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Dimethylsulfoxide enhances CNS neuronal plasma membrane resealing after injury in low temperature or low calcium

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Abstract

The inability to repair the damaged membrane may be one of the key mechanisms underlying the severe neuronal degeneration and overall functional loss seen in *in vivo* spinal cord injury and traumatic axonal injury in blunt head trauma. Promoting membrane resealing following damage may therefore constitute a potential effective therapeutic intervention in treating head trauma and spinal cord injuries. In our previous studies, we have shown that the axolemma failed to reseal following transection in clinically related situations, such as low extracellular calcium and low temperature. Our current studies indicate that DMSO is capable of rendering significant improvement in guinea pig axonal membrane resealing following transection in both 0.5 mM $[Ca^{2+}]_0$ and 25°C situations. This was demonstrated physiologically by monitoring membrane potential recovery and anatomically by conducting HRP-exclusion assays 60 minutes after injury. Further, we have shown that the addition of DMSO in normal Krebs' solution (2 mM $[Ca^{2+}]_0$ and 37°C) resulted in a decrease in membrane repair following injury. This indicates that DMSO-mediated membrane repair is sensitive to temperature and calcium. This study suggests the role of DMSO in axonal membrane resealing in clinically relevant conditions and raises the possibility of using DMSO in combination with other more established therapies in spinal cord injury treatment.

Introduction

There is strong evidence that suggests neuronal cell degeneration after physical injury results mainly from the disruption of the intracellular environment. This is due to the damage of the neuronal membrane (Gross & Higgins, 1987; Xie & Barrett, 1991; Pettus *et al.*, 1994; Fitzpatrick *et al.*, 1998). Such membrane damage could be severe and obvious, as in the case of axonal transection in traumatic brain and spinal cord injury (Xie & Barrett, 1991; Maxwell *et al.*, 1993; Shi & Pryor, 2000). On the other hand, membrane damage could also be subtle, as in the form of so-called axonal membrane "perturbation", where the axonal permeability is altered but the axons are still in physical continuity (Povlishock, 1992; Pettus *et al.*, 1994; Pettus & Povlishock, 1996; Povlishock & Pettus, 1996; Maxwell *et al.*, 1999). The latter is thought to develop, over a period of several hours, a disruption of ionic homeostasis leading to secondary axotomy. If the proximal segment of a cut axon is left unrepaired and allowed to continue to deteriorate, it will lose more intraaxonal components and "die back" from the original site of severance (Roederer *et al.*, 1983; Gross & Higgins, 1987). This reduces the possibility that future regenerated axons will reach their previous targets and form proper reconnections. In addition, it is also probable that cell death will eventually occur (Lucas *et al.*, 1985; Xie & Barrett, 1991). It is therefore reasonable to state that the earlier the axonal membrane is sealed, the better chance the axon should have to survive the mechanical insults and perhaps regain function.

Unfortunately, based on both *in vitro* and *in vivo* studies, the environment of the injury site of *in vivo* mammalian spinal cord injury does not favor axonal resealing. For example, it is generally agreed that millimolar extracellular calcium is critical for sealing (Yawo & Kuno, 1985; Xie & Barrett, 1991; Shi *et al.*, 2000). However, the $[Ca^{2+}]_0$ measured after *in vivo* spinal cord injury is significantly below this level and this reduction could last for several hours following injury (Young *et al.*, 1982; Stokes *et al.*, 1983). It is likely that this reduced level of extracellular calcium prevents damaged axons from sealing. The failure of axonal membrane repair may contribute to the axonal degeneration, severe function loss,

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and poor recovery seen in mammalian spinal cord injury.

Mild to severe hypothermia has long been believed to reduce various types of nerve injury (Hayes *et al.*, 1993; Lucas *et al.*, 1994; Koizumi & Povlishock, 1998) and hypothermia has been suggested as a therapy to improve the outcome of trauma by reducing the damage from secondary metabolic-related injuries (Clifton *et al.*, 1993). However, our recent studies have shown that membrane resealing of transected guinea pig spinal cord is inhibited *in vitro* at 25°C. This presents a dilemma when both inhibiting secondary injury and enhancing axolemma resealing are thought to be beneficial to the recovery of traumatized axons. The current study is part of the effort to search for a treatment that can enhance membrane sealing in the above-mentioned situations. Such study may contribute to the development of therapies to encourage membrane repair and reduce the axonal damage and subsequent functional loss seen in CNS trauma, and perhaps also optimize current therapies.

DMSO is an organic solvent which has long been shown to enhance cell membrane fusion in certain situations (Yu & Quinn, 1998). Since membrane resealing in certain ways resembles membrane fusion, it is possible that DMSO might also enhance membrane sealing. In fact, DMSO has already been shown to enhance membrane resealing in the dorsal root ganglion cell after axonal transection (Howard *et al.*, 1999). The current study is designed to test the hypothesis that DMSO may also enhance membrane resealing in mammalian spinal cord white matter axons. This study was carried out in two clinically related situations that are known to inhibit membrane sealing: low extracellular calcium and low temperature. We have shown that DMSO is indeed capable of enhancing membrane sealing in these conditions.

Methods

Both electrophysiological and anatomical methods have been used to evaluate membrane sealing. Sucrose gap recordings were used to monitor membrane potential changes in strips of white matter isolated from guinea pig spinal cords, by techniques that have been described previously (Shi & Blight, 1996; Shi *et al.*, 2000; Shi & Pryor, 2000). Morphological examination of sealing was carried out by studying the uptake of horseradish peroxidase (HRP) by the injured axons. The structural analysis was found to correlate well with electrophysiological findings.

ISOLATION OF THE SPINAL CORD

Adult female guinea pigs of 350–500 gram body weight were used. The experimental protocols have been reviewed and approved by the Purdue University Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. The technique for isolation of the spinal cord white matter strip was described previously (Shi & Blight, 1996; Shi *et al.*, 2000; Shi & Pryor, 2000). Briefly, guinea pigs were anesthetized deeply with ketamine (80 mg/kg), xylazine (12 mg/kg), and acepromazine (0.8 mg/kg). They were then perfused through the heart with 500 ml of oxygenated, cold Krebs' solution to remove the blood and lower core temperature. The vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae and immersed in cold Krebs' solution. 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 blade against a soft plastic block. The composition of the Krebs' solution was as follows: NaCl 124 mM; KCl 2 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.3 mM; $CaCl_2$ 2 mM; dextrose 20 mM; sodium ascorbate 10 mM; $NaHCO_3$ 26 mM, equilibrated by bubbling with 95% O_2, 5% CO_2 to produce a pH of 7.2–7.4.

RECORDING CHAMBER

The construction of the recording chamber is illustrated in Figure 1. A strip of isolated spinal cord white matter, approximately 35 mm in length, was supported in the central compartment and continuously superfused with oxygenated Krebs' solution (c. 2 ml/min) by means of a peristaltic pump. The free ends of the spinal cord strip were placed across the sucrose gap channels to side compartments filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels using fragments of plastic cover-slips and a small amount of silicone grease to attach the cover slip to the walls of the channel. Isotonic sucrose solution was continuously run through the gap channels 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), and the temperature was separately monitored across the chamber with additional thermocouple probes. The axons were stimulated and compound action potentials 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 connected to an instrument ground. Stimuli were delivered through stimulus isolation units and were usually in the form of 0.1 msec constant current unipolar pulses. Recordings were made using a bridge amplifier and Neurocorder (both from Neurodata Instruments Inc.) for digital data storage on videotape. Subsequent analysis was performed using custom Labview® software (National Instruments) on a Dell PC computer.

TRANSECTION

To study the response of the nerve fibers to transection, the tissue strip was cut at the face of the recording sucrose gap using micro-scissors. The scissors were also used to cut through the tissue at the center of the chamber, when the isolated tract needed to be transferred to an HRP solution (Fig. 1B).

DIMETHYLSULFOXIDE APPLICATION

Dimethylsulfoxide (DMSO, pure, Sigma) was dissolved directly in the perfused Krebs' solution to make a final concentration of 5%. The cord was exposed to 5% DMSO

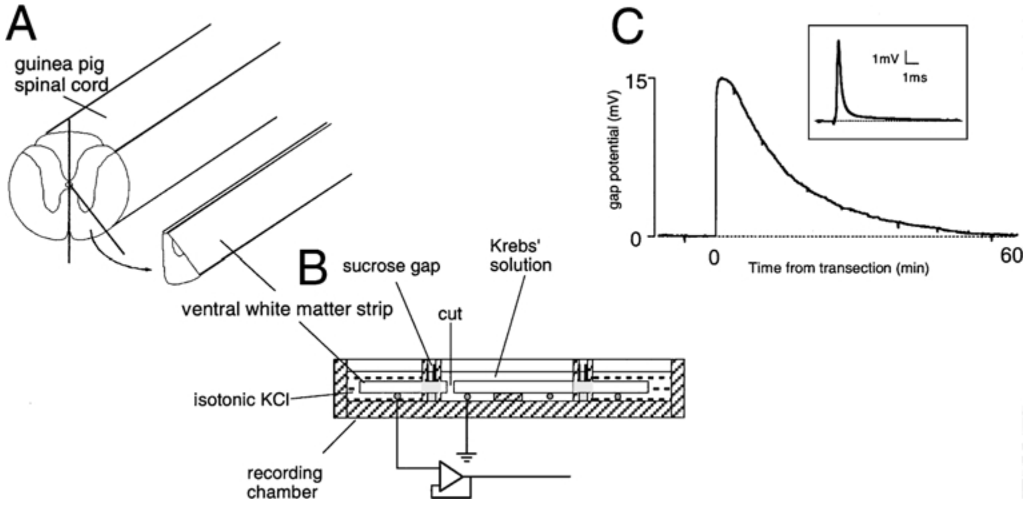


Fig. 1. Diagram to show isolated spinal cord white matter strip, recording apparatus, injury model, and an example of gap potential recording. (A) Ventral white matter strip, approximately 35 mm long, was extracted from a guinea pig spinal cord. (B) The recording arrangement, view from side, and the transection injury method. The isolated spinal cord strip is shown mounted in the apparatus, with the injury site placed on the left side of the central well, which is continuously perfused with oxygenated Krebs' solution. The two ends of the strip were placed in separate wells filled with isotonic KCl, divided from the central well by narrow channels filled with a flowing, isotonic sucrose solution. Electrodes were formed of silver/silver chloride wires. Gap potential and compound action potential were recorded with electrodes positioned within the side chamber (active) and the central chamber (ground). To examine the response to transection, the tissue was cut near the face of the recording gap. (C) The profile of gap potential depolarization is shown here in response to transection, and repolarization within the next 60 minutes. Note the inset showing the normal action potential recorded just before the cut.

20 minutes before the transection, which continued until the end of the experiments (usually 60 minutes following transection). Care was taken to block direct light projection onto the DMSO-containing solutions, as DMSO is light sensitive.

HORSERADISH PEROXIDASE HISTOCHEMISTRY

To examine the extent of sealing of disrupted axons, white matter strips were transferred after electrophysiological recording to oxygenated Krebs' solution containing 0.015% horseradish peroxidase (HRP—Sigma Type VI). After incubation for 1 hour 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 stained with a diaminobenzidine reaction to reveal the extent of HRP uptake into damaged axons. Sections near the center of the injury site and at a 5 mm distance were compared to controls for the presence of damaged fibers that might be unrelated to the deliberate injury. Sections were examined and photographed with a Nikon Optiphot microscope. Image analysis was performed using an Optronics video camera and NIH Image software on a Macintosh Quadra 950 computer.

2-D MORPHOMETRY

The method of 2-D morphometry has already been described in previous publications, and we refer the interested reader to these reports (Shi & Borgens, 2000; Shi & Pryor, 2000). The images from the HRP stained vibratome sections were digitized and captured to a Macintosh Quadra 800 Computer using a Leitz Ortoplan microscope and JVC video camera. A cross section was selected from each strip, using the 6.3 × objective. Representative area samples were chosen from peak regions of HRP uptake in transverse sections to quantify axonal sealing. The area was selected in such a way that it could be divided into three squares from pia to the gray matter: lateral, intermedial, and medial. The lateral square includes the outer surface and the medial includes the inner edge of the white matter, or the border area between the white and gray matter, where present. The size of the square varies depending on the thickness of the white matter. Then, using a 16× objective, axons were measured within the three squares. The number of unsealed axons was counted and expressed as a density. Images were first color transformed and then binarized using IP Lab Spectrum software. The axon diameter was measured. The shortest distance across the axon was taken as the diameter, based on the interpretation that deviations from circularity represent axons cut obliquely. Axons sampled in two squares were assigned to the more lateral square. Counts of individual axons were normalized per unit area of each of the three subregions and expressed as a density (axons/mm^2).

STATISTICAL TREATMENT

Throughout the paper, a student's t-test (unpaired, two tail) was used to compare electrophysiological and histological measurements between two groups in different experimental conditions. Statistical significance was attributed to values of $P < 0.05$. Averages were expressed as a mean ± standard error.

Results

THE INFLUENCES OF DMSO ON MEMBRANE POTENTIAL RECOVERY

The effect of DMSO on the maintenance of membrane potential of intact membranes

We first examined whether DMSO, at a concentration of 5%, would affect the maintenance of membrane potential of uninjured spinal cord axons. We found that a 30 minutes exposure of 5% DMSO did not cause any significant shift of membrane potential when the cord was bathed with normal Krebs'solution (2 mM $[Ca^{2+}]_0$, 37°C), 25°C (2 mM $[Ca^{2+}]_0$), or 0, 5 mM $[Ca^{2+}]_0$ (37°C) (data not shown).

The effect of DMSO on membrane potential recovery following transection in low temperature and low extracellular calcium

It has been shown previously that spinal cord axons reseal their transected membrane within 60 minutes of injury in normal physiological conditions (2 mM $[Ca^{2+}]_0$ and 37°C) (Shi *et al.*, 2000; Shi & Pryor, 2000). However, conditions of either 0.5 mM $[Ca^{2+}]_0$ or 25°C could hinder membrane resealing following transection (Shi *et al.*, 2000; Shi & Pryor, 2000). This was demonstrated by the failure of transected axons to substantially recover their membrane potential (Fig. 2) and exclude HRP molecules one hour after transection (Fig. 3) (Shi *et al.*, 2000; Shi & Pryor, 2000). However, when DMSO, was introduced to the solutions of either 0.5 mM $[Ca^{2+}]_0$ or 25°C, the membrane potential recovered significantly better (Fig. 2A–D). At 0.5 mM $[Ca^{2+}]_0$, the average membrane recovery was 63 ± 4% at one hour following transection. The addition of DMSO resulted in 84 ± 3% of membrane potential recovery, a 21% increase, which is statistically significant ($P < 0.01$). Likewise, the addition of DMSO also improved membrane potential recovery from 72 ± 7% to 95 ± 5% at 25°C, a 23% increase, when examined one hour following transection. ($P < 0.01$).

The effect of DMSO on membrane potential recovery following transection in a physiological condition (37°C and 2 mM $[Ca^{2+}]_0$)

We reason that DMSO may accelerate the sealing process when axons are transected while bathed in a physiological solution (2 mM $[Ca^{2+}]_0$ and 37°C). We have shown previously that, using the same method, guinea pig spinal cord axons are capable of resealing their cut ends within 60 minutes following injury under this condition (Shi *et al.*, 2000; Shi & Pryor, 2000). Due to this phenomenon, we were interested to study whether DMSO could accelerate the sealing. Unexpectedly, the addition of DMSO actually reduced the membrane potential recovery from 97 ± 3% to 76 ± 3%, a 25% decrease in normal physiological conditions (Fig. 2E and F, $P < 0.001$).

THE INFLUENCE OF DMSO ON MEMBRANE INTEGRITY RESTORATION BASED ON AN HRP-EXCLUSION ASSAY

Using the previously described HRP-exclusion assay, we examined the status of membrane integrity based on the ability to exclude HRP, a retrograde transported molecule (Heimer & Záborszky, 1989). Although the mechanism is not clear, the entry of HRP has been used to denote the loss of membrane integrity (Pettus *et al.*, 1994; Pettus & Povlishock, 1996; Povlishock & Pettus, 1996; Shi & Borgens, 2000; Shi & Pryor, 2000). The increased permeability to HRP has been shown to be accompanied by severe cytoskeletal damage (Pettus & Povlishock, 1996; Povlishock & Pettus, 1996) and membrane potential loss (Shi & Pryor, 2000; Shi *et al.*, 2000). In agreement with the membrane recovery profile, DMSO significantly reduced the number of axons labeled with HRP at both 0.5 mM calcium and 25°C (Fig. 3). Specifically, at 0.5 mM $[Ca^{2+}]_0$, DMSO reduced the density of HRP-labeled axons from 2132 axons/mm^2 to 440 axons/mm^2 ($P < 0.005$). Similarly, the addition of DMSO reduced HRP-labeled axons from 2956 axons/mm^2 to 245/mm^2 when the bath temperature was 25°C ($P < 0.01$). The addition of DMSO increased the density of HRP-labeled axons from 12 axons/mm^2 to 173 axons/mm^2 in normal physiological condition ($P < 0.05$), which is consistent with the electophysiological data.

DMSO-ENHANCED MEMBRANE RESEALING-THE FUNCTION OF AXONAL LOCATION

Since the location of the axons within the spinal cord correlates with their function, we would like to examine the possibility that this factor of location influences DMSO-mediated membrane resealing. Figure 4 displays the density of HRP-labeled axons grouped in three regions within the cross-section. There is a medial (adjacent to gray matter, M), a lateral (adjacent to the pial surface, L), and an intermediate position, which is between these two locations (I) (see Experimental Procedures for details). These studies were performed using 30 μm Vibratome sections labeled with HRP. First, the addition of DMSO to the axons transected in either 0.5 mM calcium or 25°C resulted in significant reduction of HRP-labeled axons in all three regions ($P < 0.05$). Second, DMSO significantly increased the HRP-labeling in axons transected at normal physiological conditions (2 mM $[Ca^{2+}]_0$ and 37°C) in all three regions. This data indicates that in the above-mentioned three conditions (low $[Ca^{2+}]_0$, low temperature, and physiological conditions), location is not a factor influencing DMSO-induced membrane resealing changes.

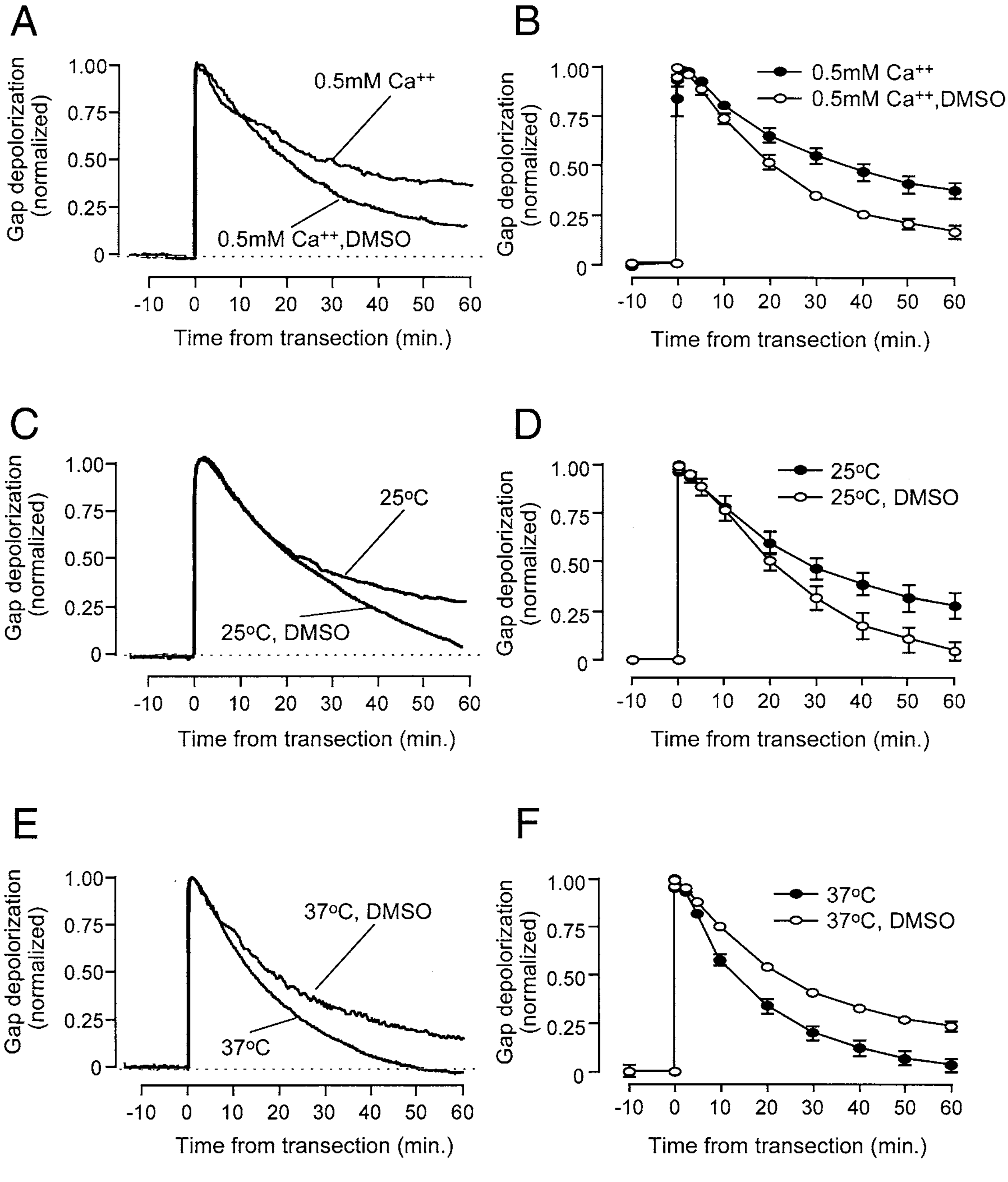


Fig. 2. Individual examples and averaged gap potential recordings in response to transection in different conditions. (A, B) There was a significant increase in the rate of recovery of the gap potential when DMSO was added to the Krebs' solution containing 0.5 mM Ca^{++} (37°C) ($n = 8$ for 0.5 mM Ca^{++} and $n = 9$ for 0.5 mM Ca^{++} plus 5% DMSO) ($P < 0.001$). (C, D) The addition of DMSO to Krebs' solution at 25°C (2 mM Ca^{++}) produced significant increase in the rate of recovery of the gap potential ($n = 8$ for 25°C and $n = 6$ for 25°C plus 5% DMSO) ($P < 0.01$). (*E*, *F*) There was a reduction in the rate of recovery of the gap potential when DMSO was added to normal Krebs' solution (2 mM Ca^{++} and 37°C) ($n = 8$ for normal Krebs' and $n = 8$ for normal Krebs' plus 5% DMSO) ($P < 0.001$).

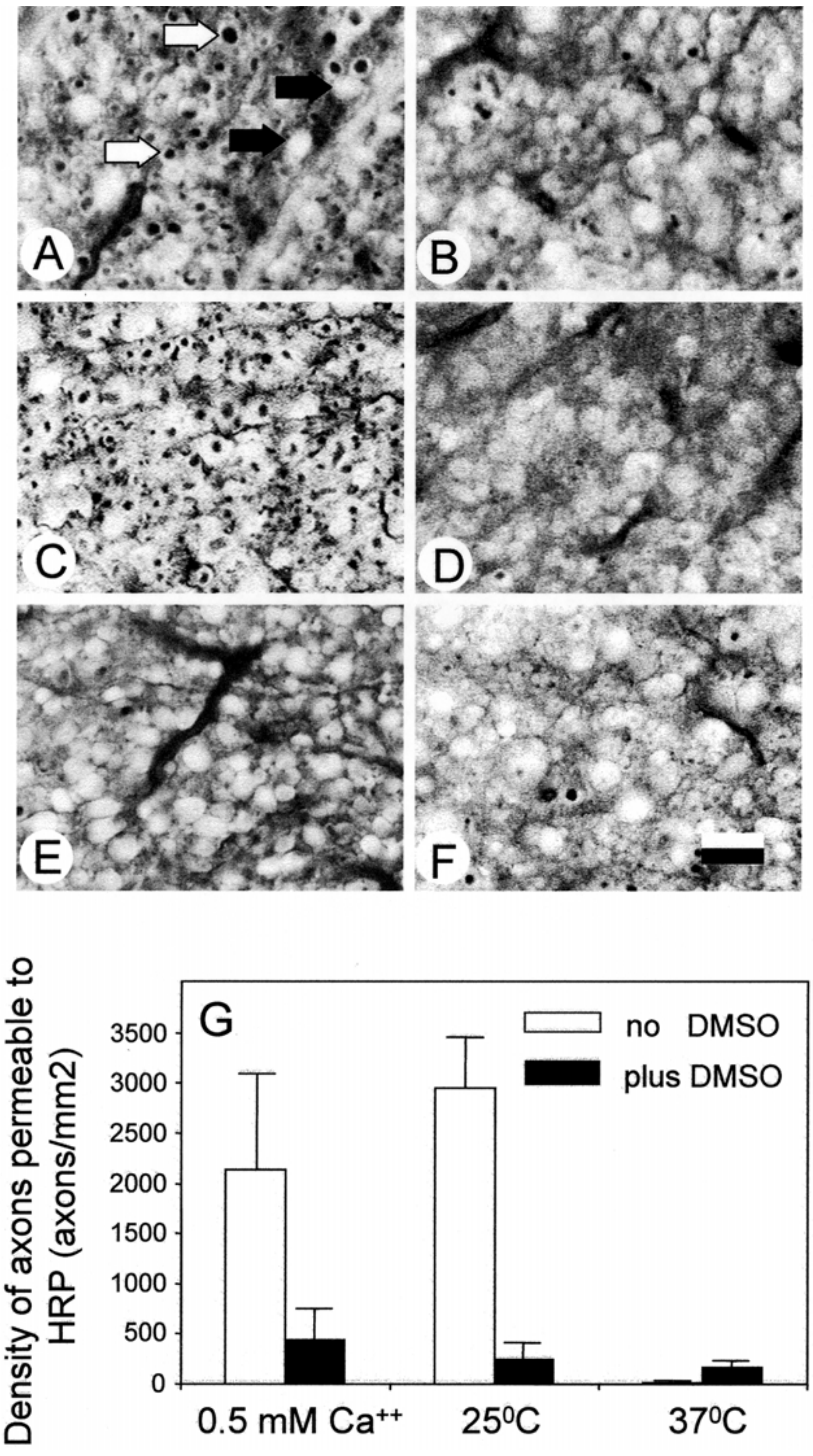
Discussion

THE POSSIBLE MECHANISMS OF MEMBRANE SEALING FAILURE IN LOW $[Ca^{2+}]_0$ AND LOW TEMPERATURE

In the current and previous studies from our group (Shi *et al.*, 2000; Shi & Pryor, 2000), we have shown that transected guinea pig spinal cord axons can completely reseal within 60 minutes when bathed in normal physiological Krebs' solutions (37°C and 2 mM $[Ca^{2+}]_0$). However, the injured axons failed to do so when the temperature of the bathing media was 25°C, or the extracellular calcium was 0.5 mM (Shi *et al.*, 2000; Shi &

Pryor, 2000). The mechanism of this effect is not entirely clear. One possibility is the role of membrane fluidity in membrane resealing.

The common feature of hypothermia and low calcium is that both manipulations could lead to a decrease in membrane fluidity. For example, when the membrane is cooled below the transition temperature, it will adopt a more rigid state (Garrett & Grisham, 1999). Therefore, lowering the temperature decreases membrane fluidity. On the other hand, low calcium concentration (0.5 mM) is thought to block activation of m-calpain, which requires millimolar calcium for activation (Croall & De Martino, 1991; Shi *et al.*, 2000). Although two forms of calpain exist in neurons, axons mainly posses m-type calpain (Hamakubo *et al.*, 1986). The calpain activity has been shown to partially



disconnect the membrane from the cytoskeleton and, therefore, increase membrane fluidity (Croall & De Martino, 1991; Gitler & Spira, 1998). This is accomplished by disrupting the components of the subcortical network that lends structural support to the plasma membrane. Calpain has been shown to proteolyze fodrin/spectrin, which is a major constituent of the subcortical cytoskeleton network (Croall & De Martino, 1991; Luna & Hitt, 1992). Based on these studies from our laboratory, and other studies as well, we hypothesize that membrane fluidity plays a critical role in the resealing of damaged axonal membranes.

Despite its role in resealing, continuous calpain activation could also underlie axonal deterioration. Whether calpain activation will lead to resealing or deterioration probably depends on the timing and the duration of its activation. It is likely that if it fails to reseal the membrane by increasing the membrane fluidity, the activation of calpain will continue and lead to axoplasm deterioration. This scenario is likely in *in vivo* CNS injury. Firstly, membrane damage has been demonstrated repeatedly in CNS axons based on HRP-uptake (Pettus *et al.*, 1994; Shi & Borgens, 2000), distribution of membrane pump activity (Maxwell *et al.*, 1995), and quantitative freeze-fracture (Maxwell *et al.*, 1999). Secondly, calcium concentration declines significantly post-injury which could prevent successful membrane resealing due to inadequate levels of m-calpain activation in axolemma (Young *et al.*, 1982; Stokes *et al.*, 1983; Hamakubo *et al.*, 1986; Croall & De Martino, 1991). Since the reduced extracellular calcium is still higher than intracellular calcium concentration, calcium influx through the unsealed membrane breach will likely persist. Eventually, continuous activation of calpain (μ and m type) will lead to axoplasmic deterioration. Povlishock and colleagues have shown that head injury could result in the pertubation of axolemma and allow passage of HRP into the axoplasm (Pettus *et al.*, 1994; Pettus & Povlishock, 1996; Povlishock & Pettus, 1996). In the same injury, calpain activity also increased which is likely due to the augmented influx of calcium through the battered membrane (Buki *et al.*, 1999). Similar calpain activation has also been noted in brain injury in another group (Saatman *et al.*, 1996). Interestingly, in both studies, there was a delay of the axonal deterioration following calpain activation, perhaps indicative of a period of unsuccessful calcpian-mediated resealing (Saatman *et al.*, 1996; Buki *et al.*, 1999). Such observations are consistent with the notion that the activation of calpain per se does not necessarily mean deterioration. On the contrary, the initial activation of calpain is probably good for resealing.

In summary, these observations highlight the importance of calpain-mediated cytoskeleton modification in axonal response to traumatic axonal injury in the brain and spinal cord. Furthermore, it also presents an initial period where medical intervention can be introduced, perhaps through the enhancement of membrane resealing by optimizing calpain activation. The medical intervention at a later stage, however, should clearly emphasize the inhibition of calpain-mediated axonal deterioration.

THE POSSIBLE MECHANISMS OF DMSO-INDUCED MEMBRANE SEALING

Since DMSO, as an organic solvent, has long been known to affect the physical status of membranes (Yu & Quinn, 1998), it is possible that DMSO may enhance sealing through the influence of overall membrane fluidity based on the following reasons. Application of DMSO has been shown to disrupt actin, another major component of subcortical cytoskeleton networks (Sanger *et al.*, 1980; Vincent *et al.*, 1990; Luna & Hitt, 1992). This could lead to the partial disconnection of the membrane from the rest of the cytoskeleton and therefore increase membrane fluidity in a manner similar to that of calpain (Sanger *et al.*, 1980; Vincent *et al.*, 1990; Croall & De Martino, 1991, Gitler & Spira, 1998). In support of this notion, Mellman and his colleagues have recently presented direct evidence that DMSO is capable of enhancing membrane fluidity in mammalian axons (Winckler *et al.*, 1999). They have shown that low levels of DMSO (0.4%) can disrupt F-actin and lead

Fig. 3. Photographs of Vibratome sections showing HRP labeling and the quantification under different conditions. The sections were taken at 1 mm from the transected end of the spinal cord strips and stained for horseradish peroxidase (HRP) using a diaminobenzidine technique. The strips were transferred to an HRP containing Krebs' solution and then incubated for one hour before fixation in glutaraldehyde. (A, B) Spinal cord strips exposed to HRP at 60 minutes following transection in Krebs' solution containing 0.5 mM Ca^{++} (37°C), without (A) or with (B) DMSO. Note the reduction of HRP-labeled axons from A to B. The open arrows indicate axons which were labeled with HRP and the filled arrows denote axons which excluded HRP. (C, D) Spinal cord strips exposed to HRP at 60 minutes following transection in Krebs' solution (2 mM Ca^{++}) at 25°C, without (C) and with (D) DMSO. Also note the reduction of HRP-labeled axons from C to D. (E, F) Spinal cord strips exposed to HRP at 60 minutes following transection in normal Krebs' solution (2 mM Ca^{++} and 37°C), without (E) and (F) with DMSO. Note the increase of HRP-labeled axons from E to F. Scale bar in F = 10 μm, for A–F. (G) The bar graph showing the quantification of HRP-labeled axons in different conditions. Six groups, 0.5 mM Ca^{++}, 25°C, and normal Krebs' solution, without or with DMSO ($n \geq 4$), were shown here. Note the addition of DMSO led to the reduction of HRP labeling in both 0.5 mM Ca^{++} and 25°C, and increase of HRP labeling in normal Krebs' solution ($P < 0.05$ for all three paired comparisons).

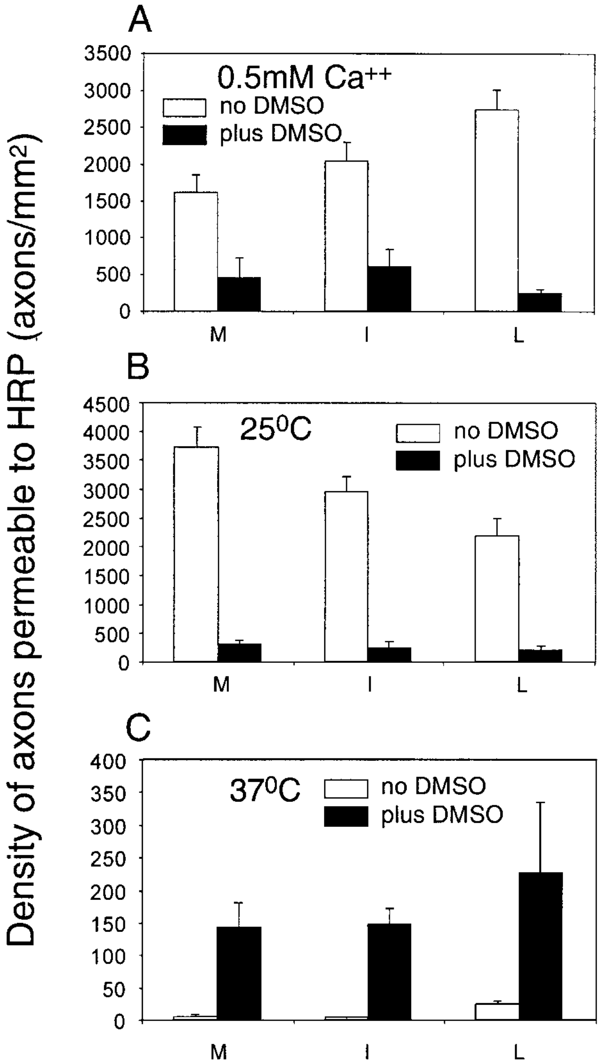


Fig. 4. The change of the density of axons permeable to HRP (axons/mm^2) as a function of axon position within the spinal cord cross section in three conditions, 0.5 mM Ca^{++} (A), 25°C (B), and normal Krebs' (2 mM Ca^{++} and 37°C) (C). HRP-labeled axons are grouped based on their position within the spinal cord cross section; M = medial (adjacent to gray matter), L = lateral (adjacent to the pial surface), and I = the intermediate position between these two locations (see Experimental procedures). Note that the addition of DMSO significantly decreased HRP-labeled axons in A and B, and significantly increased HRP-labeled axons in C at all locations ($n \geq 4$ in all groups, $P < 0.05$ for all nine paired comparisons).

to redistribution of membrane markers (an indicator of membrane fluidity), that were previously restricted to certain areas, in cultured rat hippocampal neurons (Winckler *et al.*, 1999). Based on these findings, we believe that the resealing-enhancing effect of DMSO seen in our current study was probably mediated by enhancing membrane fluidity. The fact that the DMSO inhibits resealing in normal physiological conditions could suggest that the excessive membrane fluidity induced by DMSO is detrimental to resealing in these conditions. This therefore indicates that DMSO-mediated resealing is temperature and calcium sensitive.

Another possibility is that DMSO may enhance membrane resealing by affecting vesicles fusions, a proposed critical step in membrane sealing of several cell types (Steinhardt *et al.*, 1994; Eddleman *et al.*, 1998; McNeil & Terasaki, 2001). The fusion of vesicles to each other and to plasma membrane in cortical cytoplasm surrounding the break have been shown to be a critical step leading to membrane resealing (McNeil & Terasaki, 2001; Miyake *et al.*, 2001). It is proposed by McNeil and his colleagues (Miyake *et al.*, 2001) that a prerequisite of this vesicle-plasma membrane fusion is the disassembly in the local cell cortex of the physical barrier constituted by filamentous actin. It is only after the breakdown of this "separating wall" that the vesicles can plug the break, fuse with plasma membrane and finally seal the membrane disruption (Miyake *et al.*, 2001). Considering the fact that DMSO disrupts the actin network (Sanger *et al.*, 1980; Vincent *et al.*, 1990; Luna & Hitt, 1992), it is possible that DMSO enhances membrane resealing by enhancing vesicle-plasma membrane fusion, although the existence of such a membrane resealing mechanism in mammalian CNS axons remains to be demonstrated.

THE POSSIBLE CLINICAL SIGNIFICANCE OF DMSO-ENHANCED MEMBRANE RESEALING

Based on studies from our laboratory and many others, it is well established that an extracellular calcium concentration of 0.5–1 mM is critical for axonal resealing following transection (Yawo & Kuno, 1985; Xie & Barrett, 1991; Shi *et al.*, 2000). Unfortunately, the $[Ca^{2+}]_0$ measured using *in vivo* mechanical injury is significantly below this level, which is expected to inhibit resealing. For example, $[Ca^{2+}]_0$ has been shown to decrease from 1.2 mM before injury to <0.01 mM, within minutes after injury in a cat spinal cord injury model (Young *et al.*, 1982) and a dog spinal cord injury model (Stokes *et al.*, 1983). This low level of $[Ca^{2+}]_0$ persists for several hours, especially in the central region of the compression lesion (Young *et al.*, 1982), where the membrane damage is most severe (Shi & Borgens, 2000; Shi, unpublished observation). The inability of injured axons to reseal their damaged membrane may encourage calcium influx and produce an enhanced retrograde degeneration of the surviving proximal axonal segments or increase the secondary axotomy and dissolution of damaged, but not severed, axons. If the axonal injury is physically close to the cell body, this could ultimately lead to the death of the cell itself. Furthermore, the prolonged activation of calpain may also hinder fast axonal transport by destroying the axonal cytoskeleton (Maxwell *et al.*, 1997). Since new membrane vesicles to allow repair of damaged membrane may be delivered by fast axonal transport, the deterioration of axonal cytoskeleton will contribute to the failure of membrane resealing. It is therefore reasonable to speculate that this may be one of the cellular bases for the continuing neuronal degeneration and severe functional loss following mechanical spinal cord injury. It is equally reasonable to suggest that the enhancement of axonal membrane resealing by the application of DMSO might produce an effective "acute treatment" for promoting cell survival and possibly overall functional recovery after mechanical spinal cord injury.

Hypothermia (such as 25°C) has been shown repeatedly to be able to reduce many secondary injury mechanisms (Hayes *et al.*, 1993; Lucas *et al.*, 1994; Koizumi & Povlishock, 1998). Further, a moderate hypothermic condition has been suggested to be used clinically to improve the outcome of trauma by reducing the damage from primary and secondary injuries (Clifton *et al.*, 1993). However, this has raised the possibility that while low temperature may suppress some secondary injuries, it may inhibit axonal membrane resealing (Shi & Pryor, 2000). The consequences of the injured axons' inability to reseal their damaged membrane may in some situations mask the beneficial effects of low temperature. This may explain why the study of the beneficial effect of hypothermia has a long, inconclusive, and controversial history (Hansebout *et al.*, 1984; Lucas *et al.*, 1994; Koizumi & Povlishock, 1998). As a matter of fact, there are a growing number of recent studies indicating that hypothermia alone provides little protection in treating brain or spinal cord injury when live animals or human subjects were utilized and the behavioral and functional examination were included (Shiozaki *et al.*, 1999; Robertson *et al.*, 2000; Westergren *et al.*, 2000).

Based on the findings in our study that DMSO can induce near complete axonal resealing in cold conditions, we postulate that a combined treatment of DMSO and lower temperature may be a better alternative treatment than hypothermia alone. This combined treatment is expected to preserve the beneficial effects of suppressing secondary injury while ensuring the repair of mechanically injured axons. We have also shown that DMSO can repair axons irrespective to their location within the cord (Fig. 4). This suggests that the application of DMSO is an effective treatment for axons with diverse functions. Consequently, the combination treatment of DMSO and hypothermia should produce better overall functional recovery in mechanical CNS injury.

There are some other features of DMSO supporting its use in clinical situations due to added beneficial effects, in addition to enhancement of membrane sealing. For example, independent studies suggest that DMSO can act as a free radical scavenger and anti-inflammatory agent, both of which are beneficial in treating spinal cord injury following mechanical insults (Cochran *et al.*, 1983; Badylak *et al.*, 1986; Brown *et al.*, 1988; Bruck *et al.*, 1999). In particular, DMSO has been shown to be beneficial in treating injuries caused by smoke inhalation (Brown *et al.*, 1988), gastric stress ulceration (Cochran *et al.*, 1983), kidney reperfusion (Kirpatovskii *et al.*, 1995), and hepatic failure (Bruck *et al.*, 1999). DMSO has also been shown to significantly increase the long-term survival rate following cardiorespiratory arrest and resuscitation in rats (Badylak *et al.*, 1986). We therefore conclude that, when used in combination with other more established treatments, DMSO may enhance various functional recoveries following spinal cord injury.

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