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Effects of charybdotoxin, a blocker of Ca^{2+}-activated K^+ channels, on motor nerve terminals

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1 The contribution of Ca^{2+}-activated K^+ currents ($I_{K,Ca}$) to the control of electrical excitability of motor nerve terminals and the control of acetylcholine release was assessed by studying the effects of the specific K(Ca) channel blocking toxins charybdotoxin and apamin. Electrical activity of the terminal regions of motor nerves was assessed by extracellular recording from an electrode placed in the perineural sheaths of nerves in the mouse triangularis sterni and frog cutaneous pectoris preparations. Acetylcholine release was monitored by intracellular recording of endplate potentials (e.p.ps).

2 Charybdotoxin (20–300 nM), but not apamin (10 nM–2.5 μM), selectively reduced the amplitude of an $I_{K,Ca}$ unmasked by prior blockade of the delayed rectifier K^+ current with 3,4-diaminopyridine (3,4-DAP).

3 In the combined presence of 3,4-DAP and charybdotoxin, large Ca^{2+}-dependent plateau responses developed, but only moderate and transient increases in acetylcholine release occurred.

4 In the absence of 3,4-DAP, charybdotoxin did not alter the electrical activity of, or the transmitter release from motor nerve terminals.

5 A possible role of the charybdotoxin-sensitive $I_{K,Ca}$ in the control of transmitter release is discussed.

Introduction

Motor nerve terminals innervating skeletal muscles have several distinct types of K^+ currents. Apart from the classical delayed rectifier, a second type of K^+ current, which is activated by increased levels of intracellular Ca^{2+}, has been described (Mallart, 1985b; Hevron *et al.*, 1986; Anderson & Harvey, 1988). Similar channels are also present in the presynaptic nerve terminals of rat brain (Bartschat & Blaustein, 1985). The precise nature of the Ca^{2+}-activated K^+ currents ($I_{K,Ca}$) and its contribution to the regulation of the release of acetylcholine at motor nerve terminals has not been established. The small size of nerve terminals has hindered direct electrophysiological analysis, and a lack of suitable blocking agents has restricted pharmacological studies. Activation of $I_{K,Ca}$ may be important in regulating nerve terminal excitability (either by altering the frequency of depolarizations, or by shortening the duration of depolarizations), preventing excessive accumulation of internal Ca^{2+} and thereby inhibiting transmitter release.

At least two distinct forms of $I_{K,Ca}$ have been identified in a wide variety of cells, and they can generally be distinguished on the basis of their unitary conductances and their sensitivity to different toxins (Strong & Castle, 1987). High conductance K(Ca) channels, with unitary conductances often in excess of 100 pS, are blocked by tetraethylammonium (TEA) but not by the bee venom peptide apamin; in contrast, low conductance K(Ca) channels, with unitary conductances of less than 20 pS, are blocked by apamin and are very much less sensitive to TEA. In some cells (e.g. skeletal myotubes, sympathetic ganglionic neurones, GH_3 anterior pituitary cells), the differential sensitivity to TEA and apamin has been exploited to demonstrate the co-existence of both types of channel (Romey & Lazdunski, 1984; Pennefather *et al.*, 1985; Ritchie, 1987). More recently, charbydotoxin (isolated from the venom of the scorpion *Leiurus quinquestriatus*) has been shown

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to be a high affinity blocker of the TEA-sensitive/apamin insensitive, large conductance K(Ca) channels in a variety of neuronal and non-neuronal mammalian cells (Smith *et al.*, 1986; Castle & Strong, 1986; Beech *et al.*, 1987; Guggino *et al.*, 1987; Anderson *et al.*, 1988), and it offers a more specific means to demonstrate the presence of large conductance K(Ca) channels. Charybdotoxin also blocks some K(Ca) channels in invertebrate neurones (Hermann & Erxleben, 1987).

With both perineural (Mallart, 1985a) and conventional intracellular recording techniques, we have used two K(Ca) channel toxins, apamin and charybdotoxin, to study the role of $I_{K,Ca}$ in the regulation of neuronal excitability and acetylcholine release at motor nerve terminals of frog and mouse. A preliminary account of some of this work was given to the Physiological Society, Glasgow, in June 1987 (Anderson *et al.*, 1987).

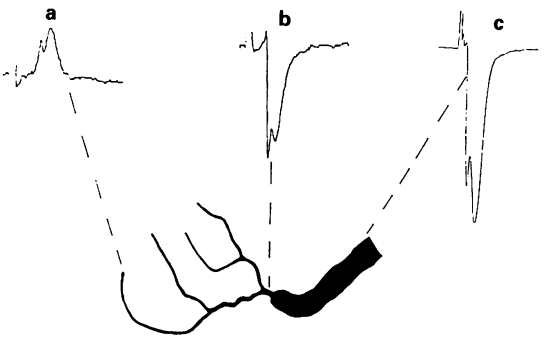

Figure 1 Schematic diagram showing the shape of the extracellularly recorded waveform associated with nerve terminal action potentials. Note that the shape depends on the specific placement of the electrode: (a) terminal region; (b) preterminal region; (c) perineural location close to an endplate. Adapted with permission, from Brigant & Mallart (1982).

Methods

Experiments were performed on the cutaneous pectoris preparation of the frog (*Rana temporaria*) (Dreyer & Peper, 1974), and on either the phrenic nerve-hemidiaphragm preparation or the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) from BALB/C mice. Frogs were stunned by a blow to the head, decapitated, and pithed; mice were killed by cervical dislocation. Frog preparations were bathed in a Ringer solution containing (mM): NaCl 116, KCl 2, HEPES 2 and $CaCl_2$ 1.8–6. The pH was 7.2. Mouse 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, $NaHCO_3$ 25, glucose, 11.1 and $CaCl_2$ 2.5–6.0) which had been previously bubbled with 95% O_2 plus 5% CO_2 (pH 7.3). All experiments were performed at room temperature (17–23°C).

For experiments performed on frog preparations, drugs and toxins were added directly to the solution in the bath. For mouse preparations, 10 ml physiological salt solution containing drugs and toxins at the required concentrations were perfused through the tissue bath and recycled after aeration. In control experiments, recycling of solutions did not affect the perineural waveforms, or the amplitude of endplate potentials (e.p.ps).

Perineural recordings

Presynaptic waveforms were recorded from frog cutaneous pectoris and mouse triangularis sterni preparations by glass microelectrodes (filled with 1–2 M NaCl, resistance 4–12 megohms) inserted into the perineural sheath of fine motor nerve bundles (Gundersen *et al.*, 1982; Mallart, 1984; 1985a; David & Yaari, 1986; Penner & Dreyer, 1986) (see Figure 1). The potential difference between the recording electrode and a silver/silver chloride reference electrode in the bath was measured by a high impedance unity gain amplifier (W-P Instruments, model M-701), displayed on a dual beam storage oscilloscope and simultaneously stored on FM tape. Waveforms were evoked by stimulating the motor nerve via a suction electrode every 2–10 s with supramaximal pulses of 50 μs duration. Muscle contractions were prevented by (+)-tubocurarine (29 μM), or by α-bungarotoxin (125 nM). Usually 10–20 waveforms were recorded at each time period. As the shape of the waveform was very dependent on the position of the recording electrode, waveforms were monitored continuously from the same site throughout the experiment (Anderson & Harvey, 1988). Recording sites were rejected if the signal amplitude fell by more than 10% in the first 10 min after impalement. Using this protocol, stable recordings could be obtained over a 60 min period in control solutions or in the presence of 3,4-diaminopyridine (3,4-DAP).

Intracellular recording

Neuromuscular junctions were identified by the presence of e.p.ps or miniature endplate potentials (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 6–10 megohms). To study evoked transmitter release, the motor nerves were stimulated supramaximally at 0.5 Hz (pulse

width 50 μs), and muscle twitching was prevented by the addition of (+)-tubocurarine (3–23 μM). In most experiments, recordings were made continuously from one endplate area before and throughout application of toxin, each endplate area (and hence each preparation) being exposed once to toxin.

Materials

Apamin was isolated from the crude venom of the European honey bee (*Apis mellifera*) (Gauldie *et al.*, 1976). Final traces of melittin were removed by chromatography on a column of heparin-Sepharose CL-6B; the resultant peptide was homogeneous by reverse phase h.p.l.c. Charybdotoxin was isolated from the crude venom of the Israeli scorpion, *Leiurus quinquestriatus hebraeus* as previously described (Castle & Strong, 1986). The sample was at least 85% pure by reverse phase h.p.l.c. and contained no toxins affecting Na^+ channels. In order to verify that the remaining u.v.-absorbing contaminants did not alter the effects of charybdotoxin, a single experiment was carried out with h.p.l.c.-homogeneous charybdotoxin; quantitatively identical results were observed to those obtained with the slightly less pure material. The molecular weight of 4–5 kdaltons as determined by SDS-PAGE (Castle & Strong, 1986) has been verified by amino acid sequence determination (Garcia *et al.*, 1988).

Crude *Apis mellifera* venom was purchased from Bulgarcoop, Sofia, Bulgaria, and crude *Leiurus quinquestriatus hebraeus* venom was from Latoxan, Rosans, France. Other compounds were obtained from the Sigma Chemical Co., Poole, Dorset, U.K.

Results

Perineural recordings

Recordings from the perineural sheaths of mouse and frog motor nerves are similar in form to those detected with a focal extracellular electrode placed on the initial nonmyelinated portion of the terminal arborization (Figure 1); they are almost a mirror image of waveforms recorded focally from the most distal terminal regions (Brigant & Mallart, 1982). Such recordings, when made close to endplate areas, consist of a small positive (upward) deflection followed by a biphasic negative (downward) deflection (Figures 1 and 2a). The first negative deflection corresponds to inward Na^+ current at the last few nodes of Ranvier and the first sections of the nonmyelinated terminal region; it is sensitive to tetrodotoxin (Konishi, 1985; Mallart, 1985a). The second negative deflection was reduced by addition of 3,4-DAP (100–400 μM) (Figure 2b), and is attributed to outward K^+ currents at the nerve terminals (Brigant & Mallart, 1982; Mallart, 1985a,b; Penner & Dreyer, 1986). These control observations are, therefore, in agreement with the previously observed components of the perineural waveforms.

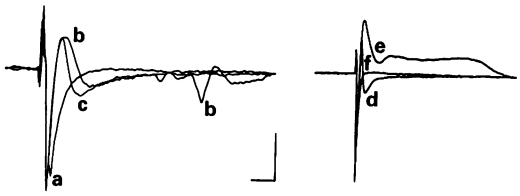

Figure 2 Effects of 3,4-diaminopyridine (3,4-DAP) and tetraethylammonium (TEA) on averaged perineural waveforms recorded from frog cutaneous pectoris preparations. (a) Control, (b) same site after 15 min application of 100 μM 3,4-DAP. Note the repetitive activity. (c) Ten min after increasing the extracellular Ca^{2+} concentration from 1.8 to 6.0 mM, in the presence of 100 μM 3,4-DAP. Note the enhancement of the second negative deflection and reduction of repetitive firing. (d–f) Perineural waveforms recorded from a different preparation bathed in 6 mM Ca^{2+}; (d) in the presence of 100 μM 3,4-DAP; (e) in the presence of a combination of 100 μM 3,4-DAP with 2 mM TEA. (f) Twenty μM verapamil and 200 μM Cd^{2+} reduced the large positive deflection produced by TEA and 3,4-DAP. Stimulus artefacts have been removed for clarity in (d–f). Vertical bar: 0.5 mV in (a–c); 1 mV in (d–f). Horizontal bar: 2 ms in (a–c), 7 ms in (d–f).

In the presence of 3,4-DAP, another negative component of frog and mouse perineural waveforms is apparent; this is smaller in amplitude, delayed in onset and dependent on the Ca^{2+} concentration in the bathing medium (Figure 2b,c). For example, increasing external Ca^{2+} to 6 mM enhanced the delayed negative deflection observed in the presence of 3,4-DAP (Figure 2c), and replacement of Ca^{2+} with Mg^{2+} abolished it (data not shown). Similar observations have been described by Mallart (1985b) and Hevron *et al.* (1986). In the presence of 3,4-DAP, the addition of TEA (1–2 mM) suppressed the delayed negative deflection and enhanced a positive component that could be reduced by the addition of Ca^{2+} channel blockers (Figure 2e–f). Thus, the delayed negative deflection seen in the presence of 3,4-DAP corresponds to a TEA-sensitive, Ca^{2+}-dependent K^+ current ($I_{K,Ca}$) of frog and mouse motor nerve terminals (see also Mallart, 1985b; Hevron *et al.*, 1986). The positive component that is enhanced in the presence of TEA represents an inward Ca^{2+} current at nerve terminals (Brigant & Mallart, 1982; Penner & Dreyer, 1986).

The actions of charybdotoxin and apamin on the motor nerve terminal $I_{K,Ca}$ were similar in both frog and mouse preparations, and therefore their effects

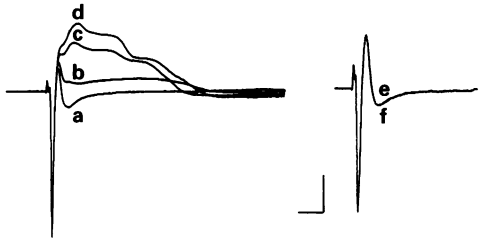

Figure 3 Effects of charybdotoxin and apamin on averaged perineural waveforms. (a–d) Perineural waveforms recorded from a mouse triangularis sterni preparation in the presence of 6 mM Ca^{2+} and 400 μM 3,4-diaminopyridine (3,4-DAP). (a) Control, (b) 70 s after 80 nM charybdotoxin, (c) 3 min after 80 nM charybdotoxin and (d) 10 min after 80 nM charybdotoxin. (e–f) Superimposed perineural waveforms from a frog cutaneous pectoris preparation in the presence of 6 mM Ca^{2+} and 100 μM 3,4-DAP. Before (e) and 15 min after (f) 1 μM apamin. Vertical bar: 2 mV in (a–d), 1 mV in (e–f). Horizontal bar: 4 ms.

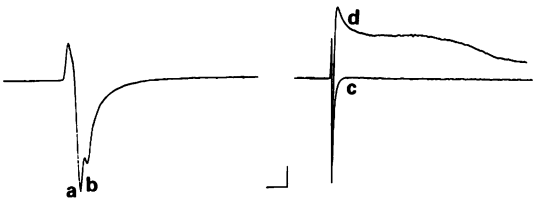

Figure 4 Effect of charybdotoxin on averaged perineural waveforms from a frog cutaneous pectoris preparation bathed in solution containing 1.8 mM Ca^{2+}. (a) Control, (b) 15 min after 80 nM charybdotoxin. (c) Same record as (b) on a different time scale. (d) Thirty s after 100 μM 3,4-diaminopyridine (3,4-DAP). Stimulus artefacts have been removed for clarity. Vertical bar: 1 mV. Horizontal bar: 1 ms in (a–b), 5 ms in (c–d).

will not be described separately for each species. In (+)-tubocurarine-paralysed preparations exposed to 100–400 μM 3,4-DAP, charybdotoxin (20 nM and above) reduced the amplitude of the delayed negative deflection (i.e. $I_{K,Ca}$) (Figure 3a–d). The reduction in amplitude of $I_{K,Ca}$ produced by charybdotoxin was accompanied by an enhancement of the large positive component (Figure 3c,d) that is sensitive to Ca^{2+} channel blockers. No repetitive firing was observed. The effects of charybdotoxin were rapid in onset: a reduction in $I_{K,Ca}$ was observed after 70–90 s exposure (Figure 3), and a maximum was reached (as judged by the amplitude and duration of the prolonged positive component) within 10–20 min of exposure. Washing with toxin-free solution for 15–45 min completely reversed these effects.

In contrast, apamin (10 nM–2.5 μM) had no effect on the amplitude of the $I_{K,Ca}$ observed in the presence of 3,4-DAP (Figure 3e–f), nor indeed on any other component of the waveform. As (+)-tubocurarine is known to block $I_{K,Ca}$ in some cells (Cook & Haylett, 1985; Goh & Pennefather, 1987), the effects of apamin and charybdotoxin were also tested in the absence of (+)-tubocurarine in mouse preparations paralysed by α-bungarotoxin, which does not affect K(Ca) channels (Cook & Haylett, 1985). Apamin (100 nM) still had no demonstrable effect on perineural waveforms recorded in the presence of 3,4-DAP, while charybdotoxin (80 nM) still blocked $I_{K,Ca}$. (+)-Tubocurarine had no effect on $I_{K,Ca}$ of mouse motor nerve terminals at concentrations of 15–150 μM.

In the absence of 3,4-DAP, charybdotoxin (80–150 nM) had no observable effect on either the first or second negative deflections of normal perineural waveforms (Figure 4a,b). When 3,4-DAP (100–400 μM) was added after 15–25 min exposure to charybdotoxin, the second negative deflection was abolished, and replaced by a large prolonged positive component that could be blocked by Ca^{2+} antagonists (Figure 4c,d). Under these conditions, the delayed negative component usually revealed by 3,4-DAP was not seen.

Effects on acetylcholine release

As the results in the preceding section showed that charybdotoxin is a specific blocker of the TEA-sensitive $I_{K,Ca}$ at motor nerve terminals, conventional intracellular recording experiments were performed to establish the role of this $I_{K,Ca}$ in the regulation of acetylcholine release.

Evoked transmitter release Under conditions when an $I_{K,Ca}$ can be observed with the perineural recording technique, i.e. in the presence of 3,4-DAP (100–300 μM), charybdotoxin (20–80 nM) produced a rapid and transient increase in the amplitude of e.p.ps in 4 out of 5 frog preparations, and in 5 out of 8 mouse preparations (Figure 5). The increase in e.p.p. amplitude produced by charybdotoxin occurred after about 90 s exposure to the toxin, and was of variable magnitude (9–65% increase) in both frog and mouse preparations. At the time of the increase in e.p.p. amplitude, there were also increases in the e.p.p. rise time (13–25% increase) and time constant of decay (6–10% increase). In addition, the latency of e.p.ps was increased in frog preparations (Figure 5). After about 3–5 min exposure to charybdotoxin in the presence of 3,4-DAP, e.p.p. amplitude started to decline (Figure 5). The reduction in e.p.p. amplitude produced by charybdotoxin ranged from 10–100%, after 15 min exposure to toxin. When $I_{K,Ca}$ was enhanced

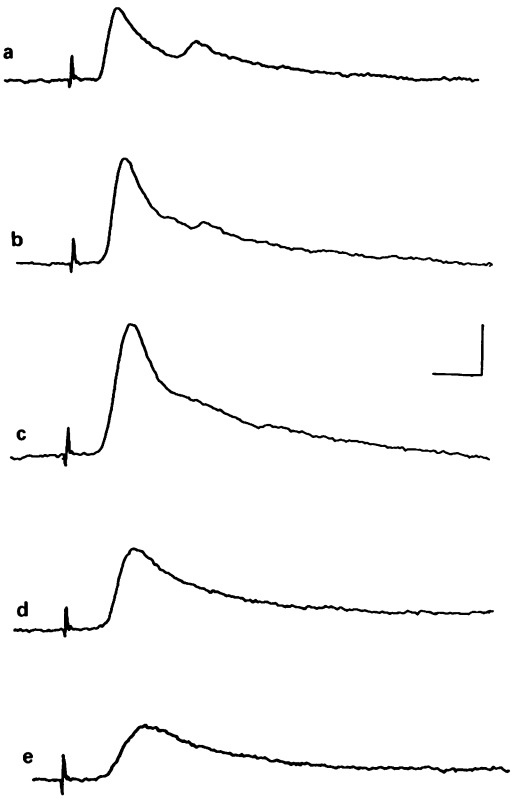

Figure 5 Effect of charybdotoxin on endplate potentials (e.p.ps) from a frog cutaneous pectoris preparation bathed in a solution containing 100 μM 3,4-diaminopyridine (3,4-DAP) and 1.8 mM Ca^{2+}. (a) Control, (b) 110 s, (c) 134 s, (d) 150 s, (e) 170 s after 80 nM charybdotoxin. Membrane potential was −76 mV, stimulation rate was 0.5 Hz, D-tubocurarine concentration was 13 μM. Vertical bar: 2 mV. Horizontal bar: 4 ms.

by raising the Ca^{2+} in the bathing medium to 6 mM, e.p.p. amplitude declined more rapidly. When e.p.ps were reduced or abolished, perineural waveforms consisted of a large monophasic negative component followed by a prolonged positive component, similar to the waveform shown in Figure 3d. The block of e.p.ps could be reversed by washing out the charybdotoxin.

TEA, which also blocks the motor nerve terminal $I_{K,Ca}$ (Figure 2), was tested for its effects on e.p.ps in two mouse preparations pretreated with 400 μM 3,4-DAP. TEA (1 mM) did not increase the amplitude of e.p.ps; instead, e.p.p. amplitude was reduced to 51–55% of control after 5–7 min exposure, and after 10 min, e.p.ps were too small to record. Further analysis of the effects of TEA in the presence of 3,4-DAP was not attempted because of the postjunctional blocking actions of TEA, which were revealed by a marked fall in the amplitude of m.e.p.ps.

In the absence of 3,4-DAP, charybdotoxin had no effect on e.p.ps; after a 10 min exposure to 80 nM charybdotoxin, the values for e.p.p. amplitude, rise time and time constant of decay were 101 ± 11, 98 ± 2 and 96 ± 13% of control, respectively (means ± s.e.mean, combined results from 2 mouse and 2 frog preparations). In contrast, on two mouse preparations exposed to 1 mM TEA in the absence of 3,4-DAP, e.p.p. amplitude was doubled after 5 min; thereafter e.p.p. amplitudes declined.

Spontaneous transmitter release In the absence of nerve stimulation and (+)-tubocurarine, charybdotoxin produced little alteration in the postjunctional sensitivity to acetylcholine, as judged by effects on m.e.p.ps. For example, after 8–10 min exposure to 80 nM charybdotoxin, m.e.p.p. amplitude, rise time and time constant of decay were 96 ± 4, 99 ± 5 and 99 ± 7% of control, respectively (means ± s.e.mean, combined results from 2 mouse and 2 frog preparations).

Discussion

We have used a perineural recording technique to characterize a K(Ca) conductance at motor nerve terminals with the aid of selective toxins. Although the interpretation of perineural waveforms can be ambiguous (the signal recorded is the sum of inward and outward currents in the vicinity of the electrode), we have preferred this technique to the alternative, terminal recording technique, because it permits stable recordings to be made continuously from the same site, over a long period of time (Anderson & Harvey, 1988). For ease of reference, the shapes of control waveforms recorded at different sites of the motor nerve are illustrated in Figure 1. For further discussion on the perineural recording techniques and interpretation of the waveforms, see Mallart (1985a) and Penner & Dreyer (1986).

Frog and mouse motor nerve endings have a current that is sensitive to block by charybdotoxin but not by apamin. This current, which is revealed after blocking the delayed rectifier K^+ current with 3,4-DAP, is Ca^{2+}-dependent, and is also blocked by TEA (Mallart, 1985b; Hevron *et al.*, 1986). Thus, it has properties in common with Ca^{2+}-activated K^+ currents in other cells (Miller *et al.*, 1985; Beech *et al.*, 1987; Guggino *et al.*, 1987; Hermann & Erxleben, 1987). The actions of charybdotoxin were rapid in onset, reversible and selective for $I_{K,Ca}$. In the presence of charybdotoxin there was no change in the

amplitude of the waveform associated with I_{Na}, and a large Ca^{2+} current could still be detected. Furthermore, when experiments were performed in the absence of 3,4-DAP, the delayed rectifier K^+ current was unaffected by concentrations of charybdotoxin that substantially reduced $I_{K,Ca}$. Although charybdotoxin has recently been demonstrated to block some voltage-dependent K^+ channels in murine lymphocytes (Lewis & Cahalan, 1988), such an effect is clearly lacking in motor nerve terminals. In other vertebrate cells, charybdotoxin selectively blocks TEA-sensitive K(Ca) channels with unitary conductances in excess of 100 pS (Miller *et al.*, 1985; Beech *et al.*, 1987; Guggino *et al.*, 1987). Although technical difficulties prevent the measurement of the unitary conductance of the motor nerve terminal $I_{K,Ca}$, we suggest that the K(Ca) channels of motor nerve terminals may be classified as the high conductance type by analogy with the actions of charybdotoxin elsewhere. In sympathetic ganglionic neurones, both high and low conductance K(Ca) channels have been characterized (Pennefather *et al.*, 1985); also, apamin-sensitive hyperpolarizations have been described in the cell bodies of cat spinal motor neurones (Zhang & Krnjevic, 1987). We found no pharmacological evidence for the co-existence of low conductance K(Ca) channels that are typically blocked by apamin.

In the absence of 3,4-DAP, charybdotoxin had no effect on the perineural waveform, and did not increase evoked transmitter release, as judged by the amplitude of e.p.ps. This suggests that, under our experimental conditions, the charybdotoxin-sensitive $I_{K,Ca}$ does not play a role in repolarizing motor nerve terminals. (The increase in e.p.p. amplitude in the presence of TEA results from TEA blocking a fraction of the voltage-dependent K^+ current.) Presumably, the delayed rectifier repolarizes the membrane so rapidly that $[Ca^{2+}]_i$ does not reach the level required for $I_{K,Ca}$ activation. In cultured rat myotubes (Blatz & Magleby, 1986) and in GH_3 anterior pituitary cells (Lang & Ritchie, 1987), the high conductance K(Ca) channels are activated by internal concentrations of Ca^{2+} greater than 5 μM, whereas the low conductance channels are activated at concentrations below 1 μM. Under normal circumstances, increases of intraterminal Ca^{2+} concentrations at neuromuscular junctions may not reach levels required to activate these K(Ca) channels. However, when the duration of the nerve terminal action potential was prolonged by blocking the delayed rectifier with 3,4-DAP, charybdotoxin initially produced a moderate increase in the evoked release of acetylcholine (as judged by an increase in the amplitude of e.p.ps, but not m.e.p.ps). Presumably, during these prolonged depolarizations, the internal Ca^{2+} concentration increases sufficiently to activate $I_{K,Ca}$, which then, in turn, helps to repolarize the nerve terminal.

Although charybdotoxin produced some increase in transmitter release (in the presence of 3,4-DAP), high levels of acetylcholine output were not maintained, despite the continued presence of large Ca^{2+} plateau responses detected by perineural recording. High levels of transmitter output could deplete the pool that is readily available for release. However, spontaneously-occurring m.e.p.ps can still be recorded at frog motor endplates when e.p.ps are blocked during exposure to a mitochondrial inhibitor (Molgo & Pécot-Dechavassine, 1988). Another possible explanation is that the large and prolonged rises in internal Ca^{2+} may act to block transmitter release by an unknown mechanism, as occurs at the squid giant synapse (Miledi & Slater, 1966; Adams *et al.*, 1985) and at frog neuromuscular junctions (Molgo & Pécot-Dechavassine, 1988). In the frog, such a failure of e.p.ps occurs while the active zones and their associated synaptic vesicles appear normal in electron micrographs (Molgo & Pécot-Dechavassine, 1988).

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