Experimental Spinal Cord Injury: Spatiotemporal Characterization of Elemental Concentrations and Water Contents in Axons and Neuroglia

RICHARD M. LoPACHIN,1 CHRISTOPHER L. GAUGHAN,1 ELLEN J. LEHNING,1 YOSHIRO KANEKO,2 THOMAS M. KELLY,3 AND ANDREW BLIGHT3
1Department of Anesthesiology, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York 10467; 2Department of Anesthesiology, Nihon University Hikarigaoka Hospital, Tokyo 179-0072, Japan; and 3Division of Neurosurgery, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7060

LoPachin, Richard M., Christopher L. Gaughan, Ellen J. Lehning, Yoshiro Kaneko, Thomas M. Kelly, and Andrew Blight. Experimental spinal cord injury: spatiotemporal characterization of elemental concentrations and water contents in axons and neuroglia. J. Neurophysiol. 82: 2143–2153, 1999. To examine the role of axonal ion deregulation in acute spinal cord injury (SCI), white matter strips from guinea pig spinal cord were incubated in vitro and were subjected to graded focal compression injury. At several postinjury times, spinal segments were removed from incubation and rapidly frozen. X-ray microanalysis was used to measure percent water and dry weight elemental concentrations (mmol/kg) of Na, P, Cl, K, Ca, and Mg in selected morphological compartments of myelinated axons and neuroglia from spinal cord cryosections. As an index of axon function, compound action potentials (CAP) were measured before compression and at several times thereafter. Axons and mitochondria in epicenter of severely compressed spinal segments exhibited early (5 min) increases in mean Na and decreases in K and Mg concentrations. These elemental changes were correlated to a significant reduction in CAP amplitude. At later postcompression times (15 and 60 min), elemental changes progressed and were accompanied by alterations in compartmental water content and increases in mean Ca. Swollen axons were evident at all postinjury times and were characterized by marked element and water deregulation. Neuroglia and myelin in severely injured epicenter also exhibited significant disruptions. In shoulder areas (adjacent to epicenter) of severely injured spinal strips, axons and mitochondria exhibited modest increases in mean Na in conjunction with decreases in K, Mg, and water content. Following moderate compression injury to spinal strips, epicenter axons exhibited early (10 min postinjury) element and water deregulation that eventually recovered to near control values (60 min postinjury). Na+ channel blockade by tetrodotoxin (TTX, 1 μM) perfusion initiated 5 min after severe crush diminished both K loss and the accumulation of Na, Cl, and Ca in epicenter axons and neuroglia, whereas in shoulder regions TTX perfusion completely prevented subcellular elemental deregulation. TTX perfusion also reduced Na entry in swollen axons but did not affect K loss or Ca gain. Thus graded compression injury of spinal cord produced subcellular elemental deregulation in axons and neuroglia that correlated with the onset of impaired electrophysiological function and neuropathological alterations. This suggests that the mechanism of acute SCI-induced structural and functional deficits are mediated by disruption of subcellular ion distribution. The ability of TTX to reduce elemental deregulation in compression-injured axons and neuroglia implicates a significant pathophysiological role for Na+ influx in SCI and suggests Na+ channel blockade as a pharmacotherapeutic strategy.

INTRODUCTION

Traumatic spinal cord injury (SCI) causes damage to myelinated axons of white matter tracts. Many axons within the trauma zone or epicenter are irreversibly injured and subsequently undergo anterograde Wallerian degeneration (Balentine 1978; Bresnahan 1978). Depending on impact force, other axons, particularly those located at the subpial rim of the epicenter, can be reversibly injured (Blight 1993; Fehlings and Tator 1995; Gruner et al. 1996). This initial axon damage can be exacerbated by additional delayed pathological mechanisms that include ischemia/anoxia, tissue edema, energy depletion, generation of reactive oxygen species, and inflammation (Hall and Braughler 1989; Young 1993). Axon dysfunction and loss are primarily responsible for paraplegia and other clinical manifestations associated with SCI (Bresnahan 1978).

Despite the obvious neurological importance of axon compromise during spinal cord trauma, the corresponding mechanism of damage is not well understood. Early studies of cell injury suggested a significant pathophysiological role for transmembrane ion shifts (Trump et al. 1979), and therefore subsequent research investigating mechanisms of SCI-induced axon injury focused on disruption of intra-axonal Ca2+ and other biological ions. Ion-selective microelectrode measurements within the immediate spinal cord trauma zone have revealed a reduction in extra-axonal Ca2+ and Na+ concentrations (Moriya et al. 1994; Stokes et al. 1983; Young and DeCrescito 1986; Young et al. 1982). Several studies have shown that tissue elemental Ca and Na concentrations were increased when measured by atomic absorption spectrophotometry (Happe1 et al. 1981; Kwo et al. 1989; Moriya et al. 1994; Young and Koreh 1986). Together, these data imply entry and accumulation of Na+ and Ca2+ in injured spinal cord axons. Evidence also suggests disruption of spinal cord K+ and Mg2+ regulation; i.e., following experimental SCI, respective tissue elemental concentrations were reduced while extra-axonal ionic K+ activity was increased (Chesler et al. 1994; Lemke et al. 1987; Vink et al. 1989; Young and Koreh 1986; Young et al. 1982). Therefore several lines of indirect evidence suggest post-SCI axon injury involves loss of transaxolemmal Na+, K+, Ca2+,
and Mg\(^+\) gradients and significant perturbation of subaxonal ion distribution. The mechanism underlying axon ion disruption is unknown, but could involve reductions in energy metabolism and Na\(^+\)/K\(^+\)-ATPase activity that develop following experimental SCI (Anderson et al. 1985; Clendenon et al. 1978; Faden et al. 1987; Goldman et al. 1983; Kurihara 1985). Regardless, defective intra-axonal ion regulation is thought to mediate many of the structural and functional consequences of SCI (see reviews by Honmou and Young 1995; LoPachin and Lehning 1997; Young 1992).

The proposed involvement of ion shifts in spinal cord axon damage is based on interstitial or tissue-level ion measurements that offer important but indirect support. Direct analyses of individual axons have not been performed heretofore in an SCI model. As a consequence, it is not known how injury influences ion distribution among various subaxonal morphological compartments nor have corresponding compartmental changes been quantitatively assessed. Therefore the purpose of the present investigation was to provide direct, detailed information regarding the effects of experimental spinal cord trauma on intra-axonal elemental concentrations and distribution. Electron probe X-ray microanalysis (EPMA) was used to measure water content and elemental concentrations (mmols/kg dry weight) of Na, P, Cl, K, Ca, and Mg in selected morphological compartments of myelinated axons (for detailed technical discussions, see LoPachin 1995). EPMA is a quantitative electron microscopy technique that has been used to characterize subaxonal elemental disturbances associated with a variety of axonopathic conditions (see reviews by LoPachin and Lehning 1994, 1997). During spinal cord compression, neuroglia might play a supportive role for damaged axons (Ransom and Orkand 1996; Sykova et al. 1992) and/or might represent additional targets of injury. Accordingly, the responses of neuroglia to SCI also were determined by EPMA. In the present investigation, controlled focal compression of isolated adult guinea pig spinal cord white matter tracts was used as a model of SCI (Shi and Blight 1996). This in vitro system excludes pathophysiological variables related to vascular damage (hemorrhage, ischemia) and thereby allows selective measurement of intrinsic axonal responses to compression injury. Finally, recent research (Agrawal and Fehlings 1996; Teng and Wrathall 1997) suggests that excess Na\(^+\) entry via tetrodotoxin (TTX)-sensitive Na\(^+\) channels might be causally involved in SCI pathophysiology. Therefore to determine how Na\(^+\) channel blockade affects elemental deregulation in damaged axons and neuroglia, compression-injured spinal strips were perfused with TTX. Results of this study provide a foundation for identifying the pathophysiological role of ion deregulation and corresponding specific and nonspecific routes of ion translocation during SCI.

METHODS

Isolation and compression injury of spinal cord

This study complied with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the local animal care and use committee. The techniques for isolation and injury of guinea pig spinal cord were similar to those described by Shi and Blight (1996). Adult guinea pigs were anesthetized (ketamine, 80 mg/kg; xylazine, 12 mg/kg; acepromazine, 0.8 mg/kg) and perfused through the heart with cold, oxygenated (95% \(\text{O}_2\)-5% \(\text{CO}_2\)) Krebs solutions containing (in mM) 124 NaCl, 2 KCl, 1.2 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 2 CaCl\(_2\), 20 dextrose, 10 sodium ascorbate, and 26 NaHCO\(_3\), pH 7.4. The vertebral column was excised rapidly, and spinal cord was removed and incubated in cold (25°C), oxygenated Krebs media. Dorsal and ventral strips of spinal cord white matter (35 mm in length) were prepared and preincubated in fresh, oxygenated Krebs solution at room temperature.

After preincubation, individual spinal cord strips (\(n = 3–4\) strips/experimental or control group) were placed in a three compartment chamber (37°C), and a double sucrose gap technique was used for stimulation (constant-current unipolar pulses, 0.1 ms duration) and recording of compound action potentials (CAPs). Axons were stimulated and CAPs were recorded at opposite ends of the white matter strip by silver-silver chloride wire electrodes (Shi and Blight 1996). Recordings were made with a Neurodata Instruments bridge amplifier, and data were stored on videotape (Neurocorder). Analyses were conducted using Labview software (National Instruments) on a Macintosh Power PC computer. To produce compression injuries, a flat raised surface in the center compartment of the recording chamber acted as a brace against which white matter strips were compressed by a Plexiglas rod (2.5 mm wide, 7 mm transverse width) attached to a motorized micromanipulator. In a limited study, tissue strips (\(n = 2\)) were removed just before compression and were immediately frozen for EPMA. These samples represent \(T = 0\) controls (see end of paragraph). After baseline measurements of CAP, the rod was advanced (24 \(\mu\)m/s), and tissue compressions were stopped at predetermined gap widths (control, 700 \(\mu\)m; severe injury, 150 \(\mu\)m; and moderate injury, 250 \(\mu\)m) (Shi and Blight 1996). The rod was then withdrawn and CAP monitored for up to 1 h. Injured spinal strips were removed from the recording chamber at several times during the postinjury period (i.e., severe injury, 5, 15, and 60 min postcompression; moderate injury, 10 and 60 min) and were then frozen immediately in liquid nitrogen–cooled isopentane. Noninjured control strips (700 \(\mu\)m gap; \(n = 2\)) were incubated for 60 min and then frozen as above (\(T = 60\) control). Control and experimental tissue samples were stored in liquid nitrogen until analyzed. Statistical analysis indicated the \(T = 0\) and \(T = 60\) controls did not differ with respect to ion distributions, and therefore data were combined to form a pooled \(T = 0\) control.

In a separate study, spinal strips (\(n = 6\)) were incubated as described above, and, after baseline determinations of CAP, strips were subjected to severe compression injury. At 5 min postinjury, CAPs were measured, and TTX (1 \(\mu\)M) perfusions were initiated. Two spinal segments were removed immediately and stored for later analysis. The remaining compression-injured samples (\(n = 4\)) were incubated in TTX for an additional 55 min (60 min total). Similar to previous findings in optic nerve (Stys and LoPachin 1998), control incubation of spinal segments with TTX for 55 min (1 \(\mu\)M, \(n = 2\)) did not alter axonal or neuroglial elemental composition (data not shown).

Cryomicrotomy and EPMA

In the following studies, frozen injured spinal cord samples were divided into two regions: the epicenter, central zone of the compression injury; the shoulder, area ~1 mm on either side of the epicenter. Methodologies for cryomicrotomy and EPMA have been published extensively (Foster and Saubermann 1991; LoPachin and Stys 1995; Saubermann et al. 1981a,b). Briefly, frozen spinal cord samples were sectioned (500 \(\mu\)m nominal thickness) on a cryomicrotome at a ambient cryochamber temperature of ~55°C. Unstained, unfixed, hydrated cryosections were then transferred under vacuum to the cold stage (~185°C) of an AMRay 1400 scanning electron microscope. The electron microscope was equipped with a Tracor Northern energy dispersive detector and pulse processor that was connected to a PC-based multichannel analyzer for collection and processing of X-rays.

For quantitative analyses of elements in spinal cord cryosections,
wet weight specimen mass was measured in frozen hydrated sections by determining continuum generation rates (Saubermann et al. 1981a,b; Saubermann and Heyman 1987). Cryosections were then dehydrated in the electron microscope column vacuum by raising the temperature of the cold stage from −185°C to −60°C for 30 min. Stage temperature was returned to −185°C for microanalysis. Morphological compartments were visualized in dehydrated cryosections using scanning transmission electron microscopy (STEM). The electron beam (20 keV, 0.4 nA current) was rastered within anatomic boundaries of the chosen structures. X-ray spectra were collected over ∼100 s of live counting time. Dry weight elemental mass fractions (mM/kg dry weight) for Na, K, Cl, Mg, P, and Ca were determined using software applying the Hall et al. (1973) method of continuum normalization (Foster and Saubermann 1991). Water content (% water) of morphological compartments was determined by the ratio of continuum counts in the hydrated and dried states (Bulger et al. 1981; Saubermann et al. 1981b). EPMA measures total elemental content and does not distinguish ionic versus bound species. Therefore symbols for each element are expressed without oxidation state (e.g., Na) when corresponding concentrations have been derived by EPMA. Oxidation states are indicated (e.g., Na⁺) for discussions of previous research involving ion-sensitive measurements or for physiological processes (e.g., membrane transport) where involvement of the ionized species is implicit (see DISCUSSION).

In cryosections of spinal cord, myelinated axons were classified as either small (<3 μm), medium (4–6 μm) or large (>7 μm) diameter fibers. For each class of nerve fiber, elemental composition and water content was determined in axoplasm, mitochondria, and myelin. The majority of axoplasm analyzed in transverse cryosections was of internodal origin. The mitochondrial compartment has been identified according to both functional (e.g., differential response to injury, Ca sequestration) and gross structural (e.g., size, shape and orientation) criteria (LoPachin et al. 1991; LoPachin and Stys 1995). Mitochondrial data represent analyses pooled primarily from organelles in large and medium fibers because mitochondria from small fibers were more difficult to identify conclusively. Swollen axons were the most obvious morphological consequence of spinal cord compression. These fibers were generally characterized by a reduction in myelin thickness, periaxonal swelling, axoplasmic compaction, enlarged mitochondria, and myelin splitting. Glial cell cytoplasm and myelin were also analyzed in compressed and noninjured white matter segments. The respective elemental compositions of myelin from large, medium and small diameter axons did not differ statistically, and the data were therefore pooled.

Statistics

Nested ANOVA was used to show that analyses from individual spinal strips of an experimental group could be pooled to derive a group mean. Therefore the descriptive parameters such as group means and standard errors of the mean are not based on number of spinal cord samples (i.e., 3–4 per time point) but rather are derived from pooled compartment data. Statistical differences (P < 0.05) between control and experimental group means were determined using one-factor ANOVA followed by Dunnett’s test.

RESULTS

Electrophysiological measurements

Electrophysiological responses of isolated white matter tracts to graded compression in the present study were similar to those reported previously (Shi and Blight 1996). Figure 1A shows several superimposed CAPs recorded before and at several times after severe compression injury. Figure 1B illustrates the effects of severe compression injury on peak CAP amplitude as a function of pre- and postcrush time. During compression of spinal cord strips, potential amplitude dropped rapidly and usually approached complete extinction. Subsequent partial recovery stabilized within 5–10 min, and overall amplitudes of residual potentials remained constant for at least 1 h, although occasional small changes in falling phase of the action potential were seen (Fig. 1A). There were, however, no gross alterations in CAP latency or form. This suggests that decreases in amplitude were produced by reductions in total number of axons contributing to the CAP, rather than changes in temporal dispersion of the signal. Amplitude was maximally depressed at 5 min postcompression (Fig. 1A), which corresponded to significant elemental deregulation in epicenter axons from the severe compression group (Fig. 3; Table 1). Evoked CAP responses of spinal strips to moderate compression were similar in form to those associated with severe injury, although mean recovery of amplitude was more extensive in the moderate group [i.e., 58.1 ± 5.8% (mean ± SE) following moderate injury vs. 35.0 ± 5.8% after severe compression].

Analyses of axons and neuroglia in epicenter of severely injured spinal cord

Although few morphological changes were evident in the epicenter zone (data not shown) 5 min after severe compres-
A, whereas P and Ca concentrations were not altered (Fig. 3, Table 1). Mean axoplasmic Mg tended to decrease, whereas Na and decreases in K concentrations occurred in all axons (Fig. 3, Table 1). However, in small axons K loss exceeded the rate of Na gain, and therefore axoplasmic water decreased significantly (Table 1). Over the next 10 min (i.e., 15 min postinjury), larger axons developed irregular profiles characterized by early granular axoplasmic changes, and there was a slight expansion of the epicenter interstitial space (Fig. 2B). At this time, the rate of mean axoplasmic Na gain and K loss slowed in all axons, whereas Mg contents decreased significantly (Fig. 3A, Table 1).

Elemental data are expressed as mean mmol element/kg dry wt ± SE; water data are expressed as mean percent water ± SE. Time = minutes postcompression; time 0 = pooled, noninjured control (see METHODS). Numbers in parentheses represent number of axons analyzed per experimental group (n = 3–4 strips/group). Epicenter refers to analyses of compartments in the central region of compression-injured spinal strips. * Significantly different (P < 0.05) from time 0 control as determined by ANOVA with Dunnett’s t test. Data from medium-diameter axons are shown in Fig. 3.

**TABLE 1. Elemental composition and water content in morphological compartments of severely injured spinal cord segments**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Na (mmol/kg)</th>
<th>P (mmol/kg)</th>
<th>Cl (mmol/kg)</th>
<th>K (mmol/kg)</th>
<th>Ca (mmol/kg)</th>
<th>Mg (mmol/kg)</th>
<th>Water (%)</th>
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<tbody>
<tr>
<td><strong>Epicenter</strong></td>
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<td><strong>Large axons</strong></td>
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</tr>
<tr>
<td>0 (41)</td>
<td>292 ± 29</td>
<td>412 ± 13</td>
<td>826 ± 39</td>
<td>1,748 ± 91</td>
<td>0 ± 1</td>
<td>18 ± 4</td>
<td>90 ± 1</td>
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<tr>
<td>5 (30)</td>
<td>970 ± 174*</td>
<td>436 ± 20</td>
<td>1,021 ± 172*</td>
<td>1,018 ± 138*</td>
<td>0 ± 1</td>
<td>8 ± 3*</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>15 (29)</td>
<td>1,182 ± 205*</td>
<td>443 ± 36</td>
<td>1,159 ± 130*</td>
<td>1,133 ± 180*</td>
<td>16 ± 5*</td>
<td>5 ± 3*</td>
<td>94 ± 1*</td>
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<tr>
<td>60 (37)</td>
<td>1,173 ± 117*</td>
<td>467 ± 24</td>
<td>1,044 ± 97*</td>
<td>235 ± 38*</td>
<td>19 ± 3*</td>
<td>0 ± 1</td>
<td>87 ± 1</td>
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<tr>
<td><strong>Small axons</strong></td>
<td></td>
<td></td>
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<tr>
<td>0 (41)</td>
<td>224 ± 18</td>
<td>477 ± 14</td>
<td>539 ± 39</td>
<td>1,508 ± 94</td>
<td>0 ± 1</td>
<td>17 ± 3</td>
<td>88 ± 1</td>
</tr>
<tr>
<td>5 (30)</td>
<td>447 ± 36*</td>
<td>524 ± 15*</td>
<td>381 ± 31*</td>
<td>664 ± 95*</td>
<td>2 ± 2</td>
<td>10 ± 3</td>
<td>82 ± 1*</td>
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<td>15 (30)</td>
<td>576 ± 91*</td>
<td>611 ± 26*</td>
<td>689 ± 75*</td>
<td>1,006 ± 94*</td>
<td>1 ± 3</td>
<td>7 ± 3*</td>
<td>88 ± 1</td>
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<tr>
<td>60 (33)</td>
<td>849 ± 97*</td>
<td>489 ± 15</td>
<td>725 ± 80*</td>
<td>226 ± 29*</td>
<td>13 ± 2*</td>
<td>0 ± 2*</td>
<td>84 ± 1*</td>
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<td><strong>Neuroglia</strong></td>
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<tr>
<td>0 (27)</td>
<td>135 ± 12</td>
<td>530 ± 26</td>
<td>141 ± 25</td>
<td>455 ± 45</td>
<td>0 ± 1</td>
<td>19 ± 3</td>
<td>68 ± 1</td>
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<tr>
<td>5 (18)</td>
<td>365 ± 30*</td>
<td>583 ± 20</td>
<td>191 ± 23</td>
<td>150 ± 13*</td>
<td>1 ± 1</td>
<td>5 ± 2*</td>
<td>63 ± 2*</td>
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<tr>
<td>15 (17)</td>
<td>824 ± 104*</td>
<td>548 ± 35</td>
<td>605 ± 84*</td>
<td>219 ± 43*</td>
<td>14 ± 6*</td>
<td>3 ± 3*</td>
<td>80 ± 2*</td>
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<tr>
<td>60 (20)</td>
<td>545 ± 85*</td>
<td>478 ± 44</td>
<td>466 ± 64*</td>
<td>230 ± 39*</td>
<td>10 ± 5*</td>
<td>4 ± 4*</td>
<td>74 ± 2*</td>
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<tr>
<td><strong>Myelin</strong></td>
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<tr>
<td>0 (24)</td>
<td>116 ± 5</td>
<td>534 ± 12</td>
<td>77 ± 6</td>
<td>147 ± 12</td>
<td>0 ± 1</td>
<td>6 ± 2</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>5 (20)</td>
<td>283 ± 19*</td>
<td>573 ± 15</td>
<td>155 ± 15*</td>
<td>116 ± 20</td>
<td>2 ± 1</td>
<td>0 ± 1*</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>15 (20)</td>
<td>455 ± 71*</td>
<td>547 ± 15</td>
<td>290 ± 55*</td>
<td>146 ± 39</td>
<td>5 ± 2</td>
<td>0 ± 1*</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>60 (22)</td>
<td>509 ± 67*</td>
<td>507 ± 14</td>
<td>428 ± 61*</td>
<td>114 ± 11*</td>
<td>10 ± 2*</td>
<td>0 ± 1*</td>
<td>50 ± 3*</td>
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</tbody>
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FIG. 2. A: scanning electron micrograph (STEM) of a frozen, unstained dehydrated transverse cryosection from noninjured control guinea pig dorsal spinal cord strip. Small (S arrow), medium (M), and large (L) diameter myelinated axons are indicated (A, 3,000 times). B: STEM image showing spinal cord morphology in the epicenter zone 15 min following severe compression injury. Several irregular axonal profiles (arrows) are evident mainly associated with larger axons (B, 3,300 times). C: STEM image showing axon morphology at the epicenter 60 min postinjury. Numerous swollen axons are present characterized by adaxonal swelling and axoplasmic fragmentation. Intramyelinic vacuoles are also evident in many swollen axons (C, 3,000 times). D: STEM image showing axon morphology of shoulder region, 60 min after severe compression injury (D, 4,500 times).
diameter fibers, large axons exhibited increases in mean axoplasmic Ca (15 min time point; Table 1). At 60 min postinjury, numerous axons exhibited periaxonal swelling, axoplasmic compaction, or granular dissolution and myelin splitting (Fig. 2C). These epicenter neuropathological changes were similar to those following in vivo contusion and in vitro compression spinal cord injuries (Balentine 1978; Blight 1991; Bresnahan 1978; Fehlings and Nashmi 1995). Corresponding elemental analyses showed that mean axoplasmic Na in all axons increased a total of four- to fivefold relative to control, whereas K concentrations decreased ~87% (Fig. 3A, Table 1). Mg was not detectable in axons, and Ca concentrations were increased in all fiber classes (Fig. 3A, Table 1). Also at 60 min, the magnitude of axonal K loss surpassed Na and Cl gain and, consequently, corresponding mean water content decreased in axons (Table 1). The pattern of postinjury mitochondrial elemental changes was similar to that of axons with the exception that mean Ca levels increased substantially at 15 min and then declined over the next 45 min (60 min time point; Fig. 3B).

Neuroglia in the epicenter zone of severely injured dorsal spinal strips exhibited a rise in cytoplasmic Na and fall in K concentrations as a function of time after injury (Table 1). Mean Ca and Cl contents also increased, whereas Mg decreased significantly (Table 1). Percent water decreased initially (5 min) and then increased significantly above control as Na/K changes developed (Table 1). Myelin mean Na and Ca concentrations increased progressively during the postinjury time, although K decreased only slightly (Table 1). Myelin water content was elevated significantly at the 60-min time point (Table 1).

Analyses of swollen axons in epicenter of severely injured spinal cord

Swollen axons with thin myelin and compacted or disintegrating axoplasm were evident at all postinjury time points, although they were most frequent at 60 min postcompression (see also Fig. 2C). Analysis of corresponding axoplasm and mitochondria indicated severe element and water deregulation as an early consequence of injury. At the 5 min time point, Na and K transaxolemmal gradients were reversed, whereas Mg was not detectable (Fig. 4A). Mean axoplasmic Cl and Ca concentrations increased significantly (Fig. 4A). Pooled data from the 15- and 60-min time points indicated a continued rise in mean axoplasmic Na and fall in K concentrations. Both Cl

FIG. 3. Effects of severe compression injury on mean ± SE dry weight elemental concentrations (mmol/kg) in axoplasm of medium axons (A) and mitochondria (B) of swollen axons in the epicenter zone of injury. For both axoplasm and mitochondria, data from the 15- and 60-min time points were not statistically different and were therefore pooled. *Mean data are significantly different (P < 0.05) from corresponding pooled control (T = 0) as determined by ANOVA followed by Dunnett’s t-test. Number of analyses per experimental time: medium axons, n = 30–40; mitochondria, n = 20–30.

FIG. 4. Mean ± SE dry weight elemental concentrations (mmol/kg) in axoplasm (A) and mitochondria (B) of swollen axons in the epicenter zone of injury. For both axoplasm and mitochondria, data from the 15- and 60-min time points were not statistically different and were therefore pooled. *Mean data are significantly different (P < 0.05) from corresponding pooled control (T = 0) as determined by ANOVA followed by Dunnett’s t-test. Number of analyses per experimental time: swollen axon axoplasm, n = 20–30; mitochondria, n = 10–30.
and Ca also increased substantially relative to 5 min postcompression (Fig. 4A). Elemental disruption in swollen axons was associated with a significant increase in axoplasmic water content (mean ± SE %water; i.e., swollen, 95 ± 0 vs. control, 90 ± 1). When compared with axoplasmic alterations in swollen axons, mitochondrial changes were more exaggerated with respect to onset and magnitude of element and water deregulation (Fig. 4B). Within 5 min after injury, mitochondrial Na, K, and Cl gradients were maximally reversed, whereas Mg levels dropped below detectability. Only mitochondrial Ca increased progressively with time (Fig. 4B). This marked elemental disturbance was associated with correspondingly large increases in mitochondrial water content; i.e., %water (mean ± SE) increased from a control level of 80 ± 2 to 88 ± 3 (P < 0.05) at 5 min postinjury and to 93 ± 1 (P < 0.05) at 15–60 min.

Effects of $Na^+$ channel blockade elemental deregulation in injured spinal axons and neuroglia

When initiated 5 min after severe spinal compression, TTX (1 $\mu$M) perfusion prevented further elemental deregulation in epicenter axons and neuroglia. Thus Fig. 5A shows that, when measured 60 min postcompression, mean Na and Cl concentrations in swollen axons from TTX-perfused spinal segments were comparable to corresponding elemental levels in non-TTX-exposed swollen axons measured at 5 min postinjury. TTX perfusion did not significantly affect the elevated water content of swollen axons (data not shown) nor did it influence the loss of axoplasmic K and Mg, and gain in Ca (Fig. 5A). In nonswollen axon regions (Fig. 5B), TTX perfusion curtailed the Na response normally associated with the 60-min postinjury time. However, unlike swollen axons, K concentrations recovered modestly, whereas Ca and Cl changes were prevented (Fig. 5B). Mitochondria from injured axons exhibited elemental responses to TTX exposure that reflected those of axoplasm (data not shown). TTX perfusion nearly preserved neuroglia element and water composition in compressed spinal strips (Fig. 5C).

Analyses of axons and neuroglia in epicenter of moderately injured spinal cord

Analyses of axons located in the epicenter of moderately injured spinal white matter demonstrated an initial (10 min postcompression) loss of axoplasmic K and Mg in conjunction with a gain in Na (Table 2). Medium- and small-diameter fibers exhibited significant increases in axoplasmic Ca concentrations, although such changes were not observed in large axons (Table 2). These elemental changes were associated with decreases in mean axoplasmic water. Mitochondrial alterations at 10 min postinjury were similar to those of axoplasm (Table 2). At 60 min after injury, both axons and mitochondria exhibited a return toward normal elemental composition and water content. Although fewer in number, swollen axons in epicenter of moderately injured spinal cord exhibited a marked perturbation of element and water regulation comparable to that of swollen fibers from severely injured cord (data not shown). Finally, neuroglia and myelin exhibited minor, transient changes in elemental composition (data not shown).

Shoulder zone analyses—severe and moderate spinal cord injury

In the shoulder region of severely injured spinal white matter, only limited morphological changes were evident throughout the experimental period; e.g., at 60 min postcompression occasional abnormal axonal profiles were observed interspersed among normal appearing fibers (Fig. 2D). Corresponding elemental analyses indicated modest changes in axons and mitochondria of the shoulder zone. Neither axoplasm nor mitochondria exhibited significant injury-typic changes in elemental composition or water content at the 5-min time point (Table 3). Swollen axons were evident throughout the experimental period, and analysis revealed that, regardless of time point, the magnitude of elemental deregulation was not comparable to that of swollen fibers in the epicenter (Table 4 vs. Fig. 4). TTX perfusion prevented compression-induced deregulation in nonswollen axon regions and neuroglia but did not affect swollen axon elemental disruption in shoulder zones (Tables 3 and 4). For moderately injured spinal strips, shoulder axons exhibited selective, transient changes in axoplasmic and mitochondrial Na and K; e.g., at 10 min postinjury, mean axoplasmic Na in medium-diameter axons increased significantly relative to control (272 ± 29 vs. 463 ± 79), whereas...
mean K levels declined slightly (1,836 ± 122 vs. 1,518 ± 134). By 60 min after compression, elemental composition had returned to preinjury levels (data not shown). Elemental compositions and water contents of nonneuril and myelin were not influenced in the shoulder zone of either moderately or severely injured spinal cord (data not shown).

**DISCUSSION**

Previous studies have provided indirect evidence that intra-axonal ion regulation is disrupted in mechanically injured spinal cord in vivo (see review by Honmou and Young 1995). In the present study, we measured concentrations and distributions of Na, K, Ca, and other elements directly in individual myelinated axons from spinal white matter strips subjected to in vitro graded compression injury. Within the epicenter zone of damage, injured axons exhibited two types of elemental deregulation: 1) rapid loss of element and water regulation in swollen axons characterized by decreased intra-axonal K and Mg with increased Na, Cl, and Ca concentrations, and 2) a more slowly developing deregulation in nonswollen axons involving initial changes in Na, K, and Mg concentrations with delayed onset Ca accumulation. The Na, K, and Ca changes in swollen and nonswollen epicenter axons are likely to be the basis of early increases in extra-axonal K$^+$ (K$_{ex}$) and decreases in Na$^{+}$o and Ca$^{2+}$o concentrations noted during previous electrophysiological studies (Chesler et al. 1991, 1994; Kwo et al. 1989; Moriya et al. 1994; Stokes et al. 1983; Young and DeCrescito 1986; Young et al. 1982). In the shoulder region, axon elemental disruption was limited to relatively small changes in K and axoplasmic water content.

**Swollen axons**

Swollen axons are an early morphological sequelae of both in vitro and in situ experimental SCI models (Balentine 1978; Bresnahan 1978; Dohrmann et al. 1972; Fehlings and Nashmi 1995). In epicenter of severely and moderately injured spinal strips, swollen axons exhibited marked loss of element and water regulation, which was comparable in magnitude to that of degenerating rat peripheral axons in vivo (i.e., induced by nerve transection or toxic axonopathy) and to the severe deregulation associated with postanoxia reoxygenation of rat optic nerve axons in vitro (LoPachin et al. 1990, 1992b; Stys and LoPachin 1996). It is possible therefore that swollen elementally deranged spinal axon regions are irreversibly damaged and, based on the significant intra-axonal Ca burden, are undergoing active Ca$^{2+}$-mediated degeneration (Balentine 1978; Banik et al. 1987). Although the mechanism of element derangement is not known, the rapid onset and complete loss of transmembrane ion gradients implicate mechanical shearing of axolemma and a nonspecific increase in membrane permeability (Blight and Decrescito 1986). A generalized loss of membrane integrity is consistent with our finding that TTX perfusion selectively reduced Na influx in swollen axons but, unlike nonswollen fibers, did not affect K, Mg, Ca, or water deregulation. Also contributing to axon demise, dissipation of mitochondrial elemental gradients (Fig. 4B) and, presumably, mitochondrial membrane potential suggest interruption of aerobic energy production and loss of Ca$^{2+}$ buffering (Guenter et al. 1994).

The magnitude and pattern of elemental disruption in swollen epicenter axons was not reproduced in swollen axons of corresponding shoulder region. The basis for this
differential expression is not understood but might be related to respective temporal patterns of swelling in each injury region. Alternatively, the mechanism of swelling might be different; e.g., in the epicenter, direct physical axolemmal rupture and abrupt loss of subaxial ion and water regulation are likely mechanisms, whereas in the shoulder region, indirect axonal stretch might produce a beading phenomenon involving redistribution of cytoskeletal constituents accompanied by moderate element and water changes (Blight and Decrescio 1986; Ochs et al. 1997). It is notable that Na⁺ influx in swollen shoulder axons was not affected by TTX perfusion, whereas influx was reduced by TTX in swollen epicenter axons. This differential response suggests different mechanisms of swelling.

Nonswollen epicenter axons

The majority of axonal areas assessed by EPMA in epicenter zones were not swollen but did exhibit significant elemental derangement. It is not clear, however, whether these nonswollen axons are irreversibly injured or are capable of eventual recovery.

### Table 3. Shoulder zone of severely injured spinal segments: elemental deregulation and TTX perfusion

<table>
<thead>
<tr>
<th>Time</th>
<th>Large axons</th>
<th>Medium axons</th>
<th>Small axons</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
<td>P</td>
<td>Cl</td>
<td>K</td>
</tr>
<tr>
<td>0 (41)</td>
<td>292 ± 29</td>
<td>412 ± 13</td>
<td>826 ± 39</td>
<td>1,748 ± 91</td>
</tr>
<tr>
<td>5 (31)</td>
<td>335 ± 57</td>
<td>412 ± 19</td>
<td>866 ± 46</td>
<td>2,105 ± 166*</td>
</tr>
<tr>
<td>15 (29)</td>
<td>688 ± 93*</td>
<td>449 ± 14</td>
<td>838 ± 77</td>
<td>1,431 ± 108*</td>
</tr>
<tr>
<td>60 (35)</td>
<td>414 ± 79*</td>
<td>414 ± 25</td>
<td>672 ± 52*</td>
<td>1,191 ± 154*</td>
</tr>
<tr>
<td>TTX (43)</td>
<td>211 ± 46</td>
<td>419 ± 19</td>
<td>732 ± 66</td>
<td>1,608 ± 129</td>
</tr>
</tbody>
</table>

**Table 4. Elemental composition and water content of swollen shoulder axons from severely injured spinal cord with/without TTX perfusion**

<table>
<thead>
<tr>
<th>Swollen Axons</th>
<th>Na</th>
<th>P</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>292 ± 29</td>
<td>412 ± 13</td>
<td>826 ± 39</td>
<td>1,748 ± 91</td>
<td>0 ± 1</td>
<td>18 ± 4</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>−TTX (14)</td>
<td>1,101 ± 126*</td>
<td>369 ± 66</td>
<td>1,278 ± 144*</td>
<td>335 ± 64*</td>
<td>18 ± 4</td>
<td>1 ± 1*</td>
<td>85 ± 2*</td>
</tr>
<tr>
<td>+TTX (26)</td>
<td>920 ± 183*</td>
<td>339 ± 22*</td>
<td>1,120 ± 124*</td>
<td>713 ± 99*</td>
<td>12 ± 3*</td>
<td>7 ± 3*</td>
<td>86 ± 2*</td>
</tr>
</tbody>
</table>

Elemental data are expressed as mean mmol element/kg dry wt ± SE; water data are expressed as mean percent water ± SE. Numbers in parentheses represent number of axons analyzed per experimental group (n = 3–4 strips/group). Control data are from large axons (see Table 1). Swollen Axons refers to analyses of shoulder axons ±1 mm to either side of the compression injury zone. −TTX, no tetrodotoxin perfusion; +TTX, tetrodotoxin perfusion initiated 5 min after severe compression injury and then continued for 55 min (60 min postinjury). * Significantly different (P < 0.05) from time 0 control as determined by ANOVA with Dunnett’s t test.
recovery. Although nonswollen axons in epicenter of moderately injured spinal segments exhibited early (10 min) deficits, both electrophysiological function (Shi and Blight 1996) and subaxonal elemental composition returned toward control values at later postinjury times. These axons, therefore appear capable of at least partial recovery following moderate compression injury. In contrast, our observations suggest that in severely compressed spinal segments, nonswollen epicenter axons, like their swollen counterparts (see Swollen axons), have undergone irreversible injury. However, unlike swollen fibers, the corresponding mechanism (see Putative mechanisms of deregulation and routes of ion entry in nonswollen axons) and temporal expression of damage differ. Nonswollen epicenter axons displayed initial disruptions of subaxonal Na, K, Ca, and Mg that worsened progressively and correlated with an absence of electrophysiological recovery (Shi and Blight 1996). The magnitude of elemental deregulation achieved was similar to that observed in degenerating axons of anoxic optic and transected peripheral nerves (LoPachin et al. 1990; LoPachin and Stys 1995). Loss of axonal Na, K, and, in particular, Ca regulation has established pathophysiological implications (Honmou and Young 1995; LoPachin and Lehning 1997; Young 1992). The substantial Mg deficit in severely compressed fibers might also contribute significantly to development of irreversible injury based on an important ubiquitous role in cell physiology (Lemke et al. 1987; Vink et al. 1989; see also Alvarez-Leefmans et al. 1987). Developing mitochondrial dysfunction also probably contributes to irreversible axonal compromise. In accordance with their presumed buffering role (Gunter et al. 1994), axonal mitochondria initially accumulate Ca; i.e., mitochondrial Ca was significantly elevated at a time (15 min postinjury) when axoplasmic Ca was normal (Fig. 3). However, at a later postinjury time (60 min), mitochondrial loss Ca, whereas Na, Cl, K, and Mg deregulation was exacerbated. A similar phenomenon was observed in mitochondria of anoxic CNS axons and of ischemic CA1 nerve cells (LoPachin and Stys 1995; Taylor et al. 1998). Release of Ca$^{2+}$ and loss of transmembrane Na, Cl, and K gradients in mitochondria might reflect pore transition formation, which is characterized by opening of a proteinaceous inner mitochondrial pore and subsequent loss of membrane potential, decreased ATP production, and rapid egress of Ca$^{2+}$ (Bernardi et al. 1994). Regardless, mitochondrial dysfunction and the eventual extent of axoplasmic elemental perturbation make it likely that the majority of nonswollen axons in severely injured spinal regions are irreversibly damaged, although the onset of injury is more protracted than that of swollen fibers.

**Putative mechanisms of deregulation and routes of ion entry in nonswollen axons**

The mechanism of elemental disruption in nonswollen axons from severely injured epicenter is unknown. The graded, sequential nature of this disruption is unlikely to be a product of immediate mechanical rupture of membranes and might be instead related to reductions in spinal cord energy metabolism and/or Na$^+$/K$^+$-ATPase activity (Anderson et al. 1985; Clendenon et al. 1978; Goldman et al. 1983; Kurihara 1985). The intra-axonal pattern of K loss coupled to Na and Cl gains is consistent with acute inhibition of membrane Na$^+$ pump activity. The route of K loss is not known, but could involve voltage-activated channels (e.g., fast or slow K$^+$ axonemal channels) and nonspecific leak. Our data, as well as several recent studies (Agrawal and Fehlings 1996; Teng and Wrathall 1997), provide strong evidence that Na$^+$ entry in injured axons occurs via TTX-sensitive, voltage-gated Na$^+$ channels. That elevated subaxonal Na$^+$ plays a pivotal role in axon pathophysiology is suggested by results from recent in vitro and in vivo SCI models (Agrawal and Fehlings 1996; Teng and Wrathall 1997) that demonstrated improved postinjury spinal structure and function following blockade of Na$^+$ influx (i.e., via procaine, TTX, or zero Na$^+$ perfusions). Microprobe analysis (current study; see also Greene and Walsh 1995) has provided direct evidence for elevated elemental Ca in mechanically injured axons. Although the route of entry has not been identified, our finding that TTX perfusion reduced both subaxonal elemental Na and Ca concentrations, suggests Na$^+$, Ca$^{2+}$, and Mg that worsened progressively and correlated with an absence of electrophysiological recovery (Shi and Blight 1997; Stys 1998; Waxman et al. 1991). As an alternative mechanism, Agrawal and Fehlings (1996) have provided evidence that elevated Na$^+$, that stimulates Na$^+$-$H^+$ exchange and subsequent intra-axonal acidosis mediates axon damage following SCI. Regardless, determining whether Na$^+$ influx is linked to excess H$^+$ or Ca$^{2+}$ entry requires further research.

**Nonswollen shoulder axons**

Axons in the shoulder region of severely injured spinal strips exhibited a transient rise in mean Na and Ca in conjunction with a late-onset decline in K. This level of elemental disruption was modest relative to respective epicenter changes (i.e., compare Table 1 vs. Table 3 data). The pattern of elemental alterations in shoulder axons 1 h postinjury is not consistent with irreversible damage. Previous studies of CNS (optic) and peripheral nervous system (tibial) nerves subjected to mechanical elongation indicated a selective loss of K and obligatory decreases in water (unpublished observations) similar to those occurring in spinal shoulder fibers. Accordingly, axons in the shoulder region might have been mechanically stretched secondary to epicenter compression (see also Blight and Decrescito 1986). Regardless, the extent of axon elemental disruption was proportional to compression because moderate injury produced initial (10 min) small but significant changes in Na, K, and Cl. Unlike severe injury, shoulder axons from the moderate injury recovered normal elemental contents at the 60-min time point.

**Elemental composition of neuroglia and myelin during in vitro SCI**

In severely injured spinal strips, elemental composition and water contents of epicenter neuroglia were significantly disturbed. This suggests that, like myelinated axons, glial cells are injured by spinal cord compression. Given the putative neuro-supportive role of glia (Ransom and Orkand 1996), direct
injury during spinal cord compression could have secondary implications for axon injury and recovery capacity. Although we could not definitively identify astrocytes versus oligoden-drocytes, the observed general loss of glial K does not implycate buffering of interstitial K\textsuperscript{+}, which is presumably increased following axonal loss (Chesler et al. 1991, 1994; Ransom and Orkand 1996; Sykova et al. 1992). Myelin osmoregulation and element distribution were also perturbed by severe compression. These changes might be related to observed myelin splitting and expansion of the adaxonal glial compartment. The finding that TTX partially preserved elemental composition in neuroglia of severely injured epicenters suggests that Na\textsuperscript{+} influx also plays a role in glial responses to compression injury.

**Conclusions**

The present study has identified two types of elemental derangement in compression-injured axons: 1) abrupt, marked perturbation of osmoregulation and element distribution in swollen axons and 2) a progressive graded deregulation of Na, K, Ca, and other elements in nonswollen fibers. These alterations largely reflect primary injury of axons because potential secondary injury of vascular or inflammatory mechanisms have been excluded in this in vitro injury model. The gross elemental deregulation of swollen axons is likely a result of immediate loss of axolemmal integrity following direct mechanical disruption. For nonswollen axons the mechanism has not been delineated but might be a product of graded membrane damage with subsequent changes in biochemical composition and activity of axolemmal ion regulatory enzymes (Demediuk et al. 1987; Faden et al. 1987; Saunders and Horrocks 1987). Elemental analysis of spinal cord neuroglia indicates probable compression-induced injury. Because glial cell interactions are critical to neuronal maintenance and function, corresponding injury might contribute to secondary axon damage. Although additional research is necessary, the finding that elevated intra-axonal Na is coupled to Ca accumulation suggests Ca\textsuperscript{2+} entry via reverse Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. The current data provide a foundation for investigating mechanisms and functional consequences of Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} deregulation in mechanically injured spinal axons. Pharmacological intervention directed toward reduction of trauma-induced transmembrane ion movement might provide an effective means of preventing degeneration and promoting functional recovery.

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Address for reprint requests: R. M. LoPachin, Montefiore Medical Center, Anesthesia Research-Moses 7, 111 E. 210th St., Bronx, NY 10467.

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