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ECTOPIC ACTIVITY IN DEMYELINATED SPINAL ROOT AXONS OF THE RAT

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SUMMARY

1. We have provoked ectopic discharges from demyelinated rat spinal roots by applying 1 mM-4-aminopyridine (4-AP), and recorded membrane currents and action potentials extracellularly by spike-triggered averaging. The demyelination was caused by intrathecal injection of diphtheria toxin, 6–9 days previously.

2. Mapping the distribution of membrane currents in the vicinity of an ectopic site showed that in most cases (eight out of twelve recorded) the impulses arose from one end of a continuously conducting internode, and conducted in both directions. In the remaining cases the impulses also arose from a site of demyelination.

3. The 4-AP-induced activity resembled the activity occurring spontaneously in some preparations, and was often highly regular (5–20 Hz). Recordings of membrane potential revealed a pacemaker potential, which was localized to the site of impulse initiation. One ectopic site was tested with applied currents and found to have a linear current–frequency relation for steady currents.

4. The time course of the pacemaker potential resembled that of the small after-hyperpolarization seen in normal fibres, due to a slow K^+ conductance (G_{Ks}). Tetraethylammonium and barium ions, which block G_{Ks}, made spontaneously active fibres fire much more rapidly, or to fire bursts of action potentials.

5. Possible mechanisms for these ectopic discharges are discussed. G_{Ks} appears to contribute to the pacing of the activity, but not its generation. The increased excitability of the active fibres could not be attributed directly to the loss of myelin, nor to extracellular K^+ accumulation. We suggest that they may have been depolarized by stretch-activated or ligand-gated channels in the demyelinated axon membrane.

INTRODUCTION

Demyelination should help to stabilize the membrane potential of an axon, both by increasing the input capacitance and by exposing internodal potassium channels. Indeed, the most serious consequences of demyelinating disease are due to the conduction failure that results from this extra stability of the membrane potential.

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On the other hand, human demyelinating diseases are also associated with 'positive' symptoms (Rasminsky, 1981), such as paraesthesiae and motor fasciculations, attributable to spontaneous ectopic discharges. This paradoxical hyperexcitability occurred only seldom in the demyelinated spinal root axons studied previously (Bostock, 1982), and reports in the literature have been sparse. Smith & McDonald (1980) recorded spontaneous and mechanically evoked activity from cat dorsal columns demyelinated with lysophosphatidyl choline (LPC). Burchiel (1980, 1981) recorded ectopic discharges from cat trigeminal roots, demyelinated by chronic implantation of chromic suture. Also Calvin, Devor & Howe (1982) recorded spontaneous activity from teased filaments of rat saphenous nerve, 7–11 days after LPC injection. However, in none of these cases were recordings of membrane current or membrane potential made at the site of generation of an ectopic impulse.

We have previously obtained evidence that human motor axons have fewer internodal potassium channels than rat motor axons, in that the accommodative properties of rat axons can be made more similar to those of human axons by exposure to 4-aminopyridine (4-AP) and tetraethylammonium ions (TEA) (Bostock & Baker, 1988). In rat spinal root axons 4-AP appears to block specifically a fast potassium conductance (G_{Kf}) and TEA a slow potassium conductance (G_{Ks}) (Baker, Bostock, Grafe & Martius, 1987; Roper & Schwarz, 1989). We have found that both of these agents, alone or especially in combination, can provoke ectopic discharges in silent demyelinated rat spinal roots. 4-AP is particularly effective, and unlike TEA its action is not readily reversible, so that even in a spinal root *in vivo* with normal circulation, brief exposure to 1 mM-4-AP can provoke steady discharges for many minutes. These ectopic discharges resemble in rate and firing pattern those occurring spontaneously in some demyelinated fibres (e.g. Smith & McDonald, 1980), and it is likely that the same mechanisms are involved. In this study we have used 4-AP to produce preparations with a single axon generating impulses at an ectopic site, and then investigated the mechanism of impulse initiation by using spike-triggered averaging to record membrane currents and membrane potentials extracellularly.

METHODS

The observations in this study were made on eighteen female Lewis rats weighing between 180 and 250 g. The rats were anaesthetized with Thiogenal (Merck, 120 mg/kg) and a minimal focal demyelinating lesion was induced in the spinal roots with diphtheria toxin, following the procedure described by Bostock & Grafe (1985). Between 6 and 9 days later, the animals were anaesthetized with sodium pentobarbitone (40 mg/kg I.P. initial dose, supplemented as required). The sacral spinal roots were exposed by laminectomy, and one at a time raised on electrodes in a pool of liquid paraffin, held within skin flaps (Fig. 1). The moist, oxygenated oil was maintained at 32 °C by a radiant heat lamp. Drugs such as 4-AP and TEA were applied to the root in droplets of Ringer solution, which were held in position by surface tension until removed. To provoke ectopic activity in a single unit, it was often sufficient to slide a drop of 1 mM-4-AP quickly along the root. In other cases more prolonged application or a higher concentration was required to activate a unit, or it was necessary to rinse the root with Ringer solution to reduce the number of active units. In some cases no spontaneous units could be activated.

When a single unit was found that discharged repeatedly, the source of the impulses was located by determining the position at which longitudinal currents reversed their sign (Rasminsky, 1981). A Gould OS4040 oscilloscope was used in pre-trigger mode to display and digitize events occurring before the impulses reached the trigger electrodes. Usually the root was kept intact, to maintain a stable circulation, and membrane currents were recorded at many positions along a root, using

a movable tripolar electrode and differencing amplifiers (see Fig. 1*B* and detailed description in Bostock, Sears & Sherratt, 1983). Polarizing currents were applied via a Ringer solution-filled glass micropipette touching the surface of the root close to the point of impulse initiation (see Fig. 2 in Bostock & Grafe, 1985). On some occasions the root was cut, so that membrane potentials could

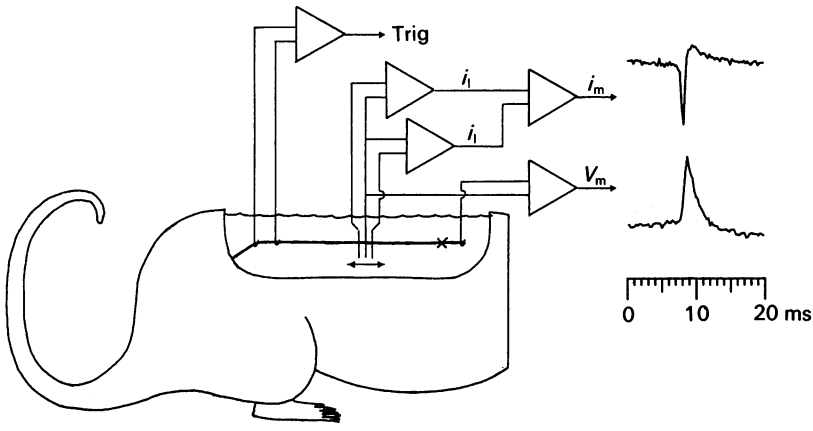

Fig. 1. Schematic diagram of arrangement for simultaneous recording of membrane current (i_m) and membrane potential (V_m) extracellularly from an ectopically active fibre at the site of impulse initiation. The movable tripolar electrode was positioned so that the longitudinal current (i_l) signals were of opposite polarity, and i_m and V_m recorded by averaging signals occurring prior to impulses arriving at the distal pair of electrodes (Trig). The spinal root was cut and crushed 5 mm from proximal V_m electrode for monophasic recording.

also be recorded extracellularly, as shown schematically in Fig. 1 (cf. Lafontaine, Rasminsky, Saida & Sumner, 1982). The digitized signals were recorded and averaged with a PDP 11/23 computer. Interspike intervals were recorded by counting the number of 1 ms pulses between action potentials. (The output of the counter was sampled at 2 s^{-1}, so that with a unit firing at 10–20 s^{-1} only a minority of intervals were recorded.)

RESULTS

Membrane current contour maps

The membrane currents recorded at sites of origin of ectopic impulses were characterized by strong inward currents that were not preceded, as at normal nodes, by any outward current (Fig. 1). From the membrane currents recorded at many positions along a root, we constructed contour maps to summarize the distribution of currents in space and time (Bostock & Sears, 1978). Figure 2*A* is a membrane current contour map for a single dorsal root axon, which discharged ectopically after brief exposure to a drop of 1 mM-4-AP. The activity was very regular (see below), which was characteristic of most units recorded. The site of origin of the impulses, where appreciable inward membrane current was first detected, is arrowed. From the distribution of the subsequent membrane currents along the fibre, we can infer that the activity started at or close to a heminode, i.e. at the transition zone between a myelinated and a demyelinated segment of the axon. To the left of the arrow in Fig.

2*A* (i.e. caudally) there was a normally myelinated internode, indicated by the presence of only a weak outward current. To the right, however, the slowly moving focus of strong inward and outward currents indicates a demyelinated internode, conducting continuously (Bostock & Sears, 1978). Once the ectopic impulse has been

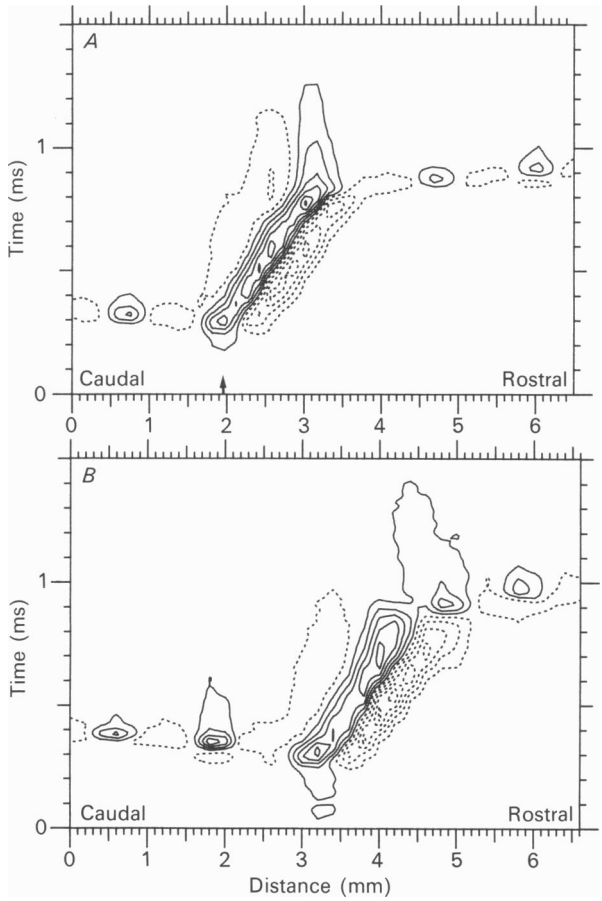

Fig. 2. Membrane current contour maps recorded for two ectopic generators provoked by 1 mM-4-AP, showing impulses arising at heminodes between segments conducting by saltatory and continuous conduction. Continuous lines indicate inward membrane current, dotted lines outward current. *A*, dorsal root fibre (6 days after injection of diphtheria toxin). *B*, ventral root fibre (7 days after injection of diphtheria toxin). (N.B. At the contour intervals chosen, the weak outward currents preceding inward current generation at the normal nodes are not visible in every case.)

initiated, the pattern of membrane current flow was similar to those recorded from other fibres demyelinated with diphtheria toxin.

The late outward currents in the demyelinated internodes were previously reported to be blocked by 5 mM-4-AP (Bostock, Sears & Sherratt, 1981). The presence of weak late outward currents in Fig. 2 is consistent with our use of a lower concentration of 4-AP for only a partial block of the fast potassium channels. The prolonged inward current at the end of the stretch of continuous

conduction, which was also seen with 4-AP in the earlier study, is reproduced by computer simulation of demyelination (Bostock, 1992) when internodal potassium conductance is reduced (H. Bostock, unpublished observations).

We recorded full membrane current contour maps as in Fig. 2*A* of eleven other ectopic impulse generators. Eight out of the twelve contour maps showed a similar

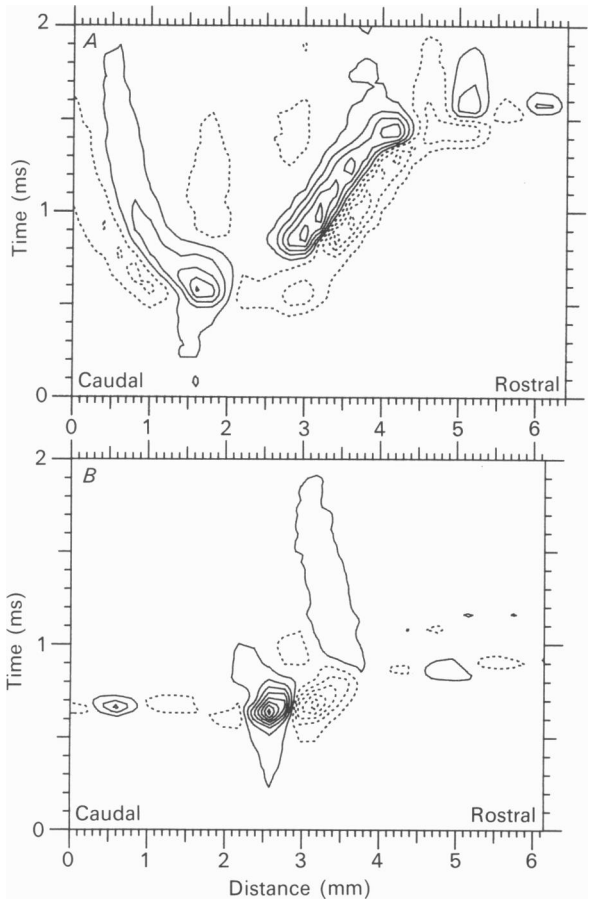

Fig. 3. Membrane current contour maps for two less usual ectopic generators. *A*, spontaneous activity in dorsal root fibre (8 days after injection of diphtheria toxin) did not require provocation by 4-AP. Impulses appeared to arise at heminode, with decremental conduction towards cell body, and saltatory then continuous conduction towards spinal cord. *B*, 4-AP-induced activity in ventral root fibre (7 days after injection of diphtheria toxin) which appeared to arise from partly demyelinated internode, not a heminode.

pattern of membrane current flow, indicating that a heminode was the source of the activity. Another example, this time from a ventral root, is shown in Fig. 2*B*. The activity arose in some cases (e.g. Fig. 2*B*) at the proximal, and in other cases (e.g. Fig. 2*A*) at the distal end of the demyelinated internode with respect to the cell body. Figure 3*A* illustrates a unit that was unusual in two respects: it fired spontaneously without the need for activation by 4-AP, and the impulses faded out in the direction

of the cell body. (Such decremental conduction is unusual in chronically demyelinated internodes, but it can be simulated by a computer model with a density of sodium channels in the demyelinated internode just insufficient to sustain continuous conduction (Bostock, 1992).)

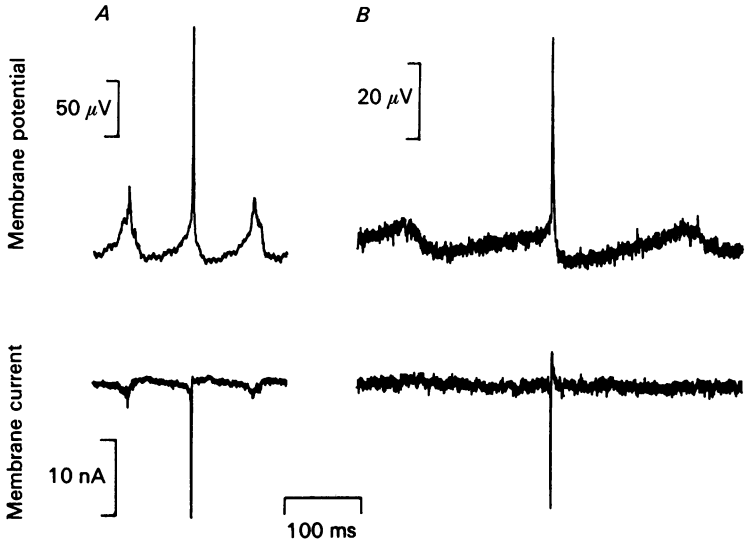

Fig. 4. Membrane potentials and currents recorded extracellularly at two sites of ectopic impulse generation, showing pacemaker activity. *A*, ventral root fibres (same unit as Fig. 3*B*). *B*, impulses arising at heminode in dorsal root fibre after treatment with 4-AP (9 days after injection of diphtheria toxin).

The remaining contour map (Fig. 3*B*) illustrates a pattern of membrane current flow seen in two other units. There was no continuous conduction, but demyelination was indicated by the very abnormal spacing of current foci. There is a focus of outward current at 3·2 mm, close to the site of intense inward current at 2·6 mm, where the impulses originate. However, from the positions of the unaffected nodes in this fibre, the mean internodal length was probably about 1·43 mm, with nodes originally close to 2·03 and 3·47 mm. In this case (and two others) the ectopic site of impulse generation appeared not to correspond to an old node. The unit in Fig. 3*B* discharged regularly from more than an hour, enabling us also to record the potentials shown below in Figs 4*A* and 5.

Pacemaker potentials

At three of the sites of ectopic impulse generation we were able to record membrane potentials as well as membrane currents, after cutting the root. Two examples, with rather different firing rates, are shown in Fig. 4. The unit in Fig. 4*A*, with an interspike interval of 84 ms, discharged very regularly, so that the spikes preceding and following the cental one (which was used to trigger the signal averager) were only partially reduced in amplitude by temporal dispersion. The unit in Fig. 4*B* fired more slowly and less regularly (interspike interval 170–200 ms), so that only the

central trigger spike is discernible in the average. The times of occurrence of the preceding and following spikes are, however, apparent from the slow undulations in membrane potential.

In all three cases, the averaged membrane potentials had prominent slow components. These slow components were much larger, in relation to the peak action

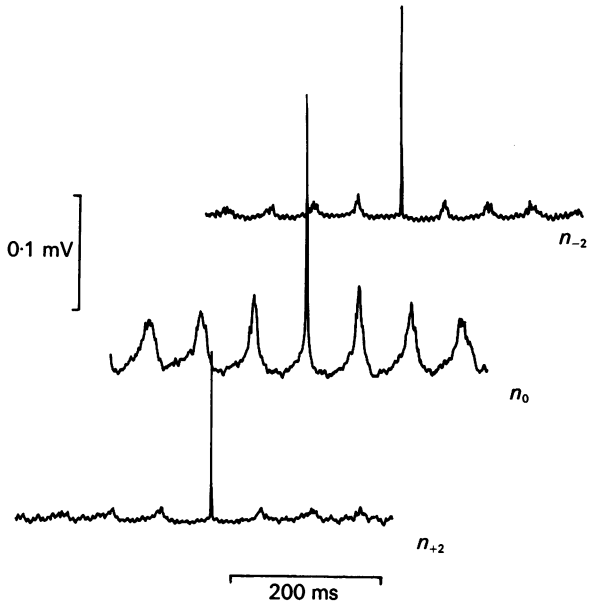

Fig. 5. Localization of pacemaker potential. Averaged membrane potentials for ectopic impulses recorded at site of impulse generation (n_0) and approximately two internodal lengths away in each direction (n_{-2}, n_{+2}). (Same unit as Figs 3*B* and 4.)

potential amplitude, than normal after-potentials. Because the slow components led directly into the rising phase of the action potentials, and appeared to trigger them, we refer to them as pacemaker potentials. For the unit in Fig. 4*A* (and Fig. 3*B*) we were also able to record membrane potential approximately two nodes away in either direction (Fig. 5). This showed that the pacemaker potential was localized to the ectopic site, and confirmed that the normal after-potentials are much smaller.

Discharge rates of ectopic sites

Most of the ectopic generators that were recorded in isolation could be described as 'tickers', firing at a fairly steady rate in the range 5–20 Hz, as in the examples above. The remainder fired in rapid bursts of two to eight impulses at intervals of 5–10 ms, repeated every 50–300 ms, but these discharge patterns were never stable enough to record satisfactory averages of the potential or current waveforms. The discharges were often acutely mechanosensitive, and could be modified by applied currents and by K^+ channel blockers.

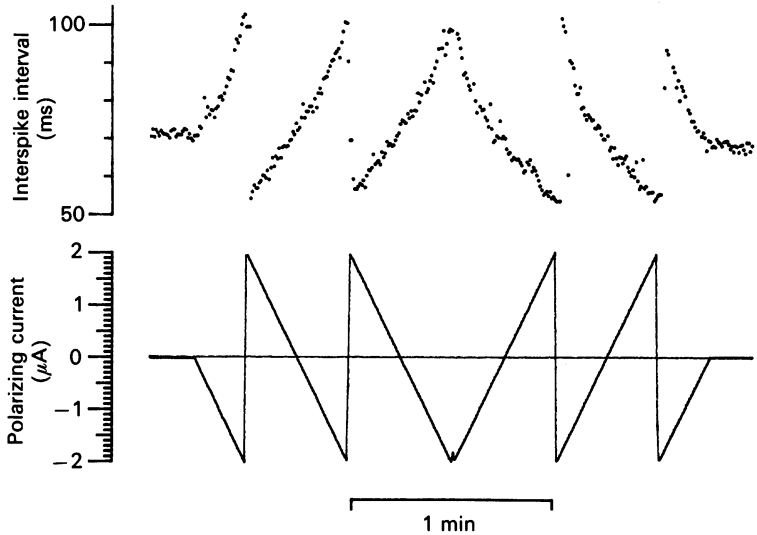

Fig. 6. Encoding properties of an ectopic generator. Variation in interspike interval with extracellularly applied polarizing current for ectopic impulses provoked by 1 mM-4-AP in dorsal root fibre (6 days after injection of diphtheria toxin). These data are replotted in Fig. 7.

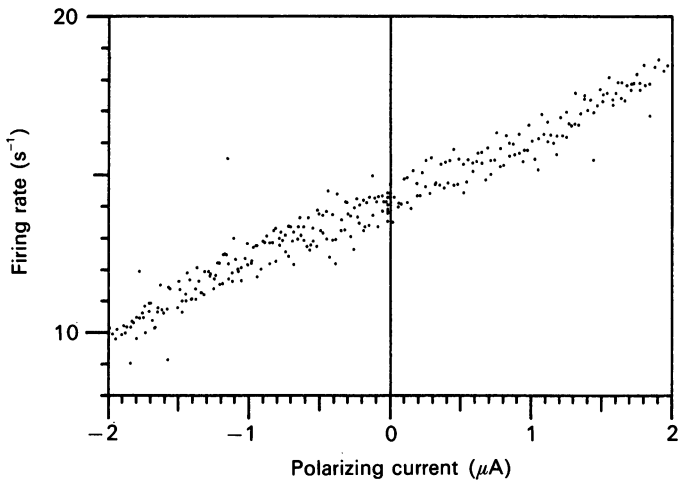

Fig. 7. Variation in firing rate of ectopic generator with applied current. Data replotted from Fig. 6. (Depolarizing current is positive.)

Modification by applied current

The slow firing rates of the 'ticking' sites are not normally recorded from mid-axon, and suggested a resemblance to the specialized encoding regions, such as the axon hillock (Hille, 1984). We investigated the encoding properties of one ectopic site, as illustrated in Figs 6 and 7. Figure 6 shows how changes in interspike interval followed ramp changes in a steady, extracellular polarizing current, without any appreciable lag or hysteresis. These data are replotted in Fig. 7 to show the

remarkably linear relationship between firing rate and polarizing current. This relationship broke down with stronger hyperpolarizing currents, which could not produce regular firing more slowly than 10 Hz in this fibre.

Effects of K^+ channel blockade

As mentioned in the introduction, the K^+ channel blockers 4-AP and TEA were both capable of provoking or increasing ectopic discharges from demyelinated spinal

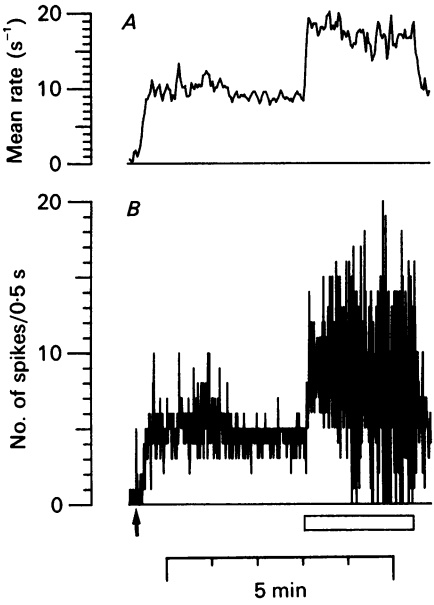

Fig. 8. Effects of K^+ channel blockers on frequency and rhythm of an ectopic generator. Impulses originated at heminode in ventral root, 6 days after diphtheria toxin injection. Firing rate is plotted as mean rate over 5 s periods (*A*), or number of spikes in each 0·5 s period (*B*). Application of drop of 1 mM-4-AP (arrow) provoked steady discharge, which was accelerated and then became irregular with 1 mM-Ba^{2+} (bar). Action of Ba^{2+} was rapidly reversed by Ringer solution (end of bar).

roots, as was the mechanical disturbance caused by applying a drop of solution to the root. When the activity was restricted to a single fibre, it became clear that the effects of different types of K^+ channel blocker were not identical. Whereas 4-AP (1–5 mM) primarily promoted the 'ticking' discharges previously illustrated, both TEA and barium ions, previously reported to block G_{Ks} (Baker *et al.* 1987), consistently and reversibly induced firing in high frequency bursts (five units). Figures 8 and 9 illustrate some recordings from a demyelinated dorsal root, in which we counted either the number of impulses per unit time (Fig. 8) or the number of milliseconds between impulses (Fig. 9). An ectopic discharge induced by 4-AP was not only increased in rate by Ba^{2+} (Fig. 8*A*), but it became markedly irregular (Fig. 8*B*). The interspike intervals recorded from the same unit show the change from ticking to bursting behaviour more clearly (Fig. 9). Partial block of G_{Ks} with 1 mM-TEA reversibly induced high frequency discharges at up to 150 Hz, separated by

intervals of up to 200 ms (Fig. 9*A*). One millimolar Ba^{2+} increased the interburst intervals to as long as a second (Fig. 9*B*). The clustering of long intervals within the range normally controlled by the pacemaker potentials in Fig. 9*A* suggests that the action potential bursts were activating residual G_{Ks}, just as trains of impulses increase the after-hyperpolarization in normal fibres (Fig. 11 in Baker *et al.* 1987).

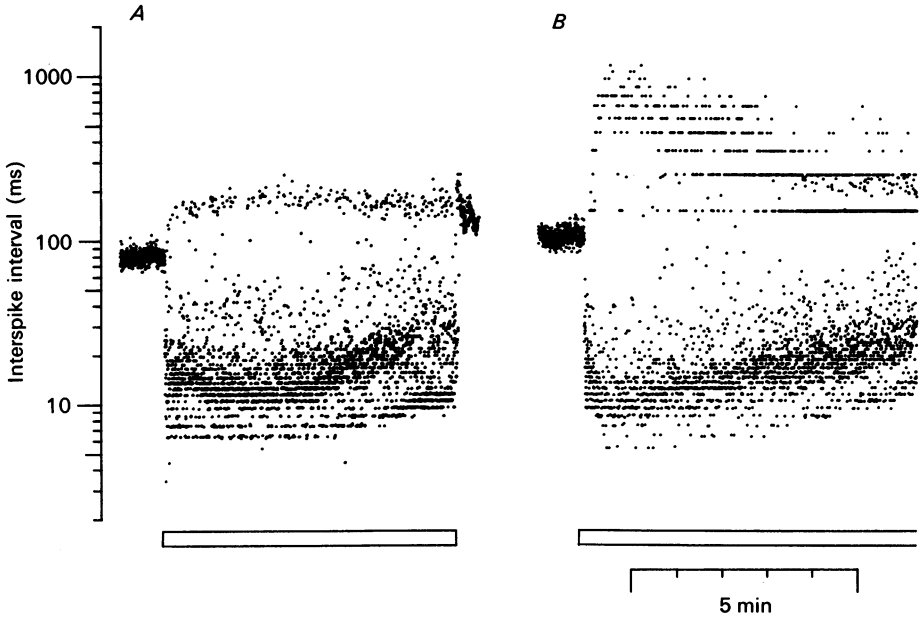

Fig. 9. Burst firing induced by TEA and Ba^{2+}. Interspike intervals for same unit as Fig. 8 plotted on logarithmic scale. Ectopic discharges were provoked by 1 mM-4-AP, then modified by 1 mM-TEA (*A*), and 20 min later by 1 mM-Ba^{2+} (*B*) as indicated by bars. (N.B. Interspike intervals to the nearest 1 ms were recorded by a pulse counter, sampled every 100 ms. Long interburst intervals in *B* were estimated to the nearest 100 ms, when count did not change.)

DISCUSSION

This paper contains the first description of membrane currents and membrane potentials recorded at sites of ectopic impulse generation in demyelinated axons. In most cases the activity was provoked by application of a minimal dose of 4-AP (i.e. the smallest dose that would induce activity in a single fibre). The question might be raised, therefore, of whether we were investigating activity induced more by the 4-AP than by the demyelination. However, even with prolonged exposure to 5 mM-4-AP, we never observed spontaneous ectopic activity in normal axons. Membrane current mapping showed that the ectopic discharges were always related to a site of demyelination. The two modes of firing recorded, steady discharges at 5–20 Hz or short bursts of two to eight impulses at intervals of 50–300 ms, have both been observed on occasion without application of 4-AP. Similar discharge patterns were also noted in spontaneously active units in cat dorsal columns demyelinated with lysophosphatidyl choline (Smith & McDonald, 1980), although their steady firing rates were somewhat faster (15–45 Hz).

Thus for a rat spinal root fibre to generate ectopic impulses, 4-AP was neither necessary in all cases, nor sufficient without demyelination. Even the combination of demyelination and 4-AP was not always sufficient to induce activity, and only a minority of demyelinated fibres could be activated by 4-AP. We excited many single units by near-threshold stimulation with needles in the tail, using membrane current recording to select fibres with segmental demyelination (cf. Bostock *et al.* 1983; Bostock & Grafe, 1985). We then stopped stimulating and applied 4-AP, in the hope that our selected single fibre would become ectopically active; but it never did, just as with earlier observations of the effects of 4-AP on single demyelinated fibres (Bostock *et al.* 1981). The minority of demyelinated fibres that were predisposed to fire spontaneously when treated with 4-AP were probably in a depolarized state. It was previously found that 4-AP only depolarizes or increases the excitability of axons that are already depolarized (Baker *et al.* 1987). This is because only the slow, 4-AP-insensitive potassium channels are significantly activated at the normal resting potential (Roper & Schwarz, 1989).

Pacemaker potentials

The pronounced pacemaker potentials which we found at sites of ectopic impulse generation (Figs 4 and 5) and the well-defined current–frequency relationship at one site (Figs 6 and 7) indicate that demyelinated axon can behave like a depolarized axon hillock or sensory terminal, where impulses are normally encoded. To what extent these encoding properties arise simply by virtue of demyelination, and to what extent they develop in response to it, is not clear. Our experiments were confined to a narrow time slot of 6–9 days after the induction of demyelination. However, our evidence suggests that the pacing is controlled by channels normally present in the axons. The first part of the pacemaker potential following the action potential is similar in time course to the slow after-hyperpolarization (AHP) responsible for the late subexcitability in normal fibres (H1 in fig. 11 of Baker *et al.* 1987), although much more pronounced. The AHP was attributed to a TEA- and Ba^{2+}-sensitive slowly activating potassium conductance, G_{Ks}. We were not able to record directly the effects of TEA or Ba^{2+} on the pacemaker potential, but the dramatic effects of these agents on the firing patterns of the ectopic generators (Figs 8 and 9) provide confirmation that G_{Ks} is involved. Although 'Ks' channels help to pace the ectopic activity, they cannot be responsible for initiating it, since they serve only to hyperpolarize the axons.

Mechanisms of ectopic discharges

Four different hypotheses have been put forward to account for the ectopic activity arising at sites of demyelination, none of which is entirely consistent with our observations. Firstly, Low (1982) found an unusually high extracellular potassium concentration at some sites in demyelinated nerves, and proposed that depolarization by potassium ions could cause the ectopic discharges. Our interpretation of the pacemaker potentials is that after each spike the axon is hyperpolarized by an increase in potassium conductance, in which case the depolarization must be caused by something other than extracellular potassium. Secondly, and contradictorally, Bergmans (1983) proposed that hyperpolarization

by the sodium pump might induce hyperexcitability by reducing accommodation, as in post-ischaemic nerve. We recently presented evidence that post-ischaemic discharges are caused by transitions between two membrane potential states, induced by a combination of increased extracellular potassium and increased activity of the electrogenic sodium pump (Bostock, Baker & Reid, 1991). However, the post-ischaemic discharges are very different from the most common type of ectopic activity induced by 4-AP in demyelination: the pacemaker potentials we have recorded fit neither with the state of being depolarized by potassium, nor with the state of being hyperpolarized by the pump, nor with transitions between the two states.

Thirdly, impulses have been observed reflected from demyelinating lesions (Howe, Calvin & Loeser, 1976) and the mechanism of repeated reflections between two sites of demyelination has been proposed for the paroxysmal discharges in *tic douloureux* (Calvin, Loeser & Howe, 1977). Such a mechanism would be clearly revealed by our membrane current maps, but we have found no evidence for it. Fourthly, W. H. Calvin (personal communication) has suggested that ectopic activity in demyelination might be due to 'hot spots' of high sodium channel density, occurring as a side-effect of the proliferation of sodium channels thought to contribute to the recovery of function in demyelinated fibres. This mechanism was also suggested by Rasminsky (1981) for the ectopic activity arising in the amyelinated spinal roots of dystrophic mice, where the focal accumulations of channels may have been visualized in freeze-fracture micrographs (Bray, Cullen, Aguayo & Rasminsky, 1979). Our observation which provides most support for this hypothesis is that ectopic activity sometimes appears to arise at a site other than a node or heminode (e.g. Fig. 3*B*). Evidence for a low threshold (though not spontaneously active) extranodal 'hot spot' was previously obtained by Bostock & Sears (1978, their Fig. 12), also in diphtheritic demyelination. Since the normal density of sodium channels in internodal membrane is considered to be rather low (Grissmer, 1986; Chiu & Schwarz, 1987) these extranodal foci generating strong inward current presumably indicate sites of sodium channel proliferation, consequent on the demyelination.

Although our membrane current recordings provide support for the idea that some ectopic foci are sodium channel 'hot spots', it is not clear that a high density of sodium channels alone would lead to spontaneous activity. In a model proposed recently for the currents in normal human motor axons, sodium channels provide only a very minor fraction of the resting inward current (Bostock *et al.* 1991). When we attempted to mimic the pacemaker activity in demyelinated axons by varying parameters in this model, we found that this could be done by adding steady inward current and reducing the potassium conductances, but not by increasing the number of sodium channels. Removal of myelin on its own would not be expected to activate an inward current, in agreement with our suggestion that only a minority of demyelinated fibres are depolarized and prone to generate ectopic action potentials. We therefore propose a fifth hypothesis for the ectopic discharges, that they are due to depolarization mediated by ion channels in the demyelinated axon membrane, such as stretch-activated or ligand-gated channels.

Patch clamp studies have shown that mechanosensitive ion channels should be regarded as normal constituents of cell membranes (Morris, 1990). Stretch-activated,

non-selective cation channels have been described in the cell bodies of dorsal root axons (Yang, Guhary & Sachs, 1986). Mechanosensitivity was a conspicuous property of many of our demyelinated spinal roots, and has been observed in other demyelinating lesions associated with spontaneous ectopic activity (Smith & McDonald, 1980; Calvin *et al.* 1982) as well as in multiple sclerosis (Nordin, Nyström, Wallin & Hagbarth, 1984). If the mechanosensitivity of demyelinated axons is due to easy activation of stress-activated channels, then the spontaneous activity may also be due to depolarization by these channels, activated by mechanical stresses from within the root or fibre due to non-uniform loss of myelin. The activity is facilitated by 4-AP, since 4-AP blocks potassium channels which normally oppose depolarization. Demyelination is also associated with inflammation and a breakdown of the blood–nerve barrier, and an alternative hypothesis for the depolarization of some demyelinated axons is that it results from exposure of axonal channels (normally covered by myelin) to inflammatory mediators or blood-borne ligands with an excitatory action.

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