Dose responses of three 4-aminopyridine derivatives on axonal conduction in spinal cord trauma

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Abstract

To explore novel treatments for enhancing conduction through traumatically injured spinal cord we have synthesized structurally distinct pyridine based compounds; N-(4-pyridyl)methyl carbamate, N-(4-pyridyl)ethyl carbamate, and N-(4-pyridyl)t-butyl carbamate. With the use of a double sucrose gap-recording chamber we perform a dose–response assay to examine the effects of these compounds on axonal conduction following an in vitro stretch injury. The tested compounds significantly enhanced axonal conduction to the stretch injured cord at 1\muM, a dose that coincides with the clinically relevant dose of potassium channel blocker 4-aminopyridine (4-AP). Methyl carbamate enhanced conduction maximally at 100\muM. This is also the most effective concentration of 4-AP in vitro. The other compounds ethyl carbamate and t-butyl carbamate enhanced conduction maximally at lower concentrations of 10 and 1\muM. At higher concentrations each of these compounds continued to increased CAP amplitude, however not significantly. Additionally, two of the compounds ethyl and t-butyl carbamate appear to have negative effects on CAP amplitude when administered at or beyond 100\muM. These compounds demonstrate the possibility that derivatives of 4-AP can retain the ability to increase axonal conduction in the injured spinal cord.

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1. Introduction

Spinal cord trauma causes severe deficits in axonal conduction. Factors involved in the dysfunction of axons following trauma partial derive from myelin disruption and increased activity of 4-aminopyridine sensitive potassium channels (Fehlings and Nashmi, 1996). Since the contiguity of the spinal cord is maintained (Bunge et al., 1993; Kakulas, 1984) in the majority of spinal cord injuries the route to enhancing conduction following trauma is possible through pharmacological therapies. Two decades ago the potassium channel blocker 4-aminopyridine (4-AP) was shown to increase conduction significantly after demyelination in mammalian nerve tissue (Sherratt et al., 1980; Targ and Kocsis, 1985). Subsequent studies have continued to explore the effects of 4-AP in vitro (Blight, 1989; Bowe et al., 1987; Jensen and Shi, 2003; Kocsis, 1985; Shi et al., 1997) and in vivo (Blight and Gruner, 1987; Blight et al., 1991). More recently 4-AP has moved into clinical trials which explore its role in conduction enhancement after spinal cord trauma (Halter et al., 2000; Hasebout et al., 1993; Hayes et al., 1994, 2003, 2004; Potter et al., 1998) as well as demyelinating diseases such as multiple sclerosis (Bever et al., 1994; Davis et al., 1991).
et al., 1990, Rossini et al., 2001; Steltski et al., 1995). Based on these and related studies it is clear that 4-AP is an effective treatment for enhancing conduction in the injured spinal cord.

While the exact function of the potassium channels targeted by 4-AP is not well characterized it is suggested that their activity is most notable when the layers of myelin surrounding them is disrupted (Chiu and Ritchie, 1980; Sherratt et al., 1980), which is a common occurrence in compressive and contusive spinal cord trauma. Immunoprecipitation and colocalization experiments suggest that the 4-AP sensitive channels are heteromultimers formed by a combination of membrane spanning a subunits Kv1.1, Kv1.2, and cytoplasmic β subunit Kvβ2 which are localized to the juxtaparanodal region (Rasband et al., 1998; Rhodes et al., 1997; Wang et al., 1993) and possibly paranodal regions (Rasband and Trimner, 2000) of myelinated axons. When exposed these channels allow efflux of potassium ions, effectively hyperpolarizing the membrane and inhibiting further responses to incoming stimuli.

Though 4-AP appears to be a successful tool in enhancing axonal conduction its clinical applicability remains modest and somewhat limited. To continue exploring treatment of spinal cord conduction deficits with potassium channel blockers, we have synthesized several compounds similar in structure to 4-AP in order to maintain the ability to enhance axonal conduction while decreasing the negative side effects. Previously we explored the effects of these compounds on conduction after an in vitro stretch induced injury. The compounds, including: methyl, ethyl, and t-buty carbamate exhibited the ability to significantly increase axonal conduction following this acute injury at a concentration of 1 mM (unpublished observations). In addition, further electrophysiological analysis revealed that unlike 4-AP these compounds have no significant effect on axonal responsiveness to dual and multiple stimuli, which may indicate less severe, albeit, decreased side effects associated with these compounds. In the present study we continue to explore these compounds in a dose-response assay to obtain a profile of their effects in axonal conduction in an in vitro injury model.

2. Materials and methods

All animals used in this study were handled in strict accordance with the National Institute of Health’s guide for the Care and Use of Laboratory Animals and the Purdue Animal Care and Use Committee approved the experimental protocol. In these experiments, every effort was made to reduce the number and suffering of the animals used.

2.1. Isolation of spinal cord

Isolation of the spinal cord was similar to that described previously (Shi and Blight, 1996). Adult female guinea pigs (obtained from Hilltop Laboratory Animals Scottdale, PA) weighing 350-500 g were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (52 mg/kg). Once anesthetized the animal was transcardially perfused with cold, oxygenated Krebs’ solution to remove the blood and lower cord temperature. The entire vertebral column was then excised for extraction of the spinal cord. To obtain ventral white matter strips the spinal cord was subdivided twice longitudinally and subsequently incubated in fresh Krebs’ solution. The composition of the Krebs’ solution was as follows (in mM): 124 NaCl, 2 KCl, 1.2 KH2PO4, 1.3 MgSO4, 1.2 CaCl2, 10 dextrose, 5.6 sodium ascorbate, and 26 NaHCO3, equilibrated with 95% O2 5% CO2 to produce a pH of 7.2–7.4. The term “ventral white matter strips” will be used interchangeably below with “cords” or “spinal cords” for ease of description.

2.2. Recording chamber

As displayed in Fig. 1, a strip of spinal cord white matter 45–50 mm in length and 2 mm in diameter was placed across the chamber with the central compartment (volume: 3.8 mL) receiving a continuous perfusion of oxygenated Krebs’ solution (2 mL/min) maintained at 37 °C via an in line heater and temperature probe (Warner Instruments). 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: 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.

![Diagram of spinal cord ventral white matter mounted in the double sucrose gap recording chamber.](image-url)

Fig. 1 – Drawing of spinal cord ventral white matter mounted in the double sucrose gap recording chamber. Five wells compose the chamber; the outer two filled with isotonic potassium chloride, the inner well with Krebs solution, and two intermediate chambers with sucrose. The four electrodes present in the chamber administer the stimulus to the cord at one end and record axonal activity in response to the stimulus at the opposite end. A side view of the injury device which consists of a nylon mesh stabilizer and a small plexiglass rod is pictured below the chamber. The nylon mesh maintains the position of the cord as the injury rod is lowered from above.
Fig. 2 – Molecular structure of 4-aminopyridine in aqueous solution (A) and the tested derivatives t-butyl carbamate (B), ethyl carbamate (C), and methyl carbamate (D). Each of the derivatives are similar in structure to 4-AP however, the side groups are modified.

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 conduction distance is in fact the whole length of the chamber. The cord was stimulated by a 1 ms constant current unipolar pulse with compound action potentials (CAP) recorded at opposite ends of the strip. Recordings were made using a bridge amplifier (Neurodata Instruments) and subsequent analysis was performed using custom Labview® software (National InstrumentsTM) on a Dell PCTM.

2.3. Compound action potential amplitude

Recordings of axonal activity were made by analysis of compound action potentials (CAP), which are formed by the spatio-temporal summation of many single unit action potentials, fired by individual axons. Therefore, the CAP amplitude is a measurement of the CAP peak response not of area under the curve. 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 digitized profile of each responding CAP was recorded continuously and stored in the computer for future analysis. To assess axonal activity during each experimental condition average action potential activity was recorded at designated time points throughout the experiment. In addition, a real time plot of CAP amplitude was displayed throughout the experiment.

2.4. Stretch injury

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 publications (Jensen and Shi, 2003; 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 (unpublished observation). A plexiglass 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, producing a strain of 50% on the isolated tissue.

2.5. Derivative synthesis and application

In order to represent the respective 4-AP classes, easily prepared amides, carbamates, and ureas were derived to determine if any of these compounds were biologically active in spinal cord injury. All prospective analogues were designed to account for 4-APs inherent acid–base equilibrium so that any potential disruption of biological activity could be avoided. 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). For a detailed description outlining the synthesis of these derivatives please refer to our earlier publication (Smith et al., 2005). The analogs were added to the oxygenated Krebs’ solution, which perfused the central well of the chamber, which is also the site of injury. Fresh solution was prepared shortly before application to the injured spinal cord.

2.6. Statistical analysis

Throughout the paper, Student’s t-test was used to compare electrophysiological data. Statistical significance was attributed to values $p < .05$. Averages were expressed as mean ± standard error.

3. Results

The ventral white matter strips were monitored in the double sucrose gap-recording chamber (Fig. 1) for approximately 30 min or until the CAP amplitude had maintained a steady response for at least 15 min before testing began. After
Fig. 3 – Response of injured spinal cord white matter to different concentrations of 4-AP derivative, methyl carbamate. CAP amplitude was increased significantly with 1 μM. CAP amplitude continued to increase linearly as the concentration was increased until reaching 100 μM, which appeared to be the most efficient concentration for enhancing conduction. Doses exceeding 100 μM did not significantly increase CAP amplitude as indicated by 1 mM.

Fig. 4 – Response of injured spinal cord white matter to various concentrations of 4-AP derivative, ethyl carbamate. Ethyl carbamate significantly increased CAP amplitude starting at 1 μM and reached its most effective concentration at 10 μM. Concentrations above 10 μM continued to increased CAP amplitude until reaching 1 mM which appeared to be detrimental to axonal conduction.

Fig. 5 – Response of injured spinal cord white matter to different concentrations of 4-AP derivative, t-butyl carbamate. This compound significantly increased CAP amplitude at 1 μM which is also the most effective concentration for increasing conduction through the injured spinal cord. As the dosage was increased above 1 μM CAP amplitude decreased progressively, possibly reaching toxic levels at 100 μM.

3.1. CAP response to analogs

Initial application of methyl carbamate at a concentration of 1 μM had no significant effect on CAP amplitude (Fig. 3). The lowest effective dose tested for the purpose of increasing CAP amplitude was 1 μM (6.78 ± 1.02%, p < 0.01, n = 6). The CAP continued to increase in a linear fashion as the concentration of methyl carbamate was increased, with the most efficient dosage reached at 100 μM (17.73 ± 2.87%, p < 0.01, n = 8). While the higher concentration (1 mM) of methyl carbamate increased CAP amplitude it was not statistically significant (1.89 ± 1.74%, p > 0.05, n = 5).

Ethyl carbamate significantly increased CAP amplitude (Fig. 4) at a concentration of 1 μM (4.47 ± 0.2%, p < 0.05, n = 5). However, ethyl carbamates most efficient dosage was reached at 10 μM (10.49 ± 0.03%, p < 0.01, n = 8). Concentrations above 10 μM continued to positively effect the CAP amplitude until 1 mM where the CAP was decreased significantly (−24.09 ± 0.03%, p < 0.01, n = 5).

t-Butyl carbamate significantly increased CAP amplitude (Fig. 5) at 1 μM which was also the most efficient dosage for this compound (14.17 ± 2.32%, p < 0.01, n = 6). Concentrations beyond 1 μM decreased the ability of this compound to positively effect CAP amplitude with toxic levels reached at 100 μM. As displayed a concentration of 100 μM significantly decreased CAP amplitude (−93.35 ± 0.03%, p < 0.01, n = 6).

4. Discussion

The purpose of the present study was to examine compounds modeled after potassium channel blocker 4-AP for the intention of enhancing axonal conduction following spinal cord injury. The results showed that methyl carbamate, ethyl carbamate, and t-butyl carbamate were effective in increasing CAP amplitude, with methyl carbamate being the most efficient at 100 μM. However, concentrations above 100 μM appeared to be detrimental to axonal conduction. The study also suggested that these compounds could be potential candidates for the treatment of spinal cord injury.
throughout the duration of the experiment, which made it system, capable of analyzing the change in axonal response.

The injury model in this study employs a whole tissue in vitro interactions; however other explanations such as p increase CAP amplitude may be explained by drug channel (Smith et al., 2005). While the source of these compounds varied effects still needs to be determined, the increase in CAP amplitude suggests that these compounds employ a similar mechanism as 4-AP. In addition the lowest achievable dosage noted in this study, 1 μM, is also the lowest achievable clinical dose of 4-AP. This may indicate that these compounds will also have clinical applicability at this lower dose however; the threshold for possible toxic effects induced by these compounds needs further study.

4.3. Model system and injury

The injury model in this study employs a whole tissue in vitro system, capable of analyzing the change in axonal response throughout the duration of the experiment, which made it ideal for characterizing the detailed effects these compounds have on CAP amplitude. While single cell analysis is beneficial for characterizing individual axonal pathology and function following injury, it is necessary in pharmacological study to utilize a system that can mimic the in vivo preparation to obtain the most relevant measure of how the test compounds will behave in the living organism. Furthermore, the increased environmental control, offered with this in vitro preparation is ideal for tracking changes in CAP amplitude through the duration of testing not just at the experimental end points.

We studied these compounds in a stretch injury based on previous research indicating that this particular type of injury may exhibit increased benefits when treated with potassium channel blockers (Jensen and Shi, 2003). One possible reason for the increased ability of this type of injury to respond to this type of therapy may be due to the extent of injury caused by this method. For instance compression injury generally involves a limited area where the tissue is in contact with the object initiating injury. However, in stretch, the force of the injury causes a differential movement between the axon and the myelin intrinsically affecting a larger more distributed area of tissue compared to compression. With the larger area of tissue exposed to stretch trauma the greater the probability of exposing the 4-AP sensitive potassium channels contained beneath the myelin, which ultimately leads to conduction block. Though this particular injury may be more responsive to this type of treatment it is equally important to remember that spinal cord injuries are multifaceted and often consist of a combination of compression and stretch trauma which both contribute to axonal dysfunction. In this regard this type of treatment is applicable to all spinal injured patients as part of treatment schedule for a fundamentally complex injury.

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References


