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PATHOLOGICAL CHANGES OF ISOLATED SPINAL CORD AXONS IN RESPONSE TO MECHANICAL STRETCH

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Abstract—White matter strips extracted from adult guinea-pig spinal cords were maintained *in vitro* and studied physiologically using a double sucrose gap technique and anatomically using a horseradish peroxidase assay. The amplitude of compound action potentials was monitored continuously before, during, and after elongation. Three types of conduction blocks resulting from stretch injury were identified: an immediate, spontaneously reversible component, which may result from a transient increase in membrane permeability and consequent disturbance of ionic distribution; a second component that was irreversible within 30–60 min of recording, perhaps resulting from profound axolemmal disruption; and a third component, which may be due to perturbation of the myelin sheath, that was reversible with application of 100 μM of the potassium channel blocker, 4-aminopyridine. The intensity of the conduction deficits correlated with the extent of initial stretch over a full range of severity. Stimulus–response data indicate that mechanical damage to axons in stretch was evenly distributed across the caliber spectrum. Morphological examinations revealed that a small portion of axons exhibited membrane damage at 2 min following stretch and appeared to be largely sealed at 30 min after injury. Further, in the entire length of the cord strip subjected to stretch, axons closer to the surface were found to be more likely to suffer membrane damage, which distinguished stretch injury from compression injury.

In summary, we have developed an *in vitro* model of axonal stretch that provides the ability to monitor changes in the properties of central myelinated axons following stretch injury in the absence of pathological variables related to vascular damage. This initial investigation found no evidence of secondary deterioration of axons in the first 30 min after stretch *in vitro*, although there was evidence of both transient and lasting physiological and anatomical damage to axons and their myelin sheaths.

Key words: axon, membrane sealing, spinal cord injury, neurotrauma, elongation, nerve repair.

The most significant structural damage in most types of spinal cord trauma and the chief contributor to the devastating clinical deficits seen in such an injury is clearly the disruption of large numbers of nerve fibers in the white matter. Better understanding of this pathology should help in comprehending the underlying mechanisms of functional damage and recovery, and perhaps the development of therapeutic interventions. Such damage is most commonly the result of mechanical insults. In the case of the spinal cord, mechanical deformation could cause various types of functional and anatomical deficits of spinal axons (Blight, 1988, 1991; Shi and Blight, 1996). Compared to compression, contusion, and transection injuries, which have been studied extensively using various kinds of models, stretch injury of axons from the CNS has received much less attention. To our knowledge, CNS axonal stretch in vertebrates has only been studied using neurons in culture (Ellis et al., 1995; Smith et al., 1999) and optic nerves in living animals (Bain et al., 2001; Maxwell et al., 1991). We believe axonal stretch is an important CNS injury, and the relationship between white matter elongation and the resultant functional and anatomical deficit in adult mammalian spinal cord merits detailed investigation.

Stretch of axons by mechanical displacement of tissue may be a significant component of the compression injury of a spinal cord (Blight and DeCrescito, 1986). Axons in the middle of the spinal cord are subjected to significant stretch during moderate compression or contusion injury (Blight and DeCrescito, 1986; Shi and Borgens, 1999), which may explain the selective axonal membrane damage in this location in a moderate compression injury revealed by horseradish peroxidase (HRP)-labeling (Shi and Borgens, 1999, 2000). Because most clinical injuries will involve a mixture of compression and stretch injury to axons (Blight, 1988; Blight and DeCrescito, 1986), it is useful to examine the effects of stretch injury alone and to determine the nature and extent of the damage that stretch can inflict in the context of spinal cord injury.

Motivated by a need to study stretch injury of the spinal cord in isolation, we have developed an *in vitro* stretch model to determine the relationship between stretch and both electrophysiological and anatomical

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Abbreviations: CAP, compound action potentials; HRP, horseradish peroxidase; 4-AP, 4-aminopyridine.

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measures of axonal integrity. This study was carried out using a previously well characterized *in vitro* adult mammalian spinal cord white matter preparation (Shi et al., 2000; Shi and Blight, 1996, 1997; Shi and Borgens, 1999). By using isolated spinal cords, we have examined this stretch factor in a tissue preparation that is not complicated by the uncontrolled effects of a damaged blood supply and/or progressive ischemia seen in whole-animal studies. By using spinal cord white matter strips containing a population of axons of different diameters, we were able to examine a more physiologically relevant response to stretch injury versus single axonal stretch studies. This has allowed us to study stretch injury without the bias of selecting either large or small diameter axons. Further, we were able to examine caliber selectivity within the population of axons of the spinal cord white matter strips. The knowledge derived from this study is likely to be relevant to *in vivo* injuries.

EXPERIMENTAL PROCEDURES

Isolation of the spinal cord

The experimental protocols have been reviewed and approved by the Purdue University Animal Care and Use Committee (PACUC). All efforts were made to minimize the number of animals used and their distress. The technique for isolation of the spinal cord was similar to that described previously (Shi et al., 2000; Shi and Blight, 1996, 1997; Shi and Borgens, 1999). Adult female guinea-pigs of 350–500 g body weight were used (Harlan, USA). The spinal cord was isolated from deeply anesthetized animals (80 mg/kg ketamine hydrochloride, 0.8 mg/kg acepromazine maleate, and 12 mg/kg xylazine, i.m.). Following anesthesia, the animal was perfused transcardially with cold (15°C) Krebs' solution, and the vertebral column was rapidly excised with bone forceps and scissors. The laminae from the lumbo-sacral to cervical levels were then removed in a continuous strip by cutting through the pedicles on either side with malleus nippers. The roots were cut carefully with microscissors as the cord was gently removed from the inverted vertebral column, and placed in cold Krebs' solution (composed of: NaCl 124 mM, KCl 2 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 1.2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, sodium ascorbate 10 mM, equilibrate with 95% O_2, 5% CO_2). The cord was separated first into two halves by midline sagittal division. The ventral white matter was then obtained by separating it from the gray matter with a scalpel against a soft plastic block (Fig. 1). White matter strips were maintained in continuously oxygenated Krebs' solution for at least an hour before mounting in the recording chamber. This was to ensure the recovery from dissection before experimentation. Based on our experience, the extracted cords continue to demonstrate normal electrophysiology responses up to 24 h after excision without significant structural and functional deterioration.

Recording chamber

The construction of the recording chamber is illustrated in Fig. 2. A strip of isolated spinal cord white matter, approximately 45 mm in length was supported in the three-compartment chamber. The central compartment, 20 mm in diameter, was continuously superfused with oxygenated Krebs' solution at 2 ml/min. The ends of the tissue were carried through the sucrose gap channels to side compartments that were later filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels using shaped fragments of glass coverslips and a small amount of silicone grease to attach the coverslip to the walls of the channel and to block the flow of fluid in the narrow gap between the coverslip and the tissue surface. An isotonic sucrose solution was run continuously through the channel at a rate of 1 ml/min. The temperature of the chamber was maintained at 37°C with an in-line solution warmer (Warner Instruments, Hamden, CT, USA), and the temperature was separately monitored across the chamber with additional thermocouple probes. The axons were stimulated and compound action potentials (CAP) recorded at opposite ends of the strip of white matter by silver–silver chloride wire electrodes positioned within the side chambers and the central bath. The central bath was similarly connected to an instrument ground.

Stimuli were delivered through stimulus isolation units and were usually in the form of 0.1-ms constant current unipolar pulses. A conventional bridge amplifier (Neurodata Instruments, Delaware Water Gap, PA, USA) was used to amplify the signal. These data were digitized and stored on videotape with a Neurodata Instruments Neurocorder for subsequent analysis. All the on-line recording and subsequent analysis was performed using LabView software (National Instruments, Austin, TX, USA) on a Macintosh G-3 power computer.

Stretching

A flat, raised surface was provided at the center well of the recording chamber, against which the isolated white matter strip could be stretched through an opening in the middle of the raised surface, using a rod attached to a motorized micromanipulator (Fig. 2). The spinal cord to be stretched was immobilized on either side of the elongation site by a nylon mesh stabilizer, which gently pressed the cord against the raised surface. The CAP was monitored to ensure that no significant reduction of CAP amplitude occurred as a result of this procedure. The strips were marked with ink near the edges of the nylon mesh (outside borders of the injury site). These marks were monitored to insure that there was no movement under the stabilizer. The end of the rod provided a surface of 3 mm along the length of the tissue, with a transverse width of 5 mm. The end of the rod where the white matter strip was contacted was made slightly concave transversely to insure proper contact during the stretching (to prevent the cord from slipping out). Along the longitudinal plane of the tissue, the surface of the rod was made slightly convex to ensure the even distribution of pressure during the stretch and to avoid transection. The stretching rod was positioned perpendicularly to the tissue and was brought to a point of contact with its surface. The vertical progress of the rod was monitored with a displacement transducer and controlled with a stepper motor control. After baseline measurements of conduction were obtained, the rod was advanced by means of a manipulator motor. Unless stated differently, the stretch rod always traveled at a speed of 25 μm/s. A small number of our experiments used speeds of 1.5 m/s to study the pattern of membrane damage inflicted by stretch. The cord was stretched until our action potential target was reached. The rod was then removed rapidly upward by means of a manipulator motor to relieve the stretch on the tissue and the recovery of the compound potential was monitored. The percent of stretch, or strain, was calculated by dividing the change of the length by the original length. This was calculated using the following equation:

$$\varepsilon = \frac{c-a}{a} = \frac{\sqrt{a^2+b^2}-a}{a}$$

ε: strain value; a: the distance between the edge of the nylon mesh stabilizer and the center of the stretching rod (pre-stretch length). b: the vertical distance between the tip of the stretching rod and the level of nylon mesh stabilizer. c: the distance between the edge of the nylon mesh stabilizer and the tip of the stretching rod (post-stretch length), which was derived with the Pythagorean theorem (see Fig. 2).

A strain value (ε) of 0.5 indicates that the cord was stretched to 50% above its initial length. For the experiments presented here, most of the cord strips were stretched to 100% beyond their initial length.

Compression

A flat, raised surface was provided at the center of the recording chamber, against which the isolated white matter strip could be compressed, using a rod attached to a motorized micromanipulator (Figs. 2D and 11F). The end of the rod provided a compression surface of 2.5 mm along the length of the tissue, with a transverse width of 7 mm, such that it was much wider than the tissue, even under compression. The compression rod was positioned perpendicularly to the tissue and was brought to a point of contact with its surface. After baseline measurements of conduction were obtained, the rod was advanced by means of the manipulator motor at a speed of 25 μm/s. The CAP and the displacement of the rod were monitored during the compression and the compression was stopped when the potential reached a set target amplitude. The rod was then removed rapidly upward to relieve pressure on the tissue and the recovery of the compound potential was monitored.

4-Aminopyridine (4-AP)

4-AP (Sigma, St. Louis, MO, USA) was dissolved in the same Krebs' solution used for normal superfusion. The pH of the solution was adjusted, where necessary, by addition of a small amount of hydrochloric acid.

HRP-exclusion test

To examine the morphology and extent of sealing of disrupted axons, white matter strips were transferred after stretch to oxygenated Krebs' solution containing 0.015% HRP. Following incubation for 1 h at room temperature, the tissue was fixed by immersion in 2.5% glutaraldehyde in a phosphate buffer. Transverse sections of the tissue were cut at 30 μm on a Vibratome and processed with diaminobenzidine by conventional methods to reveal the extent of HRP uptake in the damaged axons. Histological sections from the stretched cords and non-stretched control cords were compared. These latter sections helped to establish a control group for the presence of HRP in damaged fibers that might be unrelated to those deliberately injured. Digital images were taken using an optronics video camera and NIH Image software on a Macintosh computer.

Statistical treatment

Throughout the paper, a Student's *t*-test (two-tailed) was used to compare electrophysiological measurement between two groups (before and after stretch) in various experimental conditions. Linear correlation was expressed by a Pearson correlation coefficient (r). Statistical significance was attributed to values of $P < 0.05$. Average was expressed in mean ± S.E.M.

RESULTS

The changes of electrophysiological property in response to stretching

The form of CAP recorded from uninjured ventral white matter strips has already been discussed (Shi et al., 2000; Shi and Blight, 1996). A monophasic action potential, with the characteristics similar to intraaxonal recording, was routinely obtained after a 30-min equilibration of the spinal cord in the recording chamber (Fig. 3A). The spinal cord strips were then stretched and the amplitude of CAP was monitored (Fig. 2). The cord strips were usually stretched to a point when the CAP was completely abolished, which corresponds to an ~100% elongation of the cords. During this conduction

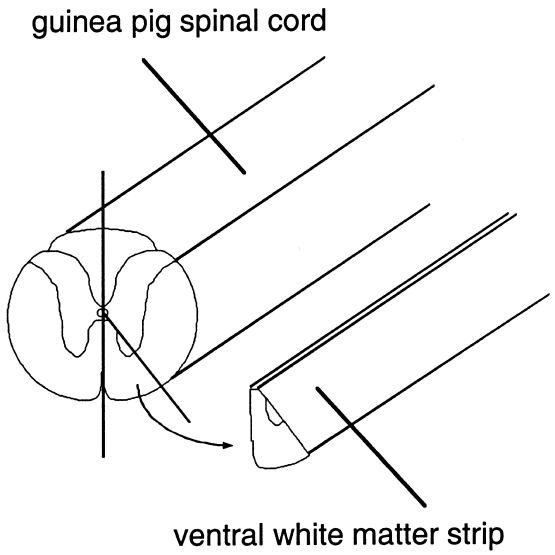

Fig. 1. Isolation of guinea-pig spinal cord ventral white matter. Diagram showing hemisection and radial incision made following removal of the spinal cord. The products are approximately 45-mm strips of ventral white matter.

decrease and loss, there was a simultaneous increase in the latency of the potential (Figs. 3 and 4). Once the CAP was completely eliminated, the stretch rod was quickly withdrawn and, subsequently, the CAP recovered rapidly. However, the amplitude of CAP was only partially recovered, usually reaching stable plateau levels within 10 min of recovery (Figs. 3 and 4). The latency partially recovered gradually after its initial increase. The extent of recovery of amplitude and latency varied with the degree of initial stretch and the potential loss at the peak of stretch. The plateau levels showed a similar degree of stability at all levels of injury (Fig. 4). Fig. 5A shows an exponential relation between the initial loss of CAP amplitude at the peak of stretch and the degree of recovery of amplitude in the plateau phase, measured at 30 min. However, a somewhat linear relation between the CAP amplitude and peak latency at 30 min post-injury was noted (Fig. 5B). This indicates that the deficits of conduction velocity generally increase with overall injury severity.

The decrease in amplitude of the response to stimulus following this stretch injury was not the result of a change in activation probability (threshold). This is evidenced by the similarity of the relationship between stimulus and response amplitude before and after injury. The response amplitude increased rapidly over a range of 300–500 μA stimulus current and then increased to a smaller degree over a further increase in stimulus (Fig. 6). A comparison of the response amplitude, before and after injury, to set stimulus currents showed no significant difference in the relative decrease in amplitude at different stimulus intensities over a wide range. Fig. 6 shows data from a white matter strip that was stimulated with increasing intensity before and after injury and the pre- and post-injury response amplitudes compared as a percentage of the maximal amplitude. The near unity slope of the latter relation, averaged from five strips,

indicates that there was no consistent selectivity of loss of conduction in fibers of lower or higher threshold after stretch (Fig. 6B). Since the large diameter axons usually have low threshold and the small diameter axons have high threshold (Kuffler et al., 1984), it is reasonable to conclude that stretch injury appeared to damage axons of a wide range of calibers. These features of the response to stretch are similar to the findings of *in vitro* compression injury (Shi and Blight, 1996; Shi and Borgens, 1999).

Refractory period was measured before and following stretch. Fig. 7A shows the classical relationship between the timing of paired stimuli and the amplitude of the two elicited CAPs. Paired stimuli, in which the interstimulus interval was between 0.3 and 15 ms, demonstrated typical reduction of CAP amplitude during the relative refractory period. Fig. 7B shows the increase in absolute refractory period to 0.6 ms in the stretched cords from the pre-injury time of 0.4 ms. This figure also shows a disturbance in the early portion of the relative refractory period, where the amplitude of the second CAP was slightly reduced when compared with that from before

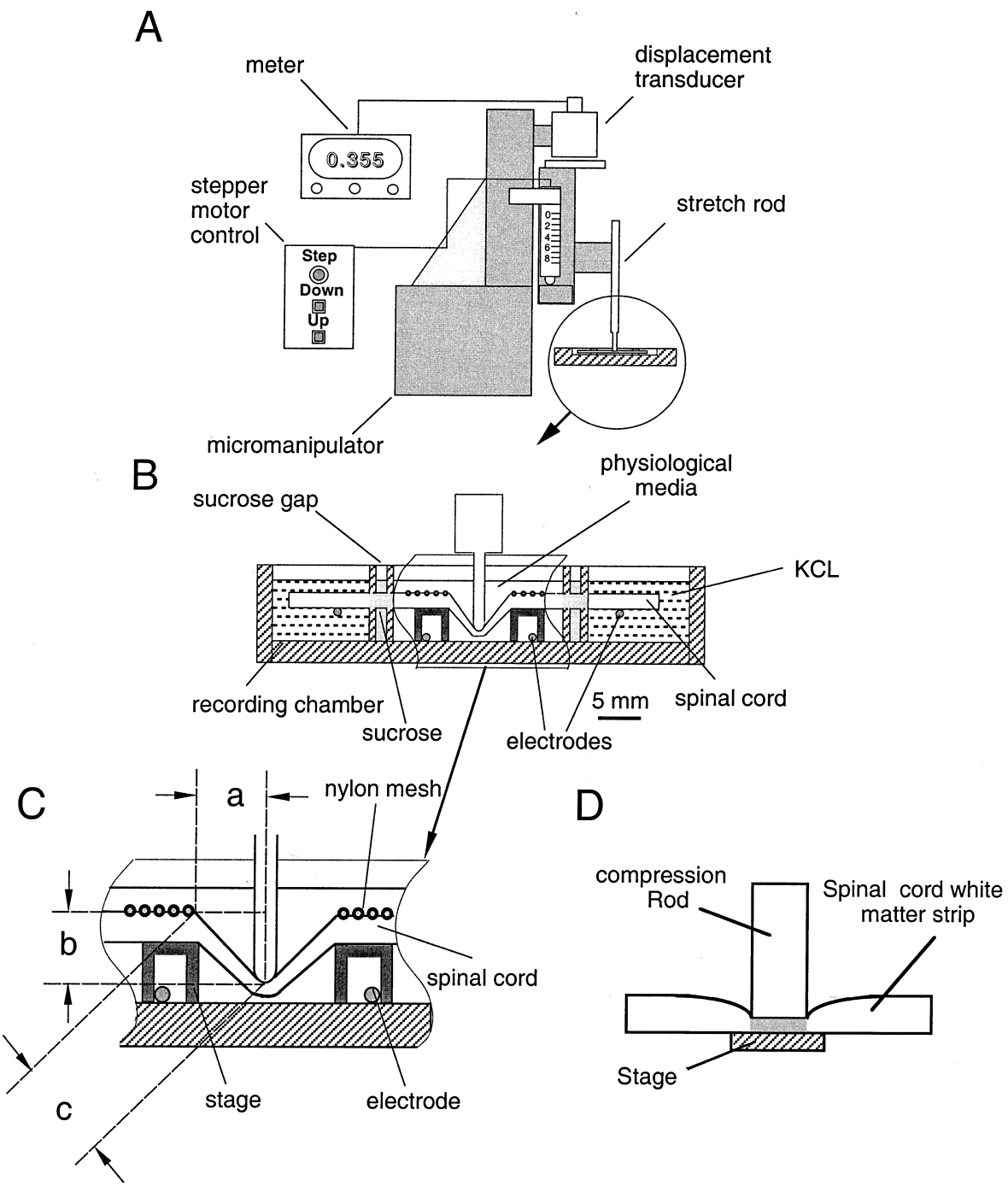

Fig. 2.

the stretch. However, this change was not statistically significant.

Since changes in refractory period may also affect the ability of the spinal cord axons to respond following a train of stimuli, we performed a train test pre- and post-injury. Fig. 8 shows the responses of spinal cord strips to a stimulus train of 500 Hz and 1000 Hz. Consistent with the result using paired stimuli, the injured cords also displayed a deficiency in response to multiple train stimuli. Fig. 8A shows an example of a typical response to a 1000 Hz/100 ms stimulus train, before and 30 min after stretch. The amplitude of post-injury response decreased by almost 50% compared to that of pre-injury. Fig. 8B shows the response of pre- and post-injury to 500 Hz/100 ms and 1000 Hz/100 ms train stimuli from an average of five cords. The difference in CAP amplitude following the train stimulation is only observed at high frequency (Fig. 8B).

4-AP effects on conduction in the plateau recovery phase

It has been previously shown that the potassium channel blocker 4-AP can improve CAP conduction in an injury where myelin is damaged, such as a severe compression injury (Shi and Blight, 1996, 1997; Shi et al., 1997). The postulated mechanism of this effect is that 4-AP blocks the activation of injury-induced fast potassium channels which otherwise short-circuit the action potential propagation (Shi and Blight, 1996, 1997; Shi and Borgens, 1999; Shi et al., 1997). It is thought that the fast potassium channels could be unmasked by the damage of myelin. Since stretch is likely to disrupt the functional integrity of myelin, the activation of the fast potassium channel is likely to contribute to the conduction block as a result of this injury model. We attempted to increase the CAP conductance with 4-AP blockade of these exposed channels. As shown in Fig. 9, application of the 4-AP to the superfusion medium produced a marked increase in the amplitude of the compound potential during the plateau phase of recovery. In this typical example, superfusion with 100 μM 4-AP at 30 min post-injury produced a 50% increase in the amplitude of the compound potential with no significant change in latency or in the overall shape of waveform, other than a slight increase in afterpotential. Using a total of five stretched cord strips, the average increase of CAP amplitude mediated by 100 μM of 4-AP is about $46\% \pm 6\%$ (data not shown). The increase in compound potential amplitude appeared to be complete within 15 min of superfusion of 4-AP, and was reversed with a similar time course after returning to normal Krebs' solution. Similar concentrations of 4-AP produce no significant increase in amplitude of the compound potential in uninjured white matter, and only a similar small increase in the amplitude of the depolarizing afterpotential (Shi and Blight, 1996, 1997).

Axon permeability changes in response to stretching

Since mechanical insult is likely to cause membrane damage, which could increase membrane permeability, we have performed an HRP-exclusion test to examine the axonal permeability following stretching injury. Before the injury, incubation of the white matter strip with HRP did not result in a significant percentage of axons taking up HRP ($0.3\% \pm 0.04\%$, $n = 4$, Fig. 10A). However, a 100% stretch injury increased the percentage of HRP-labeled axons to $20\% \pm 2\%$ when examined 2 min after stretch ($n = 4$, Fig. 10B), a significant increase compared to uninjured cords ($P < 0.0001$). Interestingly, when the cord strips were incubated with HRP at 30 min after stretch, very few axons were shown to be labeled with HRP ($0.5\% \pm 0.1\%$, $n = 4$), indicating a near complete sealing of axolemma to HRP (Fig. 10C).

Based on our previous studies and repeated in the current study, we found that spinal cord axons have a distinguished pattern of membrane damage when compressed *in vitro* (Shi and Borgens, 1999) (Fig. 11F, H). The axons located in the middle of the spinal cord cross-section are more likely to suffer membrane damage (Shi and Borgens, 1999) (Fig. 11F, H). In order to examine whether spinal cords subjected to stretch have different

Fig. 2. The stretch injury, compression injury and recording apparatus. (A, B) Apparatus used to produce a standardized stretch to the isolated spinal cord and the recording of CAP is shown. (A) Stretch injuries were produced by using a Plexiglas rod advanced at a controlled rate of 25 μm/s. Positioning of the stretch rod was accomplished with a micromanipulator. Standardized injury was controlled with a stepper motor to produce a finely graded stretch just sufficient to eliminate all of the CAP propagation (which was monitored continuously during the procedure). Injuries with lesser intensity were also produced based on the reduction of the CAP amplitude at the peak of stretch. (B) The end of the rod contacting the cord provided a surface of 3 mm along the tissue, and a transverse width of 5 mm. This surface was made convex longitudinally to minimize the pressure exerted to the cord, and concave transversely to prevent the cord from slipping out during the stretch. The spinal cord tract was immobilized on either side of the elongation site by a nylon mesh, carefully placed on the surface of the cord strips with a small amount of pressure. The vertical progress of the rod was monitored with a displacement transducer. In (B) The isolated spinal cord tract is shown mounted in the apparatus, with the injury site placed in the middle of the central well, which is continuously perfused with oxygenated Krebs solution. The two ends of the tract were placed in separate wells filled with isotonic KCl, divided from the central well by narrow channels filled with flowing isotonic sucrose solution. The electrodes were formed of silver–silver chloride wires. Action potentials were generated at the right sucrose gap, conducted through the injured part of the spinal cord, and recorded at left gap with the use of a bridge amplifier. The tissue in the central well was maintained at 37°C throughout the recording procedures. (C) A higher magnification of central well in (B) is shown. Also shown here is the schematic of the right triangle used to calculate the length of the injury segment following stretch (c). Original cord length is represented by (a) and vertical displacement by (b). (D) A diagram showing the segment of spinal cord strip that was compressed in the central well of a sucrose gap chamber. Compression of the spinal cord involved crushing it between the tip of the compression rod and a raised Plexiglas stage supporting the cord at this site. Positioning of the rod was accomplished with a micromanipulator. The compression injury was controlled with a stepper motor to produce a finely graded crush of predetermined severity.

patterns of axonal damage, we first increased the speed of stretch to 1.5 m/s which produced more axon damage, revealed by HRP labeling 2 min following injury (Fig. 11B–E). Interestingly, we found that under stretch, the axons close to the surface were most greatly damaged (Fig. 11B–E). This is in contrast with the compression injury of the same preparation using spinal cord white matter strips (Shi and Borgens, 1999) (Fig. 11F, H). This unique pattern of axonal damage revealed by HRP was found in areas between the edge of the nylon mesh stabilizer and the tip of the stretch rod (area a in Fig. 11A), as well as in the area directly below the stretch rod (area b of Fig. 11A).

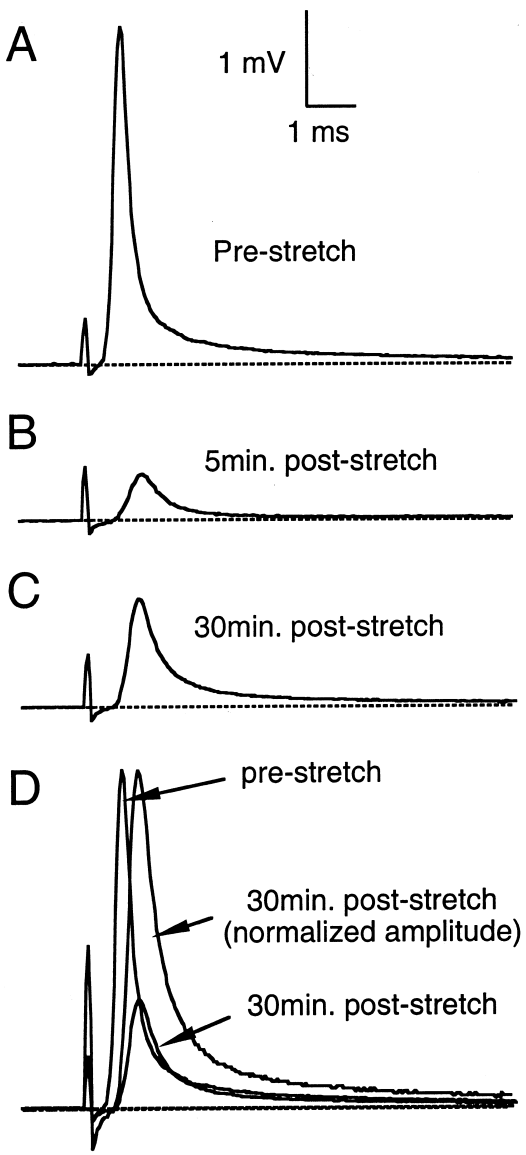

Fig. 3. Examples of loss and partial recovery of the CAP in response to stretch. CAPs recorded from a typical cord strip before and after stretch, shown (A–C) in the form of individual recordings and (D) superimposed recordings. Notice that the maximal CAP amplitude partially recovered at 5 (B) and 30 min (C) post-injury. (D) Pre- and post-stretch CAPs superimposed to demonstrate the increase in peak latency following the injury.

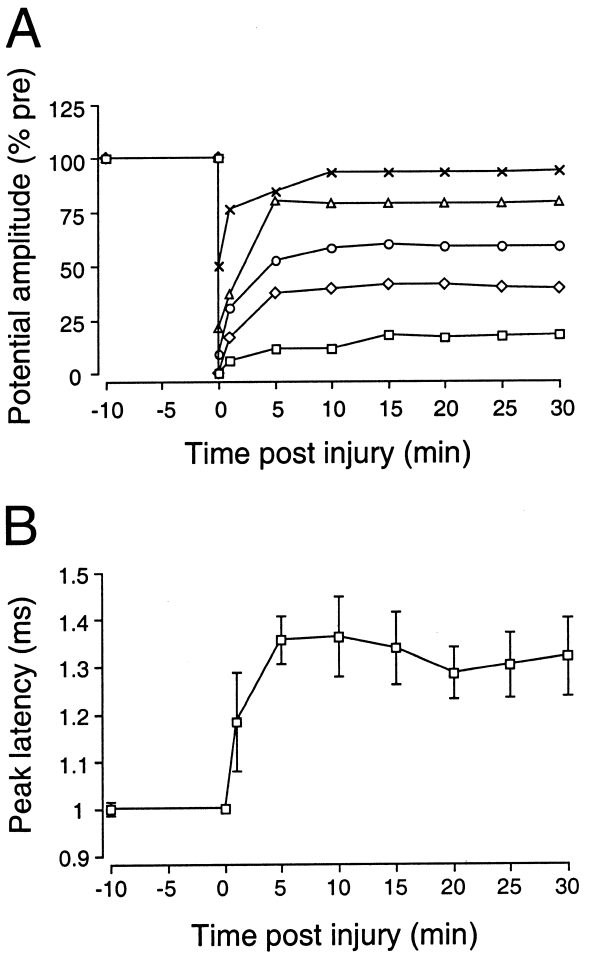

Fig. 4. Graphs showing the change in CAP amplitude and peak latency following stretch injury. (A) Five cases of varying degrees of conduction loss following stretching and recovery. The preparation represented by squares (□) subjected the most severe stretch and the preparation represented by cross (×) subjected the least severe injury. Notice the stability of conduction between 10 min and 30 min post-injury. (B) Mean ± S.E.M. of peak latency of CAP, plotted against time, for a set of 28 spinal cord strips stretched to complete loss of CAP and allowed to recover for 30 min.

DISCUSSION

In vitro model of axonal stretch

To our knowledge, our model of *in vitro* spinal cord stretch is unique in that it examines the response to stretch of many axons within an extracted spinal cord white matter strip. The studies using tissue culture are critical to examine the response of single axons subjected to stretch (Ellis et al., 1995; Galbraith et al., 1993; Smith et al., 1999). Such studies reveal information in greater detail regarding morphological and physiological changes of individual axons in response to this form of mechanical injury. However, problems emerge when one wishes to extrapolate single unit studies to the entire spinal cord. First, the examined axons could be a biased sample of the total pool of axons, based on the selectivity for larger fibers. Second, unlike monolayer tissue cultures, axons within the spinal cord white matter are

densely packed together, a factor likely affecting the behavior of axons subjected to stretch. By using isolated white matter strips, we can not only avoid such bias and examine the response of a population of axons with different sizes, but we can also injure the spinal axons in a preparation closer to an *in vivo* condition. In addition, in culture models of stretch injury, the injury site is close to or includes a cell body (Ellis et al., 1995; Smith et al., 1999). In such situations, the changes in macromolecular synthesis may occur very rapidly and make a significant contribution to neuronal responses to the physical stress. However, in spinal cord injury *in vivo* there may be a

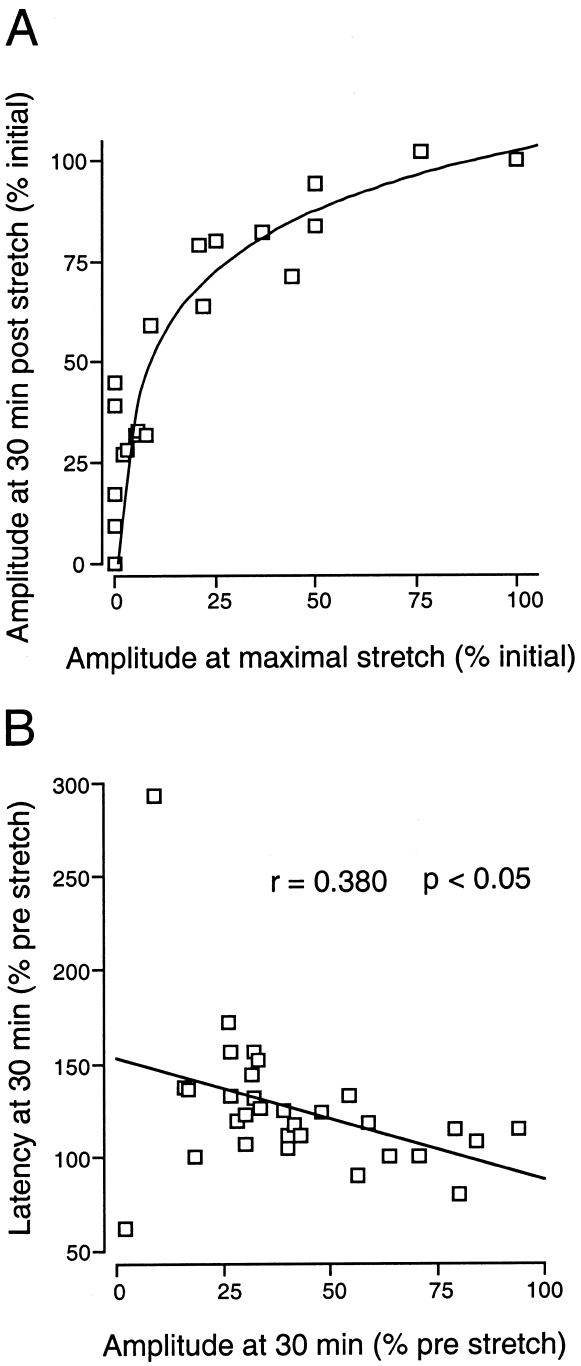

Fig. 5. (A) The relation between the initial loss of CAP amplitude at the peak of stretch and the degree of recovery of amplitude in the plateau phase, measured at 30 min. There was a clear exponential relationship between the two measurements over a range of stretch severity. (B) The relation between the CAP amplitude and peak latency at 30 min post-injury expressed as percentages of their pre-injury values, in a range of different injury intensities. It was clear that deficits of conduction velocity generally increase with overall injury severity. There was a significant negative correlation between the two measurements.

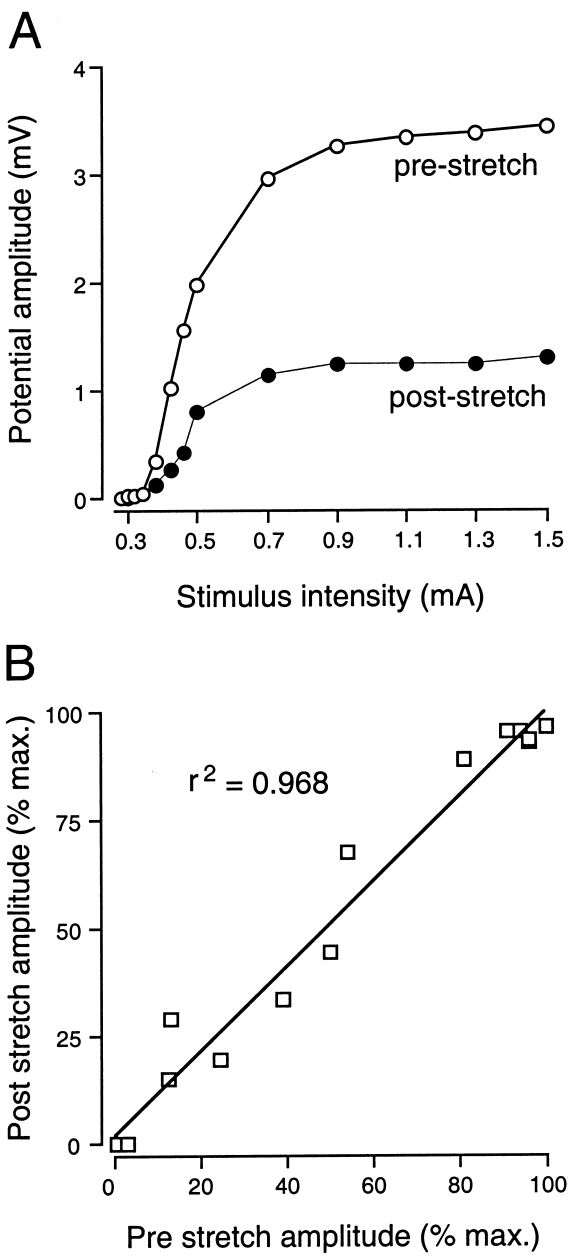

Fig. 6. The relation between stimulus intensity and the amplitude of CAP before and after injury. (A) An example of the CAP amplitude of the same spinal cord strip pre- and post-stretch. Notice that the amplitude increases rapidly with the stimulus intensity between 300 and 700 μA. Above that intensity there was only a slight further increase in amplitude. (B) Average amplitudes of CAP ($n = 5$) at 30 min post-injury plotted against pre-injury levels (each was expressed as a percentage of the maximum response, before and after injury) shows a least-squares linear regression line not significantly different than 1:1 linearity. This indicates that there is little difference in susceptibility to damage from stretching among axons with different stimulus thresholds.

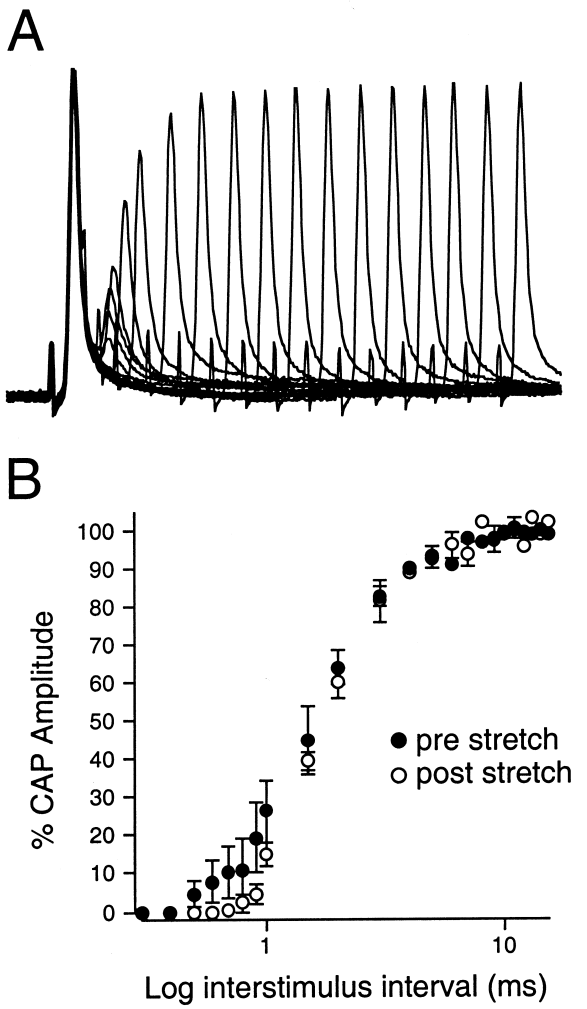

Fig. 7. Refractory period changes after twin pulse stimuli. (A) Superimposed individual recordings of CAP from a spinal cord strip in response to twin pulse stimuli with different interstimulus intervals. The first CAP shows consistent amplitude across all interstimulus intervals due to constant stimulus intensity. From left to right, the amplitude of the second CAP of each recording increases as the stimulus interval progresses through the relative refractory period. (B) The amplitude of the second CAP is plotted as a percentage of the first CAP against the log of the interstimulus interval for 5 white matter strips before (●) and 30 min. after stretch (○). Note the difference in CAP amplitude at small intervals.

significant time delay between the damage to the white matter and changes in RNA/protein synthesis in the perikarya of the injured neurons. Isolated white matter preparations such as the one in the current model allow study of myelinated axons responses to trauma in isolation from perikaryal responses. Therefore, the information derived from the current model is relevant to the *in vivo* spinal cord injury, and is a complement to the studies using tissue cultures.

One potential concern regarding this model is the potential for simultaneous compression injury. The stretch rod likely caused a disruption of axonal membrane due to the opposing normal force of inelastic elements within the strip (Figs. 2 and 11). However, we believe that the compression was minimal and did not affect the electrophysiological measurement significantly. This is mainly based on the following two factors. First, the part of the cord under the stretch rod (indicated by *b* in Fig. 11A) has an axonal damage pattern that is similar to other purely stretched segments of the spinal cord white matter strip (area a in Fig. 11A), and is distinct

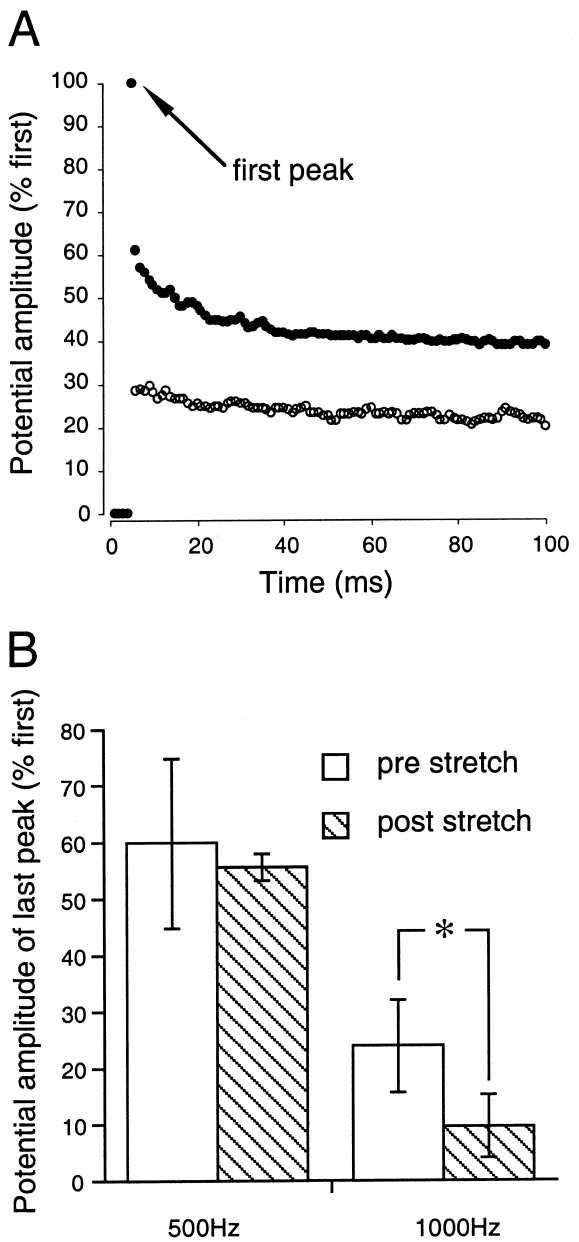

Fig. 8. Response of spinal cord strips to train stimuli at frequencies of 500 Hz and 1000 Hz. (A) A series of CAPs from a typical cord strip in response to a train of stimuli of 1000 Hz and 100 ms duration, before (●) and 30 min after stretch (○). Each CAP was plotted as a percentage of the first CAP of the responses to the train stimuli. Notice the amplitude of CAPs post-stretch was reduced by half compared to that of pre-stretch. (B) Bar graph showing the pre- and post-injury responses to 500 Hz and 1000 Hz stimuli. The data were averaged from the last CAPs of the responses of five cord strips to the train stimuli (mean ± S.E.M.). It is clear that a difference is only observed at high frequency (*$P < 0.05$, Student's *t*-test). The example shown in (A) was included in the analysis which generated the graph in (B).

from that of compression injury (Fig. 11F, H). Second, as shown in Fig. 11, the overall numbers of axons that suffered membrane damage is similar in area *a* compared to area *b* (Fig. 11B–E), which indicates the membrane damage of the stretched axons is evenly distributed along the axons. These findings suggest that the axon segment underneath the stretch rod suffered mostly stretch injury, like other parts of axons, and the compression was minimal, if any.

Three forms of conduction deficit

The principal finding of the study was that stretch injury of ventral white matter produces at least three different forms of conduction deficits, marked by distinct temporal characteristics and degrees of reversibility. First, there was a proportion of the conduction loss that was spontaneously reversible within 5 min of release of stretch for all the injuries produced within the range of severity. This may represent a transient ionic imbalance caused by a temporary increase of membrane permeability to certain ions as a result of stretch. The second conduction deficit was seen upon exposure to the potassium channel blocker 4-AP. During the stable period, it produced a reversible increase of amplitude of the compound potential without change of peak latency. This indicates that a component of the amplitude loss over more prolonged periods is based on conduction block in axons that suffered myelin damage, yet remain in continuity through the area of injury. Third, some of the amplitude loss remained irreversible within the course of the experiments performed, even during the presence of 4-AP. This portion of the conduction loss may result from more profound anatomical membrane damage inflicted by stretch. Such membrane damage could result from primary physical insult, or as a result of Ca^{2+}-mediated structure damage. It is well known that Ca^{2+} enters the cell through damaged membrane. Various researchers have demonstrated that Na^{+} influx

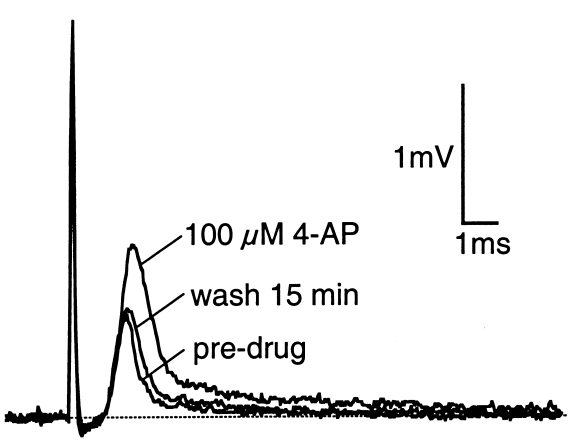

Fig. 9. Changes of CAP amplitude of a representative cord strip in response to 4-AP, 30 min following stretch injury. Superimposed traces show the increase in CAP amplitude with 4-AP and its reversal within 15 min of washing. 4-AP was added to the Krebs' perfusion to produce a concentration of 100 μM, maintained for 15 min.

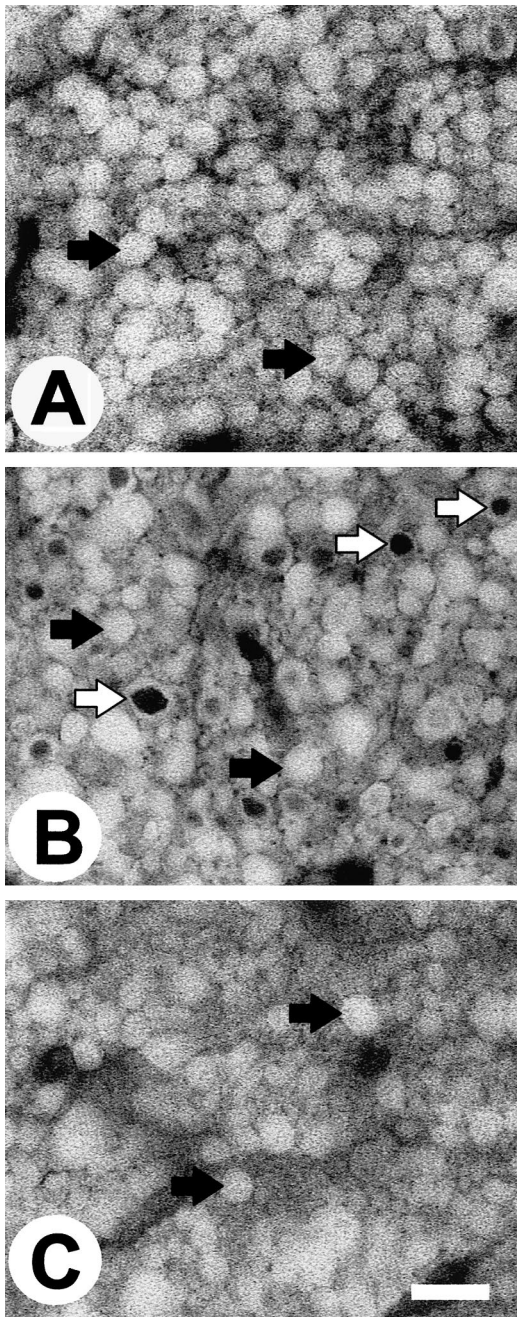

Fig. 10. Examples of HRP labeling before and after stretch. The cord strips were immersed in a solution of HRP and subsequently fixed and developed using a diaminobenzidine technique. Vibratome sections were cut in the middle of the stretched cord segment or controlled uninjured cords. (A) A cross-section of a control uninjured cord segment immersed in the HRP solution. (B) A cross-section of a cord strip that was exposed to HRP 2 min following stretch. (C) A cord strip exposed to HRP 30 min following stretch. Notice a small portion of axons was clearly labeled by HRP. It is clear that some axons, though low in number, were labeled with HRP 2 min after stretch. At 30 min following stretch, however, the number of axons labeled with HRP has reduced to a level comparable to that of pre-injury. The open arrows indicate axons which were labeled with HRP and filled arrows denote axons which exclude HRP. Scale bar = 10 μm.

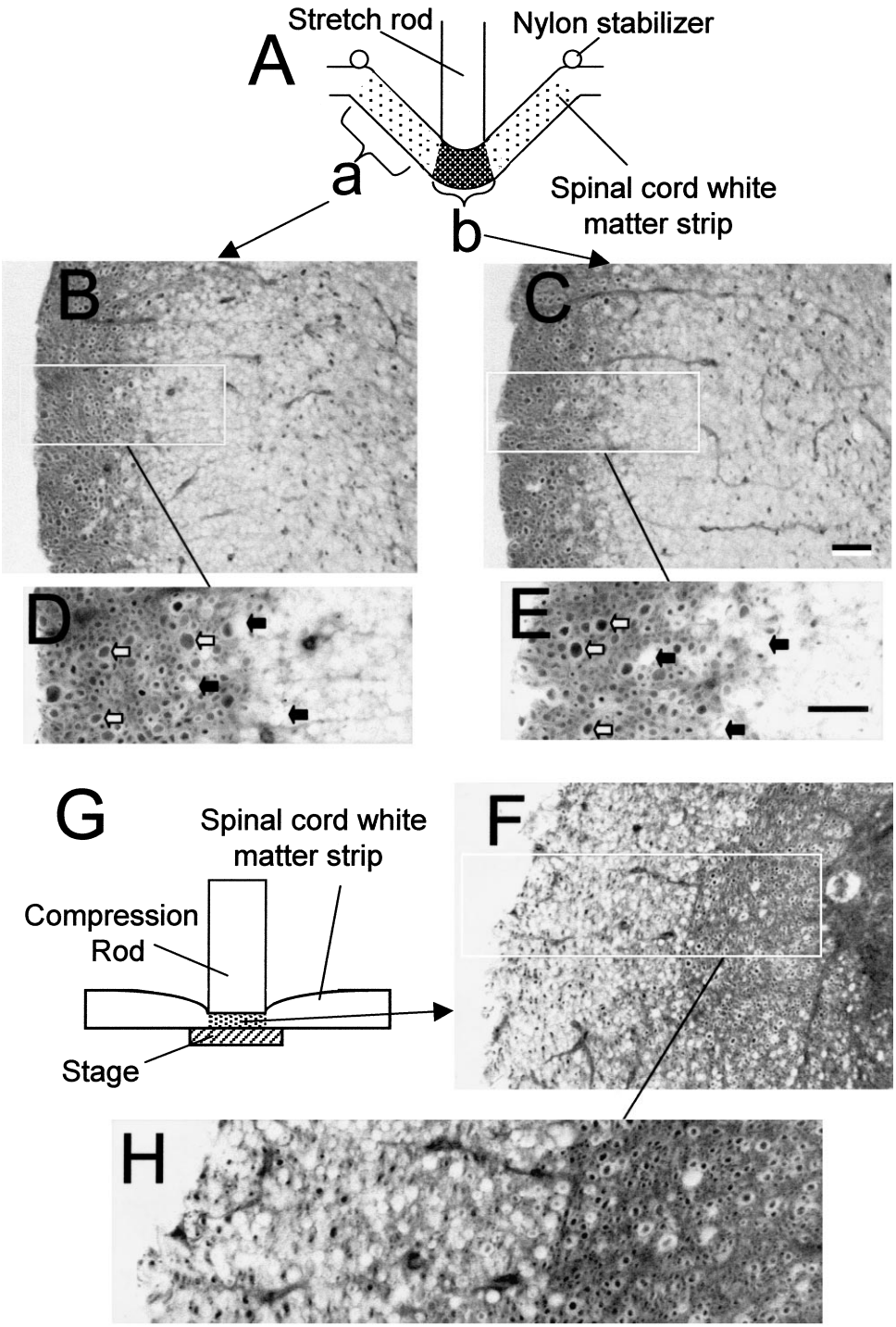

Fig. 11. The characteristics of axonal membrane damage in stretch and compression injury. (A) A diagram showing the segment of spinal cord strip that was stretched within the central well of the double-sucrose recording chamber. The letter a denotes the segment between the tip of the stretch rod and nylon stabilizer while b is the segment directly under stretch rod. (B–E) Examples of transverse sections of white matter strip with HRP-labeled axons to reveal the patterns of axolemmal damage within the spinal cord. Section B was taken from area a and section C was taken from area b. The left side of (B–E) include the surface or outside rim of the cord strip. The rectangular in (B, C) circumscribe the region shown in (D, E) at higher magnification. The open arrows indicate axons which were labeled with HRP and filled arrows denote axons which exclude HRP. (G) A diagram, similar to that in Fig. 2G, showing the segment of spinal cord strip that was compressed in the central well of a sucrose gap chamber. The part of the cord strip within the injury site was taken for the subsequent anatomical study. (F, H) An example of transverse section of cord strip with HRP-labeled axons to reveal the patterns of axolemmal damage within the spinal cord. The orientation of the section in (F) is the same as that of (B) or (C). The rectangular area in (F) circumscribes the region shown in (H) at higher magnification. The open arrows indicate axons which were labeled with HRP and filled arrows denote axons which exclude HRP. Scale bar = 50 μm (B, C and F); 20 μm (D, E and H).

could also encourage influx of Ca^{2+} through reverse operation of the Na^+/Ca^{2+} exchanger (Stys et al., 1991, 1992; Waxman et al., 1991). To support this notion, efforts to reduce Na^+ entry after anoxic and physical injury can significantly reduce tissue damage and enhance CAP amplitude (Agrawal and Fehlings, 1996; Boening et al., 1989; Goldberg et al., 1986; Rosenberg and Lucas, 1996; Rosenberg et al., 1999; Stys et al., 1992; Teng and Wrathall, 1997).

Following stretch, there was also a striking stability of conduction *in vitro* between 10 min and 30 min post-injury, indicating a lack of clear secondary deterioration or spontaneous repair in this period. During the course of stretching, a graded decrease in amplitude of the compound potential was accompanied by a small increase in conduction delay. The amount of irreversible loss related directly to the extent of elongation and initial compound potential loss. In addition to a proportion of the conduction loss that was reversible within 60 s of stretch, a further slight recovery of amplitude occurred over 3–5 min, and this was followed by a prolonged period of stable amplitude. The overall characteristic of conduction recovery following stretch is similar to that following compression injury.

The vulnerability of axons to stretch injury as a function of axonal diameter

The relation between axon caliber and electrical stimulation threshold is expected to exist in this preparation based on previous studies of spinal cord white matter (BeMent and Ranck, 1969; West and Wolstencroft, 1983). The current study indicates that there is no selectivity of vulnerability of axons to stretch based on diameter. This conclusion is based on the observation that there was no significant difference in activation probability between injured and uninjured cords (Fig. 6B). The lack of selectivity of the initial injury for large axons is also supported by previous studies of compression injury using the same preparation (Shi and Blight, 1996; Shi and Borgens, 1999). It is interesting to point out that the large axons are more vulnerable to oxygen and energy deprivation using the same preparation (M.A. Peasley and R. Shi, unpublished observation). These data strongly suggest that the selective loss of large axons seen *in vivo* (Blight, 1991; Blight and DeCrescito, 1986) following mechanical injury may result from true secondary pathological mechanisms and perhaps a selective metabolic susceptibility of large fibers.

Double and multiple pulse responses

Measurements of the refractory period at 30 min post-injury with double pulse stimulation showed no overall change in the duration of the relative refractory period, although an increase in the absolute refractory period was evident (Fig. 7). This is consistent with the multiple stimuli response examined at the same time point (Fig. 8). When the frequency of the trains of stimulation was at 500 Hz (interstimulus interval = 2 ms), no significant difference in terms of the amplitude of the responsive CAP was observed. However, when the frequency was 1000 Hz (interstimulus interval = 1 ms), during which the stimulus interval is similar to that in the early part of the relative refractory period, the CAP amplitude of post-injury strips decreased by 50% compared to the pre-injury (Fig. 8). The underlying mechanism of this phenomenon is not clear. One possibility is the over activation of Na^+/K^+ pumps following stretch, which makes the membrane more hyperpolarized and therefore more difficult to reach threshold (Gordon et al., 1990). However, an increase in the proportion of sodium channels being inactivated is also possible (Kuffler et al., 1984). In summary, these data indicate that some axons continue to propagate action potential after injury, with some conduction deficits only apparent following high-frequency multiple stimuli.

Response to 4-AP

As we have discussed in previous publications, the CAP amplitude increase induced by 4-AP, using a double sucrose gap technique, can be interpreted as an increase in the number of the axons conducting action potential through the injury site (Shi and Blight, 1996, 1997; Shi et al., 1997). To support this, 4-AP has been shown to promote recovery of conduction in chronically injured spinal cord axons using intracellular microelectrode recording from single fibers (Blight, 1989), as well as the double sucrose gap techniques (Shi and Blight, 1996, 1997; Shi et al., 1997). Recovery of conduction is also consistent with earlier studies showing reversal of conduction block with 4-AP in demyelinated peripheral nerves (Kocsis et al., 1986). Such effects on conduction can be explained as a direct result of the blockade of axonal potassium channels. This could lead to a reduced shunting of the current, although the mechanism of effect may include other factors including alteration of the resting membrane potential. Consistent with the theory that myelin damage may contribute to the activation of previously silent fast potassium channels, we have shown that both chronic and acute mechanical damage of the spinal cord clearly results in axonal myelin damage (Shi et al., 1997). In summary, there is strong electrophysiological evidence suggesting that elongation of spinal cord axons results in myelin damage. Such damage can unmask the K^+ channels and cause a conduction block.

The implication of HRP-exclusion test: membrane permeability increase

Since an HRP molecule (MW: 44 kDa) is too large to pass through any known membrane channel, we believe that exclusion of HRP molecules gives an accurate measure of membrane integrity. This is supported by the similarity between the time course of dye exclusion and the electrophysiological measurements of membrane sealing plus the recovery of membrane potential in our previous studies (Shi and Pryor, 2000). Consistent with our findings, HRP has also been successfully used to denote the increase of axonal membrane permeability in a rat brain subjected to contusion injury (Pettus et al., 1994).

Furthermore, Povlishock and his colleagues have demonstrated that membrane damage revealed by HRP correlates with profound axolemma damage compounded with many axoplasmic disruptions using the same rat brain contusion model (Pettus et al., 1994). In our current study, it was found that the permeability to HRP was increased within 2 min following stretch, but recovered within 30 min following injury (Fig. 10). This indicates that the axons have the ability to reseal the membrane damage inflicted by a stretch injury in a physiological media and temperature at the injury severity used in our current study. The exclusion is similar to that seen in transection injury where the cut end of axons were largely sealed within 30 min post-injury in the same conditions (Shi and Pryor, 2000). It is possible that more permanent structural damage may develop as a result of more severe stretch. In the current study, the injury severity, the degree of stretch (or strain), and the rate of stretch, were chosen mainly based on the feasibility of using such a model. It is unknown how closely this mimics clinical injury. Nevertheless, this could serve as a starting point and the conditions could be modified to emulate human spinal cord injury. For example, the speed of the stretch, the degree of elongation, and the composition of the media can certainly be altered to explore these variables of a stretch injury.

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