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Hexamethonium- and methyllycaconitine-induced changes in acetylcholine release from rat motor nerve terminals

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1 The neuronal nicotinic receptor antagonists hexamethonium and methyllycaconitine (MLA) have been used to study the putative prejunctional nicotinic ACh receptors (AChRs) mediating a negative-feedback control of ACh release from motor nerve terminals in voltage-clamped rat phrenic nerve/hemidiaphragm preparations.

2 Hexamethonium (200 μM), but not MLA (0.4–2.0 μM), decreased the time constant of decay of both endplate currents (e.p.cs) and miniature endplate currents (m.e.p.cs), indicating endplate ion channel block with hexamethonium. However, driving function analysis and reconvolution of e.p.cs and m.e.p.cs indicated that this ion channel block did not compromise the analysis of e.p.c. quantal content.

3 At low frequencies of stimulation (0.5–2 Hz), hexamethonium (200 μM) and MLA (2.0 μM) increased e.p.c. quantal content by 30–40%. At high frequencies (50–150 Hz) neither compound affected e.p.c. quantal content. All effects on quantal content were paralleled by changes in the size of the pool of quanta available for release.

4 The low frequency augmentation of e.p.c. quantal content by hexamethonium was absent when extracellular [Ca^{2+}] was lowered from 2.0 to 0.5 mM.

5 At the concentrations studied, MLA and hexamethonium produced a small (10–20%) decrease in the peak amplitude of m.e.p.cs.

6 Neither apamin (100 nM) nor charybdotoxin (80 nM) had effects on spontaneous or nerve evoked current amplitudes at any frequency of stimulation. Thus the ability of nicotinic antagonists to augment e.p.c. quantal content is not due to inhibition of Ca^{2+}-activated K^+-channels.

7 We suggest that hexamethonium and MLA increase evoked ACh release by blocking prejunctional nicotinic AChRs. These receptors exert a negative feedback control over evoked ACh release and are probably of the α-bungarotoxin-insensitive neuronal type.

Keywords: Neuromuscular junction; quantal analysis; acetylcholine release; nicotinic acetylcholine receptors; hexamethonium; methyllycaconitine

Introduction

The well-known neuromuscular phenomena of tetanic fade and rundown of high frequency trains of endplate potentials (e.p.ps) or endplate currents (e.p.cs) observed with muscle relaxant drugs such as tubocurarine have been ascribed, at least in part, to a prejunctional action of the compound (Liley & North, 1953; Liley, 1956; Hubbard & Wilson, 1973; Glavinovic, 1979b; Magleby *et al*., 1981; Gibb & Marshall, 1984). Radiolabel studies have shown that tubocurarine decreases [^{3}H]acetylcholine overflow from skeletal muscle/motor nerve preparations, although the detection is limited to long periods of high frequency stimulation (Wessler *et al*., 1986, 1987). Electrophysiological studies at the neuromuscular junction have shown that tubocurarine reduces e.p.c. quantal content at high frequencies of stimulation (Harborne *et al*., 1988) but can also increase the quantal content of the first e.p.p. in a train (Wilson, 1982).

Initially, the observations with tubocurarine led to the development of two apparently contradictory theories. One proposed that tubocurarine blocks a positive feedback mechanism to decrease acetylcholine (ACh) release during high frequency trains of impulses (Bowman 1980; Bowman *et al*., 1988, 1990), and the other that the drug blocks a negative feedback mechanism to increase release at the start of the train of impulses (Wilson, 1982). Both mechanisms have been proposed to involve nicotinic ACh autoreceptors on the motor nerve terminals and either, particularly when superimposed upon postjunctional neuromuscular block, would be expected to produce fade and rundown.

More recent observations, one using a wide range of stimulation frequencies (Tian *et al*., 1994) and others using drugs other than tubocurarine (Wilson & Thomsen, 1991, 1992; Tian *et al*., 1994), have opened up the possibility of the co-existence of both positive and negative feedback mechanisms at the neuromuscular junction, with differing frequency-dependence and drug sensitivities. The present study was designed to study the putative negative feedback mechanism and to confirm the type of nicotinic ACh receptor (AChR) that might be involved in its operation. For this purpose we have used the nicotinic antagonists hexamethonium and methyllycaconitine (MLA). Hexamethonium has little effect on muscle-type nicotinic AChRs, but blocks neuronal-type nicotinic AChRs in ganglia (Bowman & Webb, 1972) predominantly by an ion channel blocking mechanism (Gurney & Rang, 1984). MLA is an antagonist of neuronal-type nicotinic AChRs with a degree of selectivity for the α-bungarotoxin-sensitive α_7-type (Wonnacott *et al*., 1993; Decker *et al*., 1995; Yum *et al*., 1996). In addition, using apamin and charybdotoxin (ChTX), we have investigated the possibility that any effects of the agents to augment evoked ACh release could be related to inhibition of prejunctional Ca^{2+}-activated K^+-channels (Cook & Haylett, 1985). Our data for hexamethonium and MLA are consistent with the hypothesis that ACh release from motor nerve terminals is inhibited by ACh acting on prejunctional negative feedback nicotinic AChRs which, based on their pharmacology, are probably of the α-bungarotoxin-insensitive neuronal-type.

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Methods

Muscle preparation

Experiments utilized the rat isolated hemidiaphragm muscle preparation. Hemidiaphragms, along with 10–15 mm of their phrenic nerve were removed from male Sprague Dawley rats (150–250 g) killed by anaesthetizing to death with a gradually rising concentration of CO_2 followed by immediate cervical dislocation. Isolated preparations were cleaned of excess rib and connective tissue and pinned to the Sylgard lined base of a 10 ml tissue bath continuously perfused with a standard Krebs–Henseleit solution (see below). Preparations were immobilised for the recording of evoked responses by cutting across all fibres approximately 2 mm on either side of the central endplate region of the muscle. A cut muscle preparation was used to eliminate the need for unwanted pharmacological agents during the recording of evoked responses (Barstad & Lilliheil, 1968). For evoked signals, the motor nerve was stimulated at 0.5–150 Hz with pulses of 0.05–0.1 ms duration and voltage greater than that required to elicit maximal responses (10–20 V) using silver wire electrodes linked to a Grass S88 stimulator via a Grass SIU5 stimulus isolation unit.

Electrophysiological technique

Miniature endplate currents (m.e.p.cs) and endplate currents (e.p.cs) were recorded from motor endplates using a standard two intracellular microelectrode voltage clamp technique. All signals were recorded at a holding potential of −50 mV, chosen to avoid the complication of artefacts due to action potentials sometimes seen when clamping at higher potentials (see Prior *et al*., 1993). Voltage-clamp electronics modified from a design by Dionne & Stevens (1975) were used and microelectrodes were filled with either 3 M KCl for (voltage recording, 10–15 MΩ) or 0.6 M K_2SO_4 (current pasing, 5–10 MΩ). All current signals were recorded on FM tape (Racal Store 4DS) at a bandwidth of d.c.—5 kHz for subsequent off-line analysis. Positioning of the electrodes at the endplate was determined from the rising phase of evoked responses; less than 0.5 ms being considered as evidence for accurate placement. For evoked responses, voltage-clamp gain was adjusted so that the voltage-escape during evoked responses was less than 0.5 mV (i.e. less than 1% of the driving force). For spontaneous responses it was necessary to use slightly lower clamp gains to improve the signal-to-noise ratio of the smaller signals.

Experimental protocols

To avoid the complicating effects of dynamic changes in evoked ACh release immediately following a change in stimulation frequency, blocks of 60–100 e.p.cs were recorded at nine different stimulation frequencies ranging from 0.5 to 150 Hz and only e.p.cs within the plateau portion of each block of stimulation were considered for analysis (typically the last 60–80 e.p.cs). Prior to recording evoked responses, a recording of approximately 30 m.e.p.cs was made in the same fibre. Following collection of control data, either hexamethonium (200 μM), MLA (0.4 or 2.0 μM), apamin (100 nM) or ChTX (80 nM) was perfused through the tissue bath for 5 min. For hexamethonium and MLA, the concentrations used were selected to maximise the ratio of the pre- and postjunctional effects of the compounds. The effects of hexamethonium (200 μM) on e.p.cs and m.e.p.cs were studied with extracellular calcium ion concentrations ($[Ca^{2+}]_0$) of either 0.5 or 2.0 mM. The concentrations of apamin and ChTX used were based on those which have previously been shown to be maximally effective for the inhibition of $I_{K[Ca]}$ in hepatocytes (Cook & Haylett, 1985) and at the neuromuscular junction (Anderson *et al*., 1988), respectively. Subsequent to application of the test compound, a second set of m.e.p.cs and nine blocks of e.p.cs

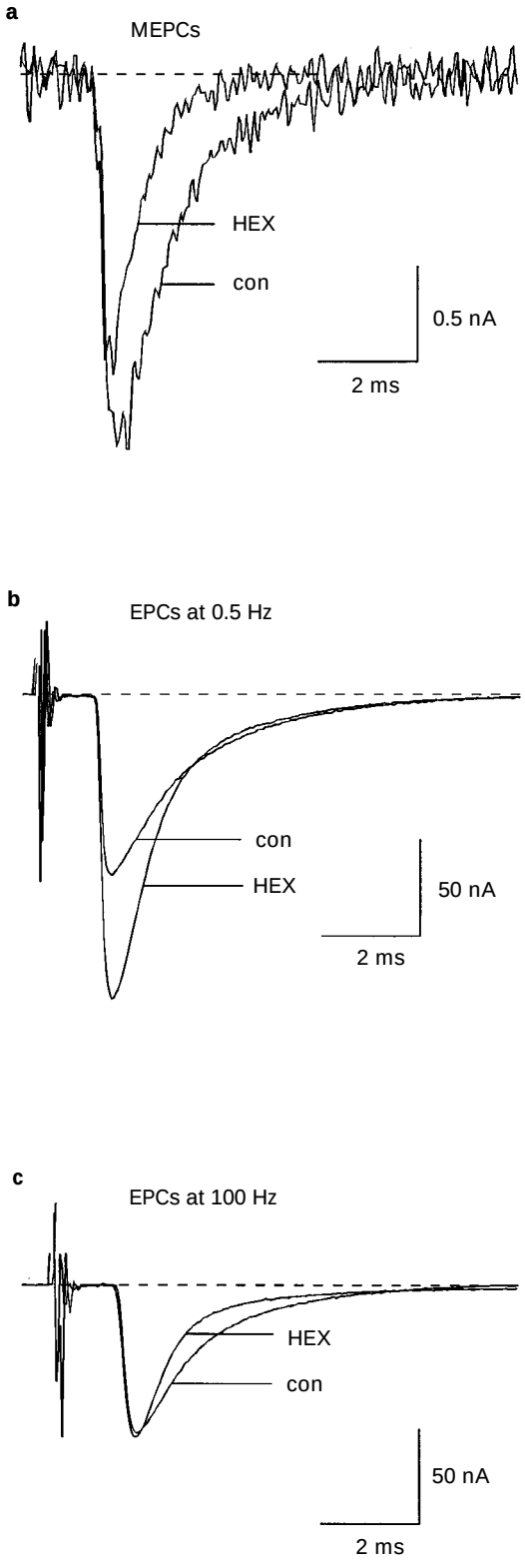

Figure 1 Representative examples of averaged m.e.p.cs (a) and e.p.cs elicited at either 0.5 Hz (b) or 100 Hz (c) recorded in the absence and presence of 200 μM hexamethonium. Note that in all cases hexamethonium decreased the decay time of the signals indicative of endplate ion channel block. Note also that while hexamethonium depressed the peak amplitude of m.e.p.cs, e.p.c. amplitudes were either increased (at 0.5 Hz) or unaffected (at 100 Hz), both indicative of a frequency-dependent hexamethonium-induced increase in e.p.c. quantal content. All records are from the same fibre voltage clamped at −50 mV and $[Ca^{2+}]_0$ was 2 mM.

(0.5–150 Hz) were recorded from the same fibre as in control. For each batch of experiments six to eight individual experiments were performed, each in a different hemidiaphragm preparation.

Analysis of electrophysiological data

All signals were analysed, using in-house electrophysiological data acquisition and analysis programs (Dempster, 1988; 1993) running on a laboratory microcomputer (Western Systems, 486SX). Signals were digitised at 25 kHz using a Lab-PC+ laboratory interface (National Instruments) and stored on hard disk for subsequent numerical analysis. Individual e.p.cs were analysed for peak amplitude as described in Prior *et al.* (1993). We determined that for the smaller spontaneous responses, better estimates of peak amplitude were obtained from the analysis of an averaged signal than from the averaging of data determined from individual signals. Therefore, 20–40 individual m.e.p.cs were averaged to give a single 'averaged' signal and this was analysed for peak amplitude in the same manner as for the individual evoked responses.

Time constants of decay of m.e.p.cs (τ_{mepc}) and e.p.cs (τ_{epc}) were obtained from averaged current signals. Decay phases were fitted with a single exponential function from 95% to 5% of their peak amplitude using an iterative non-linear least squares procedure (Brown & Dennis, 1972). Where a drug-induced change in τ_{mepc} was seen, suggesting endplate ion channel block, we subjected the m.e.p.cs and e.p.cs recorded in the presence of the drug to driving function analysis to determine the extent to which this ion channel block was affecting the peak amplitude of the currents. The time course of e.p.cs and m.e.p.cs can be considered to be the combination of a driving function, which opens postjunctional ion channels, acting upon a transfer function, defined by the ion channel open/close kinetics. The driving function is a reflection of the time course of transmitter release and is independent of ion channel kinetics; it is unaffected by changes in channel behaviour brought about by ion channel blocking drugs. Thus, the effects of ion channel block on a current signal can be eliminated by calculating the driving function from the signal and then recreating a simulated signal from that driving function using the matched control decay time constant. In this way, drug effects mediated by changes in ion channel kinetics can be discriminated from effects on transmitter release or receptor block. E.p.c. and m.e.p.c. driving functions were calculated from averaged current time courses using deconvolution analysis as described in detail elsewhere (Dempster, 1986; Henderson *et al.*, 1986):

$$dfn = \frac{\mathrm{FFT}^{-1}\left[\dfrac{\mathrm{FFT}(avg)}{\mathrm{FFT}(e^{-t/\tau})}\right]}{(V_h - V_r)}$$

where FFT(x) and FFT^{-1}(x) are the Fourier transform and inverse Fourier transform functions, respectively, *dfn* is the signal driving function, *avg* is the current signal recorded in the presence of the drug and $e^{-t/\tau}$ is an exponential function representing the decay of the endplate ion channel current with τ being taken as the time constant of decay of the matched control signal. The driving function is scaled by the current driving force, the holding potential (V_h, −50 mV) minus the reversal potential (V_r, assumed 0 mV). Reversal of the deconvolution process produces simulated currents with the same time constant of decay as their associated control signals, i.e. with the effects of drug-induced ion channel block removed. Reconvoluted currents were analysed for peak amplitude and time course in the same manner as the raw data.

Numerical and statistical analysis

E.p.c. quantal content (m), and the binomial parameters of quantal release (p, the probability of release of a quantum and n, the size of the immediately available pool of quanta) were calculated from raw m.e.p.c and e.p.c. data using a simplified version of binomial statistical analysis described in detail elsewhere (Miyamoto, 1975; McLachlan, 1978; Glavinovic, 1979a; Tian *et al.*, 1994). The resultant values were averaged over the six to eight experiments in each batch to give mean and s.e.mean values presented here. Statistical testing between control data and data recorded in the presence of each test substance was performed using a paired Student's t test (two-tailed) with the level of significance being set at $P<0.05$.

Drugs and solutions

Experiments were performed at 32°C in a physiological solution of the following composition (mM): NaCl, 118; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1; $NaHCO_3$, 25; $CaCl_2$, 0.5 or 2.0 and glucose, 11. All physiological solutions were continuously gassed with a 95% O_2 and 5% CO_2 mixture to buffer pH to 7.2–7.4. Muscle tissues were superfused with physiological solution at a rate of 10–15 ml min^{-1}. Hexamethonium bromide was purchased from Sigma Chemical Company (Poole, Dorset) and was prepared as a 1 M stock solution in distilled water. This solution was kept refrigerated at 4°C when not in use. MLA, apamin and ChTX were all purchased from Semat Technical (UK) Ltd (St Albans, Herts). Stock solutions of MLA (20 mM in 0.1 ml aliquots), apamin (0.1 mM) and ChTX (0.1 mM) were prepared in distilled water and these solutions were kept tightly sealed and frozen at −20°C prior to use.

Results

Effect of hexamethonium and MLA on m.e.p.cs

The change in the amplitude of m.e.p.cs produced by the nicotinic antagonists was taken as a measure of the postjunctional activity of the compounds. With 200 μM hexamethonium, the decrease in m.e.p.c. amplitude was around 20% irrespective of $[Ca^{2+}]_0$ (Figure 1a, Table 1). For MLA the lower concentration studied (0.4 μM) produced no statistically significant change in m.e.p.c. amplitude while the higher concentration (2.0 μM) produced a small (around 15%) but nevertheless statistically significant decrease in m.e.p.c. amplitude (Figure 2a, Table 1). No drug-induced change in the

Table 1 Effect of hexamethonium (HEX) and MLA on m.e.p.c. amplitude and τ_{MEPC} in the rat hemi-diaphragm muscle

			m.e.p.c. amplitude			τ_{MEPC}		
Compound	$[Ca^{2+}]_0$ (mM)	n	*Control* (nA)	*Drug* (nA)	*Drug* (% decrease)	*Control* (ms)	*Drug* (ms)	*Drug* (% decrease)
HEX (200 μM)	2.0	8	2.18±0.11	1.64±0.08[a]	24.4±5.6	0.77±0.08	0.59±0.06[a]	22.3±2.7
HEX (200 μM)	0.5	8	2.36±0.17	1.70±0.09[a]	26.9±3.3	0.60±0.05	0.49±0.05[a]	18.2±6.2
MLA (0.4 μM)	2.0	7	1.89±0.11	2.00±0.08	−6.8±3.8	0.54±0.04	0.45±0.04	13.7±9.1
MLA (2.0 μM)	2.0	7	2.28±0.09	1.88±0.10[a]	17.3±4.1	0.49±0.02	0.42±0.04	12.7±8.8

Data are mean and s.e.mean averaged over the number of results indicated in *n*. [a]Indicates significant drug-induced changes in m.e.p.c. parameter ($P<0.05$, two-tailed paired Student's t test vs control).

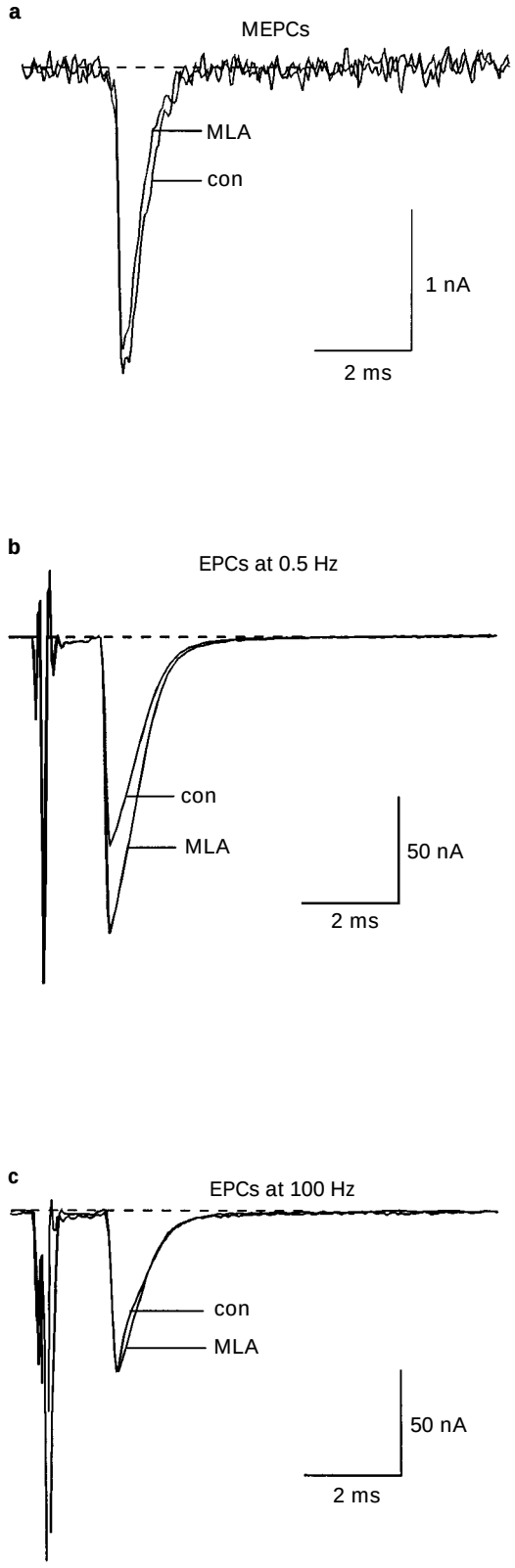

Figure 2 Representative examples of averaged m.e.p.cs (a) and e.p.cs elicited at either 0.5 Hz (b) or 100 Hz (c) recorded in the absence and presence of 2.0 μM MLA. Note that in all cases MLA had no discernible effect on the decay time of the signals indicting an absence of endplate ion channel block. Note also that while MLA had no negligible effect on the peak amplitude of m.e.p.cs or e.p.c. at 100 Hz, the peak amplitude of e.p.c. at 0.5 Hz was markedly increased, indicative of a selective low frequency-associated MLA-induced increase in e.p.c. quantal content. All records are from the same fibre voltage clamped at -50 mV and $[Ca^{2+}]_0$ was 2 mM.

Table 2 Effects of deconvolution/reconvolution on the amplitudes of e.p.cs and m.e.p.cs recorded in the presence of 200 μM hexamethonium

	Peak current amplitude (%) $[Ca^{2+}]_0 = 0.5$ mM ($n=8$)	$[Ca^{2+}]_0 = 2.0$ mM ($n=8$)
m.e.p.cs	106.8 ± 3.5	106.3 ± 1.5
e.p.cs at 0.5 Hz	109.9 ± 2.4	105.1 ± 0.5
e.p.cs at 100 Hz	107.6 ± 1.4	108.2 ± 1.3

In each fibre studied, the amplitude of the simulated current was expressed as a percentage of the amplitude of the raw current signal. Data are mean s.e.mean averaged over the number of preparations indicated.

time constant of decay of m.e.p.cs (τ_{mepc}) was detected for either concentration of MLA (Figure 2a, Table 1). However, with 200 μM hexamethonium, in both $[Ca^{2+}]_0$, there was a significant (around 20%) reduction in τ_{mepc} (Figure 1a, Table 1) consistent with the known endplate ion channel blocking activity of the compound (Milne & Byrne, 1981). Neither hexamethonium nor MLA produced a change in the frequency of occurrence of m.e.p.cs at the concentrations studied.

Effect of hexamethonium-induced ion channel block on current amplitudes

As 200 μM hexamethonium reduced τ_{mepc} it was considered necessary to examine the potential differential influence of ion channel block on e.p.c. and m.e.p.c. amplitudes with this compound. Therefore, all e.p.cs and m.e.p.cs recorded in the presence of this compound were subjected to driving function analysis (see Methods). Reconvolution of m.e.p.cs and e.p.cs recorded in the presence of 200 μM hexamethonium after subtraction of the effects of ion channel block resulted in a 5–10% increase in peak amplitude in all cases (Table 2). However, as both m.e.p.cs and e.p.cs were similarly affected and that the increase in e.p.c. peak amplitude following reconvolution was independent of the frequency of stimulation, we considered that the ion channel block would not compromise our binomial quantal analysis. The peak amplitudes of the simulated m.e.p.cs produced by reconvolution were still significantly smaller than their corresponding control currents, indicating a hexamethonium-induced depression of the m.e.p.c. amplitudes not due to endplate ion channel block.

Effect of hexamethonium on e.p.c. quantal content

At the lowest frequencies studied 200 μM hexamethonium increased m by 35–40%. This increase was statistically significant at 2 Hz and below. However, as the frequency was raised, the augmentation of m was attenuated (Figure 3a, b). At the highest frequencies tested, 200 μM hexamethonium had no discernible effect on m (Figure 3a, b). Binomial analysis of m.e.p.c. and e.p.c. amplitudes revealed that this increase in m was due to a frequency-dependent increase in the size of the pool of quanta immediately available for release (n, Figure 4a). Hexamethonium (200 μM) also produced a slight decrease in the probability of release of a quantum (p, Figure 4b). This decrease was statistically significant for five of the nine stimulation frequencies studied but was not frequency-dependent. The mechanism underlying the effect of hexamethonium on p is uncertain, although it could possibly be an artefact produced by an effect of ion channel block on the variation of individual e.p.c. amplitudes. However, a consequence of this effect is that the apparent increase in n at any frequency of stimulation is greater than the increase in m at that frequency. Indeed, for n, the increase produced by 200 μM hexamethonium was significant at all frequencies except 150 Hz.

Lowering $[Ca^{2+}]_0$ from 2.0 to 0.5 mM abolished the ability of hexamethonium to produce a low frequency-associated

augmentation of *m* (Figure 3c, d). Indeed, in a $[Ca^{2+}]_0$ of 0.5 mM the only effect of hexamethonium was a small (15%) reduction of *m* at the high stimulation frequencies; this was statistically significant at all nerve stimulation frequencies of 20 Hz and above. The depressant effect of 200 μM hexamethonium on *m* in 0.5 mM $[Ca^{2+}]_0$ at high frequencies of stimulation was due to changes in *n* (Figure 4c), rather than to changes in *p* (Figure 4d).

Effects of MLA on e.p.c. quantal content

Two concentrations of MLA were studied. The lower concentration of MLA (0.4 μM) was chosen to be a maximally effective concentration of the compound for the inhibition of α_7-type nicotinic AChRs (Decker *et al*., 1995; Yum *et al*., 1996). This concentration had no effect on *m* at any frequency studied (Figure 5a, b). In contrast, at 2.0 μM, MLA produced a pattern of changes in *m* similar to the effects of 200 μM hexamethonium. Thus there was a low frequency-associated augmentation of *m* (Figure 5c, d) which was entirely due to an increase in *n* (Figure 6a) with no change in *p* Figure 6b).

Inhibition of Ca^{2+}-activated K-channels

Neither apamin (100 nM) nor ChTX (80 nM) had any effect on m.e.p.c. amplitudes or on *m* at any frequency of stimulation studied (Figure 7). Binomial statistical analysis of e.p.cs and m.e.p.cs recorded in the presence and absence of apamin and ChTX revealed no effect of the compound on either *n* or *p* (data not shown).

Discussion

In addition to its weak competitive antagonist action on muscle-type nicotinic AChRs (Blackman, 1975), hexametho-

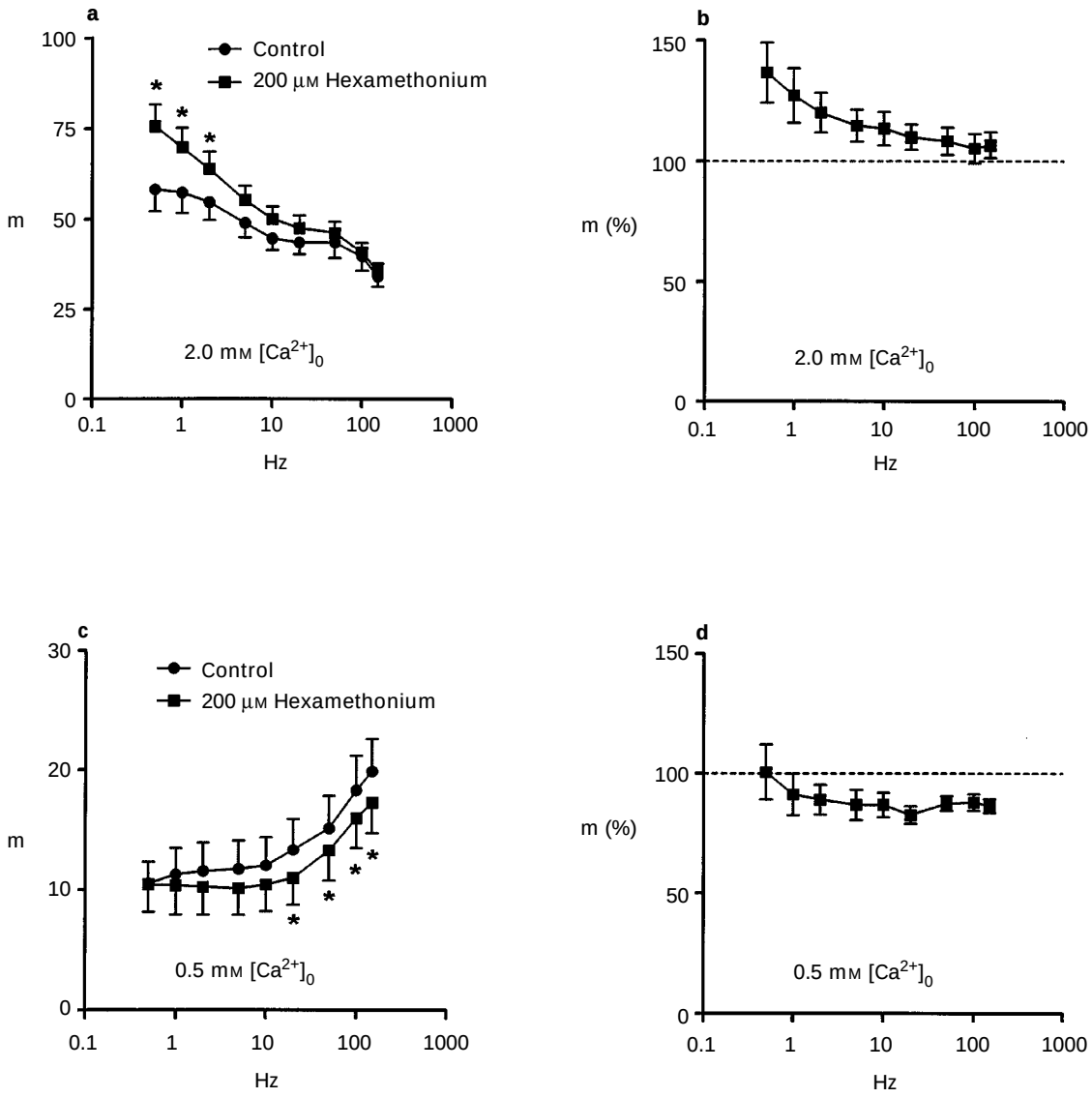

Figure 3 Plots showing frequency- and Ca^{2+}-dependence of the effect of hexamethonium on e.p.c. quantal content (*m*). (a) and (c) show *m* in the presence and absence of 200 μM hexamethonium plotted as a function of nerve stimulation frequency. (b) and (d) show the same data as in (a) and (c), respectively, with *m* in the presence of hexamethonium expressed as a percentage of its appropriate control value. Data are shown for experiments performed in the presence of 2.0 mM Ca^{2+} (a and b) and 0.5 mM Ca^{2+} (c and d). All plotted values are the mean and s.e.mean of data from 8 individual experiments. Asterisks indicate a significant difference between control and hexamethonium data ($P<0.05$, two-tailed paired Student's *t* test). Note that in 2.0 mM $[Ca^{2+}]_0$ the predominant effect of 200 μM hexamethonium is an augmentation of *m* at low frequencies of stimulation while in 0.5 mM $[Ca^{2+}]_0$ the predominant effect is a depression of *m* at high frequencies of nerve stimulation.

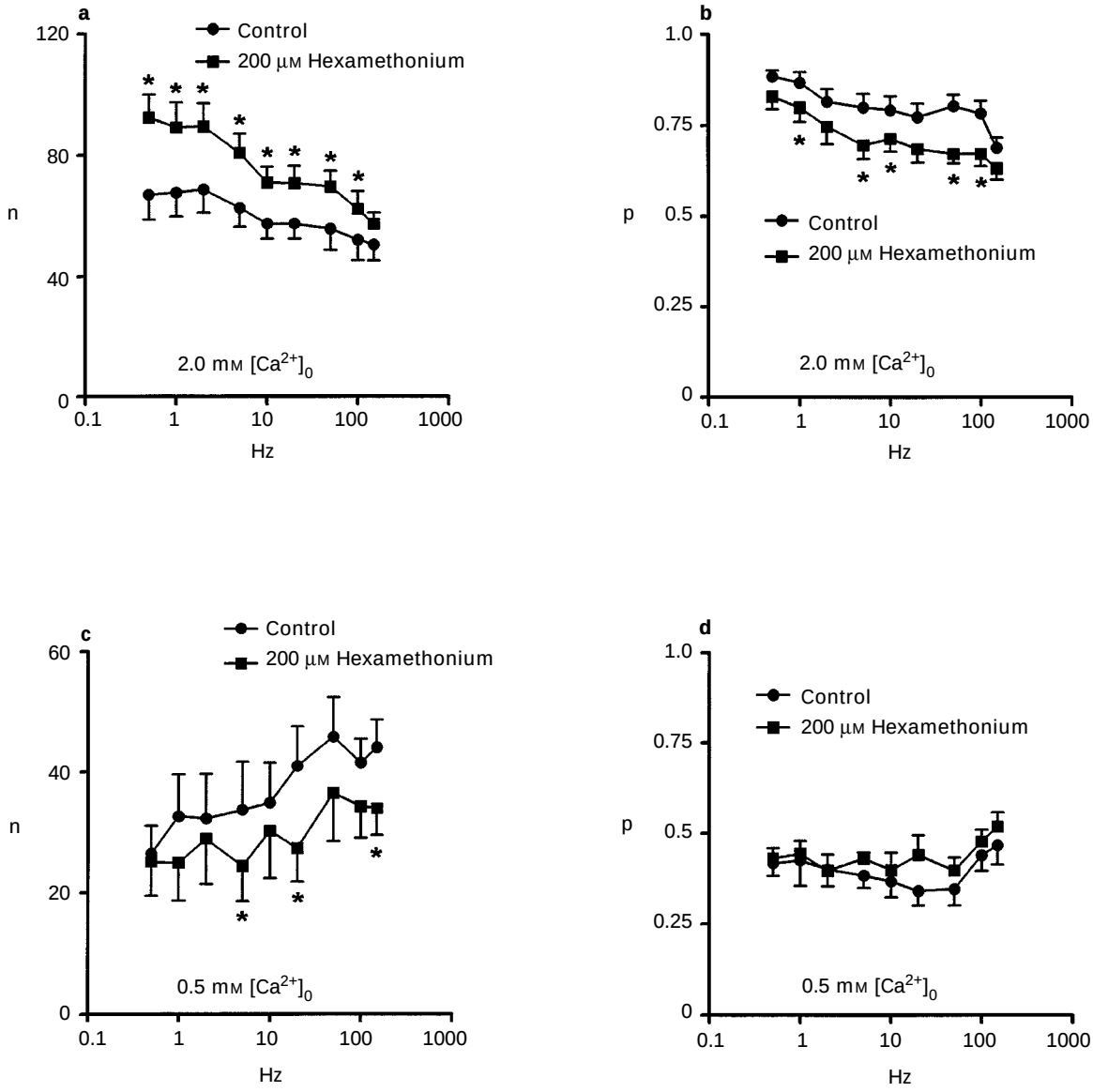

Figure 4 Plots showing frequency- and Ca^{2+}-dependence of the effects of 200 μM hexamethonium on the statistically derived binomial parameters of evoked quantal ACh release. Binomial statistical analysis (see Methods) was used to determine *n*, the size of the releasable pool of quanta (a, c) and *p*, the probability of release of a quantum (b, d). These values were determined, in each fibre studied, in the absence and presence of 200 μM hexamethonium and each plotted point is the mean and s.e.mean of data from 8 individual determinations. $[Ca^{2+}]_0$ was either 2.0 mM (a, b) or 0.5 mM (c, d). Asterisks indicate a significant difference between control and hexamethonium data ($P<0.05$, two-tailed paired Student's *t* test). Note that the changes in e.p.c. quantal content produced by 200 μM hexamethonium (Figure 1a, c) are most closely matched by changes in *n* (a, c) rather than *p* (b, d).

nium has been shown to produce ion channel block at the motor endplate (Milne & Byrne, 1981). As hexamethonium produced changes in τ_{mepc} in the present experiments, we considered the possibility that some of the observed effects of hexamethonium on *m* could be a consequence of a differential effect on e.p.c. and m.e.p.c. amplitudes due to ion channel block. However, using driving function analysis we calculated that the ion channel block produced by hexamethonium would result in the same slight depression of the amplitudes of m.e.p.cs and e.p.cs at all frequencies of stimulation. Therefore, we consider that the changes in the ratio of e.p.c. and m.e.p.c. amplitudes represents changes in the quantal release of ACh. As MLA produced no change in τ_{mepc} it is likely that the decrease in peak m.e.p.c. amplitude seen with the higher concentration of this compound, along with the residual depression of peak m.e.p.c. amplitudes seen with hexamethonium after the deconvolution/reconvolution process, represents the competitive antagonistic effects of the compounds on the muscle-type nicotinic AChRs.

We attribute the hexamethonium-induced depression of *m* at high frequencies of stimulation in a $[Ca^{2+}]_0$ of 0.5 mM to an action of the compound on the putative facilitatory prejunctional nicotinic AChRs postulated by Bowman (1980; Bowman *et al.*, 1988, 1990) and Wessler *et al.*, (1986, 1987). The absence of a hexamethonium-induced depression of *m* at high frequencies of stimulation in 2.0 mM $[Ca^{2+}]_0$ could be due to the simultaneous augmentation of *m* as a result of the Ca^{2+}-dependent action of the drug. Thus, we would argue that the effect of hexamethonium in 0.5 mM $[Ca^{2+}]_0$ represents its true prejunctional action on facilitatory nicotinic AChRs. This interpretation is supported by the finding that 200 nM tubocurarine decreases m.e.p.c. amplitude and *m* at high frequencies of stimulation in a Ca^{2+}-independent manner (Tian *et al.*, 1994).

K^+-channels and nicotinic antagonist-induced augmentation of ACh release

A number of nicotinic antagonists have been shown to block an apamin-sensitive $I_{K[Ca]}$ in guinea pig hepatocytes (Cook & Haylett, 1985; Castle *et al*., 1993). This type of K^+-channel ($I_{K[Ca]}$) is also present at motor nerve terminals (Mallart, 1985); they are activated by increased internal Ca^{2+} ($[Ca^{2+}]_i$) and are blocked by ChTX (Anderson *et al*., 1988). It has been suggested that they contribute to repolarizing the nerve terminal during high frequency activity. Thus, inhibition of these channels might be expected to produce a frequency- and Ca^{2+}-dependent increase in *m*. However, neither apamin nor ChTX affected *m*, indicating that inhibition of $I_{K[Ca]}$ does not underlie the ability of nicotinic antagonists to increase ACh release.

Nicotinic AChR and nicotinic antagonist-induced augmentation of ACh release

Our results with hexamethonium and MLA are consistent with the theory, proposed by Wilson and colleagues (Wilson, 1982; Wilson & Thomsen, 1991, 1992, that there are nicotinic AChRs on motor nerve terminals that are responsible for a negative feedback control of evoked ACh release. Block of these nicotinic AChRs by nicotinic antagonists increases ACh release. This negative feedback system is most evident at low frequencies of stimulation and, given our observed effects of hexamethonium and MLA, appears to be most sensitive to inhibitors of neuronal-type nicotinic AChRs. Conversely, the putative positive feedback nicotinic autoreceptor system described by Bowman (1980) and Bowman *et al*. (1988, 1990) and Wessler *et al*. (1986) is most evident at high frequencies of stimulation (Wessler *et al*., 1987; Tian *et al*., 1994) and is inhibited by nicotinic antagonists, such as vecuronium, with a high selectivity for muscle-type (α_1) nicotinic AChRs (Tian *et al*., 1994).

In the rat, the rank order potency for MLA against the subclasses of nicotinic AChRs is: α-bungarotoxin-sensitive neuronal-type (α_7-type) >> α-bungarotoxin-insensitive neuronal-type > muscle-type (α_1-type) (Decker *et al*., 1995). As 0.4 μM MLA, a concentration that maximally inhibits α_7-type nicotinic AChRs, did not augment *m*, we conclude that these

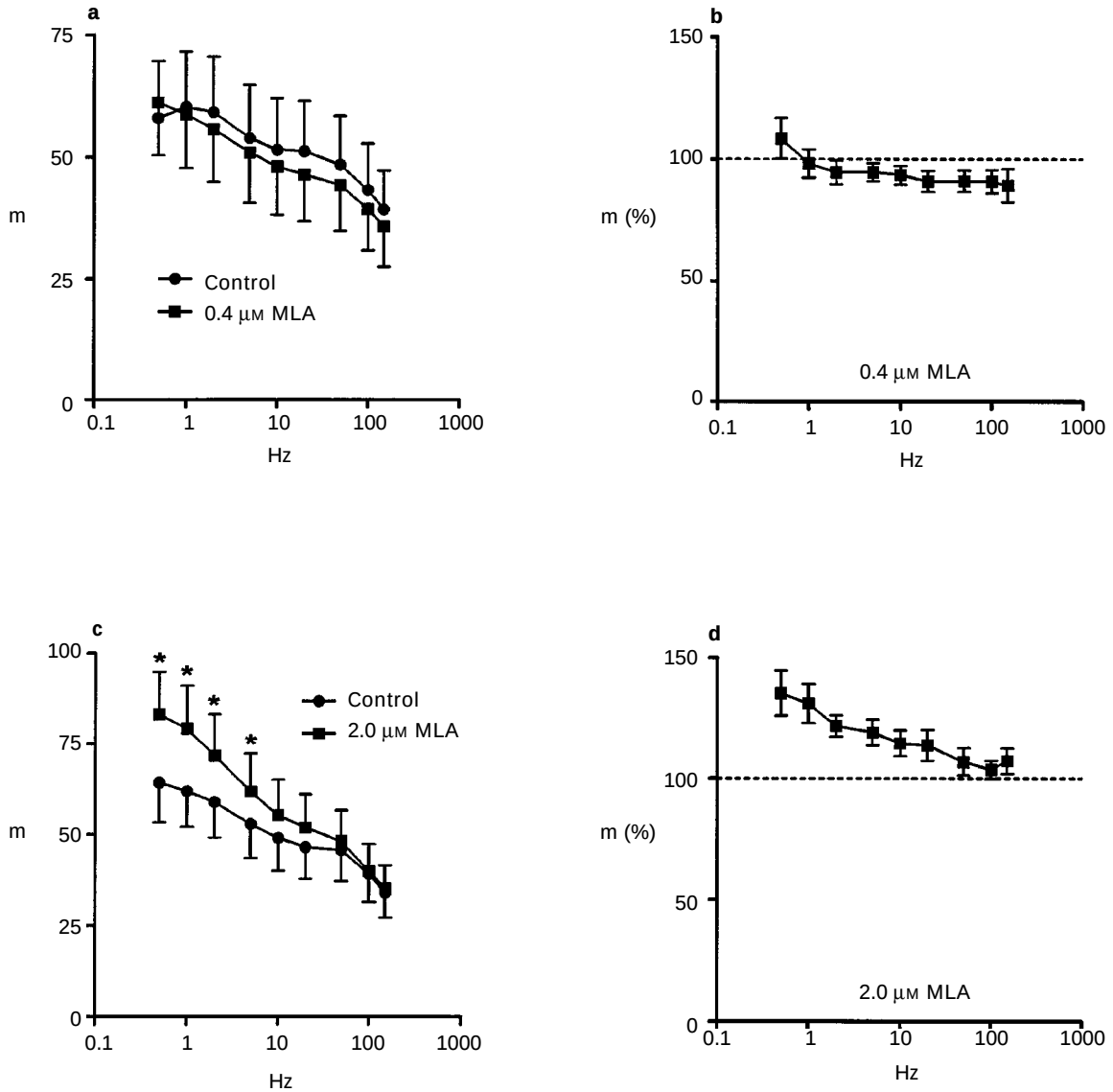

Figure 5 Plots showing concentration and frequency-dependence of the effects of 0.4 and 2.0 μM MLA on e.p.c. quantal content (*m*). (a) and (c) show *m* in the presence and absence of MLA plotted as a function of nerve stimulation frequency. (b) and (d) show the same data as in (a) and (c), respectively, with *m* in the presence of MLA expressed as a percentage of its appropriate control value. Data are shown for experiments performed using 0.4 μM MLA (a and b) and 2.0 μM MLA (c and d). All plotted values are the mean and s.e.mean of data from 7 individual experiments. $[Ca^{2+}]_0$ was 2.0 mM. Asterisks indicate a significant difference between control and MLA data ($P<0.05$, two-tailed paired Student's *t* test). Note 0.4 μM MLA has no effect on *m* while with 2.0 μM MLA the predominant effect on *m* is an augmentation at low frequencies of nerve stimulation.

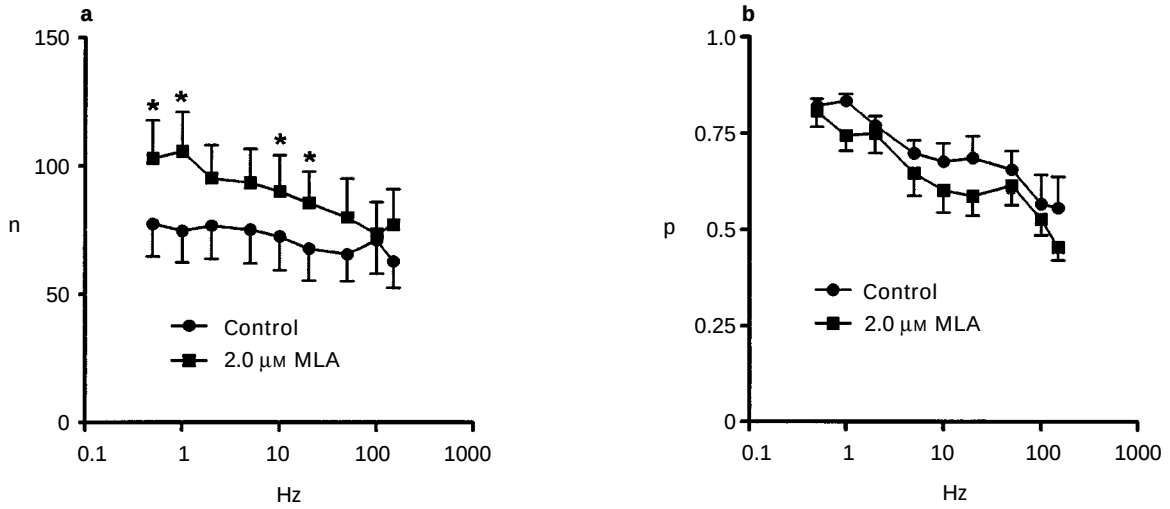

Figure 6 Plots showing frequency-dependence of the effects of 2.0 μM MLA on the statistically derived binomial parameters of evoked quantal ACh release. Binomical statistical analysis (see Methods) was used to determine n, the size of the releasable pool of quanta (a) and p, the probability of release of a quantum (b). These values were determined, in each fibre studied, in the absence and presence of 2.0 μM MLA and each plotted point is the mean and s.e.mean of data from 7 individual determinations. $[Ca^{2+}]_0$ was 2.0 mM. Asterisks indicate a significant difference between control and MLA data ($P<0.05$, two-tailed paired Student's t test). Note that the changes in e.p.c. quantal content produced by 2.0 μM MLA (Figure 3c) are most closely matched by changes in n (a) rather than p (b).

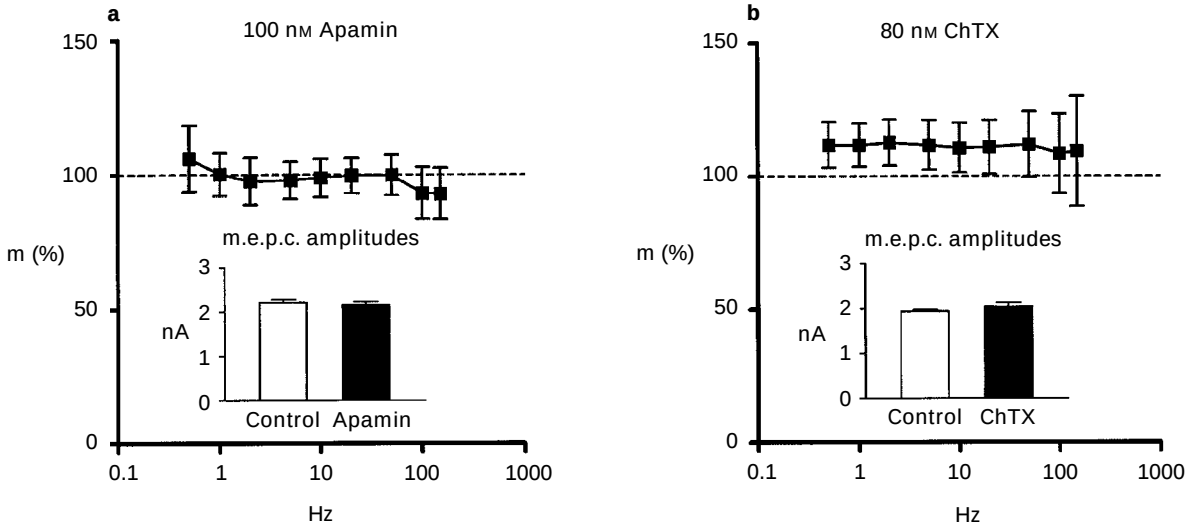

Figure 7 Plots of e.p.c. quantal content (m) vs nerve stimulation frequency in the presence of 100 nM apamin (a) and 80 nM charybdotoxin (b). All plotted values are the mean and s.e.mean of data from 6 individual experiments. In each fibre studied, m in the presence of the test compound was expressed as a percentage of its control value, recorded in the same fibre prior to the application of the agent. With either compound, and at all stimulation frequencies, m was not statistically different from 100% ($P>0.05$, two-tailed one sample Student's t test). Inset bar graphs show mean and s.e.mean of m.e.p.c. amplitudes in the absence (□) and presence (■) of each of the two compounds. $[Ca^{2+}]_0$ was 2.0 mM for both groups.

receptors are not involved in the augmenting effects of nicotinic antagonists on ACh release. In contrast, a slightly higher concentration of MLA (2.0 μM), that would be expected to produce a significant block of non-α_7 neuronal-type nicotinic AChRs with only a slight effect on muscle-type (α_1) nicotinic AChRs, produced a pronounced augmentation of m. Some block of α_1-type nicotinic AChRs by 2.0 μM MLA was evidenced by a slight decrease in m.e.p.c. amplitude. However, as vecuronium, a selective antagonist of α_1-type nicotinic AChRs, does not augment ACh release (Tian *et al*., 1994) we conclude that MLA must be acting through prejunctional non-α_7 neuronal-type nicotinic AChRs to augment ACh release at low frequencies of stimulation. Consistent with this hypothesis, Tsuneki *et al*. (1995), using immunofluorescence techniques and monoclonal antibody binding, determined that the α_3 subunit of neuronal-type nicotinic AChRs was located prejunctionally on motor nerve terminals while the β_2 and α_8 subunits were located postjunctionally on the muscle membrane. However, our present results are inconsistent with those of Domet *et al*. (1995) who report that the negative feedback system at the neuromuscular junction is sensitive to α-bungarotoxin.

How ACh, acting on prejunctional neuronal-type nicotinic AChRs, decreases its own release is unknown. That the effect of neuronal nicotinic antagonists on m is evident at low frequencies of stimulation raises the possibility that ACh derived from leakage or spontaenous release may have an inhibitory effect on its own evoked release (Tian *et al*., 1994). Further-

more, the marked Ca^{2+}-dependence, indicates a possible role for extracellular Ca^{2+} in this negative feedback system. Neuronal-type nicotinic AChRs are relatively permeable to Ca^{2+} (Sands & Barish, 1991; Vernino *et al*., 1992) and their activity is modulated by $[Ca^{2+}]_0$ (Vernino *et al*., 1992; Mulle *et al*., 1992). However, the exact link between the level of activity of prejunctional auto-inhibitory nicotinic AChRs and the size of *n* has yet to be elucidated. A decrease in *n* has been variously interpreted as a decrease in the number of active release sites at the surface of the nerve terminal or a decrease in the movement, or mobilisation, of releasable ACh from stores within the nerve terminal to the release sites. Our current data do not allow us to distinguish between these possibilities.

In conclusion, we have observed a nicotinic antagonist-induced frequency- and Ca^{2+}-dependent augmentation of evoked ACh release from rat motor nerve terminals. This effect, seen predominantly at low frequencies of stimulation, is distinct from the nicotinic antagonist-induced depression of evoked ACh release seen at high frequencies of stimulation with certain nicotinic antagonists. The nicotinic antagonist-induced augmentation of ACh release is not related to any activity of the compounds at nerve terminal Ca^{2+}-activated K^+-channels as it is not mimicked by specific toxin inhibitors of these channels. Based on the known pharmacology of nicotinic antagonists that we found to augment ACh release, we propose that this effect is due to an inhibition of prejunctional nicotinic AChRs of the α-bungarotoxin-insensitive neuronal-type. ACh acting at these prejunctional nicotinic AChRs exerts a negative feedback control on its own release.

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