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Effects of pyrethroid molecules on rat nerves *in vitro*: potential to reverse temperature-sensitive conduction block of demyelinated peripheral axons

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1 Prolongation of action potentials by cooling or pharmacological treatment can restore conduction in demyelinated axons. We have assessed the ability of pyrethroids (*in vitro*) to modify action potential kinetics and to reverse conduction block in lesioned peripheral nerve.

2 Fast Na^+ currents were isolated in mammalian neuroblastoma (NIE115). Pyrethroids (4 μM) concurrently slowed inactivation and produced a spectrum of pronounced tail currents: s-bioallethrin (duration 12.2 ± 7 ms), permethrin (24.2 ± 3 ms) and deltamethrin (2230 ± 100 ms).

3 Deltamethrin (5 μM) effected a slowly developing depression of compound action potential (CAP) amplitude in peroneal nerve trunks ($P < 0.05$). Permethrin produced no net effect on CAP amplitude, area or repolarization time.

4 s-Bioallethrin (5 μM) enhanced CAP area, time for 90% repolarization and induced regenerative activity in a subpopulation of axons.

5 Tibial nerve trunks were demyelinated by lysolecithin (2 μl) injection: 6–14 days later, slowly-conducting axons in the CAP (and peri-axonal microelectrode recordings) were selectively blocked by warming to 37°C.

6 At 37°C, s-bioallethrin (45 min, 5 μM) produced much greater after-potentials in lesioned nerves than in uninjected controls: area ($P < 0.05$) and relative amplitude ratios ($P < 0.0001$) were significantly altered.

7 In 3 of 4 cells (single-unit recording), s-bioallethrin restored conduction through axons exhibiting temperature-dependent block by raising blocking temperature (by 1.5 to >3°C) and reducing refractory period.

8 s-Bioallethrin induced temperature-dependent regenerative activity only in a sub-population of axons even after prolonged superfusion (>1 h).

9 It was concluded that pyrethroids differentially alter Na^+ current kinetics and action potential kinetics. The effects of s-bioallethrin are consistent with reversal of conduction block by demyelinated axons but regenerative/ectopic firing even in normal cells is likely to underpin its acknowledged neurotoxic actions and severely limit the clinical potential of this and related molecules.

Keywords: Multiple-sclerosis; myelin; conduction-block; pyrethroids; s-bioallethrin; lysolecithin; axons; electrophysiology; Na^+-channel; patch clamp

Introduction

The idea of increasing the safety factor for transmission of impulses in demyelinating disease by prolonging sodium conductance is based on a sound biophysical rationale. The resulting prolongation of the action potential would increase the time integral of current available for discharging the increased capacity of demyelinated membrane. This is thought to be the basis for the frequently observed effects of temperature on multiple sclerosis (MS) patients: cooling widens action potentials and can alleviate symptoms whereas increasing core temperature has been associated with relapse and, rarely fatalities (Guthrie & Nelson, 1995). *In vitro*, the efficacy of scorpion venom components to restore conduction across ventral roots demyelinated with diphtheria toxin has been demonstrated (Bostock *et al*., 1978). Such toxins have not been utilized clinically for a variety of reasons: as polypeptides they would be orally degraded, are CNS impermeant and irreversibly inhibit sodium channel inactivation. Aminopyridines, like 4-aminopyridine (4-AP) or 3,4-diaminopyridine prolong the action potential by blocking potassium channels. These molecules are effective in reversing conduction block *in vitro* (Bostock *et al*., 1981; Targ & Kocsis, 1985) and produce a demonstrable benefit in acute relapse, particularly in temperature-sensitive patients in several clinical trials (eg Van Diemen *et al*., 1992). Aminopyridines have a marginal therapeutic ratio (Reingold, 1994), almost all MS patients report paraesthesias and are convulsant at higher concentrations (Murray & Newsome-Davis, 1981). All the above molecules probably alleviate block secondary to prolongation of action potentials (as does amantadine: Schauf, 1987), but aminopyridines may preferentially promote transynaptic signalling at clinically relevant concentrations (K.J. Smith, UMDS Guy's Campus: personal communication). Clearly, then, small molecules which prolong action potentials have promise and agents like DDT have attracted theoretical attention (Schauf & Davis, 1974). However, this insecticide is notorious for accumulation in fat and for vertebrate/mammalian toxicity and is more noted for inhibition of sodium channel de-activation than interference with the fast inactivation process (Vijverberg, 1982). In recent years, newer insecticides with more toxicologically/ecologically acceptable features have been developed. Pyrethroids are structurally and pharmacologically diverse: different congeners produce a continuum of kinetic

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modifications to sodium currents ranging from milliseconds to seconds (Narahashi, 1992). They have been a commercial success because they are insect selective by virtue of avid metabolic degradation in mammals and they do not bioaccumulate as does DDT. Recently, a precedent has been set for the safe and efficacious treatment of human disease with a potent insecticidal/nematocidal molecule in the form of avermectin: used in the treatment of river blindness (Cully *et al*., 1994). The prospect that insecticides might produce symptomatic benefit in demyelinating disease has been discussed but not examined experimentally (Schauf & Davis, 1974). In this study, we describe the pharmacodynamic interaction of three pyrethroid insecticides (and, for comparative purposes, the venom from the North African scorpion, *Leiurus quinquestriatus*: see Bostock *et al*., 1978) with mammalian myelinated axons and assess their potential to reverse temperature-sensitive conduction block in demyelinated axons *in vitro*.

Methods

Lesion induction

Injection pipettes were fabricated from borosilicate glass and bevelled (at a 25° angle): the maximum diameter at the tip was *circa* 12 μm. These were then cemented onto 22 gauge luer fitting syringe barrels and backfilled with negative pressure from a hand held syringe. A 1:10 mechanical micromanipulator was used for injection (under microscopic control). Male Sprague-Dawley rats (*circa* 350 g) were anaesthetized (using an approved method under Home Office licence) and the sciatic nerve and its distal branches exposed, mid thigh, in the right hindlimb. One to four microlitres of 1% lysolecithin (Sigma) was injected in an isotonic NaCl vehicle to defined sites within the peroneal or tibial branches near the site of their bifurcation from the main sciatic trunk. Muscle and skin were then sutured and the animals monitored throughout recovery from anaesthesia. Standard procedures to maintain sterility and temperature were used throughout. One to twenty eight days later the animals were killed (by use of an approved Schedule 1 method) and the peripheral nerve trunks removed for electrophysiological or morphological assessment *in vitro*. A total of 8 animals was injected with equivalent volumes of saline alone. These were compared with contralateral (physiological) and lesioned nerves but did not exhibit morphological characteristics of Wallerian degeneration or detectable conduction deficits in control experiments (not shown).

Saline

All experiments and tissue isolation/dissection were conducted in a 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

Voltage clamp Sodium currents were isolated in the mouse neuroblastoma NIE115 which were differentiated by supplementing growth medium with 2% DMSO before electrophysiological recording by whole cell patch clamping. Cells were superfused with physiological saline comprising (mM): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.8, HEPES 10, pH 7.3 (1 M NaOH). To block competing potassium currents the following intracellular solution was used in the pipette (mM): tetraethylammonium chloride 20, CsCl 120, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES 10, pH 7.4. A List EPC7 preamplifier was used to minimize capacitive transients and >80% series resistance compensation was commonly applied by pipettes with a resistance of 3–4 MΩ. Figures represent photographic reproduction of oscilloscope sweeps without deduction of linear leak or residual capacitive currents. All observations described are based on a minimum of 3 replicated experiments.

Sucrose gap: action potential kinetics Compound action potentials were monitored from sheathed peroneal nerve trunks by use of a published technique (Lees, 1996). Stimuli (Grass S88 and optical stimulus isolation unit) were applied *circa* 1 cm from the recording site (0.25 Hz, 30–50 V, 0.01 ms) by teflon insulated platinum wire electrodes to elicit a maximal compound action potential. The killed end of the nerve was placed in an isotonic KCl based saline containing the ground electrode and separated from the test chamber by rapidly flowing isotonic sucrose and liquid paraffin seals. Test and stimulus chambers were perfused throughout with oxygenated physiological saline. The test chamber was maintained at 37±1°C by prewarming superfusing solutions in a water jacked and electrodes were isolated from thermal fluctuations by connection to the solutions via agar bridges. An Axoclamp 2B (or WPI VF-2) preamplifier was used to monitor the d.c. signals. Data were analysed as the average of six evoked responses for maximal amplitude, area and repolarization times (50% and 90%).

Single unit recording Unless otherwise stated, data presented here was obtained by peri-axonal recording (from within the peri-axonal myelin sheath) based on the relatively low resting membrane potentials and spike amplitudes of the d.c. recordings. Sharp micro-electrodes (75–150 MΩ) were mounted on the probe of a bridge pre-amplifier (Axoclamp 2B, Axon Instruments) then driven into the, locally desheathed, nerve trunks by use of 1–10 μm steps provided by a high performance piezoelectrical manipulator (Burleigh). Supramaximal stimulus pulses were applied at two sites along the nerve trunk to elicit spikes proximal or distal to the lesion site (see Figure 4c). The resulting recordings were more stable than conventional intra-axonal impalements (Lees, 1996).

Temperature dependence of axonal conduction

The experimental chamber used to monitor the temperature sensitivity of lesioned or contralateral nerves is illustrated in Figure 4c. This was used to regulate temperature in the central chamber (lesion site) between *circa* 26–38°C without affecting the temperature of the intracellular or differential extracellular recording electrodes, which were maintained at 34°C throughout. Temperature was actively controlled in three chamber lanes concurrently by an ATR4 laboratory thermoregulator (Quest Scientific, Vancouver BC). Stimuli (at 2× the intensity required to elicit a maximal spike) were applied proximal and distal to the central lane, as described above. D.C. coupled differential 'killed end' extracellular recordings were obtained by use of standard techniques and a Digitimer NL 104A preamplifier (Lees, 1996). An interface chamber was used: tissue was maintained under a stream of warmed (34°C), oxygenated and humidified air throughout these experiments.

Data acquisition and analysis

Electrophysiological data were filtered at 10 kHz then captured at 40 kHz on a digital video cassette recorder (Intracel) or digitized real-time at 25 kHz (CED 1401 plus) as sweeps of 512 or 2048 data points for analysis with WCP software (courtesy of John Dempster, University of Strathclyde). Electrophysiological waveforms and collated numerical data were plotted as graphs (Prism 2 software: Graph Pad, U.S.A.). Statistical analysis was by unpaired (two-tailed) *t* test, one-way ANOVA (for parametric data) or by a Mann-Whitney ranks test as indicated in the text or figure legends. A P value of less than 0.05 was indicative of a significant difference. All data are cited as mean $\pm$ s.e.mean.

Pyrethroid/toxin formulation

Samples of ($\pm$)-allylhydroxymethylcyclopentenone ester (s-Bioallethrin); 3-phenoxybenzyl (1**RS**)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (*cis*-permethrin) and cyano(3-phenoxyphenyl)methyl 3-(2,2-dibromo-ethenyl)-2,2-dimethyl-cyclopropanecarboxylate (deltamethrin) were obtained >98% pure (British Greyhound Chromatography and Allied Chemicals, U.K.).

Pyrethroids were formulated in dimethylsulphoxide (DMSO) and serially diluted to the stated concentration: 0.1% DMSO was present in all test solutions and treatment blanks. To facilitate permeation of tissue by *Leiurus quinquestriatus* (Sigma) venom, the nerve sheath was locally disrupted with watchmakers forceps and the desheathed segment placed in the test chamber. All compounds were administered by continuous superfusion through the test or lesion chamber.

Results

Pyrethroids differentially modified Na^+ current kinetics in neuroblastoma

Rapidly activating inward currents were seen in all cells step depolarized from holding potentials of -80 mV. Peak inward currents (ranging from 2–12 nA in amplitude) were seen with steps to -20 or -10 mV. The currents were homogeneous in their kinetics of activation and inactivation (currents inactivated completely within 3–4 ms: illustrated in Figure 1a). After equilibration with the type I pyrethroids (4 μM) residual, non-inactivating current, was seen at the end of the step and tail currents of 12.2 ± 7 ms ($n=3$) and 24.2 ± 3 ms ($n=3$) duration were produced by s-bioallethrin (Figure 1b) and permethrin (not shown), respectively. Deltamethrin (4 μM) effected a much more profound alteration of inactivation (substantial residual inward currents were still evident at the end of a 25 ms pulse) and de-activation kinetics (tail current duration 2230 ± 100 ms, $n=3$: Figure 1c). Outside-out patches taken from cells equilibrated with deltamethrin were step depolarized from holding potentials ranging from -90 to -110 mV to elicit peak inward currents (a hyperpolarizing shift in channel voltage-dependence is seen under cell-free conditions). In these experiments Na^+ channel deactivation was slow enough to follow single-channel transitions by use of the limited frequency-response of a chart recorder (Figure 1d). These results confirm that the molecules selected for study produce a spectrum of kinetic modifications in mammalian sodium channels albeit in a model preparation under voltage-clamp.

Kinetic modulation of compound action potentials

Peroneal nerve compound action potentials of 20–35 mV (pretreatment mean $\pm$ s.e. 25.5 ± 0.8, $n=20$) could be monitored by the extracellular recording technique and following equilibration with sucrose were stable for periods in excess of 90 min. The mean area was 16.5 ± 0.9 mV ms^{-1} and repolarization to 50% (t50%) and 90% (t90%) of the baseline was complete within 0.28 ± 0.01 ms and 0.845 ± 0.04 ms, respectively ($n=20$). The stability of these parameters is indicated for DMSO treatment blanks in Figure 2. D.C. potentials across the gap were monitored continuously but were found to fluctuate, or more likely, progressively increase throughout the recording period despite the apparent stability of the maximal evoked spike. No attempt was made to quantify or collate drug-induced effects on this rather unreliable indicator of compound transmembrane potential.

At 1 μM, or below, s-bioallethrin induced pronounced negative after-potentials within 5–15 min of superfusion but the response continued to increase even after 60–90 min of treatment. At 5 μM the response to s-bioallethrin developed much more rapidly and after-potential areas appeared to reach a quasi-equilibrium within 30 min (not shown). All subsequent measurements were made after 45 min exposure to this concentration. The quantitative effects of all three pyrethroids are shown in Figure 3, after being normalized to pretreatment values. s-Bioallethrin significantly enhanced the area of compound action potentials ($P<0.01$) and markedly enhanced the t90% ($P<0.01$) from 0.88 ± 0.1 ms pretreatment to 3.68 ± 0.45 ms in the presence of the pyrethroid. The effects on the early phases of repolarization were not significant and, even after averaging, the after potentials exhibited 'rippling' which was strongly suggestive of repetitive firing rather than induction of a plateau phase on the primary action potentials (see Figure 2).

The other two pyrethroids (5 μM), which were associated with more pronounced kinetic modulation of voltage-clamp currents (above) at room temperature, paradoxically, did not overtly prolong the action potential or increase its area after 45 min at 37°C. Deltamethrin ($n=5$) depressed action potential amplitude ($P<0.05$) and marginally reduced the area of the spikes ($p>0.05$). The kinetics of repolarization were not significantly altered in the residual compound action potentials ($P>0.05$, Figure 3). Permethrin did not significantly alter any of the measured parameters after 45 min at 5 μM. It is conceivable that the kinetics of onset for these molecules is different from that for s-bioallethrin or that 45 min was insufficient to attain a true equilibrium response. In this respect the depressant action of deltamethrin was still increasing between 30 and 45 min. Neither permethrin nor deltamethrin produced even transient hyperpolarizing potentials following the primary compound spike, which would be consistent with their excitatory phases on insect preparations at room temperature (GL: unpublished observations).

Scorpion venom (50 μg ml^{-1}) from *Leiurus quinquestriatus hebraeus* was applied to a total of 7 partially desheathed peroneal nerve trunks. This induced slowly-developing negative after potentials which were qualitatively and quantitatively different from the pyrethroid effects noted above. Within 7–10 min small but protracted (10–30 ms) after-potentials were noted and some rippling suggesting synchronous repetitive firing was evident. Again, onset kinetics were extremely slow and the number of modified axons (or the duration of the kinetic modification) continued to increase up to 80 min after exposure to the toxin. Effects were quantified in 2–3 cells after 60 min when t50% was 22.6 ± 6 ms and t90% was 98.5 ± 15 ms. Rate of

rise of the primary compound action potential was not notably altered (stimuli at 0.01 Hz) and the mean amplitude was not significantly changed (mean amplitude depressed by *circa* 3%; $P > 0.05$, $n = 3$). In the most profoundly modified replicate the area of the compound action potential was increased by > 140 fold after 60 min exposure (see Figure 2d).

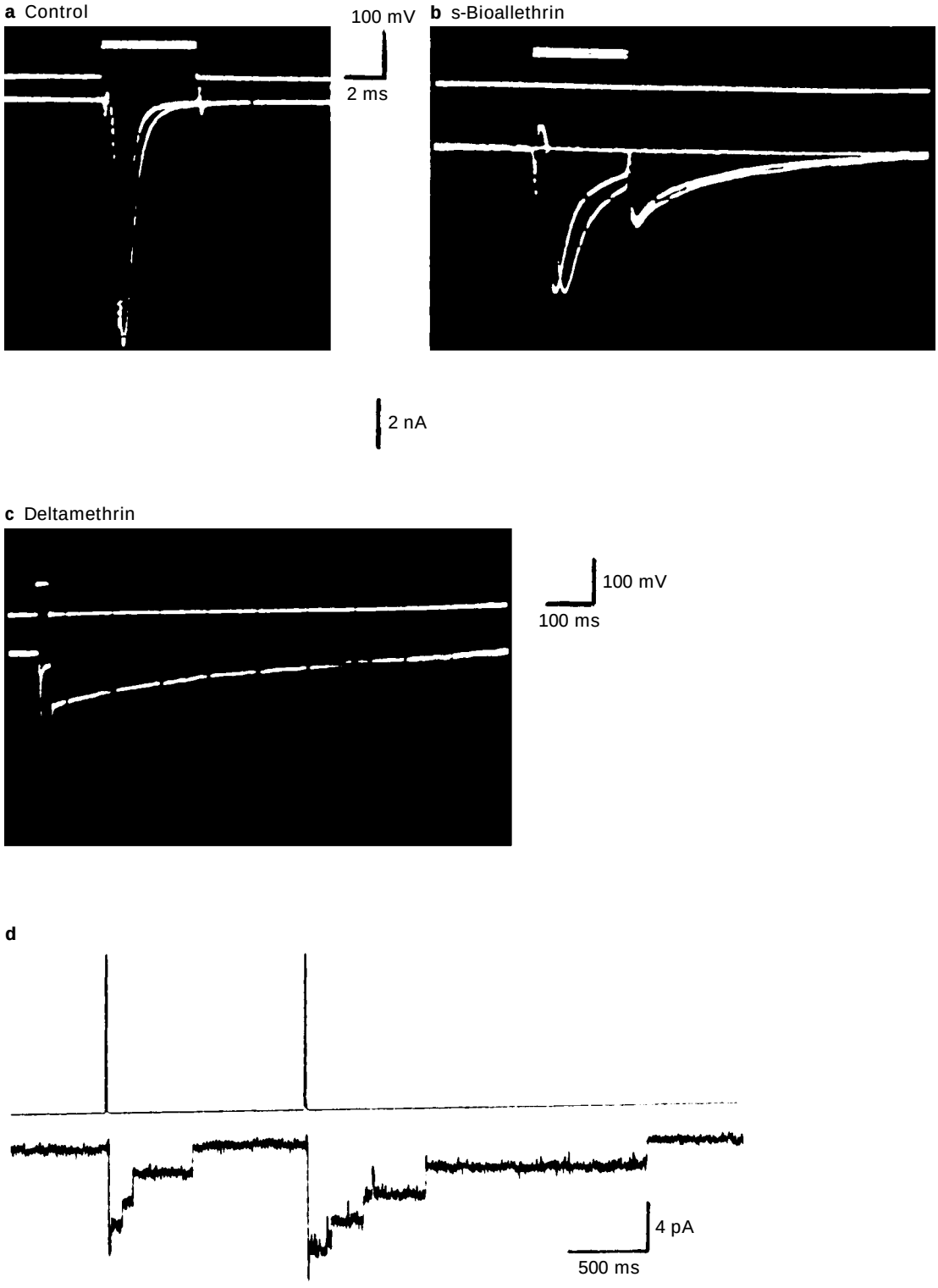

Figure 1 Kinetic divergence of pyrethroid action in the neuroblastoma cell line NIE115 under whole cell voltage clamp. Five or 25 ms step depolarizations (upper traces) were applied to elicit maximal inward currents from a holding potential of -80 mV. (a) Currents were homogeneous in untreated cells (left) and completely inactivated within 3–4 ms under these conditions. Contrast this with the effect of (b), 4 μM s-bioallerthrin (total duration of inward tails *circa* 12 ms) and (c) 4 μM deltamethrin (tails *circa* 2.2 s, note the compressed time-base). As well as producing pronounced tail currents (reflecting slowed closure of the channel's activation gate) the pyrethroids concurrently interfered with fast inactivation so that a substantial residual inward current was flowing even at the end of the depolarizing step. (d) In the presence of deltamethrin, cell-free, outside-out patches were isolated and open to closed transitions in unitary sodium channels could be resolved by use of the constrained frequency-response of a chart recorder. Upper sweep: attenuated voltage steps to activate peak inward currents from V_h -90 mV. Lower sweep: responses to consecutive stimuli show stochastic activity and de-activation of 4–5 unitary channels on the modulated timescale.

Development of an in vitro *model for temperature-sensitive conduction block*

In accord with previous studies (Smith & Hall, 1980; Smith 1994) injection of lysolecithin in volumes $\geqslant 2$ μl resulted in a marked all-or-none conduction deficit in the selected nerve trunk (lesions were perfused with saline at 22–24°C). This effect was manifest within 24 h of the injection and persisted for 3–4 days (not shown). Within 5–6 days a significant restoration of conduction was evident at ambient temperatures (Figure 4b), indicated by pronounced contributions of slowly conducting axonal populations in the compound action potential. In most cases a distinct secondary peak was evident but occasionally a very broad temporal dispersion was evident suggestive of a more diffuse longitudinal lesion. In subsequent studies at least 6 days was allowed post-injection for this 'repair' process to be affected. Attempts to lesion the peroneal trunk at a discrete location, distinct from the recording site, were not successful ($n=11$). This was attributed to the limited length of nerve which could be isolated and diffuse spread of lysolecithin towards distal portions of the trunk. Unlesioned peroneal preparations were useful to confirm the stability of killed end recordings (>90 min) and to demonstrate the lack of effect of selecting different perfusion lines on thermal stability whilst exercising all three lanes of the thermoregulator. DMSO 0.1% produced no demonstrable change in the evoked CAP after 45 min of superfusion ($n=2$ contralateral trunks, not shown).

Recording from longer segments of distal tibial nerve was feasable and the lesion site could be located in the central lane

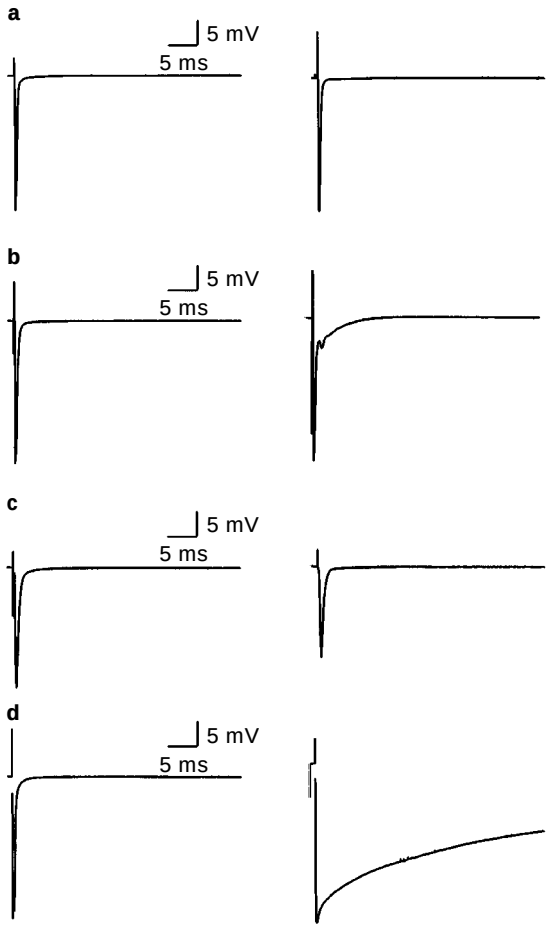

Figure 2 Representative electrophysiological waveforms (average of 6 evoked responses) comparing compound action potentials in control physiological saline (left column) and after exposure (right column) of the same preparation to the indicated treatment: (a) 0.1% DMSO blank (45 min), (b) 5 μM s-bioallethrin (45 min), (c) 5 μM deltamethrin (45 min) and (d) 50 mg ml^{-1} *Leiurus* venom (60 min).

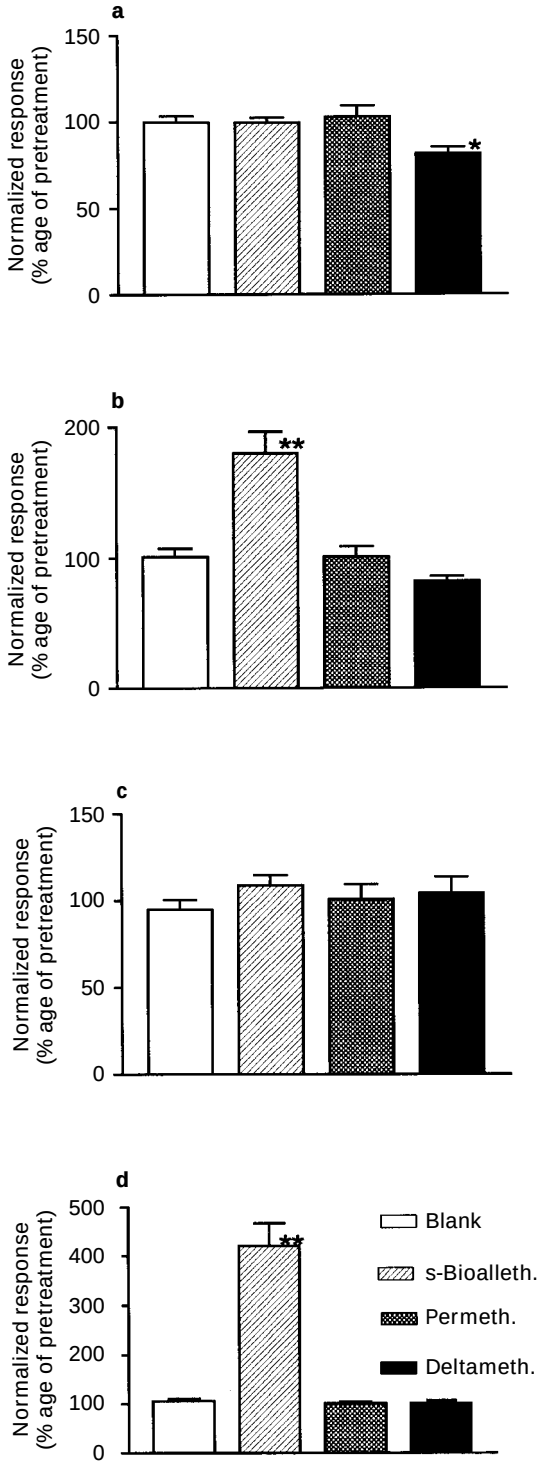

Figure 3 Histograms depicting the effect of 45 min exposure to 5 μM pyrethroid on: (a) compound action potential (CAP) amplitude C; (b) CAP area; (c) time for 50% repolarization (t50%) and (d) t90%. For each histogram treatments were (left to right) DMSO blank, s-bioallethrin, permethrin, deltamethrin. Results were tested for significance by one way. ANOVA by use of Dunnet's *post*-test to compare the ion channel modulators with treatment blanks: $^{*}P<0.05$; $^{**}P<0.01$; $n=5$ for each column.

with greater precision/certainty. Best results were obtained by injecting a total of 2 μl of lysolecithin into a site just distal to the point of bifurcation of the tibial branch from the main sciatic (which was then placed in the centre of the test/lesion chamber as shown in Figure 4c). With recording and thermal buffer lanes set to 34 ± 0.5°C the thermoregulator was able to regulate temperature at the lesion site within the range 26–38°C, with no significant oscillation or overshoot. Warming contralateral axons across this range significantly increased the conduction velocity and reduced temporal dispersion, so that peak amplitude was enhanced, (Figure 4a) but compound action potential area was constant or marginally increased (Figure 4d). The primary, fast-conducting spike in mature lesioned preparations responded in a qualitatively similar way. In contrast, the slowly conducting axons were markedly attenuated or blocked by warming to physiological temperatures (Figure 4b and d). This was later confirmed to represent a reversible temperature-dependent conduction block by peri-axonal single unit recording (see below).

s-Bioallethrin and temperature-dependent conduction block

A series of experiments were conducted to address the issue of whether the type I pyrethroid could restore conduction in axons, exhibiting temperature-dependent block at 37°C. Because s-bioallethrin can produce regenerative action potentials in normal axons we concurrently quantified the effects of the molecule on lesioned and contralateral axons to facilitate interpretation of results. Results are summarized in Figure 5. The compound action potentials in both lesioned ($n = 6$) and normal ($n = 6$) trunks were invariably enhanced in area after 45 min exposure to 5 μM s-bioallethrin. As previously noted, a subpopulation of axons in normal trunks appeared to exhibit synchronous regenerative firing (3–4 spikes). Despite the fact that the largest drug-induced peak was only *circa* 30–40% of the amplitude of the primary spike, this activity in normal axons partly obscured the effects on blocked demyelinated axons in the parallel experiments. Integrating the areas of the CAP before and after pyrethroid showed that a greater enhancement was evident in the lesioned preparations (Mann-Whitney test, $P < 0.05$). In 4 of the 6 lesioned trunks the amplitude of the primary spike was also enhanced by s-bioallethrin (see Figure 5b). This occurred in only 1 of 6 contralateral nerves under identical experimental conditions but, nevertheless, may indicate a change in electrode resistance with time. With this in mind we re-analysed the data by comparing the ratio of the peak secondary, drug-induced spike/primary spike amplitude to eliminate potential artifacts arising from electrode shunting. This data was parametric and s-bioallethrin induced a much more profound after-discharge in lesioned trunks compared to normal myelinated axons (Figure 5d: $P < 0.0001$). Overall the data suggest that s-bioallethrin can overcome temperature-dependent conduction

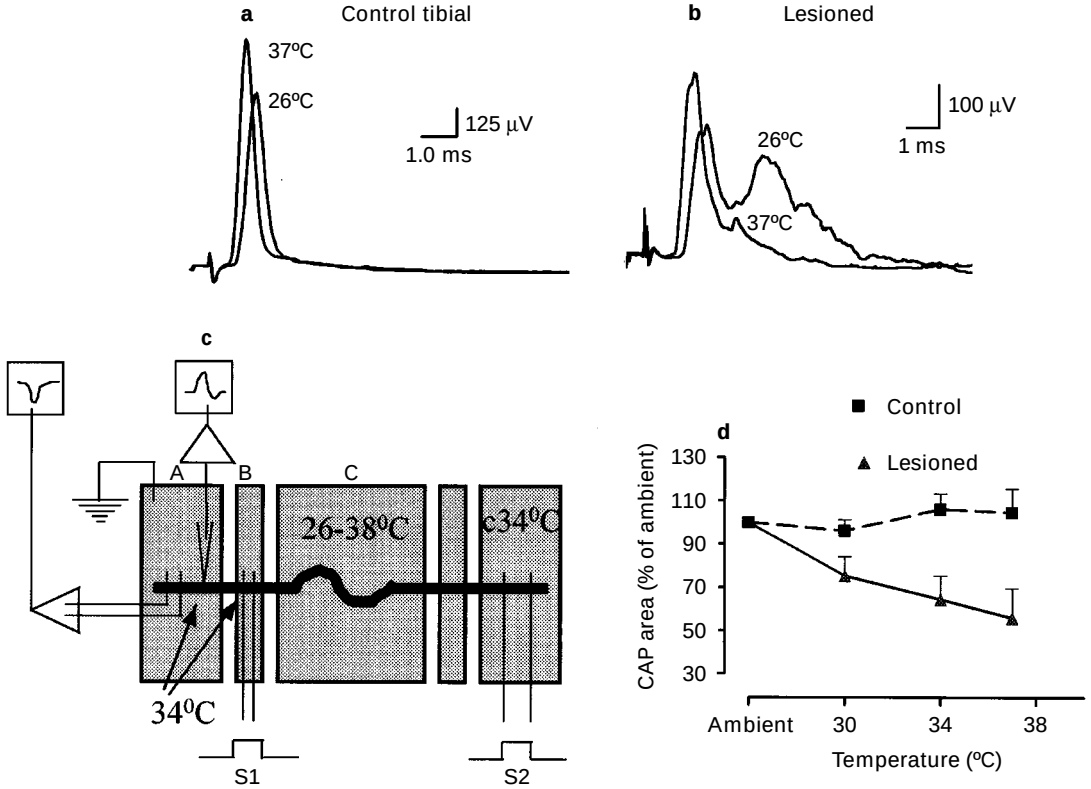

Figure 4 (a) Warming of physiological nerve trunks increased spike amplitude by reducing temporal dispersion. The area of compound action potentials was effectively unchanged. (b) Six–fourteen days after lesioning, tibial nerve trunks contained a slowly conducting population of axons which were selectively blocked by raising temperature to 37°C. (c) Cartoon depicting the thermally regulated recording chamber. Each saline lane (shaded area) was perfused at a constant rate and could be regulated at the indicated temperatures based on feedback from thermistors housed near the nerve trunk (thick black line) and the associated peltier device. The trunks were sealed in with petroleum jelly and lanes were thermally isolated by means of narrow air gaps (0.25 mm) and a thermally regulated buffer lane (B). Extracellular, intracellular and pre-amplifier ground electrodes were located in the recording lane (A). Bipolar tungsten electrodes were placed proximal (S1) and distal (S2) to the lesion lane (C) which could be maintained at the indicated temperatures with no effect on electrode temperature. (d) Quantitative description of data from 7 lesioned and 7 contralateral nerve trunks (control). Points represent means and vertical lines indicate s.e.

block in slowly conducting axons, or that residual conducting fibres in lesioned nerve trunks are more susceptible to the intoxicating effects of the pyrethroid.

Peri-axonal recordings

Twelve of twenty-six cells exposed to a 15 min protocol to examine temperature-dependence were not blocked by elevating the lesion temperature to 37°C. Fourteen of these cells exhibited all-or-none conduction block at temperatures between 29–36°C. One of these was held for a period in excess of 20 min in the absence of drugs and subjected to repeated cycling around the blocking temperature. Temperature for all-or-none block was stable throughout this period. The blocking temperature could be estimated within *circa* 1°C during the pretreatment period and averaged 33.6 ± 0.7°C. In this group, the refractory period for transmission of impulses generated distal to the lesion was greater than that for pairs of proximal stimuli (not shown). Four of these cells were held for a sufficient period of time to characterize temperature-dependent block and then to complete 45 min of superfusion with 5 μM s-bioallethrin. During this period, the lesions were maintained just below, or cycled around, the blocking temperature and the apparent refractory period for pairs of stimuli, applied distal to the lesion, was determined intermittently. In 3 of the 4, s-bioallethrin appeared to increase the temperature for all-or-none block by 1.5–>3°C and concurrently (2 of 2 examined) reduced the apparent refractory period at a fixed lesion temperature (Figure 6). Two of the 3 responsive cells exhibited after-discharges or ectopic firing after 20–30 min exposure to pyrethroid. In 1 of 4, the pyrethroid produced no discernible effect on temperature-dependence or safety factor nor did we see regenerative firing in this axon even after 45 min exposure. The bursts induced by pyrethroids were greatly prolonged by cooling from physiological to ambient temperatures (Figure 7). After perfusion for periods in excess of 60 min blind impalements suggested heterogeneity in the susceptibility of individual axons to pyrethroid-induced bursts, despite the fact that they were located within 200 μm of each other (Figure 7c). Examination of pretreatment action potential profiles (or the individual holding temperatures used) could not be used to discern the predisposition of a cell to pyrethroid-induced after-discharge.

Discussion

s-Bioallethrin represents the active isomer of allylhydroxymethylcyclopentenone ester whereas the other pyrethroids examined represent racemic mixtures contaminated with up to 50% of inert or less-active components. The allethrins and permethrin are described as Type I pyrethroids, whereas deltamethrin, in common with other molecules bearing an alpha-cyano substituent, is classified as Type II based on distinct toxicological features (Narahashi, 1992; Song & Narahashi, 1995). The clamped Na^+ currents isolated in NIE115 cells displayed similar properties to those obtained previously under similar conditions (Quandt & Narahashi, 1984). My voltage clamp experiments confirm previous studies which point out that pyrethroids kinetically modify Na^+ currents by slowing both inactivation (attenuation of current within a depolarizing step) and the de-activation process (to produce the characteristic protracted tail currents when the cell is repolarized). The action of each molecule is quantitatively distinct (see Narahashi, 1986) so that pyrethroids exert a kinetic continuum of effects. In this study, the modification was brief for s-bioallethrin, slightly more profound for permethrin and Na^+ channel deactivation was slowed >1000 fold by deltamethrin. Even by modifying a tiny

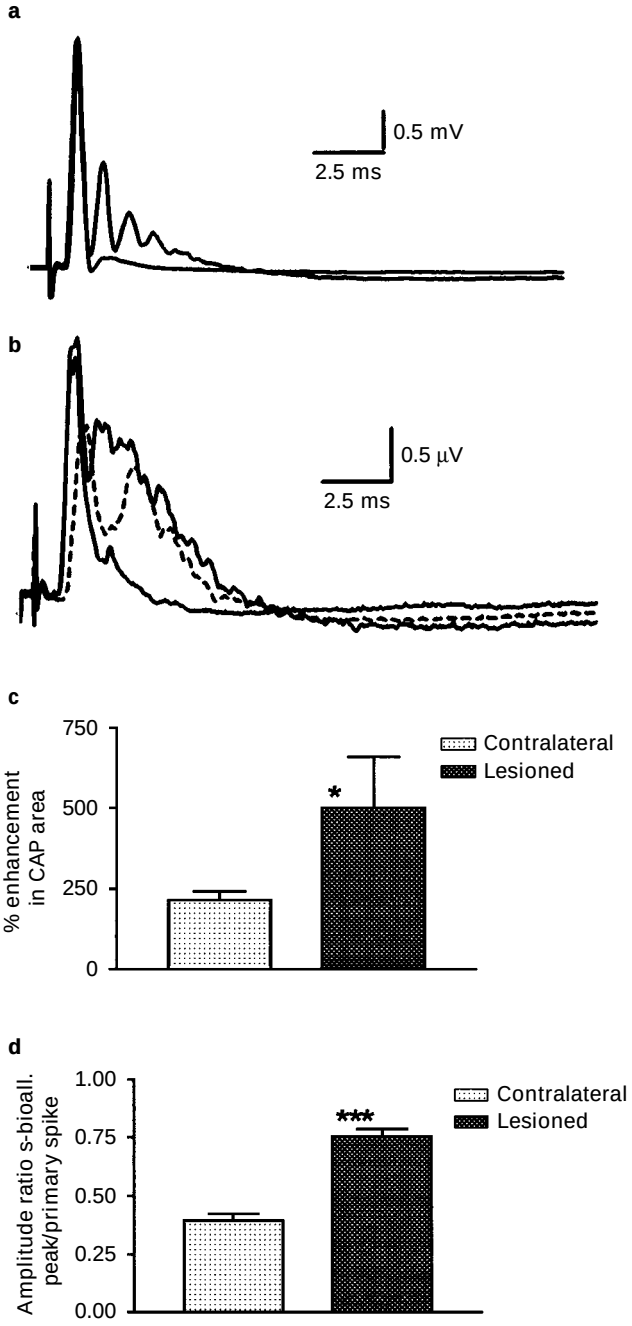

Figure 5 (a) Superimposed compound action potentials in normal control tibial nerve before and after treatment with 5 μM s-bioallethrin for 45 min. Note the pronounced rippling indicative of regenerative firing following pyrethroid exposure. (b) Similar experiment in a lesioned nerve at 37°C. Note the much more pronounced and dispersed plateau potential and the marginal increase in the amplitude of the primary spike induced by the pyrethroid. The hatched line shows the pretreatment spike at *circa* 26°C where a dispersed slowly conducting population of axons is evident. (c) Collated data from 6 cells in each group treated with 5 μM s-bioallethrin for 45 min. The lesioned preparations resulted in a much greater after-discharge (measured as an area): $*P<0.05$ (Mann-Whitney test). (d) Analysis of the ratio between the primary compound action potential amplitude and the maximum negative after potential induced by s-bioallethrin in lesioned and contralateral preparations. $***P<0.0001$, two-tailed unpaired t test ($n=6$).

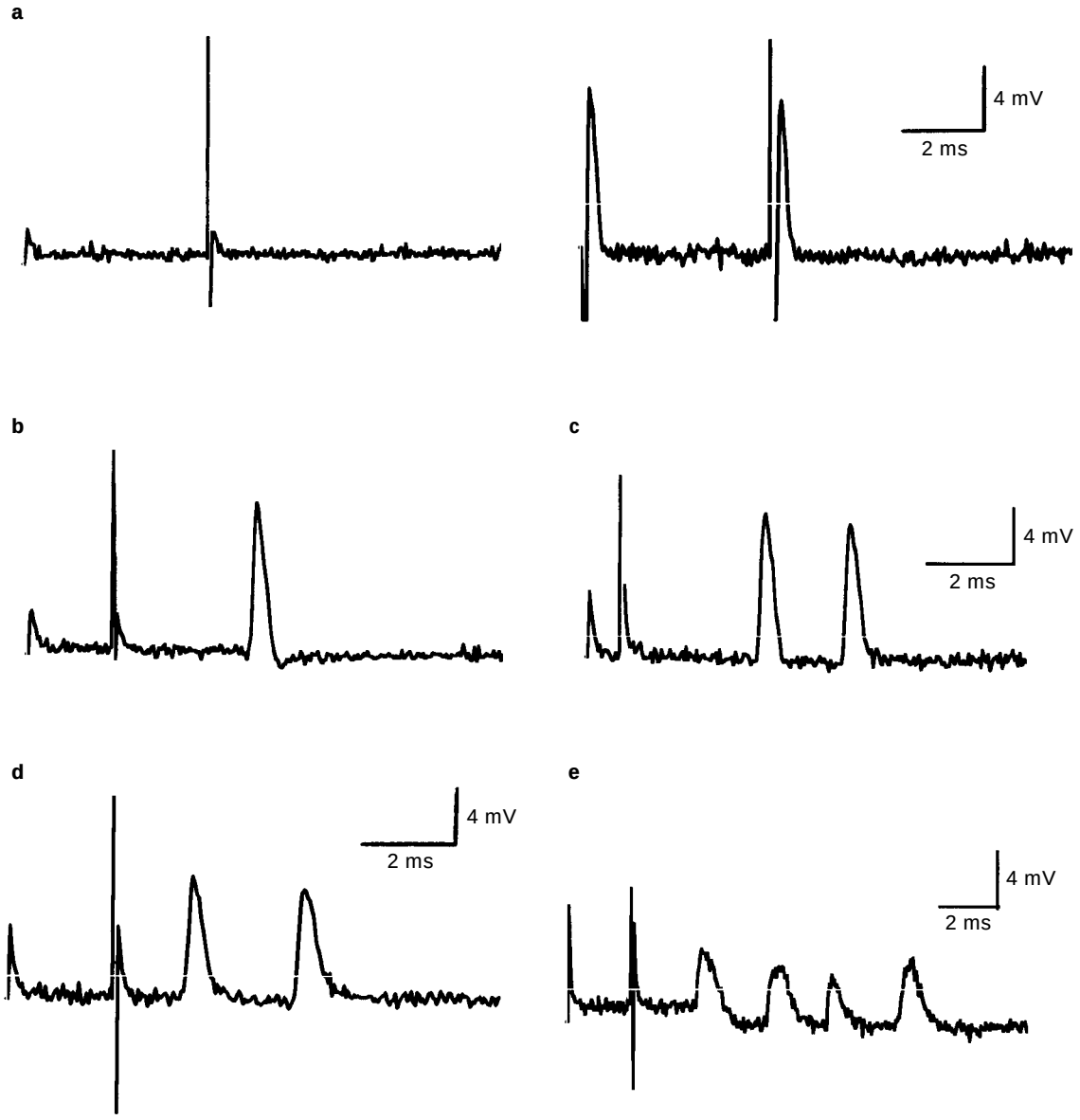

Figure 6 Responses to paired stimuli in a lesioned nerve trunk indicating that s-bioallethrin enhanced the safety factor for conduction and elevated the blocking temperature (a) Left: full block on distal stimulation with the lesion temperature at 36°C. Right: proximal stimulation resulted in full blown paired spikes at 36°C. (b) With the lesion at 34°C the refractory period for a distal stimulus was >1.84 ms. (c) After 15 min superfusion of s-bioallethrin (5 μM) at 34°C the refractory period was reduced to <0.8 ms. (d) After 25 min exposure the blocking temperature was elevated to >38°C. Spiking was secure at >39°C in this axon, indicating a rise in blocking temperature of >3°C. (e) After about 30 min intermittent ectopic spiking was evident in response to paired stimulus pulses.

proportion of channels in the cell membrane this drastically increases the steady-state Na^+ permeability of the neurone (for Type II molecules) to effect depolarization and inactivation of unliganded/unmodified channels (Vijverberg & Van den Bercken, 1979). The above spectrum of activity of the molecules would appear to explain the observed effects of the peroneal nerve. Deltamethrin, which is a very lipid soluble molecule, effected a slowly-developing depressant action: the kinetics probably reflect slow diffusion into a trunk comprising tens of thousands of myelinated axons (Schmalbruch, 1986). It is noteworthy that spike depression was still developing after 45 min, but no evidence was obtained consistent with regenerative firing (in accord with previous data obtained at room temperature in mammalian peripheral nerve: Vijverberg *et al.*, 1986). Such a molecule would clearly exacerbate existing conduction deficits in demyelinating disease. Neither deltamethrin nor permethrin were associated even with transient after-potentials or rippling (indicative or hyperexcitation or regenerative activity in these multicellular preparations) at physiological temperatures. Both type I and II molecules prolong action potentials in electrophysiological experiments where transmembrane potential is stabilized experimentally, by passing hyperpolarizing d.c. into current clamped cells, (Jacques *et al.*, 1980), thereby precluding concomitant block via the allosteric conversion of channels to inactivated conformations in depolarized axons. The lack of effect of permethrin on CAP kinetics may reflect concomitant depolarizing block in affected axons or a very pronounced negative temperature coefficient (the voltage clamp studies were conducted at room temperature). Permethrin-induced regenerative effects have been noted in frog tibial nerves after much longer exposure periods (22 h) at 19–22°C which may be

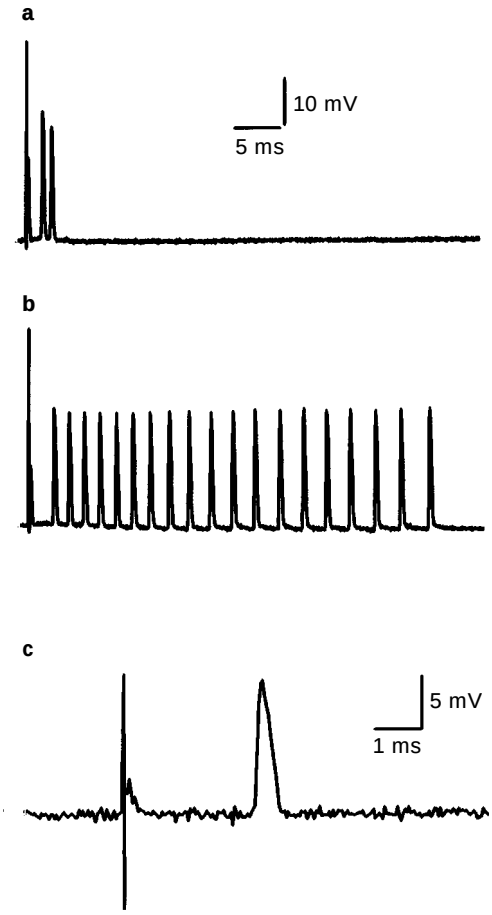

Figure 7 (a) Responses to a distal stimulus after >60 min treatment with s-bioallethrin (5 μM). A brief after discharge is evident at 37°C. (b) Cooling the same axon to 29°C resulted in a prolonged burst of action potentials. (c) Even after prolonged exposure (37°C) axons could be found which did not exhibit after-discharges.

required to equilibrate truly all membranes in the trunk with such extremely hydrophobic molecules (Vijverberg *et al*., 1986). s-Bioallethrin produced typical symptoms albeit the underlying bursts/after-potentials were brief compared to qualitatively similar responses in insect nerve trunks at room temperature (Lees, unpublished data). The sucrose gap technique does not definitively demonstrate the basis for the after-potentials. It is likely that the majority of the response reflects repetitive firing in a subpopulation of cells rather than a uniform but marginal response on primary spike repolarization in all exposed axons (consistent with the evidence from peri-axonal single unit experiments). The effects of s-bioallethrin on the peripheral nerve CAP were qualitatively similar but marginally greater in magnitude than those of 4-AP in similar experiments (Lees, 1996). 4-AP has been used as an effective symptomatic treatment in MS (eg Van Diemen *et al*., 1992) but is associated with considerable toxicity (which may reflect the induction of regenerative activity, particularly in sensory axons). It remains to be seen whether pyrethroid action is specific for a sub-populations of axons, but our results are consistent with this proposal. Alternatively, the duration of our perfusion was insufficient to effect true equilibration with all membranes in the nerve trunks. Our intention was to select a molecule with potential to reverse conduction block for further *in vitro* assessment. s-Bioallethrin was selected for further study based on the 2 fold increase in CAP area produced in this study. In contrast, the scorpion venom which has already been used to overcome conduction block in demyelinated axons *in vitro* (Bostock *et al*., 1978) enhanced action potential area by more than 140 fold. Presumably by virtue of a greater selectivity for channel inactivation, the venom did not produce concurrent depolarizing block.

The tibial nerve preparation presented here appears to represent a robust model for temperature-dependent conduction block. Over 80% of MS patients develop a panoply of neurological signs during hyperthermia which may, in part, be linked to conduction block in partially demyelinated axons (Guthrie & Nelson, 1995). In theoretical models of conduction, the temperature at which conduction block occurs is a steep function of the extent of demyelination (Schauf & Davis, 1974). A steep decline in CAP area was observed in extracellular recordings which was mirrored in all-or-none block of individual axons across a similar temperature range. The increased refractory period for transmission in the portions of the axons including the lesion is consistent with previous findings (Felts *et al*., 1995). Almost all of the decrement in the extracellular signals could be ascribed to a slowly conducting population of presumptive demyelinated, or partially remyelinated, axons. Attempts to demonstrate that s-bioallethrin could reverse the block of these fibres at 37°C were complicated by the induction of regenerative firing (even in normal axons) by the pyrethroid. Induced ectopic spiking was prevalent in the same late phases when slowed conduction in demyelinated axons would be observed, so would partially mask restored conduction in segmentally lesioned units. The pronounced and significant enhancement of both CAP area and amplitude ratios in lesioned nerves compared to contralateral trunks treated with s-bioallethrin could be interpreted as evidence for reversal of block superimposed on the 'physiological after-discharge'. Similar extracellular evidence has been published to explain the efficacy of 4-AP, which also induces regenerative activity, to reverse conduction block *in vitro* (e.g. Targ & Kocsis, 1985). The single unit recordings provide direct experimental evidence in favour of this conclusion, albeit with a relatively small sample size (reflecting the fragility of impalements and the requirement for a prolonged perfusion for pyrethroid access). Only 1 of 4 fibres did not respond with an apparent increased safety factor or elevated blocking temperature: co-incidentally it did not exhibit regenerative activity which may suggest that the exposure was too brief. The incidence of regenerative firing with this compound limits the resolution of our *in vitro* assay for conduction block and is likely to underpin the toxicity of Type I pyrethroids in mammals (hyperexcitation, ataxia, convulsions and even paralysis). s-Bioallethrin is classified as slightly toxic based on its relatively high LD_{50} (784 mg kg^{-1} in rodents). On a more constructive note the improved safety factor and elevated blocking temperatures were manifest before overt ectopic firing in all 3 responsive cells. Perhaps a non-insecticidal pyrethroid (with a greater polarity) could produce an even more marginal kinetic effect and restore conduction without inducing after discharges at physiological temperature. Until this can be demonstrated *in vitro*, or in animals models, pyrethroids as a generic class could not be used as symptomatic treatments in MS patients.

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