Effects of 4-aminopyridine on demyelinated axons, synapses and muscle tension

Kenneth J. Smith, Paul A. Felts and Gareth R. John

**Summary**
Several clinical trials have demonstrated that 4-aminopyridine (4-AP), a potassium channel-blocking agent, improves symptoms in some patients with multiple sclerosis. The beneficial effects have typically been attributed to the restoration of conduction to demyelinated axons, since this effect was previously demonstrated experimentally. However, the clinical dose is ~250–1000 times lower than that used experimentally, potentially making extrapolation of the experimental findings unreliable. To examine the action(s) of 4-AP in demyelinating disorders, the drug was administered at clinical doses, both in vivo and in vitro, to rat dorsal column axons which had been experimentally demyelinated by the intraspinal injection of ethidium bromide. 4-AP had no consistent effect in restoring conduction to demyelinated axons, even to axons which were held just on the verge of conducting by adjusting the lesion temperature. However, 4-AP had prominent effects that did not involve demyelinated axons, including the potentiation of synaptic transmission and an increase in skeletal muscle twitch tension. We propose that these latter effects may be largely responsible for the beneficial action of 4-AP in multiple sclerosis patients. If so, the dominant effects of 4-AP in multiple sclerosis patients are independent of demyelination, and it follows that 4-AP may be beneficial in other neurological disorders in which function is diminished.

**Keywords**: potassium channels; multiple sclerosis; demyelinating disease; symptomatic therapy; spinal cord injury

**Abbreviations**: 4-AP = 4-aminopyridine; CAP = compound action potential; DRR = dorsal root reflex; EBr = ethidium bromide; RPT = refractory period of transmission

**Introduction**
In 1978, Bostock and colleagues demonstrated that conduction could be restored to some demyelinated peripheral axons by prolonging the action potential through the use of scorpion venom (Bostock et al., 1978). This group later showed that a similar effect could be achieved by applying the potassium channel-blocking agent 4-aminopyridine (4-AP) directly to experimentally demyelinated axons in exposed rat dorsal roots (Sherratt et al., 1980; Bostock et al., 1981), and this finding was later extended to the rat sciatic nerve (Targ and Kocsis, 1985, 1986). 4-AP was already in clinical use for the therapy of Lambert-Eaton syndrome (Lundh et al., 1977), myasthenia gravis (Lundh et al., 1979) and botulinum poisoning (Ball et al., 1979), and the experimental demonstration that 4-AP could restore conduction to demyelinated axons spawned a number of clinical trials in patients with multiple sclerosis (Jones et al., 1983; Stefoski et al., 1987; van Diemen et al., 1992; Bever et al., 1994) or spinal cord injury (Hansebout et al., 1993; Hayes et al., 1993). After discouraging findings in an initial small trial examining multiple sclerosis patients with pronounced and long-standing symptoms (Sears and Bostock, 1981), clearly positive results were obtained in patients with milder disease of shorter duration (Jones et al., 1983; Stefoski et al., 1987). A number of subsequent trials have confirmed that administration of 4-AP, or the related compound 3,4-diaminopyridine, frequently leads to rapid improvements in a range of symptoms in multiple sclerosis patients (Davis et al., 1990; van Diemen et al., 1992; Bever et al., 1994; Polman et al., 1994a, b), and that these improvements can last for several hours (Davis et al., 1990) following each administration of the drug. Symptomatic improvements have also been reported following 4-AP treatment of patients with spinal cord injury (Hansebout et al., 1993; Hayes et al., 1993, 1994). In view of the experimental data, the improvements following aminopyridine treatment in both multiple sclerosis and spinal cord injury have often been attributed to the
restoration of conduction to demyelinated axons (e.g. Jones et al., 1983; Blight and Gruner, 1987; Stefoski et al., 1987; Davis et al., 1990; van Diemen et al., 1993; Bever, 1994).

However, there were important differences between the early experimental data and the protocols adopted in the clinical trials. First, the clinical trials examined 4-AP in multiple sclerosis, a central demyelinating disease, whereas the early experimental data were obtained in peripheral demyelinated axons. Perhaps more importantly, there were marked differences in the concentrations of 4-AP employed. In the clinical trials, the prominent proconvulsant activity of 4-AP limited the recommended maximum serum concentration to only ~1 µM (Bever et al., 1994), which is ~1000-5000 times lower than the concentrations of the drug that Sherratt and colleagues found effective when applied directly to exposed spinal roots (Sherratt et al., 1980). In view of these differences, we have examined whether clinical doses of 4-AP are effective in restoring conduction to central demyelinated axons. Perhaps more importantly, there were a number of other known effects of 4-AP by which the drug may act, including the potentiation of synaptic transmission, and also the increase of muscle tension. A brief description of some of the data has been presented previously (Felts and Smith, 1994).

**Methods**

**Induction of the demyelinating lesion**

Anaesthesia was induced and maintained in 21 adult rats (Sprague–Dawley and Wistar, male, mean weight 417 g), either with halothane (1.5–2%, the balance being O₂) or with pentobarbital (65 mg/kg intraperitoneally following premedication with 40 µg intraperitoneal atropine), and a quarter (non-irradiated lesions) or complete (irradiated lesions) laminectomy was performed at the T12 vertebral level using sterile techniques. The vertebral column was held rigidly in a clamp and ethidium bromide (EBr; 1 µl of a 0.5 mg/ml solution in saline) was injected into the left dorsal column via a drawn glass micropipette inserted through a small hole in the dura. In some animals the lesion site was immediately irradiated using a 90Sr/90Yt source (40 Gy of primarily beta irradiation through a 3 × 5 mm opening in a lead-coated shield) (Felts and Smith, 1996) to delay remyelination. The wound was closed in layers. Animals were examined daily for postoperative complications; however, none were observed. The experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

**Electrophysiological examinations**

(A) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: preparations examined in vivo in terminal experiments

Five animals with demyelinating lesions (16–35 days post-EBr injection) were anaesthetized with halothane and prepared for terminal electrophysiological examination using methods previously described in detail (Felts and Smith, 1992). The animals were intubated and mechanically ventilated to maintain end-tidal CO₂ at 4–5%, and the tail vein was cannulated for the administration of pharmacological agents. A laminectomy was performed from vertebral level T8 to level L6, the animals were placed in a frame, and a mineral oil recording pool was formed. Unless otherwise stated, the pool was maintained at 35°C using radiant heat. The dura was opened longitudinally and a pair of platinum wire stimulating electrodes attached to an isolated stimulator (DS2, Digitimer Ltd, Welwyn Garden City, UK) was placed individually over pairs of platinum wire hook electrodes attached to the headstages of differential a.c. amplifiers (Neurolog System, Digitimer Ltd). The roots were crushed between the recording electrodes to render monophasic the antidromically conducted compound action potentials (CAPs). Recordings were filtered to a bandwidth of 8 Hz to 10 kHz. Except where noted, gallamine triethiodide (Flaxedil; Davis and Geck, St Louis, Mo., USA; 10 mg, repeated as necessary to eliminate stimulus-induced muscle contraction) was administered intravenously. The experimental arrangement is illustrated in Fig. 2E.

Two electrophysiological methods were used to detect the presence of demyelinated axons, namely prolongation of the refractory period of transmission (RPT) (McDonald and either with halothane (1.5–2%, the balance being O₂) or with pentobarbital (65 mg/kg intraperitoneally following premedication with 40 µg intraperitoneal atropine), and a quarter (non-irradiated lesions) or complete (irradiated lesions) laminectomy was performed at the T12 vertebral level using sterile techniques. The vertebral column was held rigidly in a clamp and ethidium bromide (EBr; 1 µl of a 0.5 mg/ml solution in saline) was injected into the left dorsal column via a drawn glass micropipette inserted through a small hole in the dura. In some animals the lesion site was immediately irradiated using a 90Sr/90Yt source (40 Gy of primarily beta irradiation through a 3 × 5 mm opening in a lead-coated shield) (Felts and Smith, 1996) to delay remyelination. The wound was closed in layers. Animals were examined daily for postoperative complications; however, none were observed. The experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

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muscular contraction, evoked by electrical stimulation of the spinal cord, indicated that the previously injected 4-AP had indeed been delivered successfully to the vasculature.

(B) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: chronically implanted preparations

Two animals with demyelinating lesions also had chronically implanted stimulating and recording electrodes (for methods, see Felts and Smith, 1992), allowing the same population of demyelinated axons to be examined serially over several weeks in relatively intact preparations. During recording sessions the animals were anaesthetized with halothane and the ends of subcutaneous leads attached to the chronically implanted electrodes were exposed through small skin incisions. The experimental arrangement is illustrated in Fig. 3C. Animals were intubated and mechanically ventilated to maintain an end-tidal CO₂ concentration of 4–5%. A catheter was then introduced into a lateral tail-vein and gallamine (10 mg, repeated as necessary) was infused. Core temperature was monitored rectally and maintained at 35°C using a heating blanket, except when body temperature was allowed to fall in order to determine the effect of cooling on conduction. Recordings were fundamentally similar to those in the acute preparations, with CAPs and RPTs recorded before, during and after intravenous 4-AP administration.

(C) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: in vitro preparations

Preparations of demyelinated axons were examined in vitro in a brain slice recording chamber to investigate whether higher concentrations of 4-AP (i.e. concentrations above those achievable in vivo) were capable of restoring conduction to central demyelinated axons. Animals (n = 10) were reanaesthetized with halothane 28–35 days after induction of a demyelinating lesion, and the spinal cord was exposed from T8 to L2. Two additional normal animals were also examined. Following exsanguination under anaesthesia, a 3.5 cm length of dorsal column containing the lesion was promptly removed, placed in a brain slice recording chamber (Medical Systems Corporation, Greenvale, New York, USA) modified as shown in Fig. 4, and superfused with artificial CSF (containing, in mM: NaCl, 123; KCl, 3; CaCl₂, 1.5; NaH₂PO₄, 1.2; MgCl₂, 1; HEPES, 10; glucose, 10; pH 7.4, bubbled with O₂). The central and outside lanes were effectively isolated from each other so that the temperature and composition of the fluid in the central lane (which contained the lesion) could be altered while leaving conditions at the stimulating and recording sites relatively constant. CAPs conducted through the lesion were recorded in response to supramaximal stimulation, and the distribution of the RPTs of the fibres and the effects of lesion cooling were determined. By comparing the compound potentials taken at different lesion temperatures, it was possible to choose a temperature at which conduction in many demyelinated axons was just blocked due to warming. The lesion was then held at this temperature and the effects on conduction of different concentrations of 4-AP, applied selectively to the central lane, were examined.

(D) Electrophysiological examination of the effects of 4-AP on skeletal muscle twitch tension

Naïve animals (n = 12) were used in all these experiments. General anaesthesia was induced and maintained with halothane (0.8–1.5% in oxygen). Rectal and subcutaneous (proximal region of the left hindlimb) temperatures were monitored and maintained between 34.5° and 35.5°C throughout each experiment. The left tarsus and metatarsus were immobilized against a rigid metal bar using cyanoacrylate adhesive, and the fourth digit of the left hindlimb was attached to an isometric force transducer (Harvard Apparatus, Holliston, Mass., USA). The experimental arrangement is illustrated in Fig. 5B. Recordings of twitch tension were then made at intervals of 3 or 5 min in response to supramaximal stimulation of the sciatic nerve. The stimulating electrodes were either needles inserted percutaneously or platinum wires placed directly on the exposed sciatic nerve distal to the sciatic notch. This latter arrangement was adopted in experiments in which the sciatic nerve was cut at the start of the experiment in order to avoid any contamination of the data with centrally mediated reflex activity. After a control recording period (typically 30 min), aliquots of 4-AP were injected intravenously while serial recordings were made. All records obtained in these experiments were averaged responses to eight stimuli. In experiments in which the sciatic nerve was not severed proximally at the beginning of recording, it was severed proximally at the end of the experiment to discern any contribution to the contraction from central reflex activity.

(E) Electrophysiological examination of the effects of 4-AP on synaptic transmission

(i) Study of the H reflex. Anaesthesia, temperature maintenance and hindlimb immobilization were performed in five rats, as described in section D above. An ‘active’ recording electrode was inserted into the fourth dorsal interosseous muscle of the left hind foot, and a ‘passive’ recording electrode was placed subcutaneously in the same hind foot. These recording electrodes were attached to the headstage of a differential AC amplifier. The experimental arrangement is illustrated in Fig. 6B. Recordings of CMAPs were made in response to submaximal stimulation of the sciatic nerve via percutaneous needle electrodes at the sciatic
notch at 0.1 Hz. Records were taken in groups of five every 10 min. After a control recording period (typically 30 min), aliquots of 4-AP were injected intravenously while serial recordings were made.

(ii) Study of the dorsal root reflex. Naive animals \( n = 15 \) were anaesthetized and prepared for terminal electrophysiological examination as described in section A above. Stimulating electrodes were placed on the surface of the dorsal columns in a position analogous to that described in section A above. Recordings were made of the CAP obtained at the dorsal roots in response to 1 Hz supramaximal stimulation both before and at various times after the intravenous administration of aliquots of 4-AP. In some experiments \( n = 5 \) the directly conducted component of the CAP was separated from the ‘dorsal root reflex’ (DRR) (i.e. the antidromic reflex activity elicited in dorsal roots via dorsal horn synapses in response to a synchronized volley in primary afferent axons) by the addition of a conditioning stimulus preceding the test stimulus by 20 ms. The DRR is rendered refractory for \( > 20 \) ms by the conditioning stimulus, thus eliminating the DRR from the CAP produced in response to the second, test stimulus. All records obtained were averaged responses to eight stimuli, and were digitized and stored for display and analysis.

(F) Comparison of the effects of 4-AP on conduction through a central demyelinated lesion and on central synaptic transmission

In some animals \( n = 4 \) with demyelinating lesions of the dorsal columns (28–35 days post-EBr injection), both the directly conducted CAPs (recorded as in section A above) and the DRR (recorded as described in section E, part ii) were examined before and after the intravenous administration of 4-AP. This allowed the effects of 4-AP on conduction and on synaptic activity to be compared in the same preparation. As described in section E, recordings made using a single supramaximal stimulus included both the directly conducted and synaptic components. However, in alternate records the DRR was eliminated by rendering it refractory through the use of a conditioning stimulus which preceded the test stimulus by 20 ms.

Morphological investigation

After in vivo electrophysiological investigations, animals with demyelinating lesions were perfused via the left ventricle with glutaraldehyde (4% in 0.15 M cacodylate or phosphate buffer, pH 7.4) under deep anaesthesia. The spinal cord containing the lesion was removed and processed into resin, and sections were stained and examined in the light and electron microscope as previously described (Felts and Smith, 1992). Tissues used for in vitro electrophysiological examination were fixed by immersion in glutaraldehyde (4% in 0.1 M cacodylate buffer, pH 7.4) and processed and examined as above.

Results

Morphology of the demyelinating lesions

Sixteen to 35 days after injection, the EBr lesions were well circumscribed, lenticular in shape and measured 3–5 mm in rostrocaudal extent. The lesions were typically unilateral in distribution (Fig. 1) and, at their widest extent, they occupied ~50–80% of the transverse area of the ipsilateral dorsal column. The lesions contained large numbers of demyelinated axons, and these were exposed directly to the extracellular fluid, embedded in vesicular myelin debris, or totally or partly ensheathed by Schwann cells, oligodendrocytes or astrocytes. Some axons showed signs of early remyelination by Schwann cells or, less frequently, by oligodendrocytes. More detailed descriptions of the morphology of the EBr lesion have been published previously (Blakemore, 1982; Felts and Smith, 1992, 1996).

Electrophysiological examinations

Control recordings made from naive animals established the expected similarity of records obtained from opposite dorsal roots in response to stimulation of the dorsal column, even with respect to the distribution of RPTs, a sensitive measure of conduction deficits.

(A) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: acute preparations in vivo

The time course of the electrophysiological changes occurring in the EBr lesion in the rat dorsal columns has been reported previously (Felts and Smith, 1992). In agreement with the earlier findings, in all animals with demyelinating lesions the CAPs recorded from the lesioned side were much reduced in amplitude (Fig. 2A), had a prolonged latency and duration, and revealed that many of the axons had prolonged RPTs when compared with the contralateral, unlesioned side (Fig. 2B). Also, cooling of the part of the dorsal columns containing the lesion had little effect on the form of the recordings made from roots on the non-lesioned side, but the cooling resulted in a substantial increase in the area of the monophasic CAP recorded from dorsal roots ipsilateral to the lesion, particularly in the components conducting with longer latencies (Fig. 2C). Such increases in conduction through demyelinating lesions have been demonstrated previously in the peripheral nervous system, and have been attributed to the restoration of conduction in demyelinated axons (Davis and Jacobson, 1971; Rasminsky, 1973; Bostock et al., 1978).
Fig. 1 (A) Light micrograph of a transverse section through the dorsal portion of the spinal cord at vertebral level T12 orientated with the dorsal surface of the spinal cord at the top. The animal had received an injection of EBr into the left dorsal column 16 days prior to electrophysiological examination. The injected region received 40 Gy of irradiation just after the introduction of EBr. The dorsal columns are delineated by arrowheads, and there is a well-circumscribed lesion on the left side. The lesion, as demonstrated by the electron micrograph in B, contains large numbers of demyelinated axons (e.g. those labelled D). Staining: A, Richardson’s stain; B, uranyl and lead salts. Scale bar (shown in B) = 250 and 10 µm in A and B, respectively.

Although cooling of the lesion was effective in increasing the area of the monophasic CAP recorded through it, in the same preparations (at 37°C) there was never an obvious effect on the form or area of the CAP in response to the intravenous administration of 4-AP (1.4 mg, equivalent to 2.5 mg/kg) (Fig. 2D). This dose is well above that used as a bolus in clinical trials.

A potential complication of some of the experiments conducted in vivo was the use of neuromuscular blocking agents, when needed, to eliminate reflex movement of the anaesthetized preparation during the spinal stimulation. We adopted the use of gallamine triethiodide, an agent which, like 4-AP (and in common with some other neuromuscular blocking agents), has potassium channel-blocking activity (Smith and Schauf, 1981). This fact potentially raises concern when examining the role of 4-AP, but two lines of evidence indicate that gallamine neither directly improves conduction in demyelinated axons nor alters the response of demyelinated axons to the application of 4-AP. First, we have recorded CAPs from dorsal roots in response to spinal stimulation in many (n >> 100) normal and lesioned animals, both before and after the intravenous administration of gallamine (10 mg). In no preparation has an effect of gallamine on conduction been observed. Secondly, in vivo preparations examined either in the presence or absence of gallamine exhibited a similar lack of response following injection of 4-AP. Indeed, the animal used for illustration in Fig. 2 was not given gallamine until the very end of the experiment. We have also examined the effects of 4-AP on demyelinated axons in the absence of gallamine, in vitro (see section C below).

(B) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: chronically implanted preparations

From serial examination of the two chronically prepared animals, it was clear that the demyelinating lesion resulted initially in a marked reduction in amplitude of the CAP, which was then partly restored through the appearance of peaks with long latency, which then progressively shortened in latency until they merged with the early peak (if present). From our earlier studies (Smith et al., 1979; Felts and Smith, 1992) it was known that the appearance of the later peaks marked the onset of conduction in demyelinated, or very thinly remyelinated, axons, and it was during the weeks that these peaks were present that the examination of 4-AP was performed. It was also only during these weeks that temperature was found to have a marked effect on the amplitude of the CAP conducted through the lesion. Thus, recordings made either before the lesion was induced, within the few days immediately after its induction or in long-term preparations in which remyelination was complete, showed little effect of cooling on the form of the CAP. However, during the period of prominent demyelination (2–6 weeks after injection) a reduction of body temperature from 37°C to 33°C resulted in an increase in the CAP on the ipsilateral side to the lesion (Fig. 3A), just as occurred in the acute preparations: the newly conducting axons had the long latency and RPT (not shown) expected of demyelinated axons. Although these tests established the presence within the lesion of
Fig. 2 | Monophasic CAPs recorded from the right (non-lesioned side) and left (lesioned side) L4 dorsal roots in response to supramaximal stimulation (arrows indicate stimulus artefacts) of the dorsal columns rostral to a demyelinating lesion. Recordings were made in vivo (see section A of Methods) 16 days after the injection of EBr with subsequent irradiation (the lesion from which these recordings were made is illustrated in Fig. 1). (A) Antidromically conducted CAPs are shown at equal gains, demonstrating the substantial conduction block present on the lesioned side. (B) RPT distribution of the axons is illustrated by showing the response to the second of two stimuli with the given interstimulus intervals. The response to the first stimulus has been subtracted during the averaging process (Smith, 1980) and the gains have been adjusted to provide similar amplitudes in each family of records. Note the prolonged RPTs of many of the fibres on the lesioned side, as indicated by the continued increase in CAP size further back in the array. (C) Records showing the change in the form of the CAP in response to cooling of the lesion by the slow application of 1 ml of cooled saline to the surface of the lesion. The area of the CAP on the lesioned side, in contrast to the non-lesioned side, is substantially increased during the period of cooling. (D) Records showing CAPs obtained before and 10 and 35 min after the intravenous injection of 2.5 mg/kg of 4-AP. Note that 4-AP was not effective in producing an increase in the area of the CAP, even though demyelinated axons are present on the lesioned side (as indicated from the data in B), to which conduction can be restored by cooling (data in C). The recording arrangement is illustrated in E.

(C) Electrophysiological examination of the effects of 4-AP on conduction along central demyelinated axons: in vitro preparations
Study of the lesion in vitro offered the opportunity of quite precise temperature control, and under these conditions the effect of varying the temperature on the number of conducting axons was particularly clear (Fig. 4). Our recording apparatus was modified to permit the temperature of the middle segment of the dorsal column to be varied while maintaining a constant
temperature at the recording and stimulating sites, and under these conditions temperature had little effect on conduction in normal tissue (Fig. 4A, left panel). However, temperature changes had prominent effects on the form of the CAP conducted through demyelinating lesions in four of the preparations, and noticeable effects in two preparations (Fig. 4A, right panel). Such CAPs typically contained two components, an early peak, which conducted with a latency similar to the peak recorded from normal tissue (presumably reflecting conduction in axons spared by the lesion, largely those axons on the contralateral side), and a later peak. While the early peak consisted of axons conducting with normal RPT, the later peak was composed of axons with prolonged RPT, indicating that the axons were affected by the demyelinating lesion. The amplitude of the early peak varied little as the temperature of the lesion was altered, but the amplitude of the later peak varied inversely with temperature, such that it was almost eliminated at body temperature (37°C). Thus, at any temperature chosen within the range examined, there were a number of axons just on the verge of conducting. A temperature was chosen at which slight cooling produced a prominent increase in the amplitude of the second peak, and the temperature was then maintained at this 'blocking' level while the effects on conduction of exposure to artificial CSF containing different concentrations of 4-AP was determined.

The results of typical experiments are illustrated in Fig. 4B. The lesioned preparation is the same as that illustrated in Fig. 4A, and the lesion was cooled to 32°C so that there would be many nerve fibres just above their blocking temperature, and so just on the verge of conducting. Each group of data represents several superimposed, averaged records, where one of the records was obtained prior to the administration of 4-AP at a particular concentration and the other records were obtained at intervals of ~5 min thereafter (for details see legend to Fig. 4). Records from the dorsal column of a naïve animal reveal an absence of effect of 4-AP on normal central axons at concentrations of 25, 100 and 500 µM (Fig. 4B, left panel). All these concentrations are in the supraclinical range; subclinical and clinical doses were not examined in vitro since these doses had been found to have no effect on conduction in vivo. The middle and right panels of Fig. 4B reveal that 4-AP at 25 µM had no detectable effect on conduction in the axons contributing to either of the peaks. When the 4-AP concentration was increased to 500 µM (Fig. 4B, right panel), the amplitude of the early peak again remained unaffected but, in contrast,
(A) Plot presented in 3D perspective showing records of twitch tension arranged in the order in which they were obtained, with the earliest records at the front. The records show the twitch tension developed by the fourth hind digit of a naïve rat in response to supramaximal stimulation of the sciatic nerve at the sciatic notch; the sciatic nerve was cut proximally to ensure the absence of any spinal reflexes. The dose range of 4-AP employed in clinical trials is indicated. The tension curves are similar before the administration of 4-AP (0.26 mg/kg for each administration; arrows), but are increased at doses used in clinical trials. The experimental arrangement is illustrated in B.

(B) Electrophysiological examination of the effects of 4-AP on muscle twitch tension
Electrical stimulation of the sciatic nerve with single supramaximal stimuli caused a twitch contraction of the digital flexor muscles, and thereby of the digits. The curve of twitch tension developed by the middle toe was reproducible before the intravenous administration of 4-AP, but the peak tension was always increased in a dose-dependent relationship upon the injection of 4-AP, with a mean threshold dose of 0.51 mg/kg. In experiments in which the sciatic nerve was cut proximal to the site of stimulation at the start of the experiment (n = 4), the contribution to the records from the F wave or H reflex was abolished, but the twitch tension was still increased by 4-AP in a dose-related manner (from a threshold dose of 0.44 mg/kg in the illustrated preparation) (Fig. 5A). In experiments in which the sciatic nerve was severed at the end of the experiment, it was possible to discern two effects of the 4-AP. First, the drug increased the

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Although Fig. 4B shows typical results, in 30% of the experiments the supraclinical concentration of 25 µM 4-AP caused a small increase in the amplitude of the long-latency peak, and additional increments were typically observed upon the administration of higher concentrations of 4-AP (100, 500 or 5000 µM). In no instance, at any concentration of 4-AP, was the short-latency, ‘normal’ peak seen to increase, nor was the long-latency, ‘demyelinated’ peak seen to decrease. No significant effect of 25, 100 or 500 µM 4-AP was observed in normal control tissue.

Fig. 6 (A) Plot presented in 3D perspective, showing electromyographic records arranged in the order in which they were obtained, with the earliest records positioned at the front. The records show the CMAP obtained from the dorsal interosseus muscles of the fourth hind digit of a naïve rat in response to submaximal stimulation of the sciatic nerve at the sciatic notch. The timing of the stimulus, directly conducted response (M) and H reflex (i.e. the spinal monosynaptic reflex) is indicated. Five records were obtained at 0.1 Hz every 10 min before the administration of 4-AP, and 10 min after each administration of 4-AP. The 4-AP was administered (0.35 mg/kg) at the intervals indicated by the arrows, and the range of 4-AP doses employed in clinical trials is indicated. 4-AP had no detectable effect on the M response, but the magnitude of the H reflex was greatly increased, indicating that 4-AP potentiates synaptic transmission at doses used in clinical trials. The experimental arrangement is illustrated in B.
amplitude of the main contraction (due to the M response), but it also caused a magnification of a delayed contraction which was superimposed on the main contraction. The reflex origin of this delayed contraction was confirmed by observing it disappear upon severing the sciatic nerve proximally.

(E) Electrophysiological examination of the effects of 4-AP on synaptic transmission

(i) Study of the H reflex. CMAPs recorded from foot muscles in response to submaximal stimulation of the sciatic nerve at 0.1 Hz were composed of a small M response and, in some rats, a larger H reflex (Fig. 6). Administration of 4-AP had no effect on the directly conducted M response, but the amplitude of the H reflex was always greatly increased (or appeared and then increased) following 4-AP infusion (Fig. 6A). This effect was initiated at a dose of 0.35 mg/kg, and was dose-related.

(ii) Study of the dorsal root reflex. In naïve rats, supramaximal stimulation of the dorsal columns resulted in CAPs recorded from dorsal roots which consisted of a large short-latency peak and a much smaller longer-latency peak. The RPT of the large peak indicated that it represented directly conducted action potentials, while the prolonged refractoriness of the second peak indicated that it represented the DRR (this prolonged refractoriness was used to advantage to allow the elimination of the DRR from some recordings; see F below). Following 4-AP administration (from 0.43 mg/kg), the peak representing the DRR was nearly always (14 of 15 experiments) substantially exaggerated in amplitude, and this increase was dependent upon the dose of 4-AP (data not shown).

(F) Comparison of the effects of 4-AP on conduction through a central demyelinated lesion and on central synaptic transmission

In rats in which a demyelinating lesion had been induced in the dorsal column 28–35 days previously, it was possible to compare directly the effects of 4-AP on conduction along demyelinated axons with its effect on synaptic transmission, as measured by the DRR. Figure 7B shows the distribution of RPTs of axons conducting through a central demyelinating...
Discussion

Examination of the effects of 4-AP on conduction along central demyelinated axons revealed that 4-AP had no clear effects on the success of conduction through the lesion when administered at the low concentrations used clinically. Such concentrations were, however, effective in potentiating synaptic transmission and increasing muscle twitch tension, and these effects may be important in mediating the beneficial effects of 4-AP in demyelinating diseases such as multiple sclerosis. In common with their effects in peripheral demyelinated axons (Sherratt et al., 1980; Bostock et al., 1981), higher, supraclinical concentrations (500 μM) of 4-AP were effective in reversing conduction block in some central demyelinated axons.

Even though no clear effects of clinically relevant concentrations of 4-AP were observed on the success of conduction along central demyelinated axons, there were many demyelinated axons present within the lesion to which conduction could be restored by cooling. This temperature sensitivity of conduction in demyelinated axons is well documented experimentally (Rasminsky, 1973; Bostock et al., 1981; Smith, 1994), and in patients it underlies the expression of Uhthoff’s sign (Namerow, 1968; Jonas, 1989). The restoration of conduction by cooling establishes the presence within the lesion of axons which are just on the verge of conducting at normal temperature, and such axons should form an easy target for drugs such as 4-AP. The failure of 4-AP to restore conduction to such axons at clinical concentrations is therefore surprising in view of the clearly beneficial effects of the drug in patients with multiple sclerosis (Stefoski et al., 1987; Davis et al., 1990; van Diemen et al., 1992; Bever et al., 1994; Polman et al., 1994a). Several explanations for this apparent discrepancy can be considered.

First is the possibility of species differences. While these cannot be ruled out, there is evidence that rats and humans have similar sensitivity to 4-AP. The drug is a potent proconvulsive agent (Spyker et al., 1980; Murray and Newsom-Davis, 1981) and this property limits the maximum dose which can be safely administered systemically. In this study a dose of 1.9–2.0 mg/kg caused mild limb tremors in lightly anaesthetized animals, and these became pronounced at doses of 2.2–2.7 mg/kg (the maximum doses examined in vivo in this study). In the absence of anaesthesia it is reasonable to believe that the tremors may have manifested themselves as convulsions, and it is notable that a similar convulsant threshold has been reported in humans (Ball et al., 1979).

A second explanation for the apparent conflict is the possibility that the dose used in the rats was not equivalent to that employed in the clinical studies. Direct comparison of the available experimental and clinical data is hampered by the different ways of reporting the doses administered and by the different routes of administration. In the present experimental study the drug was administered intravenously across the dose range used clinically, and the maximum dose (2.7 mg/kg) clearly exceeded the maximum clinical dose. Clinical trials employing intravenous administration of 4-AP include those of Stefoski and colleagues, who administered doses of 1–5 mg every 10–60 min, to a total dose of 7–35 mg (Stefoski et al., 1987). Patient weights were not reported, but at a typical weight of 60 kg the highest intravenous doses were ~0.5 mg/kg. This maximum dose is similar to that used in the study of van Diemen and colleagues, who administered 1 mg of 4-AP every 20 min for 1 h, then up to 2.5 mg every 20 min until a maximum dose of 0.5 mg/kg had been administered or side-effects intervened (van Diemen et al., 1993). Studies employing oral administration include those by Jones and colleagues, who administered doses of between 10 and 60 mg of 4-AP (Jones et al., 1983), and Davis and colleagues, who administered a single oral dose of 10–25 mg (Davis et al., 1990). Patient weights were again not reported, but at a typical weight of 60 kg the highest oral doses were ~0.5–1 mg/kg, similar to the oral dose employed by van Diemen and colleagues (0.5 mg/kg) (van Diemen et al., 1992).

Thus, the concentrations used in the in vivo parts of the current study were typically slightly above those used clinically.

A third explanation is that the 4-AP failed to gain access to the experimentally demyelinated axons. Such a failure is most unlikely since, at the times examined, the EBv lesion lacks an effective vascular barrier (Felts and Smith, 1996). Furthermore, 4-AP has been shown to be permeable to the intact blood–brain barrier (Lemeignan et al., 1984). Another potential explanation is that the effects of 4-AP on conduction are delayed, and that the experiments were of too short a duration for the effects to be observed. 4-AP is...
known to affect K\(^+\) channels in neuroblastoma cells at a lower concentration when applied inside rather than outside the cell (Hirsh and Quandt, 1993), and Davis and colleagues have suggested that 4-AP requires time to accumulate inside the axon before it becomes effective in blocking the K\(^+\) channels, restoring conduction (Davis et al., 1995). However, Bostock and colleagues found that direct exposure of spinal roots to 4-AP for only 1 min was sufficient for responses to stabilize (Bostock et al., 1981), and patients show improvements in symptoms within only 25 min after intravenous delivery of 4-AP (Stefoski et al., 1987). Our recordings routinely lasted for a longer period following 4-AP injection (e.g. Fig. 2D); thus, this explanation seems unlikely to account for the lack of observed effect.

It is also possible that, in patients, the clinical deficit may arise not so much from conduction block but from the failure of the axons to conduct repetitively. We have not examined the effects of 4-AP on this ability, but it was examined in a previous study employing central axons demyelinated by anti-galactocerebroside antibodies (Kaji and Sumner, 1988). These findings (even at lethal (in unanaesthetized animals) doses, 4-AP had no effect on the rate-dependent conduction block.

Finally, it is possible that the axons in the experimental lesion were unlike those in multiple sclerosis lesions. However, although the mechanism of demyelination is different in the two cases, the affected axons have many similarities in their appearance. Furthermore, there are also no known differences in the electrophysiological properties of the human and experimentally demyelinated axons. It seems likely that the response of the experimentally demyelinated axons to 4-AP will be similar to that of axons demyelinated by multiple sclerosis.

If the experimental data are representative of the human lesion, then it appears that the dominant mechanisms by which 4-AP improves symptoms in patients may not include the restoration of conduction to blocked axons, as is generally believed (Jones et al., 1983; Stefoski et al., 1987; Davis et al., 1990; van Diemen et al., 1993; Bever, 1994). However, the present findings illustrate two effects of 4-AP which are manifest at clinical concentrations and which may be expected to lead to clinical improvement. The first arises from the well documented effect of 4-AP in potentiating synaptic transmission, an effect observed both within the central nervous system (Jankowska et al., 1977, 1982) and at the neuromuscular junction (Lundh, 1978; Molgo et al., 1980). These effects arise through several mechanisms, including an increase in neurotransmitter release (Jankowska et al., 1982) and an increase in the number of synaptic terminals activated by an action potential (Obaid et al., 1996; see also van Emst et al., 1996). The present findings confirm that the central effects are apparent at clinically relevant doses, as evidenced by the potentiation of both the H reflex and the DRR. In a pathway where conduction has been blocked in some, but not all, axons, the augmentation of the synaptic efficacy of the remaining active axons may tend to compensate for the conduction block. Furthermore, it is theoretically likely that at regions of inflammation, which are widespread in multiple sclerosis, there may be a reduction in synaptic function. Such a reduction may be expected due to raised levels of nitric oxide, and perhaps other inflammatory mediators, which interfere with synaptic function (Pineda et al., 1996; Yun et al., 1996); there is evidence for increased nitric oxide production in multiple sclerosis patients (Bo et al., 1994; Johnson et al., 1995; Giovannoni et al., 1997). If some symptoms are due to depressed synaptic activity, as we consider likely, then drugs such as 4-AP would be expected to improve these symptoms. For the above reasons, the effect of 4-AP on synaptic transmission may underlie a major part of the improvements seen in patients with multiple sclerosis.

The experiments conducted in vitro permitted the effects of supraclinical doses to be examined, including doses which would be lethal in vivo. These data demonstrated that 25 µM 4-AP typically had no noticeable effect on the success of conduction through a demyelinating lesion, although higher concentrations (e.g. 500 µM) were increasingly likely to restore conduction to axons affected by the lesion. Higher concentrations, in agreement with previous findings (Shi and Blight, 1997), also showed some evidence of toxicity since they became likely to impair conduction in axons conducting with short latency. Four micromolar 4-AP is the threshold for increasing the spontaneous activity recorded in the dorsal roots of isolated spinal cord (Al-Zamil et al., 1988), and spontaneous seizure-like discharges in brain slice preparations are produced by 3–10 µM 4-AP (Galvan et al., 1982). Similarly, transmitter release at both excitatory and inhibitory synapses on CA1 neurons in perfused hippocampal slices is enhanced by 10 µM 4-AP (Buckle and Haas, 1982). Thus the lowest concentration tested in vitro in this study (25 µM) is already above the threshold at which 4-AP is known to affect synaptic transmission. Supraconvolvulant doses of 4-AP have been reported to restore conduction to central axons demyelinated by anti-galactocerebroside antibodies (Kaji and Sumner, 1988).

The concentration of 4-AP encountered by axons in multiple sclerosis patients is unknown, and, as has been suggested by others (Davis et al., 1995; Shi and Blight, 1997), may be affected by the microenvironment in and around the axon. However, experimental observations have been made of 4-AP concentrations using whole brain, and CSF. Following an intravenous bolus of 1 mg/kg 4-AP, the peak concentration within homogenized rat brain was observed after 30 min and equalled ~3 µM (S. J. Roffey, R. Webster and S. G. Jezquel, unpublished observations presented at the meeting New Concepts of a Blood–Brain Barrier, London, July 1994). Although not directly comparable with brain concentration, broadly similar findings have been reported for 4-AP levels within the CSF. Using a dose higher than that normally encountered clinically, 3.5 mg/kg (approximately one-half of the LD\(_{50}\)), Lemeignan and colleagues found that the CSF concentration of 4-AP in
rats 5 min after a single intravenous injection was ~5 \mu M, falling to 2 \mu M over the next 10 min (Lemeignan et al., 1984). In dogs, Pratt and colleagues found that a dose more similar to that used clinically (0.5 mg/kg) produced a maximum CSF 4-AP concentration of ~0.6 \mu M 6 min after intravenous injection (Pratt et al., 1995). The CSF concentration then fell over the next 7 h to ~0.05 \mu M. In these two studies (Lemeignan et al., 1984; Pratt et al., 1995), serum concentration was found typically to be 5–10 times higher than the concentration within the CSF. Serum levels in multiple sclerosis patients have been examined, and sustained levels of ~100 ng/ml appear to be the maximum that can be tolerated safely (Bever et al., 1994), a concentration equal to 1.06 \mu M. From these data, a bolus intravenous injection. These 4-AP concentrations are noticeably lower than those demonstrated in this study to be effective in restoring conduction to demyelinated central axons.

However, one study has demonstrated electrophysiological effects of 4-AP on spinal white matter at concentrations within the 1–3 \mu M clinical range. Shi and Blight examined changes in conduction in vitro through crush lesions in the spinal cords of guinea-pigs. They found that the amplitude of the CAP was increased by 4-AP with a threshold of 0.5–1 \mu M and a maximal effect between 10 and 100 \mu M (Shi and Blight, 1997). This observation indicates that 4-AP can restore conduction to axons damaged by the trauma. The recordings were made 1 month or more after the spinal crush lesion, at a time when histological examination of the spinal cord demonstrated that the lesions contained a mixture of normal axons, partly remyelinated axons, widened paranodes and infrequent demyelinated axons. This heterogeneous morphology prevents the correlation of electrophysiological observations with particular axonal pathologies, but the data suggest that 4-AP may be of value in the therapy of spinal cord injury.

The second way in which 4-AP may act clinically is by increasing the tension developed by muscles (Agoston et al., 1982; Savage, 1985); the present findings show that clinical doses of 4-AP act to increase skeletal muscle twitch tension. Weakness is a common finding in multiple sclerosis, and it is clear that this effect of 4-AP will tend to reverse this symptom.

In summary, although we do not discount the possibility that 4-AP may sometimes restore conduction to demyelinated axons in multiple sclerosis, it seems likely that the dominant effect of the drug will be via other mechanisms, since these are so prominently and consistently expressed at low drug concentrations. The presence of alternative mechanisms may also help to explain the beneficial effects of 4-AP in spinal cord injury (Hansebout et al., 1993; Hayes et al., 1994; Potter et al., 1998).

It is worth noting that some of the effects of 4-AP may be mediated via effects on the immune system. Potassium channels are increased in T cells by mitogenic stimulation (Matteson and Deutsch, 1984), and potassium blockade has been shown to affect T cell mitogenesis (DeCoursey et al., 1984) and activation (Chandy et al., 1984; Radet et al., 1996). Notably, potassium channel blockers also inhibit immune responses in vivo (Koo et al., 1997), and high doses have been found to inhibit the adoptive transfer of experimental autoimmune encephalomyelitis by T cells (Judge et al., 1997). However, the prompt onset of at least some of the beneficial effects of 4-AP therapy in patients supports a role for direct effects on the nervous system, such as those reported above.

If the dominant action of 4-AP in multiple sclerosis is not to restore function to blocked axons, it follows that the use of the drug need not be restricted to patients with prominent temperature-sensitive symptoms, as it has sometimes been in the past (Stefoski et al., 1987; Bever et al., 1994). Indeed, the positive results in these patients reinforced the view that 4-AP acted by restoring conduction to demyelinated axons, although the beneficial effects of the drug were sometimes evident on symptoms where no temperature sensitivity had been noted by the patients (F. A. Davis, personal communication). A later study found that some patients benefited from the administration of 4-AP, even though they lacked the expression of temperature-sensitive symptoms (van Diemen et al., 1992).

Although the extent to which 4-AP increases the number of conducting, demyelinated central axons in multiple sclerosis patients remains uncertain, some potentially relevant observations have been made in peripheral axons. Two groups have examined the effect of a related potassium channel-blocking agent, 3,4-diaminopyridine; 3,4-DAP on conduction in various demyelinating disorders of the peripheral nervous system. 3,4-DAP was not effective in restoring conduction in patients with disorders including Guillain–Barré syndrome and chronic inflammatory demyelinating polyneuropathy (Bergin et al., 1993; Russell et al., 1995). These findings may not be directly applicable to the effects of 4-AP in the central nervous system, but, in conjunction with the results of the current study, they do suggest caution in interpreting the beneficial effects of 4-AP in multiple sclerosis as arising from the restoration of conduction to demyelinated axons. We therefore suggest that the beneficial effects of 4-AP in multiple sclerosis may arise primarily from effects which do not involve the demyelinated portions of axons, and our evidence highlights a potential role for the modulation of synaptic transmission. If this is true, it follows that 4-AP may be beneficial in some non-demyelinating central disorders.

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References


Savage AO. A comparison of the effects of 4-dimethylaminopyridine and 4-aminopyridine on isolated cardiac and skeletal muscle preparations. Arch Int Pharmacodyn Ther 1985; 273: 262–76.


