

## FUNCTION AND DISTRIBUTION OF THREE TYPES OF RECTIFYING CHANNEL IN RAT SPINAL ROOT MYELINATED AXONS

BY M. BAKER, H. BOSTOCK, P. GRAFE\* AND P. MARTIUS\*

*From the Sobell Department of Neurophysiology, Institute of Neurology, Queen Square, London WC1N 3BG and the \*Department of Physiology, University of Munich, Pettenkoferstrasse 12, 8000 Munchen 2, F.R.G.*

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### SUMMARY

1. The nature, distribution and function of rectifying channels in rat spinal root myelinated axons has been assessed with selective blocking agents and a variety of intracellular and extracellular recording techniques.

2. The electrotonic responses of roots poisoned with tetrodotoxin (TTX) to constant current pulses had fast (rise time  $\ll 1$  ms) and slow components, which were interpreted in terms of Barrett & Barrett's (1982) revised cable model for myelinated nerve. Depolarization evoked a rapid outward rectification (time constant,  $\tau \sim 0.5$  ms), selectively blocked by 4-aminopyridine (4AP, 1 mM), and a slow outward rectification ( $\tau \sim 15$  ms), selectively blocked by tetraethylammonium (TEA, 1 mM) or  $\text{Ba}^{2+}$  (0.5 mM). Hyperpolarization evoked an even slower inward rectification, selectively blocked by  $\text{Cs}^+$  (3 mM) but not by  $\text{Ba}^{2+}$ .

3. From the different effects of the blocking agents on the fast and slow components of electrotonus, it was deduced (a) that the inward rectification is a property of the internodal axon, (b) that the slow outward rectifier is present at the nodes, and probably the internodes as well, and (c) that the 4AP-sensitive channels have a minor nodal and a major internodal representation.

4. TEA and  $\text{Ba}^{2+}$  reduced the accommodation of roots and fibres not poisoned with TTX to long current pulses, whereas 4AP facilitated short bursts of impulses in response to a single brief stimulus.

5. TEA and  $\text{Ba}^{2+}$  also abolished a late hyperpolarizing after-potential (peaking at 20–80 ms), while 4AP enhanced the depolarizing after-potential in normal fibres, and abolished an early hyperpolarizing after-potential (peaking at 1–3 ms) in depolarized fibres. Corresponding to the later after-potentials were post-spike changes in excitability and conduction velocity, which were affected similarly by the blocking agents.  $\text{Cs}^+$  increased the post-tetanic depression attributable to electrogenic hyperpolarization.

6. The physiological roles of the three different rectifying conductances are discussed. It is also argued that the prominent ohmic 'leak conductance', usually ascribed to the nodal axon, must arise in an extracellular pathway in series with the rectifying internodal axon.

## INTRODUCTION

Present knowledge about the rectifying properties of mammalian peripheral nerve is based mainly on voltage-clamp data from nodes of Ranvier in isolated fibres. Such studies have indicated that apart from  $\text{Na}^+$  channels the main conductance is provided by an ohmic 'leak', with a small (Brismar, 1980; Brismar & Schwarz, 1985) or negligible (Chiu, Ritchie, Rogart & Stagg, 1979) delayed rectification playing an insignificant part in spike repolarization. On the other hand, recordings from chronically (Sherratt, Bostock & Sears, 1980; Brismar, 1981; Bostock, Sears & Sherratt, 1981) and acutely (Chiu & Ritchie, 1980, 1981) demyelinated mammalian fibres have revealed large delayed rectifier currents, which do help limit action potential duration in these axons.

If most of the rectifying channels in mammalian myelinated nerve are situated underneath the myelin sheath, it follows that techniques other than nodal voltage clamp must be used to reveal their normal functions. The first good evidence that the properties of the internodal axon are important in normal nerve physiology came from Barrett & Barrett's (1982) study with intra-axonal micro-electrode recordings from frog and lizard fibres. They produced evidence that the internodal axon normally has a long time constant ( $> 10$  ms), and that the depolarizing after-potential (d.a.p.) is caused by passive dissipation of a charge acquired by the internode during the action potential. Barrett & Barrett (1982) found a strong voltage dependence of input conductance and d.a.p. amplitude in the frog and lizard fibres, and Scappaticci & Barrett (1983) found that the  $\text{K}^+$ -channel blocker tetraethylammonium chloride (TEA) markedly reduced the input conductance and enhanced the d.a.p. In a recent micro-electrode study of the d.a.p. in rat spinal cord axons, Blight & Someya (1985) have confirmed its voltage dependence, but they did not test the effects of  $\text{K}^+$ -channel blockers, or measure rectification directly. In mammalian fibres TEA has appeared to be a less effective  $\text{K}^+$ -channel blocker than 4-aminopyridine (4AP). Unlike TEA, 4AP blocks the outward currents induced by demyelination (Bostock *et al.* 1981), and prolongs action potentials in immature and regenerating fibres (Ritchie, 1982; Kocsis & Waxman, 1983; Kocsis, Ruiz & Waxman, 1983; Bowe, Kocsis & Waxman, 1985). In immature dorsal roots, 4AP also induces bursts of action potentials in response to a single, brief stimulus (Kocsis *et al.* 1983; Bowe *et al.* 1985).

Using extracellular as well as intracellular current-injection and recording techniques, we have found that in addition to the 4AP-sensitive conductance, mammalian fibres have slow outward and inward rectifying channels, selectively blocked by TEA and  $\text{Cs}^+$  respectively. To determine the functions and probable locations of these three different channel types in normal mammalian nerve, we have compared the actions of the different  $\text{K}^+$ -channel blockers on (a) the electrotonic responses of whole rat spinal roots and single fibres to constant current pulses, (b) their accommodative properties, and (c) the after-potentials and excitability changes of single fibres following single action potentials or trains of impulses. All of our results, as well as much early unexplained data on electrotonus and after-potentials, can be accounted for qualitatively in terms of Barrett & Barrett's (1982) electrical model of the cable structure of myelinated nerve, and three types of rectifying channel with distinctive pharmacology, potential dependence and kinetics. Brief preliminary reports

have been published on some of these results (Bostock & Grafe, 1985*b*; Grafe, Martius & Bostock, 1985; Baker, Bostock & Grafe, 1985).

#### METHODS

Experiments were carried out *in vivo* (in London), with a spinal root suspended in a pool of liquid paraffin, and *in vitro* (in Munich), with the root in a chamber continuously perfused with Ringer or test solution. The *in vivo* preparation was preferred for extracellular recordings of electrotonus, and for measurements of excitability of single nodes of Ranvier. The *in vitro* preparation was used for intracellular recordings of electrotonus, accommodation and after-potentials, and for continuous monitoring of excitability changes by their effects on conduction velocity.

##### *In vivo methods*

Female Wistar or Lewis rats, weighing 200–250 g, were anaesthetized with sodium pentobarbitone (40 mg/kg, supplemented as required), and the spinal roots exposed by laminectomy. The Lewis rats required fewer supplemental doses of anaesthetic, and were preferred for that reason: no electrophysiological difference between the strains was observed. Excitability of single nodes of Ranvier was measured in intact roots as previously described (Bostock, Sears & Sherratt, 1983; Bostock & Grafe, 1985*c*). Electrotonus was recorded in cut roots suspended in liquid paraffin, by injecting current at one point, and recording the potential between a nearby point and the cut end (Fig. 1*A*). Although non-polarizable (Ag–AgCl–agar bridge) electrodes were sometimes used for current injection and recording, indistinguishable results were obtained with platinum wire electrodes, connected to a constant-current source and electrometer amplifiers (input resistance  $10^{15}$   $\Omega$ ) respectively. The wire electrodes facilitated precise replacement of the electrodes after withdrawal to apply drops of the test solutions. Potential and current wave forms were recorded with a digital oscilloscope (Gould OS4040), and averaged ( $n = 10$ ) with a PDP 11/23 computer, which was also used for controlling the current pulses, and for measuring and plotting the results. Drugs were applied in an artificial cerebrospinal fluid containing (mmol/l): NaCl, 130; KCl, 2;  $\text{KH}_2\text{PO}_4$ , 1.25;  $\text{MgCl}_2$ , 1.1;  $\text{CaCl}_2$ , 1.1;  $\text{NaHCO}_3$ , 23; glucose, 10; and gassed with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  (pH = 7.4).

##### *In vitro methods*

The *in vitro* preparation and recording techniques were described in a previous paper (Bostock & Grafe, 1985*c*). In brief, the excised spinal root was held in position in a perspex chamber (volume 1–2 ml) by two suction electrodes, which were also used for stimulation and recording of compound action potentials. The chamber was continuously perfused (flow rate 2–4 ml/min) with a standard solution, to which drugs were added. The standard solution contained (mmol/l): NaCl, 118; KCl, 3.0;  $\text{CaCl}_2$ , 1.5;  $\text{NaHCO}_3$ , 25;  $\text{NaH}_2\text{PO}_4$ , 1.2;  $\text{MgCl}_2$ , 1.0; glucose, 10; and was gassed with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  (pH = 7.4).

Intracellular recordings were made by a Dagan 8100 single-electrode system and micro-electrodes (filled with 3 M-KCl) with resistances in the range of 25–50 M $\Omega$ . Intracellular current injections and voltage recordings were performed via the same micro-electrode. Because the time constant of the electrode was comparable to that of the fast component of electrotonus, only the slow components of electrotonus could be recorded without errors due to the electrode resistance. Nevertheless, we tried to measure the fast electrotonus as accurately as possible. By testing the voltage reading of the electrode during current injections before and after impalement of an axon, we were able to estimate whether the bridge balance used in the bridge current-clamp mode, or the capacity compensation used in the switched-clamp mode (3 kHz, 50% duty cycle), were correctly set to compensate for the electrode resistance. Data were discarded when the extracellularly recorded voltage drop across the electrode resistance exceeded 20% of the intracellularly recorded fast electrotonus. Potential and current wave forms were recorded and averaged with a digital oscilloscope (Nicolet 4094), and data stored on floppy disks for off-line analysis and plotting.

Post-spike excitability changes were detected by comparing the conduction latencies for control and test compound action potentials, triggered either 10 ms apart (for assessment of superexcitability), or following more complex pulse protocols (see Fig. 13). Latencies were measured with a 2-channel latency monitor, triggered by the action potential reaching half its peak amplitude (see Bostock & Grafe, 1985*c*).

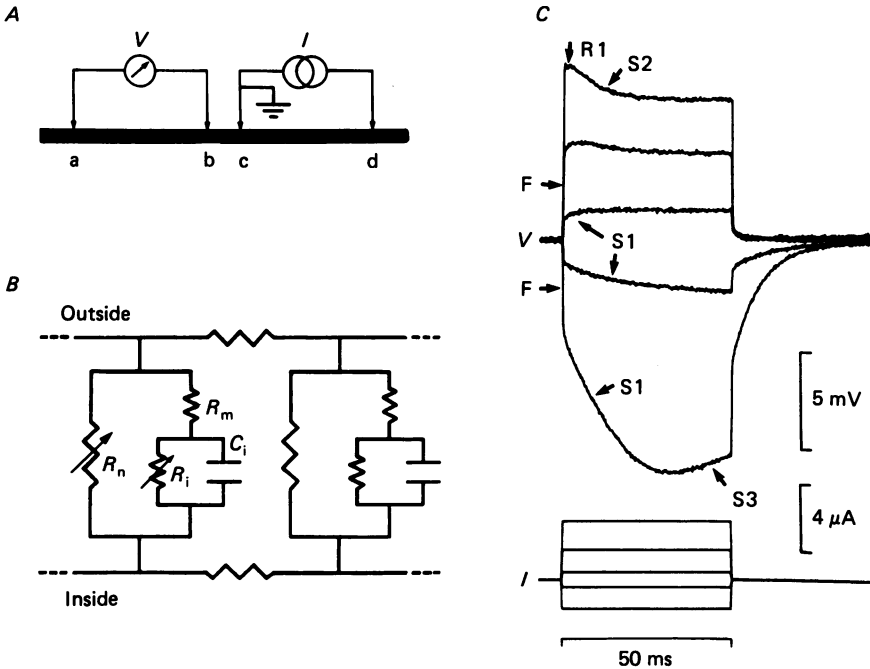


Fig. 1. Extracellular recording of electrotonus from whole spinal root. *A*, method of applying current and recording potential. *B*, simplified equivalent circuit to account for fast and slow components (cf. Barrett & Barrett, 1982, Fig. 9). ( $R_n$ , nodal resistance;  $R_m$ , myelin and internodal leakage resistance;  $R_i$ , resistance of internodal axon membrane; and  $C_i$ , capacitance of internodal axon membrane.) *C*, typical electrotonic potentials ( $V$ ) evoked by constant current pulses ( $I$ ) in a rat dorsal root treated with TTX, showing the fast (F) and slow (S1–S3) components described in the text. R1 indicates the position of an additional component, shown more clearly in Fig. 5.

## RESULTS

### *Electrotonus*

As originally observed by Lorente de No (1947), electrotonus in myelinated nerve is characterized by a fast component (rise time  $\ll 1$  ms), followed by slow potential changes, which are much more sensitive to membrane potential. Fig. 1*A* illustrates the method, and Fig. 1*C* the electrotonic responses recorded extracellularly from a rat dorsal root poisoned with TTX, showing the variation in wave form obtainable with currents of different amplitude and polarity. Electrotonus in ventral roots has the same general features, although the amplitudes of the different components differ between the motor and sensory roots. Also, because of the cable structure of the nerve, injection of constant current pulses results in potential wave-forms which depend on the distance between current injection and recording sites (Lundberg, 1951; Bostock & Grafe, 1985*a*). For the purposes of obtaining a qualitative picture of the effects of  $K^+$ -channel blockers on electrotonus, we have standardized on an interelectrode distance of 1 mm for the extracellular recordings.

The principal components of electrotonus associated with the onset of a current

pulse are arrowed in Fig. 1 *C*. The fast component (F), which appears instantaneous with this sweep duration, is almost symmetrical in the depolarizing (upwards) and hyperpolarizing (downwards) directions. Three slow components may be distinguished: S1, a slow potential change in the same direction as the fast electrotonus, seen in the responses to small current pulses in either direction, and exaggerated by

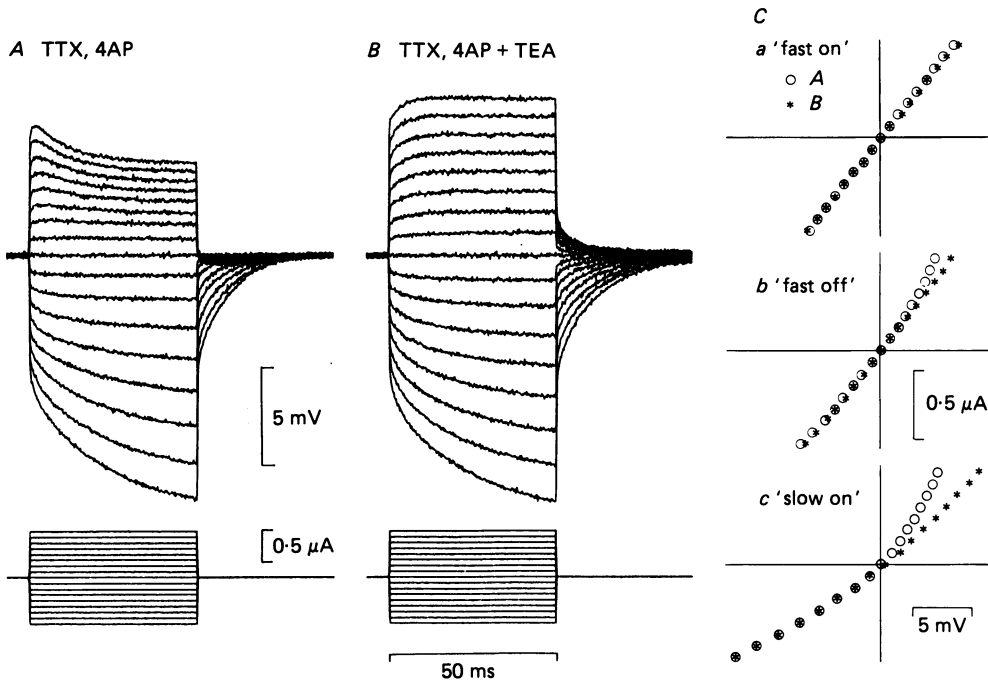


Fig. 2. Effects of TEA on electrotonus. *A*, electrotonus in ventral root treated with TTX and 4AP (5 mM). *B*, same after applying drop containing 5 mM-TEA in addition. *C*, amplitudes of components of electrotonus (see text). Temperature 30 °C.

hyperpolarization; S2, a slow sag in the responses to strong depolarizing currents, indicating an outwardly rectifying conductance; and S3, a slower, upwards 'sag' in the responses to strong hyperpolarizing currents, indicating an inwardly rectifying conductance. In addition, there is a barely discernible notch at the start of the responses to the strongest depolarizing current. With short sweep durations (see Fig. 5 *A*) this is seen more clearly as a rapid, outwardly rectifying component of electrotonus (R1), with a time course intermediate between those of the fast and slow components.

The classical models of myelinated nerve (Frankenhaeuser & Huxley, 1964; Goldman & Albus, 1968) account for only two of these components of electrotonus: F and R1. The fast component is the passive cable response, with an 'electrotonic time constant' (Bostock, 1983) determined mainly by the nodal capacitance and resistance and the capacitance of the myelin sheath. Nodal  $K^+$  channels (the classical 'delayed rectifier') account for the first rectifying component R1. On Barrett & Barrett's (1982) revised cable model (shown simplified in Fig. 1 *B*), the interpretation of the

fast component is modified, and S1 corresponds to the passive spread of charge to the internodal axolemma. The remaining components of electrotonus, the S2 and S3 sags, have not been described since the work of Lorente de No (1947) and Lundberg (1951). We will show that the rectifying components R1, S2 and S3 are selectively blocked by 4AP, TEA and Cs<sup>+</sup> respectively, indicating that they are generated by different types of ion channel.

To interpret the changes in fast and slow components of electrotonus induced by different drugs, we have used the simplified model in Fig. 1*B*, which omits the nodal and myelin capacitances (thus making fast electrotonus instantaneous). On this model, the amplitude of the fast component depends on the nodal resistance ( $R_n$ ), and also the resistance of a pathway to the internodal axolemma through or underneath the myelin sheath ( $R_m$ ), which we will assume is unaffected by the drugs or by current flow. (The probable nature of this current path is discussed by Barrett & Barrett, 1982, and by Blight, 1985.) It follows that changes in fast electrotonus indicate changes in the nodal resistance, and we will use this argument to distinguish between blockage of nodal and internodal channels.

*Effects of TEA.* Fig. 2 shows that the outwardly rectifying sag S2 is blocked by 5 mM-TEA. 4AP, as well as TTX, was present throughout, to reduce shunting by 4AP-sensitive channels and to avoid any changes in their contribution to the electrotonus resulting from the small depolarization usually caused by TEA. From the amplitude of the compound action potential recorded before TTX application (80 mV), the potentials should be increased by about 50% to obtain typical intracellular values. Below are the injected currents: we have not attempted to estimate the currents flowing within individual axons. On the right,  $I/V$  data have been plotted for three different potential measurements. 'Fast on' is the amplitude 0.5 ms after current application, when the fast component of electrotonus is virtually complete, and before the slower changes have progressed very far. The  $I/V$  curve is linear, and scarcely affected by TEA, indicating that any nodal TEA-sensitive channels take more than 0.5 ms to be activated. The 'fast off' changes were measured over the 0.5 ms after turn off of the current. In this case the  $I/V$  relationship is only linear after TEA, showing that there are nodal rectifying channels, activated between 0.5 and 50 ms, that are blocked by TEA. The 'slow on' potentials were measured at the ends of the 50 ms pulses. They show more TEA-sensitive rectification than the fast electrotonus, and it extends to smaller depolarizations. We infer, therefore, that TEA-sensitive channels are present in the internodes as well as the nodes, and that, perhaps simply because of their greater number, the internodal channels require less depolarization to generate a detectable conductance.

The time course of the change in nodal conductance blocked by TEA was explored by superimposing a 250 Hz square wave of current, as shown in Fig. 3. The amplitude of the fast electrotonus was thereby measured repeatedly during and after a 100 ms depolarization, and as we have already argued, fast electrotonus depends on the nodal resistance in parallel with a leakage pathway ( $R_n$  and  $R_m$  in Fig. 1*B*). The changes in amplitude of the fast electrotonus are seen most clearly in the bottom row of traces in Fig. 3*C*, obtained by digital subtraction of the traces without the 250 Hz square wave from those with it. The fast electrotonus declined with a time constant (*ca.* 15 ms) comparable to the slow outward rectification, and it recovered with a

similar time course on repolarization. Since the leakage pathway ( $R_m$  in Fig. 1*B*) is assumed constant, these changes should reflect faithfully the rise and fall of the nodal TEA-sensitive conductance.

After the end of the 100 ms depolarization, the peak depolarizations reached with the superimposed 250 Hz square wave (Fig. 3*B*, centre trace) were initially much reduced, because of the continuing TEA-sensitive conductance, whereas the peak

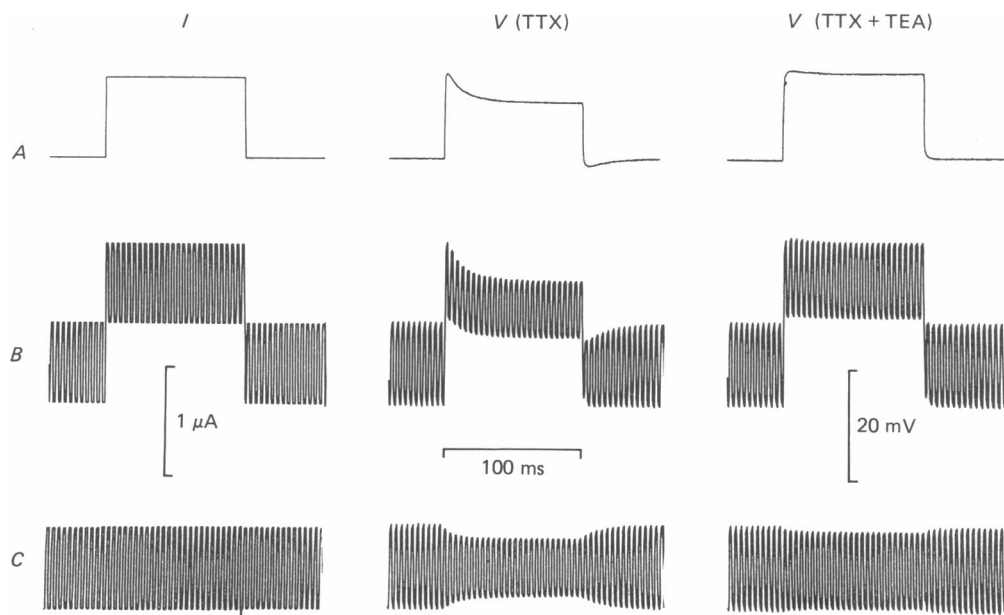


Fig. 3. Time course of slow, TEA-sensitive rectification. In this Figure only, applied current wave forms are shown in the left hand column, and recorded electrotonic potentials in the centre and right hand columns. *A*, responses of TTX-treated ventral root to  $0.8 \mu\text{A}$  depolarizing current pulse, before and after applying a drop of 5 mM-TEA. *B*, similar, but 250 Hz square wave ( $0.8 \mu\text{A}$  peak to peak, symmetrical about zero current) added to current pulse. *C*, records obtained by digital subtraction of those in *A* from those in *B*, to show changes in fast component of electrotonus. Temperature  $30^\circ\text{C}$ .

hyperpolarizations were virtually constant. Since this level of hyperpolarization was unaffected by the continuing TEA-sensitive conductance, and unaffected by TEA (compare Fig. 3*B*, right hand trace), it must have been close to the equilibrium potential for the nodal TEA-sensitive channels. This potential was 8 mV (corresponding to about 12 mV intracellularly) more hyperpolarized than the resting potential.

These extracellular recordings of the action of TEA on electrotonus in whole nerve are closely matched by intracellular recordings (see Figs. 6 and 10 below). In continuously superfused roots, 1 mM-TEA produces a near maximal effect, which is readily reversed by washing (Fig. 10).  $\text{Ba}^{2+}$ , at a concentration of 0.5 mM, mimics 1 mM-TEA (Fig. 6).

*Effects of 4AP.* Figs. 4 and 5 illustrate the actions of 4AP on electrotonus in spinal roots treated with TTX and TEA. Different duration pulses were used to demonstrate

two apparently different effects of 4AP, both quite distinct from those of TEA. Comparing Fig. 4A with 4B, the most conspicuous change is that the flat top of the depolarizing responses in Fig. 4A changes to a more rounded onset in Fig. 4B. Measurement of the fast and slow components shows (Fig. 4C) that the increase in depolarizing electrotonus nearly all occurred after the first 0.5 ms (at which the 'fast

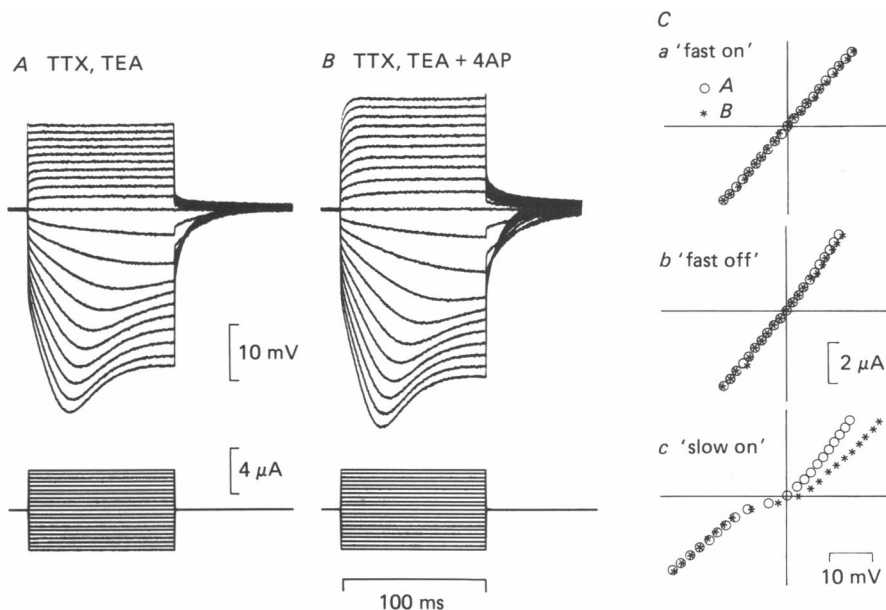


Fig. 4. Effects of 4AP on electrotonus. *A*, electrotonus in rat dorsal root poisoned with TTX and TEA (5 mM). *B*, same after applying drop containing 5 mM-4AP in addition. *C*, amplitudes of components of electrotonus (see text). Temperature 30 °C.

on' measurements were made). Also, the amplitude of the fast component at the end of the pulse ('fast off', Fig. 4Cb) was only slightly altered by 4AP, indicating that the substantial increase in slow electrotonus (Fig. 4Cc) could not be explained by block of a nodal conductance. A dorsal root is illustrated, since 4AP has more conspicuous effects on dorsal than ventral roots (cf. Bowe *et al.* 1985). The action of 4AP on the internodal axon was probably composite: block of some channels open at rest, and of others that open on depolarization. A contribution of 4AP-sensitive channels to the resting conductance has been shown directly by intracellular recording, and has been allowed for in the 'slow on' measurements in Fig. 4Cc by shifting the potential changes in 4AP 2.45 mV in the depolarizing direction so that the most hyperpolarized potentials superimpose.

There is a slight notch at the start of the plateau to the strongest depolarizing currents in Fig. 4A, corresponding to the notch labelled R1 in Fig. 1C. This rapid component of electrotonus is shown more clearly at a faster sweep speed in Fig. 5A. In this case 2 ms current pulses were applied to a ventral root, of sufficient amplitude to depolarize the root by as much as 60 mV (corresponding to about 90 mV intracellularly). R1 was abolished by 4AP application, as shown in Fig. 5B.



Comparison of the electrotonic wave forms before and after 4AP application (Fig. 5C) shows that the R1 component of electrotonus can be ascribed to a 4AP-sensitive delayed rectification, that becomes faster with increasing depolarization. The hyperpolarizing responses were unaffected by 4AP. Measurements of the fast component of electrotonus at the ends of the pulses (not illustrated) showed that

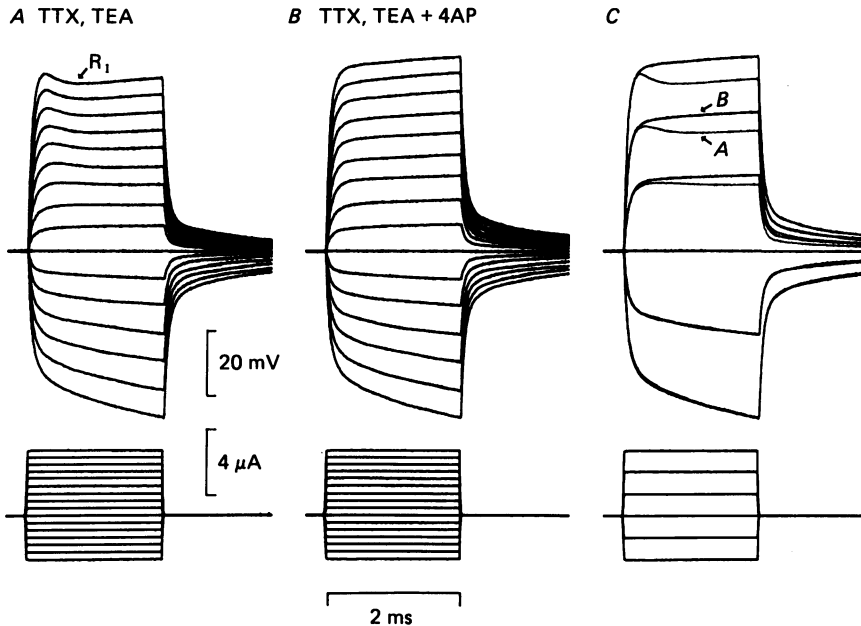


Fig. 5. Effect of 4AP on early electrotonus in rat ventral root. *A*, electrotonic responses to 2 ms depolarizing pulses in root treated with TTX and TEA (5 mM). R1 indicates the rapid component of electrotonus. *B*, responses after applying drop of 5 mM-4AP. *C*, every third trace in *A* and *B* superimposed, to show time course of 4AP-sensitive rectification. Temperature 30 °C.

R1, like the much slower sag S2, is associated with a reduction in fast electrotonus and therefore an increase in nodal conductance.  $K^+$  currents with properties to match this 4AP-sensitive conductance have been described in voltage-clamped rat nodes by Brismar (1980) and Brismar & Schwarz (1985). In other mammalian myelinated fibres such currents appear after treatments designed to loosen paranodal myelin (Chiu & Ritchie, 1981), and we have found that the R1 component of electrotonus can be increased markedly by treating a root with alternating hyper- and hypo-osmotic solutions.

*Interaction between effects of 4AP and TEA or  $Ba^{2+}$ .* Fig. 6 shows how the effects of 4AP and TEA or  $Ba^{2+}$  appear to depend on which drug is applied first. Fig. 6A illustrates an intracellular record from a rat ventral root axon. First, TEA was applied and resulted in an increase in depolarizing electrotonus with a slow onset. Secondly, application of 4AP in the presence of TEA removed the early notch (R1) and increased the slow electrotonus throughout the depolarizing pulse. A similar observation was made with an extracellular recording from a dorsal root in which  $Ba^{2+}$

was used instead of TEA to block S2 (Fig. 6C). If, however, 4AP was applied before TEA (Fig. 6D), a much weaker action of this drug, primarily on the early phase of the depolarizing pulse, was observed. TEA, on the other hand, when added in the presence of 4AP, had a much stronger action as compared to its effect when applied alone. It appears, therefore, that in each case the drug applied second has the greater

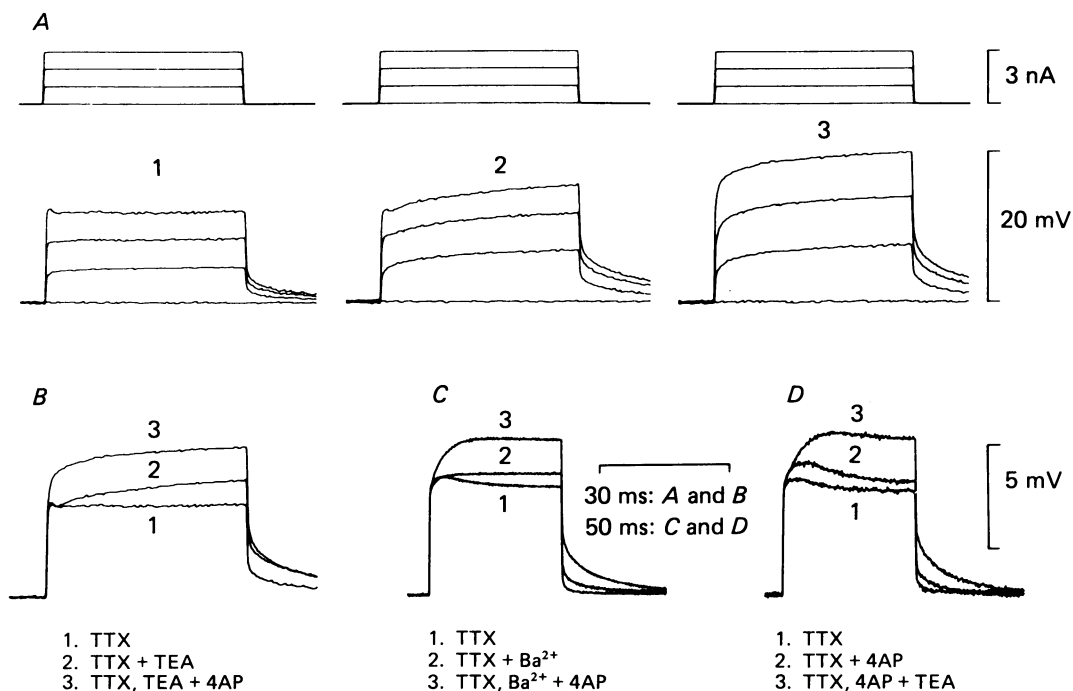


Fig. 6. Interaction between effects of 4AP and TEA or Ba<sup>2+</sup> on electrotonus. *A* illustrates intracellular recordings from a rat ventral root fibre. The electrotonic responses to three current pulses (switched-clamp mode) were recorded: 1, in the presence of TTX; 2, 3 min after the addition of 1 mM-TEA; and 3, 5 min after the further addition of 3 mM-4AP. *B* shows the three voltage responses to the strongest depolarizing pulses shown in *A*. *C* and *D* show comparable extracellular recordings from two rat dorsal roots. In *C* the current stimulus was 2.3  $\mu$ A, and 0.7 mM-Ba<sup>2+</sup> was added before 5 mM-4AP. In *D* the current stimulus was 1.8  $\mu$ A, and 5 mM-4AP was added before 5 mM-TEA. Comparing *C* and *D*, the electrotonic wave forms in TTX (1) and with the combined drugs (3) were very similar, but the effects of the single K<sup>+</sup>-channel blockers (2) were distinct.

effect on the slow electrotonus. This is only to be expected when two parallel conductance pathways are blocked in turn. We will show later that the effects of 4AP and TEA on the early (H0) and late (H1) post-spike after-hyperpolarizations can also be explained by this view (Figs. 11 and 12).

*Effects of Cs<sup>+</sup>.* Cs<sup>+</sup> rapidly and reversibly reduces S3, the inwardly rectifying component of electrotonus. Fig. 7A shows extracellular recordings from a whole ventral root previously treated with TEA and 4AP as well as TTX. Like the recordings in Fig. 4B from a dorsal root in the same solution, they show a conspicuous upwards 'sag' (S3) in response to hyperpolarizing currents. This corresponds to inward rectification, since the slope conductance increases with strong hyperpolar-

ization (Fig. 7*Cc*, open circles). The S3 'sag' was abolished by 5 mM-Cs<sup>+</sup> (Fig. 7*B*), and the *I/V* relationship became more linear in the hyperpolarizing direction (Fig. 7*Cc*, asterisks). Fig. 8 shows similar recordings made with an intracellular micro-electrode (but without TEA or 4AP). Neither the inward rectification nor the Cs<sup>+</sup> affected the amplitude of the fast electrotonus, as shown by the superimposed *I/V* plots in Fig. 7*Cb*. In this respect, the inward rectification differs from the TEA-sensitive slow outward rectification, which reduces the 'fast off' component (Fig. 2*Cb*). Similarly, superimposing a 250 Hz square wave of current as in Fig. 3 failed to show any change in the fast electrotonus related to the inward rectification. We

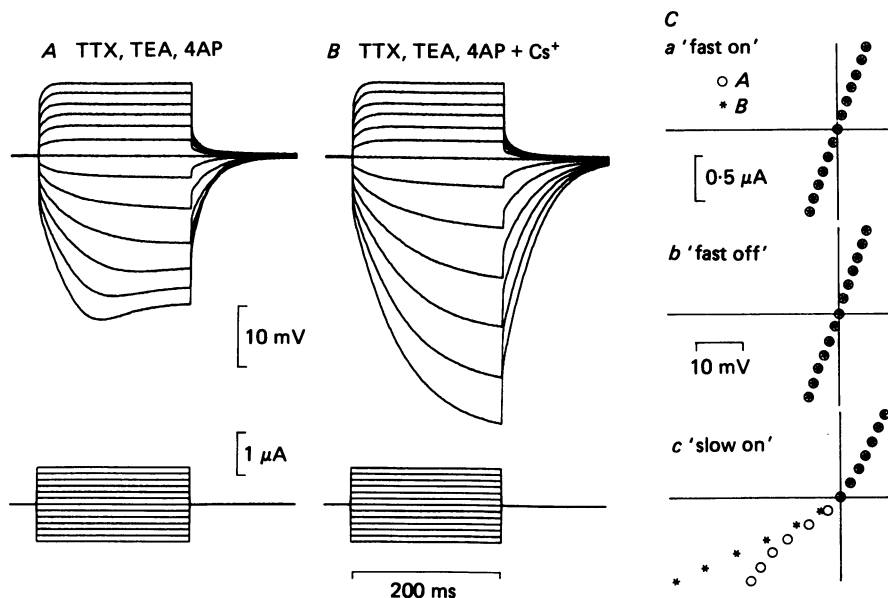


Fig. 7. Effects of Cs<sup>+</sup> on electrotonus. *A*, electrotonus recorded extracellularly from rat ventral root poisoned with TTX, TEA (5 mM) and 4AP (5 mM). *B*, same after applying drop containing 5 mM-Cs<sup>+</sup> in addition. *C*, amplitudes of components of electrotonus; the 'slow on' amplitudes were measured after 200 ms.

infer that the inward rectification is a property of the internodes alone. To aid comparisons with other inward rectifiers (see Discussion), it is worth mentioning that Ba<sup>2+</sup> did not imitate the action of Cs<sup>+</sup>.

These observations on inward rectification also provide information about the leak conductance. In Fig. 7*B* and *Cc* a strong residual rectification is evident, despite the combined actions of TEA, 4AP and Cs<sup>+</sup>. When hyperpolarized, the input resistance of the fibres is several times higher than the non-rectifying input impedance indicated by the fast electrotonus. The notion, derived from voltage-clamp experiments on isolated fibres, that there is a substantial time and voltage-independent leak conductance therefore requires revision (see Discussion).

#### *Accommodation and spike-frequency adaptation*

Motor axons are well known to accommodate rapidly to a sustained depolarization, and this is shown extracellularly in Fig. 9. With increasing strength of current

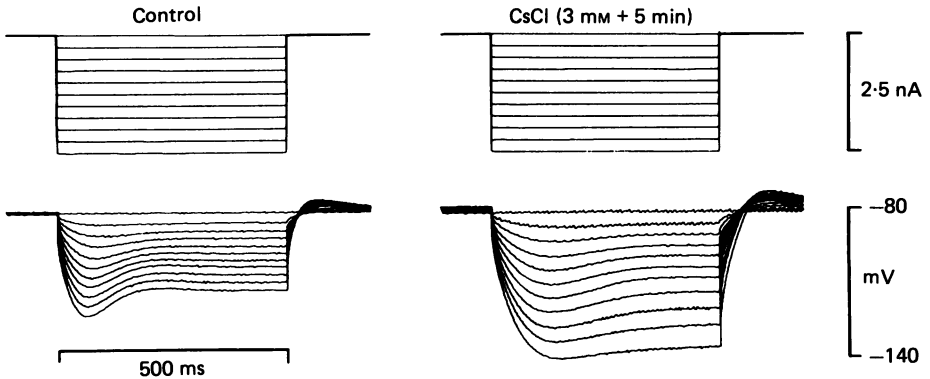


Fig. 8. Effects of  $\text{Cs}^+$  (3 mM) on electrotonus recorded intracellularly from TTX-poisoned rat ventral root fibre at  $30^\circ\text{C}$ .

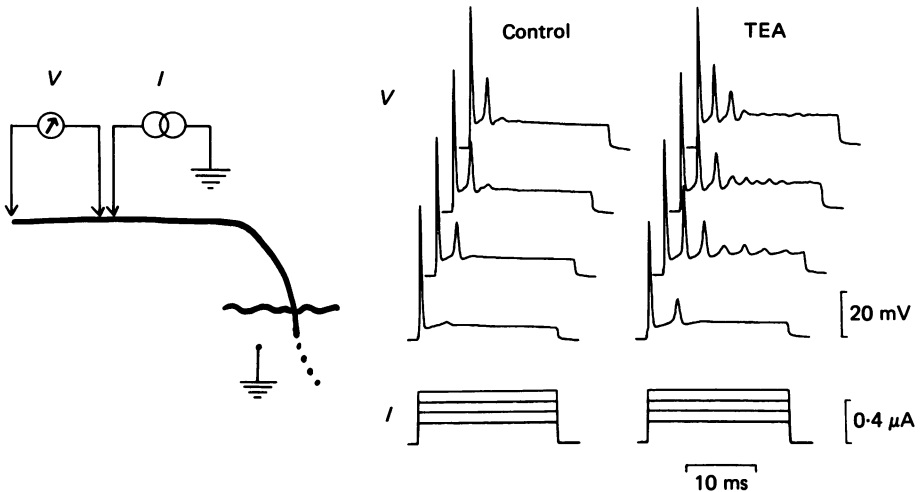


Fig. 9. Effects of TEA (5 mM) on accommodation of rat ventral root to 20 ms depolarizing pulses applied extracellularly.

stimulus, first a single compound action potential was recorded, then a second component, but no more than a trace of a third, however strong a current was applied. After treatment with 5 mM-TEA, however, a repetitive discharge became evident in at least some of the fibres. The same behaviour is shown intracellularly in Fig. 10 *A*. A typical double spike response to a standard depolarizing stimulus was changed by 1 mM-TEA into continuous firing, and the effect was reversed after washing for about 5 min. After washing off the TEA, TTX was applied to block the  $\text{Na}^+$  channels, and in *B* and *C* the effects of TEA on electrotonus in the same fibre are shown. The current was applied at a different site in *A* (the suction electrode), and therefore the electrotonus was recorded at a site different from the site of spike initiation. Nevertheless, we presume that the change in electrotonus observed was similar to that underlying the change in accommodation. In contrast to its strong action on accommodation, TEA (1 mM) did not change action potential duration (see also Fig. 7 in Bowe *et al.* 1985).

Experiments related to the effects of 4AP on accommodation and spike-frequency adaptation gave results similar to those described by Bowe *et al.* (1985) and are not illustrated. In both ventral and dorsal roots, 4AP treatment resulted in a slight prolongation of the action potential. Furthermore, an increase in amplitude of the depolarizing after-potential was observed, which resulted in repetitive spiking after

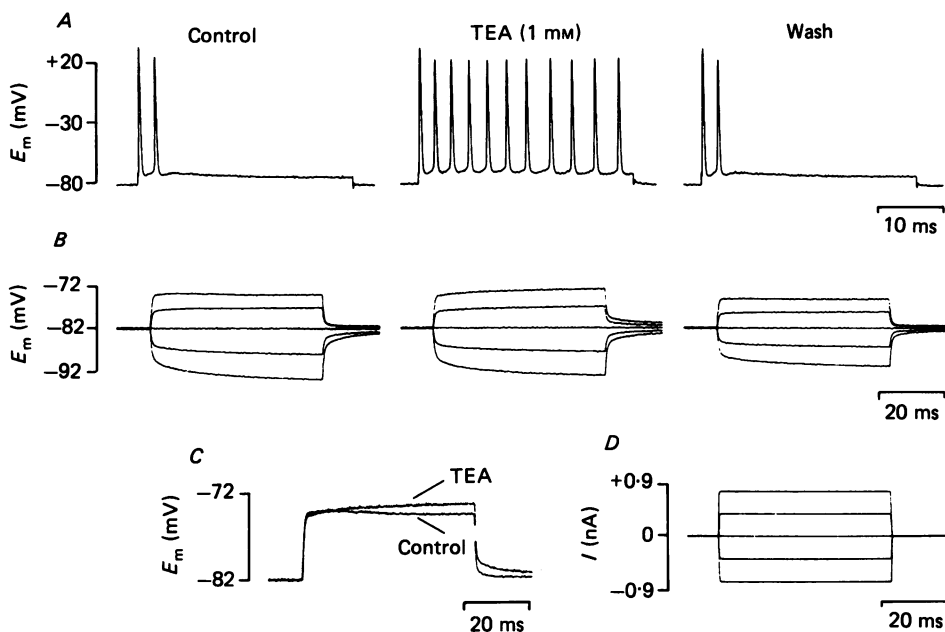


Fig. 10. Action of TEA (1 mM) on accommodation and electrotonus in single ventral root fibre. *A*, intracellularly recorded responses to a standard depolarizing pulse, applied through the suction electrode. *B* and *C*, electrotonic responses recorded in same fibre, after TTX treatment, to depolarizing currents, *D*, applied through micro-electrode. Membrane potential,  $E_m$ .

a single short stimulus in dorsal roots (and very occasionally in ventral roots). On the other hand, 4AP did not consistently facilitate repetitive firing of motor or sensory fibres during long pulses. Although, as with electrotonus, there is some interaction, our impression is that the 4AP-sensitive channels primarily prevent repetitive firing after a single spike, whereas the TEA-sensitive slowly rectifying channels underlie the accommodation and spike-frequency adaptation during long depolarizing pulses.

#### *After-potentials and impulse-dependent threshold changes*

The action potential of mammalian A fibres is followed immediately by a 'negative' or depolarizing after-potential (d.a.p.) and a subsequent hyperpolarization with at least two components (Gasser & Grundfest, 1936). Associated with these potential changes are changes in excitability, depolarization causing a decrease and hyperpolarization an increase in threshold current. Intracellular recordings from non-mammalian (Barrett & Barrett, 1982) and mammalian (Blight & Someya, 1985) axons have indicated that the d.a.p. and corresponding superexcitable period are due

to passive charge storage on the internodal axolemma. Two separate processes underlying the subsequent subexcitability have been inferred from recordings in man (Bergmans, 1970): an increase in  $K^+$  conductance, lasting up to 100 ms (H1), and an electrogenic pump component (H2) that increases almost indefinitely with a train of impulses. Micro-electrode recordings have confirmed the nature of H2

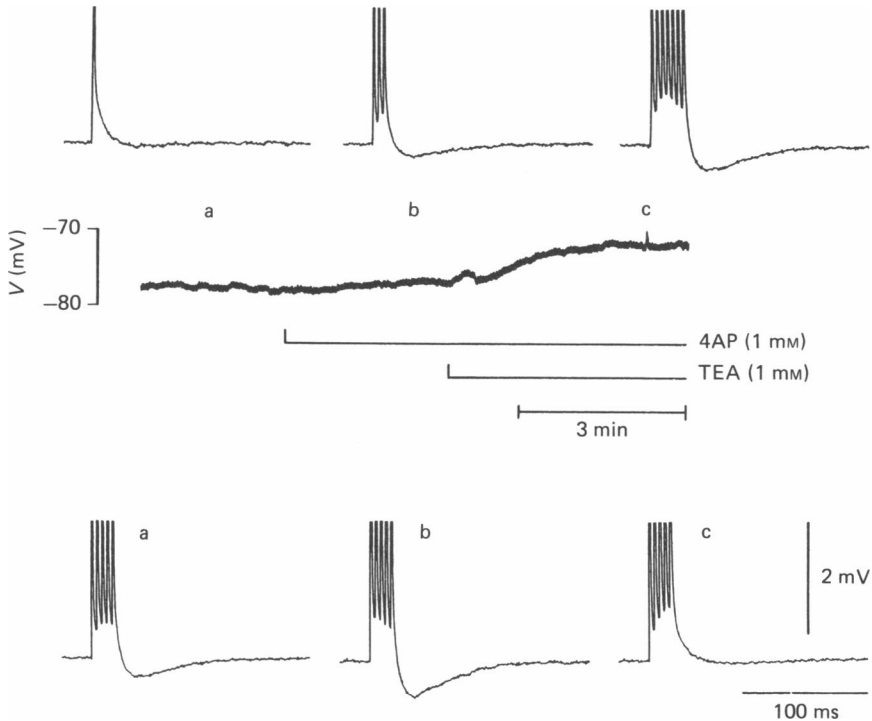


Fig. 11. Action of 4AP and TEA on the late after-hyperpolarization (H1), recorded intracellularly from a ventral root fibre. The uppermost panel shows that, following the d.a.p., a slow after-hyperpolarization (H1) is visible which increases in amplitude with the number of spikes. The bottom panel shows H1 in the normal bathing solution (a), 3 min after addition of 4AP (b), and 3 min after TEA was added to the 4AP-containing solution (c). The middle panel shows the concomitant changes in the membrane potential. In this and the following Figure, the action potentials (which are truncated) were triggered at the suction electrode.

(Bostock & Grafe, 1985c), but have not previously shown evidence of H1. Instead, micro-electrode studies have described an early hyperpolarizing current, which we shall call H0, sometimes occurring as a notch between the spike and the d.a.p., and increased by depolarization (Eccles & Krnjevic, 1959; Barrett & Barrett, 1982).

*Effects of TEA and 4AP.* We were able to record a small, but distinct after-hyperpolarization (H1) intracellularly from ventral root fibres, which was increased by short trains of impulses as described by Bergmans (1970). This after-hyperpolarization was facilitated by 4AP, but completely blocked by TEA (Fig. 11) or  $Ba^{2+}$ . *In vivo*, the H1 component can be detected as a small ( $\leq 1\%$ ) subexcitability following the superexcitable period, which is also abolished by TEA.

We could also find a 4AP-sensitive after-hyperpolarization in spinal root axons (Fig. 12). In depolarized fibres the d.a.p. was reversed to a short-lasting after-hyperpolarization (H0), which was increased in amplitude by TEA and reduced by 4AP (Fig. 12C, bottom row). The enhancement of H0 by TEA, and of H1 by 4AP, suggests an antagonism between the two drugs, but this interaction, like the facilitatory interaction previously described with electrotonus, can be explained by their blocking of two independent parallel conductances.

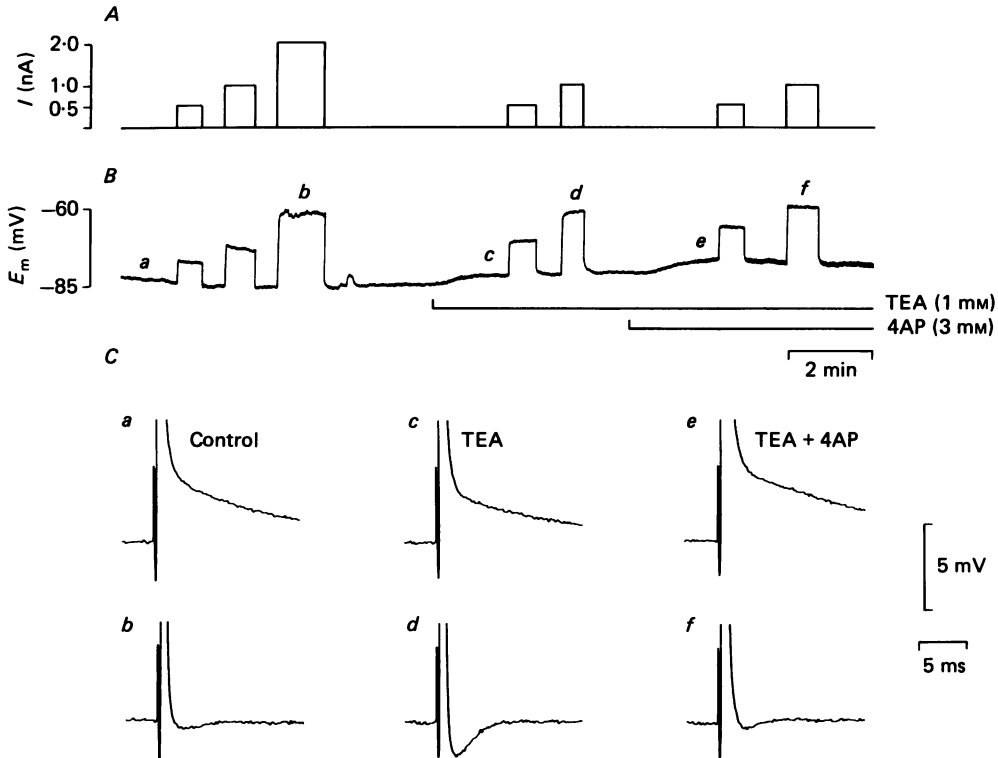


Fig. 12. Actions of TEA and 4AP on the early after-hyperpolarization (H0) recorded intracellularly from a ventral root fibre. Different membrane potential levels ( $E_m$ ) were obtained by passing currents ( $I$ ) through the micro-electrode (bridge current clamp) as shown in the top two panels (A and B). Examples of single spike after-potentials at the resting potential (a, c and e) and at approximately  $-60$  mV (b, d and f) are illustrated in the two lower panels (C). The d.a.p. is replaced, in depolarized fibres, by a short-lasting after-hyperpolarization (H0), which is increased by TEA (compare *Cb* with *Cd*) and decreased by 4AP (compare *Cd* with *Cf*).

Finally, TEA and 4AP were tested for effects on superexcitability, late subexcitability (corresponding to H1) and prolonged subexcitability (H2). As a measure of excitability changes we have used the latency of conduction of the compound action potential from one suction electrode to the other. An increase in latency is closely equivalent to an increase in threshold stimulating current (i.e. a reduction in excitability) for a uniform fibre (e.g. Bergmans, 1970; Bostock & Grafe, 1985c). Fig. 13 illustrates latency measures of superexcitability, H1 and H2, and how they

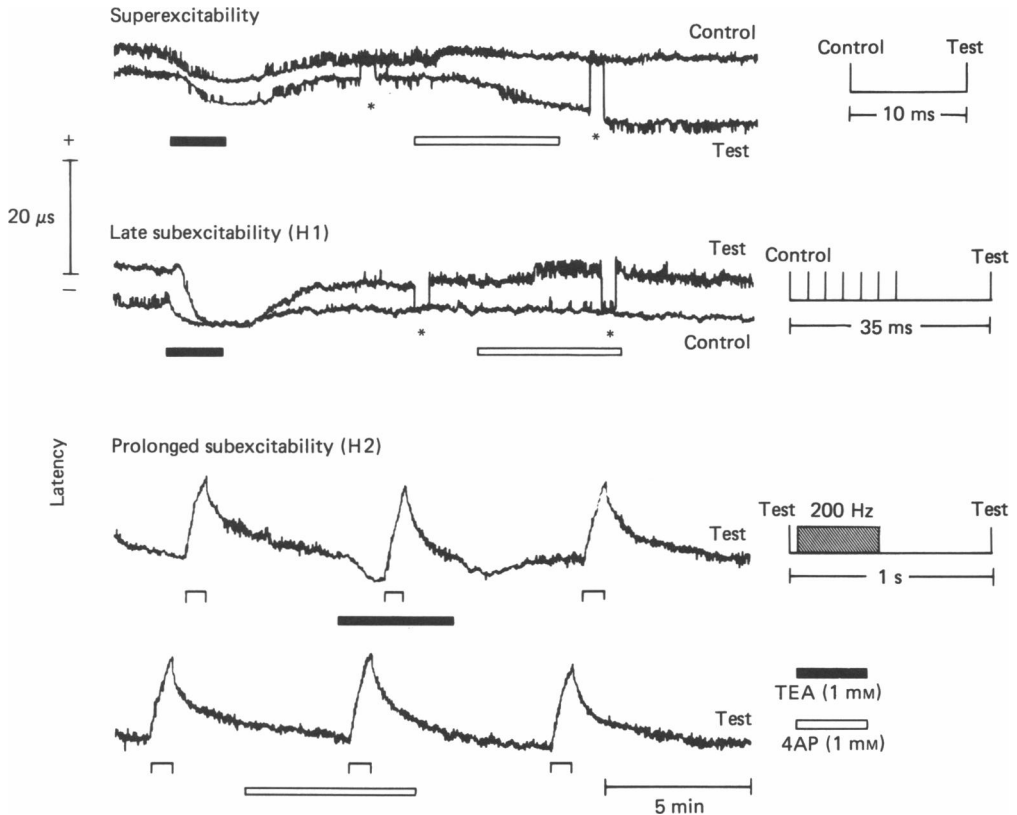


Fig. 13. Effects of TEA and 4AP on superexcitability, late subexcitability (H1), and prolonged subexcitability (H2) of a rat ventral root. The latencies were measured once per second from the compound action potentials recorded with a suction electrode. The relative timing of control and test stimuli are shown to the right: superexcitability was indicated by the reduced latency of a test spike triggered 10 ms after the control, subexcitability (H1) by the increased latency of a test spike 17 ms after a train of seven spikes at intervals of 3 ms, and prolonged subexcitability by the progressive slowing when each test spike was followed by a 400 ms, 200 Hz train (mean rate 80 Hz). The three different recordings were made from the same ventral root, which was washed for 1 h between each to allow recovery from the action of 4AP. The asterisks indicate times when the test spike was delayed (in the top panel by 90 ms, and in the middle panel by 352 ms).

are affected by TEA and 4AP. In the top panel, the control action potential was conducted about  $5 \mu\text{s}$  more slowly than the test one, travelling 10 ms later in the superexcitable period. This  $5 \mu\text{s}$  latency difference, measured every second, provides a sensitive and virtually continuous index of superexcitability. The latency of both action potentials was reduced by TEA, probably because of depolarization, but the latency difference indicating superexcitability was unchanged. 4AP, on the other hand, more than doubled the superexcitability, in agreement with its enhancement of the d.a.p.

The second panel of Fig. 13 shows the effects of TEA and 4AP on the late subexcitability (H1). The small latency difference normally observed in rat fibres was



enhanced by using a short train of conditioning spikes at 3 ms intervals, following Bergmans (1970). TEA abolished this subexcitability, just as it abolished the corresponding positive (hyperpolarizing) after-potential in Fig. 11.

The lower section of Fig. 13 shows the effects of TEA and 4AP on the prolonged subexcitability (H2) induced by a long train of impulses. In contrast to the earlier

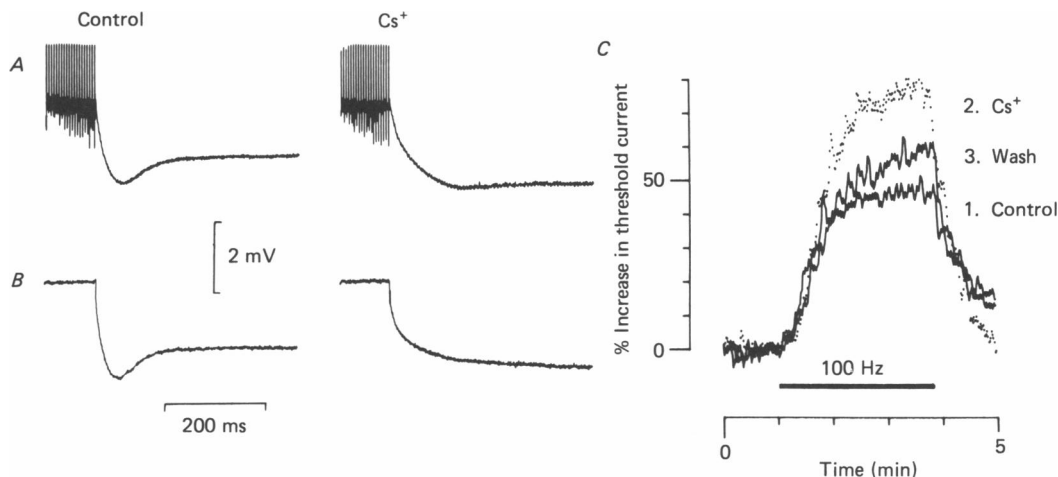


Fig. 14. Effects of  $\text{Cs}^+$  on after-potentials and excitability changes. *A*, after-potentials recorded from whole dorsal root after tetanizing at 150 Hz for 2 min, compared with electrotonic responses to a  $1 \mu\text{A}$  hyperpolarizing current (*B*) recorded after recovery from the tetanus. Responses on the right recorded similarly, after treatment with 5 mM- $\text{Cs}^+$ . (The spikes in *A* are distorted because of the limited digitizing rate and are truncated.) *C*, increases in threshold current required to excite a single node in a rat ventral root fibre during intermittent tetanization (100 Hz for 0.5 s every second), showing effect of 5 mM- $\text{Cs}^+$ .

excitability changes, H2 was not affected by either drug. We have previously shown (Bostock & Grafe, 1985c) that H2 is blocked by replacing extracellular  $\text{Na}^+$  with  $\text{Li}^+$ , and can be attributed to an electrogenic  $\text{Na}^+$  pump, activated by the rise in intracellular  $\text{Na}^+$ .

*Effects of  $\text{Cs}^+$ .* At concentrations up to 3 mM,  $\text{Cs}^+$  is without effect on the action potential or after-potentials following a single spike. Hyperpolarization by the electrogenic  $\text{Na}^+$  pump can, however, activate the  $\text{Cs}^+$ -sensitive inward rectification, as shown in Fig. 14. Electrogenic hyperpolarization of an *in vivo* ventral root by a prolonged tetanus is compared with hyperpolarization by an applied current. In each case there is a sag reduced by 1 mM- $\text{Cs}^+$ . The inward rectification blocked by  $\text{Cs}^+$  should therefore help limit the depression of axonal excitability caused by trains of impulses, and which can block conduction at sites of reduced safety factor (Bostock & Grafe, 1985c). This is shown in Fig. 14C. The threshold current required to excite a single node was tracked during identical 3 min tetanizations. The normal limit to the rise in threshold was overcome by treatment with 5 mM- $\text{Cs}^+$ .

## DISCUSSION

In this study the pharmacological agents TEA, 4AP, Ba<sup>2+</sup> and Cs<sup>+</sup> have been used to differentiate three different conductance systems in rat myelinated axons. We have performed a variety of experiments to help determine the separate functional roles of these conductances, and the probable distributions of the channels between nodal and internodal regions of axolemma. Here we bring together the different observations on each blockable conductance in turn, relating them to information from other sources, and we also consider the nature of the leak conductance.

*Slow, TEA-sensitive outward rectification*

This conductance is activated slowly (time constant *ca.* 15 ms at 37 °C) by depolarization; it reduces fast and slow electrotonus, and is important for accommodation, especially in motor fibres. Although activated too slowly to affect action-potential duration, it is responsible for the small hyperpolarizing after-potential (H 1) and subexcitability between about 20 and 80 ms. This subexcitability is probably too small (*ca.* 1%) to be of functional significance in the rat, but in human motor nerve fibres subexcitabilities as high as 10% following a single spike have been reported at comparable latency (Bergmans, 1970). Human median nerves are very similar (Stohr, 1981). It seems likely, therefore, that the slow outward rectification may be more important in man. In neither species is it clear why the mid-axon region of a nerve should be furnished with a mechanism, the most obvious effect of which is to limit ectopic firing due to a maintained depolarization. Minor trauma and ischaemia are common depolarizing agents which can induce firing in the middle of peripheral nerves, and it is possible that without the TEA-sensitive channels (and in spite of the accommodating effects of Na<sup>+</sup> inactivation), these effects would be exaggerated and prolonged intolerably.

From our observation that strong depolarizing pulses reduce the fast component of electrotonus (Figs. 2 and 3), we have concluded that a significant number of the TEA-sensitive channels are situated at or close to the nodes. It is surprising, therefore, that with one exception (Goren, Bergman & Palti, 1984), reports of K<sup>+</sup> currents in mammalian nodes under voltage clamp have not remarked on a slowly activating component. In frog nodes, however, Dubois (1981) has distinguished a slow K<sup>+</sup> (K<sub>s</sub>) current, which is sensitive to TEA and insensitive to 4AP like this one.

The cell bodies of the ventral root fibres have a slowly activating, TEA- and Ba<sup>2+</sup>-sensitive K<sup>+</sup> conductance (onset time constant 20–50 ms) (Barrett, Barrett & Crill, 1980), that is responsible for after-hyperpolarization, and which is also dependent on intracellular Ca<sup>2+</sup> concentration (Krnjevic, Puil & Werman, 1978*a*). A Ca<sup>2+</sup>-activated K<sup>+</sup> conductance, which is sensitive to TEA and Ba<sup>2+</sup> and insensitive to aminopyridines, has also been identified recently in mammalian motor nerve terminals (Mallart, 1985). We have, however, failed to influence the axonal slow conductance with dinitrophenol (used by Krnjevic, Puil & Werman, 1978*b*), or with the Ca<sup>2+</sup> ionophore A23187, which might be expected to raise intra-axonal Ca<sup>2+</sup> levels.

#### 4-AP-sensitive conductance(s)

The functional importance of 4-AP-sensitive  $K^+$  channels in mammalian fibres was demonstrated by Bowe *et al.* (1985). They found that immature dorsal root fibres in 4AP fire bursts of action potentials in response to a single brief stimulus, and even in the young adult rats we have studied this tendency was detectable. Ventral roots, stabilized perhaps by more of the TEA-sensitive channels, showed only a slight (< 5%) prolongation of the action potential in 4AP and an increase in the d.a.p. and superexcitability. For small displacements from the resting potential, fast electrotonus was not altered by 4AP (Fig. 4), so that the increase in slow electrotonus implies an increase in the resting resistance of the internodal axon, which would also account for the increase and prolongation of the d.a.p. and superexcitable period. 4AP also abolished the R1 rapid rectification (Fig. 5) seen with strong depolarizations, which affected fast electrotonus and was therefore nodal in origin. We associate this action of 4AP with its small effect on action potential duration (Ritchie, 1982), and with its block of the early hyperpolarizing potential H0 seen in depolarized fibres (Fig. 12). All these rapid 4AP-sensitive processes are consistent with the voltage-clamp evidence (Brismar, 1980; Brismar & Schwarz, 1985) for a small number of  $K^+$  channels of the classical type (Frankenhaeuser & Huxley, 1964) in rat nodes. The internodal 4AP-sensitive channels responsible for the effects on the d.a.p. and slow electrotonus may differ from the nodal ones only in quantity. We cannot say whether Dubois' (1981) distinction between two types of fast, 4AP-sensitive  $K^+$  currents ( $K_{r1}$  and  $K_{r2}$ ) applies to the rat. One difference between the species is that in the rat the 4AP-sensitive channels are quite insensitive to 1 mM-TEA.

#### Inward rectification

Lorente de No (1947) recorded from intact frog nerves a slow relaxation of the potential change induced by hyperpolarizing current, which resembled the response to post-tetanic hyperpolarization (cf. Fig. 14). His attribution of this phenomenon to the 'nerve reaction' was followed by Lundberg (1951), who recorded similar potentials from frog spinal roots and desheathed nerves, thus proving that the nerve sheath could not be responsible. Since isolated single fibres became the preparation of choice for biophysical studies of myelinated nerve, this 'nerve reaction' has disappeared from the literature. In its place the terms 'anomalous rectification' (Katz, 1949) and 'inward rectification' have been used to describe conductances activated by hyperpolarization in muscle fibres and neurones (reviewed by Hille, 1984). The inward rectification we have recorded from mammalian myelinated fibres does not affect fast electrotonus (Fig. 7C), and is therefore presumably generated by internodal axolemma. This would account for the failure of nodal voltage-clamp studies to detect it. Mayer & Westbrook (1983) have distinguished between two types of inward rectifier: one relatively  $K^+$ -specific, blocked by  $Ba^{2+}$  as well as  $Cs^+$ , and present in egg cells, muscle fibres and olfactory cortex neurones; and the other with an appreciable  $Na^+$  conductance,  $Cs^+$  sensitive but  $Ba^{2+}$  resistant, and present in Purkinje fibres and many neurones, including dorsal root ganglion and probably motor neurones. The internodal inward rectification in the spinal root axons appears to be of the second type, resembling that in their parent cell bodies. This raises the

question of how much of the inward rectification recorded in a neurone's cell body may actually arise in its axon. We can at least be sure that none of the inward rectification recorded in an isolated axon (Fig. 8) derives from its soma.

We suggest that the most likely function of the inward rectification in myelinated nerve is to limit the electrogenic hyperpolarization and consequent reduction in excitability. We have previously shown that fibres with a reduced safety factor for conduction due to demyelination can be blocked at physiological rates of impulse transmission (10–50 Hz) by activity of the  $\text{Na}^+$  pump (Bostock & Grafe, 1985c). The effects of inward rectification on excitability revealed by the  $\text{Cs}^+$  action in Fig. 14 may be of critical importance, not only in damaged nerve, but at regions of normally reduced safety factor, such as branch points (Parnas, 1972) and nerve terminals (Krnjevic & Miledi, 1959).

### *Leak conductance*

The concept of a nodal leak conductance has suffered from inconsistencies from the time that Frankenhaeuser & Huxley (1964) applied the formula  $I_L = g_L (V - V_L)$ , previously used for the squid axon, to the linear component of the voltage-clamp currents recorded from *Xenopus* nodes. Whereas in the squid axon the leak conductance ( $g_L$ ) was only 0.3 mS/cm<sup>2</sup> (Hodgkin & Huxley, 1952), the specific conductance deduced for *Xenopus* nodal membrane was 30.3 mS/cm<sup>2</sup>, and at rat nodes Brismar (1980) calculated a figure of 130 mS/cm<sup>2</sup>. Frankenhaeuser & Huxley acknowledged that their treatment was arbitrary, in that  $I_L$  was thought to be carried by both  $\text{Na}^+$  and  $\text{K}^+$ , while  $V_L$  was very close to the resting potential. Nevertheless the leak conductance has been regarded as due to ionic leakage channels, responsible for setting the resting potential of myelinated fibres (Hille, 1984) as well as providing the main repolarizing current during an action potential. We consider this view mistaken, since attempts to relate  $g_L$  and  $V_L$  have consistently failed. Hille (1973) studied the ionic basis of  $g_L$  by comparing inward leakage currents in a wide range of different solutions, but none changed  $g_L$  by more than 20%. Hille's finding that replacement of NaCl by KCl increased  $g_L$  by 19% has been taken as evidence that the leakage channels are selective for  $\text{K}^+$ , but KCl solutions have an 18% higher conductance than equimolar NaCl solutions, because of the higher mobility of  $\text{K}^+$  (Hille, 1984). In contrast, attempts to determine the ionic basis of the resting potential by ion substitution (Stampfli, 1959; Arhem, Frankenhaeuser & Moore, 1973; Bromm & Esslinger, 1974) have led to the perplexing conclusion that it is predominantly set by an unknown ion, not  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$  or  $\text{Ca}^{2+}$  (Jack, 1976). Thus all ions contribute to  $g_L$ , but none appear to be responsible for  $V_L$ .

Our conclusion from these studies and our own results is that  $g_L$  and  $V_L$  are different in origin. The resting potential is probably set mainly by the internodal axon (cf. Chiu & Ritchie, 1984), which communicates with the outside of the node via the periaxonal space. This extracellular leakage pathway gives rise to the ohmic behaviour of  $g_L$  in the voltage clamp, and of the fast component of electrotonus. (It dominates  $R_m$  in Fig. 1B.) The possible structural basis of this internodal leakage path was discussed by Barrett & Barrett (1982), who deduced its existence from their study of the d.a.p. Another manifestation of it may be the 'attenuation artifact', which Dodge & Frankenhaeuser (1958) concluded must be due to a longitudinal

current path connecting adjacent nodes, and running between the axon and the outside of the fibre. Our recordings from hyperpolarized fibres with Cs<sup>+</sup> (Figs. 7 and 8), showing that slow electrotonus can be several times larger than the fast component, make it clear that the ohmic leakage path must lie predominantly in series and not parallel with the higher membrane resistance of the axon. We conclude, therefore, that there is no evidence for ohmic leakage channels in myelinated fibres, only for an ohmic leakage pathway in series with the rectifying internodal axolemma. The axons treated with TTX, TEA, 4AP and Cs<sup>+</sup> still exhibit a pronounced outward rectification (Fig. 7). This may be due in part to incomplete blockade of internodal K<sup>+</sup> channels, but may also indicate that the residual leak conductance is outwardly rectifying, as expected from the constant field theory for a conductance that sets the resting potential. Further experiments are required, avoiding the damage or depolarization of the internodal axon that may occur in voltage-clamp preparations, to determine the nature of the resting conductance in myelinated fibres.

In conclusion, we have been able to discriminate pharmacologically between three rectifying systems in mammalian myelinated nerve, attributable to a minimum of three types of rectifying channel with different kinetics and spatial distributions. Of the different actions of these channels that we have observed, the following appear the most likely to relate to their primary physiological roles: the fast outward rectifier blocked by 4AP reduces the d.a.p., and thereby prevents a single stimulus from evoking a burst of action potentials; the slow outward rectifier blocked by TEA prevents a maintained depolarization from producing repetitive firing; and the inward rectifier acts to prevent trains of impulses from blocking conduction.

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