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THE GENERATION OF NERVE AND MUSCLE REPETITIVE ACTIVITY IN THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION FOLLOWING INHIBITION OF CHOLINESTERASE BY ECOTHIOPATE

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1 Simultaneous extracellular recordings were made from two end-plate zones of the isolated diaphragm and from the phrenic nerve of the rat in response to stimulation of the nerve. The contractions of the diaphragm were also recorded.

2 In the curarized diaphragm, the introduction of ecothiopate, a non-competitive inhibitor of cholinesterase, caused a threefold increase in the amplitude of the end-plate current and an eightfold increase in the duration at half the peak amplitude.

3 In the non-curarized diaphragm, the introduction of ecothiopate caused the generation of repetitive activity (RA) in first the phrenic nerve: this was then followed by RA in the diaphragm. At that stage, nerve RA possessed a shorter latency than muscle RA. The generation time for nerve RA was 1.6 ms and for mRA, it was 2.7 milliseconds.

4 Nerve RA was more labile than muscle RA; it was readily abolished by increasing the frequency of stimulation, by magnesium, by tubocurarine or by high concentrations of ecothiopate, whereas muscle RA was still generated. Steady exposure to acetylcholine abolished both forms of RA.

5 Two competitive inhibitors of cholinesterase, neostigmine and ambenonium, were also shown to evoke RA in nerve and muscle. The generation times for nerve RA and muscle RA were similar to those following ecothiopate.

6 It was concluded that nerve RA and muscle RA were generated after the inhibition of cholinesterase by ecothiopate as a result of the prolonged action of acetylcholine upon cholinoceptive sites on the nerve terminal and motor endplate respectively. A direct excitatory action of ecothiopate upon the phrenic nerve terminals was excluded.

Introduction

After physostigmine, stimulation of the motor nerve evokes repetitive activity in the skeletal muscle of cat (Brown, Dale & Feldberg, 1936). Masland & Wigton (1940) showed that, after neostigmine, antidromic activity also occurs in the ventral roots of the spinal cord. They concluded that, after inhibition of cholinesterase (ChE), the prolonged action of acetylcholine (ACh) upon cholinoceptive sites on the motor nerve terminal and motor endplate generated nerve repetitive activity (nRA) and muscle repetitive activity (mRA), respectively. In contrast, Eccles, Katz & Kuffler (1942) and Lloyd (1942) proposed that mRA was generated by the prolonged action of ACh at the motor endplate and this activity was conducted retrogradely to evoke nRA in the nerve terminal. A third proposal is that nRA is generated as a result of the direct action of the ChE inhibitor upon the nerve terminal. The generation of nRA, in turn leads to further ACh release from the nerve terminal and so gives rise to mRA (Riker & Okamoto, 1969; Riker, 1975). Inhibition of ChE was not considered to be an essential step in the generation of RA (Blaber, 1972).

The intention of the present study was to determine which of these proposals is correct. A preliminary account of this work has been presented (Morrison, 1976).

Methods

The methods used in this study are described more fully by Ferry & Marshall (1973). Hemi-diaphragms from albino rats, 150–250 g in weight, were

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superfused at 3 ml/min *in vitro* with physiological saline (composition (mM): NaCl 137, KCl 5, $NaHCO_3$ 12, NaH_2PO_4 1.0, $MgCl_2$ 0.1–1.0, $CaCl_2$ 2.0 and glucose 25) maintained at 37°C and gassed with 5% CO_2 in O_2. To facilitate a rapid change, the dead space in the apparatus was flushed through with fresh solution: then rapid flow over the preparation was allowed for a short period. Elapsed time was measured from the introduction of the new solution.

Simultaneous extracellular recordings were made in response to supramaximal stimulation of the phrenic nerve by 2 V pulses, 50 μs duration and frequency 0.1 hertz. Focal extracellular recordings were made from two end-plate zones (M1 and M2). The electrodes consisted of insulated silver wire, 125 μm in diameter, cut cleanly at right angles with a razor blade and supported in glass micropipettes. The recording system had a bandwidth of 10 kHz–0.8 Hz (–3 db). To locate an end-plate zone, the electrode was placed over a nerve terminal (but not in contact with the diaphragm) so that the negative spike with fastest rise time was recorded. At high gain, the positive-negative pre-synaptic spike was visible. The negative-positive muscle spike was superimposed upon the end-plate current (e.p.c.) and their summation frequently resulted in a complex waveform (see later). Small changes in the electrode's position with respect to the tissue occurred during the experiments so adjustments were made regularly to keep the recorded waveform constant. The prolongation of the e.p.c. after ecothiopate confirmed that the end-plate zone had been located. The 'matched-pair' preparation in which recordings were taken from a single nerve fibre and from the muscle fibre which it innervated (Standaert, 1963; Riker, 1966) was not employed but precautions were taken to validate comparisons between nerve and muscle recordings. The two end-plate recordings M1 and M2 were always very similar, despite having been made on opposite sides of the preparation, and were taken to be representative of activity throughout the diaphragm. In some experiments, branches of phrenic nerve, innervating parts of the diaphragm not recorded from, were sectioned. The contraction was recorded isometrically and was displayed upon a Devices M2 recorder or oscilloscope. The activity of the phrenic nerve was recorded in air with silver wire electrodes with bandwidth 40 kHz–40 Hz (–3 db). All responses were displayed upon a Tektronix 564B storage oscilloscope and photographed.

Drugs used were acetylcholine chloride (BDH); ecothiopate iodide (Ayerst Laboratories); tubocurarine chloride, neostigmine methyl sulphate (Koch-Light Laboratories); and ambenonium chloride (Sterling-Winthrop).

Results

Experiments were performed upon 142 hemi-diaphragms (both left and right). The main ChE inhibitor used in this study was ecothiopate, an agent used in eye drops for the relief of glaucoma. It is safe to use and is easily obtainable. Ecothiopate inhibits ChE irreversibly: the reaction follows pseudo first order kinetics (Ferry & Marshall, 1971; Ferry & Marshall, unpublished observations). Two competitive inhibitors of ChE, neostigmine and ambenonium, were also tested.

Effect of ecothiopate upon the curarized preparation

In the preliminary experiments the diaphragm was exposed to tubocurarine 3.0 μM in saline for one hour to give a stable block of contraction. The e.p.c. was recorded focally at high gain in response to stimulation of the phrenic nerve. In the presence of ecothiopate 500 nM, the amplitude of the e.p.c. was increased 1.2–5.1 (mean 2.5) times and the duration at half amplitude was increased 5.6–14.2 (mean 8.1) times: from 0.66 ± 0.08 ms (mean ± s.e. mean) to 5.42 ± 0.47 milliseconds. The changes were well developed after 10 min and reached a maximum after 30 min: they were not affected by removal of ecothiopate.

By the use of photographic enlargements, the transmission time across the neuromuscular junction was measured from the negative peak of the pre-synaptic spike to the start of the rise of the e.p.c., in five preparations. Transmission time was 0.15 ± 0.008 ms (mean ± s.e. mean). This agrees well with the value of 0.22 ms given by Hubbard & Schmidt (1963). Within the limitations of the measurements, there appeared to be no difference in the transmission time before and after the addition of ecothiopate.

Effect of ecothiopate upon the non-curarized preparation

After preliminary experiments upon the non-curarized diaphragm using normal physiological saline solution (in which only low levels of nRA were generated), the Mg^{2+} concentration of the saline was reduced from 1.0 to 0.1 mM in order to facilitate the generation of nRA and mRA (see later). Firstly, mRA is described. Control records from M1 and M2, are shown in Figure 1A. In these traces, the stimulus occurred at the start of the sweep while in other records (e.g. Figure 3) the stimulus artefact was present. The negative muscle spike (a) generated within the end-plate zone was followed by a positive phase (the pre-synaptic spike is not visible in records at low gain). The muscle spike had such a fast rise time that it was incompletely stored in many traces. After the introduction of 50 nM ecothiopate, progressive prolongation of the e.p.c. (c) occurred in both M1 and M2 (Figure 1B and C). After 17 min, mRA (m) was present, always superimposed upon the prolonged e.p.c. in M1 and M2 (Figure 1D). After longer

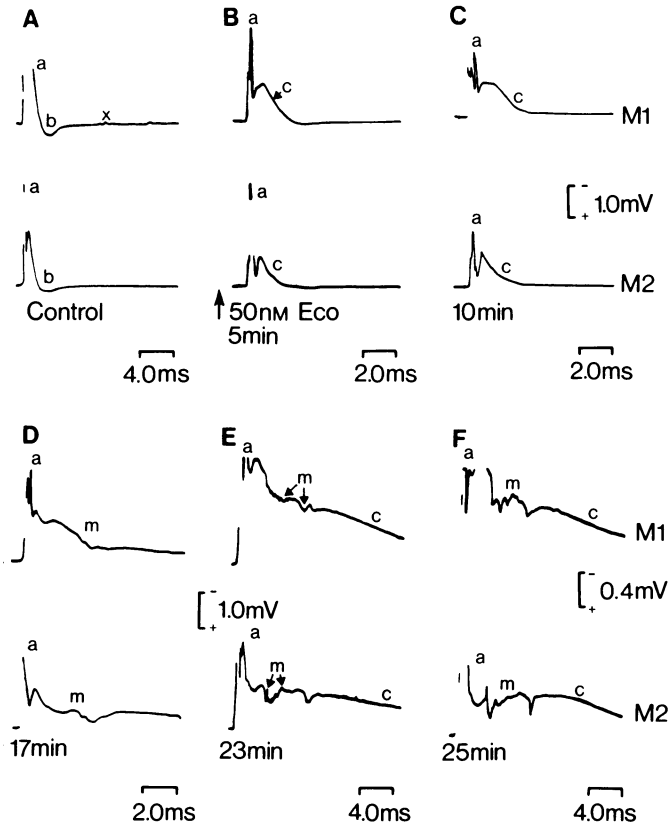

Figure 1 Simultaneous focal extracellular recordings from two end-plate zones M1 (upper trace) and M2 (lower trace). The muscle spikes were incompletely stored due to their fast rise time. (A) Control records showing the negative spike (a) followed by the positive undershoot (b) Blips on the baseline of M1 (x) were peculiar to that sweep. (B) After 5 min in 50 nM ecothiopate (Eco). Slight prolongation of the e.p.c. (c). (C) After 10 min, further prolongation of the e.p.c. (D) After 17 min, first appearance of muscle repetitive activity mRA (m). (E) After 23 min marked increases in mRA and in duration of the e.p.c. (F) After 25 min, mRA now quite pronounced. Note how M1 and M2 develop in parallel. Amplification was increased in (E) and (F).

exposures to ecothiopate, mRA increased in magnitude (Figure 1E and F). The possibility of movement artefacts in the traces was excluded when the electrode was placed near the lateral edge of the diaphragm where movement but not contraction occurred (movement was due to contraction of the main central part of the diaphragm): no movement artefacts were recorded.

The changes in the records at M1 and M2 developed in parallel (Figure 1). Thus M1 and M2 were taken to represent activity throughout the diaphragm and were compared with the records of activity in the phrenic nerve even though this may not have been generated at the two actual end-plate zones which were monitored.

Ecothiopate also increased the amplitude and duration of the contraction of the diaphragm. Furthermore the diaphragm relaxed very slowly after contraction had occurred. Figure 2 illustrates the effects of 500 nM ecothiopate: similar results were observed with 50 nM ecothiopate, except that the effects took longer to develop. After 9 min, the amplitude of the contraction had increased (but later began to decrease) (Figure 2B) and there was a persistent prolongation of relaxation (Figure 2C). Brown *et al.* (1936), using physostigmine, had noted the increased twitch duration but not the prolonged relaxation.

Simultaneous recordings from the diaphragm and phrenic nerve

Recordings were made simultaneously from M1, M2 and N in 0.1 mM Mg^{2+} saline. After the introduction

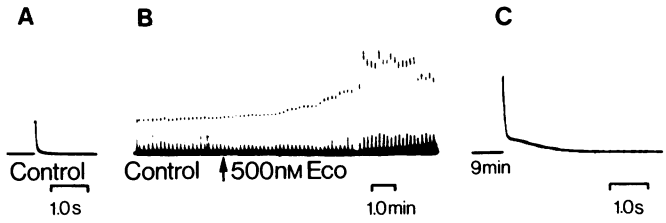

Figure 2 Effects of 500 nM ecothiopate upon isometric recordings of the contraction of the diaphragm. (A) Control contraction. (B) Introduction of 500 nM ecothiopate (Eco). A marked increase in amplitude had occurred. (C) After 9 min in ecothiopate. Prolongation of relaxation time had occurred. The amplitude of the contractions was uncalibrated.

of ecothiopate, nRA appeared within 12–20 min at 50 nM, 8–10 min at 100 nM and 4–6 min at 500 nM ecothiopate. Muscle RA started several minutes later in each case. With 5 nM ecothiopate no perceptible changes occurred even after an hour. After ecothiopate, spontaneous activity in the phrenic nerve was never observed. The antidromic discharges in response to stimulation consisted of 2–20 spikes in a train of duration less than 10 ms and with a very high frequency. The stages in the generation of RA were examined when exposures to 100 nM ecothiopate lasting 2–3 min were alternated with irrigations with saline lasting 10–15 minutes. Figure 3 illustrates part of a typical experiment. The nerve recordings may be divided into several parts. The first is the stimulus artefact (d) which was incompletely stored: it also obscured the orthodromic action potential in most records. The peak referred to as the 'electrotonic potential' (e) reflected the amplitude of the muscle activity and was thought to be the latter conducted over the surface of the phrenic nerve. However it is not a reliable indication of diaphragm activity since the conducting properties of the nerve's surface declined as the nerve progressively dried out during the experiment. For this reason, the peak e was much smaller in Figure 3C and D. Nerve RA is labelled n. The possibility of movement artefacts affecting the nerve recordings may be discounted since the contraction did not occur until after the start of nRA.

The control records in Figure 3A reveal no sign of RA in either nerve or muscle. After 3 exposures to ecothiopate which gave a total exposure time of 7 min, nRA was recorded but mRA was not (Figure 3B). After further exposure to ecothiopate for 4.5 min, mRA (m) appeared. By this time, there was marked prolongation of the e.p.c. and the nRA was well developed (Figure 3C). After further exposure to ecothiopate, nRA, mRA and the e.p.c. increased (Figure 3D). The latencies for the generation of nRA and mRA were measured from the stimulus artefact when nRA and mRA had just become well developed (at a slightly later stage than shown in Figure 3C), and

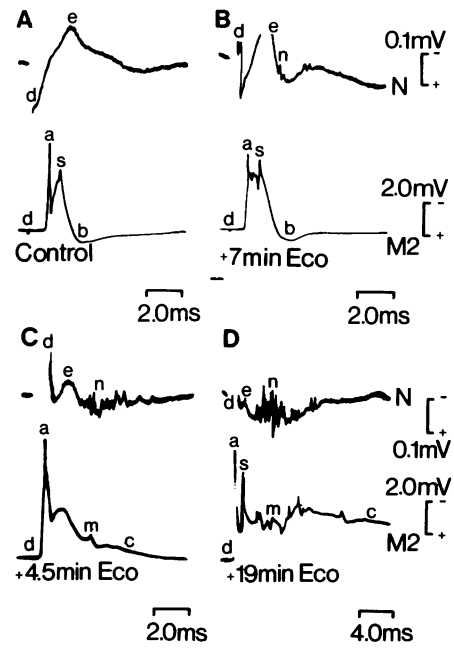

Figure 3 Simultaneous recordings from N (upper trace) and M2 (lower trace) after short exposures to 100 nM ecothiopate (Eco) followed by periods of irrigation with saline. (A) Control records: M2 shows the negative spike (a) and a smaller secondary spike (s), perhaps arising from the asynchronous discharge of muscle fibres, followed by a positive undershoot (b). N shows the incompletely stored stimulus artefact (d) followed by the electrotonic potential (e). (B) After 7 min in ecothiopate: appearance of nerve repetitive activity, nRA (n) but muscle repetitive activity (mRA) not present. (C) After a further 4.5 min in ecothiopate: prolongation of the e.p.c. (c) and appearance of mRA (m); nRA appreciably increased. (D) After a further 19 min in ecothiopate: both nRA and mRA were well developed and the e.p.c. was very prolonged. In (D) trace speed was halved.

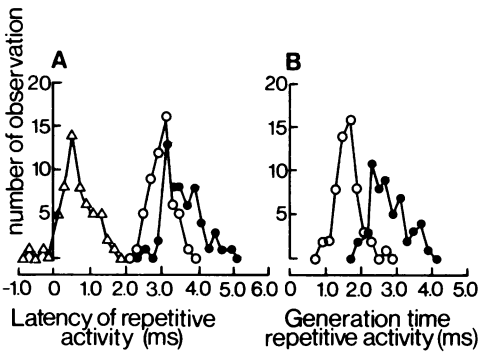

Figure 4 (A) Latencies for nerve repetitive activity (nRA) and muscle repetitive activity (mRA), after the introduction of 100 nM ecothiopate: (○) nRA; (●) mRA; (△) differences between mRA and nRA (for positive intervals nRA preceded mRA). (B) Generation times: (○) nRA; (●) mRA. Further explanation is given in the text.

are presented in Figure 4A: mean values were 2.94 ± 0.05 ms for nRA and 3.56 ± 0.08 ms for mRA. Nerve RA preceded mRA in all but 2 out of 57 diaphragms: the mean interval was 0.69 ± 0.06 milliseconds. These values did not contain a correction for conduction in the phrenic nerve (but see later). For longer exposures to ecothiopate, the nRA latency increased progressively to 6–8 ms while the latency for mRA remained unchanged. With 500 nM ecothiopate, the nRA latency was also 6–8 ms which exceeded the mRA latency of 3–4 milliseconds.

The generation time for nRA was the interval between the invasion of the nerve terminal by the orthodromic impulse and the generation of the first antidromic impulse. The generation time for mRA was the interval between the stimulus-evoked muscle spike and the foot of the first repetitive spike. These two values were calculated for 56 preparations. The generation time for nRA was calculated from the latency for nRA, as follows. The interval between the stimulus and the muscle spike comprised the conduction time in the phrenic nerve and the transmission time across the neuromuscular junction (0.15 milliseconds). So the nerve conduction time was the muscle spike latency less 0.15 milliseconds. Then, twice the conduction time (for conduction down the nerve and the return) was subtracted from the nRA latency to yield the generation time. (These calculations did not take into account the fact that conduction from the nerve terminal was to the recording electrode and not to the stimulating electrode nor that recent activity in the nerve may have reduced the antidromic conduction time, see Waxman & Swadlow, 1976; however, both corrections were very small.) The distribution of generation times for nRA is presented in Figure 4B. The mean value was 1.58 ± 0.05 ms which exceeds the value of 1.0 ms obtained by Werner (1961) in the cat, but agreed well with the value of 1.5 ms determined for the absolute refractory period of phrenic nerve terminals (Hubbard & Schmidt, 1963).

The generation time for mRA was calculated by the subtraction of the muscle spike latency from the mRA latency and the distribution is presented in Figure 4B: the mean value was 2.74 ± 0.08 ms which was appreciably longer than the value for nRA. This value exceeds the absolutely refractory period of mammalian striated muscle, 2 ms (Bowman, Rand & West, 1969). However, since diaphragm muscle may differ from other skeletal muscle in its refractory period, a comparison based upon an absolute refractory period of 2 ms is of limited value. It is of interest that, as the experiment progressed, the generation time for nRA progressively increased whilst for mRA it remained the same. This is relevant to the next section which describes the inhibitory effect which high concentrations of ecothiopate exerted upon nRA.

Effects of high concentrations of ecothiopate

After 500 nM ecothiopate nRA and mRA appeared within 4–8 min, the nRA latency gradually increased and the magnitude of the nRA gradually declined until it eventually disappeared 15–20 min after the introduction of ecothiopate. This occurred in 16 out of 18 experiments. Then, when the diaphragm was superfused with saline, nRA returned within 5–10 minutes. Muscle RA remained unaffected. A preparation treated with 50–100 nM ecothiopate developed nRA and mRA within 10–20 minutes. If the ecothiopate was washed out, the RA remained fairly stable, declining only slowly. When 500 nM ecothiopate was then added to such a preparation, there was suppression of nRA but no effect on mRA (9 out of 11 experiments). So it is suggested that ecothiopate in concentrations of 500 nM exerts a reversible inhibition of the appearance of nRA.

Effects of frequency of stimulation upon repetitive activity

The abolition of nRA by increasing the frequency of stimulation was a convenient means of checking that the recordings were not due to fluctuations in the baseline. When 'stable' RA had appeared (after 50 or 100 nM ecothiopate which was applied and then washed out) nRA was depressed when the stimulation frequency was increased to 1.0 Hz and abolished at 2.0 Hz or, in exceptional circumstances, 3.3 hertz. Under these conditions, mRA was only reduced; but was abolished at 5–10 hertz. The presence of 500 nM ecothiopate greatly increased the susceptibility of nRA

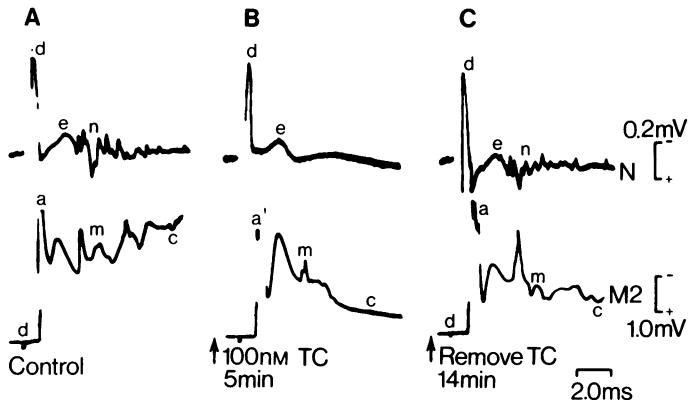

Figure 5 Effects of tubocurarine (TC) upon N (upper trace) and M2 (lower trace). Ecothiopate was removed after repetitive activity (RA) had developed. (A) Control records. N consists of stimulus artefact (d), electrotonic potential (e) and well developed nRA (n), while in M2 the e.p.c. (c) was very prolonged and mRA (m) was present. (B) After 5 min in 100 nM tubocurarine: nRA was abolished, mRA was depressed and the e.p.c. was reduced. (C) Return of nRA and mRA, 14 min after irrigation with saline. Note that the e.p.c. did not return to its control level.

to depression when 0.3 Hz or 0.5 Hz were quite sufficient to abolish nRA whilst mRA was undiminished.

Effects of magnesium upon repetitive activity

When the Mg^{2+} concentration was increased from 0.1 to 1.0 or 2.0 mM, nRA was abolished while mRA was only reduced and the duration of the e.p.c. was shortened. The effects of the Mg^{2+} were fully developed after about 10 min and were reversed over a similar period after removal of Mg^{2+}. These experiments demonstrated that mRA was recorded in the absence of nRA. The effects of the increased Mg^{2+} might be due to reduced ACh release (Liley, 1956). However, increased Mg^{2+} also stabilizes the neurolemma (Frankenhaeuser & Hodgkin, 1957); but this was unlikely to be important since reduced Ca^{2+} (0.2 mM) saline, which would labilize the membrane, also depressed both nRA and mRA.

Effects of tubocurarine upon repetitive activity

In concentrations much lower than those required to abolish the contraction of the diaphragm, tubocurarine depressed RA (Figure 5). Within 5–10 min of its introduction at 100 nM, mRA and the e.p.c. were both depressed while nRA was abolished (Figure 5B). Irrigation with saline for 5–10 min restored the nRA and mRA, although the e.p.c. had not returned to its control duration (Figure 5C). These results showed that, once again, it was possible to record mRA in the absence of nRA. (The failure of the e.p.c. to return to its control duration suggested that tubocurarine was still bound to cholinoceptive sites on the motor endplate despite prolonged irrigation with saline.)

Effects of acetylcholine upon repetitive activity

Introduction of 10 μM ACh had little effect upon the nRA and mRA evoked by stimulation of the phrenic nerve, whilst 55, 100 or 110 μM ACh abolished both nRA and mRA within 5–10 minutes. It was not possible to discern if one type of RA was more susceptible than the other. Both nRA and mRA returned after irrigation with saline for 8–10 minutes.

Effects of neostigmine and ambenonium

In order to see whether the results obtained with ecothiopate applied to other ChE inhibitors, two competitive inhibitors of ChE, neostigmine and ambenonium, were tested. On a molar basis both agents were more effective in the generation of RA than ecothiopate. The onset of nRA and mRA and prolongation of the e.p.c. occurred within 4 min of the introduction of 100 nM neostigmine or 100 nM ambenonium. These effects were well sustained for that concentration, as illustrated by Figure 6 for neostigmine. Irrigation with saline removed nRA and mRA. The e.p.c. was reduced in duration but was still very prolonged compared with the duration before neostigmine.

After the introduction of neostigmine or ambenonium, when nRA and mRA were well developed, the latency of nRA was less than the latency of mRA, and the generation times for nRA

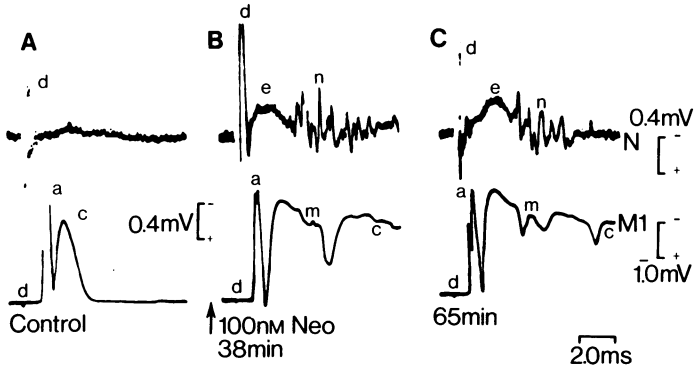

Figure 6 Effects of neostigmine upon N (upper trace) and M1 (lower trace). (A) Control records. N shows stimulus artefact (d) but electrotonic potential (e) was not visible. M1 shows muscle spike superimposed upon the e.p.c. (c). (B) After 38 min in 100 nM neostigmine (Neo): nerve repetitive activity, nRA (n) and muscle repetitive activity, mRA (m) were well developed and the e.p.c. was markedly prolonged. (C) After 65 min in neostigmine. Both nRA and mRA and prolongation of the e.p.c. were sustained. Amplification of M1 was reduced in (B) and (C).

and mRA were 1.3 ms and 2.5 ms respectively with neostigmine and 1.8 ms and 2.4 ms respectively with ambenonium: these agree well with the results for ecothiopate. When 3.3 μM neostigmine was introduced nRA and mRA were generated within seconds but several minutes later, nRA had disappeared whilst mRA was undiminished. Similar results were obtained with 500 nM neostigmine. Removal of neostigmine brought some but not complete return of nRA. Ambenonium differed from neostigmine since, after the introduction of 1.0 μM ambenonium, nRA and mRA were sustained throughout the experiment.

It may be concluded that the actions of neostigmine and ambenonium in the generation of RA were similar to those of ecothiopate.

Discussion

In the present study electrical activity at the end-plate zone has been related to antidromic activity in the phrenic nerve, after treatment of the diaphragm with ecothiopate. Some limitations of this work are as follows:

(1) Antidromic activity recorded in the phrenic nerve might not have arisen from the nerve terminals from which end-plate activity was recorded. However comparisons between the nerve and muscle recordings were in part justified by demonstrating that the two end-plate recordings M1 and M2 were similar. Hence they were presumed to be representative of end-plate activity throughout the diaphragm.

(2) Low Mg^{2+} saline was used in the present study and so the results are not directly applicable to the intact animal. The effects of reducing the Mg^{2+} may be to increase ACh release (Liley, 1956) and so the difference may be quantitative rather than qualitative. Nevertheless the results do give insight into the generation of nRA, which would not have been possible if normal Mg^{2+} had been used. Barstad (1962), Randić & Straughan (1964) and Blaber (1972) also used low Mg^{2+} solutions.

(3) The absolute sensitivities of the nerve and muscle recording electrodes are not known, so low levels of nRA and mRA may not have been detected. End-plate activity was also usually recorded at a lower gain to ensure that the recording was always focal.

(4) The electrodes M1 and M2 may have obstructed the diffusion of ACh from the end-plate zone and thus artificially prolonged the e.p.c. (Katz & Miledi, 1973). However the electrodes were placed above the nerve terminals and not actually in contact, so this effect is believed to be minimal.

(5) An *in vitro* preparation was employed. After several hours, deterioration of the preparation was readily observed by marked falls in the amplitudes of the end-plate recordings. For this reason experiments were performed usually within an hour of setting up the preparation. The nerve was stimulated only when recordings were taken.

Within these limitations, the present work has demonstrated the independence of nRA and mRA. Nerve RA had a shorter generation time than mRA and in the early stages of the action of ecothiopate low levels of nRA were recorded in the absence of mRA. This suggests that nRA was not generated by mRA. Since nRA was readily abolished whilst mRA was still

recorded, nRA *per se* did not generate mRA. Prolongation of the e.p.c. accompanied nRA and mRA (cf. Blaber, 1972) but the e.p.c. *per se* was unlikely to have generated nRA (though it generated mRA) because this would require retrograde conduction across the neuromuscular junction. This is improbable since repetitive postsynaptic activity depressed pre-synaptic activity, perhaps by accumulation of extracellular K^+ (Weight & Erulkar, 1976). So the prolonged action of ACh is proposed to be the generator of both nRA and of the prolonged e.p.c. and associated mRA. Experiments which reduce the amount of ACh released from the nerve terminal (increasing Mg^{2+} or frequency) or compete with the binding of ACh to cholinoceptive sites (tubocurarine) reduce nRA and mRA. Generally nRA was more labile than mRA. This may be attributable to a lower density of cholinoceptive sites on the nerve terminal than on the motor end-plate. This proposal would mean the probability of an ACh molecule binding with a receptor site was lower for the nerve terminal than motor end-plate. So the nerve terminal would be more sensitive to reduced ACh release. The proposal of Riker (1975) that the ChE inhibitor generates nRA directly, as opposed to indirectly via excess ACh, appears unlikely for several reasons.

(1) The present study showed that nRA was generated after ecothiopate had been washed out (although there is the possibility of ecothiopate binding to the nerve terminal).
(2) Muscle RA may be recorded in the absence of nRA. This has even been described by Riker and his associates in matched-pair experiments (Standaert & Adams, 1965; Riker, 1966). To account for this Riker & Okamoto (1969) proposed that antidromic nRA is more easily abolished than orthodromic nRA from the sites of initiation. While this proposal is difficult to refute, no evidence in support of it has been presented and so it appears reasonable to accept the results at face value, that in fact, no nRA was generated in the nerve terminal.
(3) If nRA arose as a result of the increased excitability of the nerve terminal due to the direct action of ecothiopate, nRA would be expected to be generated within the subnormal period which extended from 10 to 20 ms after orthodromic stimulation (Hubbard & Schmidt, 1963). However, the present results suggested that nRA was generated immediately after the absolute refractory period which was up to 1.5 ms after orthodromic stimulation (Hubbard & Schmidt, 1963).

In conclusion, if nRA and mRA both result from the prolonged action of ACh following ChE inhibition (as we suggest) it is no surprise that synchrony between nRA and mRA occurred in matched-pair experiments. However, nRA and mRA are nonetheless independent and thus one form of RA may be recorded in the absence of the other.

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