



Factors influencing the low-frequency associated nicotinic ACh autoreceptor-mediated depression of ACh release from rat motor nerve terminals

*¹Chris Prior & ²Shila Singh

¹Department of Physiology and Pharmacology, University of Strathclyde, Strathclyde Institute for Biomedical Sciences, 27 Taylor Street, Glasgow G4 0NR and ²Haryana Veterinary Vaccine Institute, Haryana Agricultural University, Hisar 125004, India

1 We have studied the inhibitory autoreceptor control of acetylcholine (ACh) release from rat motor nerve endings using an electrophysiological technique to quantify evoked ACh release in isolated hemidiaphragm muscles. Quantal ACh release (m) was estimated from the ratio of amplitudes of nerve evoked endplate currents and spontaneously occurring miniature endplate currents.

2 The nicotinic ACh receptor agonist cytisine (1 μM) decreased m at 0.5 Hz by around 20% but had no effect on m at 50 Hz. Changing the extracellular Ca^{2+} concentration from 1.8 mM to either 0.45 or 3.6 mM abolished the effect of cytisine on m at 0.5 Hz. The nicotinic ACh receptor antagonist hexamethonium (200 μM) increased m at 0.5 Hz by 15–20%.

3 The effects of cytisine and hexamethonium on m at 0.5 Hz were blocked by 10 μM verapamil, which itself significantly increased m . However, the effects of cytisine and hexamethonium on m at 0.5 Hz were not sensitive to 10 μM of the calmodulin antagonist, W-7. This concentration of W-7 attenuates effects on ACh release mediated by facilitatory presynaptic nicotinic ACh autoreceptors.

4 Our present observations are suggestive of actions of cytisine and hexamethonium to activate and inhibit respectively negative-feedback presynaptic nicotinic ACh autoreceptors. Further, they strengthen the case for the existence of two separate and independent autoregulatory mechanisms for the control of ACh release from motor nerve terminals and give a preliminary insight into the cellular mechanism involved in the autoinhibition of ACh release.

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Abbreviations: epc, endplate current; mepc, miniature endplate current; m , endplate current quantal content; VOCC, voltage-operated Ca^{2+} channel

Introduction

At the mammalian skeletal muscle neuromuscular junction systems exist whereby the neurotransmitter acetylcholine (ACh) can act on presynaptic autoreceptors to either enhance or depress its own evoked release (reviewed in Prior *et al.*, 1995). Evidence suggests that autoinhibition occurs predominantly at low frequencies of activation, is dependent on the extracellular calcium ion concentration ($[\text{Ca}^{2+}]_o$) and is probably mediated by a presynaptic nicotinic ACh receptor of a neuronal subtype (Tian *et al.*, 1994; 1997). Thus, the neuronal-type selective nicotinic ACh receptor antagonists hexamethonium and methyllycaconitine, by blocking endogenous autoinhibition, increase ACh release, but only at low frequencies of stimulation and not in the presence of a reduced $[\text{Ca}^{2+}]_o$ (Tian *et al.*, 1997). Conversely, the muscle-type selective nicotinic ACh receptor antagonist vecuronium decreases ACh release at high frequencies of stimulation by blocking endogenous autofacilitation but has no effect on evoked ACh release at low frequencies of stimulation (Tian *et al.*, 1994).

To date, the existence of nicotinic ACh receptor mediated autoinhibition in motor nerve terminals is based solely on the facilitatory actions of selected antagonists. However, implicit in the autoinhibition hypothesis is that low concentrations of a

neuronal-type selective nicotinic ACh receptor agonist should be capable of inhibiting evoked ACh release in a frequency and $[\text{Ca}^{2+}]_o$ -dependent manner. Further, if this were demonstrable then the possible involvement of intracellular messenger systems in the autoinhibition could be examined using appropriate agents. Therefore, the objectives of the present study were 2 fold. Firstly, to provide further evidence for the existence of presynaptic autoinhibitory nicotinic ACh receptors at the neuromuscular junction through the demonstration of an inhibitory effect of a neuronal-type selective nicotinic ACh agonist on evoked ACh release. Secondly, to gain insight into the intracellular mechanisms that might underlie the autoinhibitory effect of ACh at the neuromuscular junction.

To achieve the first objective, we determined the effects of the neuronal-type nicotinic ACh agonist cytisine on ACh release, evoked at 0.5 and 50 Hz, from rat motor nerve terminals. For the second objective, two issues were identified. An important part of identifying the cellular mechanisms involved in autoinhibition would be to demonstrate the independence of this process from high-frequency associated autofacilitation. Since the calmodulin antagonist W-7 blocks autofacilitation (Singh & Prior, 1998), it appeared logical to determine the calmodulin-sensitivity of autoinhibition. Additionally, given the $[\text{Ca}^{2+}]_o$ -dependence of the facilitatory effect of hexamethonium on ACh release (Tian *et al.*, 1997), we

*Author for correspondence; E-mail: c.b.prior@strath.ac.uk

were interested in determining the role of nerve terminal membrane Ca^{2+} -fluxes in autoinhibition. We studied the actions of verapamil on autoinhibition because verapamil-sensitive Ca^{2+} -fluxes are known to exist in mammalian motor nerve terminal membranes (Anderson & Harvey, 1987; Penner & Dreyer, 1986) that are not the primary trigger for evoked release (Uchitel *et al.*, 1992). Therefore, these Ca^{2+} -fluxes might possess a role in the modulation of ACh release by inhibitory autoreceptors.

Methods

Muscle preparation

Male Sprague-Dawley rats (150–200 g) were killed according to U.K. Home Office guidelines by dislocation of the cervical vertebrae followed by exsanguination. Both hemidiaphragm muscle preparations, along with 15–20 mm of their associated phrenic nerve, were removed from the animals immediately following sacrifice. Once isolated, each muscle preparation was dissected clear of connective tissue and fixed to the Sylgard (Dow-Corning) base of a 3–5 ml tissue bath continuously perfused ($10\text{--}12\text{ ml min}^{-1}$) with physiological solution at 32°C (see below for composition). To immobilize the muscle for electrophysiological recording, a 'cut-muscle' preparation was used (Barstad & Lilliheil, 1968). Transverse sectioning of the muscle fibres 2–3 mm either side of the main intramuscular nerve branches initially reduced the resting membrane potential in the motor endplate region to around -40 mV . This was usually sufficient to prevent generation of muscle fibre action potentials and the associated twitch of the fibres. The cut-muscle approach was preferred to the use of Mg^{2+} (to reduce ACh release), a nicotinic ACh receptor antagonist (to reduce postjunctional sensitivity to ACh) or μ -conotoxin (to selectively abolish muscle action potentials), since all these pharmacological treatments affect the motor nerve terminal and would therefore have compromised our quantitative estimates of evoked ACh release.

For evoked responses, the motor nerve was stimulated at either 0.5 Hz for 3 min or at 50 Hz for 2 s to generate around 100 individual endplate currents (epcs). Nerve stimulation was *via* a pair of silver wire electrodes over which the phrenic nerve was fixed. Pulse of 0.05–0.1 ms duration and supramaximal voltage (typically 10–20 V) were delivered by a Grass S88 stimulator linked to a Grass SIU5 stimulus isolation unit. Spontaneously occurring miniature endplate currents (mepcs) were also recorded from the same cells for 1 min immediately prior to the epcs.

Electrophysiological technique

Epc and mepc were recorded from motor endplates using a standard two intracellular microelectrode voltage clamp technique (Dionne & Stevens, 1975; Prior *et al.*, 1993). Microelectrodes were prepared from borosilicate glass (Clarks Electromedical Instruments, Pangbourne, U.K.) using a Sutter P30 micropipette puller. Voltage-recording electrodes (5–10 M Ω) were filled with 3 M KCl and current-passing electrodes (2–5 M Ω) filled with 2 M potassium citrate. The gain and bandwidth of the voltage-clamp amplifier (Axon Instruments, Axoclamp 2B) were adjusted for optimum performance and adequacy of voltage-clamping was assessed from the voltage escape during evoked responses, less than 1% of the holding potential (i.e. 0.5 mV) being deemed acceptable. In any individual fibre studied, both epcs and mepcs were

recorded with the same voltage-clamp operating parameters. All recordings were made at a holding potential of -50 mV . This depolarized potential was used to eliminate the problems associated with the reactivation of muscle Na^+ channels in fibres where the endplate is clamped close to the normal resting membrane potential (Prior *et al.*, 1993). All currents were recorded on FM-tape (Racal Store 4DS, d.c. -5 kHz) for off-line computer analysis.

Data analysis and statistics

For analysis, current signals were replayed from the FM-tape, digitized at 25 kHz (Lab-PC + laboratory interface, National Instruments, Newbury, U.K.) and stored on the hard disk of a microcomputer (Viglen 486DX). Sampling and analysis were performed using the Strathclyde Electrophysiological Software suite of programs (Dempster, 1988; 1993). All currents were individually displayed so that artefacts could be eliminated from the analysis. For each epc recording, between 80 and 100 epcs were individually analysed for peak amplitude and time course as described elsewhere (Prior *et al.*, 1993). For epcs at 50 Hz the first 20 signals were excluded from the analysis to avoid the initial rundown of epc amplitudes. Data obtained from the individual epcs were averaged to give mean values for each experimental record. For mepcs, because of the relatively low signal-to-noise of individual current signals, we have previously determined that the best estimates of amplitude and time course are obtained from a composite averaged signal (Tian *et al.*, 1994; 1997). Therefore, all mepcs from a single experimental recording (typically 20–40 mepcs) were aligned to the mid-point of their rising phase and subjected to sequential averaging of data points to give a single averaged mepc signal. This average mepc was then analysed for peak amplitude and time course in an identical fashion as to the epcs.

Evoked quantal ACh release was measured as the epc quantal content (m) and this was calculated for each experimental record as the ratio of mean epc amplitude and the amplitude of the averaged mepc. For each condition studied, 7–10 identical individual experiments were performed and the data from these were averaged to give the presented mean and standard error of the mean values. Since, in each fibre studied, both control and drug-treated data was collected, it was possible to perform statistical analysis using a paired Student's t -test ($P < 0.05$, two-tailed being taken as an indication of a significant difference). Using paired data has the advantage of reducing the influence of inter-fibre variability on the statistical tests, allowing detection of small effects of the drug treatment.

Drugs and solutions

All isolated muscle preparations were maintained (at 32°C) in a solution of the following composition (mM): NaCl 118, KCl 5, KH_2PO_4 1.2, MgSO_4 1, NaHCO_3 25, CaCl_2 0.45–3.6, and glucose 11. The solution was continuously bubbled with a gas mixture of 5% CO_2 in O_2 to provide oxygenation and to maintain solution pH at 7.2–7.4. Solutions of hexamethonium, verapamil and W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.HCl) were made fresh each day from powder (1 mM in distilled water). These were diluted in physiological solution to the desired working concentration as required. A stock solution of cytosine (10 mM) was made in distilled water and stored in 100 μl aliquots at -20°C for up to 3 months. Cytosine, hexamethonium and verapamil were purchased from Sigma

Chemical Co. (Poole, Dorset, U.K.) and W-7 was purchased from Tocris Cookson (Bristol, U.K.).

In all experiments, cytosine, hexamethonium, verapamil and W-7 were each used at a single concentration. The concentrations of hexamethonium (200 μM) and W-7 (10 μM) have been previously shown to be effective in modulating ACh release from rat motor nerve terminals (Tian *et al.*, 1994; 1997). The concentration of cytosine (1 μM) was selected, following pilot studies, to minimize complications arising from effects of the compound on muscle-type nicotinic ACh receptors (see below). The concentration of verapamil (10 μM) was one that previous studies have shown is maximal for block of verapamil-sensitive motor nerve terminal Ca^{2+} -currents (Penner & Dreyer, 1986) without significant effects on either postjunctional ACh receptors (Edeson *et al.*, 1988) or prejunctional Na^{+} -channels (Penner & Dreyer, 1986).

Results

Effect of cytosine on mepcs

Cytosine (1 μM) had no effect on the frequency of occurrence of mepcs, the decay time constant of mepcs (mepc tau) or mepc amplitude (Figure 1 and Table 1). However, at 10 μM the compound produced decreases in both mepc amplitude and the decay time constant of mepcs (Figure 1 and Table 1). In addition, 10 μM cytosine significantly (and reversibly) depolarized the motor endplate of intact hemidiaphragm muscle fibres from 72.7 ± 2.6 to 68.6 ± 2.6 mV ($n=7$, $P<0.05$ two tailed paired Student's *t*-test). These effects of 10 μM cytosine are all

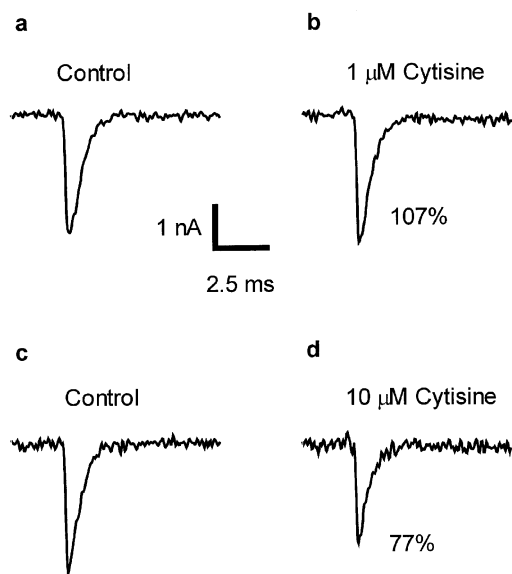


Figure 1 Examples of the effects of 1 and 10 μM cytosine on mepcs recorded from rat isolated hemidiaphragm muscle. Each trace is an average of approximately 40 individual mepc recorded before (a,c) or after (b,d) the application of 1 μM (a,b) or 10 μM (c,d) cytosine to the muscle fibre. In each case the control and drug-treated mepcs were recorded from the same muscle fibre. For all records, holding potential was -50 mV and $[\text{Ca}^{2+}]_o$ was 1.8 mM. Annotated values are the amplitude of the mepcs in cytosine (b,d) expressed as a percentage of their respective control (a,c). The apparent slight increase in amplitude following 1 μM cytosine was not a consistent observation, there being on average no overall change. However, the approximate 20% reduction in mepc amplitude produced by 10 μM cytosine was reproducible and, across all cells studied, was statistically significant (see Table 1). Calibration bars for all traces: vertical, 1 nA; horizontal, 2.5 ms.

indicative of actions of the compound on the motor endplate muscle-type nicotinic ACh receptors. Therefore, to avoid any effects of cytosine on this class of receptor, either post- or prejunctionally, from compromising our analysis of the autoinhibitory phenomenon, in all studies in which *m* was determined cytosine was used at the lower concentration of 1 μM .

Frequency-dependent effect of cytosine

The effect of 1 μM cytosine on *m* was assessed at both 0.5 and 50 Hz with a $[\text{Ca}^{2+}]_o$ of 1.8 mM. At 50 Hz, 1 μM cytosine had no effect on epc amplitude (Figure 2d,e) and consequently, given its lack of effect on mepc amplitudes at this concentration, *m* was unaffected (Figure 2f). However, at 0.5 Hz, epc amplitude was reduced by an average of around 20% (Figure 2b,e) leading to a corresponding reduction of approximately 20% in the size of *m* (Figure 2f).

Calcium-dependent effect of cytosine

The facilitatory effect of the nicotinic ACh receptor antagonist hexamethonium on *m* at a low frequency of stimulation is known to be dependent on $[\text{Ca}^{2+}]_o$ (Tian *et al.*, 1997). Thus we might expect the inhibitory effect of cytosine on *m* at 0.5 Hz also to be dependent on $[\text{Ca}^{2+}]_o$. We therefore determined the effect on 1 μM cytosine on mepc and epc amplitudes, and hence *m*, in conditions where $[\text{Ca}^{2+}]_o$ was 2 fold greater (3.6 mM) and 4 fold less (0.45 mM) than normal. Cytosine (1 μM) had no effect on mepc amplitude irrespective of the $[\text{Ca}^{2+}]_o$ used

Table 1 Effects of cytosine on mepcs in the rat isolated hemidiaphragm muscle

	1 μM Cytosine ($n=18$)		10 μM Cytosine ($n=13$)	
	Control	Cytosine	Control	Cytosine
mepc frequency (Hz)	1.13 ± 0.17	1.11 ± 0.17	0.87 ± 0.11	0.82 ± 0.09
mepc amplitude (nA)	3.23 ± 0.18	3.15 ± 0.18	2.95 ± 0.16	$2.42 \pm 0.11^*$
mepc tau (ms)	0.58 ± 0.02	0.58 ± 0.02	0.66 ± 0.04	$0.58 \pm 0.03^*$

Values are mean and standard error of the mean of data from the number of individual determinations indicated by *n*. Asterisks indicate significant differences ($P<0.05$ two-tailed paired Student's *t*-test, cytosine *versus* respective control). In all experiments $[\text{Ca}^{2+}]_o$ was 1.8 mM.

Table 2 Effect of verapamil (10 μM) on epc and mepc amplitudes and on quantal ACh release in the rat isolated hemidiaphragm muscle

	Control	Verapamil	Verapamil (% control)
mepc amplitude (nA)	2.38 ± 0.11	2.35 ± 0.13	99.0 ± 4.0
mepc frequency (Hz)	1.00 ± 0.16	1.08 ± 0.15	108.5 ± 5.3
epc amplitude (nA)	101 ± 11	122 ± 12	123 ± 12
epc quantal content (<i>m</i>)	42.8 ± 4.5	$52.0 \pm 4.8^*$	123.0 ± 7.0

All data are mean and s.e.mean of values from the same seven individual preparations. Asterisk indicates a significant effect of verapamil ($P<0.05$, two-tailed paired Student's *t*-test *versus* control).

(Figure 3a). However, epc amplitude, and hence m , was only reduced when the $[Ca^{2+}]_o$ was 1.8 mM. No effect of cytosine on either epc amplitude (Figure 3b) or m (Figure 3c) was detected when the $[Ca^{2+}]_o$ was lowered to 0.45 mM or raised to 3.6 mM.

Sensitivity of cytosine action to W-7 and verapamil

To investigate the potential intracellular signalling system involved in autoinhibition we studied the ability of two compounds, verapamil and W-7, to modulate the effects of cytosine and hexamethonium on m at 0.5 Hz. Exposure of neuromuscular preparations to verapamil (10 μ M) alone had no effect on mepc amplitude or frequency of occurrence (Table 2) but produced an approximate 20% increase in epc amplitude, and therefore a similar effect on m (Table 2). This itself suggests the possibility that there exists a degree of endogenous autoinhibition that is sensitive to a prejunctional action of the compound. As expected, neither cytosine nor hexamethonium had an effect on mepc frequency, irrespective of the presence or absence of verapamil or W-7 (Figure 4).

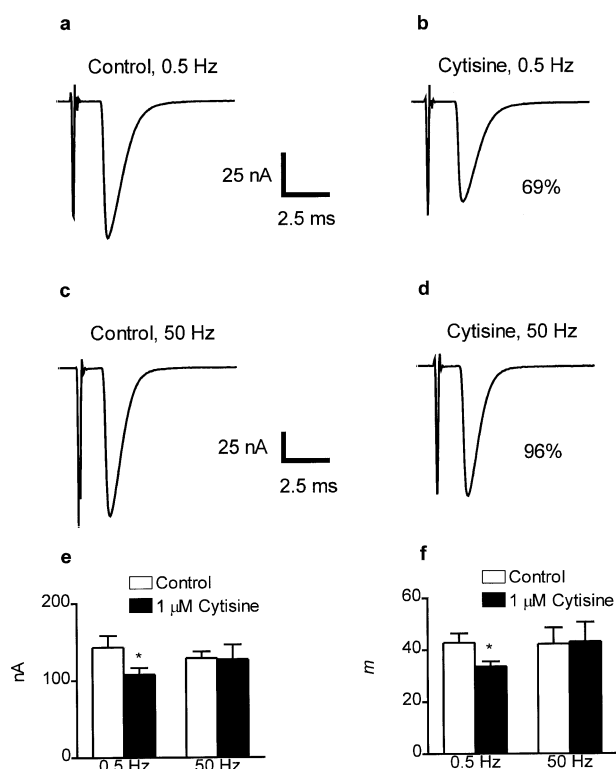


Figure 2 Examples of the effects of 1 μ M cytosine on representative epcs at 0.5 Hz (a,b) or 50 Hz (c,d) and on average epc amplitude (e) and epc quantal content (f) recorded from rat isolated hemidiaphragm muscle fibres. Each trace in a–d is an average of approximately 80 individual epc recorded before (a,c) or after (b,d) the application of 1 μ M cytosine to the muscle fibre. In each case the control and drug-treated epcs were recorded from the same muscle fibre. For all records, holding potential was -50 mV and $[Ca^{2+}]_o$ was 1.8 mM. Annotated values are the amplitude of the epcs in cytosine (b,d) expressed as a percentage of their respective control (a,c). Calibration bars for all traces: vertical 25 nA; horizontal, 2.5 ms. Panels e and f show bar chart of the average effects of 1 μ M cytosine on epc amplitude and epc quantal content respectively. Data are mean and s.e.mean of values from ten (0.5 Hz) or eight (50 Hz) individual determinations. Asterisks indicate significant differences ($P < 0.05$ two-tailed paired Student's *t*-test, cytosine versus control). Note that at 50 Hz, 1 μ M cytosine affects neither epc amplitude nor m . Conversely, at 0.5 Hz, both parameters are reduced by around 20% by this concentration of cytosine.

As already stated, in the absence of verapamil or W-7, 1 μ M cytosine depressed epc amplitude (Figure 5c) with no effect on mepc amplitude (Figure 5a). However, hexamethonium (200 μ M) depressed mepc amplitude (Figure 5b) with no effect on epc amplitude (Figure 5d). Thus cytosine increased and hexamethonium decreased m (Figure 5e,f). These are consistent with previous published data and indicate that cytosine can activate and hexamethonium can block the autoinhibitory nicotinic ACh receptors on the motor nerve terminals. In the presence of W-7, cytosine (1 μ M) had no statistically significant effect on either mepc or epc amplitudes (Figure 5a,c). However, W-7 did not prevent the inhibitory effect of cytosine on m (Figure 5e) – there was no statistically significant difference between the effects of cytosine on m in the presence

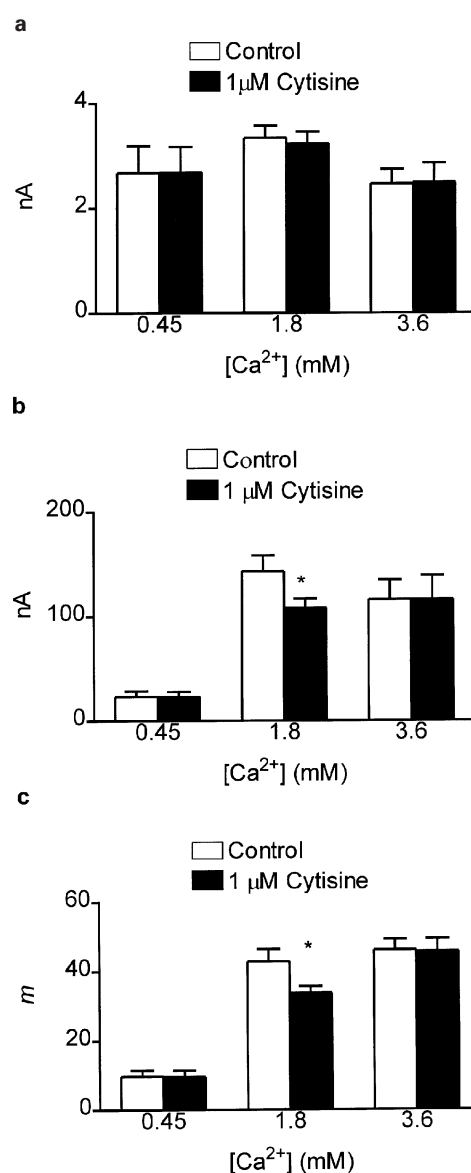


Figure 3 Bar graph showing the effects of 1 μ M cytosine on mepc amplitude (a), epc amplitude (b) and epc quantal content (c) in the rat isolated hemidiaphragm muscle. Epcs were recorded at 0.5 Hz and three different $[Ca^{2+}]_o$ were used as indicated in the figure. Data for 1.8 mM $[Ca^{2+}]_o$ are the same as in Figure 2 and are included for comparison. Note that 1 μ M cytosine had no effect on mepc amplitude, irrespective of the $[Ca^{2+}]_o$. However, 1 μ M cytosine reduced epc amplitude (and hence m) only when the $[Ca^{2+}]_o$ was 1.8 mM. All data are mean and s.e.mean of 6–10 individual determinations. Asterisks indicate significant differences ($P < 0.05$, two-tailed Student's *t*-test, cytosine versus control).

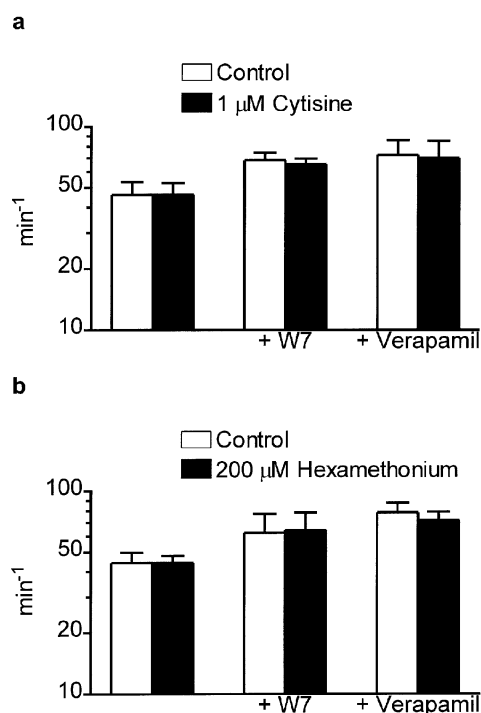


Figure 4 Bar graph showing the effects of 1 μM cytosine (a) and 200 μM hexamethonium (b) on the frequency of occurrence of mepes in the rat isolated hemidiaphragm preparation in the absence and presence of W-7 or verapamil (both 10 μM). Note that neither cytosine nor hexamethonium affected mepc frequency under any of the conditions studied. Data are mean and s.e.mean of values from 7–10 individual determinations. All data $P > 0.05$, two-tailed paired Student's t -test, control versus cytosine or hexamethonium.

and absence of cytosine ($P > 0.05$, unpaired Student's t -test). In contrast, in the presence of verapamil, cytosine was without effect on mepc and epc amplitudes (Figure 5a,c) and also m (Figure 5e). Hexamethonium (200 μM) reduced mepc amplitudes irrespective of the presence of W-7 or verapamil (Figure 5b) but only reduced epc amplitudes in the presence of verapamil (Figure 5d). Hence, verapamil, but not W-7, attenuated the facilitatory effect of hexamethonium on m (Figure 5f)

Discussion

Cytisine is most effective in activating nicotinic ACh receptors containing a $\beta 4$ -subunit (Luetje & Patrick, 1991; Covernton *et al.*, 1994; Chavez-Noriega *et al.*, 1997). However, the ability of 10 μM cytosine to depolarize the endplates of intact muscle fibres suggests that, in agreement with previous observations (Yost & Winegar, 1997), the compound is an agonist at postjunctional muscle-type nicotinic ACh receptors. Thus the decrease in the amplitudes of mepcs produced by 10 μM cytosine could be due to fast-desensitization of postjunctional muscle-type nicotinic ACh receptors as previously described for other cholinergic agonists (Wray, 1981; Pennefather & Quastel, 1982; Singh & Prior, 1998). Alternatively, this decrease might result from a concurrent competitive antagonist action of cytosine on the muscle-type nicotinic ACh receptors, as has been seen in $\beta 2$ -containing neuronal-type nicotinic ACh receptors (Papke & Heinemann, 1994). Finally, the slight decrease in the decay time constants of mepcs suggests a small degree of open ion channel block. However, at 1 μM , cytosine had no effect on either mepc amplitude or time

constant of decay, suggesting no action on postjunctional muscle-type nicotinic ACh receptors at this lower concentration. In addition, 1 μM cytosine had no effect on m at 50 Hz. This suggests that, unlike DMPP, this concentration of cytosine lacks an action on the prejunctional muscle-type nicotinic ACh receptors which mediate a facilitatory control over ACh release (Tian *et al.*, 1994; Singh & Prior, 1998). In contrast to its lack of an effect at 50 Hz, cytosine (1 μM) reduced m at 0.5 Hz by around 20%. This supports the suggested existence of prejunctional nicotinic ACh receptors that inhibit evoked ACh release and that have been reported to operate under conditions of low release (Wilson & Thomsen, 1991; 1992; Tian *et al.*, 1994; 1997). The relative pre- and postjunctional activities of cytosine reported here supports the previous published conjecture that the autoinhibitory prejunctional nicotinic ACh receptor is of a neuronal sub-type (Tian *et al.*, 1994; 1997). The possibility that the autoinhibitory receptors are muscle-type nicotinic ACh receptors of the extrajunctional (foetal) isoform is unlikely since the intrinsic activity of cytosine at these is 6–8 fold less than at the postjunctional (adult) muscle-type nicotinic ACh receptors (Yost & Winegar, 1997).

Earlier studies showed that the nicotinic ACh receptor antagonist-induced facilitation in m at low frequencies of stimulation is sensitive to a change in the $[\text{Ca}^{2+}]_o$. Thus, Tian *et al.* (1997) observed that the increase in m produced by hexamethonium at 0.5 Hz in the presence of a $[\text{Ca}^{2+}]_o$ of 2.0 mM was abolished when the $[\text{Ca}^{2+}]_o$ was lowered to 0.5 mM. Therefore to equate the results with previous studies, the effects of cytosine were also investigated on lowering the $[\text{Ca}^{2+}]_o$ to 0.45 mM. In agreement with the studies with nicotinic ACh receptor antagonists, changes in the amplitudes of epcs and m produced by 1 μM cytosine at 0.5 Hz were abolished when the $[\text{Ca}^{2+}]_o$ was lowered to 0.45 mM. This implies clearly that the inhibitory nicotinic ACh receptor agonists and facilitatory nicotinic ACh receptor antagonists share a common mechanism for their effect on evoked ACh release from motor nerve terminals.

The reduction in m produced by 1 μM cytosine in the presence of 1.8 mM $[\text{Ca}^{2+}]_o$, instead of becoming more pronounced on raising the $[\text{Ca}^{2+}]_o$, was found to be abolished when the $[\text{Ca}^{2+}]_o$ was raised to 3.6 mM. One explanation for this could be that although autoinhibition requires the presence of $[\text{Ca}^{2+}]_o$, there is a very narrow range of $[\text{Ca}^{2+}]_o$ within which the system can remain effective. This would be seen if activation of the prejunctional neuronal-type ACh receptors shifts the relationship between $[\text{Ca}^{2+}]_o$ and m to higher levels of $[\text{Ca}^{2+}]_o$, i.e. a rightward shift of the $[\text{Ca}^{2+}]_o$ - m relationship. Since in mammalian motor nerve terminals m saturates above 3 mM $[\text{Ca}^{2+}]_o$ (Cooke *et al.*, 1973), a consequence of the rightward shift would be that at high and low $[\text{Ca}^{2+}]_o$ there would be modest, if detectable, changes in m by cytosine while the largest effect of the compound would be seen in the mid-range of $[\text{Ca}^{2+}]_o$. Evidence that in these experiments the evoked release of ACh was near maximal, even at 1.8 mM $[\text{Ca}^{2+}]_o$, can be seen in the controls of Figure 3c; no statistically significant increase in m was seen when $[\text{Ca}^{2+}]_o$ was raised from 1.8 to 3.6 mM ($P > 0.05$, unpaired, two-tailed Student's t -test).

Previously, we have shown that the ability of DMPP to increase evoked ACh release and the ability of vecuronium to inhibit evoked ACh release (both at 50 Hz) represent activation and inhibition respectively of a prejunctional nicotinic ACh receptor mediating autofacilitation (Singh & Prior, 1998). In addition, the effects of DMPP and

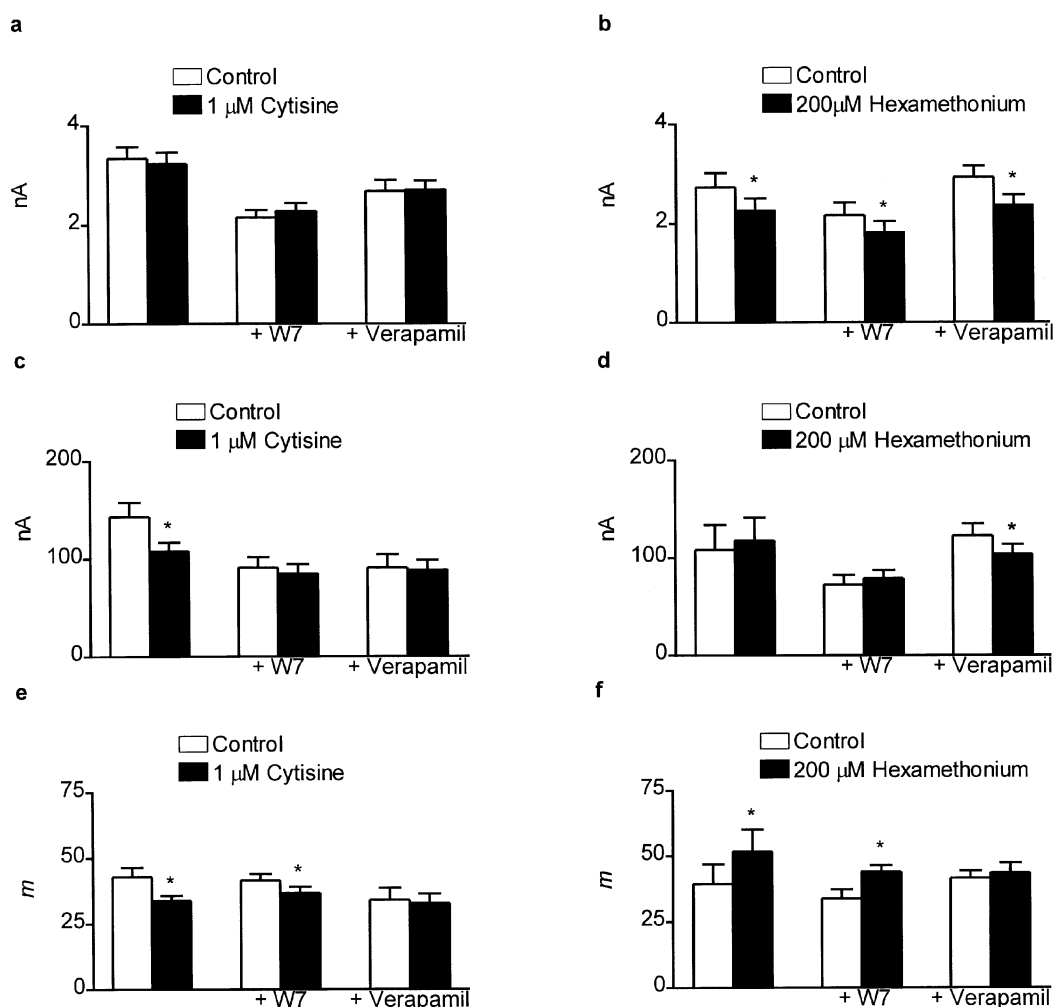


Figure 5 Bar graph showing the effects of 1 μM cytosine (a,c,e) and 200 μM hexamethonium (b,d,f) on the amplitude of mepcs (a,b), the amplitude of epcs (c,d) and the epc quantal content (e,f) at 0.5 Hz in the rat isolated hemidiaphragm preparation in the absence and presence of W-7 or verapamil (both 10 μM). $[\text{Ca}^{2+}]_o$ was 1.8 mM in all experiments. Data for cytosine alone are the same as in Figure 3 and are included for comparison. Data are mean and s.e.mean of values from 7–10 individual determinations. Asterisks indicate a significant effect of cytosine or hexamethonium on mepc amplitude, epc amplitude or epc quantal content ($P < 0.05$, two-tailed paired Student's *t*-test, control *versus* cytosine or hexamethonium). Note that the effects of cytosine and hexamethonium on *m* were unaffected by the presence of W-7. However, in the presence of verapamil, both effects were abolished.

vecuronium are attenuated by W-7 implicating a calmodulin-dependent process in autofacilitation. In this study, it was observed that both the cytosine-induced reduction and the hexamethonium-induced facilitation in *m* (at 0.5 Hz) were unaffected by the presence of W-7. However, in the presence of verapamil, neither was there any cytosine-induced facilitation nor hexamethonium-induced depression in this parameter. This suggests that mechanism by which nicotinic ACh receptor activation depresses the release of ACh at low frequencies of stimulation is not the same as the mechanism by which release of ACh is enhanced through activation of nicotinic ACh receptors at high frequencies of stimulation. Therefore, the autofacilitatory and autoinhibitory effects of ACh on evoked ACh release from rat motor nerve terminals must be separate and individually identifiable processes involving unique and distinct intracellular second messenger systems. Finally, despite the lack of an effect of W-7 on the cytosine-induced depression of quantal ACh release, W-7 attenuated the effect of cytosine on epc amplitudes. This suggests that, in addition to its calmodulin-independent effect on ACh release, cytosine may have another unidentified

calmodulin-dependent neuromuscular effect that can depress epc amplitude.

Verapamil attenuated the inhibitory effect of cytosine and the facilitatory effect of hexamethonium on *m*, suggesting that the compound blocks autoinhibition in motor nerve terminals. Further, in the absence of any other pharmacological agents, 10 μM verapamil increased *m* by around 10–20% suggesting the existence of some tonic activation of the autoinhibitory system by endogenous ACh. The question now arises as to what the underlying mechanism could be that is triggered by activation of a neuronal-type nicotinic ACh receptor, is dependent on $[\text{Ca}^{2+}]_o$ and is inhibited by verapamil? Neuronal-type nicotinic ACh receptors have been shown to be more permeable to Ca^{2+} compared to their muscle-type counterparts (Decker & Dani, 1990; Sands & Barish, 1991; Mulle *et al.*, 1992a; Vernino *et al.*, 1992), their function can be modulated by $[\text{Ca}^{2+}]_o$ (Vernino *et al.*, 1992; Mulle *et al.*, 1992b) and they can be blocked by verapamil at the concentration used here (Donnelly-Roberts *et al.*, 1995; Villarroya *et al.*, 1997). Thus a verapamil-sensitive Ca^{2+} -flux through prejunctional neuronal-type nicotinic ACh receptors may be important in autoinhibition.

Alternatively, activation of the presynaptic neuronal-type nicotinic ACh receptors may lead to the activation of nerve terminal verapamil-sensitive (L-type) VOCCs and that control autoinhibition. Verapamil-sensitive Ca^{2+} -currents do exist in motor nerve terminals (Penner & Dreyer, 1986; Anderson & Harvey, 1987). However, a direct involvement of verapamil-sensitive VOCCs in the evoked release of ACh at the neuromuscular junction is mostly denied (Nachshen & Blaustein, 1979). It is more generally accepted that the channels responsible for the evoked release of ACh from mammalian motor nerve terminals are the ω -agatoxin-sensitive P-type VOCCs (Uchitel *et al.*, 1992; Katz *et al.*, 1996). However, it is not inconceivable that presynaptic L-type VOCCs could possess a modulatory role in ACh release. Whatever the mechanism underlying the putative verapamil-sensitive Ca^{2+} -flux, it is unlikely that it would lead to a generalized rise in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) since an increase in $[\text{Ca}^{2+}]_i$ should increase the frequency of occurrence of mepcs (Hubbard & Willis, 1962), which was not seen in the present study. However, a localized rise in $[\text{Ca}^{2+}]_i$ may be sufficient enough to produce autoinhibition.

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