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N-(4-pyridyl) methyl carbamate inhibits fast potassium currents in guinea pig dorsal root ganglion cells

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ABSTRACT

Axonal demyelination is a critical pathological phenomenon associated with spinal cord injury and multiple sclerosis (MS). Previous studies demonstrated that 4-Aminopyridine, a fast potassium channel blocker, enhances impulse conduction on damaged and/or demyelinated axons, allowing for functional recovery in spinal cord injuries and MS, but with severe therapeutic limitations. To continue to explore the therapeutic value of blocking fast potassium channels while circumventing the side effects of 4-AP, we have developed three novel 4-AP derivatives that enhance impulse conduction in spinal cord trauma. In the current study, we have shown that one of these three derivatives, N-(4-pyridyl) methyl carbamates (MC), significantly inhibits a fast, I_A like potassium current in guinea pig dorsal root ganglion cells in a whole cell patch clamp configuration. This inhibition of I_A likely plays a critical role in MC's ability to restore conduction in mechanically injured spinal cord axons and may present a viable alternative to 4-AP for individuals with spinal cord injury or MS. From this, compounds with greater efficacy and perhaps less side effects will likely emerge in the near future, which will greatly enhance the functional restoration and lessen the suffering of SCI and MS patients.

1. Introduction

Disruption of the myelin sheath surrounding an axon is a well-recognized pathologic event associated with spinal cord injury (SCI) and multiple sclerosis (MS) [1–4]. The degradation of the myelin sheath exposes, otherwise silent, fast voltage-gated potassium channels and plays a critical role in conduction block, which potentially leads to severe functional deficits [2,3,5–10]. Activation of such channels is thought to lead to conduction block by allowing potassium ions to leak out of the injured axon during the events of depolarization. Loss of potassium ions prevents the membrane potential from reaching the necessary threshold required for triggering the action potential. This consequently annuls the initiation of an action potential at the node of Ranvier.

Over the last two decades, 4-aminopyridine (4-AP), a known blocker of fast voltage-dependent potassium channels, has been demonstrated to effectively enhance action potential propagation following spinal cord trauma in various experimental and clinical settings [8–15]. Specifically, 4-AP enhances action potential conductance and improves behavioural functions in both acute and chronic spinal cord trauma in rodents, dogs, and humans. For that reason, blocking these fast potassium channels may present an effective therapeutic intervention for spinal cord trauma.

Despite the potential therapeutic value, development and maturation of this line of treatment has been slow, largely due to limitations of 4-AP when applied *in vivo*. For instance, the safe dose of 4-AP achievable *in vivo* (~1 μM) is two magnitudes lower than the most effective dose of 4-AP for enhancing action potential conductance *in vitro* (~100 μM) [8,9]. High concentrations of 4-AP could evoke serious side effects such as movement hyperexcitability or even seizures [16–18].

To continue exploring the therapeutic value of blocking fast potassium channels while circumventing the side effects of high concentrations of 4-AP, we turned our attention to the development of novel fast potassium channel blockers to enhance impulse conduction in spinal cord trauma. As an initial step, we developed three compounds which are structurally similar, yet distinct from 4-AP. Our previous studies demonstrated these three derivatives, N-(4-pyridyl) methyl, N-(4-pyridyl) ethyl and N-(4-pyridyl) t-butyl carbamates, significantly enhance action potential conductance following mechanical stretch injury in guinea pig spinal cord both *in vitro* and *in vivo* [19–21]. More importantly however, we noted axons rescued by a 4-AP derivative conduct an action potential in a manner more like healthy axons than those rescued by 4-AP [10,21].

Based on the aforementioned studies regarding the therapeutic values of 4-AP derivatives, further study of the mechanisms involved

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in enhancing the electrical impulse conduction is greatly warranted. The aim of this study is to determine the ability of one of the 4-AP derivatives, methyl carbamate (MC), to block the fast voltage-gated potassium channels sensitive to 4-AP using a patch clamp configuration. Guinea pig dorsal root ganglion (DRG) neurons were used for their known expression of the fast potassium or "A" type current (I_A) [22–26]. Our data indicates that methyl cabamate is indeed capable of blocking 4-AP sensitive fast potassium channels. This capability was likely the underlying mechanism responsible for enhancing electrical impulse of guinea pig spinal cord following mechanical trauma.

2. Method and materials

2.1. Animal

The Purdue Animal Care and Usage Committee approved the following protocol using adult female guinea pigs. Guinea pigs were anesthetized with a combination of ketamine (80 mg/kg), xylazine (12 mg/kg) and acepromazine (0.8 mg/kg). Following anesthetization, guinea pigs underwent perfusion to remove the blood and lower overall body temperature using oxygenated, cold Krebs' solution (NaCl 124 mM, KCl 2 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, and sodium ascorbate 10 mM). The spinal cords were isolated as previously described [27].

2.2. Acute dissociation of guinea pig DRG cells

Dorsal root ganglia from all accessible segments of guinea pig spinal cord were collected and placed in DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich) solution. The connective tissue and nerve roots were removed from the DRGs and digested with Trypsin (Type I, 0.25 mg/ml, Sigma-Aldrich) and Collagenase (Type I, 0.5 mg/ml, Sigma-Aldrich) at 37 °C for 30 min. Dissociated dorsal root ganglion cells were triturated in culture medium containing 48.5% DMEM solution, 48.5% F-12 Nutrient Mixture (Gibco), 2% horse serum (Sigma-Aldrich) and 1% penicillin-streptomycin solution (Sigma-Aldrich), and plated on 35 mm dishes coated with Poly-L-lysine (0.2 mg/ml, coating over night, Sigma-Aldrich). Cells were incubated at 37 °C (5%CO_2 balance air) for 2.5 h before recording.

2.3. Electrophysiological recording

Whole cell patch recording was performed using the Axopatch-1D amplifier (Molecular Devices Corporation). Recording pipettes with a resistance of approximately 4 MΩ were extracted from the capillary tubing (Warner Instruments Inc.) using a P-97 horizontal puller (Sutter Instruments). The pipette solution included 140 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES titrated to pH 7.4 with KOH, while the bath solution included 145 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.03 mM $CaCl_2$, 10 mM HEPES and 1 μM TTX titrated to pH 7.4 with NaOH. The holding potential was −70 mV and currents were filtered at 1–5 kHz. Resulting data was acquired and analyzed with pCLAMP 6 software (Molecular Devices Corporation).

2.4. Isolation of voltage gated potassium currents

To ensure the major voltage-gated currents elicited during stimulation were potassium currents, Na^+ currents were blocked using 1 μM of tetrodotoxin (TTX) and both Ca^{2+} currents and Ca^{2+} dependent K^+ currents were suppressed by decreasing the Ca^{2+} concentration to 0.03 mM in the bath solution [28]. The total potassium current measured (I_{total}) consists of a transient fast activating 'A-type' current (I_A) and a slow sustained delayed rectifier type current (I_{dr}) [28]. The total outward potassium current (I_{total}) was acquired from a series of 400 ms voltage stimuli, ranging from −40 mV to 40 mV with 10 mV steps. Before the step stimuli, the cell is held at −100 mV (hyperpolarization) for 2 s. When the membrane potential is held at −30 mV (depolarization) for 2 s before the step stimuli, the fast activating component (I_A) stays inactivated during the 400 ms step stimuli, and all the collected potassium currents are slow delay rectified component (I_{dr}). 5 mM 4-AP and 5 mM N-(4-pyridyl) methyl carbamate are applied into the bath solution respectively. For the washout, a bath solution without 4-AP or N-(4-pyridyl) methyl carbamate is applied to replace the solution containing the testing compounds (4-AP or N-(4-pyridyl) methyl carbamate). 4-AP and N-(4-pyridyl) methyl carbamate are both freshly made with bath solution before recording.

2.5. Synthesis and property of N-(4-pyridyl) methyl carbamate

The manufacturing process and preparation of the methyl carbamate has been previously described in detail [19]. We also found that this compound is stable and does not degrade in the blood or CSF for at least 6 h following administration. We also noted that this compound can be detected in CSF following intravenous administration, indicating the compound crosses the blood-CSF barrier (unpublished observation).

2.6. Statistical analysis

Paired *t*-test was used for analysis of N-(4-pyridyl) methyl carbamate in inhibiting potassium currents at +30 mV. Two-way

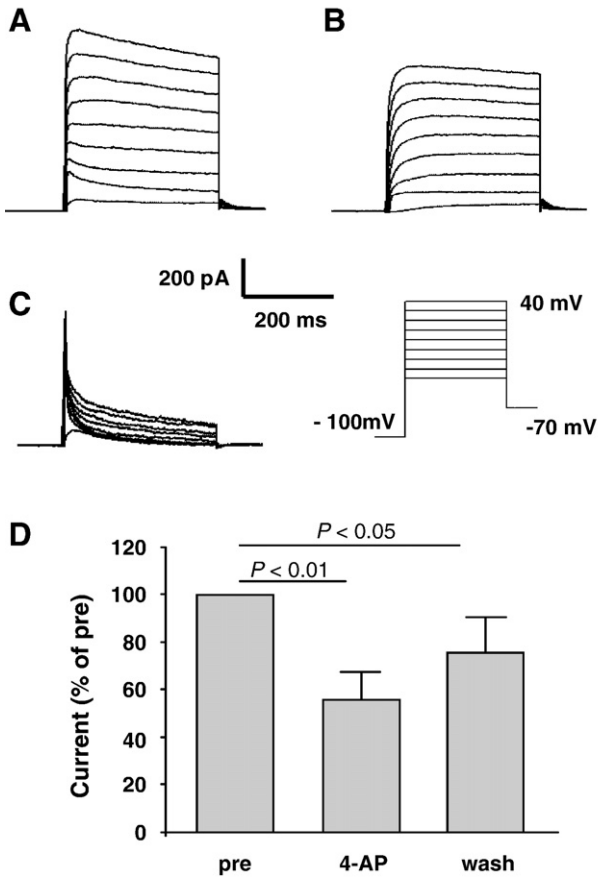

Fig. 1. 4-AP blocks fast potassium current (I_A). Representative example of isolating fast inactivating A-Type potassium current I_A from guinea pig DRG cells (A–C). Holding potential is −70 mv. Whole cell potassium currents were produced by a series of 400 ms stimuli ranging from −40 mv to 40 mv with 10 mv steps. A 2 s−100 mV pre-stimulus pulse was applied immediately before the test stimuli. A) Total potassium current (I_{total}) before the application of 5 mM 4-AP; B) Potassium current recorded after the application of 5 mM 4AP (current without A-type current); C) 4-AP sensitive A-type potassium current (I_A) was obtained by subtracting B from A. D) Bar graph showed at +30 mV for the transit peak component, only 55.69%±5.3% of the potassium current remained in the presence of 5 mM 4-AP. $N=5$, paired *t*-test, $P<0.01$.

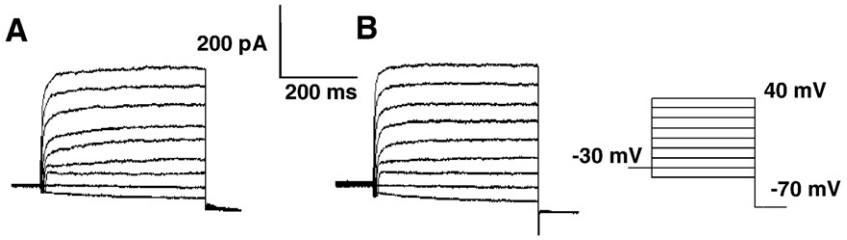

Fig. 2. Representative example of delay rectifier potassium current (I_{dr}). Holding potential is −70 mv. The stimulus steps were the same as described in Fig. 1. However, cells are held at −30 mV for 2 s before the step stimuli. A) Delay rectifier potassium current (I_{dr}) before the application of 5 mM 4-AP; B) Potassium current recorded after the application of 5 mM 4AP.

ANOVA was used to compare I–V curves before and after treatment of N-(4-pyridyl) methyl carbamate. Unpaired student *t*-test was used to compare the effectiveness between 4-AP and N-(4-pyridyl) methyl carbamate. Results are presented as means±SEM.

3. Results

3.1. Blockage of fast A-type potassium current by 4-Aminopyridine in DRG neurons

The recorded potassium current (I_{total}), represented in Fig. 1A, was rapidly activated upon depolarization but then partially decayed to a sustained level. The I_{total} likely contains both fast potassium current (I_A), which is responsible for the initial transient activation component, and delayed rectified current (I_{dr}), which provides the sustained current component [25,26,28]. After applying 5 mM 4-aminopyridine (4-AP) in the bath solution, there is obvious suppression of the initial transient part of I_{total}, while the sustained part was less affected, shown in Fig. 1B. This indicates that I_A is likely blocked by 4-AP but I_{dr} is affected less [25,29]. Fig. 1C displays the 4-AP sensitive current by subtracting the current displayed in Fig. 1B from the current in Fig. 1A. The major part of 4-AP-sensitive potassium current is the fast transient component, or I_A. However, a small but appreciable sustained component is also noted, according to Fig. 1C.

To quantify the 4-AP sensitive current, we measured the difference between peak values of the transient component with or without 4-AP at a particular command potential of +30 mV. The difference was then expressed as the peak percentage of I_{total}. As a result, only 55.69%±5.3% of the transient potassium current remained in the presence of 4-AP (Fig. 1D, $n=5$, paired *t*-test, $P<0.01$), or about 45% of the initial fast potassium current can be blocked by 4-AP at the concentration of 5 mM. In addition, the 4-AP-mediated suppression of I_A is largely recoverable upon washout for 30–45 min (Fig. 1D). To confirm that I_{dr} is not sensitive to 4-AP, the pre-step pulse potential was held at −30 mV and, as expected, I_A was inactivated before the testing pulses were delivered. Therefore, the current elicited by the testing pulse in this protocol lacks I_A and consists mainly of I_{dr}. As shown in Fig. 2, there is no appreciable difference in I_{dr} amplitude before or after the application of 4-AP. ($n=5$, paired *t*-test, $P>0.05$). This indicates I_{dr} is not sensitive to 4-AP at this level.

3.2. Partial blockade of I_A by N-(4-pyridyl) methyl carbamate

This group of experiments recorded I_{total} using the same stimulus protocol as in the previous experiment, described above. Again, the transient component of the I_{total} was significantly decreased to 86.7±8.6% of the initial level, following the application of 5 mM N-(4-pyridyl) methyl carbamate at +30 mV ($n=6$, paired *t* test, $P<0.05$), and recovered partially upon washout ($n=6$, paired *t* test, $P>0.05$) (Fig. 3). Similar to 4-AP, in addition to an initial transient current (I_A), N-(4-pyridyl) methyl carbamate blocked a noticeable sustained component of potassium currents (Fig. 3C) and did not significantly affect I_{dr} (Fig. 4, $n=6$, paired *t* test, $P>0.05$). From this, I_A was significantly inhibited by both 4-AP (Fig. 1), as shown in the previous section, and 5 mM N-(4-pyridyl) methyl carbamate (Fig. 3).

In addition to the findings regarding the effect of N-(4-pyridyl) methyl carbamate on the I_{total} elicited using a testing pulse potential of +30 mV, we also examined the effects of methyl carbamate on the initial component of potassium current elicited with testing pulse potentials ranging from −40 to 40 mV. Fig. 5 shows the I–V curves for the initial component of I_{total} in pre- and post-N-(4-pyridyl) methyl carbamate application. The findings show that N-(4-pyridyl) methyl

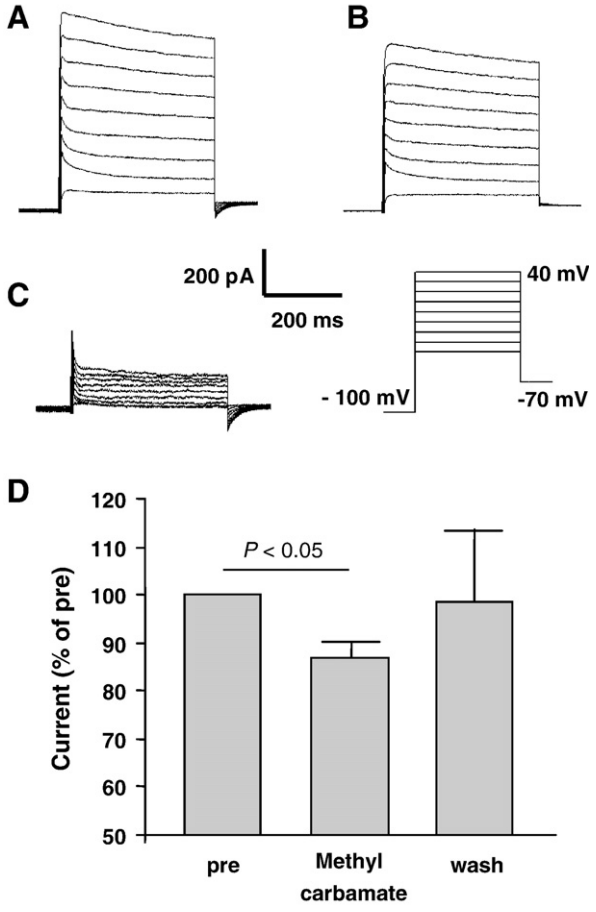

Fig. 3. N-(4-pyridyl) methyl carbamate inhibits potassium currents from guinea pig DRG cells. Holding potential is −70 mv. The step stimuli were the same as described in Fig. 1. A) Total potassium current (I_{total}) before the application of 5 mM N-(4-pyridyl) methyl carbamate; B) Potassium current recorded after the application of 5 mM N-(4-pyridyl) methyl carbamate; C) fast activating 4-AP sensitive potassium current inhibited by methyl carbamate (subtract B from A); D) Quantification showed the inhibition of potassium current by N-(4-pyridyl) methyl carbamate. Total potassium current amplitude at +30 mv command potential was significantly decreased to 86.7±8.6% ($n=6$) after the application of 5 mM methyl carbamete (paired *t* test, $P<0.05$).

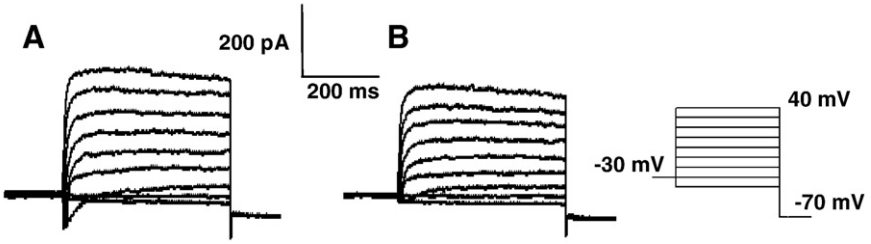

Fig. 4. Representative sample of delay rectifier potassium current (I_{dr}) before (A) and after (B) the application of 5 mM N-(4-pyridyl) methyl carbamate. Holding potential, stimulus pulse steps and pre-stimulus pulse were all the same as previously described in Fig. 2. There is no significant difference between I_{dr} amplitudes before and after application of 5 mM methyl carbamate. (Paired *t* test, $n=6$, $P>0.05$).

carbamate inhibits potassium currents at almost all testing pulse potentials (from −40 mV to 40 mV). ($P<0.01$, two way ANOVA test).

4. Discussion

In the current study we showed MC is capable of significantly inhibiting the initial transient component of potassium current. Three lines of evidence in support of this initial transient K current to be I_A are 1) the ability to be fast activated upon depolarization, 2) fast inactivated by a depolarizing pre-pulse and 3) the reported existence of I_A in guinea pig DRG. Thus, we conclude that MC can significantly block I_A. Furthermore, the fact that 4-AP, a known blocker of I_A can also block the same current in the same DRG cells further supports the notion that MC blocks I_A.

Using isolated spinal cord white matter segment extracts from guinea pig and double sucrose gap extracellular recording techniques, we previously showed that MC could significantly restore action potential conductance following mechanical injury [19–21]. However, due to limitations of the *ex vivo* preparation and extracellular recording in the previous study, it was not possible to determine whether MC was capable of inhibiting I_A. Based on the structure of MC, which resembles 4-AP, we speculated that such a possibility is likely (Fig. 6). The current study using a patch clamp confirmed our speculation and demonstrates that MC is indeed capable of inhibiting I_A which likely plays a critical role in MC's ability to enhance conduction in mechanically injured spinal cord axons.

In addition to blocking I_A, we also noticed there is a sustained potassium current also sensitive to MC and 4-AP (Figs. 1c and 3c). Voltage protocols designed to isolate I_{dr} can largely eliminate this current (Figs. 2 and 4) and indicated it is inactivated following a depolarizing pre-pulse. Based on these features, we speculate this current is likely a third component of the potassium current (in addition to I_A and I_{dr}) that exists in DRG cells, which is fast activated and long lasting. This current likely resembles a similar type of potassium current in rat DRG described by Kocsis and his colleagues [30]. Based on our knowledge, this is the first time such a current has been noted in guinea pig DRG.

Although MC blocks this fast and long lasting potassium current in DRG cells, the importance of such blockade in MC-mediated enhancement of conduction in injured axons is not clear at this point. It remains to be determined if, and how much, this current is responsible for the conduction loss in injured spinal cord axons. In contrast to I_A and a possible third component of potassium current, we found that the classic delayed rectifier I_{dr} is not sensitive to MC at 5 mM in our whole cell patch clamp study (Fig. 2). Similarly, 4-AP at the same concentration (5 mM) also has no appreciable effect on I_{dr} (Fig. 4).

In the current study, both 4-AP and MC can block I_A. However, their efficacy is different at the concentration of 5 mM. MC only block about 33% of current that is sensitive to 4-AP. This ratio is comparable to the difference in maximal effect in enhancing CAP conduction seen in ex vivo between 4-AP and MC [10,19–21,31,32]. However, the current findings using whole cell patch clamp study are based on only one concentration. It is therefore unknown whether 4-AP is still more effective in blocking I_A current in other concentrations. A more rigorous study, comparing the dose response curve in blocking I_A between MC and 4-AP in various concentrations, is needed to give a more complete picture regarding the efficacy in blocking I_A by these two compounds. It is possible, although not confirmed yet, that the difference between inhibiting I_A by 4-AP or MC may be insignificant at lower concentrations.

Despite the long history of 4-AP, a fast potassium current I_A blocker, in restoring impulse conduction and promoting functional recovery in SCI and MS [4,33–36], its effective usage has been hampered by severe side effects when the concentration is above millimolar *in vivo*. As a first step to search for an alternative I_A blocker, we synthesized several

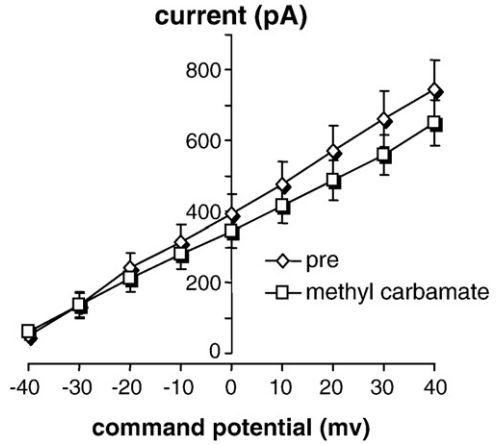

Fig. 5. I–V curve of potassium currents on guinea pig DRG cells before and after the application of 5 mM N-(4-pyridyl) methyl carbamate (◊, □ respectively). It is shown that potassium currents were significantly decreased after application of 5 mM N-(4-pyridyl) methyl carbamate at command potentials ranging from −40 mv to 40 mv with 10 mv steps.

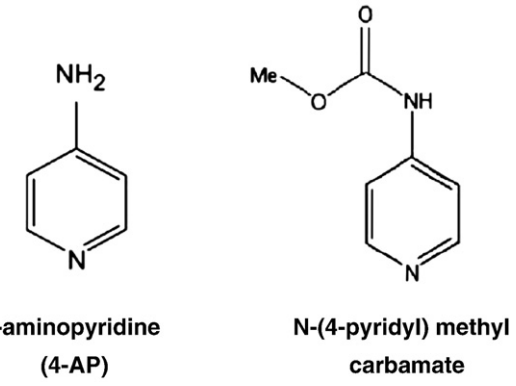

Fig. 6. Structure of 4-Aminopyridine (4-AP) and N-(4-pyridyl) methyl carbamate.

derivatives and demonstrated their ability to enhance CAP conduction in *ex vivo* preparations. Moreover, we report here that MC, is indeed a novel and effective I_A blocker. By inhibiting I_A, coupled with its ability to restore CAP conductance in injured spinal cord axons, MC emerged as a viable alternative to 4-AP to restore function in injured spinal cord.

Interestingly, we found the quality of conduction restored by MC (and two other 4-AP derivatives) is arguably better than those restored by 4-AP. For example, 4-AP is known to lengthen the absolute and relative refractory period and to degrade the ability of nerves to handle stimuli [10]. However, the conduction restored by all three novel compounds including MC, is indistinguishable from normal, healthy conduction [21]. In addition to the *ex vivo* models of SCI, MC restored conduction and/or behavioral function in *in vivo* models of SCI in guinea pigs [19]. Therefore, MC may be advantageous, compared to 4-AP, in restoring CAP conduction and hence a more appropriate choice in clinical interventions in SCI and perhaps MS as well.

We conclude that due to its ability to restore axonal conduction in injured spinal cord in guinea pig, MC represents an alternative to 4-AP in this line of research. More importantly, in our opinion, this finding will have a profound impact in inspiring major discoveries in this field of research-enhancing functional recovery by suppressing aberrant potassium current. More compounds with great efficacy and perhaps less side effects will likely emerge in the near future, which will greatly enhance the functional restoration and lessen the suffering of SCI and MS patients.

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