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The origin of the anticholinesterase-induced repetitive activity of the phrenic nerve-diaphragm preparation of the rat *in vitro*

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1 Action potentials have been recorded in contracting muscle cells of the phrenic nerve-diaphragm preparation from rats. After the organophosphorous anticholinesterase, ecothiopate, some cells fired repetitive action potentials. In 0.1 mM $[Mg^{2+}]_o$ the repetitive activity was generated presynaptically or postsynaptically, and in 1 mM $[Mg^{2+}]_o$, probably only postsynaptically.

2 The repetitive action potentials in muscle were generated ectopically about 0.2 mm away from the usual site.

3 In 1 mM $[Mg^{2+}]_o$, spontaneous action potentials in muscle were generated presynaptically. These were often followed by repetitive action potentials generated either presynaptically or postsynaptically.

4 The initiation of centripetal action potentials in the phrenic nerve was coincident with the repetitive firing in the muscle.

5 The results are discussed in relation to the manner of generation of repetitive activity in nerve and muscle after anticholinesterases.

Introduction

The effects of anticholinesterases on muscle and its innervation include potentiation of the twitch due to repetitive firing of muscle cells, the induction of antidromic activity in motor axons after conducting an orthodromic action potential, and fasciculation.

The repetitive firing of muscle *in vivo* was attributed by Brown *et al.* (1936) to the persistent presence of acetylcholine (ACh) in supraliminal concentration at the endplate and was adduced in favour of the chemical nature of neuromuscular transmission. The repetitive activity of muscle was sometimes accompanied by antidromic activity of motor axons generated at or near the nerve terminals (Masland & Wigton, 1940; Feng & Li, 1941; Eccles *et al.*, 1942; Werner, 1960; Riker & Standaert, 1966).

Considerable interest has been shown in the site of generation of this antidromic activity, and several techniques have been used to study this problem. These include the 'matched motor unit' technique of Werner (1960) and of Standaert (1963) to estimate the timing of generation of the antidromic action potentials in the motor nerve relative to the repetitive firing of the muscle; and the 'cut-fibre' preparation used to minimise the postsynaptic bioelectric phenomena *in vitro* (Barstad, 1962; Randić & Straughan, 1964). Some experiments *in vitro* involved the use of saline with low $[Mg^{2+}]$ or reduced temperatures, both of which enhanced the antidromic activity (Randić & Straughan, 1964; Morrison, 1977; Clark *et al.*, 1980; 1983; 1984). The use of such conditions helps the investigation, but their abnormality introduces some difficulties in the interpretation of the results. A different approach would be to record intracellularly from contracting muscle cells after treatment with an anticholinesterase to induce repetitive activity, for the response in muscle elicited directly by nerve stimulation can be distinguished by the clearly prolonged endplate potential (e.p.p.) from those responses elicited directly. This paper describes experiments made initially to record from contracting muscle cells to elucidate the site of the repetitive activity at the neuromuscular junction after an anticholinesterase, by use of selective stimulation and functional denervation to reduce the number of contracting cells. A similar approach was used by Clark *et al.* (1980, 1984), who weakened the contraction by stretching the muscle.

The results of some of the experiments have been

communicated to the British Pharmacological Society (Ferry, 1979).

Methods

Preparations of the phrenic nerve and diaphragm muscle were dissected from male albino rats of about 200 g weight after the animals were killed by a blow to the head and section of the cervical spinal cord. The preparations were maintained at 37 ± 0.2°C in a physiological saline of the following composition (mM): NaCl 137, $NaHCO_3$ 12, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, $NaHPO_4$ 1, dextrose 25 and gassed with 5% CO_2 in O_2. The preparation was pinned to the Sylgard floor of a Perspex bath, transilluminated and viewed at × 80 with a stereomicroscope.

Records of the membrane potential of muscle fibres were made by use of glass micropipettes filled with 3 M KCl and with a resistance of 7–10 MΩ, or of about 3 MΩ after bevelling on a settled slurry of 0.05 μm alumina powder (Banner Scientific) in 3 M KCl (Lederer *et al.*, 1979).

The dislodging of the micropipette from a muscle cell by contraction of the diaphragm was prevented by one or more of a variety of techniques: a 'floating' micropipette tip suspended on a 10 cm length of Ag wire (diameter, 125 μm); stimulation of a single terminal bundle of the phrenic nerve near the pleural surface to excite, by axon reflex, a small number of motor units each of which is distributed through about a 1 cm width of the diaphragm (Krnjević & Miledi, 1958), and causing a small contraction at any particular part of the muscle; by allowing the muscle to shorten passively, which resulted in functional denervation of the muscle cells manifest by the presence of miniature endplate potentials (m.e.p.ps) but not of endplate potentials (e.p.ps). The attenuation of the contraction assisted the recording of action potentials in the cells which were not functionally denervated.

The action potentials evoked by a single stimuli to the phrenic nerve were recorded on film or via a Datalab DL103A 100 point signal averager or a Gould OS4000 digital oscilloscope. Spontaneously occurring action potentials were recorded with the digital equipment.

Extracellular field potentials were recorded with one electrode close to the muscle and another remote from the preparation and positioned to minimize the stimulus artefact. For recording at the endplate, the first electrode was placed to record the muscle action current with minimal latency and the preceding endplate current with maximal rate of rise of negativity, and where, at high gain, a presynaptic action current could be recorded. Electrodes were either enamelled Ag wire of 0.32 mm diameter, or a broken micropipette filled with the physiological saline. Amplification was AC (−3 db points, 10 kHz, 0.16 Hz).

Records of action currents excited in the phrenic nerve were made with an array of 5 Pt wire electrodes, the central being connected to earth and the peripheral outer pair being used to stimulate the nerve and the central outer pair to record action currents. The electrode array and the nerve was positioned in a side chamber filled with liquid paraffin.

Contractile responses to nerve stimulation were recorded with a UF1 isometric transducer. In experiments where only mechanical events were recorded, the preparation was suspended in a conventional organ bath.

The anticholinesterase used was ecothiopate iodide made up from the Ecothiopate Eyedrops 0.25%, B.N.F. (Phospholine Eyedrops, Ayerst) which also contained potassium acetate to buffer the stock solution. The increase in $[K^+]$ of the physiological saline was 60 nM, which was considered negligible.

Groups of numerical data were subjected to the Mann-Whitney test to determine the probability of identicality of the groups.

Results

Effect of ecothiopate on the contractile response to nerve stimulation

The addition of ecothiopate 0.5 μM (ECO) to the bathing fluid resulted, about 7 min later, in increased contractile responses to single supramaximal stimuli to the nerve, and spontaneous twitching appeared after about 4 min. There was also a prolonged contraction at the endplate (Burd & Ferry, 1987). All of these effects waxed and waned in a repeatable pattern but with some variation of timing in individual preparations. As it was considered that ECO might have caused these effects by increasing transmitter action, experiments were made on the response to ECO in various $[Mg^{2+}]_o$. The records of a typical experiment are shown in Figure 1; in 0.1 mM $[Mg^{2+}]$, the increased 'twitch' response began and peaked sooner and the subsequent depression of the response was greater than in 1 mM $[Mg^{2+}]$. To prolong the presence of the enhanced 'twitch' and of the fasciculations, the ECO was washed out at times which varied from the first appearance of 'twitch enhancement', i.e. at about 7 min after ECO, to the time when the extracellularly recorded endplate current was maximally prolonged, i.e. at 15 min. This procedure facilitated the electrophysiological investigation of 'twitch enhancement' and of the fasciculation. The enhanced twitch was

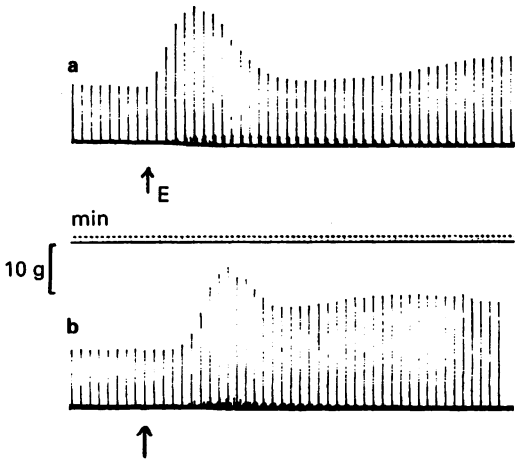

Figure 1 Rat phrenic nerve-diaphragm preparation: records of isometric contractions in response to stimulation of the phrenic nerve with single supramaximal stimuli at 0.01 Hz. (a) Preparation in 0.1 mM $[Mg^{2+}]$; (b) preparation in 1 mM $[Mg^{2+}]$. At arrow, addition of ecothiopate. Time, 1 min. Force calibration, 10 g. Note the small prolonged localized contraction recorded as a thickening of the lower part of the contraction and also the spontaneous contractions.

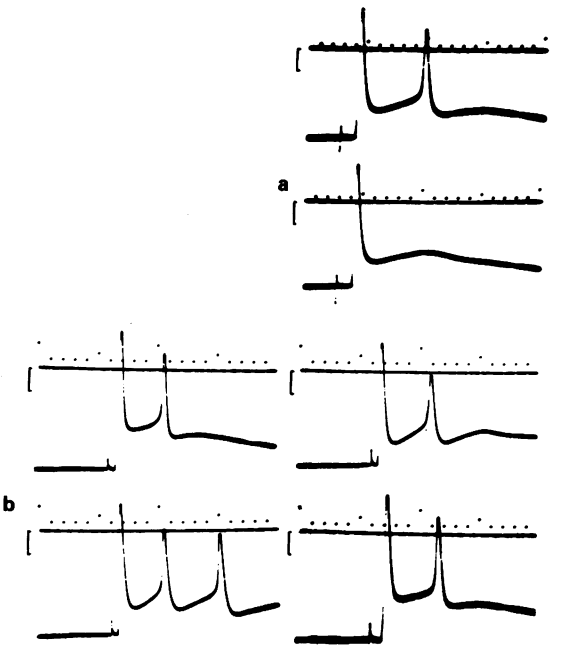

Figure 2 Rat phrenic nerve-diaphragm preparation in 1 mM $[Mg^{2+}]$ and ecothiopate. Response to a single pulse to the phrenic nerve recorded with a micropipette inserted intracellularly into the endplate region. Time, 1 ms, timetrace baseline set at 0 mV; voltage calibration, 20 mV. (a) Two responses in the same cell, the first showing the initial action potential and a repetitive action potential arising from the prolonged e.p.p., the second response without repetition and with a smaller e.p.p. (b) Responses from four cells from several preparations showing the initial action potential followed 3–5 ms later by the repetitive action potentials.

associated with repetitive action potentials in the muscle cells.

Repetitive action potentials recorded in 1 mM $[Mg^{2+}]_o$

In these experiments, the preparation was exposed to ECO for 15 min, then the bathing fluid was changed to the physiological saline only. Records were made with a micropipette inserted into the cell at the endplate, where the m.e.p.ps were larger and more prolonged than in untreated diaphragms. In all cells in which indirect stimulation provoked an action potential, the e.p.p. was prolonged, rising to a peak value of −50 mV about 6 ms after the action potential, the shape of the prolonged e.p.p. being resultant of the depolarizing effect of transmitter action and the afterhyperpolarization (Figure 2a).

In some cells, a repetitive action potential arose from the depolarizing phase of this prolonged e.p.p. and was itself followed by an after hyperpolarization. Sometimes two or even three repetitive action potentials were recorded (Figure 2b). The initial and repetitive action potentials propagated along the length of the cell and could be recorded with a micropipette inserted towards the tendinous ends where each was full size. The interval between the initial and repetitive action potentials measured at the foot of the initial and repetitive response was between 3 and 5 ms (mean, 3.82 ± 0.11 ms, $n = 62$). The repetitive action potential at the endplate arose from a membrane potential of about −50 mV and had a smaller rate of rise and overshoot than the initial action potential. In all experiments, repetitive action potentials uncomplicated by contraction artefacts were recorded from 39 cells. All of these showed repetitive action potentials arising from a depolarizing prepotential and some of these are shown in Figure 2.

Repetitive action potentials generated by outward current

In these experiments, two bevelled micropipettes were inserted into a cell, the bucking voltage adjusted to polarize the cell to −90 mV, and then a depolarizing current pulse of 10 ms duration passed through the cell. This gave rise to a series of repetitive action potentials, 3–5 ms apart and arising from

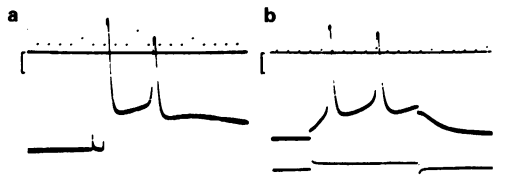

Figure 3 Rat phrenic nerve-diaphragm preparations in 1 mM [Mg^{2+}]. Records made with intracellular micropipettes. Time and voltage calibrations 1 ms, 20 mV as Figure 2. (a) Response to nerve stimulation after ecothiopate; (b) two bevelled micropipettes in the same cell, response to a 10 ms pulse of outward current 1.5×10^{-9} A, no ecothiopate.

a depolarizing prepotential. These responses were of similar appearance to those recorded after stimulation of the nerve in ECO-treated preparations (Figure 3).

Repetitive action potentials recorded in 0.1 mM [Mg^{2+}]$_o$

In these experiments the repetitive action potentials recorded at the endplate were sometimes similar to those recorded in 1 mM [Mg^{2+}] and described above, i.e. arising from a depolarizing prepotential (Figure 4b). However, in many cells the responses were different, arising abruptly from a prolonged endplate potential which was either steady or negative-going. The interval between the evoked initial response and the second type of repetitive action potential was about 4 ms (4.39 ± 0.17 ms, $n = 17$). This interval was greater ($P = 0.02$) than that recorded in 1 mM [Mg^{2+}]. Sometimes these repetitive action potentials could be seen to arise from a second endplate potential and sometimes a second endplate potential was seen which did not excite an action potential (Figure 4a). This second type of response was similar to that recorded after two stimuli to the nerve (see below and Figure 4a, lower). In some cells, both types of repetitive activity were recorded after a single stimulus to the nerve (Figure 4a, upper).

The experiments with intracellular injection of depolarizing current and with two stimuli to the phrenic nerve suggest that repetitive action potentials arising gradually from a depolarizing prepotential may have been generated by a persistent outward return endplate current i.e. by a postsynaptic mechanism and those arising abruptly might have been due to repetitive endplate potentials. The results suggest that repetition in 0.1 mM [Mg^{2+}] involved both pre- and postsynaptic mechanisms. In 1 mM [Mg^{2+}], although the shape of the response suggests a postsynaptic mechanism of repetition, it is possible that the action potential may

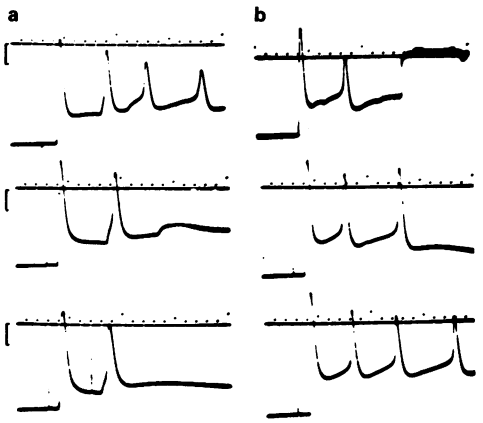

Figure 4 Rat phrenic nerve-diaphragm preparation: records made with intracellular micropipettes at the endplate in response to stimulation of the nerve. Time and voltage calibrations 1 ms, 20 mV as Figure 2. Different cells in different preparations after ecothiopate. (a) Upper and middle, responses to single stimuli in 0.1 mM [Mg^{2+}]. Note repetitive e.p.ps and mixed responses. Lower, response to two stimuli, 4 ms apart, in 1 mM [Mg^{2+}]. (b) Responses after ecothiopate in 0.1 mM [Mg^{2+}]. Note similarity to responses in 1 mM [Mg^{2+}] shown in Figure 2.

have hidden a simultaneous e.p.p. Experiments relative to this point were made by Clark *et al.* (1984) in 0.1 mM [Mg^{2+}] on muscle fibres in rat diaphragm or omohyoideus depolarized or voltage-clamped to avoid action potentials or with dithiothreitol to prevent repetitive firing of the nerve. Their records show clearly that repetitive action potentials arising from a depolarizing prepotential as in Figures 2, 3, 4b (i.e. their Figures 3, 5, 6B) were associated with only a single e.p.p. in that cell in response to a single stimulus, indicating that repetition was not presynaptic in origin but may then have been generated postsynaptically. The abruptly arising repetitive action potentials as in Figure 4a (i.e. their Figures 2, 6A) were associated with repetitive e.p.ps. Although the shape of the records in 1 mM [Mg^{2+}] is consistent with a postsynaptic generation of muscle repetition, a presynaptic mechanism cannot be excluded, for the generation of centripetal impulses in the phrenic nerve (see below) indicates this possibility. However, this centripetal discharge was scanty in 1 mM [Mg^{2+}] and the failure to record responses attributable to a presynaptic origin of muscle repetition in 39 muscle cells may be due to a low incidence of such events. Absence of responses in 39 cells could be due to a low incidence of a presynaptic mechanism of repetition e.g. of 0–9% (95% confidence limits of a binomial distribution).

It is concluded that the repetitive action potentials recorded in 1 mM $[Mg^{2+}]$ after a single stimulus to the nerve were generated mainly by a prolonged outward return current due to persistent transmitter action causing prolonged inward current through endplate channels, i.e. the repetitive activity of muscle in 1 mM $[Mg^{2+}]$ was likely to be generated by a postsynaptic mechanism. In 0.1 mM $[Mg^{2+}]$, as well as the generation of muscle repetition probably postsynaptically, there was an additional presynaptic mechanism involving synchronized release of transmitter, perhaps due to the re-excitation of the nerve terminal. The frequencies of occurrence of the two types of repetitive response were equal. These results with 0.1 mM $[Mg^{2+}]$ are similar to those reported by Clark *et al.* (1980, 1984).

Repetitive excitation of the nerve

In three experiments the nerve of an ECO-treated preparation was stimulated with two pulses and the responses recorded with a micropipette in the endplate. With a pulse interval of 4 or 5 ms the second response was an e.p.p. which always gave rise to an action potential; with an interval of 3 ms the probability of a second action potential was 0.2; there was never a second action potential with a pulse interval of 2 ms, although there was a second, smaller e.p.p. The interval between the action potentials was greater than the pulse interval, for example at a 4 ms pulse interval, the response interval was 4.5 ± 0.1 ms, $n = 13$, and due to retardation of the second response. Thus a second stimulus 2–5 ms after the first could excite the terminal part of the nerve by an orthodromic action potential even though there was depression of conduction and of transmitter release or action.

Fasciculation and repetitive firing

In phrenic nerve-diaphragms in 1 mM $[Mg^{2+}]_o$, washing after 15 min in ECO resulted in fasciculations often occurring in bursts. A micropipette was inserted into cells in a fasciculating part of the diaphragm. Sometimes action potentials were recorded like those in non-fasciculating muscle on tapping the bench and it may be that these were generated passively as the cell was moved against the electrode by a nearby spontaneously-twitching cell. Sometimes action potentials were synchronous with a spontaneous twitch observed at the point of insertion of the micropipette. These action potentials arose abruptly and continued into a prolonged e.p.p., often with repetitive action potentials (Figure 5). The shape of these repetitive responses was identical to those seen after stimulation of the nerve in 0.1 mM $[Mg^{2+}]$, i.e. sometimes arising from the prolonged first e.p.p. and sometimes arising from repetitive e.p.ps. Burd & Ferry (1987) reported that fasciculations originated presynaptically as they often had a prolonged localized contraction attributable to prolonged action of the transmitter. Thus it is concluded that the spontaneous twitching was initiated presynaptically by excitation of the terminal part of the nerve.

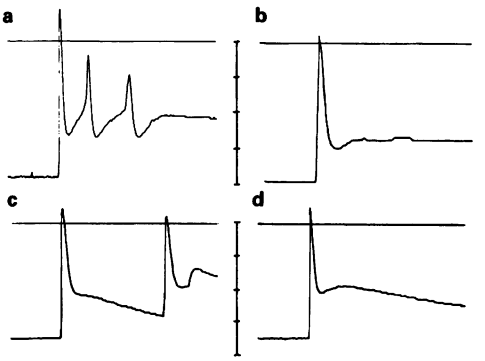

Figure 5 Rat phrenic nerve-diaphragm preparation in 1 mM $[Mg^{2+}]$ and ecothiopate. Records of spontaneous activity made with intracellular micropipettes at the endplate. Voltage calibration, 20 mV. Sweep time 20 ms in (a), 10 ms in (b), (c) and (d). Different cells in different preparations. Note single action potentials with a prolonged e.p.p., and repetitive action potentials arising from the first e.p.p. or from a repetitive e.p.p.

Extracellular field potentials after ecothiopate

In these experiments, an electrode was placed in the bathing fluid above the endplate region and after recording control responses, the preparation was exposed to ECO. The control response was a negative- then positive-going potential lasting 2 ms, and due to the inward endplate current and the excitation and propagation of a muscle action current. After ECO this sequence was followed by the prolonged inward endplate current. Superimposed on this were repetitive action currents recorded as positive-going potentials, whilst the initial action current was recorded 3–5 ms earlier as a negative then positive sequence. Spontaneous activity recorded with extracellular electrodes was similar. Such records are shown in Figure 6. In some experiments it seemed that the repetitive activity on its first appearance shortly after the onset of ECO action was initially negative-going, however a slight adjustment of the position of the electrode resulted in an increased amplitude of e.p.c. and positive-going repetitive activity (Figure 6b). Thus when the initial action current was recorded focally at its site of generation, the repetitive action current was recorded

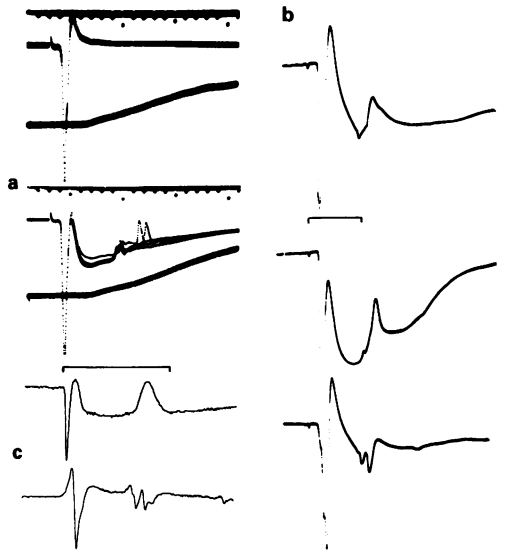

Figure 6 Rat phrenic nerve-diaphragm preparation in 1 mM [Mg^{2+}]. Records made at the endplate with wire electrodes. (a) Top, control action current and isometric contraction. Lower, after ecothiopate: note repetitive firing superimposed on prolonged endplate current. Time, 1 ms. Superimposition of five responses. (b) Three records made after ecothiopate, all at the endplate region and showing that a small movement of the electrode results in a focal recording with only positive-going repetitive activity. Time, 5 ms. (c) Spontaneous action currents and prolonged endplate currents and repetition at and away from the endplate. Time 5 ms.

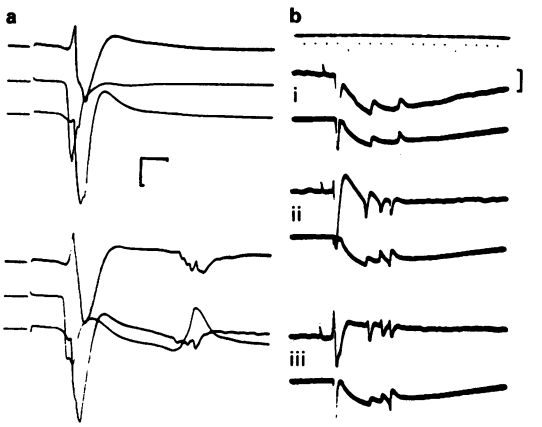

Figure 7 Rat phrenic nerve-diaphragm preparation in 1 mM [Mg^{2+}]. Responses to single stimuli to the nerve recorded with electrodes located extracellularly at the endplate region. Time, 1 ms. (a) Wire electrodes placed at the endplate region and about 0.7 mm on either side of it. Top set of 3 traces under control conditions; lower set after ecothiopate. (b) Saline-filled pipettes, one placed at the endplate, the other positioned at various distances from the first, records comprise paired traces: (i) both electrodes and traces at the endplate; (ii) lower trace at the endplate; upper trace, 0.2 mm away; (iii) lower trace at the endplate; upper trace, 0.5 mm away.

non-focally. One interpretation of this shown by the records in Figure 6 is that the initial and the repetitive responses were not generated at the same place. Another interpretation is that the preparation had moved relative to the electrode so that the initial action current was recorded negative- then positive-going at the endplate, but the repetitive action current, although generated at the endplate, was recorded non-focally as a positivity. The rise in isometric tension began just before the repetitive activity (Figure 6a), so it is possible that contraction of the diaphragm might have pulled the site of the origin of the repetitive activity away from the tip of the electrode.

Experiments with multiple extracellular electrodes

Three electrodes were positioned to record the extracellular current field relating to the activity in the same group of muscle cells of an untreated preparation. One was placed to record focally from the endplate and the others were placed on either side of it along the axis of the cells. It was expected that, if the muscle cells were to be pulled away from the electrodes, then the endplate region would be pulled away from the middle electrode towards only one of the others, and if the repetitive activity after ECO were generated at the endplate, then it would be recorded by this outer electrode as a negative-going potential and by the other two as positive-going.

The records made in such an experiment are shown in Figure 7, and it can be seen that the repetitive activity recorded with the middle electrode, placed initially at the endplate, was positive-going, whilst the same action currents recorded with both the outer electrodes were negative-going. These results indicate that movement of the muscle cannot account for the positive-going repetitive activity recorded at the endplate: thus the repetitive action potentials were generated ectopically on both sides of the endplate by the prolonged endplate potential.

In another experiment a saline-filled micropipette was positioned focally at the endplate and another was placed at various distances from the first so as to find the locus of generation of the repetitive activity, i.e. where these action currents were negative-then positive-going. Records are shown in Figure 7b, which comprises three paired records made initially with the two electrodes together at the endplate, then the second one moved 0.2 mm and finally 0.5 mm away from the endplate. From these records

it is concluded that the repetitive activity was generated about 0.2 mm away from the endplate.

Experiments on the excitability of the endplate region

The empirical observations that repetitive action potentials after ECO were not generated at the normal site suggests that the endplate region was inexcitable at the time when repetitive activity was recorded, i.e. about 3–5 ms after the initial action potential.

Therefore experiments were done on the ability of the endplate region to sustain propagation of a muscle action potential evoked by direct stimulation at various times after orthodromic activity evoked by nerve stimulation in an ECO-treated preparation. A micropipette was inserted into the endplate region to record local events in that cell and wire electrodes were used to excite the cell 1–2 mm away from the endplate. The results of a typical experiment are shown in Figure 8. The muscle action potential evoked to arrive at the endplate during the endplate potential was smaller and conducted more slowly the closer it followed the onset of transmitter action, the minimum interval between indirect and direct muscle action potentials was 3.5 ms, and propagation of the direct response into the endplate failed when attempts were made to reduce this interval to 3 ms.

It is concluded that the endplate region after ECO exhibits subnormal excitability for several tens of ms after synaptic activation and is refractory for about 3 ms.

In the experiments described above on 'Repetitive excitation of the nerve', it was found that two pulses 3 ms apart to the nerve, whilst causing two e.p.ps, did not always fire two muscle action potentials, which is consistent with this subnormality.

The centripetal repetitive activity in the phrenic nerve

Experiments have been made on the stimulus-linked centripetal activity (SLCA) in the phrenic nerve after ECO. The central cut end of the phrenic nerve was placed over a pair of recording electrodes in a pool of liquid paraffin. The muscle was immersed in physiological saline containing 1 mM [Mg^{2+}], and ECO was added. Records from the nerve after stimulation showed a large action current, the stimulated volley conducted centripetally from the more peripheral stimulating electrodes, followed about 5 ms later by a small scattered discharge of unitary action currents, the SLCA, which was carried in probably only a small percentage of phrenic axons. The response of the muscle recorded with a wire electrode at the endplate was the initial muscle action current followed by the prolonged endplate current with a positive-going repetitive activity. The aim of these experiments was to attempt to determine the time of generation of the SLCA relative to the repetitive firing of the muscle at the site of the electrode, by subtracting from the latency of the first spike of the SLCA the conduction time from the electrode on the muscle to the nerve. Such experiments are difficult to interpret because the SLCA cannot be guaranteed to arise only from the site of the recorded repetitive activity of the muscle. In these experiments an attempt was made to simplify the preparation and to record from an endplate region with minimal latency i.e. close to the point of entry of the phrenic nerve

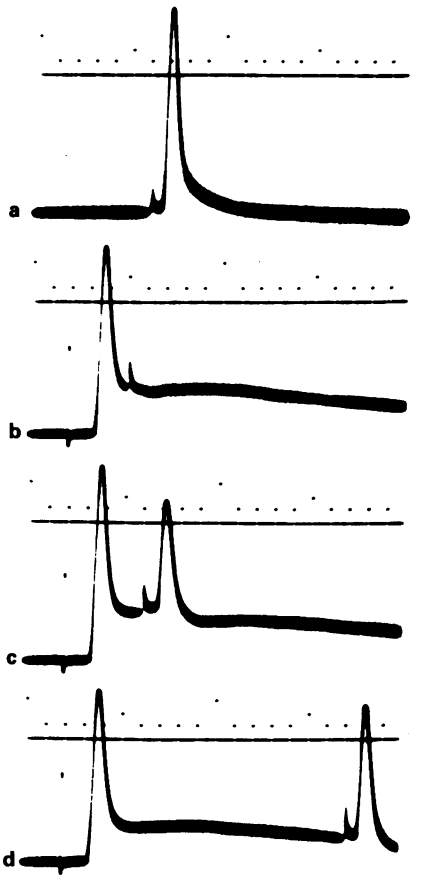

Figure 8 Rat phrenic nerve-diaphragm preparation in 1 mM [Mg^{2+}] and ecothiopate. Potentials recorded with a micropiopette inserted intracellularly into the endplate region in response to a single pulse to the muscle at various times relative to a conditioning pulse to the phrenic nerve. Time, 1 ms; timetrace baseline set at 0 mV, voltage calibration, 20 mV. (a) Directly-elicited action potential alone; (b–d) direct response preceded by indirectly-elicited action potential evoked 3, 4 or 15 ms earlier.

(Krnjević & Miledi, 1958) and to denervate the remainder of the diaphragm by cutting nearby the main intramuscular branches of the phrenic nerve. This gave an innervated strip about 3 mm wide. Typically, the centripetal conduction times of the action currents evoked by stimulation of the various terminal bundles of the peripheral arborization of the nerve within this restricted part of the diaphragm ranged from 0.9–1.3 ms with most points having a conduction time of 1 ms. At one particular site with minimal stimulus-response latency, records were made of the stimulated volley and the SLCA in the nerve and of the initial and repetitive muscle action currents. The wire recording electrode at the endplate then was used to excite the axons at the terminal part of the phrenic nerve after an orthodromic volley and it was found that these could not be excited by a stimulus applied less than 1 ms after the arrival of the orthodromic volley, and were relatively refractory for about 2 ms as indicated by a smaller response to a stimulus adequate in the absence of the orthodromic volley. With appropriate timing, the terminal axons were excited such that this peripherally-evoked centripetal activity arrived at the recording electrodes on the nerve with the same interval after the stimulated volley as the SLCA. This time of conduction of the peripherally-evoked action current after prior orthodromic activation was used to calculate the time of initiation of the SLCA in the nerve terminals and this was then compared with the time of initiation of the repetitive activity in muscle. It was found that, if it were assumed that the first part of the ECO-induced activity arose from that site on the muscle from which records were made, then both the repetitive activity in muscle and the SLCA would have been generated at the same time at the endplate region. As this site had minimum latency of the orthodromic impulse, if the first centipetal spike were to have been generated at another junction with a greater conduction time, then the latency of the time of generation of such activity would have been overestimated by 0.1 ms and exceptionally by 0.4 ms. Thus in this case, the postsynaptic repetition could have been caused by the SLCA. Because there is no guarantee that the SLCA arose at that endplate from which repetitive firing of the muscle was recorded, this type of experiment cannot provide unequivocal evidence on the relative timing of excitation of SLCA and the repetitive activity in muscle needed to resolve whether the SLCA excited the muscle repetition or *vice versa*. This conclusion is not consistent with that of Riker & Standaert (1966) who calculated that the antidromic neural activity was generated about 0.8 ms before the repetitive activity in the muscle and proposed a presynaptic origin of muscle repetition. If the neural activity arose elsewhere in the motor unit and propagated to the ventral root antidromically and by axon reflex to the endplate from which records were made, then the calculations would indicate that the nerve was excited before the muscle because no account would have been taken of the additional delay due to conduction around the axon reflex.

Thus, taken alone, these experiments on the timing of the centripetal activity in the nerve relative to the repetitive activity in the muscle do not provide unequivocal evidence for which of these events initiated the other, all that can be concluded is that the two events occur within about 0.2 ms of one another. However, when taken together with the evidence from intracellular records that the repetitive muscle action potentials in 1 mM $[Mg^{2+}]$ were likely to have been generated only postsynaptically, and the refractory period of the terminals, then the available evidence suggests that the SLCA in the nerve was generated by the repetitive muscle action potentials.

In some preparations, no ECO-induced centripetal activity was recorded after a single orthodromic volley. However, after paired stimuli to the nerve, this activity became prominent and as the interval between the stimuli was increased from 0.7 to 2.5 ms, so the centripetal activity was delayed, appearing 5 ms after the second stimulated volley. These results indicate the initiation of subthreshold hyperexcitability of the terminal part of the nerve by an orthodromic action potential and summation of this effect of the first and second action potentials to excite centripetal activity. Similar results have been reported by Eccles *et al.* (1942) and by Werner (1960).

Discussion

The results of the experiments suggest that the origin of the repetitive firing of nerve and of muscle depended upon the $[Mg^{2+}]_o$, and that the repetitive activity arose ectopically in muscle irrespective of the $[Mg^{2+}]_o$.

Ectopic site of muscle repetition

The repetitive action potentials in muscle were generated about 0.2 mm from the site of origin of the initial action potential some 3 ms earlier, and within the part of the muscle cell involved in the prolonged localized contractions (Burd & Ferry, 1987). The first repetitive action potential was later than 3 ms after the initial evoked response, i.e. after the inexcitability of the endplate region. It may be that the translocation of excitation from the subsynapse was due to local subnormality of the muscle membrane after its depolarization to about −50 mV for the previous 3 ms during the e.p.p. and the local elevation of

intracellular [Ca^{2+}]. Thus the repetitive action potentials may be generated at a site where the outward return endplate current, although attenuated by cable properties, is adequate to excite a less-inactivated part of the membrane. The recovery cycle of the endplate region thus probably determined the interval of 3–5 ms between repetitive action potentials in the muscle cell which was similar to that found *in vivo* (Brown, 1937).

The stimulus-linked centripetal activity

The SLCA in the phrenic nerve was attributed by Randić & Straughan (1964) to antidromic action potentials in the motor axons and my experiments in 1 mM [Mg^{2+}] indicate that the SLCA might coincide with the repetitive firing of muscle, i.e. 3–5 ms after the first action potential. The small SLCA indicates that re-excitation of the nerve was unlikely and this is supported by the probability that generation of repetition was postsynaptic rather than presynaptic.

There are several hypotheses for the excitation of the terminal part of the nerve to give the SLCA; that it is due to the current field of the action potential in the muscle (Eccles *et al.*, 1942; Paton, 1963), or to an action of the transmitter or to direct action of the facilitating agent (Riker, 1975) or to K^+ leaking from the subsynaptic region of the muscle (Hohlfeld *et al.*, 1981). The experiments of Aizenman *et al.* (1986) indicate that the release of transmitter is essential for the appearance of SLCA, which contradicts a direct action of the anticholinesterase. The experiments with stimulation of the terminal bundles of the nerve in ECO-treated preparations have shown that these were excitable 2 ms after an orthodromic action potential, coincident with the peak endplate current (c.f. Figure 6a, corrected for the action current), and perhaps the peak [ACh] in the cleft. However, the evidence that the antidromic activity was generated later, i.e. 3–5 ms after the first action potential and coincident with the repetitive activity of the muscle suggests that the nerve terminal was excited by the muscle action potential and not by the transmitter, although this may have had a conditioning effect. It may be that the muscle action potential can excite the terminals as suggested by Lloyd (1942), but because they were refractory for 2 ms after their orthodromic activity, excitation was by the repetitive muscle action potentials (Eccles *et al.*, 1952).

Repetitive activity of muscle

Evidence has been presented that muscle repetition in 0.1 mM [Mg^{2+}] was generated by presynaptic and postsynaptic mechanisms and in 1 mM [Mg^{2+}], perhaps postsynaptic only. Before concluding that there are two mechanisms for generating repetition, it should be considered if a single mechanism is possible.

One possibility is that the repetitive muscle action potentials generated by the prolonged e.p.p. re-excite some terminal nodes of the nerve, and that in 0.1 mM [Mg^{2+}] more axons are excited than in 1 mM. The SLCA is increased in low [Mg^{2+}] (Randić & Straughan, 1964; Morrison, 1977; Clark *et al.*, 1983), perhaps due to loss of a depressant effect of Mg^{2+} on the excitability of the terminal nodes (Hubbard *et al.*, 1965). Furthermore, in my experiments in 0.1 mM [Mg^{2+}], it was found that muscle repetition considered due to excitation of the nerve terminals occurred 0.6 ms later than repetition generated postsynaptically, which is about the latency of the axon reflex (cf Figure 2b). Thus in some junctions of a motor unit the initial e.p.p. may be large enough to generate muscle repetition which may then initiate an action potential in the terminal part of the axon to propagate to other junctions of the motor unit giving an indirectly-generated repetitive response about 0.6 ms later than that generated directly. Such a mechanism would operate in a normal physiological saline and would be enhanced in low [Mg^{2+}].

The mechanism of excitation of the terminal part of the axon

The neuromuscular synapse after an anticholinesterase differs from normal in the prolongation and intensification of the action of quantally released transmitter and in the post-synaptic effect of non-quantally released ACh (Katz & Miledi, 1977; Vyskočil & Illes, 1977) and in a local hypercontraction of the endplate region which may compress the synapse and restrict diffusion from the cleft (Burd & Ferry, 1987). There may also be leakage of K^+ from the subsynaptic area leading to elevation of [K^+ in the cleft (Hohlfeld *et al.*, 1981), and depletion of cleft [Ca^{2+}] due to its accumulation by the muscle (Burd & Ferry, 1978).

It is proposed that excitation of the nerve by the repetitive muscle action potential is due to these changes. Normally, little endplate current would be drawn through the nerve terminals because of shunting through the synaptic cleft continuous with the extracellular space. However after ECO, perhaps the local contracture increases the resistance of the shunt whilst that of the presynaptic membrane decreases due to changes of cleft [K^+] and [Ca^{2+}] and to ACh action, such that a significant amount of the current entering the subsynaptic membrane may be drawn outward through the relatively extensive presynaptic membrane after being funnelled inwards through the last nodes, which may then become hyperpolarized and superexcitable. The generation of

the repetitive muscle action potential some distance from the synapse may then cause a rapid reversal of the local currents, and the rapid resolution of anelectrotonus at the superexcitable last nodes in favour of an outward current may there excite an action potential. The phrenic nerve terminals are superexcitable 3–5 ms after the conditioning action potential (Hubbard & Schmidt, 1961; 1963; Hubbard *et al.*, 1965), and Bostock & Grafe (1985) have shown that hyperpolarization increased the superexcitability of nodes of mammalian ventral root axons. Thus there could be a state of superexcitability 3–5 ms after the conditioning action potential, at the time when a repetitive muscle action potential is proposed to excite antidromic activity in the nerve.

It would appear that the generation of action potentials in the terminal part of the motor axon is the endpoint of the summation of a number of individually subliminal processes. Of these, perhaps the current field set up by postsynaptic events is the most important and determines the timing of the SLCA. However, the vulnerability of the SLCA to small doses of drugs e.g. (+)-tubocurarine, or to temperature or frequency of stimulation that affect the release or action of transmitter or the excitability of membranes implies that excitation is multifactorial. In some terminals even in the absence of e.p.ps or muscle action potentials it would seem that the other conditioning processes are able to excite an action potential which then propagates and reverberates throughout the terminal arborization causing fasciculation.

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