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4-AMINOPYRIDINE DERIVATIVES ENHANCE IMPULSE CONDUCTION IN GUINEA-PIG SPINAL CORD FOLLOWING TRAUMATIC INJURY

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Abstract—4-Aminopyridine (4-AP), a potassium channel blocker, is capable of restoring conduction in the injured spinal cord. However, the maximal tolerated level of 4-AP in humans is 100 times lower than the optimal dose in *in vitro* animal studies due to its substantially negative side effects. As an initial step toward the goal of identifying alternative potassium channel blockers with a similar ability of enhancing conduction and with fewer side effects, we have synthesized structurally distinct pyridine-based blockers. Using isolated guinea-pig spinal cord white matter and a double sucrose gap recording device, we have found three pyridine derivatives, N-(4-pyridyl)-methyl carbamate (100 μM), N-(4-pyridyl)-ethyl carbamate (100 μM), and N-(4-pyridyl)-tertbutyl (10 μM) can significantly enhance conduction in spinal cord white matter following stretch. Similar to 4-AP, the derivatives did not preferentially enhance conduction based on axonal caliber. Unlike 4-AP, the derivatives did not change the overall electrical responsiveness of axons to multiple stimuli, indicating the axons recruited by the derivatives conducted in a manner similar to healthy axons. These results demonstrate the ability of novel constructs to serve as an alternative to 4-AP for the purpose of reversing conduction deficits.

Key words: axonal stretch, compound action potential, refractory period, repetitive stimuli, myelin damage, conduction block.

The primary functional deficits of traumatic spinal cord injury mainly stem from damage to axons of the white matter. In most human injuries the spinal cord is not completely transected, rather the axons are strained, contused, and/or compressed. Within the injured cord there is a considerable amount of anatomically continuous yet physiologically compromised axons (Blight, 1983a,b; Fehlings and Tator, 1995; Hayes and Kakulas, 1997; Kakulas, 1999). Many of these axons undergo conduction block due to ionic disturbance and progressive demyelination following injury. Alleviating conduction block from a small population of these physiologically compromised yet anatomically intact axons could produce substantial functional recovery. Therefore, identification of compounds capable of restoring conduction in surviving axons is an effective option for treatment of spinal cord trauma.

Previous studies have indicated increased activity of potassium channels as a contributor to conduction block in demyelinated nerve fibers (Chiu and Ritchie, 1980; Sherratt et al., 1980). There is increasing evidence supporting this notion. For example, α-subunits Kv1.1, Kv1.2, and cytoplasmic β-subunit Kvβ2 which form the 4-aminopyridine (4-AP) sensitive potassium channel have been documented in the juxtaparanodal region of spinal cord myelinated axons (Rasband and Trimmer, 2001; Karimi-Abdolrezaee et al., 2004; Rasband, 2004). Due to their location, these potassium channels are typically hidden beneath the myelin sheath. Consequently, myelin damage, a well-documented secondary injury subsequent to spinal cord trauma (Blight, 1983a, 1985) will likely unmask these otherwise silent channels leading to conduction block via potassium ion efflux.

The ability of 4-AP, a potassium channel blocker, to restore conduction in damaged axons has been studied extensively (Targ and Kocsis, 1985; Blight and Gruner, 1987; Blight et al., 1991; Hayes et al., 1994, 2004; Shi and Blight, 1997; Shi et al., 1997; Halter et al., 2000; Jensen and Shi, 2003). While improvements in sensory and motor function following application of 4-AP have been demonstrated in both animal and human spinal cord injuries, the overall therapeutic benefit of 4-AP remains modest (Donovan et al., 2000; Halter et al., 2000). One obvious reason is that the maximum tolerable blood level of 4-AP in both animals and humans is only 0.5–1 μM, while the most effective concentration determined *in vitro* is 100 μM (Shi and Blight, 1997; Shi et al., 1997). Concentrations beyond 1 μM produce side effects such as respiratory distress, anxiety, and epileptiform seizures (Stork and Hoffman, 1994; Pena and Tapia, 1999, 2000). Possible reasons for the negative side effects associated with higher doses of 4-AP are increased synaptic transmission or additional blockade of potassium channel currents associated with the resting membrane potential (Shi and Blight, 1997).

In order to overcome these limitations while continuing to reverse conduction block, we have developed several pyridine-based compounds whose structures are similar to, yet distinct from, 4-AP. The goal of this line of study is to search for compounds that are capable of enhancing conduction effectively as 4-AP in the injured spinal cord with fewer side effects. Here we report our preliminary results, demonstrating that, as an initial step toward our

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Abbreviations: CAP, compound action potentials; tBoc, tertbutyl carbamate; 4-AP, 4-aminopyridine.

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goal, three newly identified analogs are capable of enhancing axonal conduction following spinal cord trauma.

EXPERIMENTAL PROCEDURES

Isolation of spinal cord

All animals used in this study were handled in strict accordance with the U.S. National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and the experimental protocol was approved by the Purdue Animal Care and Usage Committee. Every effort was made to minimize the number of animals used and their suffering. The method used to isolate the spinal cord was similar to that described previously (Shi and Blight, 1996; Shi et al., 1997; Shi and Pryor, 2002). A combination of ketamine (80 mg/kg) and xylazine (12 mg/kg) was used to anesthetize adult female guinea pigs weighing 350–500 g (obtained from Hilltop Laboratory Animals, Scottdale, PA, USA). Following anesthesia the animal was transcardially perfused with cold, oxygenated Krebs' (15 °C) solution to remove the blood and lower cord temperature. Following perfusion the entire vertebral column was excised rapidly and the spinal cord was removed from the vertebrae. The cord was then subdivided to produce ventral white matter strips that were subsequently incubated in fresh Krebs' solution at room temperature for 1 h. The term "ventral white matter strips" will be used interchangeably below with "cords" or "spinal cords" for ease of description. The composition of the Krebs' solution was as follows (in mM): 124 NaCl, 2 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 1.2 $CaCl_2$, 10 dextrose, 5.6 sodium ascorbate, and 26 $NaHCO_3$, equilibrated with 95% O_2–5% CO_2 to produce a pH of 7.2–7.4.

Recording chamber

Numerous variations of the recording chamber have been described in previous publications (Shi and Blight, 1996; Shi and Borgens, 1999; Shi and Pryor, 2002). As displayed in Fig. 1 a strip of spinal cord white matter 45 mm in length and 2 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). This portion of the chamber is also the site where the tested analogs were introduced. The ends of the spinal cord strip were placed across the sucrose gap channels (volume: 0.28 mL) to side compartments filled with isotonic potassium chloride (120 mM). The sucrose gap was perfused with isotonic sucrose solution (320 mM) at a rate of 1 mL/min. To prevent the exchange of solutions the white matter strip was sealed with a thin plastic sheet and vacuum grease on either side of the sucrose gap channels. Chamber temperature was maintained at 37 °C via an in line heater and temperature probe (Warner Instruments, Hamden, CT, USA). Electrodes were present in the central and outer two wells to monitor conduction through each solution. The electrodes were not in direct contact with the spinal cord and the compound action potentials (CAP) conduction distance is in fact through the central well of the chamber. The cord was stimulated by a 0.1 ms constant current unipolar pulse with CAP recorded at opposite ends of the strip. Recordings were made using a bridge amplifier (Neurodata Instruments, Delaware Water Gap, PA, USA) and subsequent analysis was performed using custom Labview® software (National Instruments, Austin, TX, USA) on a Dell PC. Further details and a description of the original chamber can be found in our previous publications (Shi and Blight, 1996; Shi and Borgens, 1999; Shi and Pryor, 2002).

CAP amplitude

Recordings of axonal conduction were made by analysis of CAP which are formed by the spatio-temporal summation of many single unit action potentials fired by individual axons. To record the CAP amplitude, a supramaximal stimulus (110% of the maximal stimulus) was delivered at a frequency of one stimulus every 3 s. The CAP was recorded continuously and stored in the computer for future analysis. To assess axonal conduction during each experimental condition averages were recorded at designated time points throughout the experiment. In addition, a real time plot of CAP amplitude was displayed throughout the experiment.

Stretching

The injury device as well as estimation of the magnitude of stretch or strain (the degree of elongation from the initial length) is described in our previous publication (Shi and Pryor, 2002). As displayed in Fig. 1 a flat raised surface with a small hole was placed in the central compartment. The ventral white matter strip was laid across this surface and immobilized with a nylon mesh stabilizer on either side of the elongation site. The placement of the nylon mesh had no significant effect on action potential amplitude (J. M. McBride and R. Shi, unpublished observations). A Plexiglas stretch rod attached to a micromanipulator was suspended above the white matter strip. During stretch injury the rod was released traveling at a rate of 1.5 m/s from a pre-measured distance and removed from the cord immediately after application. This device produced a strain of 50% on the isolated tissue which

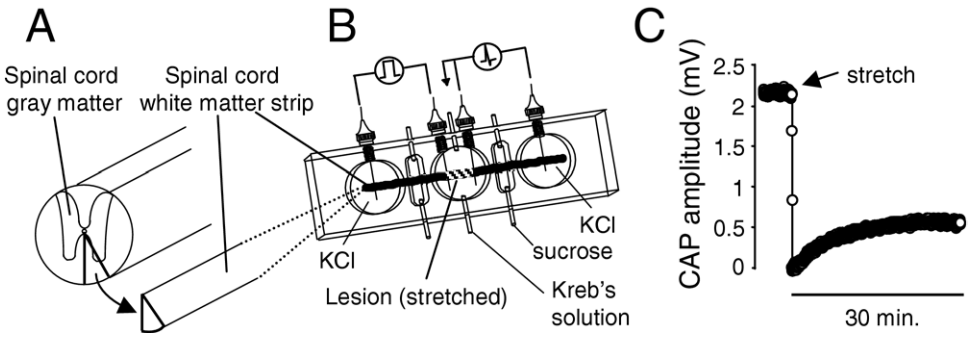

Fig. 1. Characterization of spinal cord tissue extraction, recording device, and conduction changes in response to stretch injury. (A) Drawing of tissue isolation from an extracted adult female guinea-pig spinal cord. (B) Double sucrose gap recording chamber with an isolated spinal cord sample mounted in the apparatus. The central chamber is continuously perfused with oxygenated Krebs' solution; this is also the site of oxygenated pyridine compound administration. The ends of the tissue were placed in separate wells filled with 120 mM isotonic KCl, which were separated from the central chamber by two smaller chambers containing 230 mM isotonic sucrose. (C) Example of CAP amplitude 30 min after injury to the isolated spinal cord white matter.

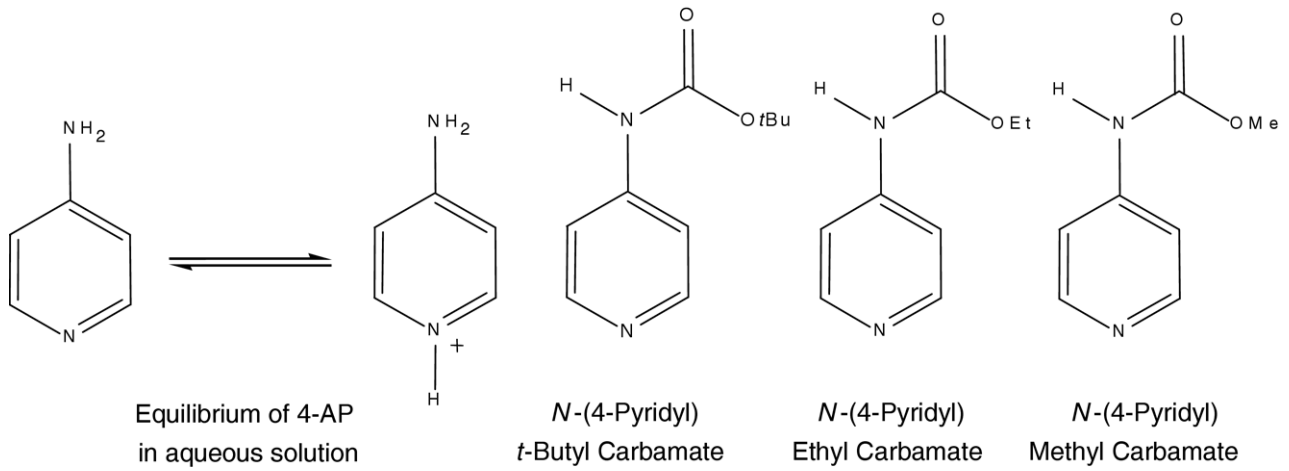

Fig. 2. Molecular structure demonstrating the equilibrium of 4-AP in aqueous solution and the tested derivatives tBoc, ethyl carbamate, and methyl carbamate. Each of the derivatives is similar in structure to 4-AP however, the side groups are modified.

indicates that the tissue is being elongated 50% of its original length.

Derivative synthesis

Examples of three chemical derivative classes of 4-AP, amides, carbamates, and ureas, were synthesized to determine if any of these compounds were biologically active in spinal cord injury (Smith et al., 2005). All prospective analogues were chosen that would not completely eliminate the pyridine–pyridinium equilibrium responsible for both transport and biological activity. In addition the pyridine nitrogen atom was not altered since the proposed mechanism of blockade arises from the ability of hydrogen bonds to form between the pyridine nitrogen and the channel pore. These compounds were synthesized to test steric requirements of the active site as well as bonding interactions between the derivatives and the channel. For this paper we will focus on conduction changes in the injured cord following application of the carbamate derivatives (Fig. 2).

Carbamates were synthesized from 4-AP which was purchased from Richman Chemical Co., Lower Gwynedd, PA, USA. Melting points were determined in capillary tubes using a Thomas Hover melting point apparatus. NMR spectra were obtained on a Bruker ARX-300 instrument using the indicated solvent. Please refer to Smith et al. (2005) for the detailed protocol of synthesizing methyl carbamate, ethyl carbamate, and tertbutyl carbamate (tBoc).

All three 4-AP analogs were added to oxygenated Krebs' solution and introduced to the injured cord through the central well of the recording chamber. For initial testing all analogs were presented to the cord at a concentration of 100 μM, the most effective concentration of 4-AP *in vitro*.

Statistical analysis

Throughout the paper, Student's *t*-test was used to compare electrophysiological data. Statistical significance was attributed to values $P<0.05$. Averages were expressed as mean$\pm$standard error.

RESULTS

After placement in the recording chamber the ventral white matter strip was monitored for approximately 30–45 min or until the CAP amplitude had maintained a stable baseline recording. An additional 10 min was utilized to record baseline measurements before the injury device was lowered. Immediately after inducing injury the amplitude of the CAP was completely eliminated. CAP response subsequently began to increase, reaching a plateau approximately 30–45 min after injury (Fig. 1C).

CAP response following derivative administration

Initial testing revealed that these compounds had no significant effect on CAP amplitude in the uninjured cord (data not shown). During presentation of the derivatives to the injured cord we observed an increase in CAP response which plateaued 30–45 min after the initial application. Fig. 3 displays the CAP response following administration of ethyl, methyl, and tBoc. Ethyl carbamate at a concentration of 100 μM resulted in a gradual increase in the CAP response which slowly declined after drug had been removed (Fig. 3A). The trend line following application of 100 μM methyl carbamate displayed a steeper CAP inclination which also progressively declined after wash was initiated (Fig. 3B). The most striking increase in CAP amplitude was observed after presentation of 10 μM tBoc (Fig. 3C). This lower concentration of 10 μM was chosen after a previous study indicated that 100 μM tBoc exhibited a significant decrease in CAP response (Smith et al., 2005). Similar to the other two compounds, the increase of CAP amplitude was largely reversible after tBoc had been removed. Imposed above the trend lines are representative action potentials for three stages of the experiment: pre-drug, drug, and wash. These individual action potential profiles showed no appreciable difference for any of the compounds in the three displayed conditions (Fig. 3).

Overall, 100 μM methyl carbamate improved CAP amplitude 16.27%$\pm$3.15 ($n=7$, $P<0.001$), 100 μM ethyl carbamate improved CAP amplitude 7.86%$\pm$1.90 ($n=7$, $P<0.002$), and 10 μM tBoc produced similar results increasing CAP response 14.88%$\pm$2.89 ($n=6$, $P<0.001$) (Fig. 4). After examining the single CAP response following administration of the derivatives, multiple response analy-

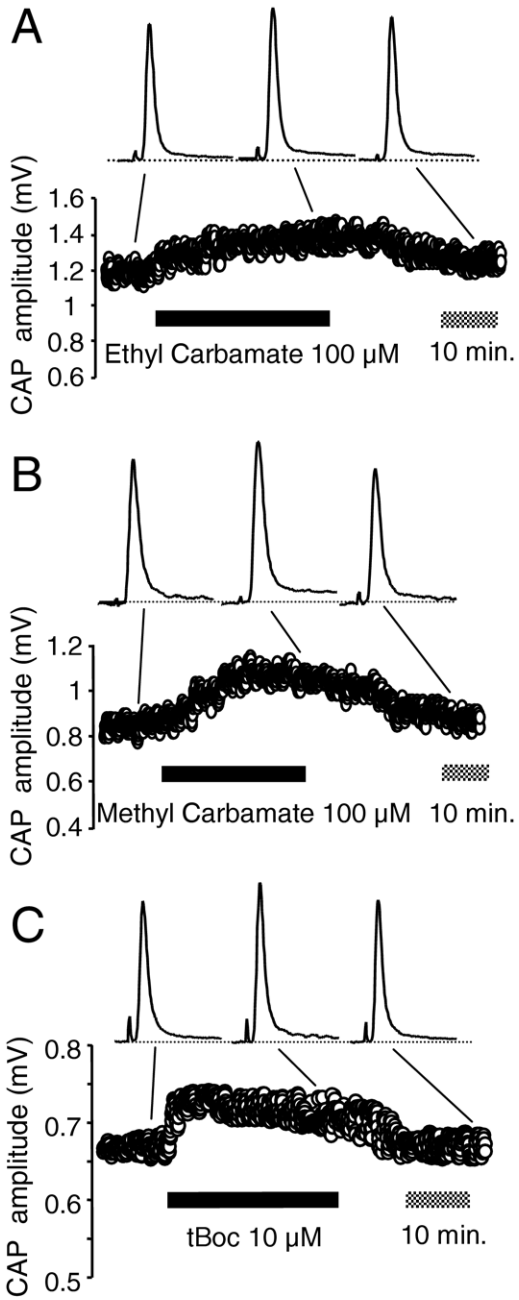

Fig. 3. Trend line representation of CAP amplitude in response to pyridine compound administration after mechanical injury to the guinea-pig spinal cord. Trend lines indicate a record of CAP amplitude over time. (A) CAP response to 100 μM ethyl carbamate. Note the slow rising phase after administration of ethyl carbamate, and gradual decline during wash. (B) CAP response to 100 μM methyl carbamate. The CAP here exhibited a steeper increase in response to drug application with a gradual decline after wash. (C) Similar trend line representation of CAP response as in A and B however, here 10 μM tBoc displayed a marked increase in amplitude similar to B at a lower concentration. A gradual decline in response was also noted after wash had ensued. Shown above the figures are examples of waveforms pre-drug, during drug administration, and after wash. All waveforms were taken when the CAP response had stabilized.

sis was performed to further define the effect of these compounds on basic nerve function.

Derivatives had no significant effect on activation threshold

Superimposed images in Fig. 5A exhibit the changes of CAP amplitude in response to 100 μM methyl carbamate at different stimulus intensities before and after drug application. During a wide range of stimulus intensities (1.85–6.5 V), CAP amplitude in the presence of methyl carbamate is proportionally higher than before methyl carbamate application (Figs. 4, 5). This relationship is demonstrated in absolute terms in Fig. 6. The near unity of the slope indicates that the enhancement in conduction in response to methyl carbamate application was not biased toward axons with low or high thresholds (Fig. 6). Similar results were found when 100 μM ethyl carbamate and 10 μM tBoc were administered. Specifically, R^2 values for methyl carbamate, ethyl carbamate, and tBoc are 0.95, 0.99, and 0.99 respectively.

Dual and multiple stimuli

Fig. 7A displays the relationship between the interstimulus interval (0.5–13 ms) and the amplitude of the two elicited CAPs. We have found that the ability of the cord to respond to dual stimuli presented at different time intervals was not altered after application of the derivatives. A plot of the second CAP against the log of the interstimulus interval, in response to 100 μM methyl carbamate, illustrates this point (Fig. 7B). The CAP response as a function of stimulus interval completely overlapped during pre-drug, drug, and wash periods (Fig. 7B). An analogous phenomenon was observed in response to 100 μM ethyl carbamate and 10 μM tBoc (data not shown). Further examination indicates that the absolute and relative refractory period in response to the three derivatives did not change significantly during pre-drug, drug, and wash periods (Fig. 8A–C).

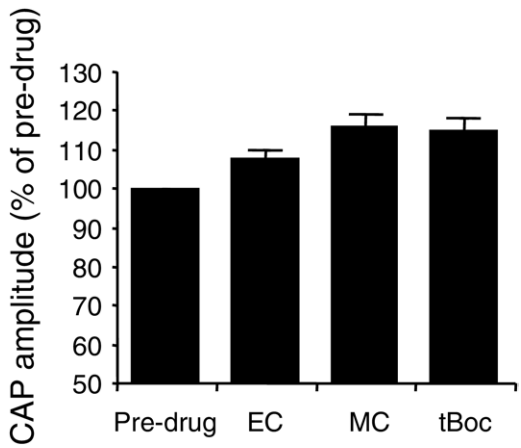

Fig. 4. Increase in CAP amplitude as a percent of pre-drug response after stretch injury to the guinea-pig spinal cord. All compounds increased CAP response significantly. Ethyl carbamate administered at a concentration of 100 μM increased CAP amplitude 7.86%±1.90 ($P<0.002$). 10 μM tBoc also increased CAP response 14.88%± 2.89 ($P<0.0005$). The most significant increase in CAP, 16.27%±3.15 ($P<0.0003$), was observed with application of methyl carbamate at a concentration of 100 μM.

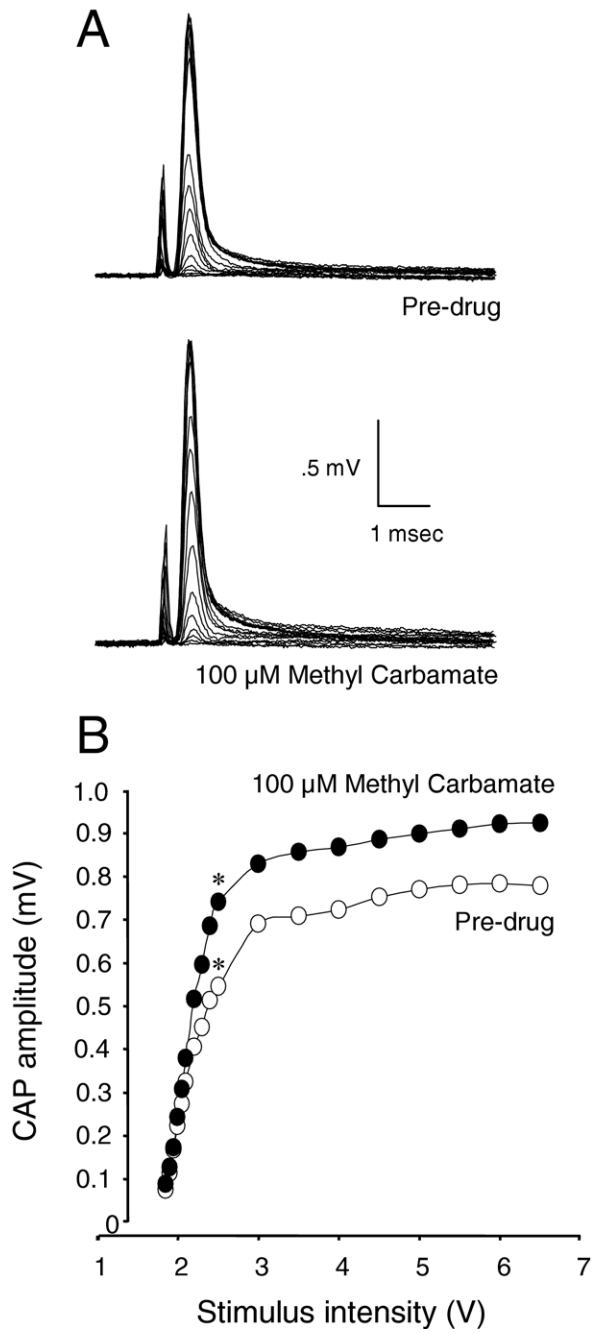

Fig. 5. Comparison of response amplitude at different stimulus intensities following stretch injury in the untreated and 100 μM methyl carbamate treated cord. Each condition is represented (A) in the form of superimposed recordings and (B) a line graph. The data points in B represent an average response of seven white matter strips. Stimulus intensities applied to the cord ranged from 1.85–6.5 V. The response amplitude of the treated cords differed significantly from the untreated cords ($P<0.03$) from stimulus intensities 2.5–6.5 V. Similar observations were made after application of 100 μM ethyl carbamate and 10 μM tBoc (data not shown).

We also examined changes in the ability of the cord to follow repetitive stimuli following application of the derivatives. An example of CAP response to a train of stimuli (500 Hz for 100 ms) is shown in Fig. 9A. The average amplitude of the last four CAPs in response to a train of stimuli at 500 Hz/100 ms or 1000 Hz/100 ms, in both pre-drug and drug conditions is displayed (Fig. 9B–D). The CAP response to stimuli of higher or lower frequency is not affected by application of the tested derivatives ($P>0.05$ in all comparisons, pre-drug vs. drug, Fig. 9B–D).

DISCUSSION

Stretch injury to spinal cord white matter

Stretch is an important component of mechanical injury to the spinal cord (Blight and Decrescito, 1986; Shi and Pryor, 2002). Compared with existing stretch models in live animals and in monolayer tissue culture system (Maxwell et al., 1991; Smith et al., 1999; Bain et al., 2001), the *in vitro*, or so called ex vivo, spinal cord stretch model employed in this study has several advantages. First, compared with the examination using monolayer cell culture; this system analyzes a whole tissue sample, which is more clinically relevant. 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 injure the spinal axons in a preparation closer to an *in vivo* condition. Therefore, the information derived from the current model is relevant to the *in vivo* spinal cord injury. Second, compared with *in vivo* stretch models, this ex vivo stretch preparation provides increased environmental control and greater accessibility of the cord. This allows us to control the degree of stretch (strain) and the speed of stretch (strain rate) in order to mimic stretch injuries in various real life situations. Furthermore, by increasing environmental control, this ex vivo preparation is

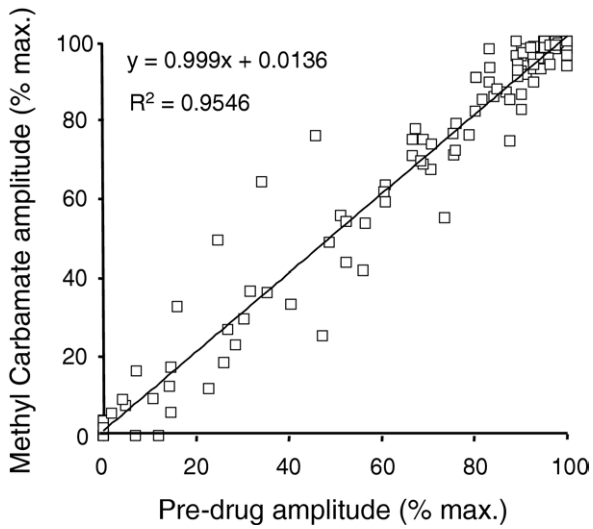

Fig. 6. Normalized CAP response of the injured spinal cord plotted before and after treatment with 100 μM methyl carbamate. Original data for this figure are the same as for Fig. 4B with seven white matter strips exposed to stimulus intensities ranging from 1.85–6.5 V. Overall axons with different stimulus thresholds display a similar response to drug-mediated amplitude enhancement. A similar trend was observed after application of 100 μM ethyl carbamate and 10 μM tBoc (data not shown).

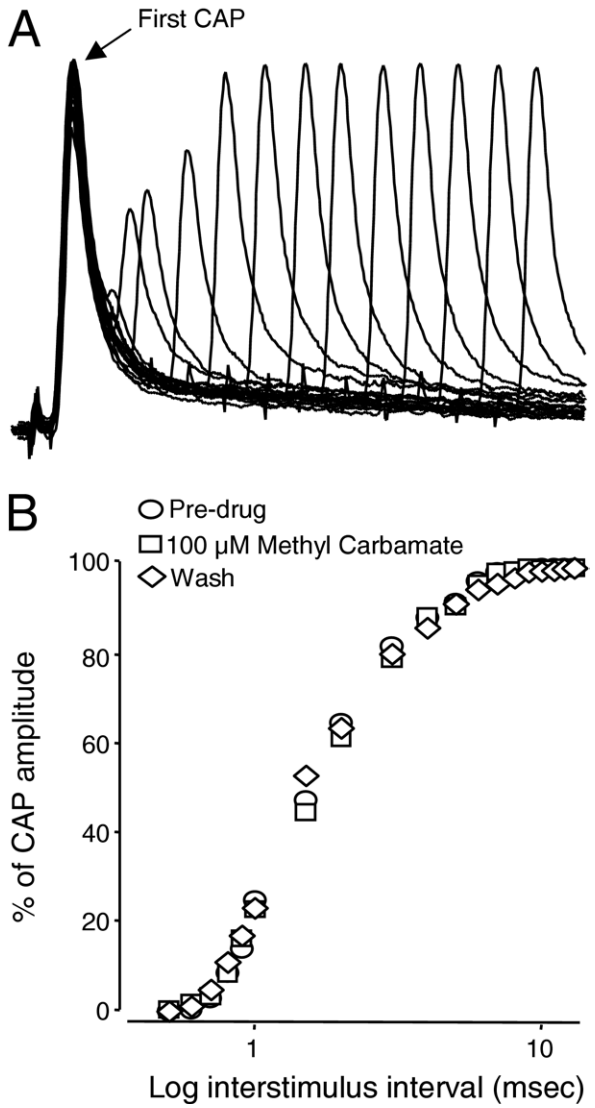

Fig. 7. Refractory period response following application of 100 μM methyl carbamate. (A) Superimposed CAP recording from a ventral white matter strip exhibiting a changing response to twin pulse stimuli with various interstimulus intervals. Due to a continuous increase in the interstimulus interval the amplitude of the second peak progressively increases. (B) Amplitude of the second CAP as a percentage of the first CAP is plotted against the log of the interstimulus interval in three conditions: pre-drug, drug-100 μM methyl carbamate, and after wash with normal Krebs' solution.

suitable for testing pharmacological interventions aimed at treating functional and anatomical deficits resulting from physical insult.

Conduction enhancement by 4-AP derivatives in spinal cord injury

4-AP has long been recognized for its ability to enhance axonal conduction in myelinated fibers by blocking A-type potassium channels (Bostock et al., 1981). This has been demonstrated in both *in vitro* and *in vivo* preparations (Targ and Kocsis, 1986; Blight, 1989; Hansebout et al., 1993; Hayes et al., 1994; Shi and Blight, 1997; Potter et al., 1998). However, despite its success in experimental injuries in animals, the use of 4-AP in human spinal cord injury severely limited by its negative side effects, as mentioned previously. Therefore, it is logical to explore new compounds that can effectively block these potassium channels with perhaps reduced side effects. As a first step toward achieving this goal, we have shown that three

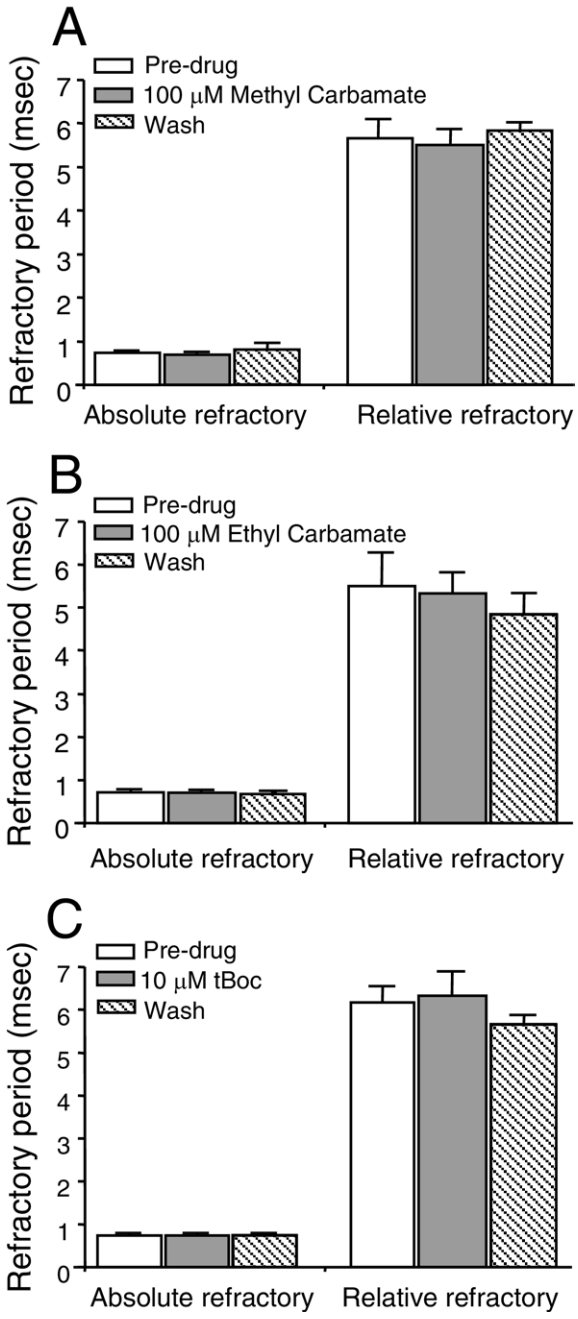

Fig. 8. Bar graph representation of changes observed in the relative and absolute refractory period following application of (A) 100 μM methyl carbamate, (B) 100 μM ethyl carbamate, (C) 10 μM tBoc. No significant changes were observed in either the absolute or relative refractory period following drug administration or wash with Krebs solution. The time when the second CAP was $\geq$95% of the first one is defined as the relative refractory period.

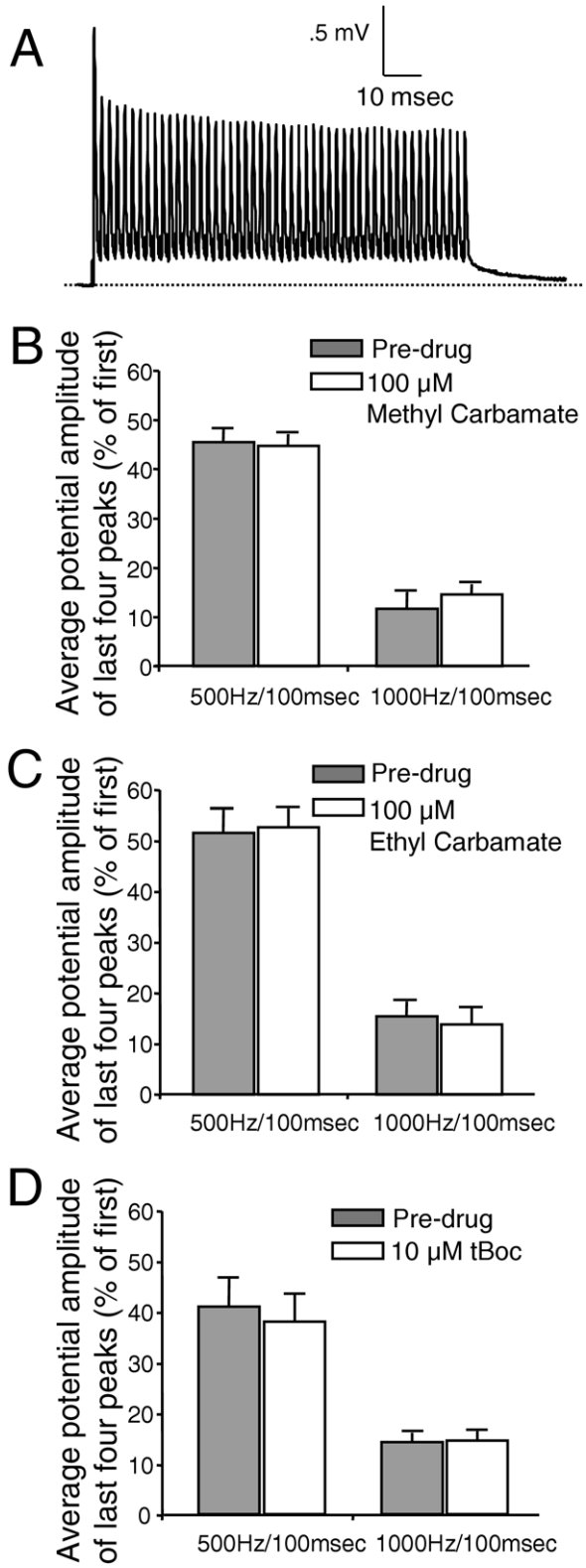

Fig. 9. White matter response to a train stimulus. (A) Series of CAPs from the typical ventral white matter strip responding to stimuli at 500 Hz for 100 ms. Bar graph representation of cord response to 500 and 1000 Hz stimuli for a duration of 100 ms before and after treatment with (B) 100 μM methyl carbamate (C) 100 μM ethyl carbamate and (D) 10 μM tBoc. Data for each graph are an average of the last four waveforms as a percentage of the first waveform for six to seven cord strips. No significant difference in response amplitude was observed at either stimulus intensity for any of the treatment conditions.

newly synthesized pyridine-based compounds with structures similar to, yet distinct from, 4-AP can significantly enhance CAP amplitude following *in vitro* stretch injury. These results are in good agreement with those obtained in earlier studies where similar compounds were examined in both *in vitro* and *in vivo* preparations (Smith et al., 2005; McBride et al., 2006). This indicates that, similar to 4-AP, these compounds are likely able to block potassium channels and enhance axonal conduction.

Individually, we observed that 100 μM, tBoc caused a significant reduction of CAP amplitude while 4-AP can cause similar suppression of conduction at 10 mM (Shi and Blight, 1997; Shi et al., 1997). For this reason, the lower dose of 10 μM was chosen for the duration of the study. While the precise reason for tBoc's differential effects is uncertain we do offer one possible explanation. The formation of the carbamate was expected to lower its pKa due to electronic factors. This would shift the pyridine–pyridinium equilibrium toward its neutral form at physiological pH. Since the neutral form of this molecule is postulated to enter the cell (Stephens et al., 1994) this may produce a much higher concentration of tBoc entering the cytoplasm.

One remaining question that is beyond the scope of this study is whether the derivatives actually block the fast or 4-AP-sensitive potassium channels. This requires a detailed electrophysiological study that can demonstrate the isolation of the potassium current and its blockade by 4-AP and these derivatives.

The selection of pyridine-based derivatives

The current understanding of how 4-AP interacts with the channel pore formed the basis for the development of the tested compounds (Smith et al., 2005). Kirsch and Narahashi (1983) revealed that 4-AP is most potent at blocking potassium channels internally in cationic form. More recently, Nino et al. (2003) proposed a functional model for potassium channel blockade by aminopyridines. This model expresses pyridine-based channel blockade as a function of energy for interaction of the ligand with the receptor, which is a function of pKa. The model also emphasizes that the pyridine ring plays a decisive role in receptor site interaction by forming hydrogen bonds with the C_4 symmetry of the inner potassium channel pore. Therefore, in the process of synthesizing these compounds, the basic structure of the pyridine ring was retained with variations made on the attached side groups.

Derivative effects on electrophysiological properties

None of the tested compounds showed preference in enhancing axonal conduction based on their calibers (Figs. 5 and 6). This indicates that axons of large or small diameter benefit equally from derivative treatment following injury. This observation is similar to a previous publication from

this laboratory regarding the ability of 4-AP to enhance axonal conduction (Jensen and Shi, 2003).

Previous study indicates that 4-AP significantly reduces axonal responsiveness by increasing the absolute and relative refractory period, as well as decreasing the ability of the cord to respond to repetitive stimuli (Targ and Kocsis, 1986; Jensen and Shi, 2003). However, the derivatives tested in this study do not adversely affect these parameters. Therefore, it appears that the 4-AP-rescued axons conduct electric impulses in a manner that is somewhat inferior to healthy axons, while the axons recruited by the derivatives conduct more like healthy axons. According to Stephens et al. (1994), 4-AP (the cationic form) probably binds near the inactivation gate. Therefore, the differential effects on axonal responsiveness may be related to the fact that 4-AP modifies channel inactivation, whereas the derivatives do not. This hypothesis can be tested through further electrophysiological studies. Another possible explanation for this phenomenon is that compared with 4-AP, derivatives are less likely to block other potassium channels that are important for axonal excitability. In summary, these data indicate that these derivatives may enable axons in mechanically injured spinal cord to conduct electrical impulses in a manner similar to healthy axons. Therefore, these derivatives may represent an alternative to 4-AP for enhancing axonal conduction in spinal cord injury victims.

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