THE NATURE OF THE PRESYNAPTIC EFFECTS OF (+)-TUBOCURARINE AT THE MOUSE NEUROMUSCULAR JUNCTION

BY C. B. FERRY AND S. S. KELLY

From the Pharmacological Laboratories, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET

SUMMARY

1. The effects of (+)-tubocurarine (TC) on tetanic run-down and quantum content of end-plate potentials (EPPs) were investigated in cut-fibre preparations of mouse diaphragm.

2. (+)-Tubocurarine, $0.15\ \mu M$, halved the amplitude of spontaneous miniature EPPs (MEPPs) and steepened the tetanic run-down of EPPs evoked at 10 Hz by increasing the quantum content of the first EPP of the train while having no effect on quantum content of plateau EPPs. With stimulation at 1 Hz, there was little run-down and the quantum content of all EPPs was increased by TC.

3. The use of binomial statistics to analyse release indicated that after TC the increase in the quantum content of the first EPP in the train at 10 Hz was due to an increase in n and that during the run-down there was a decrease in p so that plateau EPP quantum content at 10 Hz was not different from control.

4. To elucidate a possible role of cholinoreceptors in the presynaptic effects of TC, studies were made on the effects of pancuronium or of α-bungarotoxin (BTX), with concentrations and exposure times where they had postsynaptic effects equal to $0.15\ \mu M$-TC. The run-down of EPPs was unaffected by BTX, while pancuronium steepened it to a lesser extent than TC.

5. The anticholinesterase, ecothiopate, decreased the quantum content of plateau EPPs only at high frequencies of stimulation (50 Hz) and did not affect the presynaptic effects of TC at 10 Hz.

6. At concentrations which reduced MEPP amplitude, atropine ($10\ \mu M$) or hexamethonium ($50\ \mu M$) had no effect on EPP run-down.

7. These results indicate that TC could have presynaptic effects via a presynaptic acetylcholine receptor, but that such a receptor may not have the same binding specificities as the postsynaptic receptor.

INTRODUCTION

Since Masland & Wigton (1940) recorded antidromic firing of action potentials in motor nerves following the intra-arterial injection of acetylcholine (ACh) or following orthodromic stimulation after the administration of neostigmine, many workers have found pharmacological evidence for presynaptic actions of acetylcholine (ACh) at the mammalian neuromuscular junction (Hobbiger, 1976). However,

histological evidence for presynaptic ACh receptors is less certain as the use of electron microscopy to localize radio-labelled or horseradish peroxidase-labelled α-bungarotoxin (BTX) binding sites has led to debate as to how much of the apparent presynaptic binding is due to spread of radioactivity or of the reaction product from the many postsynaptic sites (Fertuck & Salpeter, 1974; Lentz, Mazurkiewicz & Rosenthal, 1977). It has also been suggested that the presynaptic effects of ACh are caused by postsynaptic actions, e.g. potassium ions released at the end-plate after activation of postsynaptic cholinoreceptors (Katz, 1962; Miyamoto, 1978), although there are arguments against this suggestion (e.g. Wilson, 1982).

Experiments made on the effects of cholinoreceptor blockers on transmitter release have yielded inconsistent results. For example, Miledi, Molinaar & Polak (1978) found that both BTX and (+)-tubocurarine (TC) increased ACh release evoked by nerve stimulation, yet Halank, Kilbinger & Wessler (1985) reported that TC decreased such release. Although evidence has been found that TC and other non-depolarizing blockers have presynaptic effects, it has not yet been firmly established that the effects are due specifically to interaction with a cholinoreceptor rather than to a non-specific effect of TC on the nerve terminal.

Electrophysiological studies on evoked release of transmitter have shown that the addition of ACh to a magnesium-treated preparation reduced quantal release (Hubbard, Schmidt & Yokoto, 1965). Also, Wilson (1982) and Chang, Hong & Ko (1986) reported that the quantum content of end-plate potentials evoked at 50–75 Hz was reduced in the presence of an anticholinesterase. Furthermore, TC has been found to steepen the run-down of end-plate potentials (Wilson, 1982) and of end-plate currents (Glavinovic, 1979; Magleby, Pallotta & Terrar, 1981) at the beginning of trains of responses to stimuli. This steepening of run-down has been interpreted as due either to decreased steady-state release of transmitter (Blaber, 1972) or to an increased initial release (Wilson, 1982).

The aims of this study were to determine the effects of an anticholinesterase or of TC on spontaneous and evoked release of transmitter at neuromuscular junctions in the mouse, and to investigate the pharmacological properties of putative presynaptic ACh receptors. The possible interaction between such presynaptic receptors and ACh released non-quantally by the nerve terminal was also investigated. The cut-fibre preparation enabled the quantum content of end-plate potentials to be calculated directly without having to block the muscle twitch by reducing quantum content with magnesium, and also enabled the calculation of the binomial parameters n and p. Although the physiological and/or anatomical correlates of these parameters are not known, it may be that n represents either the store of releasable presynaptic vesicles or the number of activated or activatable release sites and that p is the probability of release (Wernig, 1975).

METHODS

Male albino mice aged 6–7 months were killed by decapitation and the left hemidiaphragm together with its phrenic nerve was removed from each animal and pinned onto Sylgard 184 (Dow Corning) in a Perspex bath through which flowed physiological saline of the following composition (mM): NaCl, 137; $NaHCO_3$, 12; NaH_2PO_4, 1; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 25. The saline was

gassed with a mixture of 95% O_2 and 5% CO_2 and the temperature was maintained at 37.0 ± 0.5 °C. If required, the phrenic nerve was stimulated via a suction electrode with supramaximal pulses of 0·05 ms duration. In order to prevent the muscle twitching in response to nerve stimulation, the muscle fibres were cut (Barstad, 1962; Hubbard & Wilson, 1973).

Resting membrane potentials, end-plate potentials (EPPs) and spontaneous miniature end-plate potentials (MEPPs) were recorded with an intracellular glass capillary microelectrodes filled with 3 M-KCl. MEPPs and EPPs were displayed on an oscilloscope and were analysed on-line using an analog-to-digital converter and a PDP 11/03 minicomputer. The recording of MEPPs or EPPs with a rise time to amplitude ratio of less than 1·1 ms/mV was the criterion for the focal location of the microelectrode at the end-plate. Between 50 and 200 MEPPs were recorded from each muscle fibre and approximately ten fibres were sampled from each muscle. The amplitude of spontaneous and evoked EPPs was corrected for non-linear summation and to a standard resting membrane potential of -50 mV (cf. Kelly, 1978), and the mean amplitude and frequency of MEPPs were calculated at each end-plate. Martin's correction for non-linear summation (Martin, 1955) was empirically trimmed by a factor of 0·7 (cf. Discussion, McLachlan & Martin, 1981) so that corrected EPP amplitudes would not exceed the reversal potential of approximately -5 mV in these preparations (Banker, Kelly & Robbins, 1983).

Up to four trains each of fifty EPPs were recorded from each muscle fibre and in each train the mean amplitude of the last forty (plateau) EPPs was calculated. At least 1 min was allowed to elapse between trains. The mean quantum content of the EPPs in each muscle fibre was determined directly by dividing the mean corrected EPP amplitude by the mean corrected MEPP amplitude.

After recording control MEPPs and EPPs in the physiological saline, the same preparation was usually used to study the effects of one of the following drugs: ecothiopate, (+)-tubocurarine (TC) or α-bungarotoxin (BTX). In experiments with ecothiopate, the preparation was bathed in a 0·5 μM solution of the drug in physiological saline for 30–40 min and then the drug was washed out of the bath for 30 min before recording was begun. With BTX, a 0·2 μg/ml (*ca*. 10 nM) solution was left in contact with the preparation for 20 min and then the BTX was washed out of the bath for 30 min before recording. With TC, a 0·15 μM solution bathed the preparation for 1 h before recording and also throughout the recording period. During the recording period there was no change in MEPP amplitude.

Investigations were also made on the effects of the following drugs on spontaneous and evoked quantal release of transmitter: 50 μM-hexamethonium, 0·04 μM-pancuronium, 10 μM-atropine, 1 μM-tetraphenylborate and AH5183 (0·1, 0·4 or 1 μM). The last two compounds are reported to inhibit non-quantal release of ACh by at least 50% in the concentrations used (Edwards, Dolezal, Tucek, Zemkova & Vyskocil, 1985). Each of these drugs was in contact with the preparation for at least 1 h before recording began and was also present during recording. AH5183 was a gift from Glaxo Group Research and all others were obtained from Sigma Chemical Co.

Statistical analysis of the results. The binomial parameters n and p were calculated from amplitudes of MEPPs and EPPs at the plateau (Miyamoto, 1975). Values were accepted only from data which conformed to binomial statistics. For this reason, the observed and calculated distributions of EPP amplitudes were compared using the χ^2 test. The equations used to calculate the EPP amplitudes expected if the distribution were binomial (Miyamoto, 1975) were incorporated into a computer program, the validation of which has been described elsewhere (Kelly & Robbins, 1987). Unless otherwise stated, the results are routinely presented as mean ± 1 S.D. The mean of a group of experimental data was calculated from the means of the values of individual fibres in that experimental group. The probability that two groups of data were different was tested using the Mann–Whitney test. A probability level $P < 0.05$ was considered to show a statistically significant difference.

<div align="center">RESULTS</div>

Effect of drugs on spontaneous quantal release of transmitter

None of the drugs used separately or together had any effect on the resting membrane potential (49.2 ± 0.8 mV; $n = 55$) or on the frequency of MEPPs (6.65 ± 0.55 Hz; $n = 55$). The amplitude and the half-decay time of MEPPs were

TABLE 1. Effect of neuromuscular nicotinic antagonists (A), other ACh receptor antagonists (B), ecothiopate (C) or putative inhibitors of non-quantal ACh release (D) on the amplitudes and half-decay times of MEPPs and EPPs evoked at a frequency of 10 Hz

Drug	MEPP		First EPP (mV)	Plateau EPP (mV)
	Amplitude (mV)	Half-decay time		
Control	0·52±0·16	0·78±0·16 (63/6)	23·6±9·9 (60/6)	18·6±8·1 (55/6)
		A		
TC	0·32±0·07*	0·53±0·14* (49/5)	22·9±9·6 (37/4)	12·4±4·8* (37/4)
BTX	0·31±0·07*	0·53±0·14* (45/5)	14·5±7·2* (44/5)	9·3±4·6* (45/5)
Pancuronium	0·36±0·07*	0·67±0·15* (15/2)	19·7±4·7 (14/2)	11·9±2·9* (14/2)
		B		
Hexamethonium	0·34±0·08*	0·58±0·12* (17/2)	17·7±6·2* (13/2)	12·4±4·7* (13/2)
Atropine	0·43±0·11*	0·93±0·26* (15/2)	23·9±6·6 (10/2)	15·7±4·5 (10/2)
		C		
Ecothiopate	0·86±0·25*	1·8±0·51* (41/4)	44·2±18·6* (29/3)	27·4±8·8* (25/3)
		D		
Tetraphenylborate	0·44±0·10*	0·67±0·15* (24/2)	29·9±10·8* (23/2)	18·0±5·6 (23/2)
AH5183 (0·1 µM)	0·50±0·12	0·91±0·24 (17/2)	24·5±8·5 (11/2)	17·1±6·6 (11/2)
AH5183 (0·4 µM)	0·51±0·14	0·90±0·23 (9/1)	26·1±7·1 (8/1)	18·8±4·2 (8/1)
AH5183 (1·0 µM)	0·37±0·06*	0·65±0·13* (11/1)	24·5±5·7 (9/1)	15·9±3·1* (9/1)
AH5183 (0·1 µM)+TC	0·32±0·04*	0·50±0·13* (21/2)	20·8±6·2 (16/2)	12·9±4·3* (16/2)

Values are given as mean ±1 s.d. with the number of muscle fibres and number of animals in parentheses. *Significant difference from control ($P < 0.05$).

increased by ecothiopate and decreased by both TC and BTX (Table 1). The results of these experiments also show that BTX decreased MEPP amplitude and half-decay time by the same amount as did 0·15 μM-TC. Hexamethonium and pancuronium also reduced MEPP amplitude and half-decay time by a similar amount as with TC, whereas atropine and tetraphenylborate produced much smaller reductions in these two parameters.

TABLE 2. Effect of ecothiopate (0·5 μM) on plateau EPPs evoked at 20 or 50 Hz and on binomial parameters of plateau EPPs elicited at a frequency of 50 Hz

	Plateau EPP amplitudes (mV)			
	At 20 Hz		At 50 Hz	
Before ecothiopate	21·5 ± 8·3 (12)		22·6 ± 7·8 (10)	
After ecothiopate	28·6 ± 11·0 (9)	*	20·3 ± 7·9 (8)	
	Binomial parameters at 50 Hz			Number of fibres
	m	n	P	
Before ecothiopate	36·3 ± 10·4	50 ± 17	0·75 ± 0·14	13
	†	†		
After ecothiopate	26·7 ± 5·9	35 ± 11	0·81 ± 0·11	9

Values, corrected for non-linear summation (see Methods), are given as mean ± 1 S.D. of data from the same preparation. In at least six cases the same muscle fibre was used for both frequencies.

*Significant difference between the two frequencies ($P < 0.05$); †significant difference before–after ecothiopate.

Effect of ecothiopate and stimulus frequency on EPPs

Trains of stimuli at 10, 20 or 50 Hz to the nerve of preparations bathed in physiological saline produced plateau EPPs of the same amplitude at each frequency (Tables 1 and 2) without baseline depolarization during the train. After ecothiopate there was an increased amplitude of EPPs evoked at 10 Hz, which was the same for plateau EPPs and for the first EPP of trains (Table 1). Thus at 10 Hz, neither the ratio of plateau EPP to first EPP nor the directly measured quantum content of EPPs was altered by ecothiopate (Table 3). At 20 Hz, ecothiopate increased the amplitude of the first EPP and the mean amplitude of plateau EPPs, with little or no baseline depolarization during the train of responses. However, at 50 Hz and after ecothiopate a progressive depolarization was observed which reached a maximum of about 4 mV (uncorrected) by the sixth to tenth EPP and thereafter showed little or no change until the cessation of stimulation, after which it decayed with a half-time of about 2 s. Also, although the amplitude of the first EPP of the train at 50 Hz was increased after ecothiopate, the amplitude of the plateau EPPs was decreased (Table 2). This decrease was unlikely to have been due to the baseline depolarization alone because after correcting for non-linear summation at the resting membrane potential during baseline depolarization (see Methods), the plateau EPP amplitudes at 50 Hz were still significantly less than at 20 Hz even if the full correction factor was used. As the MEPP amplitude was the same before and (*ca.* 1 min) after the train, it is likely that the decreased EPP amplitude was due in part to a decrease in the number of quanta making up the EPP (Table 2).

Effects of cholinoreceptor antagonists

At a stimulus frequency of 10 Hz, the amplitudes of plateau EPPs and of MEPPs were reduced in the same proportions by TC, pancuronium or BTX (Table 1), which indicated no change in the quantum content of plateau EPPs. The ratio of plateau EPP to first EPP was decreased (i.e. 'run-down' was increased) by pancuronium and by TC but not by BTX (Table 3) and this implies that pancuronium and TC might

TABLE 3. Effect of neuromuscular blocking drugs, putative non-quantal ACh release inhibitors and ecothiopate on EPP run-down and on the quantum content of plateau EPPs elicited at a frequency of 10 Hz

Drug	Ratio: $\dfrac{\text{plateau EPP}}{\text{first EPP}}$	Quantum content (m)
Control	$0{\cdot}70 \pm 0{\cdot}14$ (53/6)	$33{\cdot}6 \pm 13{\cdot}0$ (53/6)
TC	$0{\cdot}56 \pm 0{\cdot}12$ (37/4)*	$37{\cdot}7 \pm 16{\cdot}2$ (37/4)
BTX	$0{\cdot}65 \pm 0{\cdot}13$ (45/5)	$32{\cdot}1 \pm 15{\cdot}9$ (44/5)
Pancuronium	$0{\cdot}61 \pm 0{\cdot}07$ (14/2)*	$33{\cdot}4 \pm 7{\cdot}6$ (14/2)
Hexamethonium	$0{\cdot}70 \pm 0{\cdot}07$ (13/2)	$36{\cdot}1 \pm 16{\cdot}9$ (13/2)
Atropine	$0{\cdot}66 \pm 0{\cdot}06$ (10/1)	$38{\cdot}1 \pm 14{\cdot}7$ (10/1)
Ecothiopate	$0{\cdot}66 \pm 0{\cdot}10$ (24/3)	$33{\cdot}1 \pm 13{\cdot}0$ (25/3)
TC + ecothiopate	$0{\cdot}54 \pm 0{\cdot}10$ (24/3)*	$37{\cdot}6 \pm 9{\cdot}3$ (19/3)
Tetraphenylborate	$0{\cdot}62 \pm 0{\cdot}09$ (22/2)*	$42{\cdot}5 \pm 15{\cdot}1$ (22/2)*
AH5183 (0·1 μM)	$0{\cdot}68 \pm 0{\cdot}09$ (11/2)	$36{\cdot}5 \pm 18{\cdot}0$ (11/2)
AH5183 (0·4 μM)	$0{\cdot}73 \pm 0{\cdot}08$ (8/1)	$38{\cdot}1 \pm 9{\cdot}5$ (8/1)
AH5183 (1·0 μM)	$0{\cdot}66 \pm 0{\cdot}09$ (9/1)	$46{\cdot}7 \pm 13{\cdot}2$ (9/1)*
AH5183 (0·1 μM) + TC	$0{\cdot}61 \pm 0{\cdot}04$ (16/2)*	$42{\cdot}7 \pm 14{\cdot}4$ (14/2)*†

Values are given as mean ± 1 S.D. with the number of muscle fibres and animals in parentheses.
* Significant difference from control values ($P < 0{\cdot}05$). † Significantly different from control but not significantly different from the value in TC alone.

have a presynaptic action which produces an increase in the quantum content of the first EPP in a train. In some experiments the quantum content of the first EPP was calculated by the direct method and found to be $44{\cdot}6 \pm 19{\cdot}0$ ($n = 34$) in control preparations and $68{\cdot}5 \pm 28{\cdot}6$ ($n = 23$) in TC; these values are significantly different. The ratio of the amplitude of the plateau EPP to that of the first EPP in an uncut preparation in 5 μM-TC was $0{\cdot}43 \pm 0{\cdot}05$ ($n = 26$), which suggests that the effect of TC in steepening the run-down is concentration dependent, although at least part of this difference in run-down could be due to differences between cut and uncut preparations. The effect of TC (0·15 μM) on run-down was not altered in the presence of ecothiopate.

Although TC had no effect on the directly measured quantum content of plateau EPPs, in the same fibres it increased the coefficient of variation (c.v., i.e. standard deviation/mean) of the amplitudes of these EPPs. If the distribution of quantum contents were Poisson then the value of $1/\text{c.v.}^2$ would be equal to the mean quantum content (m), but if the distribution were binomial, the relationship between $1/\text{c.v.}^2$ and m would depend upon the binomial parameters n and p. The values of $1/\text{c.v.}^2$

were 185 ± 109 ($n = 28$) in control preparations and 116 ± 69 ($n = 22$) in TC-treated preparations; these values are significantly different. Thus a reduction in $1/\text{c.v.}^2$ when the 'direct' m was unchanged indicates that TC alters the value of n and/or p.

Further experiments were made to find out if other cholinergic antagonists affected the run-down in the same way as TC or pancuronium. A concentration of hexamethonium ($50 \mu\text{M}$) sufficient to produce a decrease in MEPP amplitude similar to TC had no effect on either the quantum content of plateau EPPs or on the run-down. Atropine ($10 \mu\text{M}$) caused a small decrease in MEPP amplitude but had no significant effect on quantum content or on run-down (Tables 1 and 3). It is concluded that of the cholinergic antagonists tested only TC and pancuronium increased run-down of EPPs at 10 Hz and that with TC this was due to an increase in the first EPP of the train. This suggested that TC might act by inhibiting a tonic depressant action of ACh, possibly ACh released non-quantally.

Effect of putative inhibitors of non-quantal release of ACh

The drugs used were AH5183 and tetraphenylborate. Concentrations of AH5183 (0.1 or $0.4 \mu\text{M}$) which are reported to decrease non-quantal ACh release by at least 50% (Edwards *et al.* 1985) had no effect on either MEPP or EPP amplitudes (Table 1). Except for an increase in quantum content of plateau EPPs, the effects of TC described above were not altered by the presence of $0.1 \mu\text{M}$-AH5183. A concentration of $1 \mu\text{M}$-AH5183 produced a decrease in MEPP amplitude and half-decay time (Table 1), but no further experiments were performed with this concentration because such postsynaptic TC-like effects would have made it impossible to interpret observations of change in quantum content. Another compound reported to inhibit non-quantal ACh release, tetraphenylborate ($1 \mu\text{M}$), increased the plateau EPP quantum content and also steepened the run-down. However, this concentration of tetraphenylborate also produced a reduction in MEPP amplitude and so made it difficult to separate any effects on non-quantal release from its effects as a cholinergic antagonist.

Analysis of binomial statistics

The above results show that at 10 Hz TC steepened run-down of EPPs without changing the quantum content of plateau EPPs, and so it was decided to see if the binomial parameters, n and p, were altered by TC. Therefore, calculations of n and p were made on data from a random selection of fibres from control or drug-treated preparations. With stimulation at 10 Hz the quantum contents of EPPs from at least 70% of the fibres tested were distributed binomially (cf. Kelly & Robbins, 1987) and Table 4 is a summary of the results. Of the drugs tested, only TC had any effect on either n or p and the mean quantum content was unchanged by TC because it increased n and decreased p. The presence of ecothiopate had no effect on the changes in n and p produced by TC (Table 4).

Transmitter release evoked at 1 Hz

From the above results it would seem that in TC the value of m at the plateau is unaltered because a decrease in p offset the increase in n. This leads to the hypothesis

that the increase in quantum content of the first EPP is caused by an increase in n and the run-down is due to a decrease in p at the beginning of the train. In order to test this hypothesis and obtain an estimate of the binomial parameters which apply to the first EPP, it was decided to stimulate at as low a frequency as was compatible with obtaining sufficient data within a stable recording period. Therefore a stimulus

TABLE 4. Effects of TC, BTX, pancuronium and ecothiopate on binomial parameters of EPPs elicited at a frequency of 10 Hz

Drug	m	n	p	
Control	$33\cdot3\pm10\cdot8$	41 ± 15	$0\cdot83\pm0\cdot10$	(25/6)
TC	$38\cdot8\pm16\cdot2$	58 ± 25*	$0\cdot68\pm0\cdot15$*	(22/4)
BTX	$33\cdot4\pm15\cdot8$	41 ± 20	$0\cdot81\pm0\cdot15$	(27/4)
Pancuronium	$33\cdot4\pm7\cdot6$	44 ± 14	$0\cdot79\pm0\cdot14$	(14/2)
Ecothiopate	$34\cdot2\pm9\cdot6$	44 ± 13	$0\cdot79\pm0\cdot13$	(20/3)
TC+ecothiopate	$37\cdot6\pm9\cdot3$	58 ± 17*	$0\cdot69\pm0\cdot17$*	(19/3)

Values are given as mean $\pm$ 1 s.d. with the number of muscle fibres and animals in parentheses.
* Significant difference from control values ($P < 0\cdot05$).

TABLE 5. Effect of (+)-tubocurarine (TC) on EPPs elicited at a frequency of 1 Hz in cut-fibre mouse diaphragm preparations

EPPs at 1 Hz	Control		TC
First EPP amplitude (mV)	$23\cdot7\pm7\cdot6$		$19\cdot6\pm5\cdot9$
Plateau EPP amplitude (mV)	$22\cdot0\pm6\cdot0$		$18\cdot3\pm5\cdot5$
Ratio:plateau EPP/first EPP	$0\cdot94\pm0\cdot07$		$0\cdot94\pm0\cdot17$
Quantum content	$55\cdot6\pm11\cdot6$	*	$76\cdot2\pm22\cdot5$
Number of fibres/animals	12/2		19/2
Binomial parameters			
m	$57\cdot2\pm11\cdot4$	*	$73\cdot1\pm24\cdot3$
n	65 ± 24	*	91 ± 27
p	$0\cdot90\pm0\cdot12$		$0\cdot80\pm0\cdot12$
Number of fibres/animals	10/2		10/2

Values are given as mean $\pm$ 1 s.d. * Significant differences between values in control and curarized preparations.

frequency of 1 Hz was chosen so that sufficient time might elapse between stimuli for binomial parameters to recover almost to first EPP levels. The results obtained from those experiments are shown in Table 5. At this lower frequency about 80 % of fibres tested in control preparations had EPP amplitude distributions which conformed to binomial statistics, whereas in TC the proportion of fibres fitting binomial statistics was about 50 %. There was no significant difference between the value of m in fibres which fitted binomial statistics and the value of m in the whole sample of fibres (Tables 3 and 4).

With a stimulus frequency of 1 Hz, very little run-down of EPP amplitude was observed either in control or in curarized preparations. In control preparations the

quantum content of plateau EPPs was higher at 1 Hz than at 10 Hz and this was caused by an increase in n with no significant change in p. Although MEPP amplitude in the curarized preparations was only 55% of control values, at 1 Hz there was no significant difference in first or plateau EPP amplitudes between curarized and control preparations (Table 5). The mean quantum content, m, of these 1 Hz EPPs is therefore increased by TC and this increase is produced by an increase in the binomial parameter, n, although this is accompanied by a small decrease in p.

In summary, the effects of TC and stimulation frequency on n and p at the plateau are: (i) in both control and TC-treated preparations n is higher at 1 Hz than at 10 Hz; (ii) p is higher at 1 Hz than 10 Hz in TC, but in control preparations is not different at the two frequencies; (iii) at both frequencies, n is greater in TC than in control preparations; (iv) p is decreased by TC at 10 Hz and at 1 Hz but the decrease at 1 Hz is much less than that at 10 Hz.

DISCUSSION

Effect of stimulus frequency on quantum content

In the absence of any drugs there was no difference in plateau EPP quantum contents at 10, 20 or 50 Hz, although at 1 Hz quantum content was significantly greater. There may be a frequency-dependent process controlling quantum content at 10–50 Hz and not at low frequencies or on the first EPP. Thus at high frequencies of stimulation there would be a transition between the first EPP and plateau EPPs involving a change in the parameters controlling quantum content.

Because TC increases the quantum content of all EPPs in a train at 1 Hz, but does not affect the quantum content of plateau EPPs at 10 Hz, it is proposed that its enhancing action is seen only at low frequencies of stimulation. At higher frequencies a frequency-dependent process (e.g. mobilization) may limit transmitter output and may be unaffected by TC so that the quantum content of plateau EPPs is the same as that of control preparations. This, however, would apply only to the low concentration of TC used in these experiments and it is possible that at higher concentrations there may also be an inhibition of mobilization.

With low concentrations of TC the increased transmitter output with low-frequency or single stimuli could overcome postsynaptic receptor blockade thus leaving the twitch unaltered while tetanic tension would be depressed. Consequently, an estimate of the potency of TC might depend on whether single or tetanic stimulation was used.

Binomial analysis of transmitter release

TC increases the value of the binomial parameter n calculated from plateau EPPs at 10 Hz, but not the mean quantum content because of a corresponding decrease in p. In order to investigate further the mechanism by which TC alters the quantum content of the first EPP, a binomial analysis of EPPs elicited at 1 Hz showed that TC increased the mean plateau quantum content by increasing the value of n while producing only a small decrease in p (Table 5).

Assuming that the first EPP of trains at 10 Hz was similar to plateau EPPs at

1 Hz, it may be that in control preparations there was a decline in n and little or no change in p during the run-down at 10 Hz. In the presence of TC at 10 Hz, although the initial value of n was increased, it declined by a similar proportion as did controls during the run-down and its decline was accompanied by a decrease in p. The above results suggest that the primary effect of TC is to increase n and that subsequently p declines to a steady-state value which is determined by a rate-limiting process such as mobilization.

In order to consider further the run-down process it is necessary to present a hypothesis as to the physiological correlates of the parameters n and p. If n were to represent the number of release sites which could be activated by nerve excitation, then p would depend upon the likelihood that a quantum would interact with a release site. During run-down at higher stimulus frequencies n might decline if the release site were refractory after releasing a quantum. The value of n at the plateau would then represent the rate of recovery of release sites. Thus TC might initially increase n which, by releasing more quanta, might reduce the number of vesicles near the release site. At high frequencies such a depletion would lead to reduction in p (the probability that an activated release site has a quantum available for release), thus counteracting the increase in n. At low frequencies there would be time for local replenishment of vesicles, thus allowing a recovery of p.

Evidence for a specific presynaptic ACh receptor

At high frequencies of stimulation in ecothiopate, there was a decrease in the quantum content of EPPs, possibly because of a build-up of ACh in the synaptic cleft. This effect of ecothiopate is similar to that reported by other workers with other anticholinesterases (e.g. neostigmine) (Wilson, 1982; Chang *et al.* 1986). Both the frequency dependence and the similarity of this effect with other anti-cholinesterases indicate that the reduction of quantum content after ecothiopate may be via ACh rather than by a direct action on the nerve terminal. Further evidence that at least some of the presynaptic actions of anticholinesterases are mediated via ACh is that the production of stimulus-induced backfiring in the nerve by neostigmine requires the release of transmitter and is not a direct effect of neostigmine on the nerve terminal (Aizenman, Bierkamper & Stanley, 1986).

An action of ACh on presynaptic receptors would suggest that cholinergic antagonists might affect transmitter output. A low concentration (0·15 μM) of TC reduced MEPP amplitude to about 60 % of control and steepened run-down of EPPs at 10 Hz. This was due to an increase in the amount of transmitter released by the first stimulus rather than a decrease in plateau EPP quantum content. These results agree with those obtained by Wilson (1982), who, like us, measured quantum content directly. However, if quantum content had been estimated from $1/\text{c.v.}^2$ then it would have been concluded that there was a decreased plateau quantum content, and a different mechanism for increased run-down (cf. Galindo, 1972; Blaber, 1973).

If the increase in the first EPP were mediated by blockade of a cholinoreceptor on the nerve terminal, then that receptor appears to be blocked by TC (and to some extent pancuronium). A high (10 μM) concentration of the muscarinic antagonist, atropine, which slightly decreased MEPP amplitude, had no presynaptic effect.

Similarly, 50 μM-hexamethonium, which reduced MEPP amplitude, had no effect on quantum content. Because the various drugs causing a similar degree of postsynaptic blockade did not have similar presynaptic effects, it is possible to exclude postsynaptic potassium release as the mediator of the observed presynaptic effects of TC.

Under conditions in which its postsynaptic effects equalled TC, BTX had no presynaptic effects. Thus the putative presynaptic ACh receptor may not have the same binding properties as postsynaptic nicotinic cholinoreceptors at the end-plate. In contrast, Miledi *et al.* (1978) found that both TC and BTX increased ACh output from rat diaphragm in response to nerve stimulation at 3 Hz. However, in their experiments, the preparation had been pre-treated with anticholinesterase and then was continuously exposed to a concentration of BTX which was 25 times greater than that used in our experiments. Bierkamper, Aizenman & Millington (1986) also measured ACh output from rat hemidiaphragm–phrenic nerve preparations and found that low concentrations (1 μM) of TC increased output, whereas high concentrations decreased output. Halank *et al.* (1985) used a radiochemical method to estimate evoked release of ACh from rat diaphragm and reported that TC decreased output. However, it is difficult to make direct comparisons with the present study because they used a concentration of TC much higher than those in the present study and their stimulation protocol was very different from ours and was in the presence of hemicholinium.

The combination of the above results suggests that there is a specific presynaptic cholinoreceptor which, when activated, causes reduction of evoked release of transmitter. Thus a negative feed-back would be produced if ACh were to build up in the synaptic cleft, e.g. during high-frequency stimulation in the presence of anticholinesterase. The fact that the quantum content of the first EPP was increased by TC indicates that non-evoked ACh (i.e. MEPPs or non-quantal transmitter release) may normally activate the receptors.

As most of the spontaneous ACh release is believed to be non-quantal (i.e. non-MEPP) in origin, it might be that such non-quantal release close to the putative presynaptic receptor could inhibit evoked release. If TC increases evoked transmitter release by inhibiting the effects of non-quantal release of ACh then it might be expected that compounds reported to inhibit non-quantal release (Edwards *et al.* 1985) would have similar presynaptic effects to TC. Unfortunately, the results of the experiments described above are inconclusive because AH5183 and TPB had presynaptic effects only in concentrations which also decreased MEPP amplitude, throwing doubt on the mechanism of action of these drugs at these high concentrations. Thus these results cannot be taken as conclusive evidence either for or against the mechanism of action of TC being antagonism of the inhibitory effect of ACh released non-quantally.

In conclusion, therefore, the reduction in the mean quantum content (m) produced by ecothiopate with high frequencies of stimulation, together with the increase in m produced by TC with low-frequency stimulation indicate that there is a specific receptor for ACh at the presynaptic nerve terminal. Although the existence of such a receptor is not proven, evidence is presented that activation of this receptor by ACh causes decreased transmitter output and that this receptor, while not muscarinic,

may not be the same as the postsynaptic nicotinic receptor at the neuromuscular junction.

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