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The effects of anticonvulsants on 4-aminopyridine-induced bursting: *in vitro* studies on rat peripheral nerve and dorsal roots

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1 Aminopyridines have been used as beneficial symptomatic treatments in a variety of neurological conditions including multiple sclerosis but have been associated with considerable toxicity in the form of abdominal pain, paraesthesias and (rarely) convulsions.

2 Extracellular and intracellular recording was used to characterize action potentials in rat sciatic nerves and dorsal roots and the effects of 4-aminopyridine (4-AP).

3 In sciatic nerve trunks, 1 mM 4-AP produced pronounced after potentials at room temperature secondary to regenerative firing in affected axons (5–10 spikes per stimulus). At physiological temperatures, after potentials (2–3 spikes) were greatly attenuated in peripheral axons.

4 4-AP evoked more pronounced and prolonged after discharges in isolated dorsal roots at 37°C (3–5.5 mV and 80–100 ms succeeded by a smaller inhibitory/depolarizing voltage shift) which were used to assess the effects of anticonvulsants.

5 Phenytoin, carbamazepine and lamotrigine dose-dependently reduced the area of 4-AP-induced after potentials at 100 and 320 μM but the amplitude of compound action potentials (evoked at 0.5 Hz) was depressed in parallel.

6 The tonic block of sensory action potentials by all three drugs (at 320 μM) was enhanced by high frequency stimulation (5–500 Hz).

7 The lack of selectivity of these frequency-dependent Na^+ channel blockers for burst firing, compared to low-frequency spikes, is discussed in contrast to their effects on 4-AP-induced seizures and paroxysmal activity in CNS tissue (which is associated with large and sustained depolarizing plateau potentials).

8 In conclusion, these *in vitro* results confirm the marked sensitivity of sensory axons to 4-AP (the presumptive basis for paraesthesias). Burst firing was not preferenitally impaired at relatively high concentrations suggesting that anticonvulsants will not overcome the toxic peripheral actions of 4-AP in neurological patients.

Keywords: Aminopyridines (4-AP); carbamazepine; phenytoin; lamotrigine; multiple sclerosis; toxicity; electrophysiology; action potentials; rat sciatic nerve; dorsal roots

Introduction

Multiple sclerosis (MS) is probably an autoimmune disease triggered by environmental factors in genetically susceptible individuals that leads to myelin destruction (Johns & Bernard, 1994). Current drug therapies are geared to controlling diverse neurological symptoms as they emerge and trials of new agents have a primary goal of slowing the rate of progression of disability and lesion volume: a clinical 'cure' is unlikely to emerge in the foreseeable future (Mathews, 1993).

The biophysical consequences of MS reflect the loss of myelin in plaque tissue: exposed axonal membranes are capacitive and poor insulators so local-circuit axoplasmic currents are greatly attenuated. In recent years several electrophysiological studies have suggested that the nerve membranes of the inter- and para-nodal regions contain a class of potassium channel not expressed in developmentally mature nodes of Ranvier (Chiu & Ritchie, 1981; reviewed by Black *et al.*, 1990). These 'unmasked' channels may play a role in generating the conduction slowing/block which is characteristic of the disease. Such inhibitory channels would be expected to contribute to the resistance shunt and actively 'clamp' the internode close to the potassium equilibrium potential (E_K). These internodal potassium channels can be blocked by aminopyridines, 4-AP (Roper & Schwarz, 1991) or 3,4-diAP, which can restore conduction across demyelinated nerves *in vitro* (Bostock *et al.*, 1981; Targ & Kocsis, 1985). It has been suggested that internodal channels display a higher affinity for these compounds than a variety of related fast-transient (I_A-like) potassium currents expressed elsewhere in the nervous system or musculature (Schauf, 1987a). This is supported by the improvement in symptomatic neurological scores, with no serious side-effects, in MS patients given up to 100 mg (max single dose) of these compounds in acute or sub-acute clinical trials (Stefoski *et al.*, 1987; 1991; Jones *et al.*, 1983; Bever *et al.*, 1990; van Diemen *et al.*, 1992; Davis *et al.*, 1990; Polman *et al.*, 1994). However, most of these patients reported paraesthesias and a recent trial of 3,4-diAP in 30 patients resulted in tingling, gastro-intestinal problems and occasional seizures (Reingold, 1994). 4-AP has been used, albeit at higher doses or for longer periods of time, in a variety of other neurological conditions (including lambert-eaton syndrome, myasthenia gravis (Murray & Newsome-Davis, 1981) and botulism (Ball *et al.*, 1979)) with similar manifestations of CNS toxicity in the form of confusion, convulsions and status epilepticus. Such side-effects are unacceptable and seem likely to limit the utility of the drug in MS.

It has been suggested that aminopyridines are beneficial in conduction failure by virtue of their ability to prolong action potentials (Targ & Kocsis, 1985). Cooling (Schauf & Davis, 1974), scorpion venoms which block Na^+ channel inactivation (Bostock *et al.*, 1978) and amantidine (Schauf, 1987b) also

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increase spike duration and reverse conduction block secondary to demyelination albeit *in vitro*. The effects of 4-AP have been examined in rat peripheral nerve (Eng *et al.*, 1988): the compound induces after potentials which reflect action potential widening and after discharge. This response is pronounced in immature animals but modest in adult rats (which may be coupled to myelin development and 'masking' of paranodal channels). Electrophysiological recording from spinal roots suggest that sensory axons are much more sensitive than motor elements which may underpin the clinical incidence of paraesthesis with 4-AP (Bowe *et al.*, 1987). 4-AP is well known as an experimental convulsant (resulting in tonic-clonic seizures) (Yamaguchi & Rogawski, 1992; Cranmer *et al.*, 1994) and *in vitro* it induces epileptiform bursts in brain slices (Fueta & Avoli, 1992; Watts & Jefferys, 1993).

The anticonvulsants, phenytoin, carbamezepine and lamotrigine, all selectively depress burst firing and epileptiform discharges without producing marked depression of unitary action potentials *in vitro* or marked sedation *in vivo* (Macdonald & McLean, 1986; Lees & Leach, 1993). This preferential depression of ectopic/bursting cells is thought to reflect the compounds' frequency/voltage/state-dependent interaction with voltage-gated sodium channels (Catterall, 1987; Lang *et al.*, 1993). Such drugs are effective in preventing convulsions induced by 4-AP in mice (Cranmer *et al.*, 1994). The idea of co-administering anticonvulsants and 4-AP to neurological patients appears to be based on a sound biophysical rationale (Murray & Newsome-Davis, 1981).

The goals of the current study were to characterize burst firing induced by 4-AP, *in vitro*, at physiological temperature then to examine the potential of anticonvulsants to depress after discharges without significant impairment of unitary evoked action potentials.

Methods

Tissue preparation

Left sciatic nerves or lumbar dorsal roots were isolated from adult male Sprague–Dawley rats (340–370 g) by standard techniques at room temperature (22–24°C). The main trunk of the sciatic nerve was manually desheathed. Preparations were bathed in modified Krebs solution of the following composition (mM): NaCl 124, KCl 3, NaH_2PO_4 1.3, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, D-glucose 10, and saturated with 95% O_2 and 5% CO_2.

Electrophysiology

A portion (1–2 cm) of isolated nerve trunk was draped through a series of perspex chambers and sealed in with vaseline. The cut end (signal ground chamber) was immersed in saline: KCl 120, NaCl 7, NaH_2PO_4 2, $MgCl_2$ 2, $NaHCO_3$ 26, D-glucose 10 which was separated from the recording electrode/test chamber (thermostatic control between ambient and 37°C) by rapidly flowing isotonic sucrose (320 mM). Rectangular pulses (10–50 μs) were applied with bipolar silver electrodes to evoke maximal compound action potentials at 0.5–500 Hz. Evoked signals were monitored (via agar bridges) with WPI or axoclamp 2B preamplifiers.

Intracellular recordings were obtained by driving borosilicate glass electrodes into sciatic nerve trunks (wedged in a constricted perfusion chamber) by use of a Narishige MW203 manipulator. These were fabricated on a mecanex BBCH puller to produce tip impedances of 130–180 MΩ (backfilled with 2 M potassium acetate). Whilst seeking impalements, compound spikes were evoked at 1 Hz by the above method or individual cells were stimulated by current passage through the electrode (Axoclamp bridge mode). Only overshooting action potentials which were increased in amplitude by hyperpolarizing d.c. were accepted as viable intracellular impalements.

Drugs and data analysis

Anticonvulsants were dissolved in dimethylsulphoxide (DMSO) then serially diluted to the stated concentrations; 0.1% DMSO was present in the drug containing saline and treatment blanks but had no significant effect on action potentials or other reported parameters. All chemicals were from BDH with the exception of 4-AP, carbamezepine, phenytoin (Sigma) and lamotrigine (Wellcome Research Labs, Beckenham, Kent). Electrophysiological data was filtered at 10 kHz and recorded at 48 kHz (together with stimulus trigger signals) on a Biologic DTR-1204 digital recorder for off-line anaylsis using a CED 1401plus A/D converter and WCP software (John Dempster, Strathclyde University). Details of quantitation of 4-AP-induced after potentials are given in figure legends. Statistical assessment (Instat software) of pharmacological results was by one-way analysis of variance or Student's paired t tests as appropriate: determined means ± standard error and P values for specific comparisons are cited in the text. Graphs and figures were produced with Inplot/Prism software.

Results

Sciatic nerve: temperature and 4-AP

Sucrose gap: extracellular action potentials were stable for periods in excess of 1 h. At an ambient temperature of 23.2 ± 0.4°C ($n=7$ pilot preparations) action potential amplitude was 52 ± 5.7 mV but this was significantly reduced ($P<0.001$) to 40.6 ± 6 mV upon warming to 37 ± 0.2°C. Action potential duration was markedly decreased by warming (not quantified) but these shifts were not associated with significant changes in resting membrane potential (ambient +44.0 mV; physiological +43.3 mV: $P>0.5$). The effects of 4-AP applied at 1 mM reached steady-state within Ca. 30 min of application and were markedly temperature-dependent (Figure 1). After potentials were consistently observed ($n=41$): pronounced after-hyperpolarizations at room temperature (5–10 mV, 50–70 ms duration) were markedly attenuated at physiological temperatures (1–2 mV, 30–40 ms duration). Temporal dispersion of signals in control saline further complicated the analysis and the marginal effects were found to be poorly quantifiable for pharmacological studies at physiological temperatures.

Intracellular recording

At room temperature in blank saline, all cells ($n>60$ in a total of 12 nerve trunks) produced unitary action potentials in response to a brief stimulating pulse. Stable recordings (20–45 min) were obtained from 9 axons: resting potentials were variable (mean −50 mV, range −20 to −87 mV); action potential amplitude averaged 81 ± 6.6 mV with a duration of 0.64 ± 0.02 ms at half height. In the presence of 1 mM 4-AP (5 treated nerve trunks) axons could be placed in 2 distinct groups. The majority of cells still produced a unitary spike (marginally widened by the toxin) in response to stimulation but repetitive firing was evident in a sub-population of axons. The duration and intensity of 4-AP-induced bursts increased with exposure time: initially doublets and triplets were observed but at equilibrium (>30 min, room temperature) many cells produced 5–10 attenuated spikes per stimulus. Warming these treated preparations to approx. 37°C attenuated repetitive firing to the extent that only doublets or, rarely, triplets could be evoked. Typical traces illustrating the effects of 4-AP and temperature in these distinct groups are illustrated in Figure 2.

In 8 cells exhibiting 4-AP-induced bursting, spontaneous activity was observed, comprising either rythmic, self-limiting bursts or irregular spikes and bursts (Figure 3). Resting potentials in these cells were notably positive (range −11 to

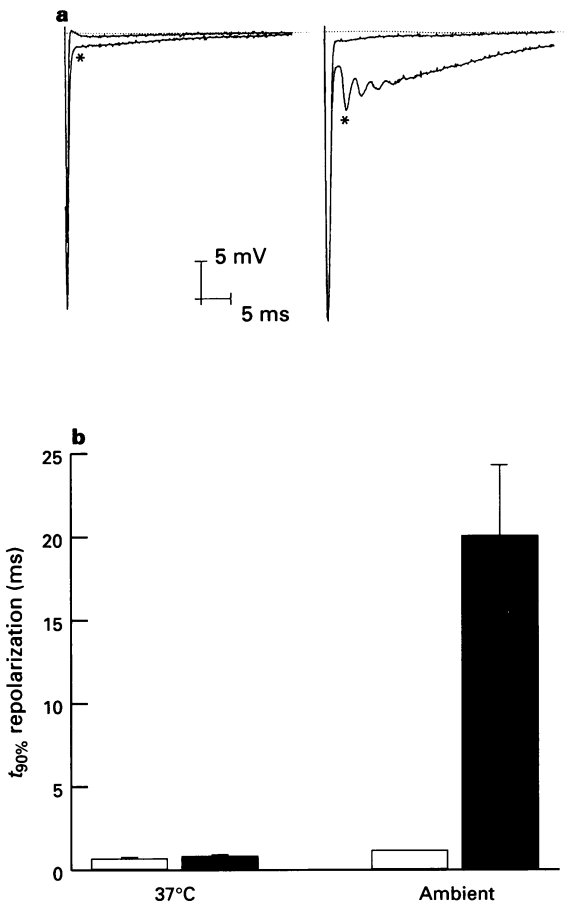

Figure 1 Sciatic nerve action potentials: the synergistic effects of 4-AP and temperature. (a) Superimposed records (averages of 10 events) of compound action potentials from the same preparation in control saline and after 1 mM 4-AP (asterisks): left records at 37°C, right at room temperature (23°C). Cooling unmasked a pronounced after potential with clear evidence of synchronized regenerative firing. (b) Compounded data from 6 sciatic nerve trunks. The time for 90% repolarization of the evoked compound action potentials was markedly and significantly increased by 4-AP (1 mM; stippled columns) at room temperature ($P<0.001$). $n=6$. At 37°C a consistent but marginal prolongation was observed in all preparations ($P>0.05$).

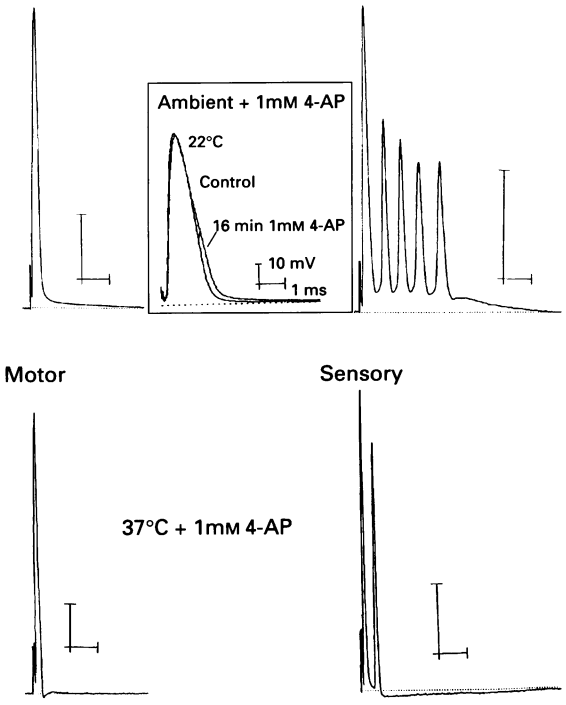

Figure 2 Intracellular recording in sciatic nerve revealed two classes of 4-AP sensitive neurone. Presumptive motor axons responded with a unitary action potential per stimulus (left records) after equilibration with 4-AP (the inset shows the onset of this response in a single cell). Contrast this with the brief burst of action potentials produced in a presumptive sensory axon at room temperature (top right). The lower traces illustrate typical responses in these two classes of cell at physiological temperatures. Note the faster spike kinetics, the attenuated 'sensory' burst and associated after hyperpolarization. Calibration bars: 10 mV, 5 ms.

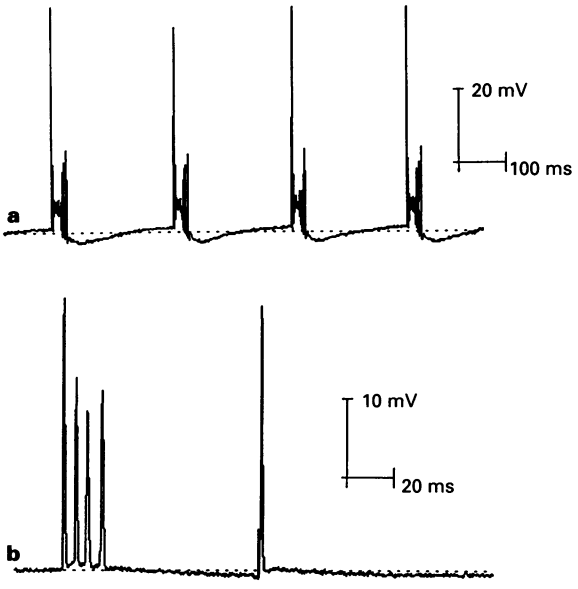

Figure 3 Intracellular recording: spontaneous activity induced by 4-AP in sciatic nerve at room temperature. (a) Rythmic self limiting bursts of high frequency spikes (attenuated in this compressed illustration). (b) Intermittent spikes and bursts in another axon.

−23 mV) and impalements were transient: spontaneous interpulse patterning of this nature was never observed in high-gain, non-invasive, sucrose-gap recording so it may have been secondary to micro-electrode-induced damage compounding the actions of 4-AP.

4-AP and action potentials in dorsal roots

Pilot experiments at physiological temperatures (36.1 ± 0.14°C) in lumbar dorsal roots ($n=21$) revealed smaller action potentials than in desheathed peripheral nerves: mean amplitude 29.8 ± 2 mV and resting membrane potential 45 ± 1.1 mV. Equilibration with sucrose and drugs was complete within 15 min for the smaller trunks and 4-AP-induced after potentials were much more pronounced (up to Ca 25–30% of evoked spike amplitude: Figure 4 inset) and quantifiable. 4-AP-induced after potentials were biphasic (Figure 4b): typically an after-discharge of 3–5.5 mV in amplitude and 80–100 ms in duration was succeeded by a slowly rising inhibitory (depolarizing) voltage shift. As before, spike amplitude and the effects of 4-AP were greatly amplified by cooling the preparations (not shown). An arbitrary method of quantifying the 4-AP-induced after discharge was devised and is outlined in Figure 4a. With this method, 1 mM 4-AP-induced after potentials were integrated and averaged 50.75 ± 5.4 mV.ms at 36.1 ± 0.14°C ($n=21$).

Anticonvulsant effects

Quantitative experiments were conducted to assess effects of lamotrigine, carbamezepine and phenytoin (at the same concentrations) on both compound spike amplitude and after-discharge area. For all three anticonvulsants after-discharges were dose-dependently depressed in parallel with evoked (0.5 Hz) spike amplitude (Figure 5): the responses were only partially reversible (see Figure 5a). The amplitude of compound spikes at differing stimulus frequencies was also examined in the presence of 4-AP and after equilibration with the anticonvulsants. All three drugs enhanced the frequency-dependent decrement of spike amplitude when normalized data (tonic block effect at 0.5 Hz) were compared to fade in the absence of drugs (Figure 5c).

Discussion

Passive properties of compound spikes in adult rat sciatic nerve were broadly consistent with previously published reports (Eng *et al.*, 1988). Warming consistently depressed action potential amplitude and duration regardless of stimulus polarity (although not analysed in depth, similar trends were observed in dorsal roots). Previous work on invertebrate axons has attributed such effects to selective modulation of rate constants governing ion permeability and to an increase in net Na^+-entry with cooling (Goldman *et al.*, 1975). The after-discharges associated with 4-AP were drastically amplified by cooling: at physiological temperatures in the adult rat sciatic nerve the changes were marginal and poorly reproducible even with signal averaging and deduction of control signals. In dorsal roots even at physiological temperatures the relative amplitude (up to Circa 25–30% of the compound spike) and duration were proportionately much greater. This reinforces previous suggestions that 4-AP selectively/preferentially activates sensory axons in these preparations (Bowe *et al.*, 1987; Eng *et al.*, 1988) and would account for the dichotomy of firing patterns observed in peripheral trunks probed with microelectrodes. Developmental and anatomical factors which might contribute to such differential sensitivity have been discussed (Targ & Kocsis, 1985; Bowe *et al.*, 1987; Eng *et al.*, 1988).

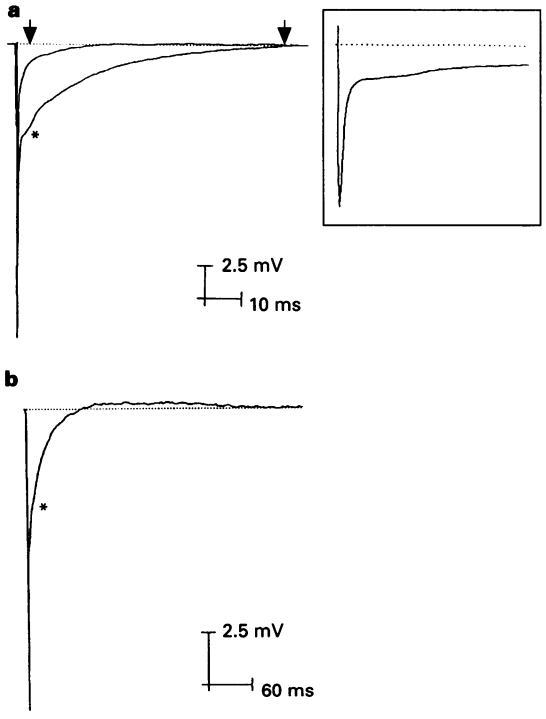

Figure 4 The pronounced effects of 4-AP on dorsal roots at 37°C. (a) Superimposed sweeps (averages of 10) of compound action potentials in control saline or 1 mM 4-AP (*) at circa 37°C. The difference between 4-AP-treated and control signals was integrated to represent drug-induced after discharge. Arrows (start and end points for integration) represent time for 90% repolarization of control spike and intersection of the after potential with resting membrane potential (dotted line). (b) Compressed high gain recording after equilibration with 4-AP: the large after hyperpolarization in the sensory axons is succeeded by a long-lived inhibitory/depolarizing voltage shift. Inset: typical 4-AP induced after potential: approximately 25% of the amplitude of this dorsal root compound spike at 36°C.

These actions of 4-AP might constitute an explanation for the consistent but untoward incidence of paraesthesias in neurological patients. Lamotrigine, carbamezepine and phenytoin all effected frequency-dependent depression of dorsal root action potentials albeit at relatively high concentrations which produced a significant degree of tonic block. However, the anticonvulsants were unable to depress after-discharges without significantly attenuating compound action potentials even at very low rates of stimulation (0.5 Hz). This is not consistent with the pronounced effects of the same compounds, applied at lower concentrations, in selectively attenuating burst firing induced by protracted depolarising stimuli (current pulses, elevated K^+ or L-glutamate) in CNS neurones *in vitro* (Macdonald & McLean, 1986; Cheung *et al.*, 1992; Lees & Leach, 1993). It is noteworthy that in all of these CNS studies, the bursts were superimposed upon sustained depolarising plateaux (analagous to paroxysmal shifts observed in epileptiform activity; 0.3–>2 s in duration) and that the depressant effect was proportional to the amplitude of evoked shifts or to positive changes in interburst transmembrane potential. In rat hippocampal slices at 34°C, carbamazepine (40 μM) completely abolished long (0.5–2 s) ictal-like bursts induced by 4-AP whilst leaving shorter (50 ms 'interictal events') intact (Watts & Jefferys, 1993). In the experiments described here, 4-AP-induced bursts comprised brief regenerative spikes which tended to accomodate/fade within 80–100 ms, even in the sensory roots, and were not associated with prolonged or pronounced plateau potentials. The assaying of these effects was conducted under arbitrary experimental conditions based on physiological salines previously used for rat nerves *in vitro* and a desire to characterize the effects of 4-AP at physiological temperatures. The preparations were desheathed, deafferented/devoid of tonic sensory input, rigourously perfused with 3 mM K^+ and stimulated at low rates throughout 4-AP application; so represent only a broad approximation to intoxicated nerve trunks *in vivo* where considerable K^+ accumulation can develop secondary to high frequency firing (in demyelinated tracts this may contribute to synchronous ephaptic transmission of excitation between adjacent axons, Rosenbluth, 1990). Extracellular K^+ is a critical determinant of neuronal membrane potential/excitability (Bear & Lothman, 1993; Jensen *et al.*, 1994) and impinges dramatically on the efficacy/affinity of use/voltage-dependent Na^+ channel blockers (e.g. Salgado, 1990). In the current study the 4-AP-induced signal (especially in dorsal roots) was biphasic: trains or after-potentials were attenuated/succeeded by a long lived inhibitory voltage shift. In rat spinal roots post-tetanic depression appears to be produced by membrane hyperpolarization secondary to electrogenic Na^+ pumping (Bostock & Grafe, 1985). In sciatic nerve this phenomenon has been attributed to a slow, tetraethylammonium-sensitive outward potassium current which limits excitability (protracted bursts and spontaneous trains were unmasked 4-AP and TEA were co-applied, Eng *et al.*, 1988). Elevations in extracellular potassium might attenuate this inhibitory mechanism and synergize both 4-AP and anticonvulsant action. Future studies in this area should be conducted *in vivo* in anaesthetized rats with sensory input intact, permitting a less invasive assessment of these interacting factors.

A recent clinical trial on 3,4-diAP produced benefit in 20 of 30 MS patients recruited but significant side effects included

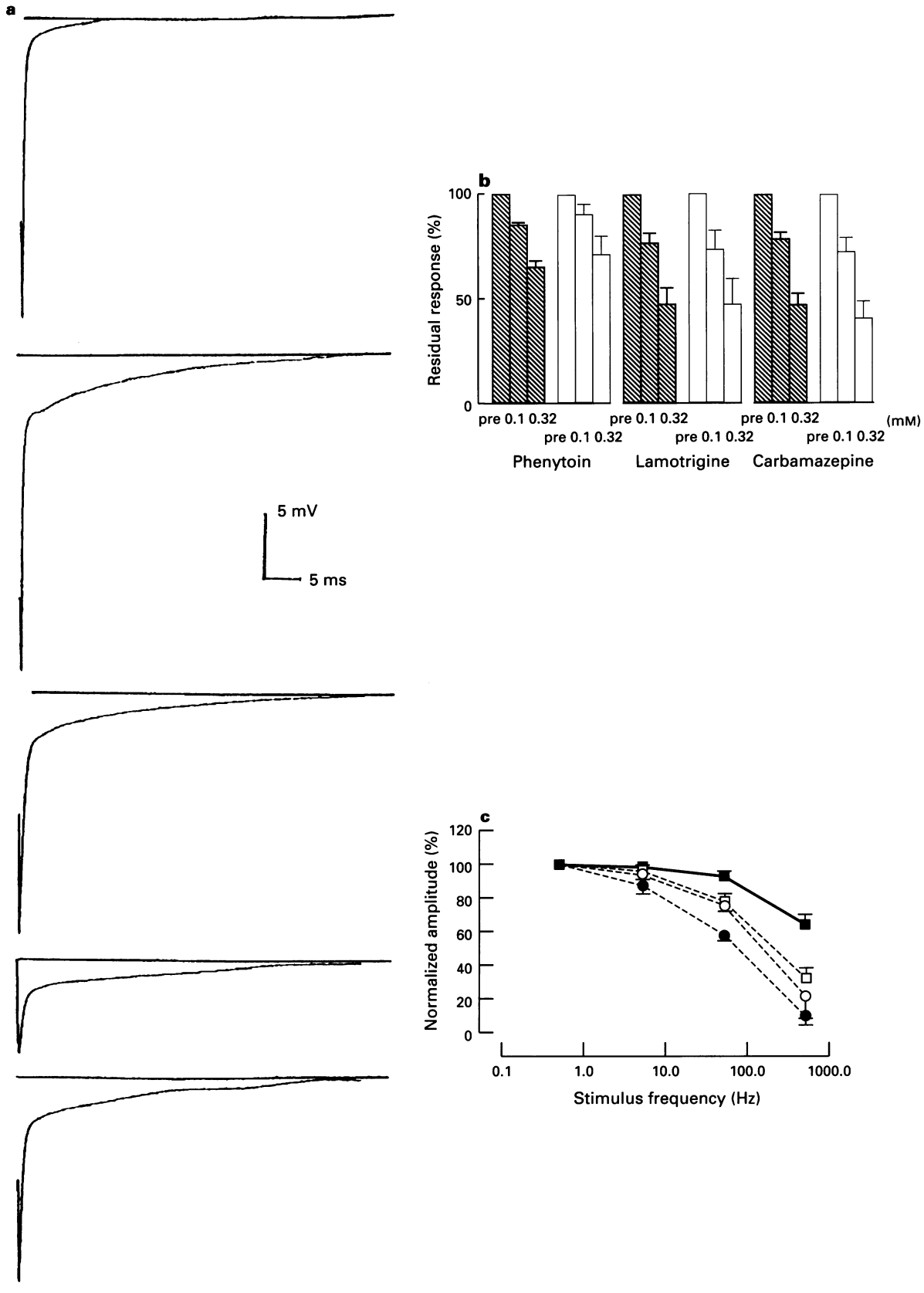

Figure 5 After-discharge and anticonvulsants. (a) Dorsal root compound action potentials after equilibration with the following salines: (from the top) control; 1 mM 4-AP; 4-AP plus 100 μM carbamazepine; 4-AP plus 320 μM carbamazepine; 4-AP wash. Note the lack of selectivity of the compound for the after discharge. (b) Normalized and compounded data from experiments on three anticonvulsant molecules. All drugs effected a significant ($P<0.01$) dose-dependent depression of both the area of after-discharge and, in parallel, the evoked compound action potential amplitude (0.5 Hz). Differential blockade of either parameter was not observed for any of the drugs ($P>0.5$). Hatched columns: CAP amplitude; open columns, after potential area, $n=4-6$. Values are mean $\pm$ s.e.mean. (c) All three drugs enhanced the degree of frequency-dependent attenuation of compound action potentials (Trains were applied until the spike amplitude reached steady-state. Data normalized to the 0.5 Hz spike amplitude; $n=3-6$ per treatment) (■) Control; (□) 320 μM phenytoin; (●) 320 μM lamotrigine; (○) 320 μM carbamazepine.

dizziness, tingling, gastrointestinal problems and, occasionally, seizures (Reingold, 1994). Another study (Polman *et al.*, 1994) suggests that, concerning both efficacy and side-effects, 4-AP is superior to 2,4-diAP in MS patients. The efficacy of phenytoin, carbamezepine and lamotrigine in suppressing seizures or epileptiform activity induced by 4-AP is well established (Yamaguchi & Rogawski, 1992; Cranmer *et al.*, 1994; Kapetanovic *et al.*, 1995). However, because of the toxic side effects of some of these drugs the decision to co-administer them with even demonstrably effective symptomatic treatments cannot be taken lightly. One of the characteristics of demyelinated axons which is thought to underlie some of the symptoms of MS in human subjects is a poor ability to transmit trains of impulses (Waxman, 1981; Bostock & Grafe, 1985). Frequency-dependent depression of already compromised axons by anticonvulsant drugs could conceivably enhance the deficit and overcome any benefit produced by the aminopyridines. The current study indicates that under physiological conditions *in vitro* the anticonvulsants are unable to impair selectively burst firing in sensory axons (the presumed basis of paraesthesias) evoked by 4-AP. Further studies *in vivo* in laboratory animals will be required to examine definitively the effects of these agents on seizure thresholds and sensory discharge in peripheral trunks induced by aminopyridines.

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