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Mechanisms of the inhibition by neostigmine of tetanic contraction in the mouse diaphragm

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1 Neostigmine (0.5–2 μM) caused fade of tetanic contractions (Wedensky inhibition) evoked by repetitive nerve stimulation. The mechanism underlying this action was studied in intact and cut isolated phrenic nerve-diaphragm preparations of mice.

2 The fade was brought about by failure to elicit muscle action potentials. During fade, the muscle was unable to conduct directly evoked action potentials across the central endplate zone. Recovery of excitability occurred in 5 s with continued stimulation.

3 In the presence of neostigmine, the resting membrane potential at endplate areas during repetitive stimulation decreased from − 80 mV to less than − 50 mV within the first 10 pulses at 75–200 Hz and thereafter recovered gradually to about − 60 mV in the following 5 s during continuous stimulation.

4 The quantal content of endplate potentials evoked by single stimulation was not reduced by neostigmine whereas that evoked by high frequency stimuli (75 Hz) was reduced to about 1/3 in 10 pulses.

5 It is concluded that the fade of tetanic contraction caused by inhibition of acetylcholinesterase is induced by the inactivation of sodium channels in the area surrounding the endplates and that the sustained fade is due to a decrease of transmitter release. Both effects are the result of acetylcholine accumulation.

Introduction

Inhibition of acetylcholinesterase (AChE) in the normal neuromuscular preparation with anticholinesterase agents, including the neostigmine-like carbamates and organophosphorus compounds, is known to enhance twitch responses to single stimuli, but on the contrary, to cause a rapid fade (Wedensky inhibition) of the tetanic contraction evoked by repetitive stimulation of the nerve (Morrison, 1977; Heffron & Hobbiger, 1979; Clark & Hobbiger, 1983; Clark *et al.*, 1983; 1984). Inhibition of butyrylcholinesterase, however, has no effect on neuromuscular transmission (Heffron, 1972). The enhancement of the single twitch has been shown to be due to repetitive firing of the muscle after single pulses because of prolongation of the endplate potentials (e.p.ps) and/or to repetitive firing of the nerve (Clark *et al.*, 1984). The fade of tetanic contraction during high frequency stimulation is generally attributed to a depolarization-induced blockade of the skeletal muscle due to an accumulation of acetylcholine in the synaptic cleft (see Bowman & Rand, 1980). Yet, how much depolarization can occur and how the depolarization might result in the fade of tetanic contraction has not been established. Akasu & Karczmar (1980) proposed that the tetanic fade in the presence of anticholinesterases is due to desensitization of the postsynaptic receptor while Wilson (1982) proposed that it is due to a negative feedback inhibition of transmitter release caused by accumulation of acetylcholine.

The present study showed that the membrane potential of the mouse diaphragm at endplate areas was rapidly depolarized beyond − 50 mV within the first 10 stimuli during train pulses at 50–200 Hz. This depolarization can account for the failure of muscle action potential generation during the early phase of tetanic fade. Subsequently a marked reduction of the transmitter release contributes to the continued failure during repetitive stimulation.

Methods

Hemidiaphragms of both sides with the phrenic nerve attached were isolated from 20–25 g mice (ICR) of either sex.

Isometric contractions

The organ bath contained 20 ml Tyrode solution (composition mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and glucose 11.2) maintained at 37 ± 0.5°C and oxygenated with 95% O_2 and 5% CO_2. The resting tension was 0.5 g. The nerve was stimulated with supramaximal pulses of 0.05 ms width at 0.1 Hz or every 100 s by 5 s trains of pulses at 25–200 Hz. Contractions were recorded isometrically with a Grass force-displacement transducer (FT. 03C). For direct field stimulation of hemidiaphragms, pulses of 0.5 ms width were delivered to the isolated bipolar electrodes, which were attached either to the costal head or to the tendon end of muscle fibres to avoid exciting the phrenic nerve while still resulting in maximal twitch tensions.

Electrophysiological recordings

The intact isolated mouse diaphragm and the cut muscle preparation (Barstard & Lilleheil, 1968) were used. In the latter, the muscle was usually depolarized to −45 mV. Intracellular recordings were performed with microelectrodes (5–10 MΩ) filled with 3 M KCl according to Fatt & Katz (1951). In some experiments, the intact muscle was immobilized by incubating the muscle with 1.8 M formamide (del Castillo & Escolona de Motta, 1978) for 5–20 min. The gross muscle action potential was also monitored by extracellular recordings with a cotton-wick electrode.

Action potentials and e.p.ps were dislayed on an oscilloscope and photographed, or directly recorded on paper with an electrostatic recorder (Gould ES 1000) or a Gould waveform recorder.

Quantal content of endplate potentials

The transmitter release in terms of quantal content was calculated according to the method of variance (del Castillo & Katz, 1954) from 30–50 e.p.ps evoked at 0.66 or 1 Hz or from e.p.ps obtained at 2.5–5 s after the initiation of high frequency stimulation. The amplitude of e.p.ps was corrected for nonlinear summation due to the change of membrane potential during repetitive stimulation, assuming 0 mV for reversal potential (Linder & Quastel, 1978), by the following equation:

$$\text{e.p.p.}_c = \text{e.p.p.}_o \times \frac{\text{m.p.}_c}{\text{m.p.}_o - 0.54 \times \text{e.p.p}_o}$$

Where e.p.p.$_c$, e.p.p.$_o$, m.p.$_c$ and m.p.$_o$ are e.p.p. corrected, e.p.p. observed, membrane potential corrected (−45 mV) and membrane potential observed, respectively. By computer simulation on the basis that the quantal size is proportional to the takeoff membrane potential (Linder & Quastel, 1978), we found that the inclusion of the factor 0.54 into the equation given by Martin (1955) gave a more accurate correction, provided that the e.p.p. does not exceed one third the membrane potential.

Data shown in the text and table are mean ± s.e.

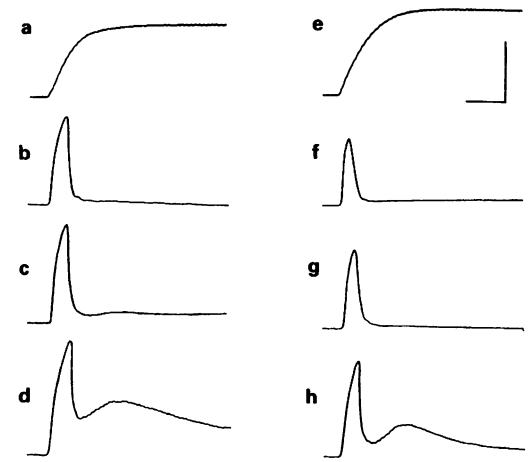

Figure 1 Effect of neostigmine on the tetanic contraction of the mouse diaphragm. The phrenic nerve was stimulated with 5 s-trains of pulses at 100 Hz every 100 s in normal Tyrode solution (left column) or in 3.6 mM $CaCl_2$ Tyrode solution (right column). (a) and (e) Control; (b) and (f) 20; (c) and (g) 60; (d) and (h) 120 min after treatment with neostigmine 0.5 μM. Calibration: 100 ms for (a)–(h); 2.5 g for (a), (e) and 1.0 g for (b)–(d) and (f)–(h). Notice that the fade was more marked in 3.6 mM $CaCl_2$ Tyrode solution.

Results

Effects on tetanic contraction

After treatment with 0.5 to 2.0 μM neostigmine which inhibits AChE by 70–90% (Chang *et al.*, 1985), the tetanic response of the mouse diaphragm to 5 s trains of repetitive stimulation at 100 Hz every 100 s was affected as shown in Figure 1. Not only was the amplitude depressed but also the tetanus faded completely in about 50 ms in spite of the continued stimulation at 100 Hz. The tetanic fade was effectively antagonized, though not completely, by (+)-tubocurarine (0.2–0.3 μM) and choline (0.03–1.0 mM) as previously reported (Stovener, 1956). The degree of fade of tetanic contraction at 50 Hz was less, but at

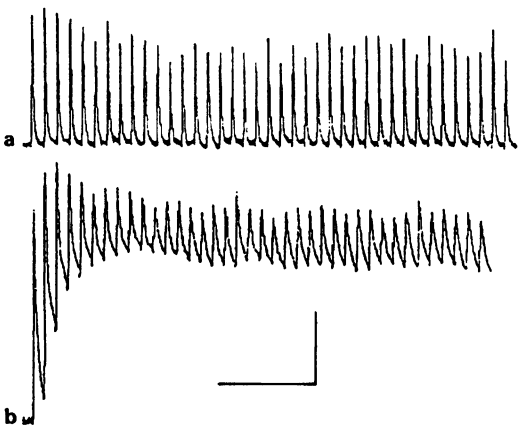

Figure 2 Membrane potential and endplate potentials of the cut mouse diaphragm during repetitive stimulation. Indirect stimulation at 75 Hz in the absence (a) and presence of neostigmine 2 μM (b). The prestimulus resting potentials were − 43 mV. Calibration: 100 ms and 4 mV.

150–200 Hz was more marked than at 100 Hz stimulation.

Change of membrane potential during repetitive stimulation

Neostigmine (2 μM) did not cause significant change of resting membrane potential at either endplate or extra-endplate areas if the nerve was not stimulated. However, during repetitive stimulation at frequencies higher than 50 Hz the membrane potential at the endplate area did not return to resting levels because of the prolongation of e.p.ps as illustrated in Figure 2.

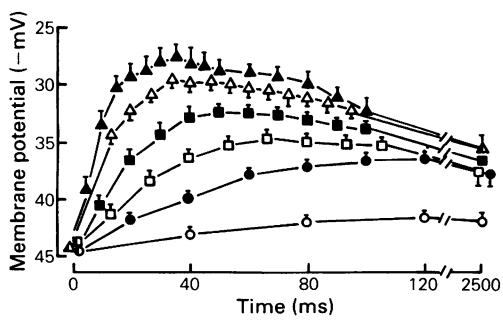

Figure 3 Effect of the stimulus frequency on the depolarization of endplate membrane in the cut muscle. The phrenic nerve was stimulated at 200 Hz (▲), 150 Hz (△), 100 Hz (■), 75 Hz (□), 50 Hz (●) and 25 Hz (○) in the presence of neostigmine (0.5 μM). Abscissa scale: time after the initiation of repetitive stimulations. Mean values of $n = 3-4$; s.e. shown by vertical lines.

Figure 3 compares the progressive depolarization of the cut muscle at various frequencies of stimulations. Maximum depolarization was attained at about the 7th to 10th pulse and the depolarization was dependent on the frequency of stimulation. A partial restoration of membrane potential occurred thereafter in the next 5 s in spite of continued stimulation.

Membrane potential was also measured in the intact muscle at rest and 2.5–5 s after the onset of stimulation at 75 Hz when the tetanic contraction had faded completely so that the insertion of a microelectrode was possible. The membrane potential decreased from -82 ± 1 mV (prior to stimulation) to -60 ± 2 mV at 2.5–5 s after the onset of stimulation. Assuming a proportional change of membrane potential occurred between the cut and intact muscles during repetitive stimulation, it is estimated that the endplate area in the intact muscle could be depolarized to about − 40 mV during the early part of the train. Direct measurement in the intact muscle immobilized with formamide revealed that the membrane potential was reduced from -76 ± 2 mV to -45 ± 2 mV ($n = 14$) at the 7th pulse at 75 Hz and then gradually repolarized to 56 ± 3 mV in 2 s.

Excitability of the muscle during tetanic fade

In preparations treated with neostigmine (1–2 μM), it was found that only the first few stimuli elicited action potentials. Whether this is due to the marked depolarization and subsequently to the inactivation of sodium channel was tested. Intact muscles treated with neostigmine (2 μM) were stimulated directly at one end of the muscle and the muscle action potential was monitored at both sides of the endplate area by means of extracellular electrodes. During the early part (<2 s) of tetanic fade at 75 Hz, the muscle action potential could be recorded only at the side where the direct simulus was applied. Afterwards muscle action potentials gradually recovered on the side opposite the muscle stimulation electrodes. In another series of experiments the contractions elicited by direct stimulation at 2 Hz at one end of the muscle were recorded in the presence of 2 μM neostigmine. When repetitive stimulation (75 Hz) of the nerve was superimposed, the direct twitch amplitude was immediately reduced to about one third and thereafter gradually recovered its original level in 5 s (Figure 4). On the other hand, no reduction of the twitch contraction to direct stimulation was observed immediately after the cessation of tetanic contraction elicited by direct stimulation at 75 Hz. The above results together indicate that the conduction of the muscle action potential from one end of the muscle to the other end was retarded at the centrally located endplate area during the early stage of repetitive nerve impulses in the presence of neostigmine.

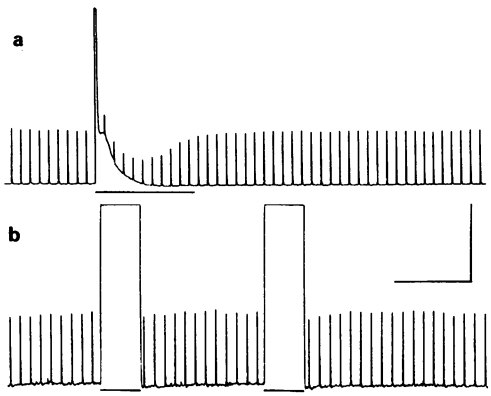

Figure 4 Effect of repetitive stimulation on the contractions evoked by single direct stimulation in the presence of neostigmine. The intact mouse diaphragm was treated with neostigmine 2 μM for 20 min and stimulated directly at 2 Hz. Stimulation of the nerve for 5 s (a) or the muscle for 2 s (b) at 75 Hz were superimposed as indicated by the horizontal bars. Note that the tetanic contractions were out of scale because of electronic cut off. Calibration: 4 s and 0.5 g.

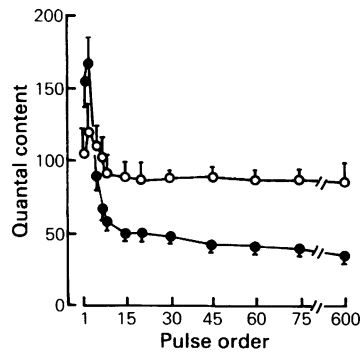

Figure 5 Effect of neostigmine on the quantal content of e.p.ps of the cut mouse diaphragm during repetitive stimulation. The phrenic nerve was stimulated at 75 Hz in the absence (○) and presence of neostigmine 2 μM (●). Abscissa scale: pulse order of e.p.ps in the train of stimulations. Mean of $n = 9-15$; s.e. shown by vertical lines.

Transmitter release during repetitive stimulation

Neostigmine (2 μM) did not cause a significant change of transmitter release when the nerves were stimulated at a low frequency (0.66 Hz) (Table 1). However, when measured after the fade of tetanic contraction in the intact muscle (2.5–5 s after the onset of stimulation at 75 Hz), the quantal content was significantly reduced (Table 1). The time-course of the change of quantal release during repetitive stimulation by neostigmine in cut muscles is illustrated in Figure 5. It is evident that the quantal content could not be maintained and declined rapidly in 150 ms to less than 50% of that of the first pulse and remained at this low level for the period of observation (3–8 s). Since the quantal size of e.p.ps is computed during trains at 2.5–5 s after starting the repetitive stimulation, an accurate quantitative estimation of quantal contents may not be reached. Nevertheless, this result confirms that of Wilson (1982) qualitatively.

Effect of carbamylcholine on the quantal release

Carbamylcholine at 100 μM depolarized the resting endplate to − 54 mV, and the quantal release evoked

Table 1 Effect of neostigmine on quantal content of endplate potentials in the mouse diaphragm

Treatment	*Resting membrane potential* (− mV)	*Quantal content* 0.66–1 Hz	75 Hz[a]
Intact muscle			
(+)-Tubocurarine (2 μM)	81.8 ± 0.4	73 ± 5 (29)[b]	46 ± 4 (14)
Neostigmine (2 μM)	82.8 ± 1.0	80 ± 8 (20)[c]	26 ± 3* (26)
Carbamylcholine (100 μM)	53.9 ± 0.8*	39 ± 4* (44)	—
Cut muscle			
Control	38–45	87 ± 5 (16)	85 ± 13 (9)
Neostigmine (1 μM)	38–45	104 ± 7 (16)	35 ± 4* (15)

[a] Measured from the e.p.ps 2–5 s after the initiation of stimulation.
[b] Number of endplates from 3–6 preparations.
[c] Measured in the presence of (+)-tubocurarine.
*$P < 0.01$ vs. (+)-tubocurarine or control (Student's t test).

at 1 Hz was only 39 ± 4 whereas the quantal content of the (+)-tubocurarine-treated control was 73 ± 5. Bierkamper & Aizenman (1984) made a similar observation. It is clear that carbamylcholine decreases the quantal content directly at a concentration which produced endplate depolarization.

Discussion

The weakness of muscle and respiratory distress induced by anticholinesterase agents are evidently due to the anticholinesterase-induced fade of tetanic contraction. There seems no doubt that this inhibition is due to effect(s) subsequent to an accumulation of acetylcholine during repetitive nerve impulses. Antagonism of the fade by the competitive antagonist (+)-tubocurarine and the weak partial agonist, choline, also support this view. However, in multiply-innervated muscles, no fade occurs during high frequency stimulation and the tetanic contraction outlasts the period of stimulation (Chang *et al.*, 1985).

What is the consequence of the acetylcholine accumulation in the synaptic cleft? The time-course of e.p.ps was prolonged markedly (Katz & Miledi, 1973; Hartzell *et al.*, 1975; Magleby & Terrar, 1975), so that during repetitive stimulation the membrane potential could not recover before the next pulse. This resulted in a progressive depolarization of the endplate area, with the shorter the interval between pulses the more marked the depolarization. In the presence of neostigmine it was found that with nerve stimulation at 75–100 Hz the membrane potential at endplate regions was reduced by more than 30 mV within 150 ms to a potential at which marked inactivation of the sodium channel should have occurred (Adrian & Marshall, 1977). Direct stimulation of the muscle also revealed that the excitation could not be conducted across the endplate area. The membrane potential thereafter gradually recovered to about −65 mV in the following 5 s and restoration of excitability occurred with the same time course. From these results, it may be concluded that, at the early stage of repetitive stimulation, fade of tetanic contraction is due to sodium channel inactivation as a result of the depolarization caused by accumulation of acetylcholine. Depolarization, however, is unlikely to be the cause for the continued fade during prolonged repetitive stimulation. It is likely that the reduced transmitter release and the reduced membrane potential act together to maintain the inhibition of tetanic contraction. Desensitization of the acetylcholine receptor during repetitive stimulation (Akasu & Karczmar, 1980) is probably not an important factor (Wilson, 1982). The restoration of membrane potential at the late stage of repetitive stimulation may be due to the decreased release of transmitter.

The rapid decline of transmitter release during high frequency stimulation might be due to a decrease of the immediately available store or to the reduced mobilization of the transmitter. However, in view of the inhibition of quantal release by carbamylcholine at a concentration that produced endplate depolarization comparable to that produced during repetitive stimulation in the presence of neostigmine, it seems more likely that the accumulated acetylcholine in the synaptic cleft inhibits the transmitter release from the nerve terminal by a negative feedback mechanism.

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