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Effects of the potassium channel blocking dendrotoxins on acetylcholine release and motor nerve terminal activity

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1 The effects of the K^+ channel blocking toxins, the dendrotoxins, on neuromuscular transmission and motor nerve terminal activity were assessed on frog cutaneous pectoris, mouse diaphragm and mouse triangularis sterni nerve-muscle preparations. Endplate potentials (e.p.ps) and miniature e.p.ps were recorded with intracellular microelectrodes, and nerve terminal spikes were recorded with extracellular electrodes placed in the perineural sheaths of motor nerves.

2 Dendrotoxin from green mamba (*Dendroaspis angusticeps*) venom and toxin I from black mamba (*D. polylepis*) venom increased the amplitude of e.p.ps by increasing quantal content, and also induced repetitive e.p.ps.

3 Perineural recordings revealed that dendrotoxins could decrease the component of the waveform associated with K^+ currents at the nerve terminals, and induce repetitive activation of nerve terminals.

4 In frog motor nerves, dendrotoxins are known to block the fast f_1 component of the K^+ current at nodes of Ranvier. Blockade of a similar component of the K^+ current at motor nerve terminals may be responsible for the effects of these toxins on neuromuscular transmission.

5 Similar conclusions can be drawn from the results obtained from mouse neuromuscular junctions.

Introduction

Neuronal membranes contain several distinct K^+ conductances that can be distinguished by their voltage-dependency, kinetic properties, Ca^{2+} sensitivity, and interactions with blocking agents (Rogawski, 1985). Different types of nerve cells have different proportions of the various subtypes of K^+ channels, thus contributing to their specialized electrical properties.

K^+ conductance help to regulate the release of transmitter from nerve terminals by controlling the time course of depolarization in response to action potentials. In order to determine if particular forms of K^+ channel play distinct roles in the regulation of transmitter release, the effects of specific channel blocking drugs or toxins can be assessed. From voltage clamp studies of the K^+ current at nodes of Ranvier of frog motor nerves, it is known that there are three kinetically distinct components: f_1, which is a fast current activated between -80 mV and -30 mV; f_2, a fast current activated between -40 mV and $+30$ mV; and a slow current (see Dubois, 1983). Both fast components are blocked by aminopyridines, but only f_1 is blocked by dendrotoxins (Benoit & Dubois, 1986). Dendrotoxins are facilitatory neurotoxins from mamba venoms (see Harvey *et al.*, 1984; Harvey & Anderson, 1985 for reviews). By recording the electrical activity of, and acetylcholine release from, frog motor nerve terminals before and after application of dendrotoxins, it was hoped to distinguish the role of f_1 K^+ conductances in the regulation of excitability and transmitter release. Similar experiments were performed on mouse neuromuscular junctions, although the pharmacology of K^+ channels in mammalian motor neurones has not been as clearly defined as in the frog.

Preliminary accounts of some of this work were given to the British Pharmacological Society, Edinburgh, September 1985 (Anderson & Harvey, 1985) and at the 6th European Society of Neurochemistry meeting, Prague, September 1986 (Anderson *et al.*, 1987).

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Methods

Experiments were performed on the cutaneous pectoris preparation of the frog (*Rana pipiens* and *R. temporaria*) (Dreyer & Peper, 1974), and on the phrenic nerve-hemidiaphragm preparation or the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) from 19–45 g male mice (A strain, Bantin and Kingman; and Balb C strain). Frog Ringer solution contained (mM): NaCl 111, KCl 2, $NaHCO_3$ 2, and $CaCl_2$ 2. The pH was 7.2, and experiments were performed at room temperature (17–23°C). Experiments on mouse preparations were performed at 37°C; preparations were continuously perfused at 5–10 ml min^{-1} with physiological salt solution (composition in mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose, 11.1) which had previously been bubbled with oxygen containing 5% carbon dioxide (pH 7.3).

Intracellular recording

Neuromuscular junctions were identified by the presence of endplate potentials (e.p.ps) or miniature e.p.ps (m.e.p.ps) with rise times of 1 ms or less. E.p.ps and m.e.p.ps were recorded using glass microelectrodes filled with 3 M KCl (resistance 4–10 MΩ) and a high impedance unity gain electrometer (W-P Instruments M-4A or M-701). A silver/silver chloride pellet was used as the reference electrode. To evoke e.p.ps the motor nerves were stimulated via a suction electrode every 2–5 s (pulse width 50 μs and supramaximal voltage). Muscles were prevented from contracting by altering the ratio of Ca^{2+} to Mg^{2+} ions (0.1–2.0. 0 mM $CaCl_2$; 1.6–16.8 mM $MgCl_2$), or by the addition of tubocurarine (1–6 μM).

For experiments performed on frog preparations, drugs and toxins were added directly to the solution in the bath. However, on mouse preparations physiological solution (10–25 ml) containing drugs and toxins at the required concentrations was perfused through the tissue bath and recycled after aeration. In three control experiments at 37°C in which toxin-free solution was recycled for 60 min, there was no change in the average quantal content of e.p.ps or in the frequency of m.e.p.ps. In most experiments, recordings were made continuously from one endplate area before and throughout application of dendrotoxins, each endplate area (and hence each preparation) being exposed to only one application of toxin. However, in some experiments, as detailed in the text, populations of 6 muscle fibres were sampled before, and at various time points after, exposure to the dendrotoxins.

Extracellular recording

To record nerve terminal activity on frog cutaneous pectoris and mouse triangularis sterni preparations, glass microelectrodes filled with 1–2 M NaCl (resistance 10–20 MΩ) were inserted into the perineural sheath of fine motor nerve bundles, as described by Gundersen *et al.* (1982), Mallart (1984, 1985a), David & Yaari (1986) and Penner & Dreyer (1986). The potential difference between the recording electrode and a silver/silver chloride reference electrode in the bath was measured as for intracellular recording. Usually 20–30 waveforms were recorded at each time period, and waveforms were monitored continously from the same area throughout the experiment. The waveforms were evoked by stimulating the motor nerve via a suction electrode every 1–5 s with pulses of 50 μs duration and supramaximal voltage. Muscle contraction was prevented by tubocurarine (20–46 μM) or by altering the ratio of Ca^{2+} to Mg^{2+} ions (0.1–0.5 mM $CaCl_2$; 1.5–2.1 mM $MgCl_2$).

Materials

Dendrotoxin and toxin I were isolated from the venoms of the Eastern green mamba, *Dendroaspis angusticeps*, and of the black mamba, *Dendroaspis polylepis*, respectively, by gel filtration followed by ion exchange chromatography as described previously (Harvey & Karlsson, 1980; 1982). Other drugs were obtained from the Sigma Chemical Co., Poole, Dorset.

Results

Effects on acetylcholine release

Evoked transmitter release Control experiments on mouse and rat diaphragm preparations revealed that dendrotoxins had little effect at room temperature or at 32°C compared to the effect at 37°C (see also Harvey & Anderson, 1985). When recordings were made at 20°C, there was no increase in e.p.p. amplitude or in the number of e.p.ps produced in response to each single nerve stimulus after 60–105 min treatment with dendrotoxin (3.5–4.9 μM). Therefore, all electrophysiological experiments on mouse preparations were performed at 37°C.

Dendrotoxin (1.4–5.6 μM) and toxin I (2.6–5.2 μM) produced two distinct effects on e.p.ps that occurred at different times after addition of toxin. The first effect, which was seen within 2–15 min (on 4 out of 7 frog preparations and on 10 out of 31 mouse preparations), consisted of a transient increase in the amplitude of e.p.ps by 20–50% (Figure 1). As the dendrotoxins did not increase the amplitude or frequency of m.e.p.ps (see below) or affect the time course of e.p.ps or m.e.p.ps, the larger e.p.ps presumably result from an increase in quantal content. The

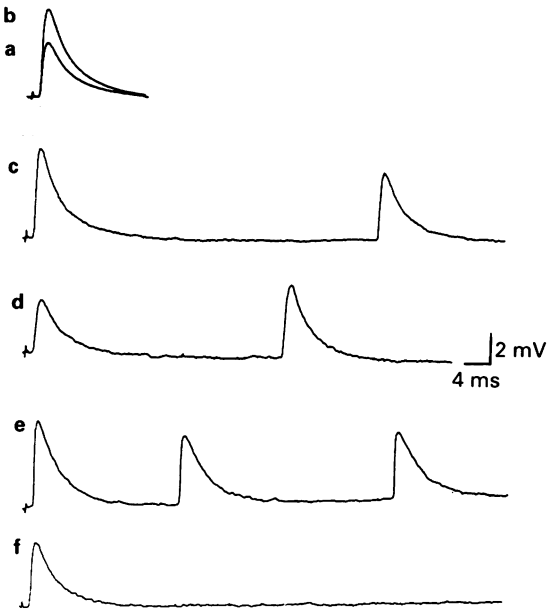

Figure 1 Endplate potentials (e.p.ps) recorded from a frog cutaneous pectoris preparation before (a) and at the same endplate after 5 min (b); 20 min (c); 25 min (d and e) and 30 min (f) exposure to 2.6 μM toxin I. The motor nerve was stimulated at a frequency of 0.5 Hz in (a-d); 0.1 Hz in (e) and 1 Hz in (f). The bathing solution contained 0.3 mM Ca^{+} and 2.0 mM Mg^{2+}; the temperature was 20°C. In (a) and (b), computer digitised averages of 20-30 e.p.ps are shown; in (c-f), typical responses to single stimuli are given. The averaged m.e.p.p. amplitude (0.4mV), and muscle fibre resting membrane potential (−80mV) remained constant throughout the recording period.

increase in e.p.p. amplitude produced by dendrotoxin was observed both in normal Ca^{2+} and low Ca^{2+} bathing media. For example, on mouse diaphragm preparations in which neuromuscular transmission was blocked by tubocurarine, the addition of dendrotoxin (4.2 μM) produced an increase in e.p.p. amplitude to 138 ± 17% control (mean ± s.e.mean, $n = 5$). Similarly, in preparations blocked with low Ca^{2+} and raised Mg^{2+} concentrations, dendrotoxin also increased e.p.p. amplitude. Such increases were rather variable (the largest being 15 times the control quantal content following exposure of a mouse diaphragm preparation to 2.8 μM dendrotoxin), and could not be determined accurately because the recording electrodes were frequently dislodged by muscle twitching. The increases in quantal content and muscle twitching were usually not maintained despite the continued presence of the toxin, and these effects often disappeared after 15–20 min exposure to the toxin.

The second effect of the dendrotoxins appeared after 20–60 min exposure. At this time, single stimulation applied to the motor nerve produced multiple e.p.ps (Figure 1). Usually two or three e.p.ps were produced in response to each single nerve stimulus, but in the frog as many as eight e.p.ps could be observed. The time to onset of the repetitive activity did not depend on the concentration of toxin used. In general, the repetitive activity appeared after 20–30 min in low Ca^{2+} solutions and 45–60 min in normal Ca^{2+} solutions. When the repetitive e.p.ps first appeared, they were separated by 50–80 ms in the frog, and by 6–8 ms in the mouse. With increasing time after the toxin the e.p.ps occurred closer together, and often the preparations began to twitch in response to stimulation. Spontaneously occurring e.p.ps could also be observed (especially on mouse preparations) in both normal Ca^{2+} and low Ca^{2+} solutions. The spontaneous e.p.ps appeared either singly or in bursts of 2–3, at a fast regular rate of 15–20 Hz. Washing for 45 min with toxin-free solution did not stop the repetitive activity from occurring. Bath application of tetrodotoxin (3–15.6 nM) abolished both the repetitive and the spontaneous e.p.ps.

Although the effects of the dendrotoxins were similar on mouse and frog preparations, there was a difference in the response of preparations to changes in stimulation frequency after toxin treatment. In the frog, lowering the frequency of stimulation from 0.5 Hz to 0.1 Hz shortened the interval between the repetitive e.p.ps, and increased the number of e.p.ps produced in response to each single nerve stimulus. Conversely, increasing the frequency to 1–5 Hz suppressed the repetitive firing (Figure 1). In the mouse, however, the repetitive activity was enhanced by increasing the frequency of stimulation from 0.2–0.5 Hz to 2–12 Hz.

Some mouse diaphragm preparations appeared to be completely insensitive to the facilitatory toxins. Thus, no increase in the amplitude of e.p.ps and no repetitive firing was seen on 7 out of 31 preparations treated with dendrotoxin.

Spontaneous transmitter release In 4 mouse diaphragm preparations in which recordings were made continuously from the same endplate areas during exposure to dendrotoxin (4.2 μM), a rapid reduction in m.e.p.p. frequency, but not amplitude, occurred during the first 5 min exposure to the toxin (Figure 2). The reduction in m.e.p.p. frequency was not maintained, and the frequency usually recovered during the following 30 min exposure to the toxin. Dendrotoxin had very little effect on the amplitude, time course, or frequency of m.e.p.ps recorded from populations of mouse diaphragm fibres 30–120 min after exposure to dendrotoxin. For example, after 105–120 min exposure to dendrotoxin (4.2 μM), the values for m.e.p.p. amplitude, time constant of decay, and

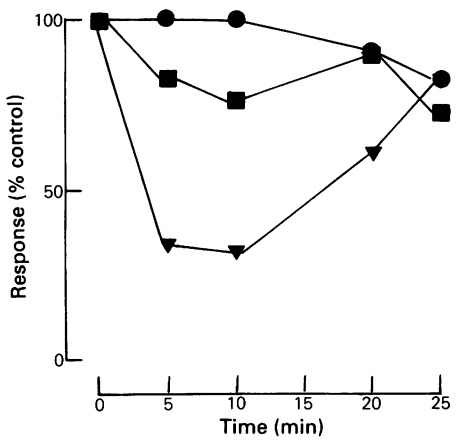

Figure 2 Changes in m.e.p.p. frequency (▼), amplitude (●) and time constant of decay (■) at one endplate in a mouse diaphragm preparation during the first 25 min exposure to dendrotoxin (4.2 μM). Each point was calculated from at least 100 m.e.p.ps obtained during a 1–2 min recording period. Control values for m.e.p.p. frequency, amplitude, and decay time constant were 16 s^{-1}, 1.2 mV, and 1.4 ms, respectively. S.e.means were about 5%.

frequency were 111 ± 19, 112 ± 4, and 97 ± 31% control (mean ± s.e.mean, $n = 3$ preparations). Similarly, the dendrotoxins did not significantly alter m.e.p.p. frequency on frog preparations.

Perineural recordings

Effects of the dendrotoxins on motor nerve terminal action potentials were assessed using the perineural recording technique. An electrode placed inside the perineural sheath of frog and mouse motor nerves, close to endplate areas, records the inverted replicas of currents leaving or entering the nerve terminal membrane following stimulation (Mallart, 1985a; David & Yaari, 1986; Hevron *et al.*, 1986; Penner & Dreyer, 1986). Perineural waveforms recorded at 20°C consisted of a biphasic negative (downward deflection) which was usually preceded by a small positive (upward) deflection (Figure 3). Increasing the temperature to 37°C shortened the time course of the mouse perineural waveforms but the components were still distinguishable (Figure 3). The first negative deflection has been ascribed to inward Na^+ current at the last few nodes of Ranvier and the first sections of the nonmyelinated terminal region (Konishi, 1985; Mallart, 1985a). The second negative deflection is selectively removed by local application of K^+ channel blockers to terminal regions of the nerves indicating that it is associated with K^+ currents at the nerve terminals (Brigant & Mallart, 1982; Penner & Dreyer, 1986). Although it is possible to record these currents focally at motor nerve terminals (Brigant & Mallart, 1982), the terminal waveforms have a poor signal-to-noise ratio, and prolonged stable recordings are not possible, especially at 37°C. Hence we used the perineural recording technique for the present experiments, because stable recordings can be made from the same site over a considerable period of time. In our control experiments, waveforms monitored continuously from the perineural sheath showed at most a 10–15% non-selective reduction in signal amplitude over a 60 min recording period. Recording sites were rejected if the signal amplitude fell by more than 10% in the first 10 min of impalement. On mouse preparations, recording sites were selected visually at 400 times magnification, and accepted if the signal had a second negative deflection of greater magnitude than the first (Figure 3). Penner & Dreyer (1986) have shown that only those signals recorded in close proximity to endplate areas have this configuration. On frog preparations, the second negative deflection was invariably smaller than the first (see also Hevron *et al.*, 1986) and so recording sites were selected to maximize

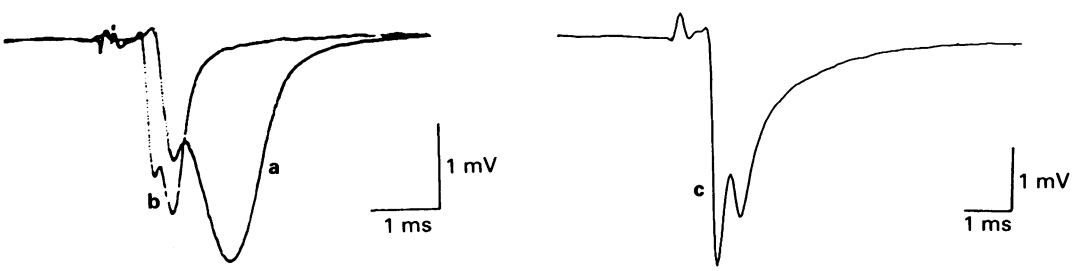

Figure 3 Averaged perineural waveforms from mouse triangularis sterni (a and b), and frog cutaneous pectoris preparations (c). In (a) and (c) the temperature was 20–21°C. The waveforms in (b) were obtained from the same site as those in (a), but at a temperature of 37°C.

the second negative deflection.

Dendrotoxins produced two effects on perineural waveforms. In experiments on frog preparations in which contractions had been prevented by altering the Ca^{2+}: Mg^{2+} ratio, the addition of toxin I (25 nM–3.9 μM) produced repetitive firing (Figure 4) without any major change in the shape of the waveform. This effect first appeared after 15–20 min addition of toxin. The repetitive activity was suppressed by increasing the stimulation frequency from 0.5 Hz to 1 Hz and by the addition of 6 mM $CaCl_2$ or $MgCl_2$ to the bathing solution. The repetitive activity was not affected by tubocurarine (44–50 μM). In some experiments, repetitive activity was monitored postjunctionally with an intracellular electrode and then an electrode was placed in the perineural sheath of a neighbouring nerve branch: the repetitive e.p.ps and the repetitive activity of the motor nerve appeared to coincide (Figure 4). On mouse preparations, dendrotoxin (5.6 μM) also induced repetitive firing in response to a single stimulus, and slightly prolonged the decay phase of the second negative deflection. Usually only one or two repetitive waveforms were observed. Following exposure of mouse preparations to dendrotoxin, 3,4-diaminopyridine (150 μM) still reduced the amplitude of the second negative deflection, and also induced more repetitive firing. Pretreatment with 3,4-diaminopyridine (100 μM) did not prevent dendrotoxin from inducing repetitive activity, even though the second negative component had apparently been abolished by the aminopyridine.

The second effect of the dendrotoxins consisted of a reduction in the amplitude of the second negative deflection (Figure 5). On frog preparations, this effect was variable both in its time to onset (2–20 min) and also in the magnitude of the reduction observed (13–100%). On the frog preparations that showed a large reduction in the amplitude of the second negative deflection, a reduction in signal amplitude together with an increase in signal duration and an enhancement of early positivity were often observed after 30–60 min (Figure 5). On mouse preparations, the second negative deflection was also reduced by dendrotoxin (4.9 μM). This effect could be reversed by washing for 30 min. Dendrotoxin did not affect the first negative component of mouse waveforms.

Discussion

The main effect of dendrotoxins at the neuromuscular junction is to induce repetitive activity in response to single pulse stimulation of the motor nerve. This happened in frog and mouse neuromuscular preparations, and it was an effect directly on nerves because it was abolished by very low concentrations of tetrodotoxin but was unaffected by high concentrations of tubocurarine.

The mechanism of action of the dendrotoxins is not fully understood. They are known not to stimulate release evoked by K^+-induced depolarization or by a Ca^{2+} ionophore (Anderson & Harvey, 1985), and they are not likely to be affecting Na^+ channel activation or inactivation for several reasons. Thus, the toxins did not consistently affect the first negative component of the perineural waveform, which is sensitive to tetrodotoxin, and they did not produce effects on perineural waveforms similar to those caused by a Na^+

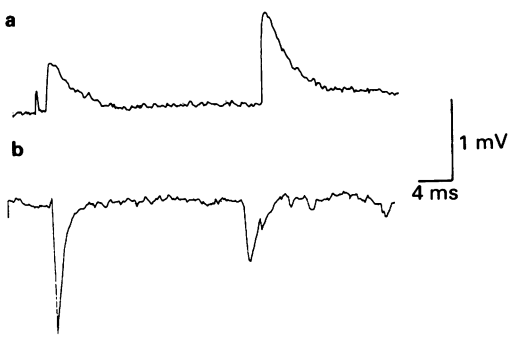

Figure 4 Endplate potentials (e.p.ps) (a) and perineural waveforms (b) recorded from a frog cutaneous pectoris preparation after 65 min treatment with toxin I (25 nM). Typical responses to single stimuli are shown. The perineural waveforms in (b) were recorded from a fine nerve branch close to the site at which the e.p.ps in (a) were recorded. The bathing solution contained 0.1 mM Ca^{2+} and 1.9 mM Mg^{2+}; the temperature was 23°C.

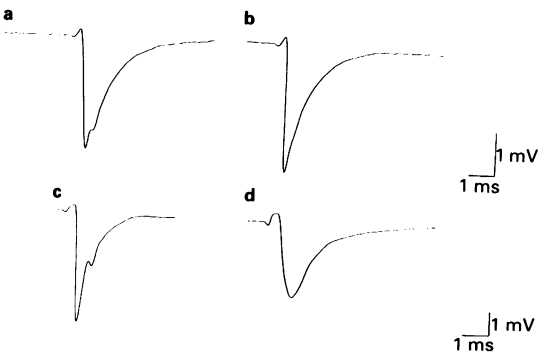

Figure 5 Averaged perineural waveforms from two frog cutaneous pectoris preparations before (a and c), and at the same sites after toxin I (b and d). (b) 20 min after 0.55 μM toxin I. The bathing solution contained 0.15 mM Ca^{2+} and 2.1 mM Mg^{2+}. (d) 30 min after 3.9 μM toxin I. The bathing solution contained 2 mM Ca^{2+} and 46 μM tubocurarine.

channel toxin, ATx II (Molgo & Mallart, 1985), or increase spontaneous transmitter release, as would happen after nerve terminal depolarization; and the toxins have been shown to have no action on Na^+ currents in mammalian and amphibian neurones (Weller *et al.*, 1985; Benoit & Dubois, 1986; Halliwell *et al.*, 1986). However, a recent study (Schauf, 1987) showed that internal application of dendrotoxin increases the time constant for inactivation of Na^+ channels in dialyzed *Myxicola* giant axons.

Dendrotoxin and toxin I have been shown to block K^+ conductances in various neurones (Dolly *et al.*, 1984; Weller *et al.*, 1985; Benoit & Dubois, 1986; Halliwell *et al.*, 1986; Penner *et al.*, 1986; Stansfeld *et al.*, 1986; Schauf, 1987). Of particular relevance to the present work is the finding that toxin I selectively blocks the f_1 component of the K^+ current at nodes of Ranvier in frog motor nerves (Benoit & Dubois, 1986). Fifty % block of the f_1 component is produced by 0.4 nM toxin I, and at least 1000 times more is needed to block other components of the K^+ current (Benoit & Dubois, 1986). As discussed by Dubois (1983), the f_1 component is probably the main contributor to the K^+ current during action potentials of myelinated nerves, and the f_2 component will play a larger part in the slower conducting nonmyelinated nerves. As dendrotoxin I produced little consistent change in the component of the nerve terminal waveform associated with K^+ current, it could be that f_1 channels contribute relatively little to the repolarizing K^+ current of motor nerve terminals. However, the repetitive firing in the presence of dendrotoxins may be a consequence of the selective block of f_1 channels, which act to stabilize the excitability of nerve terminals, although they do not contribute very much to the overall K^+ current. In mammalian hippocampal neurones, dendrotoxins have been shown to block a transient K^+ current, the A current (Dolly *et al*, 1984; Halliwell *et al.*, 1986). The function of this particular current is to prevent repetitive firing of action potentials (e.g. Connor & Stevens, 1971a,b; Segal *et al.*, 1984; Segal, 1985). Similar dendrotoxin-sensitive channels giving rise to an equivalent of the A current may, therefore, be present at mouse motor nerve endings. Segal *et al.* (1984) noted that the amplitude of the A current was more variable and consistently smaller in spinal neurones than in hippocampal neurones. Perhaps the size of this current at motor nerve endings is also small.

Dendrotoxin has also been shown to block another type of K^+ conductance. This is a non-inactivating K^+ conductance found in some cells of the nodose ganglia of the rat (Stansfeld *et al.*, 1986) and in dorsal root ganglia of guinea-pigs (Penner *et al.*, 1986). The effects of dendrotoxin on these cells is not seen after pretreatment with aminopyridines, and, at least in the nodose ganglia, the effects of the toxin are reversed by washing. Perhaps our finding of a reduction of the second negative deflection of perineural waveforms by dendrotoxins that could be reversed by washing could be a result of some terminals having toxin-sensitive non-inactivating K^+ channels.

Although the effects of dendrotoxins on frog and mouse preparations were broadly similar, there was a difference in the response to changes in the frequency of stimulation. In the mouse, increasing the frequency led to an increase in repetitive activity, whereas in the frog, the opposite occurred. The difference may be explained by different properties of the Ca^{2+}-activated K^+ channels in the two species. Mallart (1985b) showed that $I_{K,Ca}$ in mouse motor nerve terminals was suppressed by high frequency stimulation; he suggested that this might contribute to the facilitation of acetylcholine release during tetanic stimulation. In contrast, David & Yaari (1986) demonstrated that the contribution of $I_{K,Ca}$ in frog motor nerves was larger after high frequency stimulation. This would be expected to provide a more efficient repolarization of the nerve terminal, and hence, prevent repetitive firing. There is no evidence to suggest that the dendrotoxins have a direct action on $I_{K,Ca}$ at mouse motor nerve terminals (Anderson & Mallart, unpublished) or mammalian hippocampal neurones (Halliwell *et al.*, 1986).

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