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Reversals of the neostigmine-induced tetanic fade and endplate potential run-down with respect to the autoregulation of transmitter release

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1 In order to shed more light on the role of presynaptic cholinoceptors in the modulation of transmitter release, the effects of tubocurarine, choline and hexamethonium on neostigmine-induced tetanic fade and run-down of endplate potentials (e.p.ps) in response to indirect stimulation with trains of pulses were studied in the intact and cut isolated phrenic nerve-diaphragm preparation of the mouse, respectively.

2 Tubocurarine, choline and hexamethonium reduced both the tetanic fade and e.p.p. run-down caused by neostigmine, despite the fact that they themselves also induced these two effects.

3 At a given degree of postsynaptic inhibition, choline and hexamethonium caused less e.p.p. run-down and reversed the neostigmine-induced tetanic fade and e.p.p. run-down better than tubocurarine. Moreover, the e.p.p. run-down caused by choline or hexamethonium, but not that induced by tubocurarine, was reciprocally reversed by neostigmine.

4 Tubocurarine, choline and hexamethonium significantly decreased the endplate depolarization induced by repetitive nerve stimulation in the presence of neostigmine. The remaining depolarization continued to grow during repetitive stimulation in the presence of choline or hexamethonium, but not, however, in the presence of tubocurarine; a finding which suggests that choline and hexamethonium but not tubocurarine may be displaced from the receptor by the accumulated acetylcholine.

5 The mutual reversal by neostigmine and cholinoceptor antagonists of e.p.p. run-down may implicate the presence of a positive (physiological) and a negative (pharmacological) feedback regulation for evoked transmitter release via nicotinic cholinoceptors in the mammalian motor nerve, depending on the concentration of acetylcholine within the synaptic cleft.

Introduction

Anticholinesterase agents (anti-ChEs) inhibit tetanic contraction of mammalian skeletal muscles evoked by repetitive stimulation of the motor nerve (Hobbiger, 1976). Not only is the amplitude depressed, but in addition the initial muscle tension fades rapidly and markedly in spite of the continuing repetitive stimulation. This tetanic fade is due initially to the inability of endplate potentials (e.p.ps) to trigger muscle action potentials because of the marked cumulative depolarization of the endplate area and inactivation of the sodium channel (Chang *et al.*, 1986). The regenerative release of acetylcholine (ACh) triggered after a few pulses of nerve stimulation in the presence of neostigmine may also depolarize the endplate and contribute to the initial tetanic fade (Chang & Hong, 1986). The late phase of tetanic fade is due, in addition, to the decline of acetylcholine (ACh) release as evidenced by the marked run-down of e.p.ps (Wilson, 1982; Chang *et al.*, 1986). The run-down is probably a consequence of a negative feedback mechanism elicited by the accumulated ACh in the synaptic cleft. Thus, it might be pharmacologically possible to attenuate the anti-ChE-induced tetanic fade (1) by inhibiting the postsynaptic effect of ACh so as to antagonize the endplate depolarization, (2) by previously decreasing the release of ACh, and (3) by preventing the negative feedback inhibition of ACh release.

It has been known for a long time that the non-depolarizing neuromuscular blocking agents, exemplified by tubocurarine, also cause marked tetanic fade and e.p.p. run-down (cf. Bowman *et al.*, 1986),

although these agents act by a mechanism different from that of the anti-ChEs. It was of interest, therefore, to see how these two groups of agents would interact with each other with respect to tetanic fade and e.p.p. run-down. It was also hoped that such interactions might provide clues to the autoregulation of transmitter release and to the mechanism of e.p.p. run-down caused by competitive antagonists. In the present experiments, the interactions between neostigmine and tubocurarine, choline or hexamethonium were studied. Choline and hexamethonium were included for comparison with tubocurarine because they were once proposed to be antidotes for the intoxication from anti-ChEs (Stovener, 1956) and have much lower affinities for cholinoceptors (Ferry & Marshall, 1973; Blackman *et al.*, 1975).

Methods

Nerve-muscle preparations

Both the left and right hemidiaphragms with phrenic nerves attached were isolated from ICR mice (20–25 g) of either sex. The Tyrode solution contained (mM): NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and dextrose 11.2. It was maintained at 37 ± 0.5°C and oxygenated with 95% O_2 and 5% CO_2. The 'cut' muscle preparation for electrophysiological study was prepared by dissecting the muscle at its junction with the costal ribs and damaging the muscle-central tendon junction with forceps. This preparation was used for intracellular recording after equilibration in Tyrode solution for 2–2.5 h until the resting membrane potential declined to −50 to −40 mV and the muscle action potential failed.

Contraction experiments

The phrenic nerve-diaphragm preparation was assembled vertically in an organ bath containing 20 ml Tyrode solution; a resting tension of 0.5 g was applied. The nerve was stimulated with supramaximal pulses of 0.05 ms width either at 0.1 Hz to evoke single twitches or at 75 Hz for 3 s to evoke tetanic contractions. The interval between tetanic stimulations was 100 s. Contractions were recorded isometrically with a Gould 2200S recorder.

Intracellular recordings

The cut muscle preparation was used for recording e.p.ps using microelectrodes (5–10 MΩ) filled with 3 M KCl. The endplate was located by monitoring for e.p.ps with rise-times of less than 0.6 ms. Membrane potentials and e.p.ps were recorded with a d.c.-coupled Gould waveform recorder. The amplitude of the e.p.p. was corrected for non-linearity according to the method of Chang *et al.* (1986). The phrenic nerve was stimulated either with 5 pulse trains (pulse intervals ranging from 3.5 to 1500 ms) every 12 s or with 30 pulse trains (13 ms pulse interval) every 100 s, unless otherwise indicated. The ratios (%) of the amplitudes of the 5th e.p.p. (in the 5 pulse train stimulation) or the mean amplitude of the 25th–30th e.p.p. (in the 30 pulse train stimulation) in comparison with that of the first e.p.p. were taken as indicators of e.p.p. run-down and expressed as $e.p.p._5/e.p.p._1$ and $e.p.p._{25}/e.p.p._1$, respectively.

Drug treaatment

Stock solutions were made from either neostigmine bromide (Sigma Chemical Co., St. Louis) or neostigmine methylsulphate (Shionogi Pharmac. Co., Osaka); they were equipotent on a molar basis. (+)-Tubocurarine (Calbiochem, San Diego), choline chloride and hexamethonium chloride (Sigma) were used.

Statistics

All data are expressed as means ± s.e.mean.

Results

Reversal of tetanic fade

Tubocurarine and choline caused marked depression of the indirect single twitch response in the mouse diaphragm at concentrations higher than 1 μM and 3 mM respectively. The tetanic responses to stimulation at 75 Hz were depressed at lower concentrations and complete tetanic fade occurred after treatment with 0.5–0.7 μM tubocurarine or with 1 mM choline. The tetanic fade induced by tubocurarine could be partly reversed by neostigmine as previously demonstrated (Chang *et al.*, 1984). The tetanic fade produced by neostigmine (0.5 μM) was also effectively reversed by low concentrations of tubocurarine (0.2 μM) or choline (0.75 mM) as illustrated in Figure 1. Choline always appeared to reverse the tetanic fade better than tubocurarine. The effect of hexamethonium resembled that of choline, being about 40% more potent than choline.

E.p.p. run-down by tubocurarine, choline and neostigmine

The run-down of e.p.p. was assessed either by 5 pulse trains when studying the frequency-dependence or by 30 pulse trains when studying the concentration-

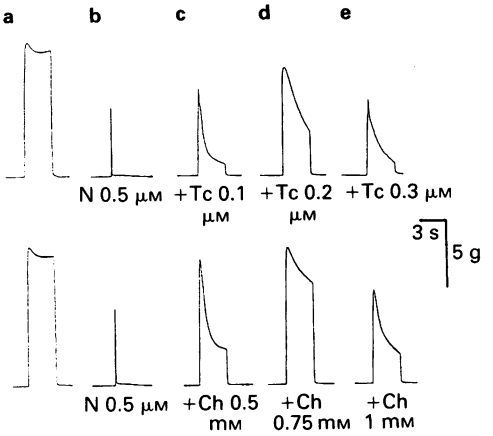

Figure 1 Antagonisms of the neostigmine-induced tetanic fade by tubocurarine and choline in the mouse diaphragm. The phrenic nerve was stimulated every 100 s with a 3 s train pulses at 75 Hz. After a control response (a) the preparation was incubated with neostigmine (N, 0.5 μM) and then further treated with tubocurarine (Tc; top panel) at 0.1, 0.2 and 0.3 μM (c, d and e), respectively, or with choline (Ch; bottom panel) at 0.5, 0.75 and 1 mM, respectively.

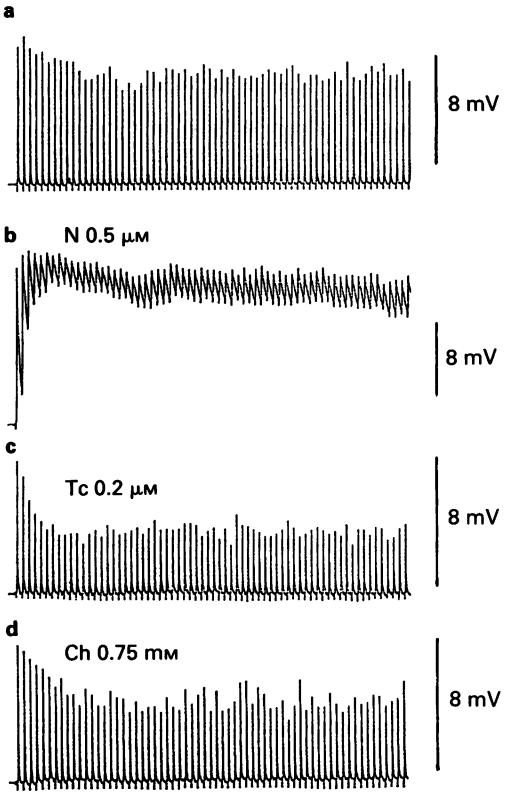

Figure 2 Effects of neostigmine (N), tubocurarine (Tc) and choline (Ch) on the e.p.p. responses in the cut mouse diaphragm. E.p.ps were elicited with 0.95 s trains of pulses at 75 Hz in the absence (a) or presence of neostigmine 0.5 μM (b), tubocurarine 0.2 μM (c) or choline 0.75 mM (d). Typical responses are shown, each from 3 to 6 different preparations.

dependence; the 30 pulse trains were given at intervals longer than 100 s in order to avoid post-tetanic potentiation. Figure 2 illustrates the typical patterns of e.p.ps evoked at 75 Hz for 0.95 s in the absence and presence of neostigmine, tubocurarine and choline. Both tubocurarine and choline simultaneously depressed the amplitude and enhanced the run-down of e.p.ps; in this respect, choline was about 1000 times less potent than tubocurarine. Unlike tubocurarine and choline, neostigmine increased and prolonged e.p.ps evoked by single stimuli and produced a cumulative and sustained depolarization upon which e.p.ps of decreased amplitude were superimposed (Figure 2). The run-down of e.p.ps at 75 Hz, in terms of $e.p.p._{25}/e.p.p._1$, in the presence of various concentrations of tubocurarine or choline correlated with the depression of the first e.p.p. (Figure 3). The latter depression could be regarded as an inhibitory effect on the postsynaptic cholinoceptor. It appeared that choline caused less e.p.p. run-down for a given degree of inhibition of e.p.p. amplitude than tubocurarine. Figures 4 and 5 show comparisons of the dependency of e.p.p. run-down ($e.p.p._5/e.p.p._1$) on the intervals of individual pulses in trains of stimulation (frequency of stimulation) for tubocurarine, choline and neostigmine. While the e.p.p. run-downs caused by tubocurarine and choline were not frequency-dependent at pulse intervals between 3.3 to 500 ms, the run-down caused by neostigmine was prominent only when the pulse interval was 20 ms or less and was very much enhanced as the pulse interval was shortened (Figure 5). The effects of hexamethonium on a single e.p.p. and trains of e.p.ps were again similar to those of choline.

Interaction with neostigmine-induced e.p.p. run-down

The typical patterns of e.p.p. evoked at 75 Hz after combined treatment with neostigmine plus tubocurarine, choline or hexamethonium are illustrated in Figure 6 and the e.p.p. run-downs at various pulse intervals are summarized for tubocurarine vs neostigmine and for choline vs neostigmine in Figure 5. Both choline (3 mM) and hexamethonium (0.6 mM) were more effective than tubocurarine (0.5 μM) in reducing the e.p.p. run-down caused by neostigmine at short pulse intervals (Figures 5 and 6). In separate experiments, the e.p.p. run-down caused by choline (Figure 5b) or hexamethonium (data not shown)

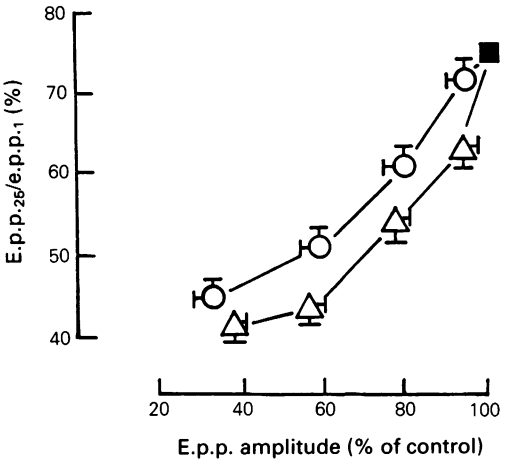

Figure 3 Effects of tubocurarine and choline on the e.p.p. run-down as a function of the depression of e.p.p. amplitude in the cut mouse diaphragm. E.p.ps were elicited at 75 Hz in the absence (■) and presence of either tubocurarine (△, 0.1–1 μM) or choline (○, 0.25–3 mM). Abscissa scale: depression of the amplitude of the first e.p.p. in the train expressed as % of control. Ordinate scale: run-down of e.p.p. in the train expressed as e.p.p.$_{25}$/e.p.p.$_1$ (%). Means ± s.e.mean are shown; $n = 4$–7.

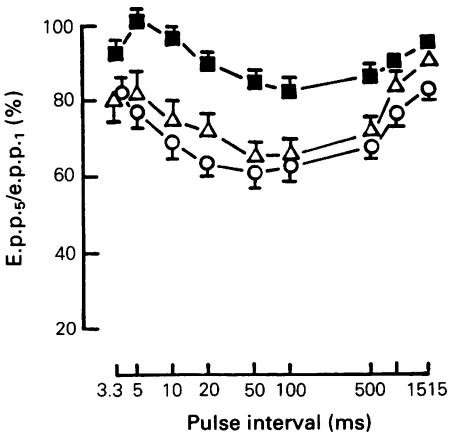

Figure 4 Effects of tubocurarine and choline on the e.p.p. run-down at various stimulation frequencies in the cut mouse diaphragm. The phrenic nerve was stimulated with a 5 pulse train every 12 s at various pulse intervals (abscissa scale) in the absence (■) and presence of tubocurarine 1 μM (△) or choline 3 mM (○). The run-down of e.p.p. is expressed as e.p.p.$_5$/e.p.p.$_1$ (%). Means are shown with vertical lines indicating s.e.mean; $n = 4$–8.

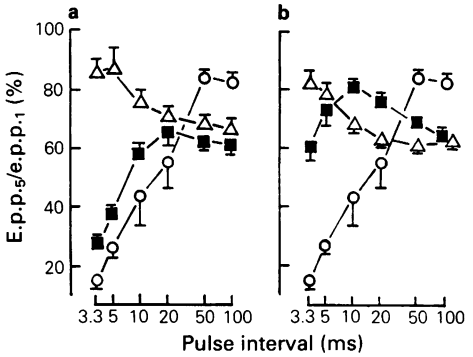

Figure 5 Interactions of tubocurarine or choline with neostigmine on the e.p.p. run-down at various stimulation frequencies in the cut mouse diaphragm. The phrenic nerve was stimulated with a 5 pulse train every 12 s at various pulse intervals (abscissa scales) in the presence of: (a) (○) neostigmine 0.3 μM; (△) tubocurarine 0.5 μM; (■) neostigmine plus tubocurarine. (b) (○) neostigmine 0.3 μM; (△) choline 3 mM; (■) neostigmine plus choline. The run-down of e.p.p. is expressed as e.p.p.$_5$/e.p.p.$_1$. Means are shown with vertical lines indicating s.e.mean; $n = 4$. Control (no drug treatment) was the same as in Figure 4.

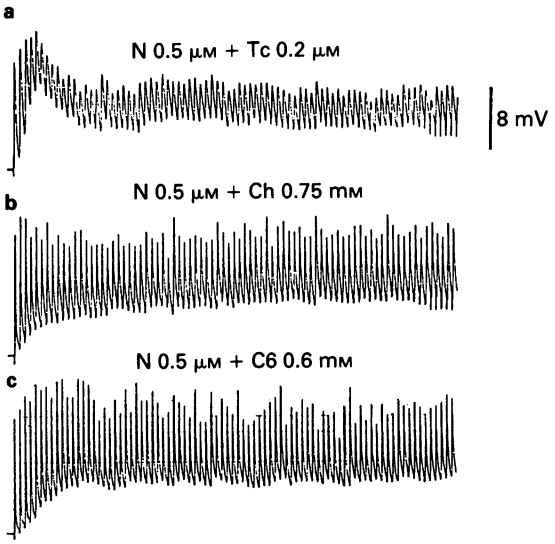

Figure 6 Effects of tubocurarine (Tc), choline (Ch) and hexamethonium (C6) on the cumulative depolarization of endplate during repetitive stimulation in the cut mouse diaphragm treated with neostigmine. The phrenic nerve was stimulated at 75 Hz in the presence of neostigmine 0.5 μM plus tubocurarine 0.2 μM (a), choline 0.75 mM (b) or hexamethonium 0.6 mM (c). Shown are typical responses, each from 3 to 6 different preparations. For the neostigmine control see Figure 2b.

alone, but not that caused by tubocurarine (Figure 5a), was reversed by neostigmine when the pulse intervals were between 10–50 ms. Interestingly, at pulse intervals of 10 and 20 ms, there was a mutual reversal of e.p.p. run-down between neostigmine and choline and between neostigmine and hexamethonium so that the e.p.p. run-down after combined use of drugs was less than that caused by any agent alone. When tested at 75 Hz (13 ms pulse interval), the e.p.p. run-down was reciprocally reversed by the combined use of neostigmine (0.5 μM) and 0.5–2 mM choline (Figure 7b) or 0.3–1.2 mM hexamethonium (data not shown). In contrast, although tubocurarine (0.2 μM) slightly reduced the neostigmine-induced e.p.p. run-down, the run-down caused by higher concentrations (0.4–0.8 μM) of tubocurarine was not attenuated by neostigmine (Figure 7a). The quantal contents of the first e.p.p., calculated by the method of variance (delCastillo & Katz, 1954), were not significantly affected by tubocurarine, choline or hexamethonium in the presence and absence of neostigmine.

Depression of the neostigmine-induced depolarization

Repetitive stimulation at 20 Hz or higher frequencies in the presence of neostigmine resulted in a cumulative depolarization of the endplate, the magnitude of which was dependent on the stimulation frequency, presumably because the residual ACh could not be dissipated before the next e.p.p. (Wilson, 1982; Chang *et al.*, 1986). Tubocurarine, choline and hexamethonium significantly attenuated this endplate depolarization evoked by repetitive stimulation (Figure 6 vs Figure 2b). There was, however, a substantial difference. The remaining depolarization still accumulated for a few pulses and thereafter subsided rapidly on continuing stimulation in the presence of tubocurarine, whereas the depolarization continued to build up during repetitive stimulation in the presence of choline, even though the initial rate of depolarization was more markedly depressed than by tubocurarine (Figure 6). The effect of hexamethonium on the endplate depolarization appeared to be similar to that of choline.

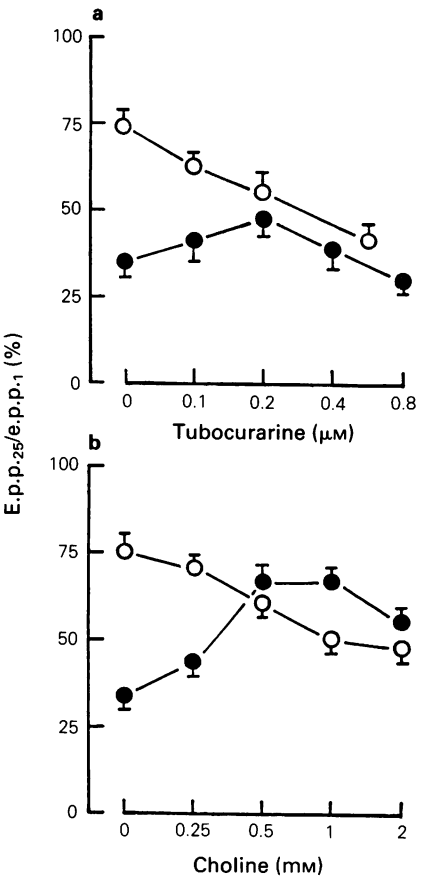

Figure 7 Interactions of various concentrations of tubocurarine or choline with neostigmine on the e.p.p. run-down in the cut mouse diaphragm. The phrenic nerve was stimulated with a 30 pulse train at 75 Hz in the presence of various concentrations of tubocurarine (a) or choline (b) with (●) or without (○) neostigmine 0.5 μM. The run-down of e.p.p. was expressed as $e.p.p._{.25}/e.p.p._{.1}$. Means are shown with vertical lines indicating s.e.mean; $n = 4$–6. The left most pairs in (a) and (b) denote the controls without tubocurarine or choline.

Discussion

It is generally believed that the rapid run-down of e.p.ps upon repetitive stimulation in the presence of tubocurarine is not due to a change in sensitivity of the postsynaptic site to ACh during nerve stimulation (Otsuka *et al.*, 1962; Magleby *et al.*, 1981). It is also not due simply to an unmasking of an inherent tendency for e.p.ps to run-down as a result of a reduction in the e.p.p. amplitude (cf. Ginsborg & Jenkinson, 1976) in view of the following considerations. First, choline induced less e.p.p. run-down than tubocurarine for a given inhibition of e.p.p. amplitude; second, the e.p.p. run-down could be dissociated from the postsynaptic depression of e.p.p. amplitude when α-neurotoxins from snake venoms were used (Chang & Hong, 1987). It is likely that the e.p.p. run-down caused by tubocurarine and similar agents has a presynaptic origin and is induced by specific binding with the nicotinic cholinoceptor (Chang & Hong, 1987; Gibb & Marshall, 1987). In agreement with Bowman *et al.* (1986) and Wessler *et al.* (1986), it may then be speculated that, under

normal physiological conditions, ACh has a positive feedback effect on the nerve terminal via nicotinic cholinoceptors to maintain the release of ACh during repetitive pulses. Blocking this positive influence should result in a more rapid run-down of e.p.p..

In contrast to nicotinic cholinoceptor antagonists, neostigmine and other anti-ChE agents cause an accumulation of ACh in the synaptic cleft during repetitive stimulation of the nerve. In this pharmacological situation, the high concentration of ACh accumulated would reduce the evoked transmitter release (Wilson, 1982; Chang *et al.*, 1986), presumably because of an excessive depolarization and this would result in a negative feedback. At one extreme of the pharmacological situation, a regenerative release of ACh may be triggered when ACh is accumulated to a threshold level by repetitive pulses in the presence of neostigmine (Chang & Hong, 1986).

Reversal by tubocurarine and other cholinoceptor antagonists of the neostigmine-induced presynaptic effects supports the concept of regulation by a cholinoceptor on the nerve terminal. In particular, the reciprocal reversal of the e.p.p. run-down resulting from the interaction between neostigmine and either choline or hexamethonium, which are much less potent 'antagonists' than tubocurarine, is of great interest. It is possible that this mutual attenuation of e.p.p. run-down between neostigmine and choline or hexamethonium could be related to the low affinity of the latter two compounds for the cholinoceptor (Ferry & Marshall, 1973). The high concentration of ACh accumulated in the synaptic cleft after the onset of repetitive stimulation might partly displace the weak antagonists from the presynaptic receptor and enhance transmitter mobilization (positive feedback) for evoked release, as in the physiological situation, and this process would result in reciprocal antagonism. Though reduced, the slowly progressive depolarization of endplate during repetitive stimulation in the combined presence of neostigmine and choline is in contrast to the rapid attainment to plateau and subsequent repolarization in the combined presence of neostigmine and tubocurarine. This may be due to a displacement of choline by the accumulated ACh at postsynaptic cholinoceptors and is analogous to the decurarizing effect of hexamethonium shown by Ferry & Marshall (1973) and Blackman *et al.* (1975). In addition to its competitive receptor blocking action, tubocurarine may produce open ion channel block, which increases with increasing agonist concentration (Colquhoun, 1980), and its interference with receptor function might be enhanced during repetitive stimulation. The more potent reversal of neostigmine-induced tetanic fade by either choline or hexamethonium than by tubocurarine parallelled the greater antagonism of the e.p.p. run-down. In this respect, choline may have a greater capacity as an antidote to the lethal toxicity of anti-ChEs. With regard to the neostigmine-induced tetanic fade, the antagonistic effects of high concentrations of Mg or hemicholinium-3, both of which decrease ACh release, was found not to be better than those of the above cholinoceptor antagonists (unpublished observations).

Finally, it may be proposed that the concentration of ACh released from the nerve under physiological conditions may facilitate its own release, presumably by an increase of transmitter mobilization, whereas higher concentrations of ACh accumulated in the cleft during exogenous application (Ciani & Edwards, 1963) or during repetitive pulses in the presence of an anti-ChE, may inhibit the evoked transmitter release, presumably by depolarization of the nerve terminal. Similar concepts have been postulated by Bowman *et al.* (1987) and Chang & Hong (1987). The paradoxical increase of ACh release found for tubocurarine and α-bungarotoxin in direct assay studies (Miledi *et al.*, 1978; Bierkamper *et al.*, 1986), which inevitably need repetitive pulses for a prolonged period and the presence of an anti-ChE, may be accounted for by the restoration from e.p.p. run-down induced by the anti-ChE used and should not be interpreted as an enhancement of transmitter release by tubocurarine or α-bungarotoxin. Indeed, Wessler *et al.* (1986) observed an inhibition of [^{3}H]-ACh release by tubocurarine when anti-ChE was not used. In this respect, direct assay of transmitter release in the presence of anti-ChEs could be misleading because anti-ChEs could have decreased the transmitter release on repetitive stimulation and this effect may vary greatly depending on the concentrations of anti-ChEs used (Chang *et al.*, 1987).

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