Effects of 4-Aminopyridine on Stretched Mammalian Spinal Cord: The Role of Potassium Channels in Axonal Conduction

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Jensen, Jennifer M. and Riyi Shi. Effects of 4-aminopyridine on stretched mammalian spinal cord: the role of potassium channels in axonal conduction. J Neurophysiol 90: 2334–2340, 2003. First published July 9, 2003; 10.1152/jn.00868.2002. Axonal conduction deficit is a major contributor to various degrees of disability after spinal cord injury. 4-aminopyridine (4-AP), a potassium channel blocker, has been shown to restore some conduction and improve neurological function in both animal and human studies. Using a double sucrose-gap recording device, we have examined the effects of 4-AP on isolated guinea pig spinal cord white matter after stretch injury. At a concentration of 100 μM, 4-AP increased the amplitude of the compound action potential by 100% while 1 μM 4-AP increased it by 43%, a larger response than seen following compression injury. The length of affected tissue is suggested as a potential explanation of this differential sensitivity to 4-AP. Plastic sections taken from the injury site revealed severe myelin damage, especially in the paranodal area, which may also partially explain why 4-AP has more effect on conduction after stretch injury than compression. In addition, we have shown that while enhancing conductivity in some axons, 4-AP significantly reduced the overall responsiveness to multiple stimuli, as evidenced by increase of the refractory period in response to dual stimuli and the decreased ability to follow repetitive stimuli. This increased refractoriness may be largely attributed to residual deficits in fibers newly recruited by 4-AP treatment.

INTRODUCTION

Disruption of axons in the white matter is the most significant contributor to the devastating clinical deficits that result from spinal cord injury. A better understanding of this pathology should help in the development of effective therapeutic interventions for both acute and chronic injuries. It is clear that axonal conduction block may result from nontranssectional damage, such as compression and stretch (Shi and Blight 1996; Shi and Pryor 2002), which are clinically more common than complete transection or partial lacerating injuries. Since varying amounts of axons in such an injury remain intact (Blight 1983, 1991; Blight and DeCrescito 1986; Shi and Pryor 2002), this provides the possibility that effective interventions may help the axons to reestablish action potential conduction and contribute to neurological function. This offers a more accessible method to regain functional activity (Blight 1989; Blight et al. 1991; Shi and Blight 1997; Shi et al. 1997; Shi and Pryor 2002; Targ and Kocsis 1985) compared with the effort to encourage severed axons to regenerate (Bähr and Bonhoeffer 1994; Schwab and Bartholdi 1996).

It is crucial to understand the mechanism of axonal conduction loss to overcome such a deficit. Recently, the contribution of axonal potassium channels as a critical factor in conduction loss after spinal cord injury in both experimental models (Blight 1989; Blight et al. 1991; Shi and Blight 1997; Shi et al. 1997; Shi and Pryor 2002; Targ and Kocsis 1985) and in human victims (Hayes 1994; Hayes et al. 1994, 1995) has been established. 4-aminopyridine (4-AP)-sensitive potassium channels have been postulated to play a significant role in conduction loss by lowering the axonal excitability. Such potassium channels are usually covered by myelin in the inter-nodal area of CNS myelinated axons (Waxman and Ritchie 1993) and therefore shielded from activation by the normal action potential. Damage to myelin, in the case of spinal cord injury, has been proposed to unmask these channels and induce conduction block (Blight 1993). In fact, 4-AP has been shown to effectively enhance action potential propagation following spinal cord trauma by blocking potassium channels in in vitro studies (Blight 1989; Shi and Blight 1997; Shi et al. 1997) and improve neurological function in human (Hansebout et al. 1993; Hayes 1994; Hayes et al. 1994, 1995) and animal clinical trials (Blight et al. 1991). These studies have established the important role of the 4-AP–sensitive potassium channel in conduction block.

Although examined repeatedly in experimental compression injury (Blight 1989; Shi and Blight 1997; Shi et al. 1997), 4-AP has not been tested in detail after isolated stretch injury (Shi and Pryor 2002). Stretch is a major component of mechanical insults and is likely to be more detrimental in axonal injury than compression (Blight and DeCrescito 1986; Shi and Borgen 2000; Shi and Pryor 2002). It is therefore important to examine the effects of 4-AP in isolated stretch injury to determine the nature and extent of potassium channel-related conduction loss. These studies are expected to elucidate the mechanism of conduction loss in stretch injury, as well as offer suggestions to overcome such functional deficit.

Despite much progress in both in vitro and in vivo studies, the ideal parameters for use of 4-AP in human spinal cord injury are not yet thoroughly established. For example, the benefit of micromolar 4-AP (0.5–1 μM), a safe level in the blood of a patient, remains modest and sometimes difficult to detect in single-dose studies (e.g., Donovan et al. 2000; Halter et al. 2000) There is still a need to dissect out the magnitude of effect and the mechanism of action of 4-AP in injured axons...
Electrophysiological recording and analysis

**RECORDING CHAMBER.** Various configurations of the basic recording chamber have been described in previous publications (Shi and Blight 1996; Shi and Borgens 1999; Shi and Pryor 2002). As illustrated in Fig. 1, a strip of spinal cord white matter approximately 42 mm in length and approximately 1.5 mm in diameter was placed across the chamber with the central compartment (volume: 3.6 ml) receiving a continuous perfusion of oxygenated Krebs’ solution (2 ml/min). The stimulating and recording electrodes were not in direct contact with the spinal cord tissue (Fig. 1, elevation view) so the available action potential conduction distance was the entire 38 mm contact with the spinal cord tissue (Fig. 1, elevation view) so the available action potential conduction distance was the entire 38 mm contact with the spinal cord tissue (Fig. 1, elevation view). Axons with different diameters have different thresholds to fire an action potential in response to stimulation (BeMent and Ranck 1969; West and Wolstencroft 1983). Current-voltage tests, which consist of a series of stimuli with increasing intensity, can gradually stimulate axons of different thresholds to fire action potentials. The larger diameter axons will theoretically tend to be activated first, at a given stimulus intensity, although in practice, the CAP appears to be dominated by large caliber axons (see DISCUSSION). This test was used to detect changes in activation threshold (probability) before and after 4-AP application (Peasley and Shi 2002). The stimulus intensities ranged from 0.5 to 13 ms. The amplitude of the first CAP was equal to or >90% of the first one was defined as the relative refractory period. The stimulus intensity level, five stimuli were repeated, and an average value was used. Throughout the test, the stimulus was always delivered at a frequency of one stimulus every 3 s.

**DOUBLE PULSE RESPONSE-REFRACTORY PERIOD.** The refractory period was examined by stimulating the white matter strip with a series of twin stimuli (110% of the maximal stimulus) at various interstimulus intervals, ranging from 0.5 to 13 ms. The amplitude of the first responding action potential remained the same for each pair of stimuli. The time when the second responding CAP was equal to or >90% of the first one was defined as the relative refractory period. The stimulus intensities ranged from 0.5 to 13 ms. The amplitude of the first responding action potential remained the same for each pair of stimuli. The time when the second responding CAP was equal to or >90% of the first one was defined as the relative refractory period.

**MULTIPLE PULSE RESPONSE.** Trains of repetitive stimuli were delivered to the white matter strip in the chamber at both 500 and 1,000 Hz for 100 ms. The last three CAPs were averaged and expressed as a percentage of the first CAP.

**Stretching**

The device employed to induce stretch injury and the estimation of the magnitude of stretch or “strain” (the degree of elongation from the initial length) are described in our previous publication (Shi and Pryor 2002). As shown in Fig. 1, a flat raised surface with a small hole was
but stabilized over a period of 30 min after the cord strips were mounted in the recording chamber.

Conduction deficit as a result of mechanical stretch

RESULTS

Statistical analysis

Throughout the paper, Student’s t-test was used to compare electrophysiological and histological measurements between different groups. Linear correlation between some electrophysiological measurements were expressed by Pearson correlation coefficient (r). Statistical significance was attributed to values \( P < 0.05 \). Averages were expressed as mean \( \pm SE \).

RESULTS

Conduction deficit as a result of mechanical stretch

The amplitude of the CAP gradually increased in the initial stage after the cord strips were mounted in the recording chamber, but stabilized over a period of 30–60 min. Following an additional 10-min recording of CAP baseline, the cord was stretched, and the amplitude of the CAP was monitored continuously. As illustrated in Fig. 2, such an insult initially completely abolished CAP conduction. Once the target strain level was reached, the stretch rod was quickly withdrawn, and subsequently, the CAP slowly but steadily recovered, reaching a plateau at 30 min following injury (Fig. 2). The amplitude of CAP following 30 min recovery was about 19% of the prestretch level.

4-AP enhances axonal conduction after stretch

Application of potassium channel blocker 4-AP through the perfusion medium resulted in a striking increase in the amplitude of the CAP at 30 min after injury. An example is illustrated in Fig. 3, where 100 \( \mu M \) 4-AP produced a 100% increase in the amplitude. The increase of CAP appeared to be largely reversible after wash with normal Krebs’ solution. Overall, the application of 4-AP increased CAP amplitude from 0.44 \( \pm \) 0.06 to 0.91 \( \pm \) 0.11 mV (Fig. 3, \( n = 20, P < 0.001 \)). At a concentration of 1 \( \mu M \), 4-AP produced an average of 43% increase in the amplitude of CAP in the stretched axons (\( n = 6, P < 0.005 \)). In addition, we performed experiments to examine the effect of 4-AP on the cords subjected to nylon mesh pressure, without stretch. The CAP amplitude in the presence of 4-AP (101.4% of pre–4-AP) was not significantly altered (\( n = 5, P > 0.05 \)).

The increase of CAP amplitude as a result of 4-AP application was not the consequence of a change in activation probability (threshold), because the relation between stimulus and response amplitude was similar before and after 4-AP application (Figs. 4 and 5). It is obvious during a wide range of stimulus intensities that CAP response in the presence of 4-AP is proportionally larger than before 4-AP application. Both before and after 4-AP application, the response amplitude increased rapidly over a range of 1.85–2.5 V stimulating voltage and increased to a smaller degree over an extended increase in stimulus intensity (Fig. 4). A comparison of the...
nearly all the stimulus intensities (\textit{matter strips. Clearly the response amplitude increased with 4-AP exposure at fi
voltages showed no signi
response amplitude, with and without 4-AP, to a set of stimulus voltages showed no significant difference in the relative increase of amplitude at different stimulus intensities over a wide range. Figure 5 shows data from nine different cord specimens that were stimulated at similar intensities before and after 4-AP and compared in absolute terms. The near unity slope of the relation indicates that there was no consistent selectivity in the enhancement of conductivity in fibers with low or high thresholds in response to 4-AP application.

Changes in responsiveness to dual and multiple stimuli as a result of 4-AP exposure

Figure 6\textit{A} shows the relationship between the interstimulus interval and the amplitude of the two elicited CAPs. The amplitude of the second CAP was plotted against the log of the interstimulus interval revealing the expected sigmoidal relationship (Fig. 6\textit{B}). The exposure to 4-AP increased the absolute refractory period from 0.83 ± 0.03 to 1.05 ± 0.04 ms (\textit{n} = 15, \textit{P} < 0.0005) and relative refractory period from 4.77 ± 0.29 to
7.54 ± 0.65 ms (\textit{n} = 13, \textit{P} < 0.005). Washing with normal Krebs’ solution reversed the change completely (Fig. 6\textit{C}).

Changes in the ability to follow repetitive stimuli were also tested in response to 4-AP application. Figure 7\textit{A} shows an example of responses to the train stimuli administered to the white matter strip. Figure 7\textit{B} indicates that the average amplitude of the last three CAPs in response to train stimuli of 500 Hz and 100 ms was 42.2 ± 3.7% of the first CAP for pre-4-AP and 22.2 ± 2.9% with 4-AP. This 4-AP-mediated decrease of the last CAP amplitude in response to train stimuli is significant (\textit{n} = 16, \textit{P} < 0.001). The results for the train test of 1,000 Hz and 100 ms were 18.7 ± 1.6% for pre-4-AP and 6.2 ± 1.5% with 4-AP (\textit{n} = 16, \textit{P} < 0.0005). These data indicate that there is a significant decrease in amplitude responsiveness in lower and higher frequencies after 4-AP treatment.

Myelin damage revealed by anatomical examination

The acutely stretched cords were examined anatomically using toluidine blue on 1-μm sections taken from the lesion site. The uninjured cords were also processed in the same manner and observed for comparison. In the uninjured cords (Fig. 8\textit{A}), the myelin is adherent to the axolemma at the paranode regions on either side of the nodes of Ranvier. However, there are several conspicuous changes of individual axons, as well as the overall structure, in the stretched cords. There are frequent cases of significant damage in the node of Ranvier, marked by increased length of the node and the separation of myelin and axonal membrane in the paranodal area (Fig. 8\textit{B}). A lesser degree of myelin damage is also visible in Fig. 8\textit{C}, where large vacuoles developed between myelin and the axolemma in the paranodal area, perhaps from a condition preceding severe myelin damage (Fig. 8\textit{B}). Notice also the undulating course along the longitudinal axis of the axons. There also appears to be more extracellular space compared with that of uninjured cords. Overall, both axons and myelin have an irregular appearance along the longitudinal axis compared with the normal tissue (Fig. 8\textit{D}).

DISCUSSION

In vitro model of axonal stretch

The in vitro spinal cord ventral white matter stretch model described in the current study and in a previous publication...
(Shi and Pryor 2002) from this group has several unique features compared with other existing in vivo (Maxwell et al. 1991) and in vitro mechanical stretch models (Ellis et al. 1995; Smith et al. 1999). This in vitro, or ex vivo, model enables us to gain control of experimental conditions while preserving the local environment similar to in vivo conditions (Shi and Pryor 2002). Since the spinal cord tissue is studied in ventral white matter, not single axons, the local extracellular matrix that may affect the behavior of axons subjected to stretch is preserved,

FIG. 6. Refractory period changes in response to 4-AP and is reversed following washout. A: individual CAP recordings from ventral white matter are superimposed, revealing a changing response to twin pulse stimuli with varying interstimulus intervals. Due to constant stimulus intensity in the 1st recording, the initial CAP peaks at a consistent amplitude; however, as the interstimulus interval progresses, the amplitude of the 2nd CAP as a percentage of the 1st one is plotted against the log of the interstimulus interval in 3 conditions: pre–4-AP, with 100 μM 4-AP, and wash with normal Krebs. Note the increase of both the absolute refractory period and the relative refractory period on exposure of 4-AP and their reversal on wash with normal Krebs’ solution (n = 19). C: bar graph showing the changes of absolute and relative refractory period as a result of 4-AP application and their reversal on wash (***P < 0.0005, *P < 0.001, n = 13–16). The time when the 2nd responding CAP was equal to or >90% of the 1st one is defined as relative refractory period.

FIG. 7. Response of spinal cord strips to train stimuli. A: series of CAPs from a typical ventral white matter strip responding to train stimuli at 1,000 Hz with 100-ms duration. B: bar graph showing the pre–4-AP and 4-AP-treated responses to 500- and 1,000-Hz stimuli. Data for this graph was obtained from the average of the last 3 waveforms as a percentage of the first of 16 cord strips. Noticeably, there is a clear increase in amplitude observed at the lower frequency (*P < 0.001) as well as at higher frequency (**P < 0.0005) as a result of 4-AP application.

FIG. 8. Photographs of toluidine blue–stained, 1-μm sections of uninjured and stretched spinal cord white matter strips. A: longitudinal sections from an uninjured ventral white matter showing the node of Ranvier (arrow) and paranode region; notice the symmetry of the myelin in either side. Axons appear to be densely packed. B: longitudinal section from acutely stretched ventral white matter displaying disruption of the paranode region; notice the increased length of node of Ranvier (arrow). Also shown here is the apparent separation of myelin and axolemma. C: similar section from a stretched cord showing the myelin damage in the paranodal region (arrow). Notice the large vacuole formed in this region. D: another longitudinal section from a stretched cord strip showing the characteristic feature of axons following stretch. Notice the undulating course developed along the longitudinal axis of the indicated axon (arrow).
which makes this investigation more relevant to an in vivo situation. Furthermore, by using a sucrose-gap extracellular recording device, the CAP can be monitored continuously, even during the stretch (Shi and Pryor 2002). By varying the speed of loading, magnitude of loading, and the condition of the experiment, including physical, chemical, and pharmacological variations, this model has the potential to elucidate the detailed mechanisms of primary and secondary injury related to stretch as well as pharmacological therapies to reverse the damage.

Similar to our previous published recordings, the CAP generated from the double sucrose-gap apparatus is monophasic. The relationship between this monophasic potential and the underlying axonal conduction has not been examined in detail, although some considerations may be useful. The white matter of the guinea pig spinal cord contains a range of myelinated axons with diameters from <1 μm to about 12 μm in diameter (in fixed tissue) (Blight 1991). The ventral white matter shows a monophasic distribution, with approximately 100 times more axons in the 1- to 2-μm range than in the 9- to 10-μm range. The expected conduction velocities of these two ranges at physiological temperature would average about 15 and 95 m/s, respectively, given a myelination index of about 0.6 and a conduction velocity of about six times the fiber diameter. The corresponding latencies of the action potentials would then be expected to be 2.5 and 0.4 ms. The very largest axons would be expected to produce a CAP latency of about 0.3 ms. This corresponds to the recorded latency at very low stimulus intensities (Fig. 4), indicating that the very largest fibers are surviving, and conducting at a normal physiological speed. Even shorter action potential latencies at higher stimulus intensities may be explained by the expected occurrence of current spread down the axons at supramaximal stimulus strengths. The smaller axons that would still be expected to produce much longer latencies apparently contribute relatively little to the CAP.

This minor contribution of small axons to the CAP may be explained in terms of the relative cross-section of these fibers, which not only reduces the amount of current that the action potential in each fiber generates but also increases the resistance of the pathway for the passive conduction of current across the sucrose gap. Since the cross-sectional area of the axon is proportional to the square of its caliber, the reduction in signal amplitude expected in the gap recording will be inversely related to the cube of the caliber. Therefore although the largest fibers are >100 times fewer in number (Blight 1991), they still contribute much more to the magnitude of CAP. Since there is a continuous gradation of axon calibers from the smallest to the largest, the most we would expect is a broadening of the downward face of the CAP as more and more small fibers are recruited. This is exactly what is seen in Fig. 4, for example, or in a number of equivalent published figures from uninjured preparations (Shi and Borgens 1999; Shi et al. 1996). Furthermore, the fact that large axons are the major contributors to the CAP indicates that, although we are clearly recording axons with different stimulus thresholds, the wide range of threshold is not representative of an equally wide range of axon calibers.

Based on our experience using this sucrose-gap technique with spinal cord white matter preparations, it is usual for compound potentials to be in the range of 1–4 mV (Shi and Borgens 1999; Shi and Pryor 2002; Shi et al. 2000). Therefore the example chosen for the illustration in Fig. 2 was one of the smallest amplitude recordings. We do not know of examples of this technique giving rise to recordings that are substantially larger in amplitude. In this regard, there is clearly a difference between this tissue and peripheral nerve or optic nerve recordings, as has been documented by others (Utschneider et al. 1991). It seems likely that the relatively small amplitude of the compound potentials seen in the spinal cord preparation is the result of current shunting through the extra-axonal components of the tissue and the fact that we are recording primarily the effects of conduction in the relatively sparse, large caliber axons.

It is unlikely that the small size of these potentials reflects mechanical damage. We have seen that the slight compression produced by the nylon mesh did not alter the amplitude of the recorded potential and that white matter strips subjected to nylon mesh pressure but no stretch showed little or no change in CAP amplitude when exposed to 4-AP.

Compared with our previous report (Shi and Pryor 2002), the current study used a faster loading rate (1.5 m/s)—closer to the kind of injury suffered during high-speed movements, such as sports and motor vehicle accidents. Consequently, more severe conduction deficits were seen, even though overall recovery profile was similar to that following slower loading rates (25 μm/s) (Shi and Pryor 2002) (Fig. 2). This indicates that in addition to strain, the stress, related to the rate of loading, also plays an important role in the initial damage and axonal dysfunction.

### 4-AP enhances CAP conduction following stretch

Similar to our previous reports using compression injury (Shi and Blight 1997; Shi et al. 1997), the current study showed that the isolated spinal cord following stretch injury is also subject to 4-AP–induced conduction enhancement. At a concentration of 100 μM, 4-AP doubled the CAP amplitude following stretch (Fig. 3), whereas the same concentration only increased the compound potential amplitude by 40% in acute compression injury (Shi et al. 1997). Likewise, at 1 μM, a clinically achievable concentration in spinal cord injury victims (Donovan et al. 2000; Halter et al. 2000), 4-AP improved the CAP amplitude by 43%, which is also more effective than that in acute compression injury (Shi et al. 1997). The cause of this differential sensitivity is not clear. One simple explanation may be the length of the tissue over which damage is distributed. The length of the cord under direct compression was only 2.5 mm, while the current stretch injury was produced over a 5.5 mm length. Another possible factor is that myelin damage may be more severe following stretch compared with focal compression. Our preliminary morphological observations seem to support this impression. We have found more severe myelin damage, especially in the paranodal region following acute stretch (Fig. 8) compared with what was seen following acute compression injury (Shi et al. 1997). It is evident that the damage in stretch injury separates the myelin sheath from the underlying axolemma in the paranodal area (Fig. 8) where 4-AP sensitive potassium channels are believed to exist in high density (Waxman and Ritchie 1993). This would unmask a great number of potassium channels that are otherwise largely silent in uninjured axons. A surge of potassium leakage through these channels would likely cause conduction block by shunting the positive current that is necessary to depolarize the...
membrane potential and trigger the opening of sodium channels. From our previous study, we have learned that the stretch injury is not a function of axonal diameter (Shi and Pryor 2002). It appears from the current study that 4-AP–induced conduction enhancement is not likely to be a function of axonal diameter (Figs. 4 and 5). This indicates that large and small diameter axons should equally benefit from 4-AP treatment.

Response to double and multiple pulse stimuli

Even though 4-AP at a concentration of 100 μM induced a significant conduction recovery, there is a concomitant increase in refractoriness. This is evident in the absolute and relative refractory periods, which were significantly increased following 4-AP exposure (Fig. 6). In addition, the ability to follow multiple stimuli was also depressed (Fig. 7). It is likely that the newly recruited axons are responsible for this change compared with the healthier axons that conduct action potentials before the application of 4-AP.

The mechanism of the enhanced refractoriness is not clear. The simplest explanation would be that, although some axons are able to conduct with blockade of voltage-dependent potassium channels, the safety factor for conduction remains significantly compromised by damage to paranodal structures and the increased capacitative load provided by the internodal membrane (Blight 1985). It is also possible that, while enhancing conductance by blocking fast potassium channel, 4-AP at 100 μM may also block some resting potassium conductance. The subsequent depolarization of axons might increase the inactivation of sodium channels in the nodal membrane and therefore reduce the responsiveness to dual and repetitive stimuli, although this seems unlikely, given that the response to the initial stimulation is enhanced.

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DISCLOSURES

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REFERENCES


