Slow sodium-dependent potential oscillations contribute to ectopic firing in mammalian demyelinated axons

R. Kapoor, Y.-G. Li* and K. J. Smith

Summary
Ectopic action potentials can arise at regions of axonal demyelination, and are believed to contribute to a range of symptoms in patients with demyelinating conditions such as multiple sclerosis. The mechanism(s) by which the ectopic impulses are generated are uncertain. We have previously shown that such ectopic activity can result from inward potassium currents. Paradoxically, the potassium channel blocking agent 4-aminopyridine (4AP) can sometimes cause ectopic activity in demyelinating lesions. To study this phenomenon we have made intra-axonal recordings of ectopic activity in demyelinated axons, both in the presence and absence of 5 mM 4AP. 4AP promoted a pattern of firing which was also observed, albeit less frequently, in demyelinated axons in the absence of this drug, namely trains of single impulses, or trains of short, high-frequency bursts of impulses. When recorded close to the demyelinated lesion, the impulses were generated by an underlying, almost sinusoidal oscillation of the membrane potential. This oscillation was abolished by the sodium channel blocking agent tetrodotoxin (0.1–1 µM). We conclude that the ectopic spiking induced by 4AP is generated by membrane potential oscillations associated with the site of demyelination. The sodium-dependent current underlying these oscillations, together with the prolonged inward potassium currents which we have previously described, may contribute to the generation of ectopic discharges in a range of disorders of myelinated axons.

Keywords: sodium channels; paraesthesiae; spontaneous activity; 4-aminopyridine

Abbreviations: 4AP = 4-aminopyridine; TTX = tetrodotoxin

Introduction
Demyelinating disorders can lead to the production of ectopic action potentials in the affected fibres, and the resulting impulses can result in a wide range of positive clinical phenomena. Some of the mechanisms which generate the ectopic activity have been identified. For example, ectopic impulses can arise from ephaptic transmission, which has been demonstrated in the spinal roots of dystrophic mice (Rasminsky, 1980). Ectopic impulses can also arise from the effects of internodal potassium currents which can become excitatory in normal myelinated fibres if the internodal, periaxonal space is loaded with potassium. Such loading can generate bursts of ectopic action potentials and can occur following ischaemia (Bostock et al., 1991) or tetanic stimulation (Bostock and Bergmans, 1994; Felts et al., 1995), or can be obtained artificially by ionophoresis (Kapoor et al., 1993). Ectopic firing has also been found to arise from a slow inward potassium current in potassium loaded normal myelinated rat axons (David et al., 1993) and in experimentally demyelinated axons in the rat dorsal column and sciatic nerve (Felts et al., 1995).

In the initial work on normal myelinated axons both the inward currents, and the prolonged bursts of ectopic action potentials associated with them, were inhibited when potassium currents were blocked using 4-aminopyridine (4AP) or, to a lesser extent, tetraethylammonium (Kapoor et al., 1993; see also David et al., 1993). Paradoxically,
however, it is also known that exposure to 4AP can potentiate ectopic firing in some demyelinated axons (Targ and Kocsis, 1986; Bowe et al., 1987; Baker and Bostock, 1992). In order to clarify the mechanism underlying this latter effect, we have now applied intra-axonal recording techniques to study the ectopic firing which is induced by 4AP in demyelinated axons of rat dorsal columns and sciatic nerves. We have identified small oscillations in membrane potential which are dependent upon sodium channels and which generate the ectopic activity.

**Methods**

**Lesion induction**

Demyelinated lesions were induced in adult rats (Sprague–Dawley or Wistar, 250–350 g, male). Briefly, central lesions were induced in the dorsal columns by the intraspinal microinjection of ethidium bromide (2×0.5 µl, 0.5 mg/ml in saline) following a partial laminectomy at the T10 vertebral level under general anaesthesia (2% halothane, 70% nitrous oxide in oxygen) (for detail, see Felts and Smith, 1992). Peripheral demyelinating lesions were induced by the microinjection of lysophosphatidyl choline (2 µl, 10 mg/ml) into the tibial and peroneal branches of the sciatic nerve in the mid-thigh (Smith and Hall, 1980). The wounds were closed in layers. The animals were allowed to recover and were examined daily for evidence of any complications: none were observed.

**Electrophysiological examination**

The animals were re-anaesthetized when the demyelinating lesions had developed to a stage when remyelination was present in some fibres (3–4 weeks post-injection in the CNS and 1–3 weeks in the PNS) and a 3 cm length of tissue containing the lesion was removed and the animals then sacrificed by overdose of anaesthetic. For CNS lesions, the dorsal column was dissected free from the spinal cord, and for PNS lesions, the sciatic nerve was desheathed. The tissues were then placed in an interface recording chamber (Medical Systems Corporation, New York, USA) perfused with artificial extracellular fluid [containing (mM): NaCl 123, KCl 3, CaCl₂ 1.5, MgCl₂ 1.0, HEPES 10, glucose 10; pH 7.4; flow rate 0.5 ml/min bubbled with O₂]. The chamber was modified to permit the medium bathing the lesion to be changed independently of the media bathing the tissue at the stimulating sites (Fig. 1). Bath temperature was maintained at 35°C using a feedback control mechanism and thermistors located near the tissue. It took between 2 and 10 min to exchange solutions around the tissue depending upon the configuration of the apparatus. If necessary, solutions containing drugs were made isosmolar by substituting the drug for the equivalent amount of NaCl. The dorsal column or sciatic nerve was placed on pairs of platinum wire stimulating electrodes situated at least 5 mm from the recording site in order to elicit either rostrally or caudally propagating action potentials. Intra-axonal recordings were made using micropipettes (DC resistances 30–60 MΩ) filled with 3 M KCl, and an Axoclamp 2A amplifier (Axon Instruments, Foster City, Calif., USA) in bridge mode. Recordings were either made in preparations perfused with control extracellular solution, or following at least 30 min of perfusion with a similar solution containing 5 mM 4AP. The experimental protocol for these animal experiments was approved by the Home Office, UK.

**Results**

The detailed histology of the demyelinating lesions produced by ethidium bromide and lysophosphatidyl choline have been described previously (Hall and Gregson, 1971; Gregson and Hall, 1973; Felts and Smith, 1992). At the stage studied, these lesions consist of circumscribed, focal areas of primary demyelination in which varying numbers of demyelinated axons are either ensheathed by glial or Schwann cells, or are in the early stages of remyelination.

In the absence of 4AP, intra-axonal recordings obtained at or near demyelinating lesions revealed the presence of ectopic firing in only ~5% of impalements (Felts et al., 1995). In 95% of these examples, the discharges took the form of prolonged intermittent bursts of action potentials, while the remaining 5% of recordings demonstrated a second form of ectopic firing, namely regularly spaced single action potentials or brief bursts of action potentials. Exposure of the
Ectopic firing in demyelinated axons

Demyelinating lesions to 4AP in the current study resulted in a considerable increase in the incidence of the second form of ectopic firing, such that examples were observed in virtually every electrode track. It is possible that the ectopic firing was confined to particular functional subclasses of dorsal column axons, but this was not investigated.

The increase in ectopic activity which occurred following exposure to 4AP was common to recordings made in both sciatic nerve and dorsal column axons. Although it is known that 4AP can lead to ectopic impulse generation in immature, but otherwise normal myelinated axons (Kocsis et al., 1983), the drug caused no ectopic activity in the normal adult myelinated axons examined in this study. The results obtained from intra-axonal recordings in central and peripheral demyelinating lesions were similar, and they will therefore be described together.

In all of 36 stable intra-axonal recordings obtained from axons exhibiting ectopic activity in the presence of 4AP, the discharges consisted of evenly spaced brief bursts of two to four impulses, or of continuous trains of relatively evenly spaced impulses at a frequency of 10–25Hz (Fig. 2; compare with record in Fig. 3 obtained in the absence of 4AP). The records were of two types. Those obtained several millimetres from the lesion (n = 10) showed impulses which arose from a flat baseline, not preceded by any depolarizing prepotentials (Fig. 2A). Those obtained near the lesion (n = 26) showed action potentials which arose from underlying, regular oscillations in the membrane potential (Fig. 2B and C). Since the oscillations were only observed in impalements made near the lesion, we infer that they were generated by membrane currents associated with the demyelinated axolemma. In some recordings (n = 7) the subthreshold membrane potential oscillations sometimes failed to initiate ectopic action potentials, and in these circumstances the potentials had an almost sinusoidal waveform (Fig. 3). Subthreshold oscillations, similar to those in Fig. 3B and C, were observed in recordings obtained from both central and peripheral demyelinated lesions. Similar oscillations were observed in demyelinated fibres in the presence of 4AP and in its absence. Some of the axons concerned had a refractory period of transmission including the lesion in the conduction pathway which was approximately double that obtained in the same axon when the lesion was excluded, providing good evidence that the axons were, indeed, demyelinated (McDonald and Sears, 1970).

The input resistance of the axon at different points during the sinusoidal oscillation was studied using small, brief hyperpolarizing current commands. The changes in membrane potential resulting from these commands were indistinguishable, such that averaged responses were exactly superimposable irrespective of whether the current commands were applied at the peak of the oscillation or at its trough. This result implies that there was little change in the axolemmal membrane conductance during the course of the membrane potential oscillation. Interestingly, the amplitudes of the oscillations increased when the axonal membrane was hyperpolarized using injections of constant hyperpolarizing currents through the recording electrode. With further hyperpolarization, both the oscillations and the ectopic activity were abolished. These findings suggest that the
Fig. 3 Intra-axonal recording from a central demyelinated axon in the absence of 4AP showing oscillations in membrane potential which either initiate ectopic action potentials (A, similar in appearance to those in Fig. 2B), or which sometimes fail to do so (B and C). The almost sinusoidal waveform of the oscillations is clearly observed where there is an absence of superimposed spiking activity (B and C). Resting potential = −55 mV.

Oscillations were generated by an inward membrane current located in the axolemma close to the recording site.

To determine whether sodium currents were involved in the generation of the oscillations we attempted to inhibit them with tetrodotoxin (TTX). In recordings made near the demyelinating lesion, and in which a regular pattern of firing was observed before exposure to TTX, the drug inhibited both the ectopic action potentials and the underlying subthreshold membrane depolarization at a concentration of 0.3–1.0 µM (Fig. 4). Similar concentrations of TTX were required completely to block conduction of the whole compound action potential in this preparation and apparatus.

In the intra-axonal recordings the firing became intermittent prior to complete block by TTX, and at this stage the underlying oscillations of the membrane potential became apparent. Several seconds after the loss of the spikes, the oscillations were also blocked. Using a range of concentrations of TTX between 0.1 and 1.0 µM we were unable selectively to abolish the action potentials while allowing the subthreshold oscillations to continue unaffected. Following the blockade of ectopic activity with TTX, membrane potential oscillations could not be triggered by depolarizing or hyperpolarizing current commands. The results obtained from intra-axonal recordings in four sciatic nerves and two dorsal columns were similar.

Discussion
In this study, we have investigated the increased ectopic activity elicited in demyelinated axons by the inhibition of potassium currents using 4AP. Our recordings demonstrate that exposure to 4AP favours a regular pattern of ectopic firing which can also be observed in a small number of recordings in the absence of 4AP. The intra-axonal recordings made near demyelinating lesions included small oscillations in the membrane potential which were found to initiate the ectopic activity. The oscillations were similar to the pacemaker potentials previously described by Baker and Bostock (1992) in demyelinated spinal roots exposed to 4AP, and we have extended their observations through the use of intra-axonal recording techniques. In both studies the oscillations were significantly also found, albeit infrequently, in recordings made in demyelinated axons in the absence of 4AP. However, the fact that such oscillations were encountered more frequently in the presence of 4AP implies that the inhibition of potassium currents promotes a firing mechanism which is generally present in demyelinated fibres, but whose effects are usually suppressed.

Neither the ectopic activity, nor its pattern, can be dependent upon micropipette impalement, since a similar pattern of ectopic firing was observed in intra-axonal impalements made near the lesion site, in impalements made distally, and in extra-axonal recordings. Furthermore, our recordings are similar to those obtained in intact spinal roots (Baker and Bostock, 1992). Because the oscillations were only observed in recordings made near lesions, it is likely that they were generated by membrane currents associated with sites of demyelination: in contrast to the action potentials, the subthreshold oscillations would be expected to attenuate rapidly with passive propagation from their site of origin.
Ectopic firing in demyelinated axons

Intra-axonal recording from a peripheral axon near the site of the lesion, in the presence of 4AP.

The axon initially shows a regular pattern of firing (A), but after the addition of tetrodotoxin (TTX; 1 µM) the firing becomes intermittent (B): the continuing membrane potential oscillations, some of which are marked with asterisks, may be seen. Soon the TTX abolishes the spiking (C), and after several seconds the underlying potential oscillations are also inhibited. Resting potential = −55 mV.

In the earlier study by Baker and Bostock (1992) of rat spinal roots demyelinated using diphtheria toxin, evidence was presented that a slow potassium conductance was responsible for pacing the ectopic activity, although the ionic mechanism by which the oscillations were generated was unclear. In the present study, the membrane potential oscillations and the ectopic action potentials were found to be blocked by TTX. This observation implies that the oscillations are driven by an axonal sodium current which, given the spatial localization of the oscillations, is associated with regions of central or peripheral demyelinated axolemma. It seems unlikely that other inward currents contribute to the membrane depolarization, since active depolarizing responses could not be elicited following exposure to TTX using current commands which elicited sodium dependent action potentials before such exposure. Voltage clamp studies of acutely demyelinated axons have only revealed evidence of sodium and potassium currents (Chiu and Ritchie, 1981; Roper and Schwarz, 1989; Schwarz et al., 1995), and the inward potassium currents which we and others have previously described (David et al., 1993; Kapoor et al., 1993) would have been inhibited by exposure to 4AP.

It is notable that the slow oscillations of the membrane potential were inhibited by the same bath concentration of TTX which inhibited the action potentials, although the oscillations were inhibited some seconds after the action potentials, during which time there was presumably a build-up in the tissue concentration of TTX. However, this delay does not necessarily imply that the spiking and oscillations arose from sodium currents with different TTX sensitivities. The ‘all or none’ phenomenon of the action potential might simply be more sensitive to a smaller reduction in the total level of a single sodium current. On the other hand, there is evidence that multiple sodium currents occur not only in neuronal cell bodies but also in axons. Thus, both fast-inactivating and slow, non-inactivating sodium currents may be present in optic nerve axons (Stys et al., 1993) and cutaneous sensory afferents (Honnou et al., 1994), and it also appears that fast-activating, persistent sodium currents are active at the resting potential in peripheral axons and sensory neurons (Bostock and Rothwell, 1995; Baker and Bostock, 1996). In this respect, it would be of interest to know whether ectopic firing elicited by 4AP is a property of particular functional subtypes of dorsal column axons. Sodium channels of unknown physiological properties are also known to be inserted into demyelinated axolemma at sites of contact with glial cells (Black et al., 1991).

The precise relationship between these various sodium currents and the inactivating sodium current which generates action potentials remains unclear, and our recordings do not allow us to suggest which of these currents may be responsible for the oscillations in the membrane potential. In addition, it is possible that currents may be active in both the depolarizing and hyperpolarizing phases of the oscillation, since the axonal input resistance was found to be similar during these two phases. The hyperpolarizing phase of the oscillation may therefore be associated with an inactivation of sodium current and the activation of a hyperpolarizing current, such as a slow potassium current (Baker and Bostock, 1992).

In summary, the blockade of potassium currents in
demyelinated axons appears to expose small, sodium-dependent oscillations of the membrane potential, similar to those we have observed in the absence of potassium channel blockade. These oscillations can give rise to regularly spaced ectopic action potentials which may occur singly, or in small groups. We conclude that ectopic activity and, thereby, positive clinical phenomena can arise from the excitatory effects of slow, sodium-dependent potentials, as well as from ephaptic interactions (Rasminsky, 1980), and disturbances of potassium buffering leading to inward internodal potassium currents (Felts et al., 1995). Each of these mechanisms is likely to be associated with the demyelinated portion of the axonal membrane.

Acknowledgements
The work was supported by the Scarfe Trust (to R.K.), the Multiple Sclerosis Society of Great Britain and Northern Ireland (to K.J.S.) and the Wellcome Trust (to K.J.S.).

References

Baker M, Bostock H. Sustained inward currents in large neurones cultured from adult rat dorsal root ganglia. J Physiol (Lond) 1996; 491P: 141P–2P.


Bostock H, Rothwell JC. The time constants of motor and sensory axons in human peripheral nerve. J Physiol (Lond) 1995; 487P: 47P.


Rasminsky M. Ephaptic transmission between single nerve fibres in the spinal nerve roots of dystrophic mice. J Physiol (Lond) 1980; 305: 151–69.


Received June 3, 1996. Revised October 11, 1996.
Accepted December 3, 1996