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NATURE OF THE ANTICHOLINESTERASE-INDUCED REPETITIVE RESPONSE OF RAT AND MOUSE STRIATED MUSCLE TO SINGLE NERVE STIMULI

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SUMMARY

1. The action of the anticholinesterase Paraoxon on neuromuscular transmission in rat diaphragm and mouse omohyoideus preparations was investigated.

2. In both preparations, when Paraoxon potentiated the twitch in response to a single nerve stimulus, repetitive muscle action potentials were recorded with an intracellular electrode placed at the motor end-plate region.

3. At end-plates of Paraoxon-treated rat diaphragm preparations where the membrane potential was not sufficiently negative to support muscle action potentials, repetitive end-plate potentials were recorded in response to a single nerve stimulus. No repetitive end-plate potentials could be recorded under such conditions in preparations which had been exposed to dithiothreitol before being treated with Paraoxon, although twitch potentiation and repetitive muscle action potentials were still observed in these preparations.

4. In Paraoxon-treated mouse omohyoideus preparations only single end-plate potentials were recorded from end-plates where the membrane potential was not sufficiently negative to support muscle action potentials. This applied whether or not the preparation had been treated with dithiothreitol before being exposed to Paraoxon.

5. In voltage-clamped rat diaphragm preparations which had been treated with Paraoxon, repetitive end-plate currents were frequently recorded in response to a single nerve stimulus. Under the same conditions mouse omohyoideus preparations responded with a single end-plate current.

6. It is concluded that Paraoxon-induced twitch potentiation in rat diaphragm and mouse omohyoideus preparations is caused by repetitive muscle action potentials being triggered by a single nerve stimulus. Under the conditions stated, the repetitive muscle action potentials in rat diaphragm preparations arose from a prolonged end-plate potential or repetitive end-plate potentials or a combination of both. In mouse omohyoideus preparations the repetitive muscle potentials were the consequence of a single prolonged end-plate potential.

INTRODUCTION

In mammalian skeletal muscle exposed to an anticholinesterase, there is a potentiation of the twitch in response to a single stimulus to the motor nerve. This potentiation is associated with repetitive muscle action potentials, as recorded with extracellular electrodes (Brown, 1937). Under certain conditions, the potentiation is also associated with repetitive antidromic nerve action potentials (Masland & Wigton, 1940; Feng & Li, 1941). Antidromic firing might thus be a mechanism for twitch potentiation but it cannot be the only mechanism since when antidromic firing is abolished by the disulphide bond reducing agent, dithiothreitol, peak twitch potentiation can be either unaffected or only slightly reduced (Clark, Hobbiger & Terrar, 1979, 1983). A reduction in antidromic firing with little influence on twitch potentiation can also be obtained by varying the concentrations of Ca and Mg in the solution bathing the muscle (Clark, Hobbiger & Terrar, 1979, 1983).

If antidromic firing is involved in twitch potentiation, it should be associated with repetitive end-plate potentials in individual muscle fibres. To obtain direct evidence for the occurrence of repetitive end-plate potentials as well as muscle action potentials in anticholinesterase-treated muscle, an intracellular micro-electrode was used to record from the region of the motor end-plate. In some experiments a voltage clamp was applied to obtain information on end-plate currents. The anticholinesterase used was Paraoxon (an organophosphate), and the experiments were done on two muscle preparations (the isolated rat diaphragm and the isolated mouse omohyoideus muscle).

A preliminary account of this work has been presented to the Physiological Society (Clark, Hobbiger & Terrar, 1980).

METHODS

Rat diaphragm strip preparation

Left hemidiaphragms were obtained from male Sprague–Dawley rats, weighing 150–250 g. Strips were prepared by cutting parallel to the muscle fibres, 1–3 mm on either side of the point where the phrenic nerve aligns with the muscle. Each preparation included a portion of rib, and the muscle fibres together with their nerve supply.

Mouse omohyoideus preparation

Omohyoideus muscles, obtained from male T.O. strain mice, weighing 30–35 g, were dissected free with their nerve supply and portions of scapula and hyoid bones (Dreyer, Muller, Peper & Sterz, 1976).

Muscle tension recording

Preparations were mounted horizontally in a flow chamber (1 cm wide, approximately 1 ml capacity). A thread connected the central tendon of the diaphragm strip or the hyoid bone of the omohyoideus muscle to a Grass F.T.-03C force–displacement transducer which was coupled to a Grass 7D polygraph. A resting tension of 0·4–1 g was applied. The nerve was stimulated via a suction electrode (frequency 0·2 Hz, pulse duration 50 μs, supramaximal voltage).

Intracellular recording from the motor end-plate region

Diaphragm and omohyoideus preparations were mounted in a chamber which was similar to that described above, except that it contained a curved elevated section over which the muscle was stretched to reduce or abolish movement when the nerve was stimulated. The amount of stretch

was controlled by a rack and pinion device attached to the central tendon of the diaphragm strip or the hyoid bone of the omohyoideus muscle. Nerve stimulation was as for tension recording, except that the stimulus strength was not maximal for the whole preparation but only sufficient to give a consistent response in the fibre under study. In this way the number of contracting fibres and any associated residual contraction were minimized.

Conventional micro-electrodes containing 3 M-KCl were used (resistance 6–30 MΩ). The potential difference between micro-electrode and bath electrode was measured differentially, using a system with FET amplifiers (Burr-Brown 3522) for the input stage. The recorded voltages were displayed on a Tektronix 565 oscilloscope and a Gould OS 4000 digital storage oscilloscope, and were recorded with a Racal Store 4 FM tape recorder, usually at 7·5 in./s (1 dB down at 2·5 kHz).

For locating motor end-plate regions a compound microscope fitted with a long working distance objective (total magnification 320× or greater) was used. Electrodes were positioned for fastest rise and largest amplitude of miniature end-plate potentials (Fatt & Katz, 1952).

In muscles which had been stretched to minimize movement, intracellular recording at the end-plate during stimulation of the motor nerve typically showed muscle action potentials. Recording became more difficult after Paraoxon treatment of preparations because stretching became less effective in reducing movement, particularly at the end-plate where a local contracture could be observed to occur. This was more pronounced in the diaphragm than in omohyoideus muscles. After Paraoxon treatment of the preparation several muscle action potentials were initiated by a single stimulus to the motor nerve. When movement of the muscle occurred, the recorded membrane potential became less negative, presumably as a consequence of damage around the tip of the micro-electrode, producing a low resistance pathway to 'shunt' the membrane potential in that region of the muscle fibre. Under these conditions, muscle action potentials declined as a consequence of inactivation of the fast Na conductance (Adrian & Marshall, 1977; Pappone, 1980), revealing the underlying end-plate potentials. At membrane potentials less negative than approximately −55 mV the end-plate potentials would be expected to be uncontaminated by active Na currents. In experiments where the membrane potential remained more negative than this, a second current-passing micro-electrode was sometimes inserted into the muscle fibre to depolarize the membrane sufficiently to abolish muscle action potentials.

Recording of end-plate currents

Another method for avoiding muscle action potentials, and consequently simplifying the analysis of repetitive responses to single nerve stimuli after Paraoxon, is to voltage clamp muscle fibres at a steady membrane potential. In some experiments a conventional two-micro-electrode voltage-clamp system (Takeuchi & Takeuchi, 1959; Connor & Stevens, 1971; Terrar, 1978) was used to record end-plate currents.

Solutions

In all experiments the chamber was perfused continuously with Tyrode solution (composition (mM): NaCl, 137; $NaHCO_3$, 12; KCl, 2·7; $CaCl_2$, 2·0; $MgCl_2$, 0·1; glucose 11), pre-warmed to 35–37 °C, bubbled with oxygen containing 5% CO_2 and flowing at a constant rate of 10–15 ml min^{-1}. Drug-containing solutions were kept in separate reservoirs. Speedy changeover of solutions was made possible by a tap connecting the reservoirs to the flow chamber via a short silicone rubber tube; the delay for drug-containing solutions to reach the bath was less than 20 s.

Drugs

The following drugs were used: diethyl-4-nitrophenylphosphate (Paraoxon, Koch-Light); dithiothreitol (Sigma); 5,5′dithiobis (2-nitrobenzoic acid) (DTNB, Sigma).

RESULTS

Rat diaphragm strip preparation

Effect of Paraoxon on twitch tension. When rat diaphragm strip preparations were exposed to Paraoxon (0·5 μM) for 5–15 min, the response of the muscle to low frequency (0·2 Hz) stimuli to the motor nerve was potentiated. Peak twitch poten-

tiation amounted to an increase in tension to $3{\cdot}0 \pm 0{\cdot}3$ ($n = 5$) times pre-Paraoxon tension, and little or no decline of the potentiation was seen after terminating exposure of the preparation to Paraoxon.

Effect of Paraoxon on responses recorded from the motor end-plate region. In the absence of Paraoxon, the recorded membrane potential was in the range -60 to -90 mV, and a single stimulus to the motor nerve resulted in a single muscle action potential at the end-plate region, with a peak depolarization to 0 mV or beyond. The

Fig. 1. Effect of Paraoxon on responses recorded from the motor end-plate region following single nerve stimuli in a rat diaphragm strip preparation. *A–C*, responses recorded from the same motor end-plate after 30 s (*A*), 1 min (*B*) and 2 min (*C*) exposure to Paraoxon (0·5 μM). *D*, response recorded from another motor end-plate in the same preparation 12 min after a 5 min exposure to Paraoxon. Vertical scales represent the recorded membrane potentials.

action potential lacked any obvious sign of the end-plate potential which initiated it, either in the form of a step at the foot of the action potential or as a discontinuity during repolarization, because of the brevity of end-plate potentials under these conditions.

When Paraoxon (0·5 μM) was added to the Tyrode solution, the recorded response to nerve stimulation first developed a prolonged repolarization phase, which became progressively more marked, presumably reflecting a prolonged end-plate potential, and then repetitive muscle action potentials were recorded in response to a single stimulus to the motor nerve (Fig. 1). Exposure to Paraoxon frequently resulted in some movement of the muscle despite stretch, and there was a consequent progressive depolarization of the muscle membrane close to the recording site (see Methods). Records from such an experiment are shown in Fig. 2. It can be seen that when the membrane had depolarized to approximately -40 mV (Fig. 2*D*), when muscle action potentials could no longer be triggered by the end-plate potential, repetitive end-plate potentials were recorded in response to a single stimulus to the motor nerve. Fig. 2*B*

and *C* show responses at intermediate stages where active Na currents were impaired but could nevertheless still contribute to the repetitive response.

When the period of exposure to Paraoxon was limited to 5 min, repetitive end-plate potentials could be recorded from a partially depolarized muscle fibre for 30–60 min after removal of Paraoxon. In seventy-six end-plate regions investigated under these conditions where the membrane potential had declined to −55 mV or less (see Methods), forty-eight showed repetitive end-plate potentials in response to a single

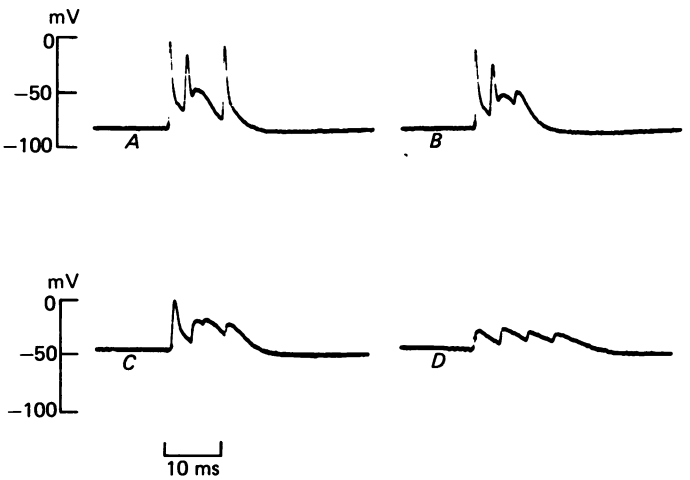

Fig. 2. Effect of progressive depolarization at the motor end-plate region on the repetitive response to a single nerve stimulus in a rat diaphragm strip preparation exposed to Paraoxon. *A–D*, responses recorded from the same motor end-plate, at intervals during the first 4 min of exposure to Paraoxon (0·5 μM). The recorded membrane potential declined from −72 mV (*A*) to −41 mV (*D*). Vertical scales represent the recorded membrane potentials.

nerve stimulus. For the remainder, identification of repetitive end-plate potentials was not so clear, and this point is taken up in the Discussion. Successive end-plate potentials were separated by variable intervals, ranging from approximately 2 to 20 ms.

Effect of Paraoxon in dithiothreitol-treated preparations. In preparations which had been exposed to dithiothreitol (1 mM for 10 min), a single nerve stimulus produced a single muscle action potential similar to that observed in untreated preparations. Subsequent exposure to Paraoxon (0·5 μM) progressively prolonged the repolarization phase of the muscle action potential, and produced repetitive potentials in response to a single nerve stimulus (Fig. 3). In contrast to preparations which had not been treated with dithiothreitol, there was less muscle movement, largely because of the absence of local contractures, and a correspondingly greater stability of recording. In those muscle fibres which did nevertheless depolarize at the end-plate region to a membrane potential of −55 mV or less, only single end-plate potentials were seen (Fig. 3).

In dithiothreitol-treated preparations, a 10 min exposure of the preparation to Paraoxon (0·5 μM) consistently produced repetitive muscle action potentials at

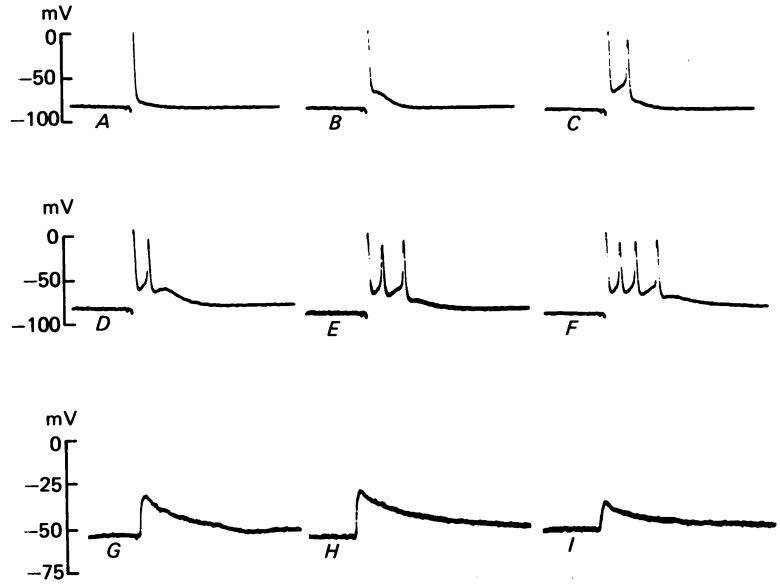

Fig. 3. Effect of Paraoxon on responses recorded from the motor end-plate region following single nerve stimuli in a rat diaphragm strip preparation treated with dithiothreitol (1 mM for 10 min). *A*, single action potential recorded after dithiothreitol treatment alone. *B–F*, records obtained at intervals from the same motor end-plate during a 3–5 min exposure to Paraoxon (0·5 μM). *G–I*, records obtained from three other motor end-plates following exposure to Paraoxon and after partial depolarization at the motor end-plate region. Vertical scales represent the recorded membrane potentials.

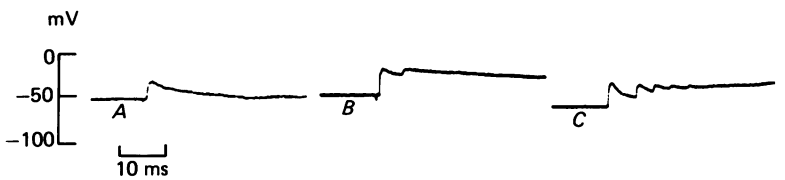

Fig. 4. Effect of dithiothreitol and DTNB on responses recorded after partial depolarization at the motor end-plate region in rat diaphragm preparations exposed to Paraoxon. *A*, record obtained following treatment with dithiothreitol (1 mM for 10 min) and Paraoxon (0·5 μM for 10 min). *B* and *C*, records obtained from two other motor end-plates from the same preparation after subsequent exposure to DTNB (1 mM for 10 min). Vertical scale represents the recorded membrane potential.

end-plate regions where the membrane potential was sufficiently negative for active Na currents to occur ($n = 19$). In sixty-three fibres with membrane potentials less negative than −55 mV, a single end-plate potential only was recorded in response to a single nerve stimulus.

Addition of the oxidizing agent DTNB (1 mM for 10 min), which reverses the effect of dithiothreitol on disulphide bonds, led to a reappearance of repetitive end-plate potentials in a proportion of the muscle fibres investigated (Fig. 4). Of ninety-three end-plate regions sampled following exposure to DTNB, twelve clearly showed repetitive end-plate potentials. Movement of the muscle causing displacement of the

electrode was more pronounced following exposure to DTNB and consequently the true number of end-plate regions showing repetitive end-plate potentials was probably underestimated (see Discussion).

Mouse omohyoideus preparation

Effect of Paraoxon on twitch tension. Exposure to Paraoxon (0·5 μM) for 5 to 15 min produced a peak twitch potentiation which amounted to an increase in tension to $6{\cdot}4 \pm 1{\cdot}2$ ($n = 7$) times pre-Paraoxon tension. Twitch potentiation was well maintained for periods up to 60 min following removal of Paraoxon from the Tyrode solution.

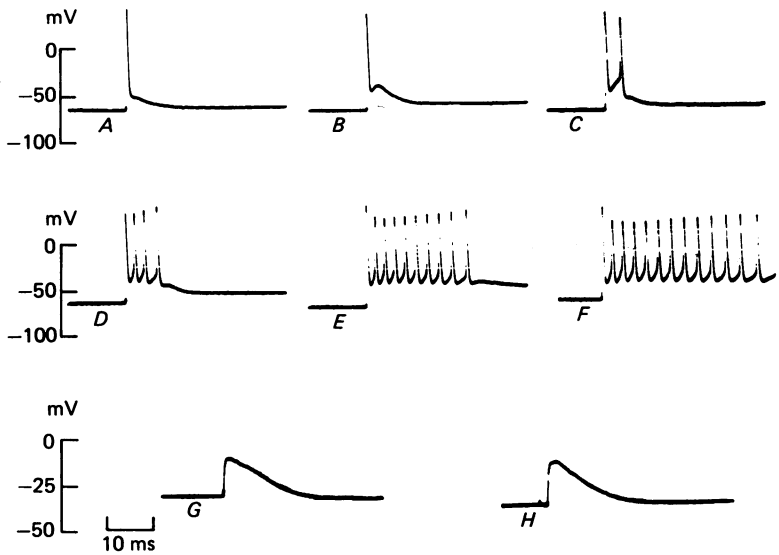

Fig. 5. Effect of Paraoxon on responses recorded from the motor end-plate region following single nerve stimuli in a mouse omohyoideus preparation. *A*, single action potential recorded before exposure to Paraoxon. *B–F*, responses recorded from the same motor end-plate, between 2 and 8 min exposure to Paraoxon (0·5 μM). *G* and *H*, records obtained from two other motor end-plates following exposure to Paraoxon and after partial depolarization of the muscle fibre at the end-plate region. Vertical scale represents the recorded membrane potential.

Effect of Paraoxon on responses recorded from the motor end-plate region. As in the diaphragm strip preparation, when Paraoxon (0·5 μM) was added to the Tyrode solution, the muscle response to a single nerve stimulus first developed a prolonged repolarization phase and then repetitive muscle action potentials were recorded at the motor end-plate region of muscle fibres with membrane potentials in the range -60 to -90 mV (Fig. 5). There was less movement of the muscle following exposure to Paraoxon than was seen in the diaphragm strip preparations. A further difference from diaphragm strip preparations was the observation that at end-plate regions where the membrane was partially depolarized (membrane potential less negative than -55 mV) only a single end-plate potential was seen ($n = 20$) (Fig. 5). The only exception to this was an experiment in which two end-plate potentials were recorded in response to a single nerve stimulus.

Effect of dithiothreitol on Paraoxon-treated preparations. The effects of dithiothreitol on responses recorded from end-plate regions of Paraoxon-treated preparations of omohyoideus muscle was investigated in three experiments. In one preparation Paraoxon (0·5 μM) applied for 10 min increased the number of muscle action potentials recorded from the same motor end-plate from one to seven; dithiothreitol (1 mM) applied for 10 min then decreased the number of muscle action potentials recorded at the same end-plate to one, and DTNB (1 mM) applied for 10 min restored the number to seven. In the other two preparations comparable results were obtained.

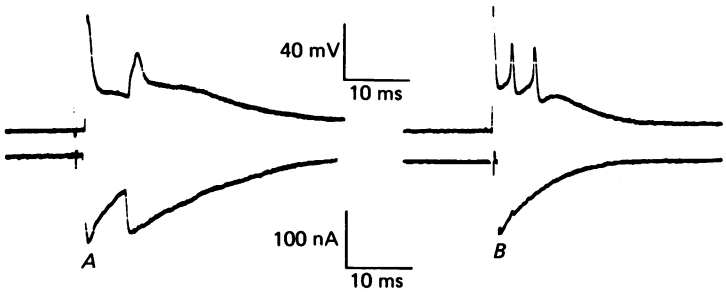

Fig. 6. Responses recorded from the motor end-plate region following single nerve stimuli. In each panel the upper record shows the membrane potential response, and the lower record shows end-plate currents recorded from the same fibre after voltage clamping to −50 mV. The steady level of membrane potential before applying the voltage clamp was −68 mV in *A* and −69 mV in *B*. *A*, rat diaphragm strip preparation. *B*, mouse omohyoideus preparation.

Following DTNB treatment seventeen additional motor end-plate regions were sampled in the three preparations. At motor end-plates where the membrane potential was more negative than −55 mV repetitive muscle action potentials were observed whereas at twelve partially depolarized fibres where the membrane potential had become less negative than −55 mV only a single end-plate potential could be recorded.

End-plate currents in rat diaphragm and mouse omohyoideus preparations.

In experiments in which muscle fibres were voltage clamped to avoid muscle action potentials, a single nerve stimulus after Paraoxon (0·5 μM for 5 min) frequently caused repetitive end-plate currents in rat diaphragm preparations (Fig. 6). In contrast, in mouse omohyoideus preparations only a single end-plate current was recorded in response to nerve stimulation after Paraoxon at end-plate regions where multiple muscle action potentials had been recorded before the voltage clamp was applied (Fig. 6).

DISCUSSION

The experiments reported here show that, after exposure of rat diaphragm strip and mouse omohyoideus preparations to the anticholinesterase Paraoxon, individual muscle fibres respond to a single nerve stimulus with repetitive muscle action potentials. The timing of these action potentials (approximately 2–20 ms apart) would be expected to lead to a contraction comparable to a brief tetanus rather than a twitch, and thus to twitch potentiation.

The experiments also show that in Paraoxon-treated rat diaphragm preparations, repetitive end-plate potentials can occur in response to a single nerve stimulus. The occurrence of repetitive end-plate potentials was confirmed by experiments where muscle fibres were voltage clamped at the end-plate region; repetitive end-plate currents were recorded under experimental conditions similar to those when repetitive end-plate potentials had been observed. Repetitive end-plate potentials, like antidromic firing of nerve action potentials in Paraoxon-treated rat diaphragm preparations (Clark *et al.* 1979, 1983), were abolished by dithiothreitol. This is taken as evidence that the antidromic nerve action potentials are also conducted orthodromically to release acetylcholine. Further evidence in support of this interpretation is the observation that the interval between the first end-plate potential or current and subsequent potentials or currents corresponds with the timing of antidromic firing under similar experimental conditions (Clark *et al.* 1979, 1983).

The observation that repetitive muscle action potentials, like twitch potentiation (Clark *et al.* 1979, 1983), were not abolished by dithiothreitol in Paraoxon-treated rat diaphragm preparations indicates that they can occur also in the absence of repetitive end-plate potentials. It can therefore be concluded that there are two mechanisms for initiation of repetitive muscle action potentials and for the accompanying twitch potentiation in Paraoxon-treated rat diaphragm preparations. Repetitive muscle action potentials can arise from a single end-plate potential as a consequence of its prolongation by Paraoxon. When repetitive end-plate potentials occur, these would be expected to contribute an additional influence for repetitive muscle action potentials, partly in their own right, and partly from the further prolongation of the end-plate response which would be expected to occur under these conditions (see Magleby & Terrar, 1975).

The quantitative assessment of the frequency with which repetitive end-plate potentials occur in Paraoxon-treated rat diaphragm preparations is difficult because repetitive end-plate potentials can be unambiguously identified only when the membrane is sufficiently depolarized to prevent muscle action potentials. Even in these conditions, recognition of repetitive end-plate potentials in response to a single nerve stimulus may be impaired for the following reasons. The second and subsequent end-plate potentials occur during the early part of the decay of the first stimulus-induced end-plate potential, and are small (because the membrane is at a more depolarized level close to the reversal potential and perhaps because of local saturation of receptors by acetylcholine when acetylcholinesterase is inhibited (Hartzell, Kuffler & Yoshikami, 1975)). A further problem is that muscle movement, in particular local contractures in the end-plate region, sometimes dislodged the electrode. Local contractures were more obvious in conditions where repetitive end-plate potentials occurred. With these problems in mind, when muscle fibres were grouped for quantitative purposes, only those showing clear repetitive end-plate potentials were counted and so their number is likely to be an underestimate.

When mouse omohyoideus preparations were exposed to Paraoxon, the occurrence of repetitive end-plate potentials in response to a single nerve stimulus appeared to be a rare event. Similarly in voltage-clamp experiments repetitive end-plate currents were never observed. In this muscle the repetitive muscle action potentials responsible for twitch potentiation thus appear to arise from a single prolonged end-plate

potential under our experimental conditions. Twitch potentiation was larger in mouse omohyoideus than in diaphragm strip preparations. This might perhaps occur because more muscle action potentials (fourteen in Fig. 5) were triggered by a single nerve stimulus in mouse omohyoideus than in the rat diaphragm strip preparations, but this point was not studied systematically.

The reason for the difference between rat diaphragm strip and mouse omohyoideus preparations in their response to Paraoxon is unknown. It also remains to be established to what extent the findings are typical for other types of skeletal muscle, and for *in vivo* conditions.

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