Run-down of neuromuscular transmission during repetitive nerve activity by nicotinic antagonists is not due to desensitization of the postsynaptic receptor

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1 Whether the function of the postsynaptic acetylcholine receptor is use-dependently affected by repetitive nerve stimulation in the presence of competitive antagonists was studied in the mouse phrenic nerve-hemidiaphragm preparation.

2 For electrophysiological experiments, the preparation was immobilized by synthetic μ -conotoxin, which preferentially blocks muscular Na-channels causing neither depolarization of the membrane potential, inhibition of quantal transmitter release, nor depression of nicotinic receptor function.

3 High concentrations of cobratoxin depressed indirect twitches and endplate potentials (e.p.ps) without inducing waning of contractilities or run-down of trains of e.p.ps evoked at 10–100 Hz. However, waning and run-down were accelerated after washout of the toxin despite diminished postsynaptic receptor blockade. Once the run-down of e.p.ps was produced by washout or low concentrations of cobratoxin, further depression of e.p.p. amplitude with high concentrations of cobratoxin did not attenuate the e.p.p. run-down.

4 The degrees of waning of tetanus and trains of e.p.ps produced by a very high concentration of tubocurarine (20 μ M) were also less than that caused at a 100 fold lower concentration, albeit the amplitudes of twitches and the first e.p.p. were depressed more rapidly and markedly.

5 Tubocurarine, like cobratoxin, depressed the amplitude of miniature endplate potentials (m.e.p.ps) more than e.p.ps.

6 In contrast to the steepened run-down of successive e.p.ps in the presence of low concentrations of either nicotinic antagonists, the amplitude of m.e.p.ps observed during repetitive stimulation was uniform and was not different from that before stimulation.

7 The results suggest that the e.p.p. run-down and tetanic fade induced by nicotinic antagonists are due to a slow kinetic blockade of presynaptic receptors and confirm that the e.p.p. run-down is not produced by a use-dependent failure of postsynaptic nicotinic receptors. The roles of the presynaptic nicotinic receptor in positive or negative feedback modulations of transmitter release are discussed.

Keywords: μ -Conotoxin; tubocurarine; acetylcholine release; endplate potential; nicotinic receptor; tetanic fade

Introduction

Characteristic phenomena observed after neuromuscular blockade by competitive nicotinic antagonists are the waning to instantaneous fade of already reduced tetanic contraction and the rapid run-down of successive endplate potentials (e.p.ps) or endplate currents during repetitive stimulation (Paton & Zaimis, 1952; Blaber, 1973; Hubbard & Wilson, 1973; Glavinovic, 1979; Magleby *et al.*, 1981). The degree of e.p.p. run-down caused by tubocurarine and other competitive antagonists is somehow in parallel with the depression of the amplitude of the first e.p.p., at least in *in vitro* experiments after equilibrium incubation (Gibb & Marshall, 1984; Chang *et al.*, 1988). The extent of the tetanic fade and the train-of-four inhibition, however, differs between antagonists and between the phases of drug action, being more prominent at the phase of recovery than at the phase of ongoing of drug action (Bowman & Webb, 1976; Sugai *et al.*, 1976; Bowman *et al.*, 1986). The most typical is the group of peptides known as α -neurotoxins isolated from snake venoms which often do not cause fade of tetanus when neuromuscular transmission is blocked by high concentrations of toxins (Chang & Lee, 1963; Bowman *et al.*, 1986; Gibb & Marshall, 1986). Interestingly, however, the fade becomes more marked after washout of α -neurotoxins (Bradley *et al.*, 1987; 1990; Chang & Hong, 1987). This unique action of α -neurotoxins can be substantiated by

their effect on trains of e.p.ps, i.e., little run-down despite the depressed amplitude of the first e.p.p. at the phase of ongoing action vs marked run-down but with restored amplitude of the first e.p.p. on washout of the toxin (Chang & Hong, 1987).

The run-down of e.p.ps in the presence of a nicotinic antagonist has been attributed to a successive decline in the release of acetylcholine (ACh) (Bowman, 1980; Bowman *et al.*, 1986; Chang *et al.*, 1988) since the postsynaptic response to exogenously applied ACh remains unchanged immediately after the run-down of e.p.ps as compared to that before high frequency stimulation (Otsuka *et al.*, 1962). Moreover, tubocurarine depresses uniformly the responses to trains of ionophoretically applied ACh without causing any run-down (Gibb & Marshall, 1984). The presynaptic hypothesis suggests that the release of ACh during repetitive stimulation is automodulated by the released ACh acting through presynaptic nicotinic receptors. A blockade of this positive feedback modulation by nicotinic antagonists decreases the quantal contents of successive e.p.ps and results in run-down of e.p.ps. The differences in the degree of tetanic fade or e.p.p. run-down caused by different nicotinic receptor antagonists could be due to differences in the affinities or binding kinetics of pre- and postsynaptic receptors (*cf.* Bowman *et al.*, 1986; Chang & Hong, 1987). Recently, these findings have been explained by an agonist-antagonist cooperation at the postsynaptic site (Bradley *et al.*, 1987; 1990). It is hypothesized that when the high affinity site of the postsynaptic nicotinic receptor is selectively occupied by a low concentration of antagonist, not

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agonist, subsequent binding of the agonist to the low affinity site initiates, after activation of the receptor, a chain of events which leads to desensitization of the receptor.

These hypotheses have been tested by measuring any changes in the activity of spontaneous miniature endplate potentials (m.e.p.ps), which are reliable indicators of the sensitivity of the postsynaptic nicotinic receptor, during repetitive stimulation when antagonists had induced run-down of trains of e.p.ps. Changes of m.e.p.p. amplitudes rather than changes in the amplitude of iontophoretic ACh potentials were followed because alterations of junctional nicotinic receptors may not be equally manifest in the response to endogenous and exogenous neurotransmitters (Pennefather & Quastel, 1981).

Methods

Muscle contractions

Phrenic nerve-hemidiaphragm preparations were isolated from ICR strain mice 20–30 g. The organ bath contained 8–15 ml Tyrode (composition in mM: NaCl 137, KCl 2.8, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.33, NaHCO₃ 11.9 and glucose 11.2) maintained at 37°C and oxygenated with 95% O₂ and 5% CO₂. The phrenic nerve was stimulated with 0.03 ms rectangular pulses at the frequencies indicated. Muscle contractions were recorded via an isometric transducer (BG 25) on a physiorecorder (Gould 2200S).

Intracellular recordings

Intracellular recordings of m.e.p.ps and e.p.ps and current injections were carried out by conventional techniques with a high impedance amplifier (Dagan 8100). Microelectrodes were filled with 3 M KCl and resistances of 3–10 MΩ. The potentials were d.c.-coupled (Gould PB200) and hardcopied onto an electrostatic recorder (Gould ES2000). The recording unit has a bandwidth of 35 kHz (–3 dB). Diaphragms were immobilized with synthetic μ -conotoxin (1.2 μ M), which like the natural one (Cruz *et al.*, 1985; Hong & Chang, 1989) blocks the Na-channel of muscle preferentially against that of nerve. Undistorted e.p.ps without the complication of muscle action potential can be evoked from the normal non-depolarized resting membrane potential. M.e.p.ps can be monitored accurately even after binding of about 50% of the ACh receptors with nicotinic antagonists. The effect of μ -conotoxin on evoked quantal release was studied on the cut diaphragm muscle preparation (Barstad & Lilleheil, 1968) prepared by damaging both ends of muscle fibres (Hong & Chang, 1989).

M.e.p.ps were recorded continuously from 10 s before and for up to 10 s after 15 s trains of pulses. For the sake of accuracy of measurement only those endplates with averaged m.e.p.p. amplitude (before trains of pulses) greater than 0.3 mV (> 2 fold noise level) were selected. M.e.p.ps with amplitude greater than 2.5 fold average amplitude (giant m.e.p.ps) were discarded. Quantal contents of e.p.ps were estimated from e.p.ps and m.e.p.ps recorded in the same endplate according to the direct method (del Castillo & Katz, 1954). The amplitudes of e.p.ps in these calculations were corrected for non-linear summation (McLachlan & Martin, 1981; Chang *et al.*, 1986) with the correcting factor set at 0.56.

Statistics

In order to correlate changes of m.e.p.ps with e.p.ps during trains of stimulation, statistical analyses of data were sampled from two groups of endplates based arbitrarily on the extent of run-down of e.p.ps in trains: endplates with plateau e.p.ps (the average amplitude of the 51st to the 150th e.p.ps) maintained at greater than 75% of the first e.p.p. (see Results) were classified as those with a normal physiological run-down, while those which declined to less than 70% were classified as

significant run-down. According to this sorting criterion, the majority of endplates (19 out of 21) belonged to the physiological run-down group when the preparations were not treated with nicotinic antagonists. The degree of e.p.p. run-down is defined as $(e.p.p._f - e.p.p._p)/e.p.p._f$, where $e.p.p._f$ and $e.p.p._p$ represent amplitudes of the first and the plateau e.p.ps, respectively.

All data are expressed as means $\pm$ s.e. of recordings obtained from more than 15 endplates in at least 3 preparations. Differences between group means were analysed by Student's *t* test. *n* refers to the total number of endplates studied.

Drugs

Tubocurarine chloride and tetrodotoxin were purchased, respectively, from Sigma (St. Louis, U.S.A.) and Calbiochem (San Diego, U.S.A.). μ -Conotoxin GIIIB (a 22 amino acid protein) was synthesized by the Peptide Institute (Osaka, Japan). Cobratoxin (a 62 amino acid protein) was purified from crude venom of *Naja naja atra* to homogeneity by repeated chromatography on CM-Sephadex columns (Pharmacia, Sweden).

Results

Effect of synthetic μ -conotoxin

Like the natural geographutoxin II, synthetic μ -conotoxin, at a concentration of 1.2 μ M, changed neither the resting membrane potential (-80.7 ± 0.2 vs -83.4 ± 0.2 mV, $n \geq 65$) nor the m.e.p.p. amplitude (1.4 ± 0.1 vs 1.7 ± 0.2 mV, $n \geq 87$) or frequency of occurrence (1.8 ± 0.2 vs 1.4 ± 0.2 /s, $n \geq 87$) while providing good recordings of e.p.ps with amplitude as large as 26.3 ± 1.0 mV ($n = 20$). After μ -conotoxin, the muscle membrane exhibited little, if any, active regenerative properties when the intracellularly injected current, up to 40 nA, induced a large electrotonic membrane depolarization of 25–30 mV and this was verified by further treatment with tetrodotoxin 0.5 μ M. The amplitudes of successive e.p.ps evoked at 10–100 Hz declined to a plateau of 20.8 ± 0.9 mV ($n = 20$) within 20 pulses. The relative amplitude of e.p.ps at plateau in comparison to the first e.p.p. was not different from that obtained in cut muscle preparations, the degree of run-down being 0.22 ± 0.01 vs 0.24 ± 0.02 ($n = 29$). In cut muscle preparations, μ -conotoxin affected neither the amplitude nor the run-down of e.p.ps. The pharmacological profile and potency of synthetic μ -conotoxin on skeletal muscle are generally the same as those of natural geographutoxin II (Hong & Chang, 1989).

Effects of cobratoxin

It is known that short-term after washout of cobratoxin or erabutoxin the tetanic fade and run-down of e.p.ps were more prominent than before washout despite a significant recovery of the twitch response, peak tetanic tension and e.p.p. amplitude (Bradley *et al.*, 1987; 1990; Chang & Hong, 1987). On the other hand, similar tetanic fade and rundown of e.p.ps could be produced if the preparation was treated with very low concentrations of cobratoxin (0.0015–0.0045 μ M) for a prolonged time (3–5 h), suggesting that the process of washout of toxin is not essential for the development of fade and run-down. To save time, waning was initially induced by incubating preparations with 0.45 μ M cobratoxin for 30 min, a procedure which completely abolished e.p.ps and muscle contractions. Preparations were then washed twice with an interval of 5 min between washes. Further washing resulted in a too rapid and more complete recovery. Typical indirect twitch experiments are illustrated in Figure 1. A greater degree of waning of contractility was found after washout of cobratoxin than before washing. For intracellular recordings, μ -conotoxin at a con-

Figure 1 Effects of cobratoxin on wanings of indirect contractility of mouse phrenic nerve-hemidiaphragm preparation. The phrenic nerve was stimulated with trains of pulses (10 Hz for 7 s, left column) and 60 s later, at 100 Hz for 3 s (right column). (a) Control; (b) 12 min after treatment with cobratoxin $0.45 \mu\text{M}$. (c and d) 10 and 50 min, respectively, after washout of cobratoxin (the preparation had been incubated with cobratoxin for 30 min). Note the more marked wanings of contractility after washout of cobratoxin in spite of the restoration of the initial contractions. The paired upper and lower numerals to the right of each panel represent the force calibrations for twitches at 10 Hz and tetanus at 100 Hz, respectively. The time scales on the right top corner of each column represent 3 s.

centration of $1.2 \mu\text{M}$ was added immediately following wash. After these treatments, the response of the nicotinic receptors recovered gradually and m.e.p.ps began to appear 15–20 min after the last wash. At endplates where m.e.p.ps were greater

than 0.3 mV, repetitive nerve stimulation at 10 Hz was applied for 15 s to see the effect of stimulation on m.e.p.ps and e.p.ps. In agreement with previous reports, most of the endplates (27 out of 30) exhibited significant pharmacological run-down of e.p.ps 30–50 min after washout of cobratoxin (Figure 2 and Table 1). The degree of e.p.p. run-down increased from 0.22 ± 0.01 to 0.42 ± 0.01 . In contrast, there was no run-down of m.e.p.ps during nerve stimulation (Figure 2 and Table 2).

With time, the amplitudes of e.p.ps and m.e.p.ps grew while the degree of run-down of e.p.ps decreased. Sixty to 80 min after washout of cobratoxin, the amplitude of both m.e.p.ps and the first e.p.p. in a train of stimuli recovered to 65 ± 6 and $84 \pm 5\%$ of controls, respectively, and the population of endplates showing significant e.p.p. run-down at 10 Hz decreased to about 42% (14 out of 33 endplates). Two h after washing, less than 20% of endplates (4 out of 27) showed significant run-down of e.p.ps. Whether there was run-down of e.p.ps or not, repetitive nerve stimulation did not induce a progressive decline of m.e.p.p. amplitude.

M.e.p.p. frequency was also studied to determine whether some m.e.p.ps might be depressed and lost in the noise during repetitive nerve stimulation which might have been inadvertently excluded. Nerve stimulation at 10 Hz increased m.e.p.p. frequency by about 30% either in control or in cobratoxin treated preparations, indicating that the unchanged amplitude of m.e.p.ps during repetitive stimulation is not due to an overestimate (Table 2). It is unlikely that a use-dependent block of the postsynaptic receptor occurred. When the stimulation rate was increased to 50–100 Hz, cobratoxin still caused a run-down of e.p.ps. Under these experimental conditions, m.e.p.ps were difficult to measure because of the increased incidence of superimposition of m.e.p.ps on e.p.ps. Nevertheless, measurements indicated that m.e.p.p. amplitude was not reduced during stimulation.

The above results, together with those obtained with tubocurarine (see next heading), indicate that high concentrations of nicotinic antagonists not only depress e.p.p. amplitude rapidly but also prevent run-down of trains of e.p.ps. High concentrations of cobratoxin ($0.45 \mu\text{M}$) did not attenuate the already established run-down of trains of e.p.ps that was produced either after washout of a high concentration of cobratoxin (Figure 3) or by prolonged incubation with low concentrations ($0.0045 \mu\text{M}$) of the toxin (not shown).

Effects of tubocurarine

From the results obtained with α -neurotoxins it appears that a rapid blockade of the receptor provoked little tetanic fade or e.p.p. run-down. It is of interest to see if tubocurarine, a competitive antagonist with rapid association/dissociation kinetics, produced less waning at high than at low concentrations like cobratoxin. The preparation was pretreated with

Table 1 Effects of nicotinic antagonists on the amplitude of endplate potentials (e.p.ps) evoked by repetitive nerve stimulation

Antagonist	Treatment (μM)	(min)	The first e.p.p. ^a (mV)	E.p.ps at plateau ^b (mV)	E.p.p. run-down	n
Control			25.1 ± 0.7	$19.8 \pm 0.4^{**}$	0.22 ± 0.01	44
Cobratoxin ^c	0.45 washout	30	0	0	0	15
		0–5	0	0	0	15
		30–50	$16.9 \pm 0.5^*$	$9.9 \pm 0.4^{**}$	$0.42 \pm 0.01^*$	27
		60–80	$21.3 \pm 0.9^*$	$13.9 \pm 0.6^{**}$	$0.35 \pm 0.02^*$	14
Tubocurarine ^d	0.1–0.2	60–120	$19.1 \pm 0.6^*$	$12.0 \pm 0.8^{**}$	$0.37 \pm 0.02^*$	43

^a E.p.ps were evoked by trains of pulses (10 Hz for 15 s) at various times after washout of cobratoxin or addition of tubocurarine as indicated.

^b The amplitude of e.p.ps at plateau was the mean of e.p.ps generated during the last 10 s of stimuli. The degree of e.p.p. run-down was defined as the fractional depression of the plateau e.p.p.

^c Mouse phrenic nerve-hemidiaphragm preparations were pretreated with cobratoxin $0.45 \mu\text{M}$ for 30 min, washed twice to remove cobratoxin and finally immobilized with μ -conotoxin $1.2 \mu\text{M}$.

^d The nerve-muscle preparations were pre-immobilized with μ -conotoxin $1.2 \mu\text{M}$ for 60 min.

Control experiments followed identical procedures but without addition of cobratoxin or tubocurarine. The results from these two groups were not different and were pooled together.

n: endplates studied. * $P < 0.05$ vs control; ** $P < 0.05$ vs the first e.p.p.

Figure 2 Effects of repetitive nerve stimulation and cobratoxin on amplitudes of endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps). Mouse phrenic nerve-hemidiaphragm preparations were treated with cobratoxin $0.45 \mu\text{M}$ for 30 min, washed twice and immobilized with μ -conotoxin $1.2 \mu\text{M}$ (b, d). Control experiments (a, c) followed the same procedure but in the absence of cobratoxin. (a) and (b) show e.p.ps evoked by a train of pulses at 10 Hz. (c and d) show high gain amplification recordings of m.e.p.ps just prior to and during the last 3 s of a 15 s train of pulses. The constant downward and upward deflections indicate stimulus artifacts and evoked e.p.ps (amplitude cut off), respectively. The resting membrane potentials of the 4 different endplates were in the range of -78 to -86 mV. Note that cobratoxin induced significant run-down of e.p.ps but not of m.e.p.ps. Calibrations: 2 s and 10 mV (a, b) or 1.25 s and 5 mV (c, d).

$1.2 \mu\text{M}$ μ -conotoxin for 60 min and then with $20 \mu\text{M}$ tubocurarine, a concentration that rapidly depressed e.p.p. amplitude at a rate one thousand fold faster than cobratoxin. The degree of e.p.p. run-down on tetanic stimulation was far less prominent compared to that after washout or after treatment with a 100 fold lower concentration of tubocurarine (Figure 4 and Table 1). The results are compatible with *in vivo* experiments in which tubocurarine caused virtually no tetanic fade when administered by close-arterial bolus injection (Bowman *et al.*, 1986). In order to record e.p.ps as well as m.e.p.ps at steady states and to avoid blocking the ACh ion channel (cf. Colquhoun, 1986) lower concentrations of tubocurarine were used in the following experiments. After 60 min of equilibration with

Figure 3 Effects of a high concentration of cobratoxin on run-down of endplate potentials (e.p.ps). The mouse phrenic nerve-hemidiaphragm preparation was immobilized with μ -conotoxin $1.2 \mu\text{M}$. E.p.ps were evoked by a train of pulses at 100 Hz. (a) Control; (b) about 5 min after incubation with a high concentration of cobratoxin $0.45 \mu\text{M}$; (c) 80 min after washout of cobratoxin (the preparation had been incubated with cobratoxin $0.45 \mu\text{M}$ for 30 min); (d) the same endplate as (c) about 5 min after a further addition of a high concentration of cobratoxin $0.45 \mu\text{M}$. Note that cobratoxin depressed e.p.p. without attenuating run-down of e.p.ps (b). The resting membrane potentials at the time of recordings were -78 , -84 , -84 and -82 mV, respectively. Calibrations: 100 ms and 10 mV (a, c) or 2.5 mV (b, d).

0.1 – $0.2 \mu\text{M}$ tubocurarine, the amplitudes of sampled e.p.ps and m.e.p.ps were 76 ± 1 and $46 \pm 1\%$ ($n = 43$), respectively, of those measured before tubocurarine. Repetitive stimulation at 10 Hz induced a significant run-down of e.p.ps (0.37 ± 0.02) (Table 1). Again, the amplitude of m.e.p.ps was not suppressed by nerve stimulation (Table 2). These results were similar to those obtained with cobratoxin.

Quantal release before and during stimulation

The effect of nicotinic antagonists on evoked transmitter release was estimated (Table 3). Repetitive stimulation in the presence of antagonists caused a progressive decline in the

Table 2 Effects of repetitive nerve stimulation on the amplitude and frequency of miniature endplate potentials (m.e.p.ps).

Antagonist	Treatment ^a		Amplitude (mV) ^b		Frequency (s ⁻¹)		n
	(μM)	(min)	before stimulation	during stimulation	before stimulation	during stimulation	
Control			1.41 ± 0.05	1.34 ± 0.05	1.39 ± 0.06	1.76 ± 0.06	44
Cobratoxin	0.45	30					
	washout	30–80	$0.63 \pm 0.02^*$	$0.58 \pm 0.02^*$	1.09 ± 0.03	1.42 ± 0.06	41
Tubocurarine	0.1–0.2	60–120	$0.65 \pm 0.01^*$	$0.61 \pm 0.02^*$	1.02 ± 0.06	1.37 ± 0.06	43

^a Mouse phrenic nerve-hemidiaphragm preparations were treated with μ -conotoxin, cobratoxin or tubocurarine as described in Table 1.

^b M.e.p.ps were taken 10 s before or during the last 10 s of trains of pulses (10 Hz for 15 s).

* $P < 0.05$ vs control.

Figure 4 Effects of a high concentration of tubocurarine on run-down of endplate potentials (e.p.ps). The mouse phrenic nerve-hemidiaphragm preparation was immobilized with μ -conotoxin $1.2 \mu\text{M}$. E.p.ps were evoked by a train of pulses at 100 Hz. (a) Control; (b) 10 s after a bolus addition of $20 \mu\text{M}$ tubocurarine (the organ bath contained 8 ml Tyrode solution). (c) 200 s after washout of tubocurarine (the preparation had been incubated with tubocurarine for 25 s). (a), (b) and (c) were from the same endplate. The resting membrane potentials at the time of recordings were -86 , -86 and -78 mV, respectively. Note that tubocurarine depressed e.p.p. rapidly without causing significant run-down of e.p.ps (b). Calibrations: 300 ms and 10 mV (a, c) or 5 mV (b).

amplitude of trains of e.p.ps but had no effect on m.e.p.ps. It is evident that the quantal content of plateau e.p.ps declined. Cobratoxin and tubocurarine, on the other hand, appeared to increase the quantal content of the first e.p.p. by about 50% since both antagonists depressed the amplitude of m.e.p.ps more than e.p.ps.

Discussion

We have shown that the synthetic μ -conotoxin affected neuromuscular transmission qualitatively and quantitatively in a manner similar to natural geographutoxin II. This toxin has proven to be a useful tool for studies of neuromuscular transmission in that it does not depolarize the muscle, change the cellular ion composition or block ACh receptors (Hong & Chang, 1989).

The present results demonstrate that, in contrast to the enhanced run-down of trains by e.p.ps by nicotinic antagonists, the quantal response (m.e.p.p.) of the postsynaptic ACh receptor was not depressed further after repetitive bombardment of 'junctional' receptors with nerve-released ACh. If nicotinic antagonists do, in cooperation with nerve-released agonists, cause a use-dependent failure of the postsynaptic receptor ion channel (desensitization) as suggested by Bradley *et al.* (1990), the amplitude of the m.e.p.p. should be depressed more or less in parallel to the run-down of e.p.ps. From the present experiments, it seems evident that junctional receptors did not functionally change during the period of repetitive stimulation. The finding that further applications of high concentrations of cobratoxin did not attenuate the e.p.p. run-down once it was produced, seems to disfavor the idea that e.p.p. run-down results from selective binding of nicotinic antagonists to high affinity sites on ACh receptors with slow dissociation kinetics (Marchot *et al.*, 1988) as proposed by Bradley *et al.* (1990). Voltage clamp data also indicate that the concentration of tubocurarine used is too low to produce a significant open-channel block of the ACh receptor (Magleby *et al.*, 1981; Gibb & Marshall, 1984; Colquhoun, 1986). The unchanged function of the postsynaptic site throughout repetitive stimulation supports the hypothesis that e.p.p. run-down originates in the presynaptic site (Hubbard *et al.*, 1973; Glavinovic, 1979; Bowman, 1980; Magleby *et al.*, 1981; Wilson, 1982; Gibb & Marshall, 1984; Bowman *et al.*, 1986; Chang *et al.*, 1988; Ferry & Kelly, 1988). The relatively slow induction and dissipation of e.p.p. run-down and tetanic fade vs the rapid depression and restoration of e.p.p. amplitude and twitch tension suggest that the presynaptic receptor has a higher affinity but slower association/dissociation kinetics than the postsynaptic one.

The present findings seem to give further support to the positive feedback hypothesis that an appropriate activation of the nerve terminal nicotinic receptor enhances the transmitter release under physiological conditions, while a negative feedback modulation predominates when the concentration of ACh in the synaptic cleft piles up in the presence of anticholinesterase agents (Bowman *et al.*, 1986; Chang *et al.*, 1988). The apparent increase of the quantal content, estimated by the direct method, of either single e.p.ps or the first e.p.p. in the train by nicotinic antagonists (Wilson, 1982; Ferry & Kelly, 1988; present experiment) seems to contradict the above hypothesis. The lesser inhibition of single e.p.ps than of m.e.p.ps by nicotinic antagonists can be accounted for partially by the non-linear summation of ACh responses (McLachlan & Martin, 1981). This view is supported by the finding that tubocurarine suppressed the amplitudes of single e.p.ps and m.e.p.ps proportionately if the amplitude of e.p.ps was first reduced to less than 4 mV by lowering the Ca^{2+} concentration in Tyrode to 0.2 mM (unpublished). Moreover, in voltage clamp experiments (Harbone *et al.*, 1988) tubocurarine did not increase the quantal content of the first endplate current but reduced those of subsequent endplate currents. The view concerning the negative-feedback role of presynaptic nicotinic receptor during a low rate of nerve stimulation in the presence of intact acetylcholinesterase implies that a majority of the nicotinic receptor is tonically stimulated by the non-

Table 3 Effects of nicotinic antagonists on the estimated quantal content of trains of endplate potentials (e.p.ps)

Antagonist	Treatment ^a		Quantal contents ^b		
	(μM)	(min)	The first e.p.p.	E.p.ps at plateau	n
Control			21.3 ± 0.9	$16.9 \pm 0.7^{**}$	44
Cobratoxin	0.45	30			
	washout	30–80	$33.7 \pm 0.9^*$	$21.2 \pm 0.6^{**}$	41
Tubocurarine	0.1–0.2	60–120	$34.4 \pm 0.7^*$	$21.9 \pm 0.6^{**}$	43

^a Mouse phrenic nerve-hemidiaphragm preparations were treated with μ -conotoxin, cobratoxin or tubocurarine as described in Table 1.

^b Quantal contents of e.p.ps were estimated from data in Tables 1 and 2 by dividing e.p.p. with m.e.p.p. The amplitudes of e.p.ps were normalized to a standard resting membrane potential of -82 mV with the correcting factor set at 0.56 (see Methods).

* $P < 0.05$ vs control; ** $P < 0.05$ vs the first e.p.p.

evoked leakage of ACh from the nerve terminal which is unlikely. It is also incompatible with the observations that anticholinesterase agents, which can cause a remarkable build up of ACh in the synaptic cleft (Zemkova *et al.*, 1990), did not reduce transmitter release to the first stimulus in a train (Wilson, 1982; Ferry & Kelly, 1988). Taken together, the inference that blockades of presynaptic nicotinic receptors may enhance evoked transmitter release (Wilson, 1982; Ferry & Kelly, 1988) should be reserved.

Paradoxical to the overwhelming electrophysiological findings that trains of e.p.s or endplate currents run down within 20 pulses in the presence of nicotinic antagonists (Hubbard & Wilson, 1973; Glavinovic, 1979; Magleby *et al.*, 1981; Gibb & Marshall, 1984), the neurochemical data indicate that tubocurarine causes essentially no effect on [³H]-ACh release pooled from 100 pulses but 50% depression if 200 pulses were studied (Wessler *et al.*, 1987). If this result were correct, it would mean that the release of ACh up to the 100th pulse was unchanged whereas that from the 101st pulse on was suddenly and completely abolished. This is unlikely and is not substantiated by

any electrophysiological studies. For the collection of nerve released [³H]-ACh to be feasible hemicholinium-3 was used which, alone, has undesired pharmacological actions such as alterations of ACh synthesis and receptor response (Takagi *et al.*, 1970) and may bring about complicated drug interactions. In addition, the measurement of [³H]-ACh suffers from a low signal to background ratio, the evoked release of [³H]-ACh being only some 20–100% greater than the background leakage. In these respects, one should be circumspect in interpreting the neurochemical results. Indeed, it is difficult to reconcile that the time course of inhibition of evoked [³H]-ACh release by tubocurarine should lag behind the occurrence of e.p.s run-down (Wessler *et al.*, 1987) and that α -bungarotoxin but not cobratoxin or erabutoxin reduced evoked [³H]-ACh release (Wessler *et al.*, 1990).

We appreciated the generous gift of μ -conotoxin from Dr K.T. Wang (Taipei). The work was supported by the National Science Council (NSC80-0412-B002).

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(Received October 16, 1990)

Revised November 20, 1990

Accepted November 26, 1990)