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Conduction Block in Acute and Chronic Spinal Cord Injury: Different Dose–Response Characteristics for Reversal by 4-Aminopyridine

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The effect of the potassium channel blocker, 4-aminopyridine (4-AP), on conduction of action potentials in injured guinea pig spinal cord axons was measured using isolated tracts in oxygenated Krebs' solution at 37°C. The dose–response characteristics of acutely and chronically injured axons were compared. The maximal improvement of conduction occurred in acutely injured axons at a concentration of 100 μM 4-AP, but in chronically injured spinal cord at 10 μM. The threshold for significant response to 4-AP was between 0.5 and 1 μM in chronically injured cords, and between 1 and 10 μM following acute compression injury. The difference in susceptibility to potassium channel blockade may be related to underlying differences in the mechanism of conduction block at the two stages of injury. Initially, junctions between axons and myelin are acutely disrupted, altering primarily the leakage resistance of the myelin sheath and periaxonal space. In chronically injured cords, there is widespread but incomplete process of repair in the lesion site, which leaves many axons partially myelinated. The difference in sensitivity to 4-AP suggests there is also some modification of the accessibility of axonal potassium channel or a change in their affinity for the drug.

Key Words: **demyelination; trauma; axon; action potential; potassium channel**

INTRODUCTION

The extent to which conduction block in axons of the white matter contributes to the deficits that follow spinal cord injury has not been fully explored. Chronic deficits of myelination are seen histologically in both animal models (2, 3, 7, 15, 17, 20) and human injury (11, 27). The physiological correlates and clinical significance of these structural abnormalities have been more difficult to define. Conduction block at physiological temperature has been demonstrated in isolated animal spinal cord preparations (1, 4), but this kind of temperature-sensitive block is difficult to establish unequivocally in more intact animal preparations or in the clinical setting in human subjects with spinal cord injury (23).

The potassium channel blocker, 4-aminopyridine (4AP), restores conduction in some chronically injured spinal cord axons *in vitro* (4), and this observation led to a series of animal (8, 9) and human studies (19, 21, 22, 24, 25) which have indicated functional benefits from administration of the drug in chronic, "incomplete" injury. Although there has not been a large-scale definitive trial of efficacy, several small studies have shown similar clinical benefits in a significant subset of spinal injured subjects, including improvements in sensory, motor, and autonomic function that may be explained by recovery of conduction in axons, by enhanced transmission at synapses, or a combination of both effects. Clinically acceptable doses of 4-AP produce very low serum and CSF concentrations, and the relative sensitivity of conduction and transmission mechanisms to potassium channel blockade with 4-AP may determine the relative contribution of these different sites of action (14, 31).

Acutely injured spinal cord axons have also been shown to be affected by 4-AP *in vitro* (13, 30) from a few minutes to hours postinjury, but the relative efficacy and sensitivity have not been compared systematically with chronic injuries. There has been little evaluation of functional efficacy at early stages of injury in animals (18), none comparable to the initial chronic studies, and there have been no clinical trials in acute injury. The present study was undertaken to compare the effect of 4-AP on acutely injured spinal axons in guinea pigs with the previously established dose–response in chronic injuries (31), as part of a continuing exploration of the mechanism and natural history of demyelination and conduction block in mammalian spinal cord trauma.

METHODS

Animals and Injury Technique

Studies were carried out using tissue from adult, female, Hartley strain guinea pigs (Sasco), and were approved by the Institutional Animal Care and Use

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Committee of the University of North Carolina at Chapel Hill. The techniques of injury to the spinal cord have been described elsewhere in full (5, 30). Briefly, for *in vivo* injury, guinea pigs were anesthetized for sterile surgery by intramuscular injection of ketamine 60 mg/kg, xylazine 10 mg/kg, and acepromazine maleate 0.6 mg/kg, supplemented as required. A T12 dorsal laminectomy exposed the spinal cord, with dura intact. A special tool, constructed from watchmakers' forceps, was inserted on either side of the spinal cord and used to compress it to a thickness of 1.2 mm, over a length of 5 mm, for 15 s. The wound was closed in layers and the animal allowed to recover. The animals were maintained in individual cages, with free access to food and water. Spinal cords were isolated for recording between 1 and 6 months postinjury (average 2.8 months).

Isolation of the Spinal Cord

The technique for isolation of the spinal cord and compression injury *in vitro* is described elsewhere (30). Briefly, guinea pigs were anesthetized and perfused through the heart with 500 ml of oxygenated Krebs' solution (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 6 mM, equilibrated with 95% O_2, 5% CO_2) at room temperature. The spinal cord was rapidly exposed by excision of the vertebral column over ice, and then the vertebral laminae were cut away as a continuous sheet by cutting through the pedicles on either side with fine bone-cutters. The spinal roots were cut, and the cord was removed carefully from the spinal canal and placed into cold Krebs' solution. Subdivision of the spinal cord into four separate longitudinal tracts was carried out on a cooled Plexiglas block. Laid on its dorsal surface, the ventral fissure was opened along the length by pulling gently on the midline pia-arachnoid with two pairs of watchmakers' forceps. The dorsal column was then divided into left and right halves with a scalpel blade, which was also used to cut through the saggital midline of the injured part of the cord. The two lateral halves of the cord were then subdivided longitudinally, using a scalpel blade, to make ventral and dorsal white matter strips, which were placed into 15 ml Krebs' solution, bubbled with oxygen at room temperature. Most recordings were made from the ventral strips, which contain somewhat larger, faster-conducting fibers than the dorsal tracts.

Sucrose-Gap Recording

The recording arrangement is illustrated in Fig. 2. An isolated strip of white matter, approximately 35 mm long, was laid in the recording chamber, with the injured or central part of the tissue in a central well, superfused with Krebs' solution (2 ml/min), which could be changed rapidly to superfusion with Krebs' solution containing 4-AP. The two ends of the strip of white matter were isolated in separate wells, containing 120 mM potassium chloride, by sucrose-gap channels that contained flowing isotonic sucrose solution (1 ml/min), and which were sealed along their edges with mineral grease. Silver/silver chloride wire electrodes were present in the two end wells and in the central superfusion chamber for stimulation and recording of conduction in the white matter strips. The chamber was constructed on a Peltier plate (Cambion) that allowed control of the temperature, which was recorded with thermocouples. The tissue was maintained at 37°C throughout the recording procedures.

Stimulation was in the form of 0.1 ms constant current unipolar pulses, delivered to the stimulating electrodes through a stimulus isolation unit (WP Instruments). Recordings were made using a Neurodata Instruments bridge amplifier and Neurocorder, for digital data storage on videotape. Subsequent analysis was performed using Labview software (National Instruments) on a Macintosh PowerPC computer.

The compound potential was recorded continuously at a stimulation frequency of 0.7 Hz. Stimulation current intensity was set to produce maximal compound action potential amplitude. To measure changes in compound potential amplitude, 10 responses were averaged before drug application, at approximately 30 min post drug application, and at 30 min or more after washing with normal Krebs' solution. The amplitude change was defined as the difference in amplitude during the drug application and the average of the amplitude before drug application and after washing with normal Krebs' solution. The 4-aminopyridine (Sigma) was dissolved in the same Krebs' solution used for normal superfusion, and the pH of the solution was restored to 7.3, where necessary, by addition of a small amount of hydrochloric acid.

In Vitro Injury

The technique of *in vitro* compression injury has been described in full elsewhere (30). Briefly, 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. 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. 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, the rod was advanced by means of the manipulator motor at a speed of 24 μm/s. The compound action potential and the displacement of the rod were monitored during the compression and the compression was stopped when the potential reached a target amplitude of approxi-

mately 10% of the initial amplitude. The rod was then removed rapidly upward to relieve pressure on the tissue and the recovery of the compound potential was monitored.

Histology

At the end of the experiment, the spinal cord strips were fixed by immersion in 5% glutaraldehyde in phosphate buffer. Transverse and longitudinal blocks were cut from the center and immediate surround of the lesion site, respectively. These were washed in buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in plastic. Semithin (1 μm) sections from these blocks were stained with 1% toluidine blue, observed, and photographed with the light microscope (Nikon Optiphot).

RESULTS

Sucrose Gap Recording of Action Potentials

The form of the compound action potential recorded from uninjured ventral white matter strips has been described elsewhere (30). A monophasic action potential, typically several millivolts in amplitude was followed by a long-lasting depolarizing afterpotential. Similar action potentials, but of smaller initial amplitude could be recorded from white matter isolated from chronically injured spinal cord (31). During acute compression of previously uninjured spinal cord, the maximal amplitude of the compound potential decreased with compression (30). The potential was almost completely abolished with about 80% compression, but following immediate relief of such compression, the compound potential recovered partially in amplitude, usually attaining a stable amplitude, smaller than that in the uninjured state, within 5–10 min of recovery (30).

4-AP Effects on Conduction in Injured Cord

Addition of the potassium channel blocker, 4-AP, to the superfusion medium usually produced a marked increase in the amplitude of the compound potential in both acutely and chronically injured cords, but only over a narrow range of concentration. The dose–response relations for the two types of injured tissues are shown in Fig. 1. Examples of two individual cases where 10 and 100 μM solutions produced reciprocal effects between chronic and acute injury are shown in Fig. 2. The increase in compound potential amplitude appeared to be complete within 15 min of superfusion and was reversed with a similar time course on washing with normal Krebs' solution. Application of a similar concentration of 4-AP to uninjured white matter strips produced no significant increase in amplitude of the compound potential (31). The depression of compound potential amplitude seen at concentrations of 4-AP above 1 mM was indistinguishable between chronic and acute injury, as it is between chronically injured and uninjured cords (31). There were no clear effects of 4-AP on conduction speed.

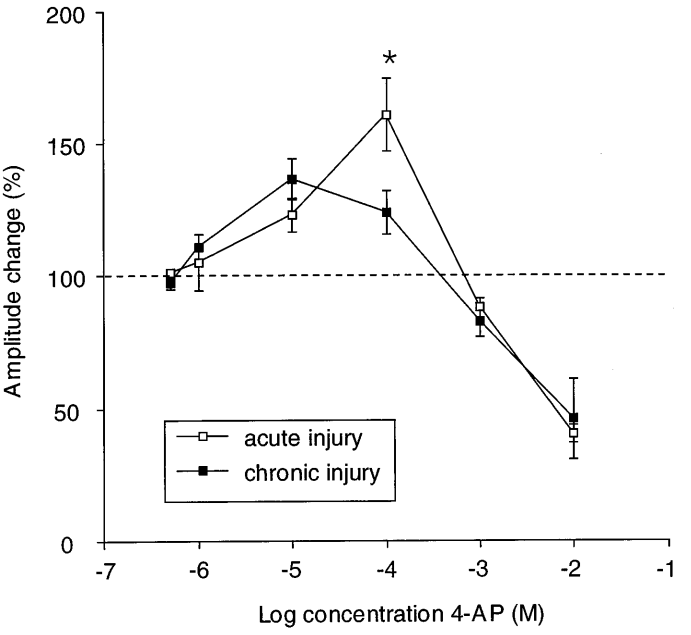

FIG. 1. A graph to show the relation between 4-AP concentration and the mean (±SEM) change in compound potential amplitude recorded in acutely and chronically injured spinal cord strips. The plots were derived from a total of 5–15 measurements from each group at each concentration, with larger samples in the middle of the range. At 0.5 μM, there was no significant change in amplitude and no difference between acutely and chronically injured cords. At 1 and 10 mM, the significant depression of compound potential amplitude was also not different between acutely and chronically injured tissues. At 10 and 100 μM a significant increase in amplitude occurred in both acutely and chronically injured tissue, with the peak increase for acute occurring at 100 μM and that for chronically injured at 10 μM. At 1 μM 4-AP concentration, the chronically but not the acutely injured cords showed significant increase in compound potential amplitude. The relative amplitude change differed significantly between the two groups at 100 μM (ANOVA, $*P < 0.05$).

Histology of the Injury Site

Some details of the histopathology of the chronically injured spinal cord in the guinea pig model have been illustrated elsewhere (5, 6, 31). Axons in the lesion site range between apparently normally myelinated fibers and regions of complete demyelination, with the most extensive abnormalities being thin myelin sheaths and particularly characteristic are nodes of Ranvier with minimal or no myelination of one paranodal over tens of microns in length. A typical area of transition at the edge of the lesion between myelinated and demyelinated axons is illustrated in Fig. 3B. The acutely injured spinal cords were quite different in appearance and were characterized by more subtle changes in surviving

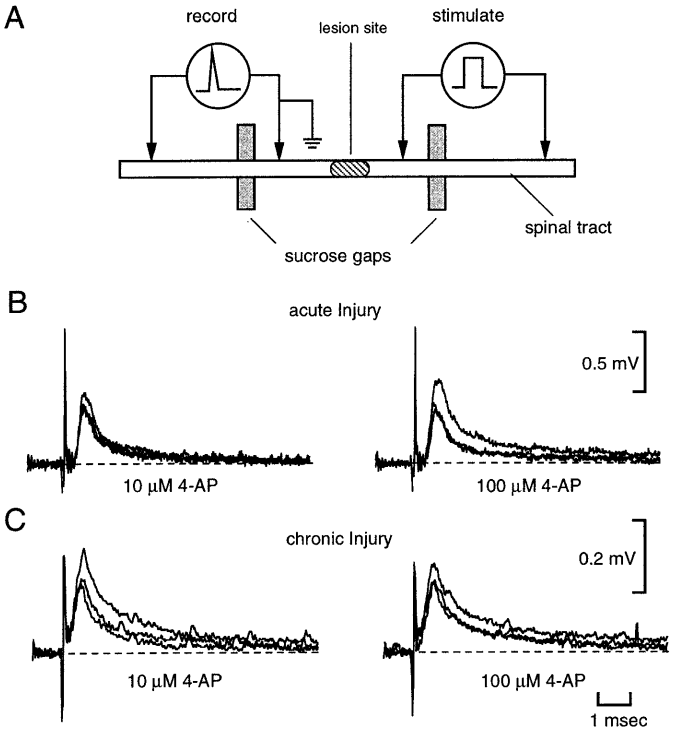

FIG. 2. (A) Diagram of the recording arrangement. The isolated spinal cord tract is shown mounted in the apparatus, with the chronic lesion site placed in the middle of the central well, which was continuously perfused with oxygenated Krebs' solution. The two ends of the tract were placed in separate wells filled with isotonic KCl, which was divided from the central well by narrow channels filled with flowing isotonic sucrose solution. The tract was sealed at both edges of each sucrose gap with coverslips held in place with mineral grease. Silver/silver chloride electrodes were used for recording and stimulation. (B) Three superimposed, averaged compound potential recordings from an acutely injured strip of spinal cord white matter, recorded before, during, and after superfusion with 4-AP containing Krebs' solution. The amplitude of the response increased more dramatically following 10 min exposure to 100 μM 4-AP than following similar exposure to 10 μM 4AP. (C) An equivalent set of recordings from a strip of white matter from a spinal cord injured 4 months previously. In this case, the amplitude of maximal response was larger during exposure to 10 μM 4-AP than 100 μM. In all cases, the amplitude returned close to the initial value after washing in Krebs' solution.

axons, though there was frank disruption of parts of the tissue. Figure 3A shows two adjacent nodes of Ranvier from one such lesion, in which the smaller of the two fibers appears quite normal, but the larger shows signs of significant damage, with some displacement of the paranodal loops of myelin and an accumulation of intracellular organelles in one paranode. This kind of pathology is consistent with acute stretch injury in other contexts.

DISCUSSION

Demyelination of surviving axons may contribute significantly to long term functional deficits in spinal cord injury, and the degree to which this plays a role may vary considerably from one type of injury to another (11, 27). However, the mechanism of posttraumatic demyelination is not well understood, and the precise relation between the structural deficits in the myelin sheath, seen histopathologically, and alterations in axonal physiology is not known. From animal

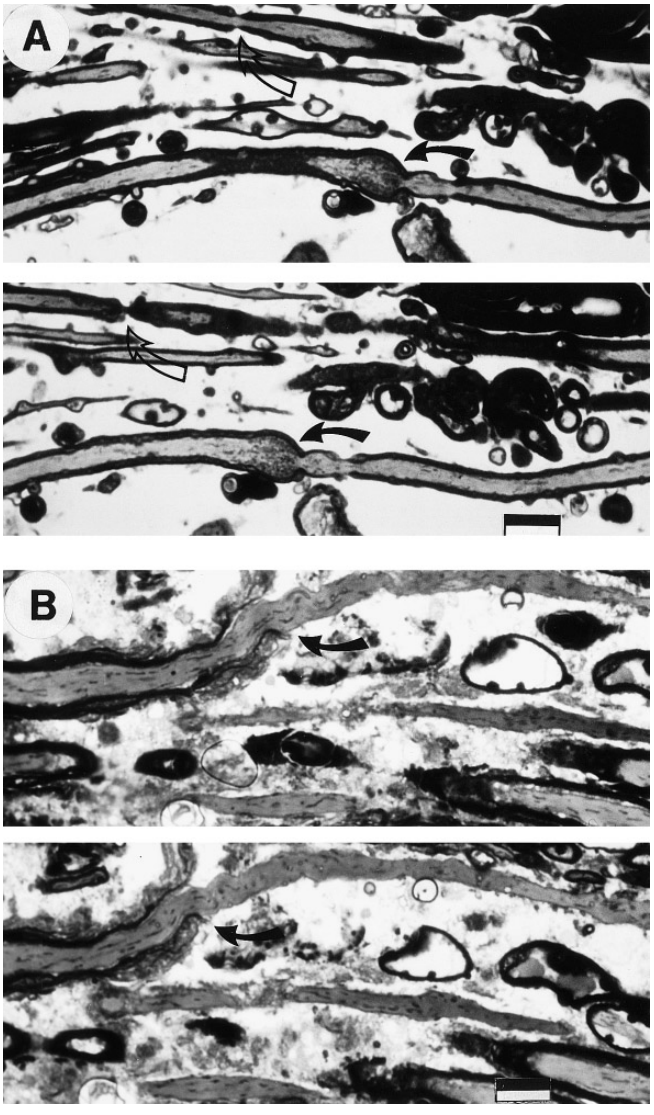

FIG. 3. Photomicrographs of toluidine blue-stained, 1-μm longitudinal sections through the center of the lesion site in two different spinal cord tracts. (A) Two adjacent serial sections through an acutely injured white matter strip, showing disruption of paranodal in one large fiber, with accumulation of organelles in the axoplasm (solid arrow), and maintained normal morphology in a medium sized node of Ranvier nearby (open arrow). (B) Similar sections from a spinal cord at 4 months postinjury, showing an area of complete myelin loss in several adjacent fibers. The demyelinated segment in the central fiber (beginning at arrow) could be traced for a distance of 105 μm, to the next myelin sheath. This section is from the same tissue from which the data described in the legend to Fig. 2C was collected. Scale bar, 10 μm.

models, we do know that demyelination of surviving fibers appears to be delayed by a number of days from the breakdown of myelin around axons that are immediately disrupted (3, 7). It was therefore surprising to find that conduction in acutely injured spinal cords is improved by blockade of potassium channels (30), as had been previously shown to be the case for chronically injured cords (4), and a variety of other neurological models that involve peripheral or central axon demyelination (10, 26, 32). The purpose of this study was therefore to compare the 4-AP sensitive conduction deficit in acutely and chronically injured spinal cords, based on the evidence that indicates the structural alterations in these two conditions are quite different. Beyond the direct interest in the relation between structure and function, there is also a question regarding the applicability of 4-AP as a symptomatic treatment for spinal cord injury, and the time at which such a therapy might usefully be applied.

The terms "acute" and "chronic" in spinal cord injury are practically useful but are applied arbitrarily in different contexts, and can be confusing. For the purposes of this study, the two stages of injury are considered to be temporally separate. "Acute" is applied to the immediate consequences of compression injury, including the initial mechanical disruption and the consequent disturbances of membrane permeability that produce shifts in intracellular and extracellular concentrations of electrolytes and other bioactive substances. These more physicochemical processes are assumed to be completed within a few hours of the initial trauma. Later, more biologically based processes of inflammation and tissue repair are concentrated within the period from 24 h to 3–4 weeks postinjury in the animal model, and can be referred to as "subacute." This period is not considered here, but is likely to be pharmacologically distinct in itself. Stabilization of tissue pathology and behavioral recovery is largely completed within 4–6 weeks postinjury in small animal models of spinal cord injury. The histopathology of the lesion and functional status of the animals usually changes relatively little beyond this point, so that the period of "chronic" injury can be taken to proceed from this time, though a more conservative definition would use a longer period, as is usually the case in clinical application of the term.

The experiments reported here rely on the recording of compound potentials as measures of conduction in myelinated axons, as they have been used previously to compare injured with uninjured spinal cord (30, 31). Increase in amplitude of the maximal short-latency component of the compound potential is interpreted as an increase in the number of axons that conduct through the lesion site. This interpretation, elaborated in previous publications (30, 31), is based on a number of factors, including: its consistency with unambiguous restoration of conduction in single fiber studies (4); the fact that there was no significant increase in the peak latency of the response, which would have been expected if there was simply an increase in duration of the action current in the vicinity of the recording gap, without increase in the number of fibers conducting; and the previous observation that 1–100 μM 4-AP has no effect on the amplitude or duration of the single fiber action potential or compound potential in uninjured spinal cord axons. The recordings were made from axons sufficiently remote from the site of injury (more than 2 length constants) that the potentials represent essentially intact fibers.

The lack of obvious effect on conduction speed in these studies may be related to the relatively short length of tissue involved in the lesion, though it should be noted that compound potential measurements of this type are always heavily biased to recording of conduction in large and fast-conducting fibers.

The two models of posttraumatic conduction block compared in this study were different in their sensitivity and appeared slightly different in their degree of response to 4-AP. Conduction in the chronically injured cords was significantly increased at 1 μM, while the acutely injured cords were not reliably affected at this concentration. While the chronically injured axons showed maximal increase in conduction at 10 μM concentration, the acutely injured axons were more strongly affected at 100 μM (Figs. 1 and 2). The requirement for higher concentrations in the acute injury seems to be more consistent with dose–response reported in chemical (lysolethicin) demyelination (14).

The differences between the injuries examined in this study include not only the acute and chronic nature, but also the fact that the acute injuries were performed in isolated tissue. This compromise was necessitated by the fact that spinal cords injured acutely *in vivo* are very difficult to extract for *in vitro* recording. The tissue is sufficiently weakened by the compression injury that it is not generally possible to record any conduction following isolation, and the tissue frequently falls apart at the injury site during transfer to the recording chamber. The absolute width of the compression site was also smaller in the acute injury, though it was similar relative to the diameter of the tissue being compressed, the whole cord being 2.5–3 mm wide, compared to the 0.5–0.7 mm of the isolated tract. Nonetheless, the differences seen between the acute *in vitro* injury and the chronic *in vivo* are most reasonably explained by the pathological progression rather than the details of the injury itself, since the damage to individual axons caused by the gross tissue compression/extrusion should be mechanically quite similar in the two models.

As pointed out in the previous study (31) the response characteristics seen in the injured spinal cord axons appear comparable to the fast delayed rectifier of

the amphibian node of Ranvier (12). Mammalian neuronal A-currents usually show K_d values in the range of 1–2 mM (28, 29), but a recent study of rat dorsal root ganglion cells has shown the presence of six different K+ currents, among which the I_{Af} current, predominantly in large diameter dorsal root ganglion cells, shows a K_d for 4-AP of 59 μM at +45 mV and a threshold sensitivity of about 1 μM (16). This behavior seems compatible with the response of acutely injured cord, especially if the upper range of effect is masked by the depression of conduction with millimolar concentrations of 4-AP, which appears to result from a depolarizing effect of blocking the fast potassium channel of the internode, since an identical depression is found in normal, uninjured fibers (31). The distinct response to low concentrations of 4-AP seen in chronic injury, as opposed to acute injury, might result from molecular changes in the channel or reflect normal physiology that is revealed by the consequences of structural alterations in the myelin sheath. Among these alterations might be a greater susceptibility to the depolarizing effect of potassium channel blockade.

The clinical implications of this study are limited. While it appears that the acutely injured cord is less sensitive to the drug, it may still be possible to increase conduction in injured pathways to some extent with clinically acceptable doses. However, the application of any experimental procedure is not without some risk, and such studies are likely to be more safely and successfully conducted at less stressful times in the course of the condition. Also, the benefits of 4-AP administration in chronic injury, while clinically significant, have been relatively subtle and may be even more difficult to quantify during the more unstable early course of acute injury. The most prudent approach may be to postpone acute studies until application of the drug in chronic injury is more fully investigated and matters of dosage, safety, and efficacy are well established.

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