armstrong and Protasi, 1997; Leong and MacLennan, 1998; Melzer et al., 1995). A functional interaction between L-type Ca\textsuperscript{2+} channels and RyR has also been suggested in the CNS (Chavis et al., 1996; Mouton et al., 2001).

In CNS white matter, removal of extracellular Ca\textsuperscript{2+} is strongly protective against anoxic and traumatic injury (Stys et al., 1990; Tekkoc and Goldberg, 2001; Waxman et al., 1993), suggesting that control over internal Ca\textsuperscript{2+} stores is not lost during these insults. However, less is known about how a more severe ischemic insult may affect the release of intracellular Ca\textsuperscript{2+} pools in this tissue. We hypothesized that a more profound energy deficit, as occurs during ischemia compared to anoxia alone, may trigger release of intracellular Ca\textsuperscript{2+} stores, particularly since there is evidence that ER Ca\textsuperscript{2+}-ATPase may be preferentially fuelled by glycolytic ATP (Hüsler et al., 2000; Xu et al., 1995), and this source of energy would be depleted during ischemia. Our results indicate that spinal cord white matter tracts are capable of transducing membrane depolarization to Ca\textsuperscript{2+} release from ER.
stores, whereby L-type Ca²⁺ channels function to gate RyR, analogous to “excitation-contraction coupling” in muscle. Under pathological conditions such as ischemia, this mechanism releases toxic amounts of Ca²⁺ capable of severely injuring central axons, which cannot be rescued solely by control of extracellular Ca²⁺ influx.

Results

Electrophysiological recordings of compound action potentials (CAPs) in dorsal column of spinal cord showed that 1 hr oxygen glucose deprivation (OGD) at 37°C followed by 3 hr recovery caused severe injury and CAP propagation failure. The CAP was abolished following OGD exposure (1.4% ± 1.6% of preinjury CAP area, n = 8), in contrast to time-matched control experiments without OGD where CAP area did not significantly change (Figures 1A and 1B; 106% ± 11%, n = 12, p < 0.001). Surprisingly, in contrast to 1 hr of anoxia alone (CAP area recovery: 41% ± 6% of control following reoxygenation, n = 4), where removal of external Ca²⁺ was highly protective (73% ± 10% recovery, n = 3; see also Imaizumi et al., 1997), omission of bath Ca²⁺ (+0.5 mM EGTA) was not protective after 1 hr OGD, with CAP area remaining severely depressed (Figures 1C, 1D, and 1E; 2% ± 2%, n = 9).

Role of Intracellular Ca²⁺ Stores

The inability of extracellular Ca²⁺ removal to prevent injury suggested that either this injury was Ca²⁺ independent or that another source of Ca²⁺ was recruited. Mobilization of intracellular Ca²⁺ stores has been suggested during anoxia in axons of the optic nerve and in a model of dorsal column traumatic compression (Stys, 2004; Thorell et al., 2002). Nonspecific chelation of intracellular Ca²⁺, regardless of source, using the membrane-permeable Ca²⁺ chelator BAPTA-AM during OGD was examined to confirm whether OGD-mediated injury was still Ca²⁺ dependent, even in zero bath Ca²⁺. Animals were administered BAPTA-AM (20 mg/kg intracardiac) 5 min before excision of dorsal columns for in vitro study. Pretreatment with BAPTA-AM improved CAP area recovery to 29% ± 5% (Figures 2A, 2B, and 2E; n = 5, p < 0.01) after 1 hr OGD in normal external Ca²⁺. In zero-external Ca²⁺ (+EGTA), recovery of mean CAP area further improved to 66% ± 4% versus 2% ± 2% in zero-external Ca²⁺ alone (Figures 2C and 2E; n = 7, p < 0.001). These results suggested that Ca²⁺ is a common mediator of injury and that during OGD an important source of deleterious Ca²⁺ originates from an intracellular source. Depletion of ER-derived intracellular stores with thapsigargin (1 μM), an inhibitor of ER Ca²⁺-ATPase (Lytton et al., 1991), in the absence of external Ca²⁺ also conferred robust protection (52% ± 6% versus 2% ± 2% without drug, p < 0.001, n = 10; Figures 2D and 2E), further implicating the release of Ca²⁺ from internal stores during OGD.

Depolarization-Induced Ca²⁺ Release

To determine whether depolarization and activation of Na⁺ channels contributes to ischemic dorsal column injury in the absence of external Ca²⁺, we used the Na⁺ channel blocker tetrodotoxin (TTX; 0.1 μM). Direct inhibition of Na⁺ channels significantly improved recovery (54% ± 9% compared to 2% ± 2% without TTX, p < 0.001, n = 11; Figures 3A, 3B, and 3E), as did substitution of Na⁺ with impermeant NMDG (Figures 3C and 3E; CAP area recovery: 66% ± 9%, p < 0.001, n = 8) (both in the absence of external Ca²⁺). These results suggested that either ischemic membrane depolarization, Na⁺ influx, or both played a role in triggering Ca²⁺ release from stores. To rule out Na⁺ influx per se, Na⁺ was replaced with Li⁺, a monovalent cation that permeates Na⁺ channels well and that allows injured axons to depolarize (Leppanen and Stys, 1997). Under these conditions, after 1 hr OGD (in zero-external Ca²⁺), CAP area recovered to only 14% ± 2% (Figures 3D and 3E; p < 0.01, n = 7) compared to 54% ± 9% in normal bath Na⁺ plus TTX (a treatment that reduces axonal depolarization; Leppanen and Stys, 1997) and zero-external Ca²⁺, suggesting that ischemic membrane depolarization (which
proceeds in Li⁺-substituted perfusate), rather than Na⁺ influx, promoted release of Ca²⁺ from stores.

To study the link between depolarization and Ca²⁺ release, possibly analogous to excitation-contraction coupling in skeletal muscle where gating of L-type Ca²⁺ channels activates RyR, we used the L-type Ca²⁺ channel blockers nimodipine (10 μM) (Figure 4B) or diltiazem (100 μM) (Figure 4C), known inhibitors of the voltage sensor on these channels (Rios and Brum, 1987) (there was no Ca²⁺ flux through the channels, as all experiments were performed in zero-Ca²⁺/EGTA solution). CAP area recovered to 62% ± 8% (n = 8) and 54% ± 7% (n = 4) with nimodipine and diltiazem, respectively, compared to 2% without Ca²⁺ channel blocker in zero-external Ca²⁺/EGTA perfusate (p < 0.001). In contrast, Cd²⁺ (100 μM; Figures 4D and 4E), known to block Ca²⁺ entry through Ca²⁺ channels but without effect on the voltage sensor (Garcia et al., 1994) or ryanodine receptors (Woo et al., 2003), had a much weaker protective effect against 1 hr OGD (Cd²⁺: 20% ± 6%, n = 7, versus 62% ± 8% with nimodipine). Taken together, these results strongly suggested a voltage-gated release of Ca²⁺ from intracellular stores; indeed, direct inhibition of ryanodine receptors (ryanodine 30 μM, also in zero-external Ca²⁺/EGTA) was also significantly protective (46% ± 25% versus 2% without drug, p < 0.01, n = 6; data not shown). Combining nimodipine and ryanodine was not additive (52% ± 19%, n = 7, versus 62% ± 22% in nimodipine alone, p > 0.05), indicating that L-type Ca²⁺ channels and RyR are interdependent and sequentially involved in the same pathway controlling release of intracellular Ca²⁺.

Ultrastructure of ER in Dorsal Column Axons
Electron microscopic inspection of rat dorsal column revealed the presence of ER profiles in the cortical as well as the central axoplasm (Figures 5A–5C). These
Figure 4. Activation of L-type Ca\(^{2+}\) Channels and RyR during OGD in Spinal Cord Dorsal Column
(A) In the absence of external Ca\(^{2+}\), CAPs recorded in dorsal column were completely abolished after 1 hr OGD.
(B–D) Blockers of the voltage sensor on the L-type Ca\(^{2+}\) channel (nimodipine or diltiazem) were significantly protective (B and C), but a pore blocker (Cd\(^{2+}\)) had only a weak effect (D).
(E) Quantitative summary of CAP area recovery after 3 hr of reperfusion following 1 hr OGD.

Figure 5. Distribution of the “Axoplasmic” Reticulum in Dorsal Column Axons
Ultrastructural examination of dorsal column axons revealed endoplasmic reticulum profiles in the cortical as well as the central axoplasm.
(A) Lower magnification showing subaxolemmal cisternae parallel ing the axolemma (arrows).
(B and C) Circular, elongated, or irregular cisternae abutted the axolemma. MY, myelin; AX, axoplasm; AL, axolemma. Scale bars, 200 nm.

displayed a range of morphological appearances corresponding to those described in the squid giant axon by Metuzals et al. (1997). Circular, elongated, or irregular subaxolemmal membrane-bound cisternae abutted the adjacent axolemma. Many of these were continuous with tubular or “tethered” profiles of ER which extended into deeper aspects of the axoplasm or, alternatively, paralleled the axolemma for some distance.

Coimmunoprecipitation and Immunohistochemistry of L-type Ca\(^{2+}\) Channels and RyR
The above results strongly suggest a close functional relationship between L-type Ca\(^{2+}\) channels and RyRs. To determine whether RyRs and voltage-gated Ca\(^{2+}\) channels are part of a protein complex in dorsal column, we carried out a co-IP assay with specific antibodies to RyR1 and RyR2 and used specific antibodies to Ca\(^{1.2}\) and Ca\(^{1.3}\) for Western blot analysis. As shown in Figure 6, RyR1 coimmunoprecipitated with Ca\(^{1.2}\), but not with Ca\(^{1.3}\), in both whole brain as well as in dorsal column. In contrast, RyR2 selectively coprecipitated with Ca\(^{1.3}\) channels, again both in brain and in dorsal column. These data suggest that both whole brain and dorsal column contain RyR1-Ca\(^{1.2}\) and RyR2-Ca\(^{1.3}\) protein complexes. Immunoprecipitation with Ca\(^{1.2}\) and Ca\(^{1.3}\) antibodies resulted in detection of RyR1 and RyR2 (respectively) in Western blots (data not shown). Taken together, these results support the notion that L-type Ca\(^{2+}\) channels are physically associated with RyR in rat brain and spinal cord, potentially allowing the former to activate the latter in a voltage-dependent manner.

Immunohistochemistry was performed to spatially localize any associated Ca\(^{2+}\)-RyR clusters. As illustrated in Figure 7, there were numerous clusters of Ca\(^{1.2/1.3}\) and RyR1/2 located near the periphery of NF-positive axon cylinders and beyond (the latter likely associated with glial cells). A fraction of these clusters were colocalized (arrowheads, Figure 7A); when such pairs occurred at edges of axon cylinders (and presumably in the axolemma, confirmed by immunoelectron microscopy; Figure 7K), they were frequently found to overlie “lacunes” devoid of neurofilament, suggestive of a cisternal structure lacking cytoskeletal components (arrowhead, Fig-
Ca\textsubscript{2+}-Ryanodine Receptor Coupling in White Matter

57

Western antibody: Cav1.2 Cav1.3

IP with RyR1

1 2 3 4 1 2 3 4 220kd

IP with RyR2

1 2 3 4 1 2 3 4 220kd

Figure 6. Physical Interaction between L-type Ca\textsuperscript{2+} Channels and Ryanodine Receptors in Dorsal Columns

Coimmunoprecipitation of ryanodine receptor and L-type Ca\textsuperscript{2+} channels in whole rat brain and spinal cord dorsal columns. Solubilized proteins from brain or spinal cord dorsal column were immunoprecipitated using the RyR1 or RyR2 antibody and then probed with anti-Ca\textsubscript{1.2} or Ca\textsubscript{1.3} antisera. Ca\textsubscript{1.2} coimmunoprecipitated with RyR1, but not RyR2, in spinal cord dorsal columns and whole-brain homogenate. In contrast, Ca\textsubscript{1.3} does not coimmunoprecipitate with RyR1, but appears to specifically associate with RyR2 in both tissues. Lanes 1 and 2 are whole brain (100 μg and 10 μg total protein), lane 3 is dorsal column (100 μg total protein), and lane 4 is control with RyRX antibodies omitted from the coimmunoprecipitation.

ure 7C). On the YZ projections, such clusters appeared narrow but oblong, applied as pairs of bands along the outer surface of the axon cylinders (Figure 7D). Some cluster pairs were located near but not immediately adjacent to axon cylinders, possibly situated on glial processes or the outer layer of the myelin sheath (Figures 7H–7J). Double-immunogold labeling confirmed that some clusters were located right at the axolemma (Figure 7L).

Ca\textsuperscript{2+} Imaging in Dorsal Column Axons during Ischemia

Direct measurements of free Ca\textsuperscript{2+} changes in axon cylinders were performed using fluorescent Ca\textsuperscript{2+} indicators and confocal microscopy of live dorsal column slices exposed to chemical ischemia (Figure 8). Axon cylinders loaded with red Ca\textsuperscript{2+}-insensitive dye were well visualized (Figures 8A and 8D); these regions were used to generate ROIs for measuring the green Ca\textsuperscript{2+}-sensitive fluorescence. Control axons displayed very weak green fluorescence, indicative of a low resting [Ca\textsuperscript{2+}], and all responded to ischemia with an average increase of 78% ± 26% in Ca\textsuperscript{2+}-containing perfusate after 20–30 min. In Ca\textsuperscript{2+}-free (with 0.5 mM EGTA) bath, 114 of 140 (81%) axon segments (from 15 dorsal column slices) responded with an average fluorescence increase of 24% ± 17%, whereas 26 axons did not exhibit an increase in axoplasmic Ca\textsuperscript{2+} (<5% change over baseline). When the latter experiment was repeated with the addition of 60 μM ryanodine to block RyR, only 28 of 53 axons (53%; 5 slices) responded, with a mean Ca\textsuperscript{2+} fluorescence increase of 17% ± 10% (p < 0.01 compared to zero-Ca\textsuperscript{2+}/ischemia without ryanodine). Care was taken to exclude an extracellular Ca\textsuperscript{2+} source as a confounder in the latter experiment; in a separate study, when Ca\textsuperscript{2+}-dependent fluorescence was monitored with a 2% agar bead impregnated with Ca\textsuperscript{2+} indicator, the free [Ca\textsuperscript{2+}] within 20 μm of the surface of the bead (the same depth used for imaging axons within dorsal column slices) dropped to ~200–300 nM after 3–4 min of switching solutions from one containing 2 mM Ca\textsuperscript{2+} to zero-Ca\textsuperscript{2+}/EGTA (data not shown). Dorsal columns were exposed to zero-Ca\textsuperscript{2+}/EGTA for 10–30 min prior to ischemia; therefore, we are confident that any observed Ca\textsuperscript{2+} increase originated from intracellular sources.

Discussion

Pathophysiological studies of white matter injury emphasize the importance of intracellular Ca\textsuperscript{2+} overload in triggering the cascade of events leading to axonal damage (for review, see Stys, 2004). Removal of extracellular Ca\textsuperscript{2+} from anoxic dorsal columns was very protective (Imazumi et al., 1997), indicating a role of external Ca\textsuperscript{2+} influx in inducing injury. A surprising finding in our in vitro ischemia (as opposed to anoxia) model was the observation that removal of external Ca\textsuperscript{2+} failed to protect dorsal columns; however, direct buffering of intracellular Ca\textsuperscript{2+} using a Ca\textsuperscript{2+} chelator was protective in the same paradigm, confirming that a rise in intracellular [Ca\textsuperscript{2+}] remains a key event. In the absence of bath Ca\textsuperscript{2+}, therefore, this cation must have been sourced from an intracellular compartment. Axoplasm of CNS myelinated axons contains substantial amounts of total Ca\textsuperscript{2+}, with concentrations approachng 1 mM (LoPachin and Stys, 1995; Stys et al., 1997). The precise localization of this Ca\textsuperscript{2+} pool is unknown, but association with Ca\textsuperscript{2+} binding proteins in the axoplasm and storage within ER are likely reservoirs (Due and Keen, 1978; Henkart, 1980). Indeed, [Ca\textsuperscript{2+}] in neuronal ER may reach extremely high levels, particularly after electrical activity (Pozzo-Miller et al., 1999). We suggest that under Ca\textsuperscript{2+}-free conditions, axonal ER was an important Ca\textsuperscript{2+} pool released during ischemia, causing Ca\textsuperscript{2+}-dependent injury in dorsal columns, and residual extracellular Ca\textsuperscript{2+} did not play a significant role for the following reasons: there remained a robust axoplasmic [Ca\textsuperscript{2+}] rise even in Ca\textsuperscript{2+}-free perfusate (Figure 8), which was significantly reduced by ryanodine, indicating that a substantial portion of internal Ca\textsuperscript{2+} release occurred through opening of RyR. Inhibitors of the voltage sensor on the L-type Ca\textsuperscript{2+} channel (nimodipine, diltiazem) were far more protective than blocking the pore itself (Cd\textsuperscript{2+}), indicating that permeation of ions through the channel was much less important than gating of the channel by transmembrane electrical field changes during ischemic depolarization. Finally, preischemic depletion of intracellular Ca\textsuperscript{2+} stores by inhibiting ER Ca\textsuperscript{2+-}ATPase was very protective (Figure 2), as was direct inhibition of RyR-mediated Ca\textsuperscript{2+} release, consistent with the notion that ER releases damaging amounts of this ion during injury. This finding is in agreement with a recent observation of a protective effect of ryanodine in an in vitro model of traumatic spinal cord injury (Thorell et al., 2002). In addition, ryanodine decreases action potential-evoked rises in intracellular Ca\textsuperscript{2+} in cerebellar basket cell axons (Liano et al., 2000), suggesting a physiological role.

Three RyR isofoms have been identified in mammalian tissues, and all three are expressed to various degrees in the CNS (Furuichi et al., 1994; Giannini et al., 1995; Kuwajima et al., 1992; Lai et al., 1992; McPherson...
Figure 7. Immunolocalization of Cav and RyR in Spinal Dorsal Columns

(A–D) Triple labeled sections (Ca\textsubscript{1.3}/RyR2/neurofilament) show many Cav and RyR clusters, which are occasionally colocalized. Clusters were found associated with the surfaces of axon cylinders and elsewhere, likely within glial structures. Deconvolution (C and D) reveals a more accurate localization of cluster pairs at the surface of an axon cylinder, overlying a neurofilament-poor area, possibly representing a cistern. YZ projection (D) shows elongated “fingers” of associated Cav/RyR complexes. Similar distributions of Cav\textsubscript{1.2}/RyR1 profiles are shown in (E)–(J), also associated with axon cylinders, and in neurofilament-free areas presumably beyond the axon cylinder per se, probably localized to glial regions ([I]; deconvolved: [J]). Control sections with primary antibodies omitted but otherwise processed and imaged identically to the above showed absence of nonspecific labeling (K). Double immunogold staining (L) using pan-RyR (small grains) and Cav\textsubscript{1.3} (large grains) shows close association of both proteins at the axolemma (see text for details).

and Campbell, 1993). To date, only RyR1, known as the skeletal muscle isoform, does not require influx of extracellular Ca\textsuperscript{2+} for activation. Instead, surface membrane depolarization sensed by L-type Ca\textsuperscript{2+} channels is transduced to RyR1, likely by direct protein-protein interaction. Such mechanical gating of RyR1 in turn causes release of Ca\textsuperscript{2+} (so called depolarization-induced Ca\textsuperscript{2+} release) from the sarcoplasmic reticulum and skeletal muscle contraction (Franzini-Armstrong and Protasi, 1997; Leong and MacLennan, 1998; Melzer et al., 1995). In contrast, RyR2 activation appears dependent on an elevation of intracellular Ca\textsuperscript{2+}, which occurs mainly by flux through L-type Ca\textsuperscript{2+} channels; this Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release underlies excitation-contraction coupling in cardiac muscle (Niggli, 1999). Our Western blots on spinal dorsal columns (Figures 6A and 6B) confirm the presence of RyR in this tissue as well.

The unexpected finding here was the mechanism leading to RyR-mediated Ca\textsuperscript{2+} release in ischemic dorsal columns, which appears identical to “excitation-contraction coupling” in skeletal muscle (i.e., Ca\textsuperscript{2+} release is induced by axonal depolarization rather than requiring Ca\textsuperscript{2+} influx as in cardiac myocytes). Several lines of evidence support this conclusion. First, in the absence
of extracellular Ca\(^{2+}\), inhibiting voltage-sensitive Na\(^+\) channels or substitution of Na\(^+\) with an impermeant ion were very protective, whereas substituting Na\(^+\) with the permeable Li\(^+\) ion was far less protective. This suggests that it is membrane depolarization, rather than Na\(^+\) influx, that triggers release of Ca\(^{2+}\) from internal stores. Second, blocking the voltage sensor on the L-type Ca\(^{2+}\) channel with nimodipine or diltiazem (Rios and Brum, 1987) was also highly protective in zero-external Ca\(^{2+}\), mirroring the effects of these agents on inhibition of voltage-gated Ca\(^{2+}\) release in skeletal muscle, whereas the pore blocker Cd\(^{2+}\) was far less effective (the minor protective effect may have been due to its known Na\(^+\) channel blocking actions; Kuo et al., 2002). Moreover, combined blockade of RyR and L-type Ca\(^{2+}\) channels was not additive in our paradigm, indicating that these two systems are interdependent. Third, RyR coimmunoprecipitated with L-type Ca\(^{2+}\) channels, providing direct evidence for physical interaction and, by inference, for functional coupling between these two proteins. This association parallels what was originally discovered in muscle (Marty et al., 1994) and more recently in whole rat brain (Mouton et al., 2001), where a functional interaction has been demonstrated in neurons (Chavis et al., 1996). The specific interaction of RyR1 with Ca\(_{1.2}\) and RyR2 with Ca\(_{1.3}\) (Figures 6A and 6B) was unexpected. Even more unusual was the observation that Ca\(_{1.2}\) associates with RyR1 in spinal white matter, when classically this Ca\(^{2+}\) channel isoform coordinates with RyR2 to support Ca\(^{2+}\)-dependent cardiac-type E-C coupling. However, another group also found that Ca\(_{1.2}\) interacts with RyR1 in rat brain (Mouton et al., 2001); perhaps this is a unique design difference between the CNS and muscle. Whether the traditional Ca\(_{1.1}\) isoform associates with RyR1 (the pair that supports Ca\(^{2+}\)-independent E-C coupling in skeletal muscle) in CNS is unknown and will require further study. Taken together, it is highly likely that RyR1 mediates release of damaging quantities of Ca\(^{2+}\) from ER, precipitated by ischemic depolarization which is sensed by axolemmal Ca\(_{1.2}\). What the role is for the Ca\(_{1.3}\)/RyR2 coupling is less clear, as this RyR isoform would require Ca\(^{2+}\) influx for activation. It is conceivable however that this pair could mediate additional release of stored Ca\(^{2+}\) in a Ca\(^{2+}\)-dependent man-
ner under “regular” conditions where extracellular Ca\(^{2+}\) has not been artificially depleted and is available to move into cells through Ca\(_{\text{1.3}}\) and other voltage gated Ca\(^{2+}\) channels (see below).

Electron micrographs of dorsal column axons reveal a network of profiles consistent with ER cisternae, often abutting the axolemma (Figures 5A–5C) (Lambert, 1967), similar to what was observed in other axons from different areas in the peripheral and central nervous systems (for review, see Berthold and Rydmark, 1995; Hirano and Llena, 1995) and in squid giant axons (Metzals et al., 1997). Such apposition is essential if the ER-localized RyRs are to be activated by Ca\(^{2+}\)-sensitive channels resident on the axolemma where the latter would be in a position to sense voltage changes. Double immunogold labeling (Figure 7L) confirmed occasional clusters of Ca\(_{\text{i}}\) and RyR located at the axolemma, a prerequisite for sensing transmembrane voltage changes and transducing Ca\(^{2+}\) release. Confocal microscopy revealed numerous Ca\(_{\text{i}}\) and RyR clusters, with only a minority appearing colocalized, and sparsely distributed along axon cylinders. Nevertheless, communoprecipitation confirmed that at least some of the detected Ca\(_{\text{i}}\) and RyR proteins were physically associated. If the Ca\(_{\text{i}}\)-RyR complexes do indeed contribute to focal Ca\(^{2+}\)-dependent axonal injury (possibly leading to eventual biochemical transaction as is seen in cerebral ischemia and trauma), it is important to note that a single locus of disruption along the entire length of an axon is sufficient to render the fiber nonfunctional. Therefore, even infrequently distributed Ca\(_{\text{i}}\)-RyR complexes could contribute to catastrophic failure of the fiber if the potentially large, albeit localized, reservoir of Ca\(^{2+}\) stored in the underlying organelle is released inappropriately. This may explain why our Ca\(^{2+}\)-imaging experiments showed that not all axon segments exhibited an increase in [Ca\(^{2+}\)]\(_{\text{ax}}\) in zero-Ca\(^{2+}\) perfusate; given the sparse distribution of RyR clusters along the fibers, it is quite probable that random sampling included regions devoid of RyR complexes and therefore not expected to respond to ischemia with a release of Ca\(^{2+}\) into the axoplasm. Another intriguing possibility is that not all axons or segments are subject to depolarization-induced Ca\(^{2+}\) release (skeletal muscle type), but may require a finite Ca\(^{2+}\) influx to trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release (cardiac type); whether clusters of RyR1 and RyR2 (respectively) underlie these distinct modes of Ca\(^{2+}\) release requires more detailed study. Taken together, it appears that the architecture of axonal ER and distribution of the main proteins required to support voltage-dependent Ca\(^{2+}\) release from this organelle are properly organized to support “excitation-contraction coupling”-like Ca\(^{2+}\) release in spinal cord axons. Interestingly, some Ca\(_{\text{i}}\)-RyR clusters were observed to clearly lie beyond axonal profiles (Figures 7A and 7J), raising the possibility that this mechanism of Ca\(^{2+}\) release also occurs in glial regions.

Why central myelinated axons should be endowed with such a mechanism is unknown. The mode of Ca\(^{2+}\) release we describe here almost certainly was designed to support a physiological role. For example, increasing axoplasmic [Ca\(^{2+}\)] in response to electrical traffic may be a signal to stimulate Ca\(^{2+}\)-sensitive mitochondrial matrix dehydrogenases (Robb-Gaspers et al., 1998) in order to increase energy production in an active fiber.

Axonal transport is known to be stimulated by Ca\(^{2+}\) (Breuer et al., 1992), so it is possible that the interaction we have demonstrated subserves an activity-dependent transport role, setting transport rates of signaling molecules to distal parts of the axon or terminal commissure with neuronal activity. Modulation of Ca\(^{2+}\-)sensitive ion channels known to be present on myelinated axons (e.g., K\(_{\text{Ca}}\) channels; Jonas et al., 1991) may be required to shape firing patterns of action potential trains. The myelinated fiber has a unique limitation imposed by its myelin sheath, which blocks the axon cylinder’s access to the extracellular space from all but a small fraction of its length, at the nodes of Ranvier. If Ca\(^{2+}\) is indeed required to regulate various processes as suggested above, such Ca\(^{2+}\) would presumably need to be delivered not only at the nodes, but to intermodal regions as well; relying on passive diffusion from single entry points at nodes would be impractical because of the slow diffusion of this cation, especially in healthy, energy-replete fibers (Al-Baldawi and Abercrombie, 1995). Instead, utilizing Ca\(^{2+}\) that is stored in ER cisternae distributed along the intermodal axon would be a logical solution, allowing rapid local initiation and termination of Ca\(^{2+}\) signaling. One potential problem involves the ability of intermodal Ca\(^{2+}\) channels to sense electric field changes in an area covered by many wraps of myelin, where the full amplitude of an action potential would be greatly reduced, leaving sensors inserted into the intermodal axolemma able to perceive only small voltage swings. Physiological studies on peripheral (Barrett and Barrett, 1982) and central (Blight and Someya, 1985) myelinated axons suggest that parallel low-resistance leakage pathways exist through or under the sheath, so that myelin supports saltatory conduction more by a reduction of intermodal capacitance rather than increased resistance. Indeed, gap junction-forming connexins have been localized to both central and peripheral myelin sheaths (Altevogt et al., 2002), suggesting a deliberate attempt to reduce the resistivity of this structure. It is interesting to speculate that one effect of such a low resistance pathway would be to expose intermodal voltage sensors to much larger electric field fluctuations, precisely the solution required to allow intermodal proteins located on the axolemma, such as L-type Ca\(^{2+}\) channels, to transduce and integrate ongoing action potential traffic. Under pathological conditions where axonal energy is limited and depolarization prolonged, this mechanism may be overdriven, leading to a damaging release of stored Ca\(^{2+}\). This notion has fundamental implications for the design of neuroprotective measures, where much work has focused on controlling aberrant fluxes of Ca\(^{2+}\) from the extracellular space through voltage- and ligand-gated channels. We suggest that at least in white matter, such measures in isolation will likely be futile without coordinated intervention designed to maintain control over intracellular Ca\(^{2+}\) pools.

**Experimental Procedures**

Experiments were performed on spinal cord dorsal columns in vitro from adult Long Evans male rats (200–250 g) prepared as previously described (Li et al., 1999). Thoracic spinal cord was removed and placed in cold oxygenated zero-Ca\(^{2+}\) solution containing 126 mM NaCl, 3 mM KCl, 2 mM MgSO\(_4\), 26 mM NaHCO\(_3\), 1.25 mM NaH\(_2\)PO\(_4\),
2 mM MgCl2, 10 mM dextrose, and 0.5 mM EGTA, oxygenated with 95% O2/5% CO2. One centimeter-long dorsal column slices were gently dissected and placed in an interface recording chamber perfused with oxygenated aCSF containing normal Ca2+ ([Ca2+]o) (composition similar to above but with 2 mM CaCl2 instead of 2 mM MgCl2, and without EGTA) gradually warmed to 37°C over 1 hr.

Orthodromic CAPs were evoked by supramaximal constant-voltage pulses using a suction electrode at the caudal end of the dorsal column. Recordings, compensated for electrode resistance fluctuations that alter amplitude, were made from the opposite end by suction electrode as previously described (Stys et al., 1991). OGD was induced for 1 hr by changing to 5% CO2/95% N2 atmosphere and perfusing with a solution containing 10 mM sucrose replacing glutamate (glu) and 0.15 M NaCl, and 6 mM sucrose and 2 g/L glucose (OGD solution). CAP recovery after OGD was measured 3 hr after reoxygenation and superfusion with glucose-containing aCSF. Zero-Ca2+ experiments were performed by first preapplying zero-Ca2+/10.5 mM EGTA per fusate to the slices for 60 min prior to any manipulations to remove the majority of Ca2+ from the extracellular space.

Electron Microscopy
Deeply anesthetized rats were perfused transcardially with a fixative containing 1.6% glutaraldehyde in cacodylate buffer. The spinal cord was then removed, and fragments of dorsal columns were immersed in the same fixative and then processed routinely for electron microscopy. Tissue was placed in capsules containing Spurr-resin and polymerized for 24 hr at 80°C. Blocks were trimmed, and the tissue was cut at 60 nm thickness onto copper grids, stained with uranyl acetate and lead citrate, and examined using a Hitachi 7100 transmission electron microscope.

Immunoprecipitation
Spinal cord dorsal columns and whole brain were separately frozen in liquid nitrogen, ground, and homogenized in 1 ml RIPA buffer (50 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 7.5], and a mixture of protease inhibitors). The solution was incubated on ice for 1 hr and centrifuged for 15 min (4°C) at 15,000 g, and the supernatant was collected for overnight dialysis against the binding solution (300 mM NaCl, 50 mM Tris [pH 7.5], and 0.1% Triton-X-100). Total protein concentration was determined with the modified Lowry assay, and 100 μg or 10 μg total brain protein and 100 μg of dorsal column protein was mixed with one antibody directed toward Cav1.2, Cav1.3, RyR1, or RyR2 (1:100 dilution). The mixture was incubated overnight at 4°C with rotation. 100 μl of precleared protein A Sepharose (Pharmacia) was added to the polyclonal antibodies (anti-Ca,1.2 and anti-Ca,1.3), and 100 μl of precleared protein G Sepharose was used for the monoclonal antibodies (anti-RyR1 and anti-RyR2). The mixture was incubated at room temperature for 1 hr, and then the solution was washed two times with buffer B (0.2% NP-40, 10 mM Tris [pH 7.5], 0.15 M NaCl, and 2 mM EDTA), one time with buffer C (0.2% NP-40, 10 mM Tris [pH 7.5], 0.5 M NaCl, and 2 mM EDTA), and one time with buffer D (10 mM Tris [pH 7.5]). The complex was centrifuged and resuspended in 25 μl 3 M urea/10 mM Tris (pH 7.5) plus an additional 25 μl of 2x sample buffer added to each sample, boiled 5 min, and loaded onto a 5% acrylamide SDS-PAGE minigel with the bis-acrylamide ratio changed to 8.5%. The samples were separated and transferred to nylon membranes, and Western analysis was performed using the Ca,1.2, Ca,1.3, RyR1, or RyR2 antibodies diluted 1:500 and detected using ECL detection methods.

Immunohistochemistry
For light microscopy, deeply anesthetized adult rats were perfused with saline and then 4% paraformaldehyde in 0.1 M phosphate buffer. Dorsal columns were excised, postfixed, treated with methanolf for 30 min on ice, and then blocked with 10% NGS in Tris buffer for 1 hr. Primary antibodies (RyR1, Upstate Biotechnology; RyR2, Sigma clone c3-33; Ca,1.2/1.3, Alomone Labs) were applied at 1:100–1:200 dilution. Secondary antibodies (Texas Red or Cy2 labeled) were applied at 1:100, while anti-neurofilament 160 (1:50; Sigma) was directly conjugated with Alexa 647 (Molecular Probes kit). Slides were imaged on a Nikon C1 confocal with a 60x 1.4 NA oil immersion objective. Images were analyzed and deconvolutions performed using ImageTrak software written by PKS (http://www.ohri.ca/stys/imagetrak).

Tissue for double immunolabeling was prepared from deeply anesthetized adult rats perfused with 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.08 M Sorrenson’s buffer. The cervical spinal cord was removed, infiltrated with 2.3 M sucrose and 30% polyvi- nylpyrrolidone, placed on specimen stubs, and frozen in liquid nitrogen. Ultrathin cryosections at longitudinal orientation were cut on glass knives in a Reichert Ultracut S (Leica Instruments) at −110°C. The sections were placed on carbon- and Formvar-coated grids and immunostained as previously described (Trapp et al., 1995).

Briefly, sections were stained by floating the grids sequentially on drops of the following solutions: 10% ovalbumin, 3% normal goat serum, and PBS for 30 min; primary antibodies (anti-pan-RyR, Alomone, clone 34-C, Alexis; anti-Ca,1.3, Alomone), 1% ovalbumin, and 0.3% normal goat serum (PBS-1) overnight at 4°C; PBS rinse (6 × 5 min); colloidal gold-labeled secondary antibodies and PBS-1 for 1 hr; and PBS rinse (6 × 5 min). The grids were then placed in 2.5% glutaraldehyde in PBS for 15 min and rinsed in PBS (4 × 5 min) and distilled water (4 × 2 min). The sections were then stained with neutral uranyl acetate and embedded in 2% methylcellulose containing 0.3% uranyl acetate. Grids were examined on a Philips CM-100 electron microscope.

Confocal Ca2+ Imaging
Freshly excited dorsal columns were loaded with a Ca2+-insensitive reference dye (red dextran-conjugated Alexa Fluor 594), to allow identification of axon profiles (e.g., Figures 8A and 8D), and the dextran-conjugated Ca2+ indicator Oregon Green-488 BAPTA-1 (both from Molecular Probes), as previously described for optic nerve (Ren et al., 2000). Tissue was transferred to a custom-built chamber on a Nikon C1 confocal microscope and imaged every 60 s at 37°C with a 60 × 1.0 NA dipping lens, itself maintained at 37°C to reduce local cooling of the imaging field. For technical reasons, ischemia was induced chemically with the glycolytic inhibitor iodicetic acid (1 mM) + Na2EGTA (2 mM), a blocker of oxidative phosphorylation, which we have shown to be a reliable and reproducible model of ischemic injury in white matter (Leppanen and Stys, 1997; Malek et al., 2003). Green Ca2+-dependent signal was ratioed against the Ca2+-insensitive red channel, and then percent change during ischemia compared to control was calculated individually for each axon segment using ImageTrak.

Acknowledgments
This work was supported by NINDS R01 NS40087-01 (operating), CIHR 90408 (equipment), and Heart and Stroke Foundation of Ontario Career Investigator Award (salary) grants to P.K.S.; Premier’s Research Excellence Award, Province of Ontario (M.O.D.); Canadian Stroke Network (W.W.), G.W.Z. is a CIHR Investigator and an AHCPR Senior Scholar. This work was supported in part by a CIHR grant to G.W.Z. and NINDS R01 29818 to B.D.T. The authors thank Drs. J.F. MacDonald, M. Tymianski, and S.G. Waxman for critical reading of the manuscript.

Received: December 6, 2002
Revised: June 25, 2003
Accepted: August 8, 2003
Published: September 24, 2003

References
Barrett, E.F., and Barrett, J.N. (1982). Intracellular recording from

Ca2+-Ryanodine Receptor Coupling in White Matter 61


