Cellular/Molecular

Traumatic Axonal Injury Induces Proteolytic Cleavage of the Voltage-Gated Sodium Channels Modulated by Tetrodotoxin and Protease Inhibitors

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We demonstrated previously that dynamic stretch injury of cultured axons induces structural changes and Ca$^{2+}$ influx modulated by tetrodotoxin (TTX)-sensitive voltage-gated sodium channels (NaChs). In the present study, we evaluated potential damage to the NaCh α-subunit, which can cause noninactivation of NaChs. In addition, we explored the effects of pre-injury and post-injury treatment with TTX and protease inhibition on the proteolysis of the NaCh α-subunit and intra-axonal calcium levels ([Ca$^{2+}$]i) over 60 min after trauma. After stretch injury, we found that [Ca$^{2+}$]i continued to increase in untreated axons for at least 60 min. We also observed that the III-IV intra-axonal loop of the NaCh α-subunit was proteolyzed between 5 and 20 min after trauma. Pre-injury treatment of the axons with TTX completely abolished the posttraumatic increase in [Ca$^{2+}$]i and proteolysis of the NaCh α-subunit. In addition, both pre-injury and post-injury inhibition of protease activity attenuated long-term increases in [Ca$^{2+}$]i as well as mitigating degradation of the NaCh α-subunit. These results suggest a unique “feed-forward” deleterious process initiated by mechanical trauma of axons. Na$^{+}$ influx through NaChs resulting from axonal deformation triggers initial increases in [Ca$^{2+}$]i and subsequent proteolysis of the NaCh α-subunit. In turn, degradation of the α-subunit promotes persistent elevations in [Ca$^{2+}$]i, fueling additional pathologic changes. These observations may have important implications for developing therapeutic strategies for axonal trauma.

Key words: axon trauma; diffuse axonal injury; sodium channels; calcium; proteolysis; protease inhibitors; noninactivation; tetrodotoxin; traumatic brain injury

Introduction

Diffuse axonal injury (DAI) is thought to be the most common and important pathology in mild, moderate, and severe traumatic brain injury (Adams et al., 1982, 1989; Graham et al., 1988; Povlishock, 1992; Maxwell and Graham, 1997; Smith and Meaney, 2000). In severe cases of DAI, shearing forces can cause primary disconnection of axons. However, the vast majority of posttraumatic axonal pathologies evolve over time because of a series of deleterious cascades that include activation of proteases, second messengers, and mitochondrial failure (Povlishock et al., 1983; Banik et al., 1987; Povlishock, 1992; Gitler and Spira, 1998; Buki et al., 1999, 2000). We demonstrated previously that dynamic mechanical stretch injury of cultured axons replicates many of the morphological and ultrastructural changes found in DAI in vivo (Smith et al., 1999). With this model, we found the first evidence that rapid stretch of axons induces an immediate increase in intra-axonal calcium levels ([Ca$^{2+}$]i), and that this response could be completely reversed with the voltage-gated sodium channel (NaCh) blocker tetrodotoxin (TTX) (Wolf et al., 2001). Thus, although sustained elevated [Ca$^{2+}$]i may be an important mediator of secondary damage to axons after trauma, as proposed previously (George et al., 1995; Saatman et al., 1996, 2003; Buki et al., 1999; Wolf et al., 2001), this increase in [Ca$^{2+}$]i is dependent on trauma-induced Na$^{+}$ influx through NaChs. However, the disposition of NaChs after dynamic stretch injury has not been examined previously.

Noninactivation of NaChs has been shown to cause pathological Na$^{+}$ influx and membrane depolarization, a state that could potentiate Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels and reversal of the Na$^{+}$–Ca$^{2+}$ exchanger (Stys et al., 1991, 1992, 1993; Fern et al., 1995; Stys and Lopachin, 1998; Imaizumi et al., 1999; Wolf et al., 2001). Persistent noninactivation of NaChs can result from damage to their α-subunit (Armstrong et al., 1973; Vassilev et al., 1988; Stuhmer et al., 1989; Benz et al., 1997). In the present study, we used the model of dynamic stretch injury of axons from primary cortical neurons to investigate potential proteolysis of discrete regions of the NaCh α-subunit after trauma. In addition, we examined the role of potential NaCh proteolysis on prolonged posttraumatic increases in [Ca$^{2+}$]i.
Materials and Methods

Cell culture. In the present study, we used rat primary neuronal cell cultures in place of the N-Tera 2 cl/D2 (NT2) neurons used previously in the axon stretch-injury model (Smith et al., 1999; Wolf et al., 2001). The primary neurons were purified from neocortices of embryonic day (E) 17 Sprague Dawley rats (Charles River, Wilmington, MA). The cells were maintained in culture with NeuroBasal media (Invitrogen, Gaithersburg, MD) supplemented with B-27 neural supplement (Invitrogen), 5% fetal bovine serum (HyClone, Logan, UT), and 1% penicillin-streptomycin (Invitrogen). These primary neurons were seeded on a poly-L-lysine- and laminin-treated deformable substrate (Specialty Manufacturing, Saginaw, MI) in custom-designed culture wells (Smith et al., 1999). A 2 × 16 mm clear silicone barrier (specialy modified from flexiPERM slide; Sigma, St. Louis, MO) was placed on the membrane in the center of the well before plating of the primary neurons to create a 2 mm “gap” through the center of the membrane. Cells were allowed to attach for 24 hr before the barrier was removed. The temporary barrier prevented neurons from seeding in the gap region, creating a cell-free area for growth of isolated axons. After the barrier was removed, axons traversed the gap, ultimately integrating with neurons on the other side (see Fig. 1A). The cells were plated at 375,000 cells/cm². The experiments were performed at 12 d in vitro (DIV).

Support cell culture. To maintain the cultured axons until testing, support cultures were prepared from neocortices of Sprague Dawley E17 rat embryos (Charles River). The cells were plated at 300,000 cells/cm² on 12 mm Millicell wells (Millipore, Billerica, MA) 1 week before the neuronal cultures for stretch injury (SILASTIC cultures) were plated on custom-designed culture wells. The support cell cultures (10 DIV) were transferred onto the SILASTIC cultures (3 DIV). Once the Millicell wells were placed over the SILASTIC cultures, they received the same feeding and media as the SILASTIC culture.

Identification of axons in the cell-free zone. We performed immunocytochemistry to detect the identity of the processes traversing the cell-free 2 mm gap in the wells, evaluating immunostaining for microtubule-associated protein 2 (MAP2), a specific marker for the dendrites and neuronal somata, and NaCh protein, which stains the axons in addition to the dendrites and neuronal somata. Cultures were fixed in 4% paraformaldehyde and 0.1 M PBS for 20 min, permeabilized with 0.1% Triton X-100 (PBST) for 20 min at room temperature (RT), and double labeled with monoclonal mouse anti-MAP2 antibody (Ab) (AP20; 1:500; Sigma) and 5% fetal bovine serum (HyClone, Logan, UT), and 1% penicillin-streptomycin (Invitrogen). These primary neurons were seeded on a poly-L-lysine- and laminin-treated deformable substrate (Specialty Manufacturing, Saginaw, MI) in custom-designed culture wells (Smith et al., 1999). A 2 × 16 mm clear silicone barrier (specialy modified from flexiPERM slide; Sigma, St. Louis, MO) was placed on the membrane in the center of the well before plating of the primary neurons to create a 2 mm “gap” through the center of the membrane. Cells were allowed to attach for 24 hr before the barrier was removed. The temporary barrier prevented neurons from seeding in the gap region, creating a cell-free area for growth of isolated axons. After the barrier was removed, axons traversed the gap, ultimately integrating with neurons on the other side (see Fig. 1A). The cells were plated at 375,000 cells/cm². The experiments were performed at 12 d in vitro (DIV).

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Axonal stretch injury. In this study, we used a specially designed axon stretch injury apparatus and technique that we characterized previously (Smith et al., 1999). This method closely mimics mechanical loading conditions of DAI in humans, using dynamic uniaxial stretch or “tensile elongation” of axons to induce injury at strains and strain rates below those that might induce disconnection. To induce stretch injury, the culture wells were placed in a device that consists of an aluminum cover block, a stainless steel plate with a machined 2 × 18 mm slit, and an air pulse-generating system. The culture well was inserted into the cover block and then placed on the slit plate so that the area of the deformable substrate contained the cultured axons. The top plate was attached to the microscope stage, creating a sealed chamber. The top plate had a quartz viewing window in the center, an air inlet for compressed air, and a dynamic pressure transducer (model EPX-V01–25P-16F-RF; Entrant, Fairfield, NJ) to monitor internal chamber pressure. The introduction of compressed air into the chamber was gated by a solenoid (Parker General Valve, Elyria, OH). The solenoid and the pressure transducer were controlled and monitored by an analog-to-digital board (Metabyte; Keithley Instruments, Cleveland, OH) integrated with a computer data acquisition system (Capital Equipment Corporation, Bellerica, MA). The device was mounted on the stage of a Nikon inverted microscope (Optical Apparatus, Armonda, PA), allowing for continuous observation of the axons throughout the experiments. A controlled air pulse was used to induce stretch to only the cultured axons traversing the gap in the well (see Fig. 1A). A rapid change in chamber pressure deflected downward only the portion of the substrate that contained the cultured axons, inducing tensile elongation (see Fig. 1B). The rate at which this strain was applied to the axon was between 20 and 35 sec⁻¹, well within the range for traumatic injury experienced by the human brain during rotational acceleration. Measurement of nominal uniaxial strain (ε) was calculated by determining the centerline membrane deflection (δ) relative to the slit width (w) and substituting into the geometric relationship:

\[ ε = \frac{w^2 + 4δ^2}{4wδ} \sin^{-1}\left(\frac{4δw}{w^2 + 4δ^2}\right) - 1.0. \]

For the experiments presented here, peak internal chamber pressure was set at 13 psi to induce a transient uniaxial strain on the axons calculated at 1.70–1.75 or 70–75% beyond their initial length.

Immunocytochemical detection of NaCh proteolysis and its modulation. We used specific antibodies to the intracellular I-II loop on the NaCh α-subunit (aa 476–485 of full length aa 1–2005; P04775), polycional rabbit anti-brain type II NaCh Ab (AB5206; 1:50; Chemicon, Temecula, CA) (Noda et al., 1986; Gordon et al., 1987; Westenbroek et al., 1989), and intracellular III-IV loop of the NaCh α-subunit (aa 1491–1508 of full length aa 1–2005; P04775), and polycional rabbit anti-pan NaCh Ab (66–811; 1:80; Upstate Biotechnology) (Miller et al., 1983; Dugandzija-Novakovic et al., 1995; Vabnik et al., 1996; Meier et al., 1997; Rasband et al., 1999) to evaluate immunoreactive changes with and without pharmacologic modulation. Treatment groups included control saline solution (CSS), TTX (Sigma), and a protease inhibitor (PI) mixture tablet Complete (Roche Diagnostics, Indianapolis, IN), which inhibits >90% of each protease activity of serine and cysteine (including calpain I and II) proteases, metalloproteases, Pronase, thermolysin, chymotrypsin, trypsin, and papain. TTX and PI were solubilized separately and added to the culture 10 min before injury or 5 min after injury. Injuries with no treatment were performed using CSS with no modifications (n = 24 wells; no treatment). TTX was used at 1 μM (n = 12, pre-injury treatment; n = 12, post-injury treatment). The PI was used according to the manufacturer instructions (n = 12, pre-injury treatment; n = 12, post-injury treatment). Sham injuries (no stretch injury) with no treatment (n = 6), and pre-injury TTX (n = 3), post-injury TTX (n = 3), pre-injury PI (n = 3), and post-injury PI (n = 3) treatments also were performed.

After injury, the cultures were fixed at 0, 5, 20, and 60 min after injury (n = 3 wells/group/fixed time point) and permeabilized. Sham-injured wells had a single fixation time point of 20 min after sham injury (n = 3 wells/group). After fixation, the cultures were incubated with polycional rabbit anti-pan NaCh Ab (66–811; 1:80) specific for a segment of the III-IV intracellular loop of the NaCh α-subunit or polycional rabbit anti-brain type II NaCh Ab (AB5206; 1:50) specific for a segment of the intracellular I-II loop on the NaCh α-subunit. Fluorescent labeling of these antibodies was performed as above.

Western blot analysis of NaCh proteolysis and its modulation. To corroborate immunocytochemical evidence of a posttraumatic loss of immunoreactivity to the III-IV loop of the NaCh α-subunit, we performed Western blot analysis. For this analysis, we used protein exclusively extracted from the 2 × 16 mm cell-free zone (i.e., almost entirely derived from axons). Because of the extremely low yield of protein from this region, the number of experimental groups was limited to include only those relevant to the observed changes in immunoreactivity found with immunocytochemical analysis. For each experimental group, extracts from 15 wells were pooled to produce sufficient protein to run three immunoblots for each analysis. Protein extractions of injury with no treatment were evaluated 5 and 20 min after injury. Protein extractions to evaluate the effects of pre-injury TTX treatment and post-injury PI treatment were performed at 20 min after injury. Protein extractions from
sham injuries were also collected from no treatment, TTX treatment, and PI treatments at a timepoint equivalent to the duration of treatment for the injured groups at 20 min after injury.

To collect the protein at the appropriate time point, cultures were frozen on dry ice, and only the axons on the 2 × 16 mm cell-free gap were collected under the microscope. The collected samples were lysed in 150 μl of radioimmunoprecipitation assay buffer (1% PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with a mixture of protease inhibitor (Roche Diagnostics). After sonicating cells until clear, the protein concentration was determined by DC Assay Kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. The samples were frozen at −80°C until use. The samples were heated to 37°C for 30 min in 2 × SDS sample buffer (1 ml of glycerol, 0.5 ml of β-mercaptoethanol, 3 ml of 10% SDS, 1.25 ml of 1 M Tris-HCl, pH 6.7, and 2 mg of bromophenol Blue) and run on NuPAGE gel (Invitrogen). The polypeptides were electrotransferred to immunobilon membranes (Millipore). Non-specific binding was blocked using 5% nonfat milk in PBST (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, and 0.1% Tween 20) for 30 min at RT. Membranes were incubated with the polyclonal rabbit anti-pan NaCh Ab (06–811; as above), specific for a segment of the III-IV intracellular loop of the NaCh α-subunit at 1:200, the polyclonal rabbit anti-NaCh type II Ab (AB5206; as above), specific for a segment of the I-II intracellular loop of the NaCh α-subunit at 1:200, or the monoclonal mouse anti-NaCh type II Ab (K69/3; Upstate Biotechnology), specific for a segment of the IV loop to carboxyl terminal of the NaCh α-subunit (aa 1882–2005 of full length aa 1–2005; P04775) at 1:200 overnight at 4°C and then by biotinylated secondary Ab (1:250; Vector Laboratories, Burlingame, CA) as well as Vectastain ABC kit (1:1000; Vector Laboratories) at 1: 1000 for 1 hr each at RT. The membranes were rinsed three times with Tween TBS (100 mM Tris, 0.9% NaCl, and 0.1% Tween 20) for 5 min at RT and then visualized by DAB (Vector Laboratories).

The relative immunoreactivity of NaCh blots was examined using NIH Image software. The optical densities of the bands corresponding to 220 kDa NaCh protein were determined, and the background (optical density of the membrane between lanes) was subtracted from those of each band of NaCh protein. The optical density measurement was performed five times for each of three blots per group and averaged. All values are presented as the means ± SE. Statistical analysis was performed using one-way ANOVA followed by Fisher’s test for multiple comparison. A P value <0.05 was considered significant.

Analysis of changes in [Ca2+]i after injury. Intra-axonal Ca2+ levels were determined using a method for NT2N cells that we previously described in detail (Wolf et al., 2001). The cortical cells were loaded with 2 μM fluo-4 AM ester (Molecular Probes) solubilized in DMSO (0.05% final) with pluronic F-127 [0.004% (w/v) final] in a CSS (120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 15 mM glucose, and 25 mM HEPES, pH 7.4, adjusted to 330 mosM with sorbitol) in which all experiments were run (Takahashi et al., 1999). Because of the small diameter and volume of the axons, fluo-4 AM, an analog of the widely used fluo-3 AM, was used to achieve the maximum fluorescence after binding Ca2+. Fluoro-4 is superior in this system because of its increased fluorescence excitation at 488 nm compared with fluo-3 AM. The ion dissociation constant Kd (Ca2+) is reported to be similar for the two dyes under identical conditions (fluo-3, 325 nM; fluo-4, 345 mM; manufacturer specifications). In addition, we evaluated axonal fascicles of 5–10 μm in diameter for total fluorescence. Pluronic F-127 (Molecular Probes), a non-ionic detergent, was used to further disperse the dye in the CSS and allow greater access to the cytoplasm by altering membrane fluidity. The dye solution was loaded at 37°C for 30 min; the cells were rinsed, allowed to sit for another 30 min to allow for additional de-esterification of the dye, and then rinsed once more before injury. The ionophore 4-bromo-A23187 at 50 μM (Molecular Probes) served as a positive control for dye response to Ca2+ influx and was used under all treatment conditions to ensure that proper dye loading had occurred and that the treatments had not altered the Ca2+ affinity of the dye. Because of the brightness of the dye at high Ca2+ concentrations and the sensitivity setting of the camera necessary to image axons, the ionophore-treated axons uniformly reached the maximum detectable fluorescence in our system. Fluorescence microscopy was performed on a Nikon Diaphot inverted microscope with a Hamamatsu Orca CCD camera attached (Optical Apparatus, Ardmore, PA). A xenon light source excited the dye at 488 nm, and the emitted fluorescence was collected at 515 nm. Fluorescence images (1024 × 768 pixels) were collected and analyzed using the MetaFluor software package on a personal computer to which the camera was attached (Universal Imaging, West Chester, PA). Images were taken at 1 sec intervals for 20 sec, at which time the injury was induced (see above). Sampling continued at 1 sec intervals for the first minute after injury. Sampling continued once per minute until the end of 60 min experiments. During the experiments, the temperature on the microscope stage was controlled with a specially designed heater at 37°C. Analysis of changes in fluorescence of the fluo-4 dye was performed on six representative axons for each group. Axons were excluded from analysis if they had undergone primary axotomy (<5% of injured fibers). Three random regions from each axon were analyzed and then averaged. The axons were continuously sampled, except for a 2–10 sec period after injury during which the microscope was refocused. To account for potential variation in dye loading among axons or experiments, we used a standard procedure for nonratio indicators in which self-ratios were taken (∫F(t)/∫F0) between the measured fluorescence (F) and initial fluorescence (F0). Background fluorescence subtraction was accomplished by continuously sampling three areas in the field that had no axons in them for the duration of the experiment. The mean of these values was obtained at every time point and subtracted from the raw value obtained at each analyzed region of the axon before analysis.

Modulation of [Ca2+]i changes after injury. To evaluate modulation of [Ca2+]i relative to the degradation of the NaCh, we used the same treatment groups (CSS, TTX, and PI) and time points of treatment (10 min before, 5 and 20 min after injury). Injuries were performed using CSS (n = 6 wells; no treatment), TTX treatment (n = 6, pre-injury treatment; n = 6, 5 min post-injury treatment; n = 6, 20 min post-injury treatment), and PI treatment (n = 6, pre-injury treatment; n = 6, post-injury treatment). Sham injuries were also performed (n = 3 for each group). To compare experimental groups, mean values for three areas of each axon analyzed in the culture were then averaged to obtain a mean value for each experiment. Mean values of fluorescence over initial fluorescence (∫F(t)/∫F0) for the experimental groups were then compared, and statistical significance was calculated using the post hoc Newman–Keuls test.

Results
Morphological response to stretch injury
The neurites clearly visualized by light microscopy in the cell-free gap (region of injury) were comprised only of axons, demonstrated by a complete absence of MAP2 immunoreactivity while staining for NaCh proteins (Fig. 1CD). In comparison with previous studies using NT2 neurons (Smith et al., 1999; Wolf et al., 2001), axons in the gap region from primary cortical neurons were of smaller caliber (0.5 to 0.9 μm in diameter). In addition, whereas axons of the NT2 neurons that crossed the cell-free zone rarely formed fasciculations, axons from primary cortical neurons formed many fasciculations crossing the gap (5–10 μm in diameter). Because of the larger diameter of fasciculations, changes in calcium fluorescence or immunofluorescence were easier to visualize. In corroboration with our previous observations using NT2 neurons immediately after stretch injury, the axons of primary neurons demonstrated a delayed elastic response, becoming severely undulated but gradually regained most of their pre-stretch orientation by 20–60 min. However, the fasciculated axons appeared less undulated than single axons. Few axons were found disconnected (primary axotomy) immediately after the stretch, limited to approximately <5% per well.

Sodium channel protein immunoreactivity in untreated stretch-injured axons
Immunocytochemical analysis in sham-injured axons without treatment demonstrated a distinct pattern for both the intracellu-
lular III-IV and I-II loops of the NaCh α-subunit. In axons that underwent injury without treatment, the III-IV loop of the NaCh α-subunit was detected at 5 min after injury, but it subsequently almost completely disappeared at 20–60 min after injury (Fig. 2A). However, in axons that underwent injury without treatment, immunoreactivity for the I-II loop of the NaCh α-subunit did not appear to change after stretch at any time point within the first hour (t = 5, 20, 60 min) (Fig. 2C). Nonetheless, the axonal fibers appeared more irregular and tortuous over time after injury, with some displaying swollen regions.

On Western blot analysis of the III-IV loop of the NaCh protein, we found 220 kDa molecular weight (MW) bands, consistent with the size of α-subunit of NaCh protein. In axons that underwent injury without treatment, immunoblotting appeared to follow the same temporal pattern as was found with immunocytochemical analysis. Staining intensities significantly decreased at 20 min after injury compared with those in sham-injured axons (p < 0.01) (Fig. 2B). In contrast, Western blot analysis of the I-II loop of the NaCh protein revealed 220 kDa MW bands, consistent with the size of α-subunit of NaCh protein in sham axons and at 5 min after injury. At 20 min after injury, this band almost completely disappeared with a significant reduction in staining intensity (p < 0.01). However, at this time point, a new immunoreactive band was found that ranged in MW of 45–55 kDa, not found in sham axons or those 5 min after injury (p < 0.01) (Fig. 2D). Similar to the results with the I-II loop of the NaCh, the 220 kDa MW band found with Western blot analysis of the IV loop of the NaCh protein for sham axons also almost disappeared at 20 min after injury (p < 0.01), whereas a new 55 kDa immunoreactive band was found (p < 0.01) (Fig. 2E). Thus, by 20 min after injury, proteolysis resulted in the production of large fragments of the NaCh.

Sodium channel immunoreactivity in stretch-injured axons treated with TTX
On immunocytochemical analysis for the III-IV loop of the NaCh α-subunit, in axons that were pretreated with TTX (1 μM) and then injured, immunoreactivity remained for at least 60 min after injury (Fig. 3A). However, in axons that were injured and treated with TTX beginning at 5 min after injury, immunoreactivity almost completely disappeared at 20–60 min (Fig. 3B).

On Western blot analysis of the III-IV loop of the NaCh α-subunit, axons that were pretreated with TTX and then injured, distinct immunoreactive bands at 220 kDa were still visible from protein preparations taken 20 min after injury (Fig. 3C). No significant difference in staining intensities was found at 20 min after injury compared with those in sham-treated axons.

Sodium channel immunoreactivity in stretch-injured axons treated with protease inhibitors
On immunocytochemical analysis for the III-IV loop of the NaCh α-subunit, axons that were either pretreated with PI (Fig. 3D) or post-treated starting 5 min after injury (Fig. 3E), staining was preserved for at least 60 min in both groups.

On Western blot analysis of the III-IV loop of the NaCh α-subunit, axons that were injured and then treated with PI at 5 min after injury, strong immunoreactive bands at 220 kDa were found for at least 20 min after injury. No significant difference in staining intensities was found at 20 min after injury compared with those in sham-treated axons (Fig. 3F).

Calcium response in untreated stretch-injured axons
In noninjured axons, baseline Fluo-4 fluorescence remained stable for at least 60 min. In nondisconnected axons, we observed a large increase in the measured fluorescence immediately after injury (F/F₀ = 1.96; p < 0.01). After immediate increase in the mean fluorescence, there was a downward trend 2 min after stretch (t = 2 min; F/F₀ = 1.85). At 20 and 60 min after stretch, however, the fluorescence significantly increased (t = 20 min, F/F₀ = 2.41; t = 60 min, F/F₀ = 2.94; p < 0.01) (Fig. 4A, B).

Calcium response in stretch-injured axons with pharmacological manipulation
In axons that were pretreated with TTX (1 μM) and then injured, we found a complete attenuation of the Ca²⁺ influx (F/F₀ = 1.02; NS) response immediately after injury compared with untreated axons that underwent stretch injury (F/F₀ = 1.96; p < 0.01). In axons that were injured and then treated with TTX at 5 min after injury, we found significant attenuation of the Ca²⁺ influx response at 60 min (F/F₀ = 2.11) (Fig. 4B) after injury compared with untreated axons that underwent stretch injury (F/F₀ = 2.94; p < 0.05), whereas there was no significant attenuation of the Ca²⁺ influx response at 20 min (F/F₀ = 1.96) (Fig. 4A). Surprisingly, in axons that were injured and then treated with TTX at 20 min after injury, we also found significant attenuation of the Ca²⁺ influx response at 60 min (F/F₀ = 2.11) (Fig. 4A) after injury compared with untreated axons that underwent stretch injury (F/F₀ = 2.94; p < 0.05). Importantly, there was no significant increase of the Ca²⁺ influx between 20 and 60 min after injury (p > 0.05) in 20 min post-injury TTX-treated axons.

In axons that were pretreated with PI and then injured, we found no attenuation of the Ca²⁺ influx response 2 min after injury compared with untreated axons that underwent stretch injury. However, pretreatment with the PI did result in a significant attenuation of the Ca²⁺ influx response at 60 min after injury (F/F₀ = 1.86) (Fig. 4B) compared with untreated axons that underwent stretch injury (F/F₀ = 2.94; p < 0.05) (Fig. 4A). In axons that were injured and then treated with PI at 5 min after injury, we found a significant attenuation of the Ca²⁺ influx response at 60 min (F/F₀ = 1.98) after injury compared with untreated axons that underwent stretch injury (F/F₀ = 2.94; p <
0.05), whereas there was no significant attenuation of the Ca\(^{2+}\) influx response at 20 min (F/F\(_0\) = 1.98) (Fig. 4A).

**Discussion**

In this study, we report the first evidence that dynamic stretch injury of axons induces selective proteolysis of Na\(_V\) channels linked to progressively increasing levels of intra-axonal Ca\(^{2+}\). A key region of the Na\(_V\) channel that was degraded after axonal trauma included intracellular domains III and IV of the \(-\) subunit. Blockade of the Na\(_V\) channel with TTX before injury completely inhibited posttraumatic increases in \([\text{Ca}^{2+}]_i\) and prevented proteolysis of the Na\(_V\) \(-\) subunit. In addition, protease inhibition attenuated long-term increases in \([\text{Ca}^{2+}]_i\) and degradation of the III-IV linker of the Na\(_V\) \(-\) subunit. These data suggest a sequence of deleterious events after axonal trauma that is coupled to Na\(_V\) channel damage. Mechanical trauma of axons results in Na\(^+\) influx through the Na\(_V\), triggering rapid increases in \([\text{Ca}^{2+}]_i\). This initial increase in \([\text{Ca}^{2+}]_i\) then induces proteolysis of the Na\(_V\) \(-\) subunit, which in turn pushes already high \([\text{Ca}^{2+}]_i\) to continue to increase. These observations may have important implications for the development of therapeutic strategies for axonal damage in traumatic brain injury.

It has been shown previously that proteolysis of the \(-\) subunit at the intracellular loop of the III-IV linker domain of the Na\(_V\) will prevent the normal inactivation of Na\(_V\) channels leading to persistent leakage of Na\(^+\) (Vassilev et al., 1988; Stuhmer et al., 1989; Benz et al., 1997). Without inactivation, pathologic Na\(^+\) influx can induce sustained depolarization, opening of voltage-gated Ca\(^{2+}\) channels (Fern et al., 1995; Imaizumi et al., 1999; Wolf et al., 2001), and reversal of the Na\(^+\)-Ca\(^{2+}\) exchanger (Stys et al., 1992, 1993; Stys and Waxman, 2000). The resulting increased intracellular Ca\(^{2+}\) concentrations may induce several deleterious cascades including activation of proteases and degradation of cytoskeletal elements (George et al., 1995; Saatman et al., 1996, 2003; Buki et al., 1999). Based on the current results, a similar process initiated by loss of normal inactivation of Na\(_V\) channels in axonal trauma with one important distinction. Initial Na\(_V\) dysfunction after stretch injury is caused by direct or indirect mechanical deformation rather than through enzymatic cleavage, because the initial TTX-dependent Ca\(^{2+}\) rise could not be blunted by broad spectrum protease inhibition.

It has long been suggested that elevated intra-axonal Ca\(^{2+}\)
levels play a pivotal role in the secondary damage to axons after mechanical deformation (Povlishock et al., 1983; Banik et al., 1987; Povlishock, 1992; Gitler et al., 1998; Buki et al., 1999, 2000). However, we only recently found direct evidence that Ca\(^{2+}\) enters axons shortly after dynamic stretch injury in culture using the neuronal cell line NT2N (Wolf et al., 2001). It was observed that the posttraumatic rise in intra-axonal Ca\(^{2+}\) was completely dependent on Na\(^+\) entering through TTX-sensitive NaChs, as corroborated in the present study using primary cortical cells in the same model. It was also found that after trauma, intra-axonal Ca\(^{2+}\) increased by entry through voltage-gated Ca\(^{2+}\) channels and reversal of the Na\(^+\)–Ca\(^{2+}\) exchanger. These observations are consistent with mechanisms of Ca\(^{2+}\) influx in models of noninactivation of NaChs after proteolytic damage to the \(\alpha\)-subunit.

The present data suggest that although mechanical deformation is the watershed event triggering increases in \([\text{Ca}^{2+}]_{i}\), proteolysis of the NaCh \(\alpha\)-subunit plays an important role in the pathologic sequelae of axonal trauma. These sequelae appear to include a unique feed-forward process after axonal trauma, whereby mechanical trauma leads to NaCh proteolysis, in turn, perpetuating pathologic Ca\(^{2+}\) influx (Fig. 5). Specifically, increases in \([\text{Ca}^{2+}]_{i}\) were found shortly after injury, yet loss of immunoreactivity to domains III and IV of the \(\alpha\)-subunit was not observed until between 5 and 20 min after trauma. Both the posttraumatic increase in \([\text{Ca}^{2+}]_{i}\) and proteolysis of the NaCh \(\alpha\)-subunit could be abolished by pre-injury treatment with TTX. This demonstrates that Na\(^+\) influx through NaChs after axonal trauma induced initial increases in \([\text{Ca}^{2+}]_{i}\), triggering degradation of the NaCh \(\alpha\)-subunit. Although TTX treatment at 5 or 20 min after injury had no effect on proteolysis of the NaCh \(\alpha\)-subunit, both completely abolished any additional increases in \([\text{Ca}^{2+}]_{i}\) until at least 60 min after trauma. Thus, although Ca\(^{2+}\)-dependent proteolysis of the NaCh \(\alpha\)-subunit is a rapid event after trauma, attenuation of additional Ca\(^{2+}\) influx into the axon can still be achieved with post-injury blockade of the NaCh. The observation that TTX eliminated Ca\(^{2+}\) influx even after proteolytic damage to the NaCh had occurred suggests that the overall NaCh structure remained sufficiently intact to allow pathologic Na\(^+\) influx.

It was also found that pre-injury and post-injury inhibition of protease activity attenuated delayed increases in \([\text{Ca}^{2+}]_{i}\) as well as mitigating degradation of the NaCh \(\alpha\)-subunit. These data demonstrate that protease activity plays an important role in maintaining increases in \([\text{Ca}^{2+}]_{i}\) after axonal trauma. In particular, proteolytic degradation of the III and IV linker of the NaCh \(\alpha\)-subunit may ensure long-term influx of Na\(^+\) into axons known to be linked with persistent elevations in \([\text{Ca}^{2+}]_{i}\) (Stys et al., 1991, 1992, 1993; Fern et al., 1995; Stys and Lopachin, 1998; Imaizumi et al., 1999; Wolf et al., 2001). This feed-forward cycle may proceed with the high \([\text{Ca}^{2+}]_{i}\) fueling progressive proteolysis, in turn, inducing even additional increases in \([\text{Ca}^{2+}]_{i}\). It is important to note that we found \([\text{Ca}^{2+}]_{i}\) continued to increase over at least 1 hr after axonal trauma. Although we did not identify specific calcium-mediated proteases responsible for NaCh degradation, one likely candidate is calpain, which has been
shown to play an important role in generalized proteolysis in axonal trauma (George et al., 1995; Saatman et al., 1996, 2003; Buki et al., 1999, 2000).

It is not presently clear why the NaCh α-subunit is so rapidly proteolyzed after axonal trauma. One possibility is that traumatic deformation may induce a conformational change in domains III and IV, rendering this region a target for rapid proteolysis. Previously and in the current study, we found direct evidence that the mechanical loading conditions of axon stretch injury can induce immediate changes to other axonal structures (Smith et al., 1999; Wolf et al., 2001). In particular, the loss of axon elasticity after trauma demonstrates an immediate transformation of cytoskeletal elements. Likewise, conformational changes of the NaCh may be responsible for both the pathologic influx of Na+ immediately after injury and predisposition to proteolytic degradation of the α-subunit. However, direct physical disruption of the NaCh at the time of trauma remains to be determined.

To date, all phase III clinical trials evaluating treatments for human brain trauma have failed to identify an efficacious agent (Bullock et al., 1999; Morris et al., 1999; Narayan et al., 2002). Reasons for this are certainly multifactorial, but it must be taken into account that few of these therapies specifically targeted one of the most important pathologic features of human brain injury, DAI. The development of therapeutic strategies for DAI rests primarily on the understanding of the initiating events of axonal trauma. Our current results support previous proposals that sustained intra-axonal Ca2+ increases may initiate a series of deleterious cascades such as activation of proteases (George et al., 1995; Saatman et al., 1996, 2003; Buki et al., 1999, 2000). However, it remains to be established whether NaCh dysregulation and proteolysis play a global role in the pathogenesis of axonal trauma in vivo. For example, differences in NaCh distribution between axons of varying caliber and extent of myelination may result in a differential response to trauma.

Although TTX and generalized protease inhibition used in the present study may not be good candidates for treatment in humans, their collective ability to suppress Ca2+-induced intra-axonal proteolysis with post-injury application demonstrates a therapeutic opportunity. It has been reported that protease inhibition suppressed the degradation of neurofilament proteins at the site of mechanical insult and secondary axonal degeneration and improved motor function in acute spinal cord injury in rats (Iwasaki et al., 1985, 1987; Iizuka et al., 1986; Schumacher et al., 2000). Likewise, calpain inhibition suppressed axonal pathology in traumatic brain injury (Buki et al., 1999), anoxic optic nerve (Jiang and Stys, 2000), and traumatic optic nerve stretch injury (Witgen et al., 2001). It has also been shown that NaCh blockers

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Figure 4. Graphic representation of changes in intra-axonal Ca2+ fluorescence at 2, 20, and 60 min on axonal fascicles after stretch injury in CSS compared with pre-injury treatment of TTX (TTX-pre), 5 min post-injury treatment of TTX (TTX-5 min), 20 min post-injury treatment of TTX (TTX-20 min), pre-injury treatment of PI (PI-pre), and 5 min post-injury treatment of PI (PI-5 min). A, $F/F_0 = \text{change in Ca}^{2+} \text{fluorescence over initial fluorescence.}$ Comparison with 2 min after injury in CSS: *$p < 0.05$; **$p < 0.01$. Comparison with 20 min after injury in CSS: $p < 0.05$. Comparison with 60 min after injury in CSS: *$p < 0.05$; **$p < 0.01$. B, Representative photomicrographs demonstrate changes in Ca2+ fluorescence before (left) and 60 min after (right) axonal injury in CSS, with pre-injury treatment of TTX and with pre-injury treatment of PI.

Figure 5. Proposed feed-forward pathway of Ca2+ entry and NaCh proteolysis resulting from traumatic mechanical deformation of axons. VGCCs, Voltage-gated Ca2+ channels.
improve functional outcome and reduce axonal pathology in models of spinal cord crush injury (Teng and Wrathall, 1997; Rosenberg et al., 1999; Schwartz and Fehlings, 1999) as well as functional outcome in traumatic brain injury (McIntosh et al., 1996; Zhang et al., 1998). Taken in context with the present results, these therapeutic approaches may show benefit by interrupting the feed-forward process initiated by NaCh dysfunction after traumatic axonal injury.

In summary, these experiments are the first to demonstrate the exquisite sensitivity of NaChs to axonal trauma. As a consequence of mechanical deformation of axons, sustained Na\(^+\) influx through NaChs triggers initial increases in [Ca\(^{2+}\)] to subsequent proteolysis within domains III and IV of the NaCh α-subunit. In turn, this degradation of the α-subunit maintains long-term increases in [Ca\(^{2+}\)], potentially fueling additional pathology. The observation that these deleterious responses can be mitigated after injury suggests a therapeutic window of opportunity and potential strategies to treat traumatic axonal injury in vivo.

References


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