THE EFFECT OF ANTICHOLINESTERASE ACTION ON MAMMALIAN SKELETAL NEUROMUSCULAR TRANSMISSION

Submitted by:

Paul Frank Burd, B.Sc. Hons.

to the "University of Aston in Birmingham", in accordance with the requirements for the degree of PhD.

September, 1980

SUMMARY

A brief outline of skeletal neuromuscular transmission as a sequence of events leading to muscular contraction and the actions of anticholinesterases is presented in the Introduction.

The work has been largely concerned with the irreversible inhibition of cholinesterase by ecothiopate (phospholine) at the neuromuscular junction, thereby altering the time course of transmitter action, and the subsequent effect on the contractile response of isolated mammalian diaphragm preparations.

Ecothiopate (5×10^{-7}M) evokes a complex and, apparently, time-dependent series of changes in the contractile response, one of which, the prolonged endplate-localised contraction, was believed to be a new observation and was to be the main subject of further study.

Electrophysiological, radioisotopic and histological methods have been employed to determine the cause of these prolonged contractions. They have been found to be associated with prolonged currents and with an accumulation of calcium, both at the endplates. It has not been possible to exclude a direct effect of membrane depolarisation or an effect of post-junctional calcium entry as the cause of the prolonged localised contraction. It has been concluded that ecothiopate (5×10^{-7}M) is unlikely to have a significant direct effect on the changes in contraction and endplate calcium accumulation.

A biochemical method to determine cholinesterase activity has been devised which is believed to give results which represent endplate cholinesterase activity. An attempt has been made to correlate the ecothiopate-mediated changes in contractile response with the degree of cholinesterase inhibition. It has been concluded that the time-dependent changes in the contractile response after ecothiopate are mainly due to a time-dependent inhibition of cholinesterase in non-perfused muscles.

Finally, the waning of the ecothiopate-mediated changes in contraction have been suggested to be due to a reduction in transmitter action, perhaps, by receptor desensitisation. The association between desensitisation and endplate calcium accumulation has been discussed.

Key Words: Anticholinesterase Calcium Diaphragm
Prolonged Contraction

Paul Frank Burd
Submitted in accordance with the requirements for the degree of
Ph.D. 1980

ACKNOWLEDGEMENTS

The work was supervised by Professor Brian Ferry, to whom I am indebted for giving me the opportunity to study for a higher degree. I am also grateful for the advice and never-ending supply of ideas which he has provided during the practical work, and for his helpful discussions during the preparation of this thesis.

I am also indebted to Mr David Briggs for his technical assistance and forthright opinions, particularly concerning the electrophysiological equipment. I would also like to extend my thanks to Alan and Marion Richardson for their very helpful general technical assistance and also to Mr John Bird and the staff of the Neurology Department of the Midland Centre for Neurosurgery and Neurology, Smethwick, West Midlands.

My thanks also to Dr John Smith for his practical advice and helpful discussions during the latter part of my research period at Aston.

A special thankyou to my mother for the careful and patient typing of this thesis and also for the support she has given me during this period.

Financial support was provided by the Medical Research Council.

Lastly, I am deeply indebted to my wife, Alexis, without whom I doubt whether this thesis would ever have been completed.

CONTENTS

FIGURES PAGE

TABLES PAGE

ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AChR(s)	acetylcholine receptor(s)
antiChE(s)	anticholinesterase(s)
β - Butx	β - Bungarotoxin
Ca	calcium
Ca^{2+}	calcium ions
ChE	cholinesterase
DFP	diisopropylfluorophosphonate
dTC	D - tubocurarine
epp(s)	endplate potential(s)
eepp(s)	extracellular endplate potential(s)
GBHA	glyoxal bis (2 - hydroxyanil)
mepp(s)	miniature endplate potential(s)
Mg	magnesium
Mg^{2+}	magnesium ions
2 - PAM	pyridine - 2 - aldoxime methiodide
plc(s)	prolonged localised contraction(s)
SC(s)	spontaneous contraction(s)
SR	sarcoplasmic reticulum
TEPP	tetraethylpyrophosphate

INTRODUCTION

Evidence of cholinergic transmission at the neuromuscular junction of skeletal muscle was obtained by Dale, Feldberg and Vogt (1936) who recovered acetylcholine (ACh) from the perfusate of neuromuscular preparations following nerve stimulation and produced twitch-like contractions in the muscle following close arterial injection of ACh. That ACh was released from the nerve terminals and not from the muscle was shown by Dale et al. (1936) when they could not obtain ACh from a denervated muscle. Detailed study of the transmitter release process at individual synapses began with the experiments of Fatt and Katz (1952) who observed that ACh appeared to be released in small packages or quanta. These correspond to the synaptic vesicles (del Castillo and Katz 1955b) which are clustered on the pre-synaptic side of neuromuscular junctions (Robertson, 1956; Birks, Huxley and Katz, 1960a).

The actual release process, however, remains uncertain. It seems clear that calcium ions (Ca^{2+}) are involved, for, without calcium, release of ACh ceases (del Castillo and Stark, 1952; Jenkinson, 1957; Elmquist and Feldman, 1965a). Depolarisation of the nerve terminal membrane is believed to cause an increase in membrane conductance to Ca^{2+} (Katz and Miledi, 1967; 1968). Following Ca^{2+} entry, it is now thought that synaptic vesicles fuse with the terminal membrane and release their contents. The exact mechanism by which Ca^{2+} entry leads to the fusion of vesicles with the membrane remains unknown.

The passage of the nerve impulse along the motor nerve and its arrival at the nerve terminal results in the release of a large number of quanta (several hundred). The ACh released

diffuses across the synapse and is recognised and bound by receptors on the (normally polarised) post - synaptic membrane. If this happens within 1 msec or so, a chain of events is triggered that leads to membrane depolarisation, that is, the generation of an endplate potential (epp) and the subsequent triggering of a muscle action potential.

The reaction of ACh with ACh receptor (AChR) results in the opening of ion channels thus increasing the conductance of the membrane to sodium (Na^{+}), potassium (K^{+}) and, to a lesser extent, calcium (Ca^{2+}) ions (Fatt and Katz, 1951; del Castillo and Katz, 1954; Takeuchi and Takeuchi, 1960).

The action of ACh at the neuromuscular junction is terminated by either diffusion or destruction by an enzyme. The latter was postulated as early as 1914 by Dale. This enzyme has subsequently been identified as cholinesterase (ChE).

The close proximity to the AChR and the rapid turnover rate of ChE is believed to be responsible for limiting the number of reactions of ACh molecules with AChRs to 1, or 2, at the most when ChE is working normally.

The actions of inhibitors of ChE (i.e. anticholinesterases) at the skeletal neuromuscular junction have been extensively reviewed, particularly by Werner and Kuperman (1963) and more recently by Bowman and Webb (1972).

An action of physostigmine on skeletal muscle was first demonstrated by Pal (1900). It is generally assumed that the increase in muscle contraction is due to a prolonged transmitter effect that gives rise to a prolonged epp, which in turn fires off a series of muscle action potentials. The augmented contractions are therefore tetani of short duration (Brown, 1937a).

Feng (1940, 1941) and Eccles, Katz and Kuffler (1942) both showed that physostigmine increased and prolonged epps in curarised and non-curarised skeletal muscles which was accompanied by repetitive firing in the muscle fibres of the latter. Similar results have been obtained with DFP in the isolated phrenic nerve-diaphragm preparation of the rat (Meeter, 1958). Masland and Wigton (1940) showed that antidromic nerve impulses accompanied the repetitive muscle response evoked by a single shock applied to the motor nerve after injection of neostigmine. Since similar effects were produced by intra-arterial injection of acetylcholine, they concluded that ACh, persisting as a result of ChE inhibition, stimulated the nerve endings directly as well as the motor endplate of the muscle. Since K^+ are not thought to initiate antidromic firing (Feng and Li, 1941), there would appear to be AChRs at pre- as well as post-junctional sites (Eccles, 1964 and Hubbard, 1965, and reviewed by Miyamoto, 1978).

In doses smaller than those necessary to block neuromuscular transmission, tubocurarine (dTC) abolishes the effects on motor nerve endings of anticholinesterases (antiChEs) (reviewed by Bowman and Webb, 1972) thus indicating that dTC combines with nerve terminal receptors as well as endplate receptors. This provides strong evidence that the prejunctional action of antiChE drugs is mediated by ACh. However, the action of antiChEs is not restricted to inhibition of ChE. For example, edrophonium has been described as having potent anti-ACh actions (Katz and Thesleff, 1957; Kuperman and Okamoto, 1964). The effect of any antiChE drug on neuromuscular transmission will be dependent upon (i) the relative affinities of the antiChE for the ChE and the receptors, (ii) the concentration of ACh with which the antiChE competes and

(iii) the concentration of the antiChE.

Organophosphates are potent inhibitors of ChE (10^{-8} - 10^{-5}M) (Silver, 1974). Among these compounds are many widely used insecticides and the "nerve gases", potential chemical warfare agents. The action on the enzyme, first discovered in the 1940s, is considered to be irreversible. Due to the presumably similar structure of the active sites of AChR and ChE, higher concentrations of organophosphates (e.g. ecothiopate, 10^{-3}M) have been observed to have an effect on the AChR (Bartels and Nachmansohn, 1969). Ecothiopate is a non-selective and, apparently, irreversible inhibitor of ChE (Silver, 1974). It has been used previously in the treatment of Glaucoma and Myasthenia Gravis.

In the fast striated twitch fibres of vertebrate skeletal muscle, the normal sequence is for one impulse in the nerve to give rise to one impulse in the muscle. In the endplate region of fast mammalian muscle fibres a depolarisation of about 10 - 20 mV (Boyd and Martin, 1956b; Liley, 1956a) is sufficient to initiate an all-or-none conducted action potential. Intracellular electrical recording near the endplate of these fibres shows a complex membrane potential change which is the result of the localised epp acting in parallel with the propagating muscle action potential. A few millimetres away, only the latter is seen, and it is this conducted action potential that triggers the contractile component to produce its mechanical response, the twitch.

The rapid sequence of changes in the membrane potential which influence the state of the contractile system comprise the process known as Excitation -Contraction Coupling. The effect of more prolonged changes of potential, by increasing the external K^+ concentration, on the contraction of fast skeletal muscle fibres

suggests that depolarisation causes the release of a substance which activates the contractile component. The extremely rapid changes in the action potential do not give rise to simultaneous changes in force developed. The existence of a minimum degree of depolarisation of the membrane potential in order to reach the mechanical threshold for tension development (Hodgkin and Horowicz, 1960) implies that a minimum amount of activator must be released within a certain time so that its concentration at the contractile sites can reach a value sufficient to produce a perceptible amount of tension. Restoration of the membrane potential to its resting level results in the removal or depletion of the activators leading to relaxation.

Ca^{2+} has long been regarded as a likely candidate for the role of activator (e.g. Heilbrunn and Wiercinski, 1947). Ca^{2+} injected into the interior of a normal, non-depolarised muscle fibre, or applied directly to a preparation of isolated myofibrils, freed of the sarcolemma, produces a local, non-propagated contraction. Jobsis and O'Connor (1966) using the Ca^{2+}-sensitive dye, murexide, and Ashley and Ridgeway (1968) using aequorin, a protein that luminesces in the presence of free Ca^{2+}, have directly demonstrated the release of Ca^{2+} when a muscle fibre is stimulated to produce either a twitch or a tetanus. They also found that the free Ca^{2+} disappeared during relaxation. Their results strongly support the view that Ca^{2+} is indeed the "activator" of contraction in vertebrate striated muscle.

Heilbrunn and Wiercinski (1947) hypothesised that the ionic currents associated with the conducted action potential might release Ca^{2+} from structures located within the cell membrane or allow external Ca^{2+} to enter the cell. The observation that skeletal

muscle contractility can be observed in the absence of extracellular Ca^{2+} has generally been interpreted to indicate a lack of critical role of extracellular Ca^{2+} or Ca^{2+} fluxes across the sarcolemma in regulating skeletal muscle contraction and/or relaxation (Sulakhe and St. Louis, 1980).

In addition, studies on the influx of Ca^{2+} during the contraction of skeletal muscle show that, although Ca^{2+} influx does occur following muscle activation (Bianchi and Shanes, 1959), the amount entering was less than 1% of what is required to fully saturate the contractile system (Carlson and Wilkie, 1974). The time taken for Ca^{2+} to enter and reach the myofibrils (100 msec) is too slow since peak tension in fast skeletal muscle at $20^{o}C$ is achieved in 20 msec also (Carlson and Wilkie, 1974). A. V. Hill (1948) suggested that some process other than diffusion is responsible for the inward conduction of the activating stimulus. A system of transverse tubules (T-system) have now been discovered which run across the muscle cell (along the Z-lines) eventually coming into close contact with the sarcoplasmic reticulum (SR) and connecting with the exterior through openings on the cell surface. Huxley and Taylor (1958) showed that these tubules could provide the means for the inward spread of the stimulus to the contractile machinery.

There is, therefore, good physiological evidence for believing that in fast skeletal muscle fibres the stimulus for contraction consists of

(i) the propagated action potential with its accompanying depolarisation of the membrane,

(ii) the movement of excitation inward along the T-system,

(iii) the release of Ca^{2+} from sites somewhere within the muscle, very likely located in the SR, and

(iv) the diffusion of Ca^{2+} to the contractile component and the activation of this component.

It is plausible to associate the relaxation phase of the contractile cycle with the removal of Ca^{2+} from the contractile component, thus allowing it to return to its resting state.

The development of tension is believed to result from the cyclic attachment and detachment of cross-bridges projecting from the thick (myosin) filaments and interacting with the thin (actin) filaments in such a way as to draw them toward the centre of the sarcomere. The SR maintains the sarcoplasmic Ca^{2+} concentration in resting muscle at a level (i.e. $< 10^{-7}$M) below that needed for activation of actin-myosin interaction. Upon stimulation, an electric signal is transmitted along the T-tubule system to the SR, thereby causing a rapid release of Ca^{2+} and an elevation of sarcoplasmic Ca^{2+} concentration to approximately 10^{-5}M. Cross-bridge attachment is promoted by the binding of Ca^{2+} to the actin filaments. During the relaxation phase Ca^{2+} is reaccumulated within the SR and the cross-bridges detach from the thin filaments (Fuchs, 1974).

Reviews by Sandow (1970), Fuchs (1974), Ebashi (1976), Endo (1977) and Tada, Yamamoto and Tonomuro (1979) comprehensively cover the topic of excitation-contraction coupling. How depolarisation, induced in the T-system, releases stored Ca^{2+} from the SR remains the least understood step in this coupling process.

Endo (1977) came to the conclusion that only Ca^{2+} themselves or depolarisation of the SR membrane might play an important role in the physiological excitation - contraction coupling. Calcium-induced calcium release requires an amount of Ca^{2+} to trigger the further release of Ca^{2+}. Weiss and Bianchi (1965) and Bianchi

and Bolton (1966) proposed that the small amount of Ca^{2+} entering during an action potential may trigger the further release of Ca^{2+}. However, the observation that single muscle fibres can twitch normally for at least 20 mins in a solution from which free Ca^{2+} has been virtually removed and in which no Ca^{2+} influx is expected (Armstrong, Bezanilla and Horowicz, 1972; Sandow, Pagala and Sphicas, 1975) suggest that the trigger Ca^{2+} is unlikely to be extracellular. Calcium-induced calcium release may still be important if the trigger calcium comes from the internal surface of the T-system membrane, where it is bound at rest and is released on depolarisation (Endo, 1977).

It is not yet known whether calcium release, induced by depolarisation of the SR (i.e. depolarisation-induced calcium release) is important under physiological conditions.

Whatever the mechanism, the end result of the sequence of events beginning with the invasion of the motor nerve terminals by action potentials is an increase in sacroplasmic Ca^{2+} concentration sufficient to activate the contractile component.

METHODS

METHODS

1. Preparation of the Phrenic Nerve Hemi - Diaphragm of the Rat or the Mouse.

Phrenic nerve hemi - diaphragms from albino rats or mice were used in all the experiments.

All diaphragms were dissected according to the method of Bülbring (1946). The animals were killed by a blow on the back of the head followed by dislocation of the neck. The skin and muscle overlying the thorax and abdominal viscera were cut away. The ventral part of the rib-cage was now removed. The left and right phrenic nerves leading to the diaphragm were dissected out and ligatured as far away from the diaphragm as possible (usually 2-3 cm). On removal from the animal, the diaphragm was placed in cooled physiological saline and was then divided into right and left halves by section of the medial tendon and the ribs were trimmed back to the costal margin. Each hemi - diaphragm was immersed in physiological saline for the experiment.

2. Composition of Physiological Saline

The physiological saline used was identical with that described by Liley (1956a) except for a greater dextrose content of 25 millimolar (millimoles litre^{-1}) as compared with 11 millimolar in Liley's solution. This modification was similar to the one made by Krnjević and Miledi (1958) who used 28 millimolar dextrose in an attempt to minimise the transmission block well known in isolated preparations (Adrian and Lucas, 1912; Brown and Harvey, 1938). The solution used, therefore, had the following composition:-

NaCl 137mM, $NaHCO_3$ 12mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, KCl 5mM, NaH_2PO_4 1mM, dextrose 25mM

being made up in distilled water and exerting an osmotic pressure of 315 milliosmoles and having a pH of 7.3 - 7.38 when gassed with 95% O_2 / 5% CO_2.

Variations in $[\ Mg^{2+}\ ]$

In some experiments a $[\ Mg^{2+}\ ]$ of 10^{-4}M was used. These solutions were made by adding 10% of the normal addition of $MgCl_2$, no other changes being made. In some experiments a $[\ Mg^{2+}\ ]$ of 3.5×10^{-3}M was used. These solutions were made by adding 3½ times the normal quantity of $MgCl_2$, again no other changes being made.

Drugs Used

Ambenomium Chloride (Winthrop)

β-Bungarotoxin, 50μg ml^{-1}, 95% pure (Boehringer Mannheim Gmbh)

Ecothiopate Iodide (Phospholine Iodide) (Ayerst). Dry powder containing 12.5mg (0.25% w/v) ecothiopate and 40 mg potassium acetate for eye drop preparation. Usually made up to 10^{-2}M stock solution with 3.2 ml distilled water.

Neostigmine (Inj. B.P.), 2.5 mg ml^{-1} (Roche)

d - Tubocurarine Chloride (Koch-Light)

Pyridine - 2 - Aldoxime Methiodide (Sigma)

3. Recording of the Contractile Response of the Hemi - Diaphragm .

Each phrenic nerve hemi - diaphragm , dissected as previously described, was attached to the recording system (isometric transducer) by either a thread of cotton tied around the central tendon or a length of stainless steel wire (diameter approx. 0.5 mm) hooked through the central tendon. The costal margin of the diaphragm was either attached to a perspex holder or pinned to a wax or Sylgard (a transparent, encapsulating resin) layer, depending on the type of tissue bath used. Each hemi - diaphragm was usually placed under an initial tension of 10g.

The preparations were immersed in physiological saline in one of two types of tissue bath used to record the contractile responses:-

(i) A 'vertical' tissue bath in which the diaphragm was held vertically, tendon uppermost, by a perspex holder. The bath was surrounded by a water-jacket circulated with water maintained at $37^{o}C$ ($\pm$ 0.5) (Fig.A).

(ii) A 'horizontal' tissue bath made of perspex in which the diaphragm was held horizontally with the costal margin pinned to a wax or Sylgard layer at the bottom of the bath. This perspex bath was the same as that used for the electro-physiological experiments and was similar in construction to that described by Boyd and Martin (1956). This bath was of 20 ml. capacity approximately and the physiological saline was made to circulate over the preparation and through a heating coil by a bubble-lift of the O_2/CO_2 mixture used for aeration. The temperature was maintained at $37^{o}C$ ($\pm$ 0.5) by means of an electronic feed-back system. (Fig. B).

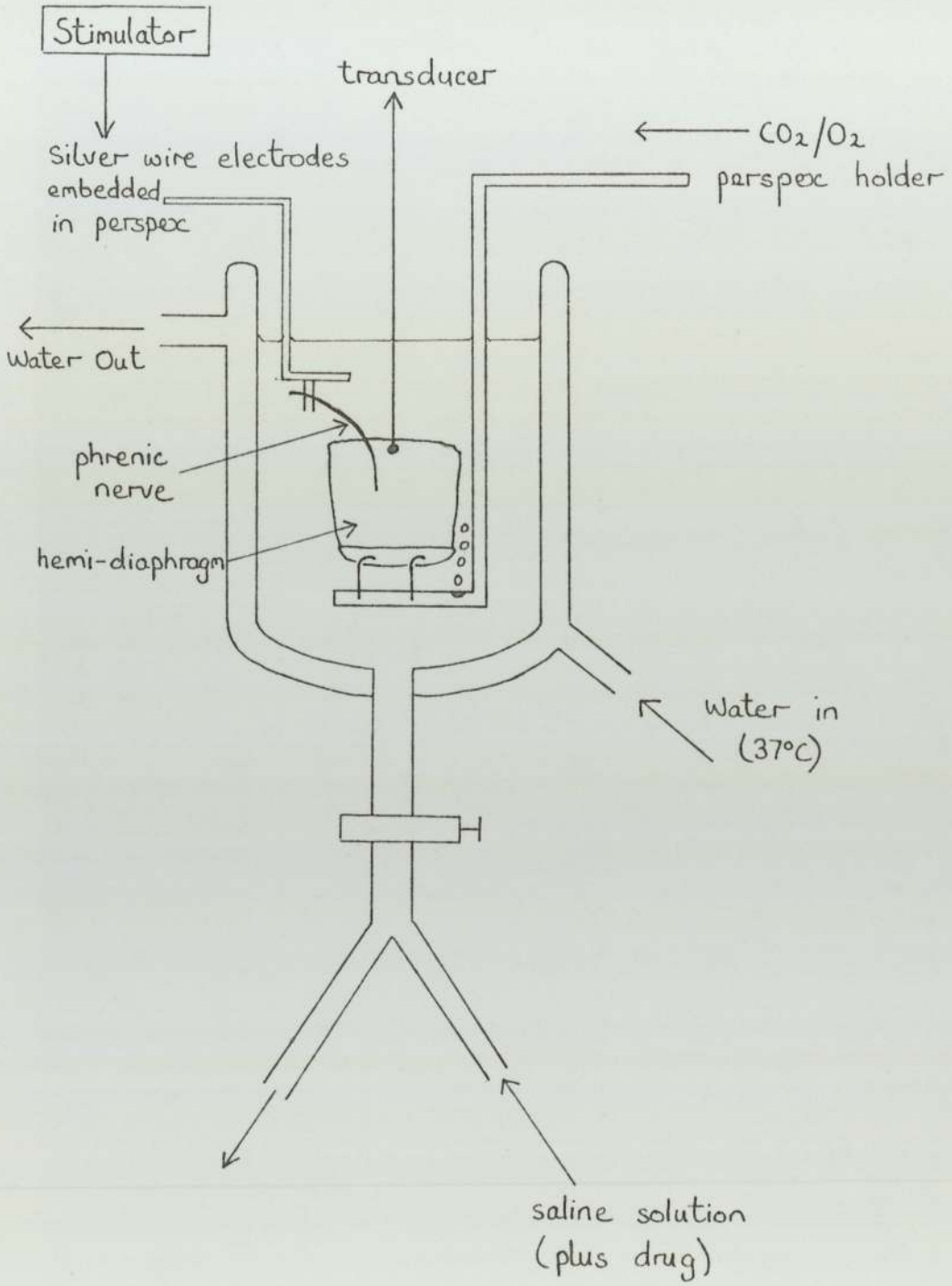


Fig. A 'Vertical' Tissue Bath incorporating a water-jacket (x 1 approx.)

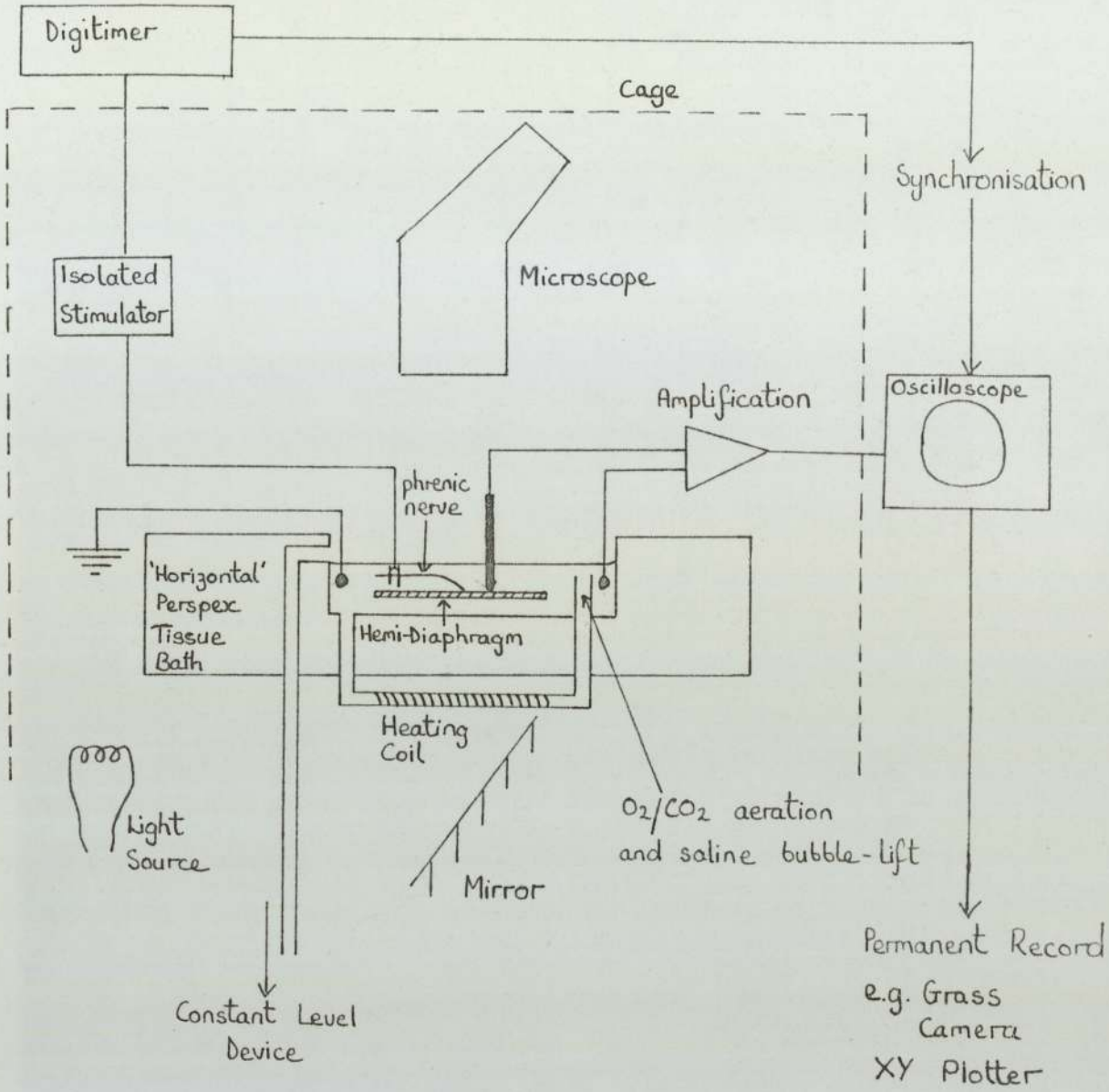
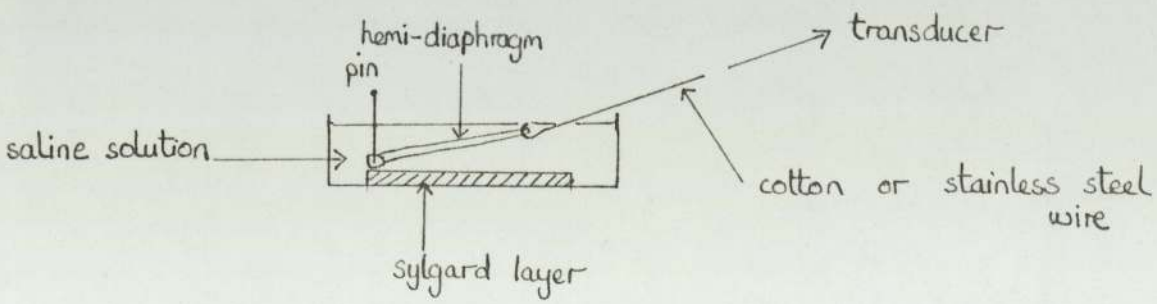


Fig. B Above: Layout of apparatus for electrophysiological recording

Below: Modification of diaphragm position for contraction recording.



In most experiments the physiological saline was added continuously at an approximate rate of 6 ml.min^{-1} and the volume of fluid kept fairly constant by overflow or a suction device. This constant replacement of fluid offset any changes in osmotic pressure by evaporation and also removed protein, lipids, waste-products, etc. released from intact or cut cells of the diaphragm.

Drugs were made up in physiological saline at the required concentration and were brought into contact with the preparation by infusing them through the circulation system. It was possible to achieve a rapid change in the concentration of drugs at the preparation by increasing the flow of the physiological saline to a rate of 30 ml. min^{-1} approximately. Bath temperature was observed to fall by about 2^{o}C only during this period of rapid flow.

Stimulation

The phrenic nerve was stimulated by current pulses derived from a rectangular wave isolated voltage stimulator (Digitimer) through silver wire electrodes embedded in perspex. The stimulator had an output impedance of approximately 10^3 ohms. A pulse of duration 0.05 msecs and of voltage at least five times the threshold, which was usually 1v, was used to stimulate the nerve. The stimulator was triggered by a Digitimer, sometimes via a gated pulse generator. (Fig. B).

Recording and Display of the Contractile Response

This was done in one of three ways:-

(a) by using a Devices UFI Isometric Transducer (Range 0-30g.) which was usually set with a load of 10g. The contractions were recorded on a Devices M2 hot-pen recorder and used to compare the amplitude of responses throughout the experiment.

(b) by using the same transducer and Devices recorder and relaying the signals to a Tektronic Type 564B Storage Oscilloscope and photographing the stored traces with an Edixa 2MTL 35mm Camera. This system was used in early experiments before (c) became available.

(c) by using the same transducer and Devices recorder and relaying the signals to a Gould Advance Digital Storage Oscilloscope OS 4000. The responses could then be played out on to a Bryans XY Plotter. This system allowed for time course analysis of each contractile response. In some experiments a signal averager was used to record spontaneous contractions (See Signal Averaging P.26).

Note:

Although an isometric transducer was used to record tension developed, the preparation did shorten because of the compliance of the intercostal muscle and the ribcage between the hemi - diaphragm and the pins used to fix the preparation to the holder.

4. Electrophysiological Methods

Bioelectrical potentials were recorded using either extracellular or intracellular electrodes. After appropriate preamplification (see below) the bioelectric potentials were displayed on an oscilloscope and permanent records were made.

Apparatus for extracellular recording

Potentials due to current flowing in the extracellular space were led from the preparation by a pair of suitably located electrodes. These electrodes were either

a) a pair of silver wire electrodes, 3.2. x 10^{-4}m in diameter, and Diamel insulated except at the cut end (Johnson and Mathey)

b) a glass micropipette filled with either physiological saline or sodium chloride (4M), continuity between the electrode contents, the bathing fluid and the amplifier being made with Ag/AgCl pellet electrodes (see also p. 20 Intracellular Recording).

The potentials detected with the silver wire electrodes were amplified by either (a) a Tektronix 122 preamplifier which had an input impedance of 10^6 ohms when used differentially, a maximum bandwidth of 0.2Hz - 40kHz (-3dB) and a maximum gain of 10^3 and/or (b) a Devices 3160 AC amplifier which had a maximum bandwidth of 0.16Hz to 10kHz (-3dB).

Signals picked up by high impedance glass micropipettes were amplified by High Performance Operational Amplifiers with input impedance of 10^{11} ohms, maximum gain of 3 x 10^3 and frequency response of 1.3Hz to 30kHz (-3dB). The signals from the above

amplifiers were then normally passed through a Tektronix 5A18N dual-trace amplifier which had an input impedance of 10^6 ohms, a maximum bandwidth of DC to 2MHz (-3dB) and a maximum gain of 5×10^3 and then displayed on an oscilloscope.

Signals due to action potentials in cells were 1 - 10mV in amplitude and signals due to endplate potentials only, had amplitudes of approximately 0.1 - 0.5mV. In some experiments the bandwidth was extended to 0Hz by using a differential DC preamplifier (Fenlow AD55) which had an input impedance of 10^{11} ohms, a set gain of 10^2 and a frequency response of 0 - 30kHz (-3dB). Output impedance was only 10 ohms.

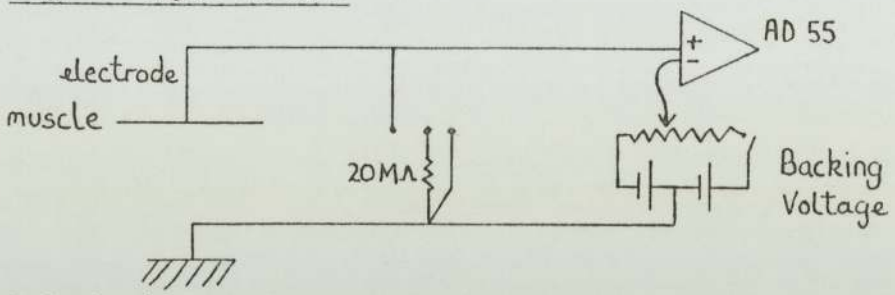
Input to these preamplification stages was through screened cables which were kept as short as possible in order to minimise the input time constant.

Apparatus for Intracellular Recording

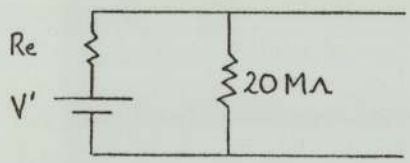
Micropipettes were made from borosilicate glass tubing of external diamater 2mm and internal diameter 1mm (manufactured by Plowden Thompson) by the use of a solenoid-assisted vertical electrode puller (Scientific and Research Instruments Ltd.). The force on the solenoid and the temperature of the platinum wire furnace of the electrode puller could be adjusted so as to produce the required shape and/or resistance of the electrode. Micropipettes were filled with KCl (3M) by injecting into the shank, the KCl being conducted to the tips by capillarity which was aided by the presence of glass wool fibres. The electrode was connected to an AD55 preamplifier (see Extracellular Recording p. 19) with a silver/silver chloride pellet electrode. Satisfactory electrodes had a resistance of

between 7 - 15 Megohms which was calculated from the change in recorded tip potential produced by shorting the inputs through 20 Megohms. Fig. C below shows the input circuit of the apparatus. The Backing Voltage could be adjusted to balance out the tip potentials.

Fig. C Input Circuit



Calculation of electrode resistance



Re = resistance of electrode
V = potential when circuit open
V^1 = potential when circuit connected through 20MΩ

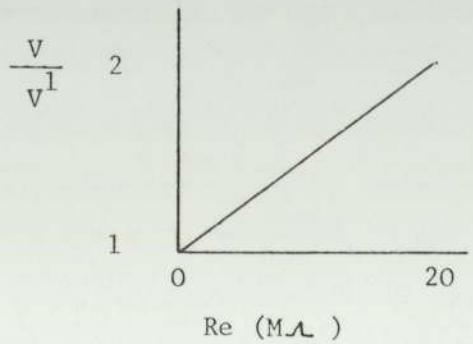
$$V^1 = \frac{20V}{Re + 20}$$

$$\frac{V}{V^1} = \frac{Re + 20}{20}$$

When $\frac{V}{V^1} = 1$, Re = 0 MΩ

When $\frac{V}{V^1} = 2$, Re = 20 MΩ

Plotting the ratio $\frac{V}{V^1}$ against Re



. Knowing $\frac{V}{V^1}$, Re can be measured from the graph

Intracellular recording was used to measure

(i) membrane potentials, using a high impedance digital voltmeter at the output of the AD55 amplifier and

(ii) mepps and action potentials, where the signals from the AD55 amplifier were further amplified (Devices 3160 AC amplifier, see Extracellular recording, p. 19) before display.

Note. In both extracellular and intracellular recording the bath fluid was earthed by means of a silver/silver chloride pellet electrode.

Display and Recording

The recorded signals were displayed in one of five ways:

(a) On a Tektronix D12 dual-beam oscilloscope of input impedance 10^5 ohms. Input was either differential A.C. or D.C. Permanent records of the oscilloscope trace were made on orthochromatic film with a Grass Kymograph camera.

(b) On a Gould Advance Digital Storage Oscilloscope OS4000 of input impedance 10^6 ohms. Permanent records were made on paper using a Bryans XY Plotter.

(c) Membrane potentials were displayed via a Digital Voltmeter.

(d) Permanent records of the averaged eepps and mepps were made on paper using a Bryans XY Plotter.

Ancillary Apparatus

In all methods of electrical recording mechanical stability was essential and, therefore, the muscle-bath (previously described) and the Leitz micromanipulators (used for location of electrodes) were fixed on to a metal platform which rested on spring shock-absorbers. The whole apparatus rested on a strongly constructed table and was surrounded by an earthed cage of wire mesh. Observation of the diaphragm, particularly the endplate region, was by means of a Zeiss Binocular Microscope mounted on a swivel-arm (maximum magnification = 125). Transillumination of the preparation was obtained by reflecting the focussed beam of a microscope lamp with a mirror placed beneath the tissue bath (see Fig. 8).

Electronic Apparatus

The following equipment was used in the elicitation and recording of bio-electrical potentials. As high levels of amplification were involved, care was taken in the construction of the apparatus to minimise the possibility of spurious pick up of A.C. (mainly mains 50Hz) by the amplifiers. All transformers were encased in earthed metal boxes and power to the equipment was supplied through screened cables. Input cables had an earthed screen and connections to the amplifiers were made by U.H.F. plugs. To prevent earth-loop current, all screens were connected to a single ground point.

Stimulator

A devices Isolated Voltage Stimulator was used as described previously. This isolated stimulator minimised the stimulus artifacts produced by earth-return currents.

Synchronisation

Synchronisation of the various pieces of apparatus, for example, the stimulation of the preparation with the triggering of the oscilloscope and camera, was achieved by using a MkIV Devices Digitimer.

Technique for recording extracellularly from the endplate region.

(i) Extracellular Field Potentials

One electrode was positioned as near to the endplate as possible. This was achieved by applying the following criteria to the resultant extracellular field potential obtained after stimulating the nerve.

that (a) the spike in the muscle was recorded as a negative then positive going potential

(b) the rate of rise at the base of the spike was maximal

(c) the latencies between the stimulus artifact, the base and the peak of the field potential were minimal

(d) if possible, a pre-synaptic spike was present. This was only observed when the other criteria above had been satisfied and the electrode was lowered so as to be in contact with the muscle surface.

The electrode however, was generally situated so as to give as simple a shape of potential as possible since extracellular recording necessarily involves recording from more than one muscle cell. The end of the electrode normally did not touch the surface of the preparation but was positioned just clear of the pleural membrane.

The second electrode was positioned away from the preparation and located to minimise the stimulus artifact.

(ii) Extracellular Epps (eepps)

The electrode was positioned at the endplate using the following criteria:

that (a) the amplitude of the epp was maximal

(b) the rate of rise was maximal

(c) the latencies between the stimulus artifact, the base and the peak of the epp were minimal

(d) as previous (see Extracellular Field Potentials p. 24).

In order to record epps, excitation of the muscle was prevented by reducing the size of the epps with dTC ($1 - 2 \times 10^{-6}$M). (For full details see Results p. 199).

In some experiments, eepps were averaged ($\bar{x} = 8$) (see Signal Averaging p. 26).

Technique for recording intracellularly from the endplate region

The tip of the micropipette was placed in the bathing fluid and the backing voltage was adjusted to balance out the tip potentials and the potential between the Ag/AgCl electrodes in the bathing fluid and 3M KCl. This also zeroed the digital voltmeter. The micropipette was advanced until it penetrated a cell as indicated by the rapid development of potential of about -70mV. The usual criteria used to position the micropipette at the endplate were:

that (a) the amplitudes of miniatures were 0.5mV and greater

(b) the rise times of the miniatures were less than 0.5 msecs.

In some experiments, mepps were averaged ($\bar{x}$ = 32) (see below Signal Averaging).

Signal Averaging

Signals were recorded via the signal averager for one or more of three reasons:-

(i) to provide a mean of a variable response

(ii) to improve the signal to noise ratio and thereby to improve the records of responses

(iii) to capture, on a fast time basis, spontaneous events occurring infrequently.

The equipment used was a Datalab DL 102A Signal Averager with facilities for editing and pre-trigger recording. This was used to average stimulus evoked eepps, spontaneous mepps and spontaneous contractions.

Voltage signals derived from amplifiers or a transducer described previously (pp. 19-20) were fed into a variable gain DC amplifier with variable DC offset and then into the averager. The purpose of this variable amplification was to ensure that the signal was of sufficient amplitude, that is, near full scale deflection, so that the instrument was digitising the analogue signal as accurately as possible. Calibration of the output of the averager was made with signals of known amplitude (1g or 1mV),

5. Histochemical Methods

Rat hemi - diaphagms were investigated for the accumulation of calcium ions (Ca^{2+}) in the endplate region using histochemical techniques. Two staining techniques were used:-

(a) for endplate cholinesterase in order, primarily, to determine endplate sites and

(b) for free intracellular Ca^{2+} at the same sites. The procedures were applied, in turn, to alternate serial sections from the same muscle.

After the first part of the experiment, rat hemi - diaphragms were prepared for staining by incubating them in Ca^{2+} - and Mg^{2+} - free physiological saline for periods of up to 15 minutes in order to remove Ca^{2+} and Mg^{2+} from the extracellular space. The muscle tissue was then mounted on a cryostat chuck with O.C.T. embedding medium (Ames) and frozen either with cold carbon dioxide or liquid nitrogen ready for sectioning. Alternate co-planar serial sections, $15 - 25 \times 10^{-6}$m thick were cut with a Bright Freestanding Cryostat incorporating a Cambridge Rocking Microtome. Initially, the sections were freeze-dried overnight before staining using a Virtis Automatic Freeze Drier. Later, it became apparent that there was little difference in appearance after staining between the freeze-dried sections and those air-dried at room temperature. Therefore the latter easier procedure was adopted.

(a) Method for Staining Cholinesterase.

Initially, the ChE was stained by the method described by Koelle and Friedenwald (1949) and modified by Evans (1974). Sections were immersed in physiological saline containing 0.1% $CuSO_4 . 5H_2O$, 0.2% glycine and 5 x 10^{-3}M acetylthiocholine iodide and which had been adjusted to pH 6.5 with a 10% solution of 2 - amino - 2 - methyl-propan - 1 - ol. After 15 min. in this solution at room temperature the sections were rinsed in distilled water and placed in a 1% solution of yellow ammonium sulphide at pH 9 for 5 sec; this was followed by a rinse in distilled water and immersion in 70% ethanol. After counterstaining with methylene blue (0.25% in 70% ethanol) the sections were completely dehydrated with increasing concentrations of ethanol and finally cleared with xylene.

The thiocholine generated as a result of cholinesterase activity is captured by cuprous (Cu^{2+}) ions, precipitating as colourless copper thiocholine. The latter becomes converted to brownish copper sulphide on the addition of yellow ammonium sulphide and this is located near to the enzyme.

However, this method proved to be unsuccessful, the fault being possibly associated with the adjustment of the pH of the incubation medium with 10% 2 - amino - 2 - methylpropan - 1 - ol. The latter was found not to adjust the pH of the medium as it was meant to. In fact, it increased the pH. No explanation for this variation from previous work could be found.

A modification of the method described by Karnovsky and Roots (1964) was suggested by the Neuropathology Department of the Midland Centre for Neurosurgery and Neurology, Smethwick, West Midlands and proved more successful.

In this method, the sites of cholinesterase activity become stained (brown/black) during the incubation itelf and not as a stepwise development as in Evans's modified method. This has the advantage that the development of the stain is under visual control and can be terminated when appropriate. The thiocholine, generated by cholinesterase action, is believed to reduce ferricyanide to ferrocyanide, the latter combining with cuprous (Cu^{2+}) ions to form insoluble copper ferrocyanide. The cuprous ions in the medium are complexed with citrate to prevent formation of copper ferricyanide. The addition of iso-OMPA (below) to the medium is to block the action of butyryl cholinesterase.

Incubation Medium

Acetylthiocholine Iodide 12.5 mg

0.06N (0.82%) sodium acetate 15.8 cm^3

0.1N (0.6%) acetic acid (0.5 cm^3)

0.1M (2.94%) sodium citrate 1.2 cm^3

30mM (0.75%) cupric sulphate 2.5 cm^3

4mM (0.137%) iso-OMPA (tetraisopropylpyrophosphoramide) 0.5 cm^3

5mM (0.165%) potassium ferricyanide 2.5 cm^3

Distilled water 2.0 cm^3

The medium was prepared no earlier than 30 minutes before use by adding to the substrate (acetylthiocholine iodide) the solutions in the order in which they are listed. Distilled de-ionised water was used for all the solutions and the rinsing of glassware. The medium had a clear green colour. Individual solutions were kept in stock in the refrigerator at $4^{o}C$.

Procedure

(i) The air-dried sections were incubated in the above medium at 37°C for periods ranging from 60 - 135 minutes.

(ii) They were then rinsed with 70% ethanol and

(iii) counterstained with 0.25% methylene blue in 70% ethanol for approximately 1 minute.

(iv) The counterstained sections were rinsed with 70% alcohol and then with 95% alcohol.

(v) The sections were then completely dehydrated by immersing in absolute ethanol for two minutes and were

(vi) finally cleared by immersing in xylene, also for two minutes.

(vii) The sections were then mounted in DPX and covered with a cover-slip.

(b) Method for Staining Calcium Ions

Of the various histochemical methods currently available that might have been used for determining cellular calcium the method developed by Kashiwa and Atkinson (1962) and modified by Evans (1974) appeared to be relatively simple, specific and sensitive enough to detect amount of Ca^{2+} entering endplates during prolonged transmitter action whilst preserving cellular morphology.

Incubation Medium

0.4% GBHA - 0.4 g. of Glyoxal bis (2-hydroxyanil) (GBHA) in 100 cm^3 of absolute ethanol.

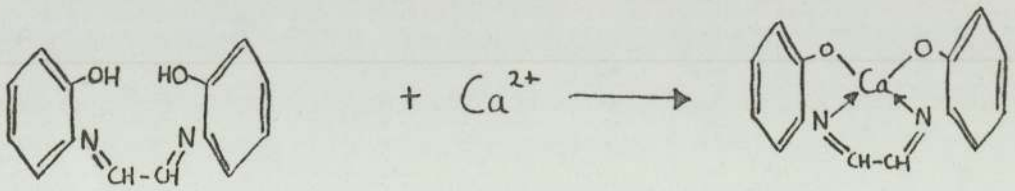
5% NaOH solution - 5 g. of sodium hydroxide in 100 cm^3 of distilled deionised water.

Immediately prior to staining, 16 cm^3 of GBHA solution were added to 7.2 cm^3 of NaOH solution and thoroughly mixed.

Basis of Method

The GBHA molecule, in alcoholic solution made alkaline with sodium hydroxide, chelates with calcium, barium, strontium, cadmium, copper, cobalt and nickel ions forming coloured precipitates. All the coloured GBHA complexes, with the exception of the red Ca^{2+} - GBHA complex, decolourise when immersed in a solution containing carbonate and cyanide. Evans (1974) and myself both decided to miss out this latter stage of the process since it apparently made little, if any, difference to the resultant stain. This was, presumably, because the other above metal cations were not normally present.

Suggested mechanism:



GBHA

- ionic bonds

→ co-ordinate covalent bonds

Procedure:

(i) Sections were flooded with the freshly mixed stain for 3 minutes after which

(ii) they were mixed with 70% alcohol

(iii) they were then counterstained with methylene blue (0.25% in 70% ethanol) for approximately 30 seconds and then

(iv) rinsed in 95% ethanol.

(v) They were finally completely dehydrated by immersing in absolute ethanol for 2 minutes and

(vi) cleared by immersing in xylene, also for two minutes

(vii) the stained sections were then mounted in DPX and covered with a cover-slip.

The presence of free Ca^{2+} was indicated by the reddish granular appearance of the GBHA-Ca^{2+} complex.

Note:

1) It was important that, during the 3 minute period of incubation in the GBHA medium, enough oxygen reached the sections so as to fully develop the stain. This was achieved by draining the medium off the sections, so leaving them exposed to the air for short periods. Blowing on the sections was also found to help.

2) Due to the sensitive nature of this method for staining free Ca^{2+}, water used for preparing solutions and washing up glassware was free, as far as possible, of Ca^{2+}. Distilled, deionised water was used throughout.

Photographic Recording of all Sections

The sections were viewed with a Zeiss standard 18 Microscope under a magnification of x 160 and photographs were taken using Kodak High Speed Ectachrome (Type B - Artificial Light) Film with an Olympus PM 6 Microscope Camera. The exposures ranged from 0.2 - 1.0 seconds, dependent on the amount of stain taken up by, and the thickness of, the sections, and at times between 24 and 72 hours after staining. After reversal developing, the film was mounted as lantern slides for projection and examination. The prints shown in the results (Section IV) were made commercially from the slides.

6. Radioisotopics Methods I - Determination of Endplate Accumulation of Calcium

In order to determine any accumulation of Ca at hemi - diaphragm muscle endplates, the experiments were made with the hemi - diaphragms incubated in ^{45}Ca - labelled physiological saline. After experimentation and the removal of any extracellular ^{45}Ca, the resultant radioactivity will give some indication as to the extent of Ca accumulation in the muscles.

Mouse diaphragms, dissected in the same way as rat diaphragms, but not cut into two halves at this stage, were placed into a known volume of physiological saline, approximately 25 ml. per diaphragm, which contained sufficient $^{45}CaCl_2$ in trace amounts (specific activity of stock solution, 18-22mCi/mg ^{45}Ca ml i.e. 0.8 - 1.0mCi/mmol; The Radiochemical Centre, Amersham) to give reasonable final counts ($3 - 8 \times 10^5$ cpm ml^{-1} physiological saline). This added approximately 0.5µmole of ^{45}Ca to the 2mmoles of Ca already present per litre of physiological saline.

Initial experiments included stimulation of some of the diaphragms via the phrenic nerves. In these experiments diaphragms were pinned out on circular cork discs before immersion in physiological saline. Usually one half of the diaphragm was stimulated, using silver wire electrodes and a rectangular wave isolated voltage stimulator. The frequency of stimulation was 0.02 Hz and the pulses were 0.05×10^{-3}s in duration with a minimum voltage 5 x threshold activation. The other half of the diaphragm was unstimulated.

After incubation in ^{45}Ca under various experimental conditions the diaphragms were rinsed in tracer-free physiological saline. usually for 30 mins, and placed in acetone. After two changes of acetone over a minimum period of two hours. the diaphragms were cut into two halves.

Each hemi - diaphragm was dissected free of the rib-cage and then divided into a junctional region, to include the area 1×10^{-3} m on either side of the intramuscular nerve branches approximately, and a non-junctional region (see Fig. D) in the manner described for the rat diaphragm by Hebb, Krnjević and Silver (1964). Immersion in xylene made it easier to see the intramuscular nerve branches.

The junctional and non-junctional pieces of muscle were placed separately in 20 cm^3 polyethylene scintillation counting vials which were then loosely capped and subsequently placed in a water bath at 60°C for approximately 30 minutes to remove any trace of xylene. The dried muscle samples were then weighed on a Torsion Balance. The weights of the junctional and non-junctional pieces of a single hemi - diaphragm were usually each in the range 1.0 - 3.0 mg. The weighed muscle samples were replaced in the vials and 0.5 cm^3 of concentrated hydrochloric acid was added to each vial and the muscle samples were digested to completion, usually 30 minutes at 60°C or 12 hours at 25°C. Absolute ethanol (2 ml) and either Toluene fluor (10 ml) [PPO (2,5 - Diphenyloxazole), 5g $litre^{-1}$; POPOP (1,4 - Di (2 - (5 - phenyloxazolyl)) benzene), 0.3g litre; made up in toluene] or Bray's fluor (10 ml) [PPO, 4g $litre^{-1}$; POPOP, 0.2g $litre^{-1}$; Napthalene, 100g $litre^{-1}$; Methanol, 100µl $litre^{-1}$; made up in 1,4 - Dioxan] were added to each vial and the radioactivity of the ^{45}Ca was determined by liquid scintillation counting (Beckman

LS 230) at an average efficiency of approximately 30%. Standards which consisted of a known volume of the ^{45}Ca - labelled physiological saline were prepared for each experiment and counted in the same way as the muscle samples.

Calculation of Ca accumulation

The junctional pieces will contain non-junctional parts of muscle cells as well as the endplates. To make allowance for non-junctional uptake, it was assumed that the resultant accumulation of Ca occurred evenly along the length of the muscle cells. A corrected value for the Ca accumulating at the endplates of hemi-diaphragms was obtained by subtracting the value of Ca entering per mg. dry weight of the non-junctional pieces of muscle fibres of the junctional pieces from the total value of Ca per mg. dry weight entering the junctional pieces, that is,

endplate Ca = (endplate calcium + non-junctional calcium) − non-junctional calcium

(endplate calcium + non-junctional calcium): junctional piece of muscle

non-junctional calcium: non-junctional piece of muscle

The result was expressed as n-moles of Ca accumulated at the endplates per mg. of dry muscle.

$$\text{i.e. n-moles of Ca at the endplates } mg^{-1} \text{ dry muscle} = \left[\frac{\text{cpm junctional pieces} - \frac{\text{cpm non-junctional pieces} \times \text{wt. of junctional pieces}}{\text{Wt. of non-junctional pieces}}}{\text{Total Wt. of muscle}} \right] \times \left[\frac{\text{n-moles of Ca in standard (50)}}{\text{cpm standard}} \right]$$

The amount of $^{45}CaCl_2$ added to the physiological saline was negligible (i.e. approximately 10 - 20 p-moles) and, therefore, the total concentration of Ca in the standard volumes (25µl) was assumed to be the normal | Ca | of the physiological saline (i.e. 50 n-moles).

The quantity of Ca accumulating in the non-junctional pieces of the hemi-diaphragms was calculated as follows:

$$\text{n-moles of Ca in the non-junctional pieces } mg^{-1} \text{ dry muscle} = \frac{\text{cpm non-junctional pieces}}{\text{Total wt. of muscle}} \times \frac{\text{n-moles of Ca in standard}}{\text{cpm standard}}$$

Note that (i) the values calculated represent the total Ca accumulated and not only ^{45}Ca accumulation.

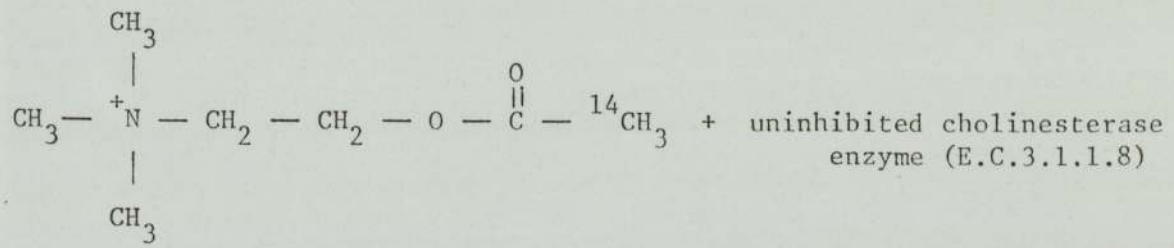
(ii) the results did not, apparently, show any significant differences between left and right hemi-diaphragms and so the latter were not treated separately.

6. Radioisotopic Methods II - Assay of Cholinesterase Activity of Rat Diaphragm Muscle

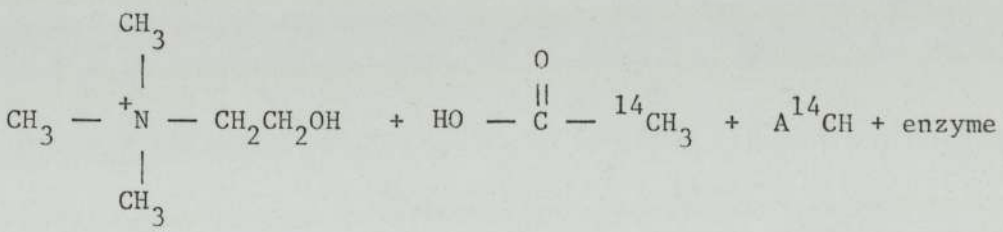
Basis of Method

The ability of homogenates of muscle to hydrolyse acetyl choline (ACh) by the action of the enzyme, cholinesterase (ChE), was determined by the method described by Siakotos, Filbert and Hester (1969).

In this procedure, the amount of hydrolysis of ACh is measured by the quantitative determination of the radioactive acetate product of the hydrolysis of ^{14}C - labelled ACh, viz.

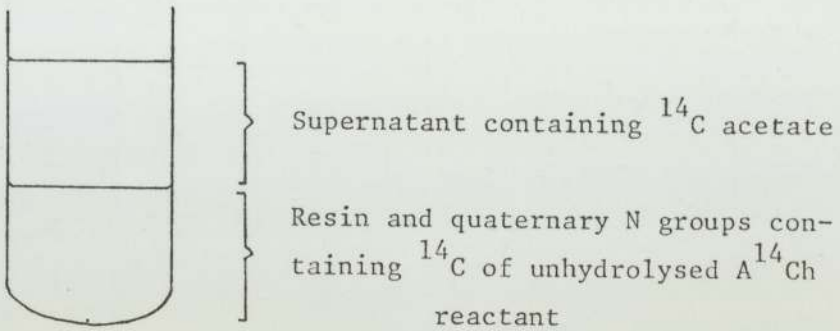


ACh



Choline ^{14}C Acetate Unhydrolysed ACh

The unhydrolysed $A^{14}Ch$ is removed from the reaction mixture by the addition of an Amberlite resin - dioxan mixture which stops the reaction by attracting the quaternary - $\overset{|}{\underset{|}{N^+}}$ - group thus also removing choline from the medium. The free acetyl group, $H - \overset{O}{\overset{\|}{C}} - {}^{14}CH_3$ remains in the supernatant on centrifugation.



The radioactivity of the supernatant is a measure of the amount of hydrolysis per unit time and therefore can be used to determine the activity of the enzyme.

Retrograde Perfusion of Circulation of Diaphragm

This technique was developed by Burgen, Dickens and Zatman (1949) and Paterson (1965). Albino rats were killed as before and after exposing the chest and abdominal cavities, removing the rib-cage and dissecting out the right phrenic nerve leading to the right hemi-diaphragm, a polythene cannula was placed in the thoracic right inferior vena cava pointing retrogradely towards the diaphragm. The hepatic veins and the inferior vena cava below the diaphragm were tied. The complete diaphragm with cannulated right inferior vena cava and right phrenic nerve was removed from the animal and the diaphragmatic blood vessels were flushed out with physiological saline. The diaphragm was placed in a perspex bath containing oxygenated (5% CO_2 in O_2) and continuously circulating physiological saline maintained at 37°C and then perfused with physiological saline for 30 minutes, the saline escaping from the cut arteries at the costal

margin after flowing retrogradely.

The left hemi-diaphragm was dissected free of the perfused right hemi-diaphragm after ligating the interconnecting blood vessels and was removed from the bath. This then acted as a control to measure uninhibited cholinesterase activity. The right hemi-diaphragm was subsequenly perfused with ecothiopate for varying periods. The drug was then washed from the system using physiological saline for 30 minutes before assay for cholinesterase.

Preparation of muscles for assay

Both right and left hemi-diaphragms were treated as follows. The rib-cage and tendons were removed and the wet weight of the remaining muscle was obtained. At this stage, in order to determine the surface area of each muscle, a contact print was made by placing the muscle on to a piece of photographic paper and exposing it to light for a few seconds. From the developed print the outline of the muscle was cut out of the paper and its surface area was calculated by comparing its weight with the weight of a previously determined $1cm^3$ of photographic paper.

Each hemi-diaphragm was now dissected into endplate (junctional) and non-junctional areas (as previously described, see p. 35) and the strips of muscle were weighed. Each strip was divided into 5 or 6 pieces (approximately 2mm x 2mm) and placed in a polythene vial containing $2.5cm^3$ of 0.1% Triton X-100 containing KCl (200mM, $10cm^3g^{-1}$ wet weight). The latter was used to disrupt membrane structure by lipid solubilisation and osmotic shock. The vial was

now kept in ice or a refrigerator. The muscles were then homogenised using a Ten Brock Homogeniser and the homogenates were stored frozen until assayed. This storage procedure had no measurable effect on cholinesterase activity (B.A. Hemsworth, personal communication).

Assay

Incubation mixture :

$A^{14}Ch$ (Radiochemical Centre, Amersham; specific Activity 54μCi/mg or 9.86mCi/mmol) 100μl

Muscle Homogenate 100μl

Buffer Solution (NaCl 17.53g,
NaH_2PO_4 2.62g,
Na_2HPO_4 11.5g,
Triton X-100 10 ml,
per litre of distilled water,
final pH = 7.4) 100μl

The above incubation mixture was placed in a $10cm^3$ graduated test-tube and kept at 37^oC for ten minutes. Blank values were determined in which the muscle homogenate was replaced with distilled water. This would represent any non-enzymatic or spontaneous hydrolysis of the ACh. Hydrolysis was terminated by the addition of 5 ml. of a suspension of Amberlite CG - 120 resin in 1,4 - Dioxan. This was followed by a further 5 ml. of 1,4 - Dioxan. The function of the 1,4 - Dioxan is to act as a solvent for the ^{14}C product, acetic acid, of ACh hydrolysis. The tubes were shaken thoroughly and centrifuged at 2000 rpm for 5 minutes in a bench centrifuge to separate the layers.

5 ml. of the supernatant was removed and placed in a glass counting vial together with 5 ml. of Brays (1960) scintillation fluor. The vials were then counted using a Beckman LS - 230 Liquid Scintillation Counter.

Cpm values for the blanks were subtracted from both the junctional and non-junctional counts, the results of which will represent enzymatic hydrolysis only.

Intracellular ChE of striated muscle fibres has been shown by both histochemical and biochemical methods to be associated with the contractile proteins in the A bands (Karnovsky, 1964), on myosin (Varga, Konig, Kiss, Kovacs and Hegedus, 1955), in the sarcoplasmic reticulum(Barrnett and Palade, 1959), on microsomes, in mitochondria and in the ribonucleoprotein fraction of muscle (Namba and Grob, 1968). It is assumed that this intracellular pool of AChE is distributed evenly along the whole muscle fibre. To make allowance for this intracellular AChE, non-junctional counts, adjusted for junctional weights, were subtracted from junctional counts. This was assumed to give counts

which represented external endplate ChE activity and, perhaps, any internal ChE closely bound to the post-junctional membrane. The values obtained in the absence of any cholinesterase inhibitors were taken to represent the control values, that is, 100% enzyme activity or, alternatively, zero cholinesterase inhibition.

Expression of Cholinesterase Activity

This was to be calculated for each endplate and, therefore, an estimation of the number of endplates per unit weight of hemi-diaphragm muscle was required.

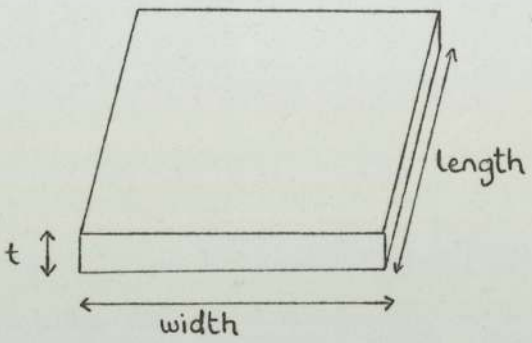
Estimation of Endplates

Assumption (1) : In rat diaphragm muscle

no. of endplates = no. of muscle fibres.

Weight of hemi - diaphragm (m) = volume x density

$\therefore$ m = width x length x thickness(t) x density



Assumption (2) : For rats of the same age and size the mix of muscle fibre types will be the same and so, therefore, will be the density

$\therefore$ m $\propto$ surface area x t.

If t could be estimated and the number of fibres mm^{-2} of the cross section of the diaphragm were known then the total no. of muscle fibres and, therefore, the total no. of endplates in each piece of hemi - diaphragm could be calculated.

$$\text{i.e.} \quad t \propto \frac{m}{\text{surface area}}$$

No. endplates per piece of hemi - diaphragm = t x no. of fibres mm^{-2} x width (mm)

This formula could be applied to pieces of rat hemi - diaphragm from rats of similar age and size on the assumption that the density of, and the extracellular space between, the muscle fibres were the same.

Determination of no. of fibres mm^{-2} of cross-section

A small piece (1cm x 2mm approximately) from each of 3 diaphragms, where t had been estimated as shown previously and where m was known, was embedded in O.C.T. embedding medium (Ames), mounted on a Cryostat chuck and frozen with carbon dioxide.

Transverse sections, 6 - 10 x 10^{-6}m thick, were cut using the Bright Cryostat incorporating a Cambridge Rocking Microtome as previously described (p. 27). The sections were then differentially stained with Haematoxylin and Eosin as follows:

(i) The section was covered with haematoxylin for 5 - 10 minutes

(ii) rinsed with distilled water and placed in tap water for 5 - 10 minutes.

(iii) The excess water was removed and the section covered with an aqueous solution of eosin for 1 minute.

(iv) After careful rinsing with distilled water, the section was mounted in glycerol and covered with a cover slip.

The sections were then photographed, using Ilford FP4 film in an Olympus PM 6 Microscope Camera, while being viewed with a Zeiss Standard 18 Microscope under a magnification of 160. A transparent graticule consisting of 1mm squares had been placed underneath the slide. The number of fibres per mm^2 were counted on enlarged prints obtained from the film negative.

The total number of fibres per endplate strip of muscle were calculated by multiplying the number of fibres mm^{-2} by the width of the muscle strip. Allowance for any shrinkage of the muscle during the procedure did not, therefore, have to be made since the total number of fibres per wet weight of muscle will still equal the total number of fibres after any shrinkage.

7. Statistics

The following statistical procedures were applied at various times to the results:-

(i) Mean used as an estimate of the average value of a number of measurements.

$$\text{Mean} = \frac{\Sigma x}{n}$$

where x represents a single measurement

n represents the number of measurements

and Σ is a symbol (sigma) meaning 'sum of'.

(ii) Standard Deviation (S.D.) used as an estimate of the scatter of the individual measurements which make up the mean.

$$\text{S.D.} = \sqrt{\frac{\Sigma (x^2) - \frac{(\Sigma x)^2}{n}}{n - 1}}$$

(iii) Standard Error of the Mean (S.E.) used as a measure of the variability of the mean. Just as a single measurement is subject to variation, so the average of a small number of measurements will vary in repeated experiments. The variability of the mean will depend on the variability of the individuals but will be less than it. The S.E. provides an estimate of the accuracy (or inaccuracy) of the mean

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{n}}$$

(iv) Independent t values used as an estimate of the two-tailed difference between 2 sets of data (unpaired) using the Null Hypothesis that the treatment applied to one of the samples has had no effect.

$$t\ ind = \frac{\overline{X} - \overline{Y}}{\sqrt{\left[\frac{(Nx - 1)\ S.D.x^2 + (Ny - 1)\ S.D.y^2}{Nx + Ny - 2}\right]\left[\frac{1}{Nx} + \frac{1}{Ny}\right]}}$$

where $\overline{X}$ and $\overline{Y}$ are the means of the two sets of data X and Y.
Nx and Ny are the number of measurements in each set of data.

Independent t values and probabilities were calulated using the above formula on a HP2000 Digital Computer.

If the t values were greater than the designated value for probabilities of less than 0.05, allowing for the latter's variation with degrees of freedom, the differences between the two sets of data was considered statistically significant. Any results not significant were merely regarded as 'not proven'.

RESULTS AND DISCUSSION

SECTION 1

The Effect of Anticholinesterase Drugs on the Contractile Response of the Rat Phrenic Nerve Hemi - Diaphragm Preparation to Nerve Stimulation

Introduction

When mammalian skeletal muscle preparations are treated with anticholinesterases (antiChEs), a maximal stimulus to the motor nerve may be followed by a contraction which is larger than that in the untreated muscle both in amplitude and rise time (Brown, Dale and Feldberg, 1936; Bülbring, 1946). This is because some of the treated muscle fibres sustain repetitive action potentials in reponse to a single stimulus (Brown, 1937a). Hence, the resulting contraction is, in effect, a brief tetanus.

In this section, the effects of three antiChEs on the contractile response of the isolated rat phrenic nerve hemi - diaphragm have been determined. The majority of the experiments, however, were made using the antiChE, ecothiopate iodide.

Results

Experiments were made on the phrenic nerve diaphragm preparation of rats maintained in vitro in physiological saline at 37°C (see Methods p. 14-18). After treatment with ecothiopate (5×10^{-7}M), the contractile responses to nerve stimulation with single maximal pulses at 0.1 - 0.005 Hz were recorded on a slow time base ($0.025 mms^{-1}$) with a Devices Hot-Pen Recorder. The record showed the following apparently time-dependent changes (see Fig.1) :

(a) an initial increase in the amplitude of contraction followed by a decrease. In some experiments, particularly with physiological saline containing a low $[Mg^{2+}]$ (10^{-4}M), a further secondary increase in amplitude was observed before the response finally began to wane.

(b) the development of spontaneous contractions (SCs) and (c) an, apparently, late prolongation of the contractile response, observed as dark thickenings on the trace at the base of each recorded response. These were observed to last several seconds (Fig. 6)and are different to the increase in duration of time course of the earlier major component of the contractile response (early contraction) as seen on a faster time base and described in Fig. 3.

Similar results were obtained following treatment with ambenonium chloride and neostigmine bromide (Table 1). The time course and extent of the changes are detailed in Tables 1 and 2. The data exhibited in Table 2 originates from the same 10 experiments used to measure the various changes due to ecothiopate (5×10^{-7}M) in physiological saline ($[Mg^{2+}]$ 10^{-3}M), as shown in Table 1 .

Experiments made in physiological saline ($[Mg^{2+}]$ 10^{-3}M) showed that the early contraction became enhanced approximately 5 mins

(mean 10 experiments) after ecothiopate (5×10^{-7}M) addition. The maximum amplitude of the early contraction was achieved 13 mins (mean 10 experiments) after ecothiopate and was on average 76% greater than control responses. The late prolongation of contractions began 9 mins after ecothiopate, the prolongation apparently reaching a maximum at 16 mins and disappearing completely by 35 mins (means of 10 experiments).

The development of spontaneous and, apparently, random contractions of small groups of muscle fibres was very variable both in time course and numbers observed (see Table 2). They developed approximately 5 mins after ecothiopate and had waned approximately 15 - 20 mins later in most experiments (i.e. 6 out of 10). These spontaneous contractions, which appeared to be unrelated to nerve stimulation, were sometimes too small to be recorded although they could still be observed through the microscope.

Experiments made in physiological saline ($[Mg^{2+}]\ 10^{-4}$M) showed similar results except that the late prolongation of contractions continued to 79 mins (mean 3 experiments) after ecothiopate before disappearing.

The antiChEs ambenonium and neostigmine both affected the contractile response of rat hemi-diaphragm in a similar manner to ecothiopate, that is, they caused enhancement of the early part and prolongation of the late part of the contractile response and caused the development of spontaneous contractions.

It was interesting to note that with ecothiopate, the late prolongation of contraction began shortly before the enhancement of the early contraction had reached maximum (see Table 1 and Fig. 1). Additionally, the progressive prolongation of the late part of the

contraction frequently coincided with a rapid decrease in the amplitude of the early contraction towards control values. The latter was more noticeable in experiments using $[Mg^{2+}]$ (10^{-4}M). The maximum prolongation of the late part of the contraction at 16 mins ($[Mg^{2+}]$ 10^{-3}M) coincided approximately with the maximum decrease in the amplitude of the early contractions (Figs 1 and 3).

The changes in each contraction after ecothiopate could be elucidated further by recording on a fast time base ($1ms^{-1}$). The time-dependent changes in amplitude, time to peak and time from peak to half-maximum amplitude of the early part of the contractile responses, are detailed in Table 3 and Figs 3, 4 and 5. Representative records of the contractions from which the above parameters were measured are shown in Fig. 2 . It was found that the values of these parameters varied from one hemi - diaphragm to another and in order to apply statistical methods to them, that is, to determine means and standard deviations, it was necessary to determine their values relative to controls.

The extent and duration of the prolonged late part of each contractile response were clearly observed when recorded on a time base of, for example, $0.2cms^{-1}$ (Fig. 6). In some experiments the late part of the contractile response was prolonged by up to 4.5 secs. (These experiments are described fully in Section II on pp. 70-77).

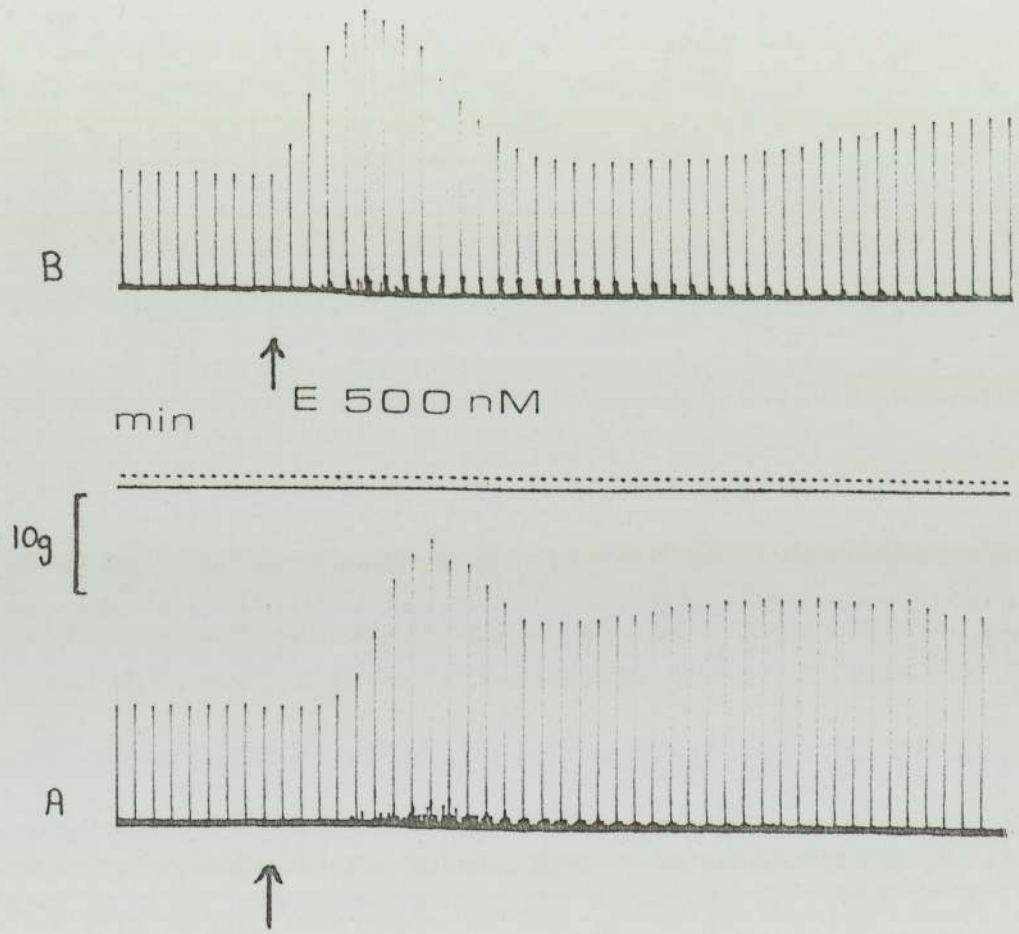


Fig. 1 Rat hemi - diaphragm. 37°C. Physiological saline. Record of contraction to nerve stimulation (0.01Hz). Ecothiopate (5×10^{-7}M) added at arrows.

A $[Mg^{2+}]$ 10^{-3}M

B $[Mg^{2+}]$ 10^{-4}M.

Note the longer time course of occurrence of the late prolongation of contractions (illustrated by the dark thickening of the traces near the base line).

Drug and Experimental Conditons		Latency of Twitch Enhancement (mins)	Latency of maximum amplitude of contraction (mins)	% increase in amplitude of contraction	Late Prolongation of Contractions		Latency of maximum development of prolonged contractions (mins)
					START (mins)	FINISH (mins)	
Ecothipate(5×10^{-7}M)	mean	5.6	13	76	9.2	35	16.2
($[Mg^{2+}]$ 10^{-3}M)	S.D.	2.2	2.9	38.8	1.7	8.6	2.1
10 expts.	range	1-7	9-20	30-136	7-12	28-55	13-19
Ecothiopate(5×10^{-7}M)	mean	1.5	11	99	10	79	18
($[Mg^{2+}]$ 10^{-4}M)							
3 expts.	range	1-2	9-16	92-108	8-12	70-90	18-19
Ambenonium (5×10^{-7}M)							
($[Mg^{2+}]10^{-3}$M)		6	17	117	11	45	17
* 1 expt.							
Neostigmine(4×10^{-7}M)							
($[Mg^{2+}]$ 10^{-4}M		1-2	3	90	6	140 +	20
* 1 expt.							
Ecothiopate(5×10^{-7}M)	mean	1.3	5	59	5	84	13
($[Mg^{2+}]$ 10^{-4}M)							
* 3 expts.	range	1-2	4 - 6	45-69	3-7	60-100	12 -14

* Physiological saline and drug solutions not continuously added to tissue bath

Table 1

The time courses of the changes in the contractile response brought about by various anticholesterases. Drugs added at 0 mins. Frequency of nerve stimulation, 0.01 or 0.017Hz.

Drug and Experimental Conditions	Time course of occurrence of spontaneous contractions (SCs)	
	Experiment	(x) indicates the approximate number of SCs observed
Ecothiopate (5×10^{-7}M) ($[Mg^{2+}]$ 10^{-3}M) 10 expts	(i)	none recorded
	(ii)	21 - 26 (3)
	(iii)	7 - 11 (3)
	(iv)	11 - 21 (9)
	(v)	6 - 13 (15)
	(vi)	10 - 17 (13)
	(vii)	6 - 46 (40+)
	(viii)	8 - 46 (65+)
	(ix)	none recorded
	(x)	none recorded

Table 2. The time course of the ecothiopate-induced spontaneous contractions in isolated rat hemi - diaphragm. Nerve stimulation 0.01 or 0.017Hz. The experiments used to compile the data were the same as those used in Table 1, i.e.

ecothiopate (5×10^{-7}M) $[Mg^{2+}]$ (10^{-3}M)

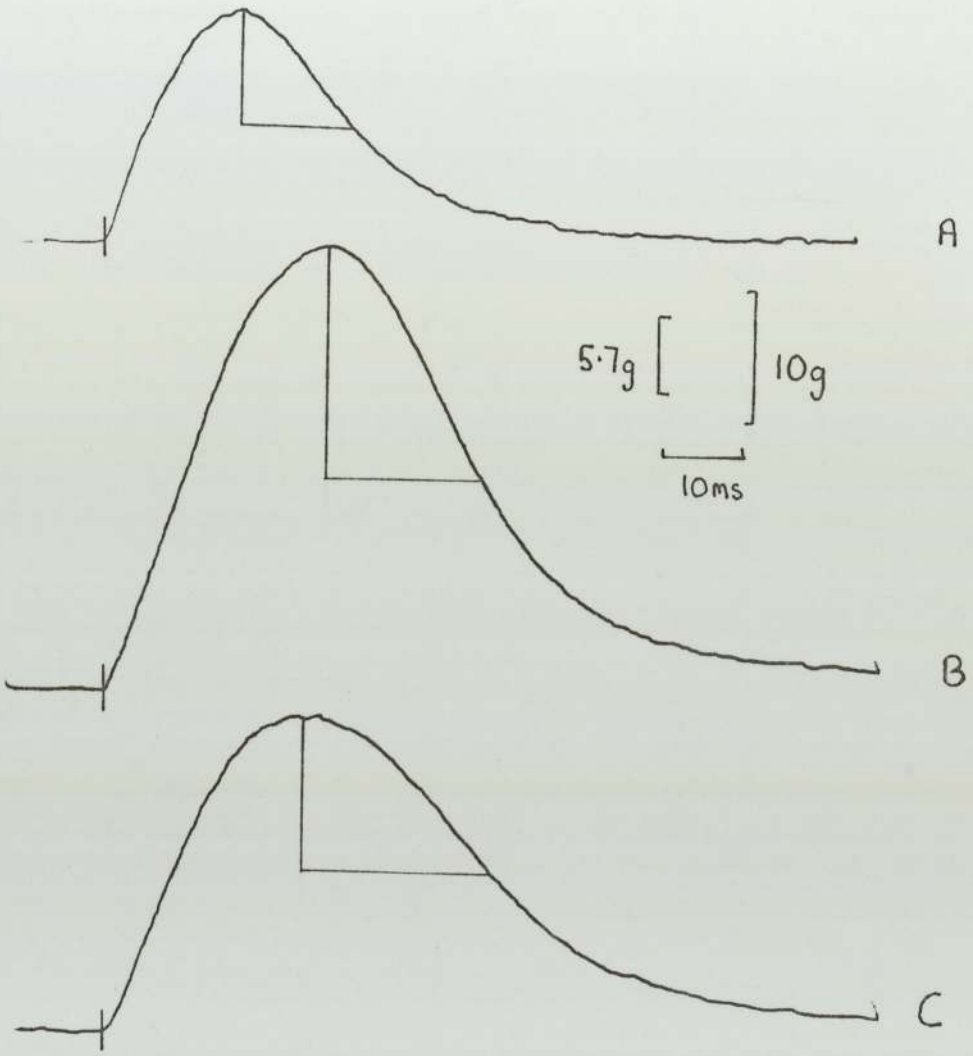


Fig. 2

Representative records of the contractions from which the amplitude, time to peak and time from peak to half-maximum amplitude were measured (see Table 3). Records made on paper using an XY Plotter. Calibration, 1cm = 10ms, 5.7g.

A. Control Response to nerve stimulation

B. Response 11 mins after ecothiopate (5×10^{-7}M). Note the obvious increases in amplitude, time to peak and time from peak to half-maximum amplitude.

C. Response 23 mins after ecothiopate. Note the reduction in amplitude and time to peak but in this experiment, a slight increase in the time from peak to half-maximum amplitude. The latter usually had waned by this time.

Time after ecothiopate (mins)	Amplitude of Contraction RTC		Time to Peak RTC		Time from Peak to half-maximum amplitude RTC	
	Mean	S.D.	Mean	S.D	Mean	S.D.
0	1.0	0	1.0	0	1.0	0
5	1.01	0.05	1.11	0.09	1.40	0.14
6	1.04	0.06	1.16	0.13	1.58	0.28
7	1.12	0.13	1.29	0.20	1.60	0.35
9	1.34	0.25	1.40	0.31	1.74	0.33
11	1.51	0.40	1.41	0.24	1.37	0.32
12	1.49	0.41	1.37	0.26	1.34	0.26
13	1.42	0.42	1.35	0.21	1.37	0.28
14	1.44	0.39	1.29	0.22	1.29	0.18
15	1.39	0.31	1.30	0.22	1.21	0.18
19	1.21	0.22	1.28	0.19	1.31	0.18
20	1.21	0.23	1.23	0.17	1.32	0.14
21	1.21	0.25	1.22	0.19	1.32	0.17
23	1.22	0.25	1.27	0.20	1.32	0.20

Table 3.

Rat hemi-diaphragm in physiological saline ($[Mg^{2+}]$ 10^{-3}M) at 37°C. Amplitudes, times to peak and times from peak to half-maximum amplitude of contractions. Nerve stimulation 0.017Hz. Means of 6 experiments (Relative to Controls,RTC) and Standard Deviations. Data plotted against time after ecothiopate in Figs. 3, 4 and 5.

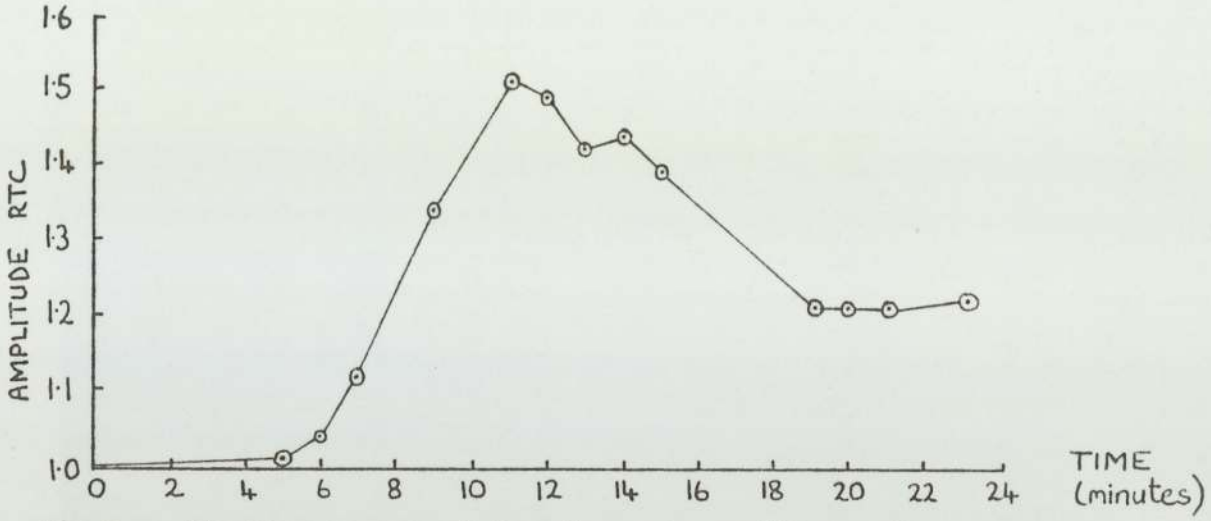


Fig.3. The time course of the change in amplitude of contraction. Values are relative to controls and are the means of 6 experiments.

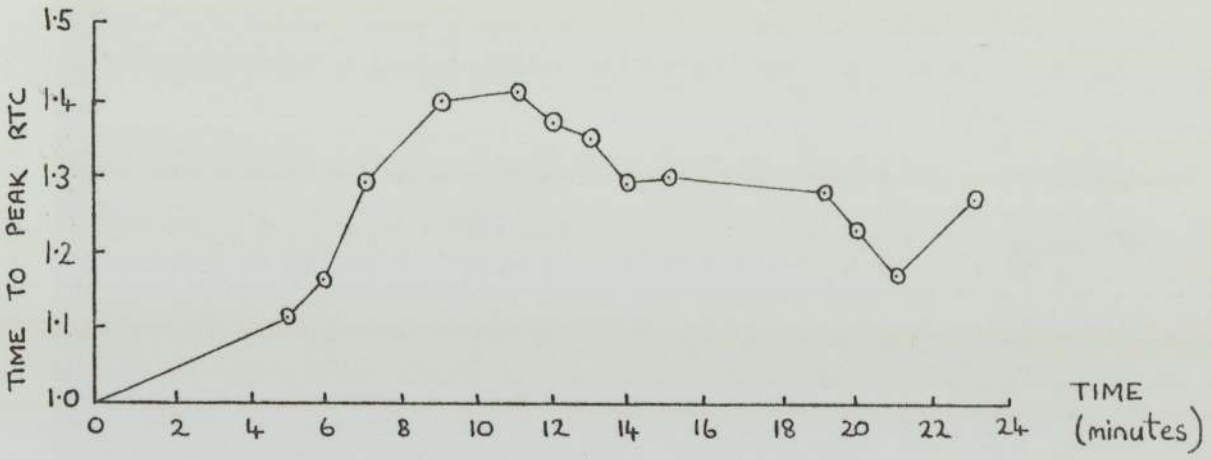


Fig. 4. The time course of the change in time to peak of contraction. Values are relative to controls and are the means of 6 experiments.

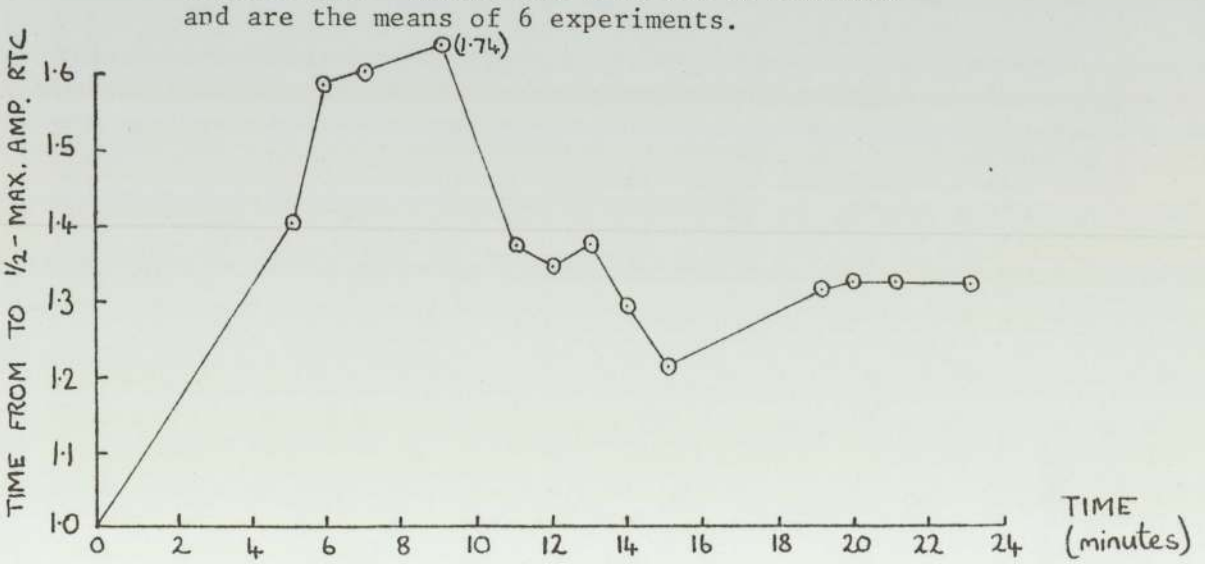


Fig. 5. The time course of the change in time from peak to half-maximum amplitude of contractions. Values are relative to controls and are the means of 6 experiments.

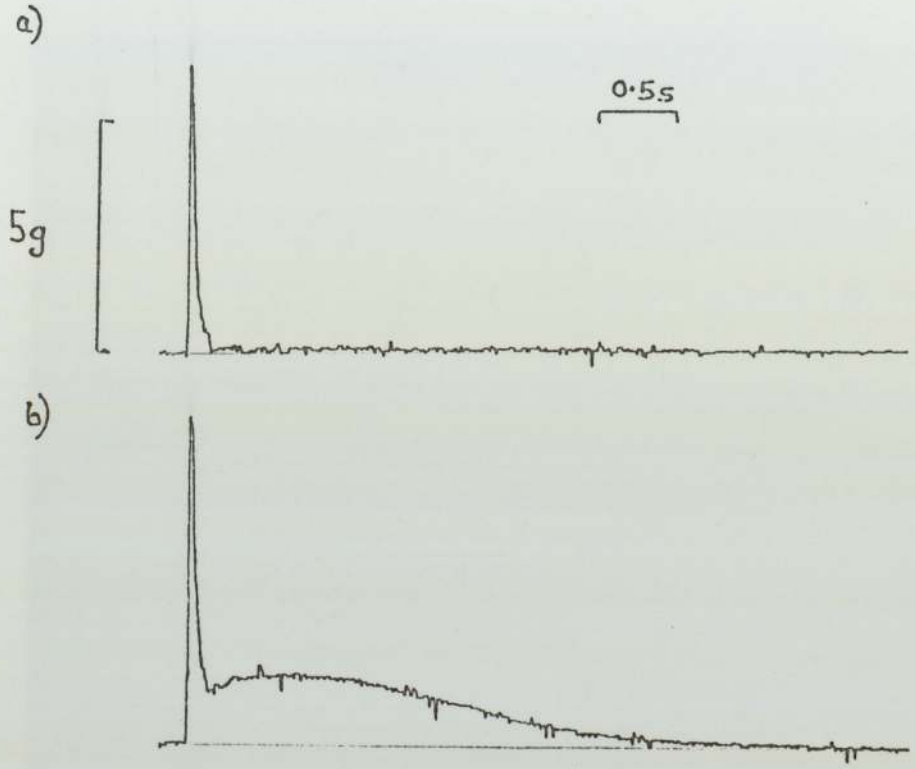


Fig. 6

Rat hemi-diaphragm. 37°C Physiological Saline. Contractile response to nerve stimulation at 0.0056Hz. Records were made using a storage oscilloscope and an XY Plotter. Initial tension 10g.

a) Control Response. Note the time course of the contraction (approx. 0.15s).

b) Response 21 min. after ecothiopate (5×10^{-7}M). Note the enhanced amplitude of contraction followed by the prolongation of the response (approx. 3.5s).

Discussion

Since increases in the amplitude and time course of the early part of the contractile response were observed in the presence of the antiChE, ecothiopate, beyond that produced as a result of supra-maximal nerve stimulation only, it was probable that the response to the latter resulted from a sub-maximal transmitter - receptor interaction. It would seem reasonable to conclude that the maximum amplitude of the early contraction achieved with ecothiopate coincided with maximum transmitter action in the neuromuscular junction under the conditions of these experiments. Assuming ecothiopate acted, at this concentration (i.e. 5×10^{-7}M), primarily to inhibit functional ChE, that is, that ChE terminating transmitter action, then the subsequent increase in transmitter action was believed to be due to an intensification and prolongation of transmitter action.

In effect, the inhibition of the enzyme normally responsible for terminating transmitter action, would allow for suprathreshold transmitter activity to persist at the endplate longer than the muscle refractory period and thus initiate further (repetitive) action potentials (Brown et al., 1936). The increase in amplitude of the early contraction above controls (Fig.1), presumably, reflected the amount of repetitive firing in the muscle cells, the increase in amplitude being proportional to the amount of repetitive activity in the whole population of muscle fibres comprising the hemi - diaphragm.

Theoretical basis of a possible interpretation of the contraction of muscle in the presence of antiChE.

It is assumed that 1) the overall contraction of a muscle is the sum of the contractions of its individual fibres and 2) the individual fibres are arranged into groups or motor units.

If, for simplicity, it is considered that one motor unit comprises 5 muscle fibres, indirect stimulation via the motor axon will elicit similar muscle action potentials and subsequent contractions in all 5 fibres (Fig 7 a). However, in the presence of an antiChE, more than one muscle action potential may result due to prolonged transmitter action. The number and frequency of repetitive muscle action potentials being, at least, partially a post-synaptic event and, therefore, dependent on the individual muscle fibres, will, thereby, lead to variation in the contractile response of the individual fibres and, therefore, the motor unit and, consequently, the whole muscle. Five hypothetical responses to 1 - 5 repetitive action potentials (very rarely more than 5 observed, Ferry, personal communication) are shown in Fig. 7 (b - e).

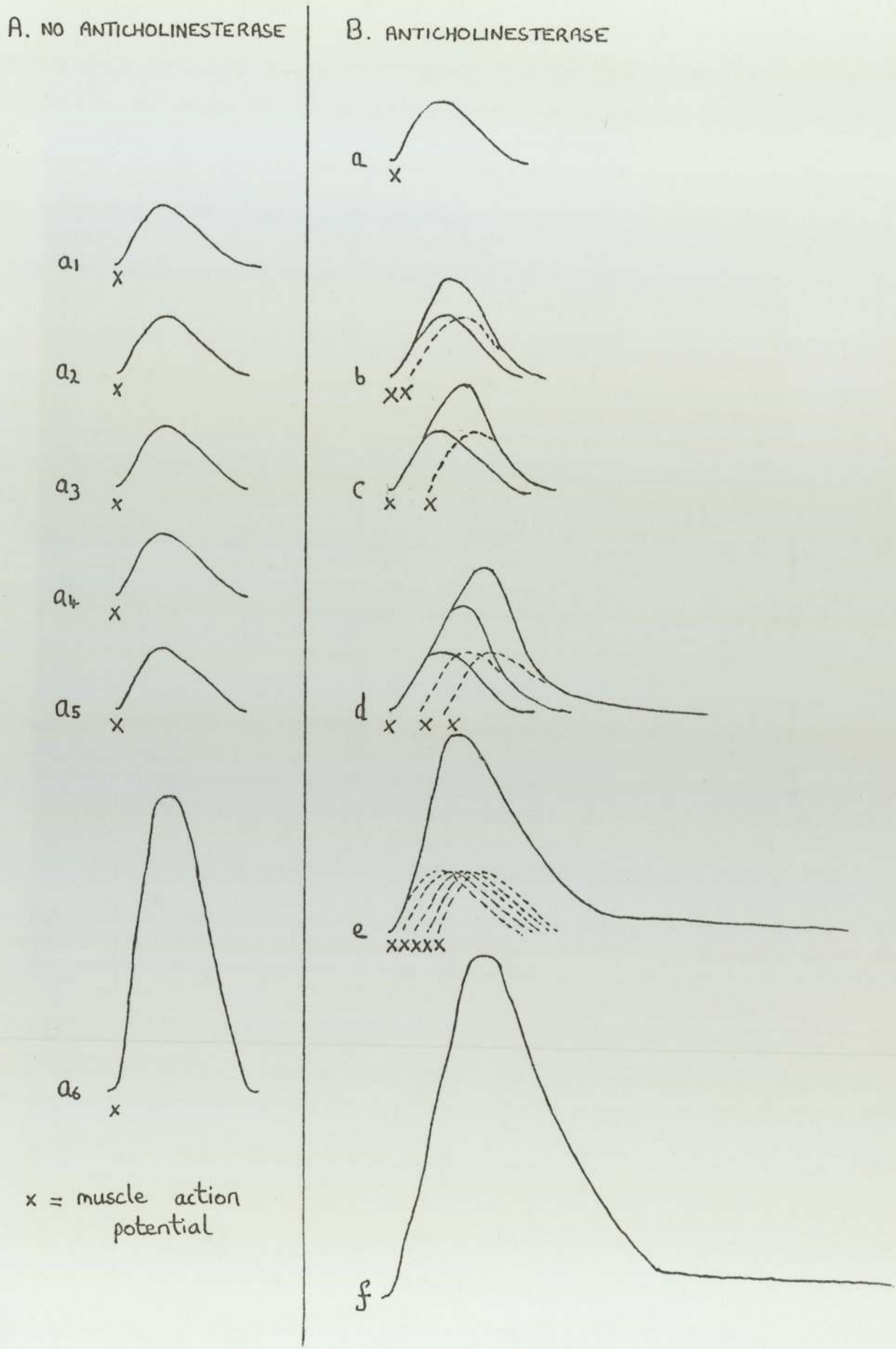
It is assumed possible that any one of the individual fibres can respond in any one of the five ways illustrated at any one time.

The overall contraction of the whole muscle at any one time is considered to be a mix of the various states of response individual fibres can make.

From Fig. 7 , it is suggested that

1) in the absence of an antiChE, the contractile responses of individual fibres would resemble state a.

2) subsequent amplitudes of the early contraction, in the presence of an antiChE, reflect the number of repetitive action


A. NO ANTICHOLINESTERASE
B. ANTICHOLINESTERASE
a1
a2
a3
a4
a5
a6
a
b
c
d
e
f
x = muscle action potential

potentials and that increases in amplitude would involve the transition of individual fibre responses from state a towards e. 3) times to peak and times from peak to half - maximum amplitude of the early contraction reflect both number and frequency of repetitive action potentials and that increases in both times would also involve the transition of individual fibre responses from state a towards e. A decreasing frequency of repetitive action potentials with time after drug addition has been observed with ecothiopate (Ferry, unpublished) and diisopropylfluorophosphonate (DFP) (Van der Meer and Meeter, 1956a).

Ecothiopate caused increases in the amplitude, the time to peak and the time from peak to half - maximum amplitude of the early contraction achieving the maxima at approximately 11 - 13 mins, 11 mins and 9 mins respectively after treatment (Table 3 and Figs. 3, 4 and 5). It is suggested that these broadly similar maximal times coincide with the majority of individual fibre early contractions exhibiting states b, c, d and e. The summed response would reflect a mix of these states (Fig. 7).

Maximum amplitudes were not maintained for more than 1 or 2 mins and rapidly diminished in size, but never back to control size (in physiological saline with $[Mg^{2+}]$ 10^{-3}M), reaching a post - ecothiopate minimum at approximately 19 mins after ecothiopate. Similar observations were made by Barnes and Duff (1953) using paraoxon (4×10^{-7}M), Burgen, Keele and Slome (1949) using tetraethylpyrophosphate (TEPP) and Van der Meer and Meeter (1956a) using DFP all on the isolated rat hemi - diaphragm preparation. Times to peak and from peak to half-maximum amplitude were also not maintained, diminishing to post - ecothiopate minima at approximately 21 mins and 15 mins respectively. It is suggested that the

diminishing enhanced amplitude, time to peak and time from peak to half-maximum amplitude of the early contraction reflect a transition in the majority of the individual fibre contractile responses from states e back towards a, that is, a reduction in the frequency and number of repetitive action potentials in the whole population.

The observation that amplitudes, times to peak and times from peak to half-maximum amplitude of early contractions apparently always remained above controls suggests that some muscle repetitive activity was maintained throughout the time courses of the experiments. Presumably, the majority of the individual fibre responses resemble states above a, after ecothiopate. Repetitive activity in muscle fibres waxes and wanes but some cells usually show repetitive spikes even 1 hr after ecothiopate (Ferry, unpublished).

Further Discussion

The other apparently time-dependent changes in the contractile response, that is, the development of spontaneous contractions and the late prolongation of contractions were also believed to be due to the antiChE action of ecothiopate resulting in the prolongation of transmitter action. Barnes and Duff (1953) also observed irregular twitchings of the diaphragm following paraoxon treatment (4×10^{-7}M) and noted them to diminish as the indirect contractile responses returned to control size. However, the number and time course of occurrence of spontaneous contractions observed with ecothiopate varied considerably from one hemi-diaphragm to another (Table 2) and did not always wane in a time corresponding to the decrease in amplitude of the indirect early contraction.

Spontaneous contractions arising as a consequence of ecothiopate action might be generated as a result of a post-

synaptic event, for example, increased excitability of a number of muscle fibres perhaps caused by the intensification of transmitter action. Alternatively, spontaneous contractions might be a consequence of a pre-synaptic event, for example, by excitation of the nerve terminal or some other action culminating in a synchronised release of transmitter quanta, perhaps resulting in multiquantal (the so-called 'Giant') mepps or just a number of mepps being released in close proximity. In any case, the resultant post-synaptic transmitter action would become prolonged as a result of ChE inhibition, the normally sub-threshold spontaneously released transmitter becoming supra-threshold in certain groups of endplates. Such excitation of the pre-synaptic membrane in the course of antiChE action, that is, the occurrence of antidromic backfiring following single stimuli to the nerve has been well documented (e.g. Masland and Wigton, 1940) and reviewed recently (Miyamoto 1978). That ecothiopate leads to such backfiring has been confirmed by Morrison (1977). It is speculated that spontaneous contractions reflect synchronous contractions involving one or more motor units and are brought about by the re-excitation of nerve terminals, perhaps as a result of prolonged synaptic transmitter action, causing antidromic firing to spread to the other terminals of the motor units, by axon reflex action, thereby recruiting the entire motor units and subsequently resulting in further excitation of these motor units. Morrison (1977) found that with ecothiopate (5×10^{-7}M), repetitive firing in the nerve developed after about 5 mins and waned completely by about 20 - 25 minutes after ecothiopate application. This time course agrees well with the time course of spontaneous contractions in the majority of the experiments. It, therefore, seems likely that spontaneous contractions of motor units were caused by axon reflex excitation initiated by a pre-synaptic action of the

prolonged transmitter action.

The late and greatly prolonged contraction (up to 4.5s) observed after ecothiopate may correspond to the "after-contractures" observed by Riesser (1921) in some of the skeletal muscles of the rabbit following the indirect contractions in the presence of physostigmine. He found that single contractile responses of the semitendinosus muscle were followed by residual muscle shortening of several seconds duration (see Final Discussion).

In my experiments, the inconsistency in the time course of the time from peak to half-maximum amplitude of the early part of the contractile response (maximum at 9 mins) and the late prolongation of the contractions (maximum at 16 mins) confirms the observation (Fig. 6) that the prolongation involves only the final part of the contraction and by consequence, is not directly attributed to the repetitive firing in the muscle fibres, since this presumably has waned considerably by this time. This waning of repetitive firing has been used to explain the accompanying decrease in the amplitude of the early part of the contractile response which, itself, has frequently coincided with the progressive development of the late prolongation of the contractions. In addition, once maximal prolongation of the late part of the contractile response had apparently been achieved, there was no further decrease in the amplitude of the early part of the contractile response and, indeed, in some experiments, the amplitude had apparently increased coincident with the waning of the late prolongation of the contractile response (see also p.232). These observations lead to speculation that, if the late prolongation is due to prolonged transmitter action as is believed, then the waning of muscle repetitive firing and, subsequently, the amplitude of the

early part of the contractile response, might be due to a reduced sensitivity of the post-junctional membrane to further transmitter action. However, if the membrane was in some form of desensitised state, it is difficult to envisage the late part of the contractile responses becoming prolonged as a result of persistent transmitter action.

Alternatively, ecothiopate action may eventually lead to a reduction in transmitter release which would, presumably, effectively lead to a reduction in muscle repetitive firing and, subsequently, a reduction in the amplitude of the early part of the contraction. This would also, however, surely lead to a shortening in the prolongation of the contraction.

Experiments made in physiological saline ($[Mg^{2+}]$ 10^{-4}M) showed fairly similar results except that the late prolongation of contractions continued to 79 mins after ecothiopate before disappearing. It is difficult to explain how the lower $[Mg^{2+}]$ would bring about this change in prolongation since Mg^{2+} could be exerting its effects pre- and/or post-synaptically and, indeed, may be directly interfering with ecothiopate's action in inhibiting ChE action (see later discussion, p. 134).

One of the effects of a reduced $[Mg^{2+}]$ would be to increase transmitter release slightly (Hubbard, Jones and Landau, 1968) in the presence of an antiChE, e.g. ecothiopate, this would presumably lead to an even further prolongation of transmitter action in the synapse. It is possible, therefore, that this further increase in prolongation of transmitter is responsible for extending the time course of occurrence of the late prolongation of contractions. However, the waning of the amplitude of the early contraction following maximum enhancement was even more extensive in $[Mg^{2+}]$ (10^{-4}M) -

physiological saline.

It was thought that the progressive changes in the contractile response as a result of ecothiopate action were due to a progressive potentiation of transmitter action resulting from differences in ChE inhibition. However, it was also possible that the changes were due to the adaptation of the preparation to a higher synaptic transmitter concentration following substantial cholinesterase inhibition. This is discussed further following experiments made to chart a progress curve after cholinesterase inhibition (Section IX).

After the initial experiments with a number of anticholinesterases it was decided to continue experimentation with ecothiopate only. Ecothiopate was chosen as it was in use in the laboratory because (a) it was an irreversible inhibitor which would have allowed for the possibility of determining acetylcholinesterase inhibition by biochemical methods obviating the dissociation of the inhibitor from the enzyme.

(b) it appeared to have the greatest and most interesting effect in changing the contractile response. The enhancement of contraction and the spontaneous activity had been well documented. The late prolongation of each contraction was a new observation which was to be the subject of further study.

Summary

AntiChE drugs have been shown to evoke a complex series of changes in the contractile response of rat hemi - diaphragm to low frequency nerve stimulation (0.01 - 0.017Hz). These changes, apparently time - dependent, were an enhancement of the early part and a prolongation of the late part of the response together with the development of a variable number of spontaneous contractions. These changes have been suggested to be due to the antiChE action of the drugs resulting in a prolongation and intensification of transmitter action. The increased action of transmitter on (i) the post - synaptic membrane is believed to cause the repetitive firing responsible for the enhancement of the early contraction, (ii) the pre - synaptic membrane is believed to cause the antidromic firing in the nerve resulting in the axon reflex excitation of motor units and, subsequently, spontaneous contractions and (iii) the post- and/or pre- synaptic membranes is believed to cause the late prolongation of contraction.

SECTION II

The Visualisation of the Prolonged Part of the Contractile Response

Introduction

The late prolongation of the individual contractions, described in Section 1, could be observed with the stereomicroscope (see Methods p. 16), when using the apparatus described in Fig. 8 . The prolongation of each contraction appeared to be centred around the endplate region of the hemi-diaphragm. This section includes a description of what was seen and details attempts to quantify the area of muscle exhibiting prolongation.

Results

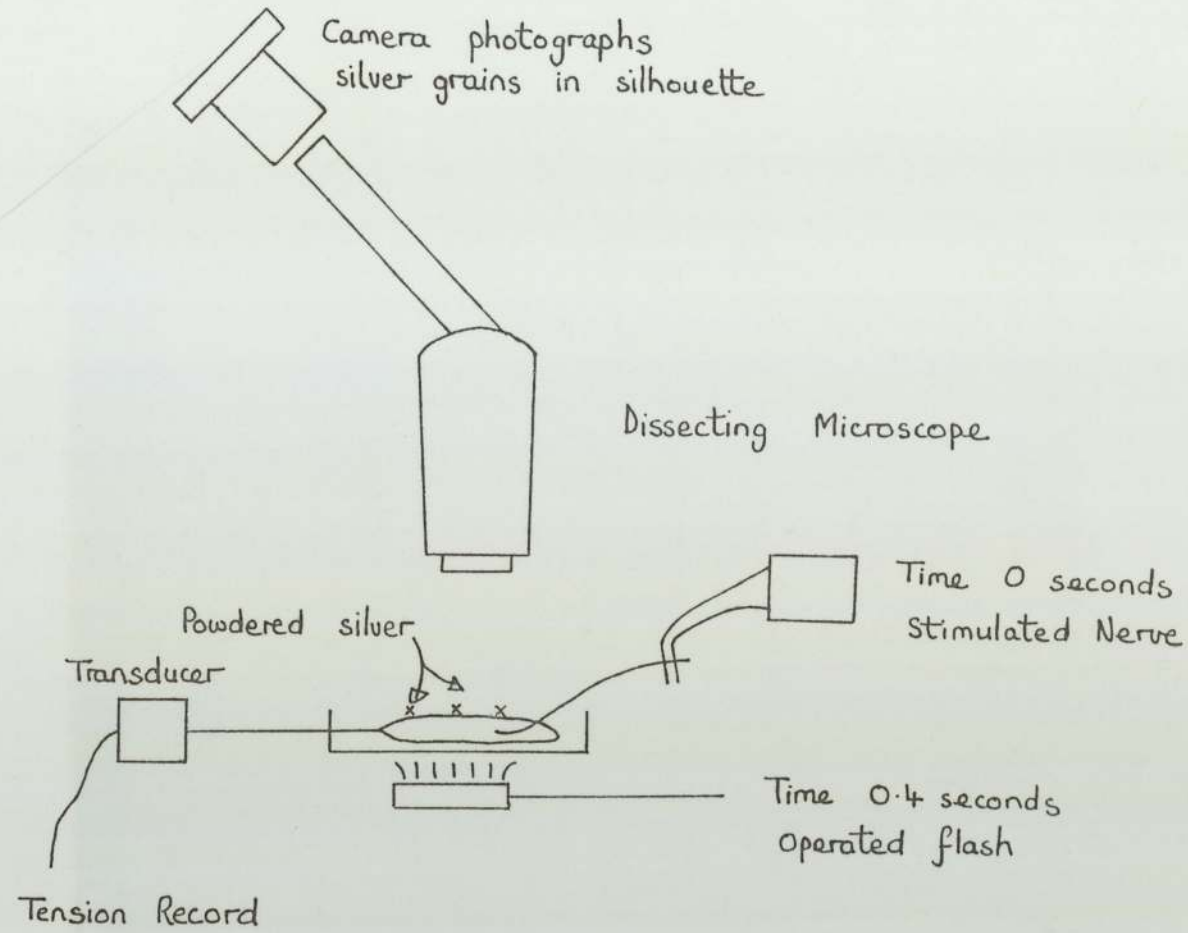
Observation of a hemi - diaphragm muscle with a stereomicroscope after stimulation of the nerve in the presence of ecothiopate (5×10^{-7}M) showed it to shorten very slightly at first even though it was attached to an isometric transducer. This was followed by the relaxation of the muscle except for a small region either side of the nerve running through the muscle (i.e. the endplate region). This region not only remained in a contracted state for some considerable time after the relaxation of the remainder of the muscle (up to 4.5 secs at the peak) but was observed to shorten so developing even more tension following the relaxation of the rest of the muscle, reaching a maximum approximately one second after the start of the initial contration (see Fig. 6). To try and record this localisation of the prolonged contraction in the endplate region the following method was employed. Silver grains were sprinkled on to the surface of the hemi - diaphragm to which they adhered. A camera was fitted on to the microscope and an electronic flash gun was placed under the tissue bath so as to be able to illuminate the grains. This system (see Fig. 8) allowed photographs to be taken of the hemi - diaphragm before (i.e. at rest) and a set time after stimulation of the phrenic nerve in the presence of ecothiopate (5×10^{-7}M) (see Fig. 9). Note that in the absence of drug the muscle fibres return to their resting state by approximately 0.15s after stimulation of the nerve

In Fig 9 photograph A shows the hemi - diaphragm in the resting state. The muscle fibres running top to bottom, a blood vessel and the phrenic nerve entering the diaphragm can be seen in silhouette. Since each silver grain has a characteristic shape it was possible to locate individual grains, for example, those enclosed by circles and

indicated by arrows.

Photograph B has been 'trimmed', by locating the same silver grains in each photograph, to show the same region of muscle 0.4s after stimulation of the nerve in the presence of ecothiopate. Photograph B is somewhat shorter than photograph A indicating that at least part of the muscle is in a contracted state. The photographs clearly show that in the region of the endplates the grains are closer together 0.4s after nerve stimulation in the presence of ecothiopate compared with the resting state whereas, in the regions away from the endplates, the grains remain at resting distance.

Plotting the distance between selected grains along the length of certain muscle fibres 0.4s after stimulation in the presence of ecothiopate, l_s , over the resting distance, l_r , against distance from the endplate region, 0, (see Fig 9) shows that the resultant prolonged contraction is, indeed, localised to approximately 1mm either side of the endplate region of the muscle. Fig. 10 shows the results of a number of experiments confirming this measurement.


Camera photographs
silver grains in silhouette
Dissecting Microscope
Time 0 seconds
Stimulated Nerve
Powdered silver
Transducer
Time 0·4 seconds
Operated flash
Tension Record

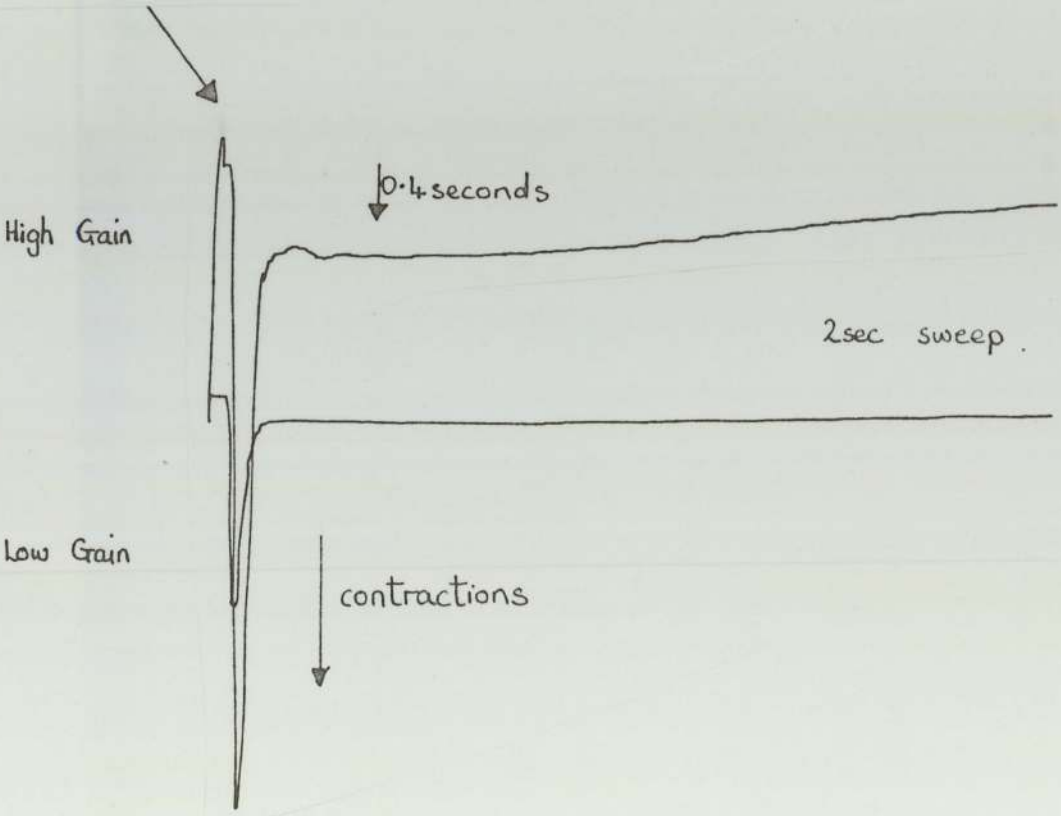

0·4 seconds
High Gain
2sec sweep
Low Gain
contractions

Fig. 9

Rat hemi - diaphragm in physiological saline 37°C. Initial tension 10g.

I Photographs of hemi - diaphragm at rest and 0.4 seconds after single nerve stimulation in the presence of ecothiopate (5×10^{-7}M). Note the dark shapes of the silver grains.

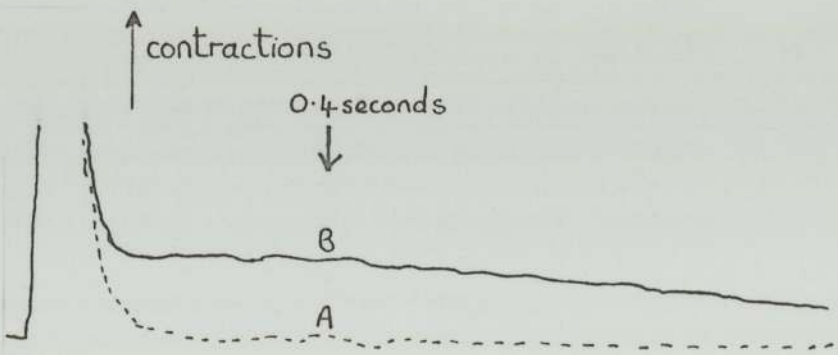
II A record of the tension in response to nerve stimulation showing the point at which the photographs were taken. The dotted line represents the tension in the absence of ecothiopate.

III Graph of $\frac{ls}{lr}$ against distance from the endplate region (designated 0mm) to show the distribution of the prolonged contraction. The 'unshaded' circles show the results from the above photographs. The 'shaded' circles show the results from other responses.

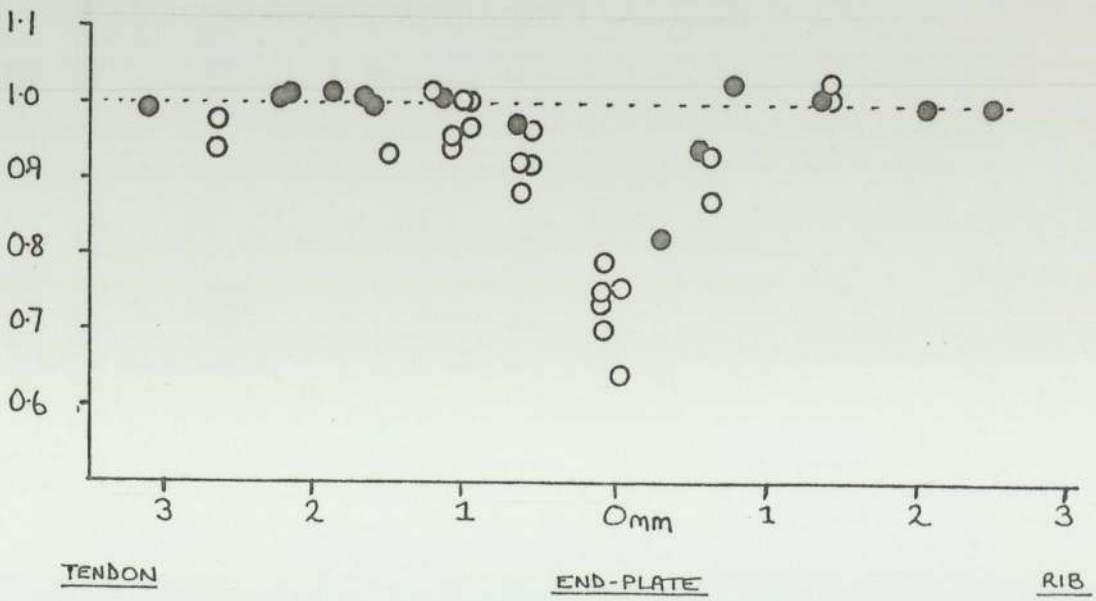
I

RESTING STIMULATED

II



III



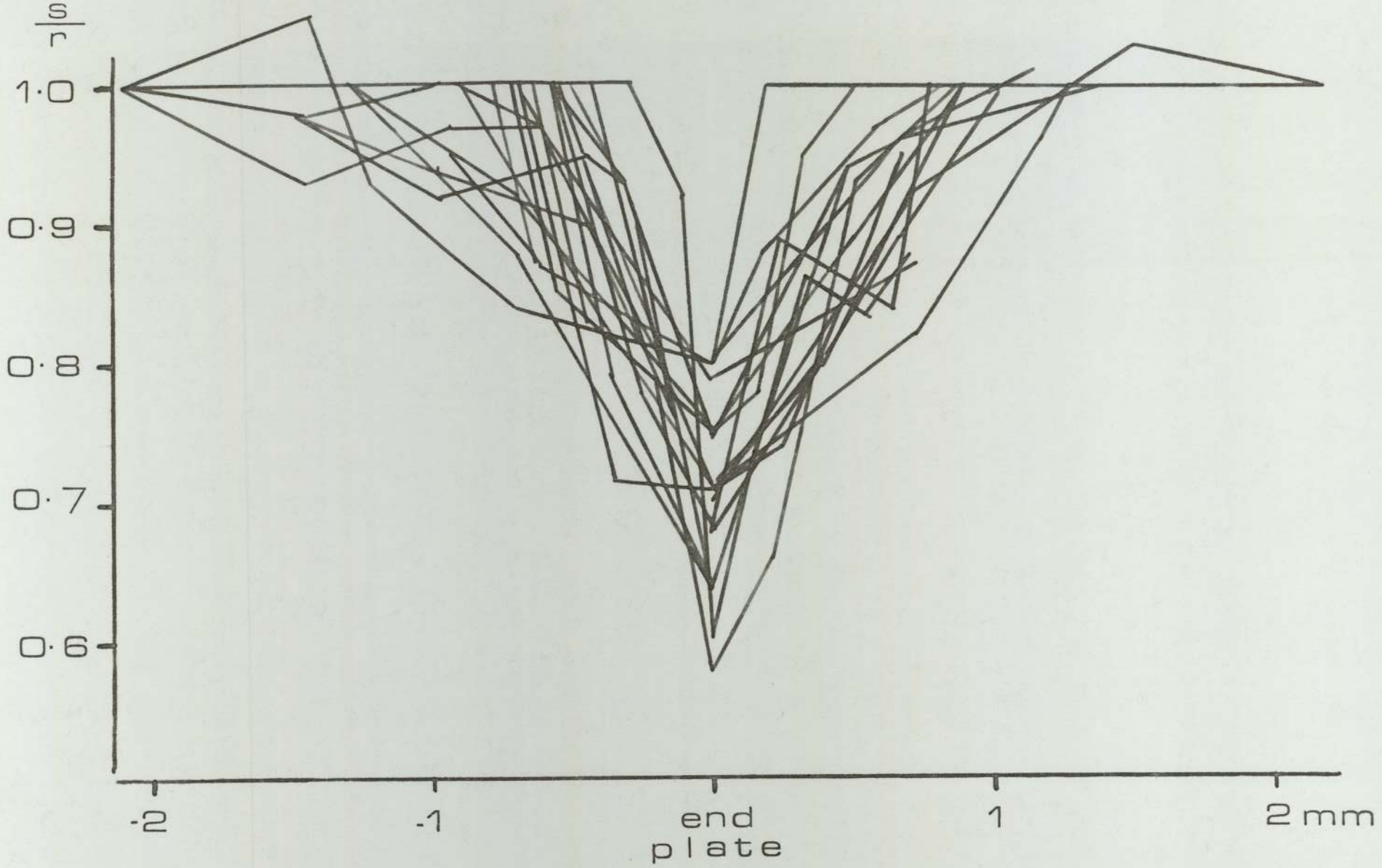

s/r
1·0
0·9
0·8
0·7
0·6
-2
-1
end plate
1
2 mm

Fig. 10. Graph of $\frac{ls}{lr}$ against distance from the endplate region to show the distribution of the prolonged contraction. Measurements made from 4 experiments.

Discussion

It is concluded that the late prolongation of the contraction as a result of ecothiopate action is localised at the endplate region of the muscle and is, hereafter, referred to as the prolonged localised contraction (plc).

The maximum tension developed in the plcs occurred approximately 0.5 - 0.85 secs (e.g. Fig. 6) after the relaxation of the rest of the muscle. This might have been due to the waning contraction in the non-junctional parts of the muscle fibres thus allowing maximum tension to be developed in the endplate region. The latter tension could not have been achieved until the whole muscle had stopped shortening whence the tension would have been finally transmitted to the transducer, presumably, approximately 0.5 - 0.85 secs after the relaxation of the rest of the muscle.

Presumably plcs are due to a prolonged and, perhaps, more intense action of Ca^{2+} in the sarcoplasm of the endplate regions of the muscle fibres.

How is the Ca^{2+} - activation of the contractile machinery at the endplates prolonged ?

Activation of the contractile proteins in vertebrate skeletal muscle is induced by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) into the sarcoplasm (see Ebashi 1976 for recent review). Activation is initiated by depolarisation beyond a threshold (Hodgkin and Horowicz, 1960). The parameters of the muscle action potential, that is, the duration of the action potential and the threshold depolarisation for mechanical activity, determine the duration and intensity of the mechanical activity (Sandow, Taylor and Preiser, 1965; Taylor, Preiser and Sandow, 1972).

Therefore, in order for Ca^{2+} action at the endplates to be prolonged, as is suggested to cause plcs, endplate depolarisation presumably should be prolonged as well.

It is possible that the maximum development of tension in the plcs might have coincided with the maximum accumulation of local intracellular Ca^{2+} resulting from the increase in transmitter action. The use of the indicator dye, Arsenazo III, might have proved useful in determining this relationship if it was not for the fact that movement artifacts appear to make the results of this, and other Ca^{2+} - sensitive dyes, unreliable.

Summary

A technique for measuring the late prolongation of the contraction observed after ecothiopate has been developed. This technique shows that prolongation is localised to approximately 1mm either side of the endplate region.

It has been suggested that plcs are due to a prolonged Ca^{2+} action in the sarcoplasm of the endplate region. The latter has been suggested to be caused by the prolonged transmitter action thought to occur after ecothiopate.

SECTION III

The Effect of Ecothiopate On The Electrical Response As Recorded Extracellularly At The Endplate Region

Introduction

It was previously suggested that plcs were due to prolonged Ca^{2+} - activation of the contractile machinery in the endplate regions. In addition, this prolonged Ca^{2+} action should be initiated by a prolongation of the endplate depolarisation which will presumably be due to the prolonged transmitter action resulting from the inhibition of ChE by ecothiopate.

Feng (1940, 1941) and Eccles, Katz and Kuffler (1942) both showed that physostigmine increased and prolonged extracellular endplate currents indicating that transmitter action had been prolonged as a result of ChE inhibition.

In this series of experiments, attempts were made to relate the plcs with electrical activity recorded in the endplate regions of the rat hemi - diaphragm in order to, primarily, determine whether the prolongation of transmitter action as a result of ecothiopate action was associated with plcs.

Results

An extracellular electrode was located at the endplate (see Methods p. 24) around which the plc has been shown to reside, the nerve was stimulated and the extracellular currents and contraction were recorded simultaneously (Figs. 11 and 12). These extracellular currents comprise the inward- and then outward-going action currents, due to the initation and propagation of an action potential, together with the inward-going localised endplate currents (Fig. 13).

Fig.11(A) shows the action currents preceding the development of tension. Maximum tension is achieved approximately 20msec after stimulation. Fig.11(B) shows the records 9 minutes after ecothiopate (5×10^{-7}M). The action currents remain of similar size but there are now prolonged endplate currents lasting at least 40msec with superimposed repetitive action currents at about 5msec intervals. The greater maximum tension is now achieved approximately 30msec after stimulation. These records are a typical response to ecothiopate. However, in one experiment, repetitive firing occurred much later than the usual limit of approximately 15 - 20msecs after stimulation. The records of this experiment are shown in Fig.12. In (C) the repetitive firing appears to continue until at least 60msec after stimulation.

In other experiments, alternate records of the extracellular currents and contractions, in response to nerve stimulation at 0.017Hz, were made with sweep times of 100msec and 5 secs respectively. The records of one such experiment are shown in Fig.14. These particular records show (1 - 3) that the prolongation of the endplate currents precede the development of plcs and that at their respective peaks, approximately 18 mins after the addition of

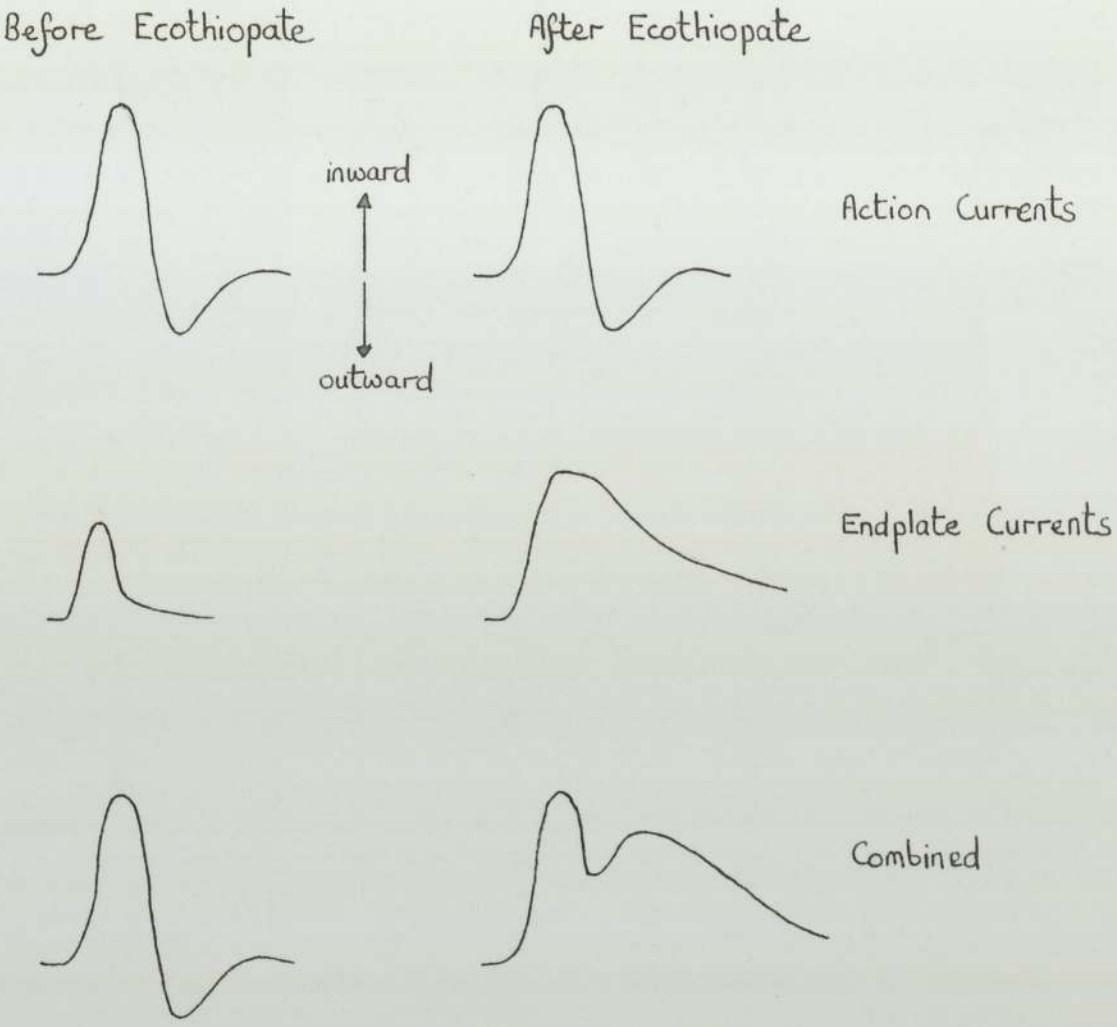


Fig. 13. Diagrammatic representation of the electrical activity recorded extracellularly at the endplate.

ecothiopate (5×10^{-7}M) which agrees with the value in Table 1 , the endplate currents are prolonged up to 65msec and the localised contraction is prolonged up to 4.5sec approximately.

Since AC amplification was used to record the previous extracellular currents, then these might be distorted due to the attenuation of the low frequency components of the signals. Therefore, DC records of the extracellular currents were made in an attempt to determine the true time course of these currents in the presence of ecothiopate (5×10^{-7}M) (Fig.15). These records apparently show that the endplate currents are sometimes prolonged beyond 20 secs at approximately 20 mins after ecothiopate addition. It is, however, possible that these DC - coupled prolonged endplate currents were the result of either biological changes in the preparation or artefactual changes arising from recording during the time course of the plcs. The latter might lead to the records becoming distorted due to the movement of the preparation as it contracts and/or to the movement of the electrode by the preparation, particularly if the electrode is quite close to the surface of the muscle. To eliminate the latter, DC records, and also the majority of the AC records, were made with the extracellular electrode a sufficient distance vertically away from the muscle surface. Figure 15(3) shows endplate currents lasting a minimum of 2 secs.

A number of experimental records made have shown that prolonged endplate currents could be recorded in the absence of plcs either before these start or after they have waned. Fig. 14 shows the development of prolonged endplate currents preceding plc development. Fig. 16 shows prolonged endplate currents after the waning of the plcs.

Similarly after ecothiopate was treated with Pyridine - 2 - aldoxime methiodide (2-PAM), which reactivates cholinesterase, prolonged endplate currents were observed in the absence of plcs (Fig. 14). Lastly, experiments made with d-Tubocurarine (dTC) (5×10^{-8}M) which blocks transmitter action (partially at this concentration) showed prolonged endplate currents recorded in the absence of plcs (Fig. 17)

Fig. 11

Rat hemi-diaphragm. 37°C. Physiological saline ($[Mg^{2+}]10^{-4}M$). Nerve Stimulation 0.01Hz. Simultaneous records of extracellular currents (E) at the endplate (AC amplification) and associated contraction (C).

Ⓐ Upper : records made under control conditions

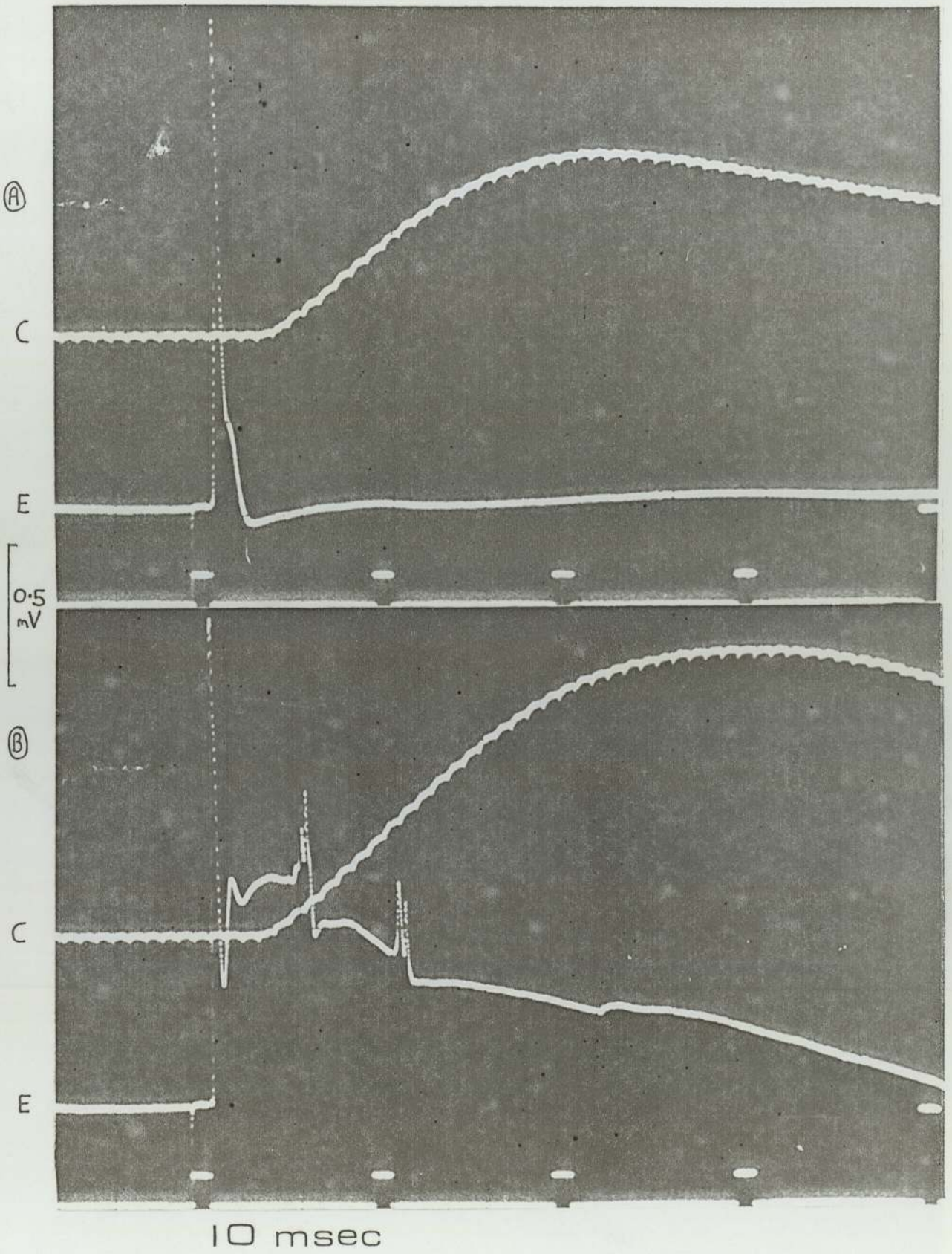
Ⓑ Lower : records made 9 mins after ecothiopate ($5 \times 10^{-7}M$)

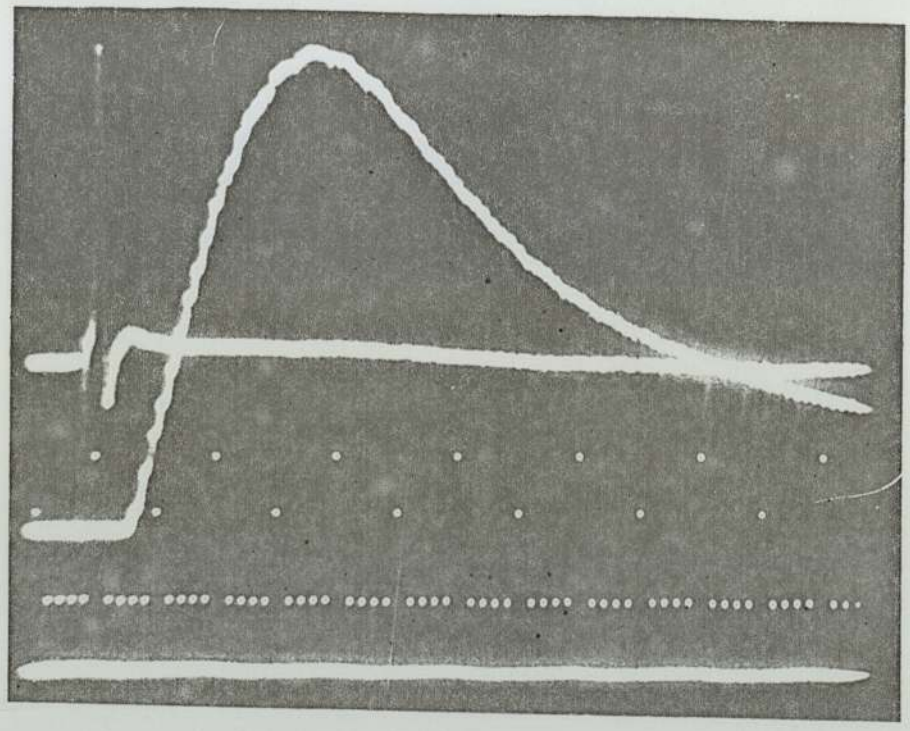
Note the increase in (i) the maximum tension achieved
(ii) the time to maximum tension
(iii) the time course of endplate currents
and (iv) repetitive action currents

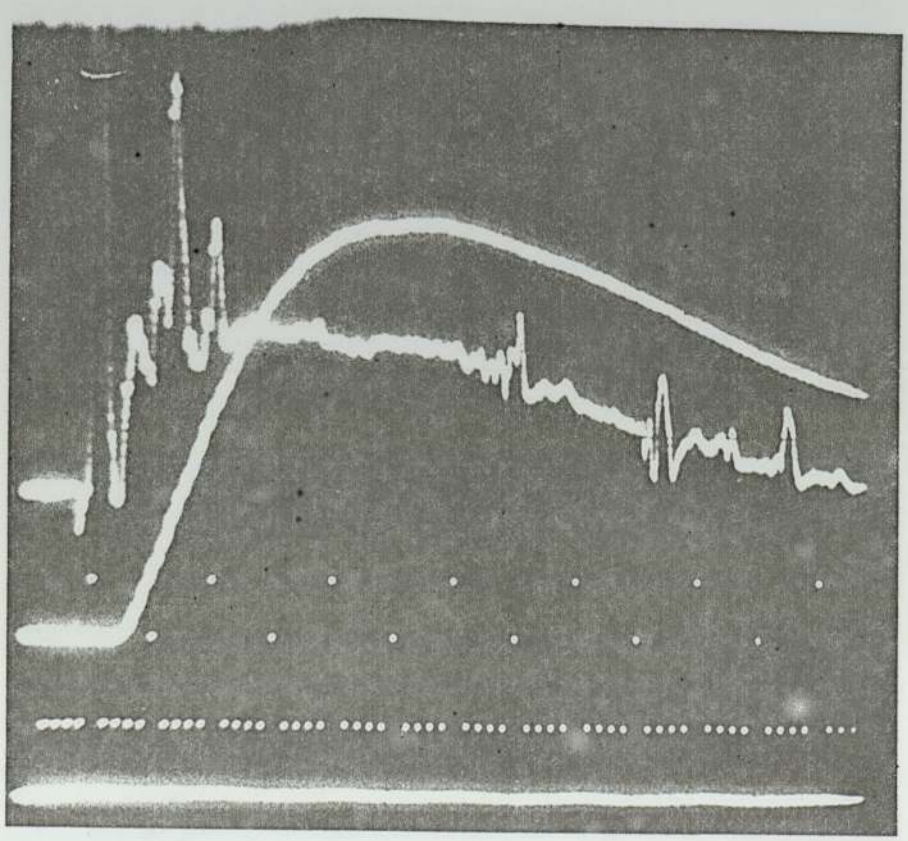
in the presence of ecothiopate.

Time Bars are at 10 msec intervals

Calibration of extracellular currents $0.2mVcm^{-1}$


A
C
E
0·5 mV
B
C
E
10 msec


0·1mV
E
C
A
5msec


E
C
B

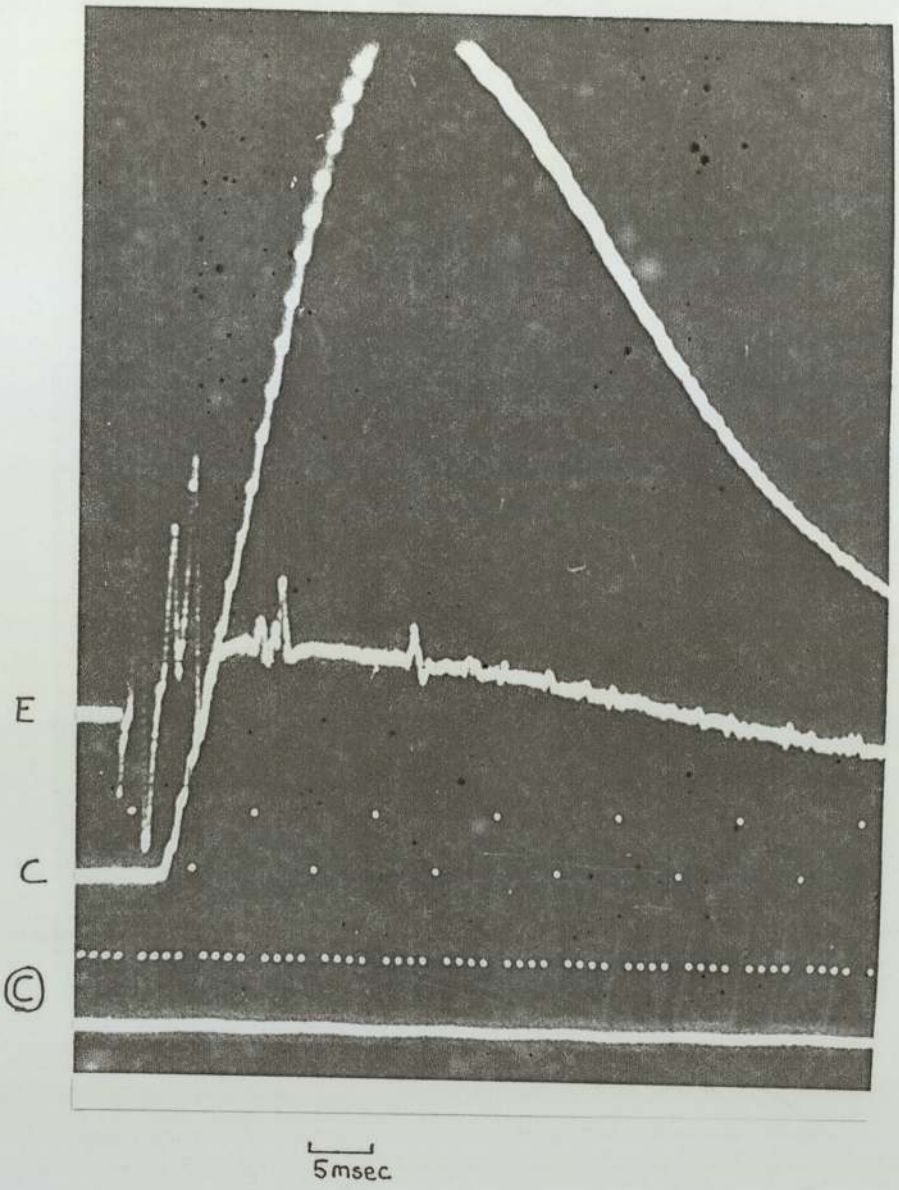


Fig. 12

Rat Hemi - diaphragm 37^{oC} Physiological saline ($[Mg^{2+}]10^{-4}$M) Nerve stimulation 0.1Hz. Simultaneous records of endplate extracellular currents (E) (AC amplification) and associated contractions (C)

(A) Control records

(B) Records 9 mins after ecothiopate (5×10^{-7}M)
Note the much increased maximum tension of contraction and the presence of repetitive firing lasting about 20msecs after stimulation

(C) Records 16 mins after ecothiopate
Note the presence of repetitive firing lasting at least 60msecs after stimulation and also the lower maximum tension of contraction achieved at this stage of drug action.

Note the time scale is in milliseconds.

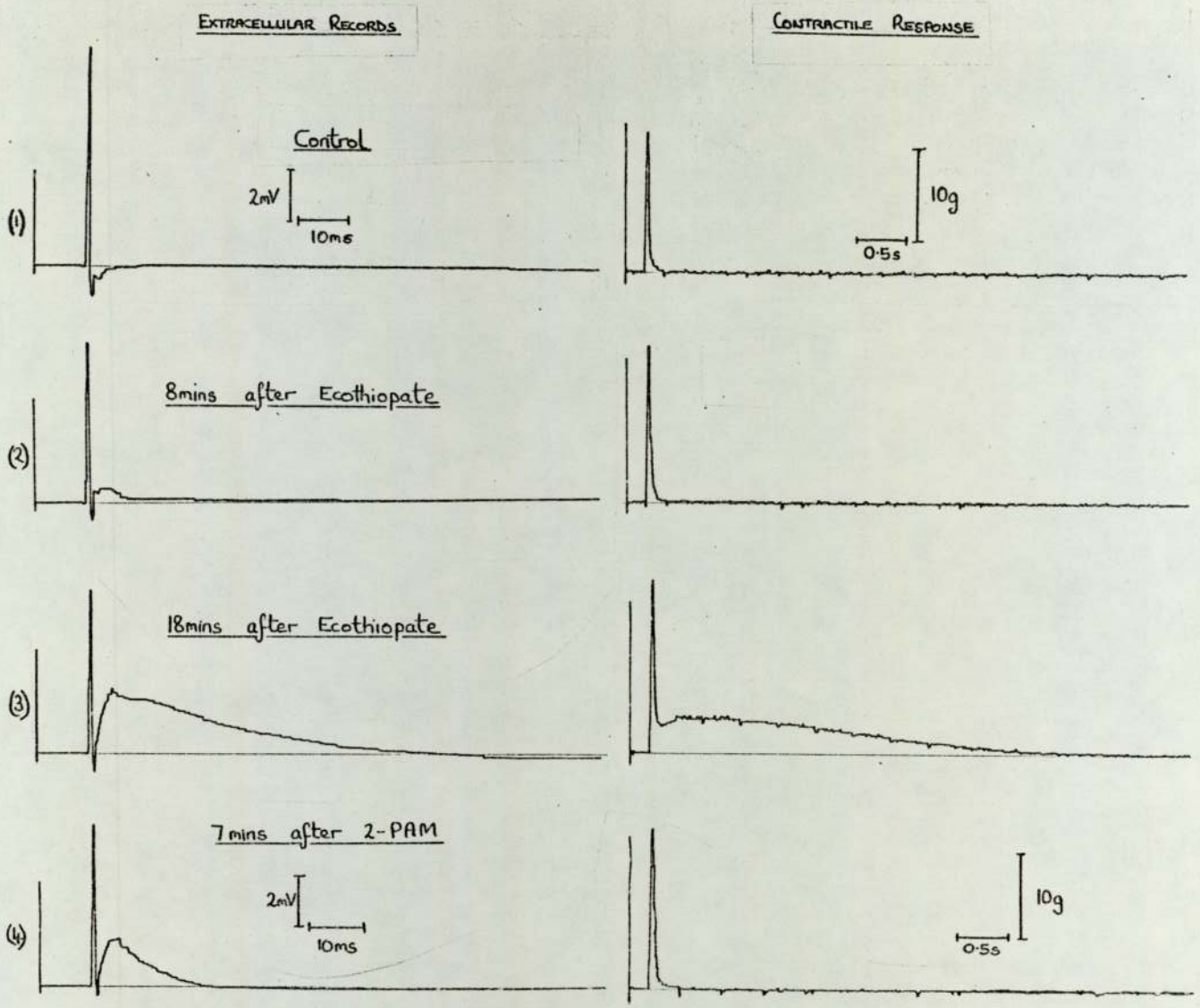

Extracellular Records
Contractile Response
Control
2mV
10ms
10g
0·5s
(1)
8mins after Ecothiopate
(2)
18mins after Ecothiopate
(3)
7mins after 2-PAM
2mV
10ms
10g
0·5s
(4)

Fig. 14

Rat hemi - diaphragm Physiological saline 37°C

Records of contraction and of extracellular currents at the endplate (AC amplification). Nerve stimulation, 0.017Hz.

Records made using storage oscilloscope and XY Plotter

(1) Control Records

(2) Records 8 mins after the additon of ecothiopate (5×10^{-7}M), Note the slight prolongation of the endplate currents and the absence of any prolongation of the contraction.

(3) Records 18 mins after the addition of ecothiopate(5×10^{-7}M). These records show the maximum prolongation of both the endplate currents and the localised contraction.

(4) Records 7 mins after the addition of the cholinesterase re-activator 2 - PAM (10^{-4}M). Again note the prolongation of the endplate currents in the absence of any prolongation of contraction.

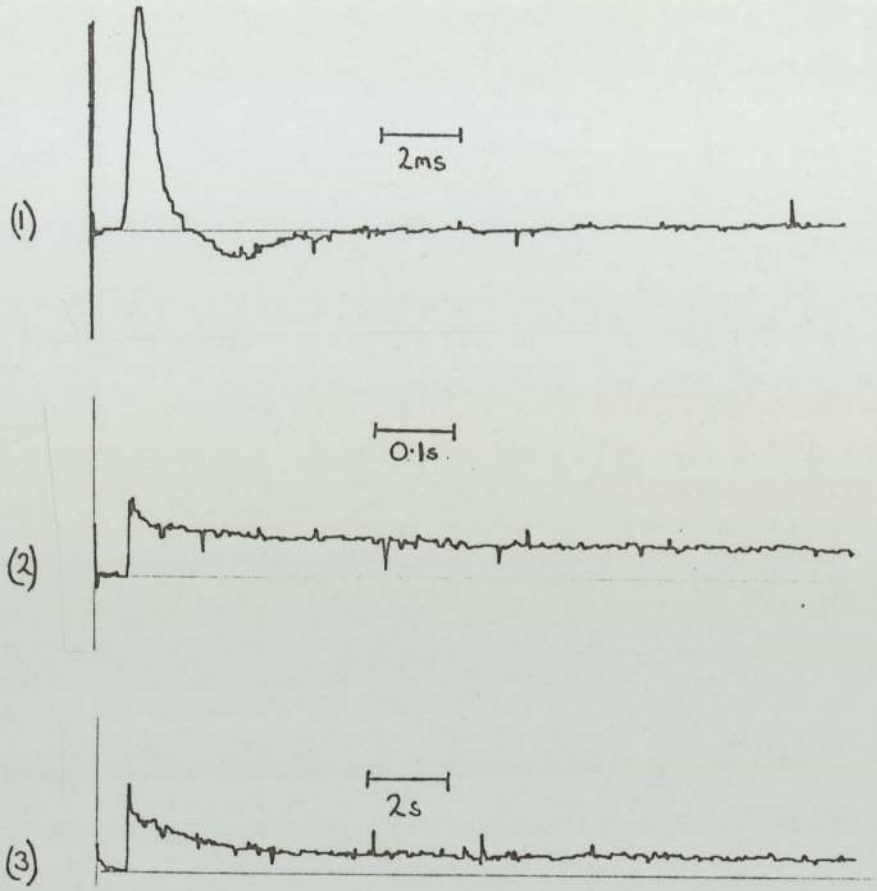


Fig. 15

Rat hemi - diaphragm. Physiological saline. 37°C.
Records of extracellular currents at the endplate (DC amplification)
Nerve stimulation 0.017Hz Records made using storage oscilloscope and XY Plotter.

(1) Control record

(2) Record 12 mins after the addition of ecothiopate(5×10^{-7}M)

(3) Record 20 mins after the addition of ecothiopate(5×10^{-7}M)

Note that in the records (2) and (3) the time course was such that the amplitude of the action potential was not accurately recorded by the Storage Oscilloscope.

Also note the different time bars on each trace.

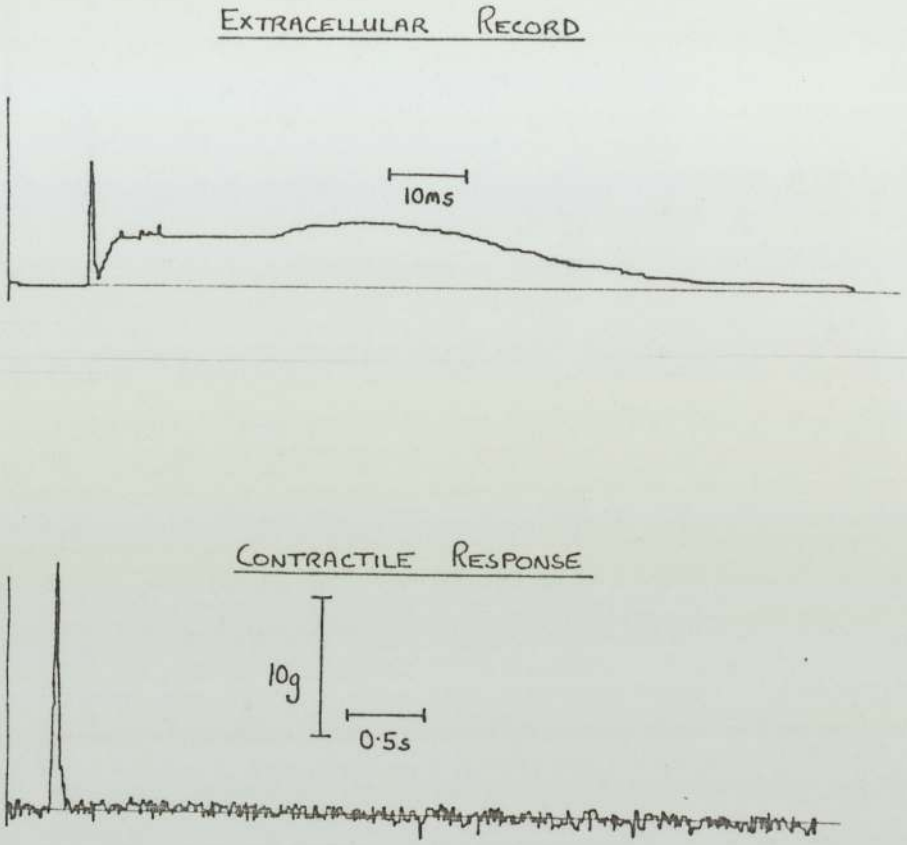


Fig. 16

Rat hemi - diaphragm. Physiological saline. 37°C.

Nerve stimulation, 0.017Hz

Records of contraction and extracellular currents at the endplate (AC amplification) 29 and 30 mins respectively after the addition of ecothiopate (5×10^{-7}M).

Note the presence of prolonged endplate currents and the absence of any prolongation of the contraction. Records made using storage oscilloscope and XY Plotter.

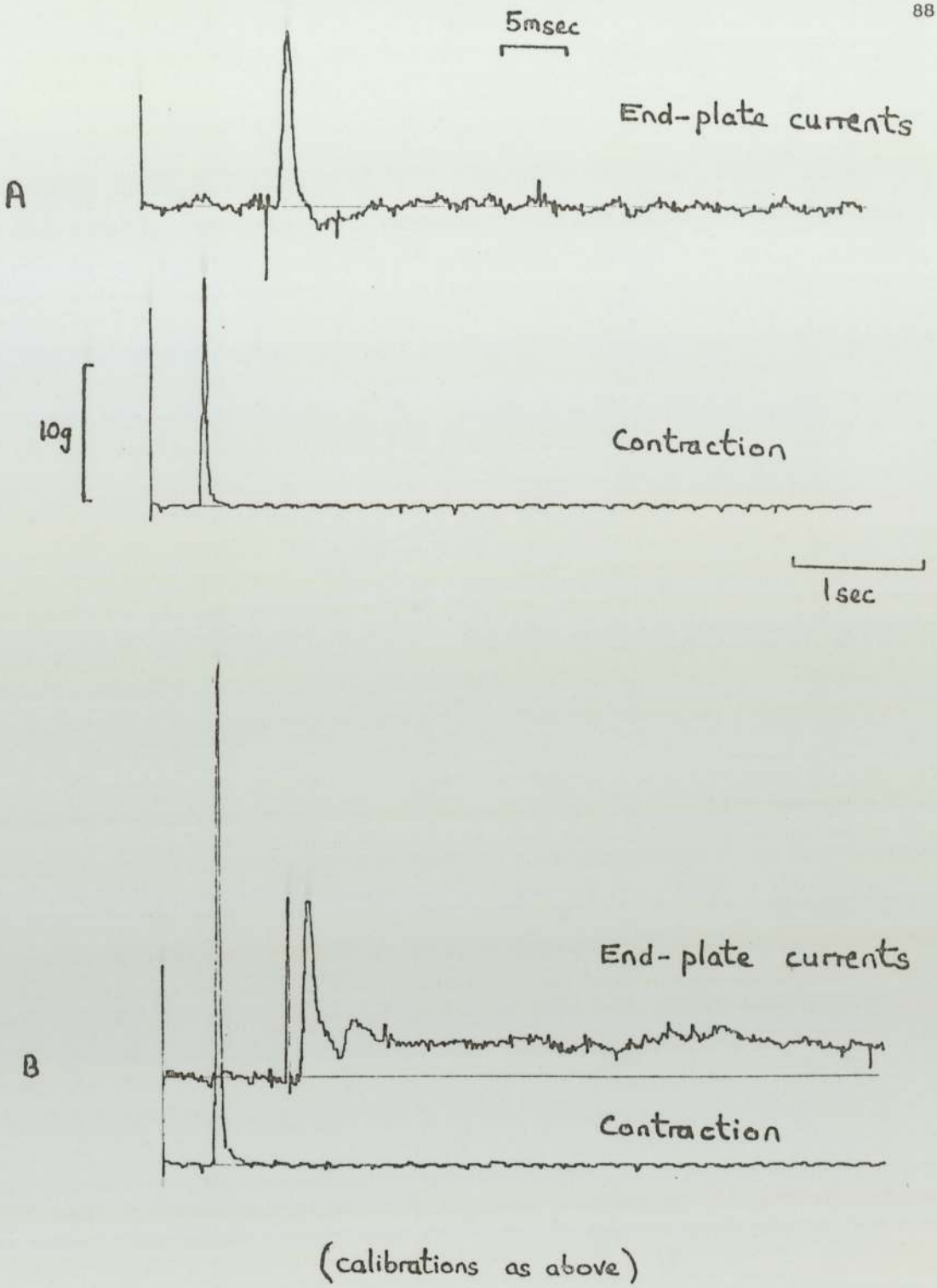


Fig. 17

Rat hemi-diaphragm. Physiological saline ($[Mg^{2+}]$ 10^{-4}M).

Records of contraction and extracellular currents at the endplate (AC amplification).

Nerve stimulation, 0.017Hz .

A. Control responses

B. Responses 11 mins. after the addition of ecothiopate (5×10^{-7}M) and dTC (5×10^{-8}M) to the saline.

Note the prolongation of the endplate currents and absence of any localised prolongation of contraction.

Records made using storage oscilloscope and XY Plotter .

Discussion

The results show that, generally, the action of ecothiopate in increasing transmitter action, firstly leads to the prolongation of the endplate currents followed after a short interval (1 - 3 mins) by the endplate-localised prolongation of each contraction (Fig.14). In addition, the maximum prolongation of depolarisation was usually observed to coincide with maximum prolongation of the late part of the contraction (Fig.14(3)). This suggests that plcs are, at least associated with the prolonged electrical activity in the endplate region.

From AC - amplified records, the duration of the prolonged endplate currents is of the order of tens of milliseconds (approximately 60msec) with repetitive action currents usually having waned by approximately 20msec. It is difficult to suggest, on this basis, that the plcs, which can be up to about 5 seconds in duration, directly result from the prolonged endplate currents. However, since the DC - amplified records apparently show that the endplate currents may be prolonged by at least 2 secs, (Fig.15) it is reasonable to suggest that the plcs are related to, and, indeed, may even directly result from, the prolonged endplate currents.

Sandow et al (1965), in their experiments on frog sartorious muscle, found that the duration of the mechanically effective period of the action potential, that is, that which determines the subsequent mechnanical activity, was 1.5msec, while the duration of the mechanically active period, that is, the contraction, was much longer (about 100msec). If their analysis is correct and can be applied to rat diaphragm muscle then for an endplate localised contraction lasting 4.5 sec, the mechanically effective period of the action potential and subsequent endplate depolarisation would need to be

approximately 65 - 70msec. That the prolonged endplate currents last, at least, approximately 60msec have been reliably demonstrated using AC - amplification. This obviates the reliance on the less reliable DC - amplified records.

It is also thought that it is the prolonged endplate currents, rather than the repetitive action currents which initiate the prolongation of the endplate-localised contractions of hemi - diaphragm muscle. This is because, firstly, the repetitive action currents would be transmitted through the muscle fibres and so would lead to a late prolongation of the whole muscle whereas the endplate currents are restricted to the endplate region and secondly, the repetitive currents have usually waned at approximately 15 - 20msec after nerve stimulation which is insuffient, even according to Sandow's guidelines, to prolong the late part of the contractile response to that observed.

It has, though, been possible to record quite substantially prolonged endplate currents in the absence of plcs when experiments were made in dTC, or after 2 - PAM or after the waning of plcs which might suggest that there is no causal relationship between prolonged endplate currents and plc development.

However, since the onset of plcs in the presence of ecothiopate only is always associated with the prolongation of the endplate currents, it remains possible that plc development is associated with the prolongation of endplate currents. It maybe that the waning of plcs in ecothiopate only is due to some mechanism other than the shortening of the prolonged endplate currents. It is possible that plcs are initiated when the prolonged endplate depolarisation remains above a certain mechanically effective threshold level. The waning of plcs could then simply be explained in terms of the depolarisation falling below the mechanically effective

threshold level. It may be that dTC and 2 - PAM act by reducing the mechanically effective period sufficiently to prevent plc development.

Presumably, as a result of the progressive inhibition of functional ChE by antiChE action, transmitter action at the neuromuscular junctions after each impulse is prolonged, perhaps progressively (i.e. time - dependent inhibition) which perhaps also involves the penetration of ACh to receptors not normally reached. It is plausible to suggest that this progressive prolongation of transmitter action, seen as a progressive prolongation of endplate currents, is somehow responsible for the plcs.

On the basis that plcs are due to a prolonged action of Ca^{2+} in the sarcoplasm of the endplate regions, the prolonged endplate currents might exert their effect in a number of ways.

Prolonged transmitter action may lead to post-junctional Ca^{2+} entry and subsequent accumulation at the endplates. This may, in turn, be directly responsible for prolonging the endplate localised contraction. Nastuk and Gissen (1965) speculated that exposure to depolarising drugs enhances Ca^{2+} uptake into the endplate regions of muscle. Numerous workers (e.g. Jenkinson & Nicholls, 1961, and Evans, 1975) have shown a post-junctional entry of Ca^{2+} into rat diaphragm muscle during prolonged depolarisation. Alternatively, sufficient Ca^{2+} may enter the endplate regions during the prolonged transmitter action to trigger the release of further Ca^{2+} from the SR. Influx of external Ca^{2+} into muscle fibres during the action potential has been suggested as a possible mechanism controlling Ca^{2+} release from the SR (Ford and Podolsky, 1972; Chiarandini and Stefani. 1973, Stefani and Chiarandini, 1973).

It is possible that, in the presence of an antiChE, calcium release from the SR may be triggered by a post-junctional entry of Ca^{2+}.

The presently more fashionable theory of excitation-contraction coupling is that membrane depolarisation is believed to directly increase the permeability of the SR membrane to Ca^{2+} thus causing release (Costantin and Podolsky, 1966 and 1967). Presumably membrane depolarisation initially results in the release of Ca^{2+} from the SR and contraction, and repolarisation results in the uptake of Ca^{2+} back into the SR and relaxation. It is suggested that the prolonged endplate currents, produced in the presence of ecothiopate might exert their effect more directly by delaying the sequestration of Ca^{2+} back into the SR. Presumably, once depolarisation had ceased, the Ca^{2+} would be returned to the SR.

Summary

It has been shown that the progressive prolongation of the endplate localised contraction after ecothiopate is accompanied by a prolongation of endplate currents following stimulation of the nerve with single maximal pulses at low frequency. It has been suggested that the prolonged depolarisation directly or indirectly via a post-junctional entry of Ca^{2+} prolongs Ca^{2+} action in the sarcoplasm of the endplate region.

SECTION IV

Histochemistry of the Endplate

Introduction

In order to determine the possible involvement of a post-junctional entry of Ca^{2+} in directly or indirectly leading to plcs, it was decided to investigate the effect of inhibiton of ChE by ecothiopate on the accumulation of Ca^{2+} in the endplate regions of diaphragm muscle. It was possible that any post-junctional entry of Ca^{2+} would accumulate at the endplate and that a progressive accumulation of Ca^{2+} might explain the progressive development of the plcs. Lièvrement, Czajka and Tazieff - Depierre (1968) have reported that stimulation of the phrenic nerve in the presence of the antiChE neostigmine caused staining in the junctional region of mammalian skeletal muscle.

Histochemical staining techniques were employed to

(a) locate endplate regions and

(b) to determine any accumulation of free Ca^{2+} (i.e. in solution) in the same regions resulting from ecothiopate (5×10^{-7}M) action.

Results

Rat diaphragm muscles, incubated in physiological saline containing Ca^{2+} ($2-5 \times 10^{-3}$M) and carbachol (10^{-4}M) for 30 minutes and then frozen and cut into sections for staining (for full details of methods see pp. 27-33), showed red granular patches of Ca^{2+} - GBHA complex at the endplate regions, as illustrated in Fig. 18 , indicating an accumulation of free Ca^{2+} in these regions. The upper photograph shows the location of the endplate regions (the dark, almost black, stained areas of cholinesterase) whilst the lower photograph shows the corresponding Ca^{2+} accumulations in a serial section.

Muscles incubated in physiological saline ($2-5 \times 10^{-3}$M Ca^{2+} and $10^{-3} - 10^{-4}$M Mg^{2+}) in the absence of any drugs showed that whether stimulated via the nerve or not, no such accumulation of Ca^{2+} could be demonstrated as illustrated in Fig. 19 .

Muscles incubated in physiological saline ($2-5 \times 10^{-3}$M Ca^{2+} and $10^{-3} - 10^{-4}$M Mg^{2+}) and stimulated via the nerve (0.02Hz) in the presence of ecothipate for approximately 25 minutes (i.e. long enough for the plcs to develop) generally did not show (4 out of 5 experiments) any accumulation of Ca^{2+}. The lower photograph in Fig. 20 shows the small accumulations in endplate regions obtained in one experiment only.

In addition, certain experiments were made where the hemi-diaphragm muscles were rapidly frozen in liquid nitrogen as soon as possible after a contraction with a fully developed plc had occurred. This was done in order to test the hypothesis that any increased uptake of Ca^{2+} during transmitter action did not subsequently become bound thereby making the Ca^{2+} unavailable for staining by this procedure. These muscles did not show any

accumulation of Ca^{2+} either.

Fig. 21 shows the differences in staining of cholinesterase in the presence of carbachol (upper photograph) and ecothiopate (lower photograph).

Fig. 22 shows most clearly the accumulation of Ca^{2+} in the presence of carbachol as stained by GBHA.

Figs. 18 - 20. Rat hemi - diaphragm. Transverse sections of muscles stained for Ca^{2+}. The upper photograph of each pair is a serial section, stained for cholinesterase, which shows approximately the same field of view as the lower photograph.

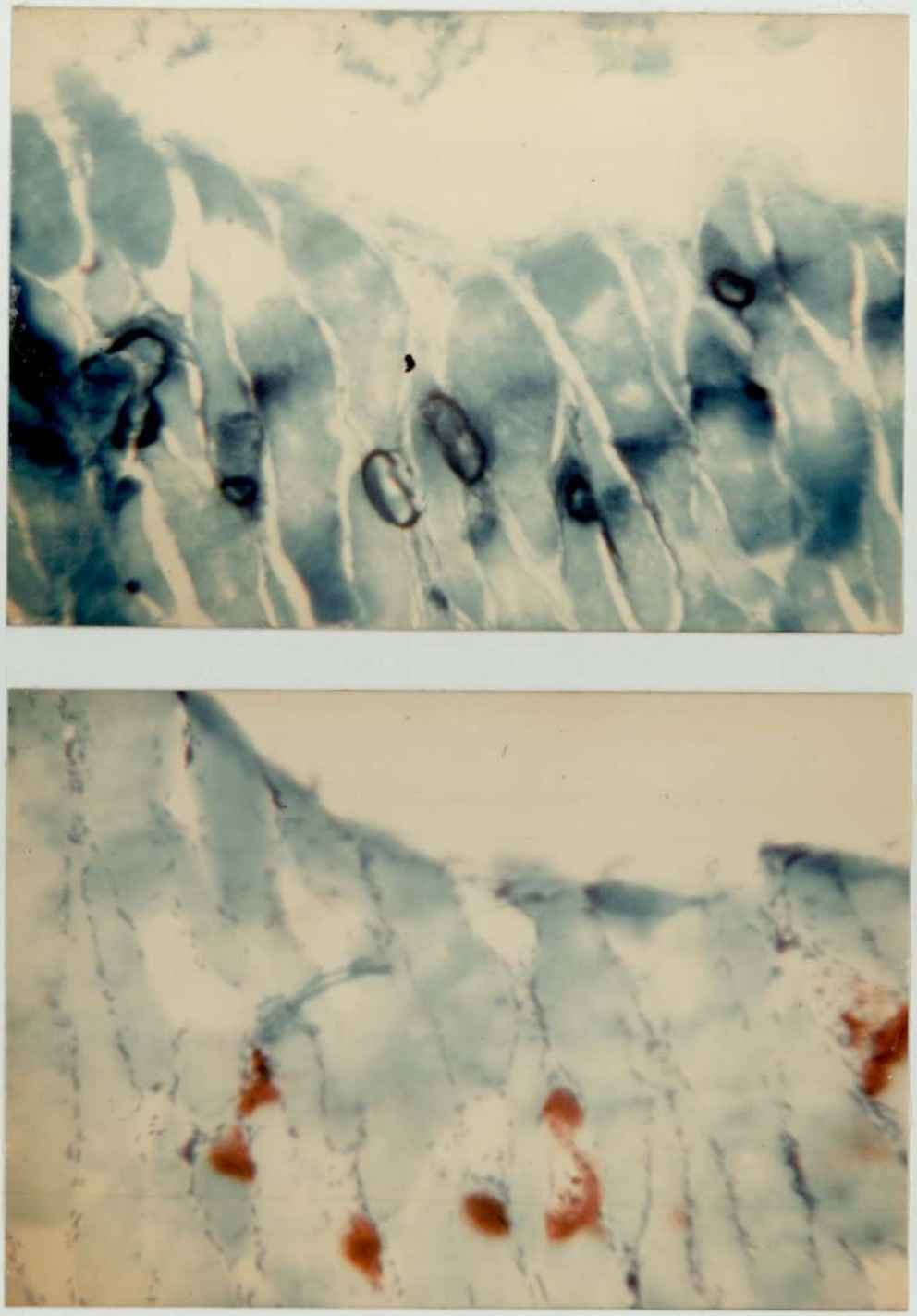


Fig. 18. Carbachol (10^{-4}M), Ca^{2+} (5×10^{-3}M).

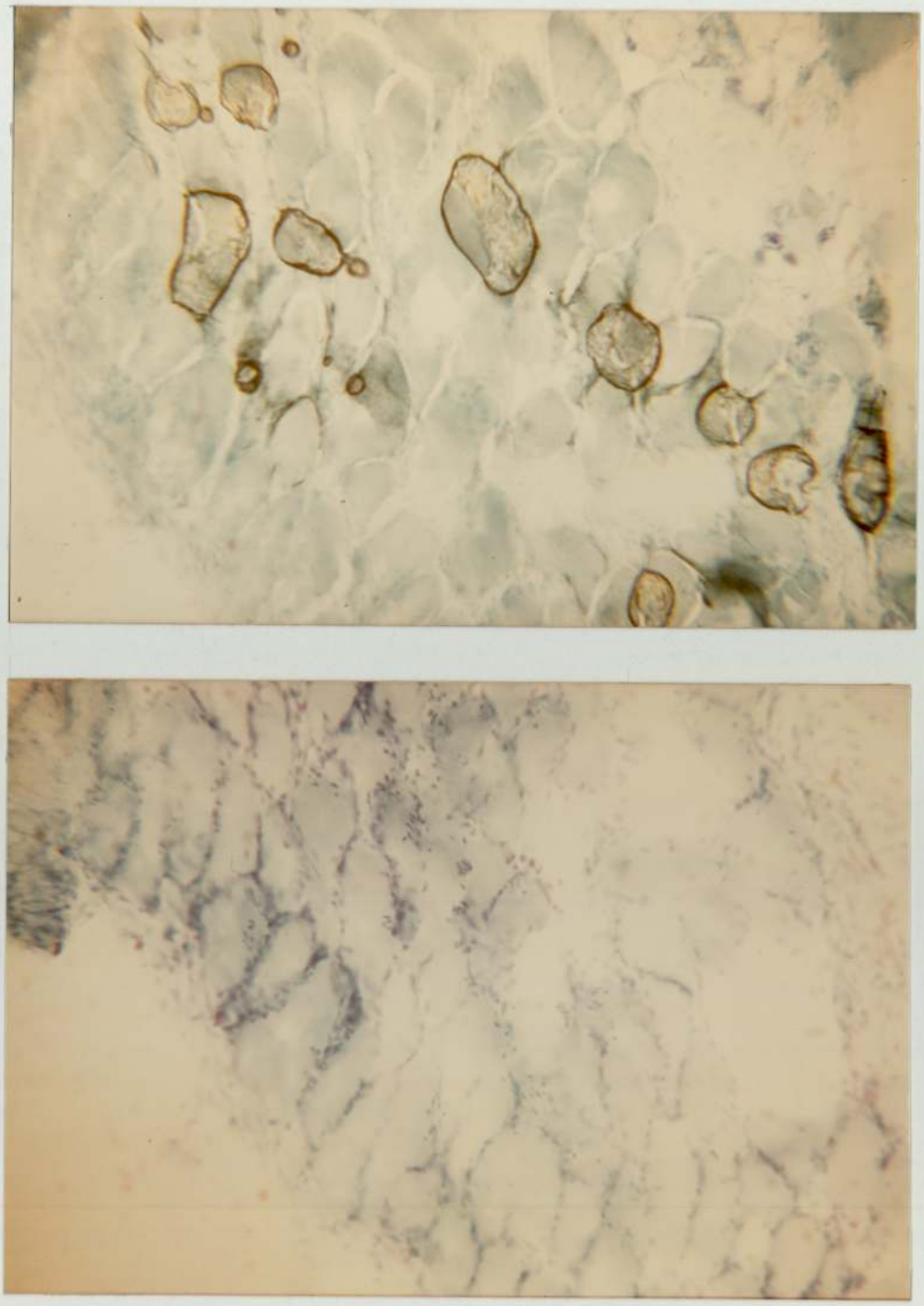
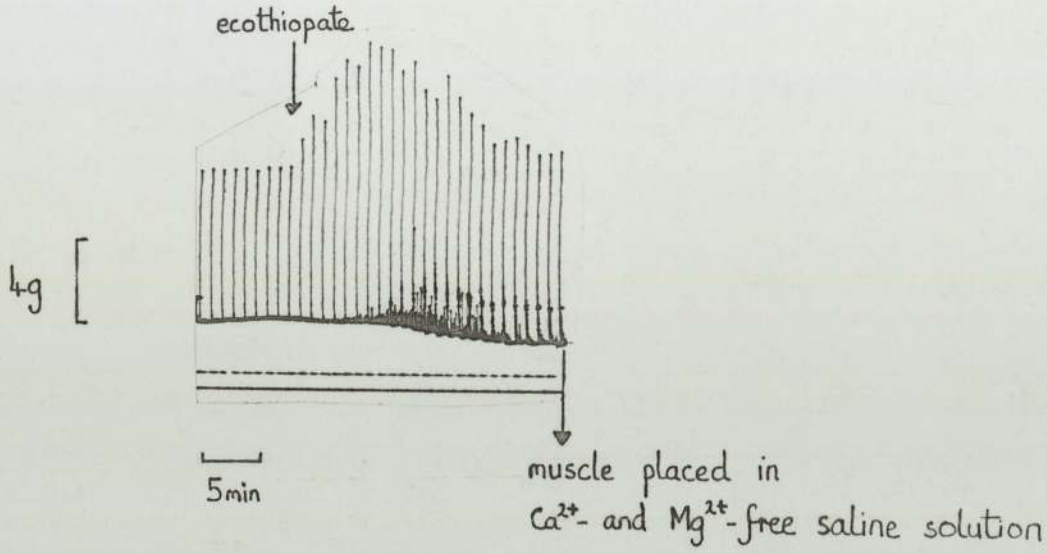


Fig. 19. Physiological saline only, Unstimulated.


ecothiopate
4g
5min
muscle placed in
Ca2+- and Mg2+-free saline solution

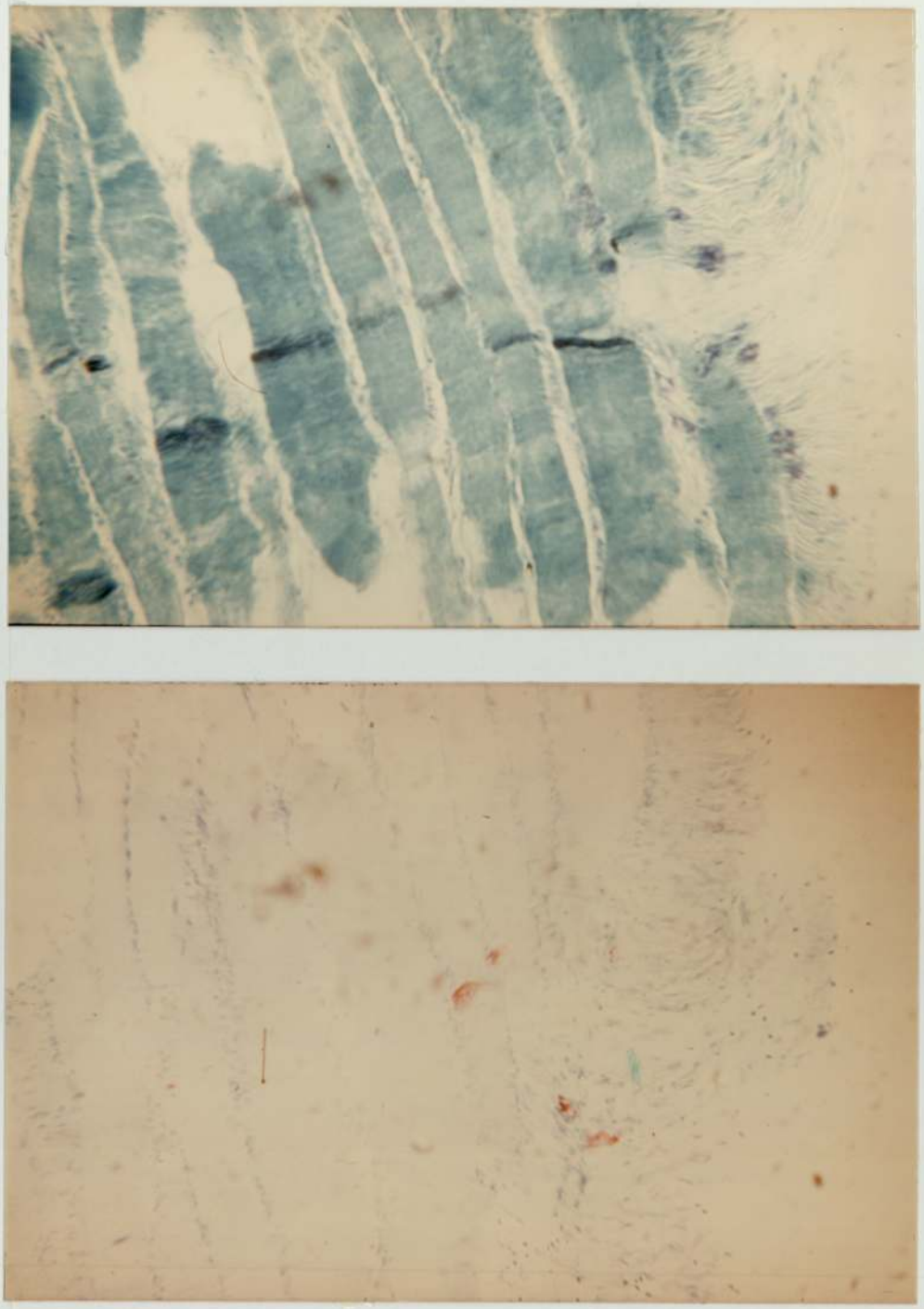


Fig. 20. Ecothiopate (5×10^{-7}M), Mg^{2+} (10^{-4}M). Stimulated (0.017Hz).
Opposite: the accompanying record of contraction.
note the point at which the muscle was placed in Ca^{2+} - and Mg^{2+} - free physiological saline for 5 mins before being frozen.

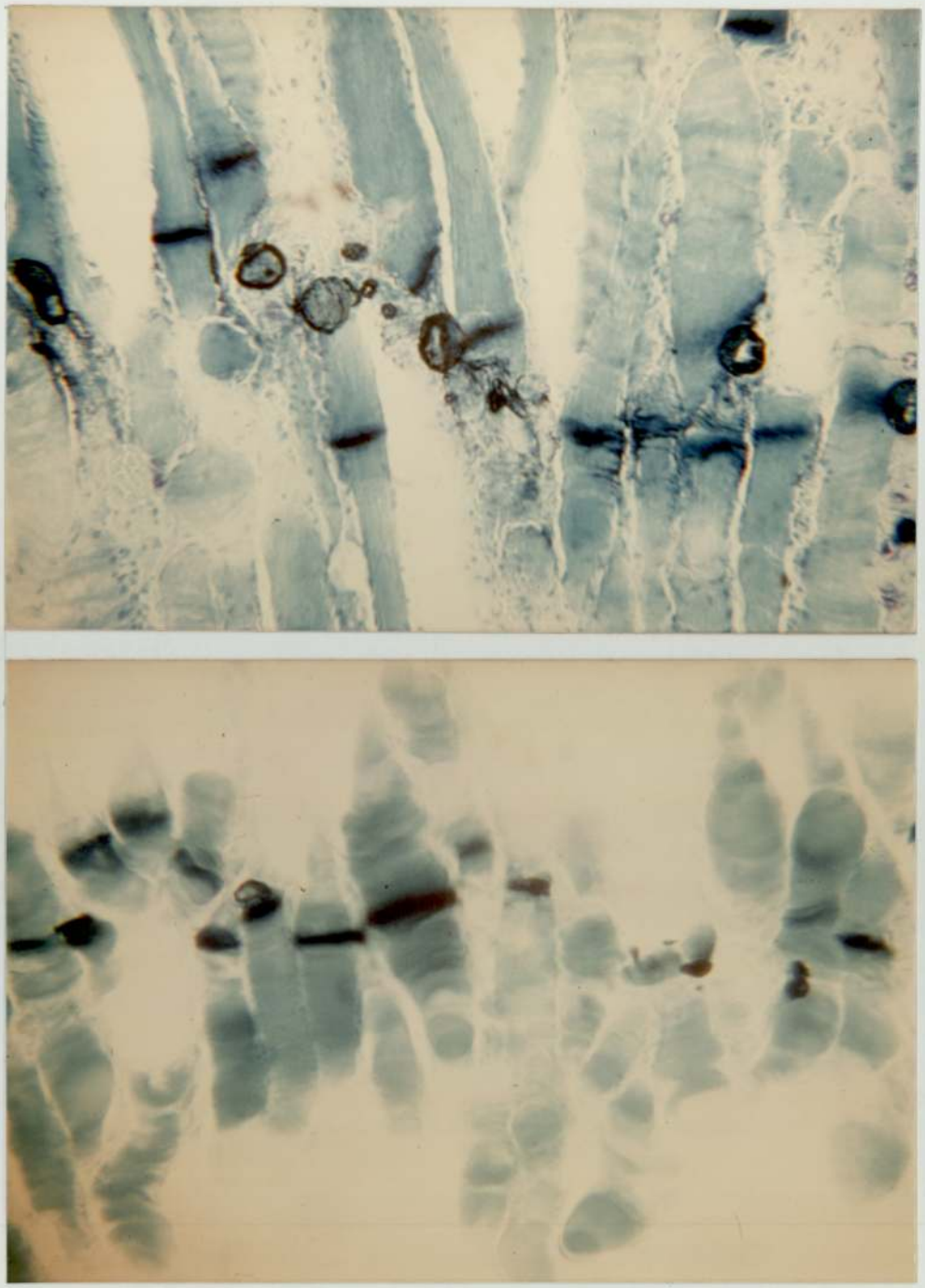


Fig. 21. Rat hemi - diaphragm. Transverse sections of muscles stained for cholinesterase.

Upper photograph: Carbachol (10^{-4}M), Ca^{2+} (5×10^{-3}M). Note the presence of 'open circular' and 'striped' stained areas of cholinesterase.

Lower photograph: Ecothiopate (5×10^{-7}M). Stimulated (0.017Hz). Note the presence of only the 'striped' stained areas of cholinesterase.

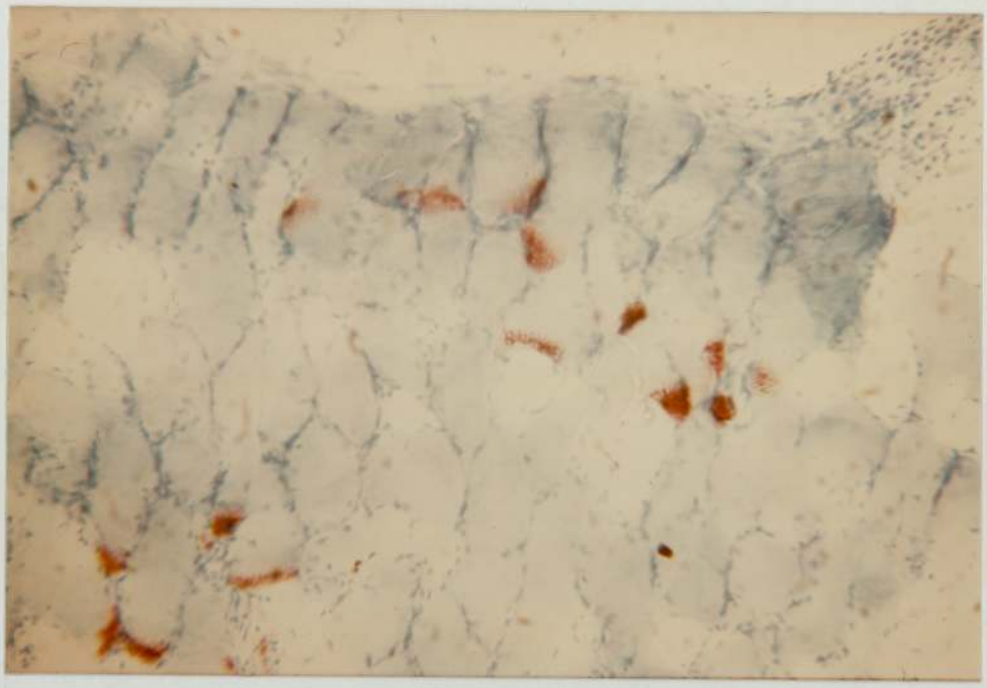


Fig. 22. Rat hemi - diaphragm. Transverse section of muscle stained for Ca^{2+}. Carbachol (10^{-4}M), Ca^{2+} (5×10^{-3}M)

Discussion

The results obtained from the experiments made with carbachol are in agreement with those of Evans (1974), who used mouse diaphragm muscle in his experiments, and serve as an indicator that the technique is reliable.

On the basis of the results obtained it would appear that the progressive development of plcs is not due to the progressive accumulation of Ca^{2+} in the endplate regions of ecothiopate-treated muscle. However, there are a number of possible reasons which might explain the absence of Ca^{2+} accumulation using these histochemical techniques:

(i) that the accumulation of Ca^{2+} is too small for this technique to be able to detect it. This would suggest that any such accumulations of Ca^{2+} arising from ecothiopate treatment are smaller than those resulting from carbachol treatment. Since it is believed that ecothiopate (5×10^{-7}M) does not have any direct depolarising action on the post-junctional membrane and that its only action is to intensify and prolong transmitter action following a nerve impulse as a result of its anticholinesterase action and since carbachol does have a direct depolarising action on the post-junctional membrane, then the latter would presumably result in a greater accumulation of Ca^{2+}.

(ii) that because the technique is only able to detect free Ca^{2+}, it was possible that any accumulation of Ca^{2+} during the prolonged depolarisation was subsequently lost as a result of

(a) efflux before freezing and/or

(b) becoming bound in a way which makes the calcium unavailable for staining.

Evidence from Evans (1974) and from later radioisotopic experiments (see p. 165) suggests that any accumulated Ca^{2+}

would have a slow rate of efflux. It is, therefore, believed that little, if any Ca^{2+} is lost from the endplate regions during the washing of the muscles in Ca^{2+} and Mg^{2+} - free physiological saline. This, in itself, might imply that any Ca^{2+} accumulated becomes bound in some way. However, why should free Ca^{2+} become bound during ecothiopate action and not during carbachol action? It is possible that the Ca^{2+} influx due to carbachol action saturates the uptake mechanism and subsequent Ca^{2+} influx accumulates and becomes stained. Whereas, Ca^{2+} influx due to ecothiopate action, is thought to be less than that due to carbachol and does not saturate the uptake mechanism and, therefore, does not accumulate sufficiently to be stained (see also pp. 167-). It is, therefore, possible that any net increase in Ca^{2+} uptake after ecothiopate, as a result of prolonged depolarisation, becomes sequestered into, for example, the local sarcoplasmic reticulum. This may prevent its' staining by GBHA (i.e. Ca^{2+}).

Alternatively, post-junctional Ca^{2+} entry may be negligible in the presence of ecothiopate and, therefore, would play no part in prolonging the late part of the contractions. In addition, the absence of a progressive accumulation would, therefore, suggest that the progressive development of plcs is due to prolongation of a direct depolarisation - induced Ca^{2+} release and subsequent delay in sequestration resulting from the progressive prolongation of transmitter action. That progressive prolongation of transmitter action has been observed to coincide with the progressive prolongation of the late part of the contraction, both achieving maximum prolongation at similar times, would appear to suggest this.

Presumably, if this technique does only detect free Ca^{2+}, the amount of staining must reflect the state of activation/

contraction of the isolated muscle preparation (i.e. resting tension). It would therefore have been expected that the staining of carbachol - treated muscles would have reflected an increase in such tension. In effect, the muscle perhaps should show some shortening. Experiments to investigate this possibility, however, were not made. Evans (1974) could not visually detect any movement of mouse diaphragm muscles in response to perfusion with carbachol (10^{-4}M). In the majority of experiments made to record the effect of ecothiopate on the contractile response, there was no overall change in tension of the isolated hemi - diaphragm preparations.

In conclusion, if ecothiopate action does result in an increased Ca^{2+} entry through the post-junctional membrane, subsequent accumulation of free Ca^{2+} is either too small to be detected by this technique or does not occur. It was now, therefore, necessary to try a different technique which could determine if there was an increased entry of Ca^{2+} into the endplate regions of the muscle fibres resulting in an accumulation of Ca, whether free or bound, due to ecothiopate action.

Summary

An attempt has been made to detect any accumulation of free Ca^{2+} (i.e. in solution) at the endplates after ecothiopate which might reflect a post - junctional entry of Ca^{2+}. The evidence presented indicated that there was no accumulation of free Ca^{2+}.

SECTION V

Radioisotopic Determination Of Calcium At the Endplate

Introduction

ACh and other neuromuscular depolarising drugs have been found to increase the uptake of labelled calcium into the endplate regions of mouse diaphragm (Evans 1974).

The purpose of these experiments was to determine, using the radioisotope $^{45}Ca\ Cl_2$, whether or not there was an accumulation of calcium at the endplate resulting from a post-junctional entry of Ca^{2+} as a consequence of ecothiopate action.

Results

The procedures used for dissecting the diaphragms, for incubating them in tracer and for preparing them for counting (i.e. washing out in tracer-free physiological saline, solubilisation of the muscle tissues and the actual counting) are detailed in the Methods Section (pps 34-37).

Initially, rat hemi-diaphragms were used for these experiments but the thickness of these muscles made their dissection into endplate and non-junctional pieces very difficult because the endplate region, more specifically, the nerves did not show up very clearly when treated with xylene. This difficulty may have accounted for the variable and inconclusive results obtained and was, therefore, deemed unsatisfactory. Hence, the thinner hemi-diaphragms of mice were used for easier recognition of the endplate regions. The method used was basically as described by Evans (1974).

Effect of ecothiopate on the contractile response of mouse hemi-diaphragm

Experiments were performed on mouse hemi-diaphragms to determine whether their contractile response to nerve stimulation (0.017Hz) was changed by ecothiopate (5×10^{-7}M) in a similar manner to that of rat hemi-diaphragms.

The results were found to be very comparable particularly the time course of the development of plcs (Fig.23). Maximum prolongation of the late part of the contraction was achieved approximately 16 mins after ecothiopate which agrees very well with the value obtained for rat hemi-diaphragm (Table 1).

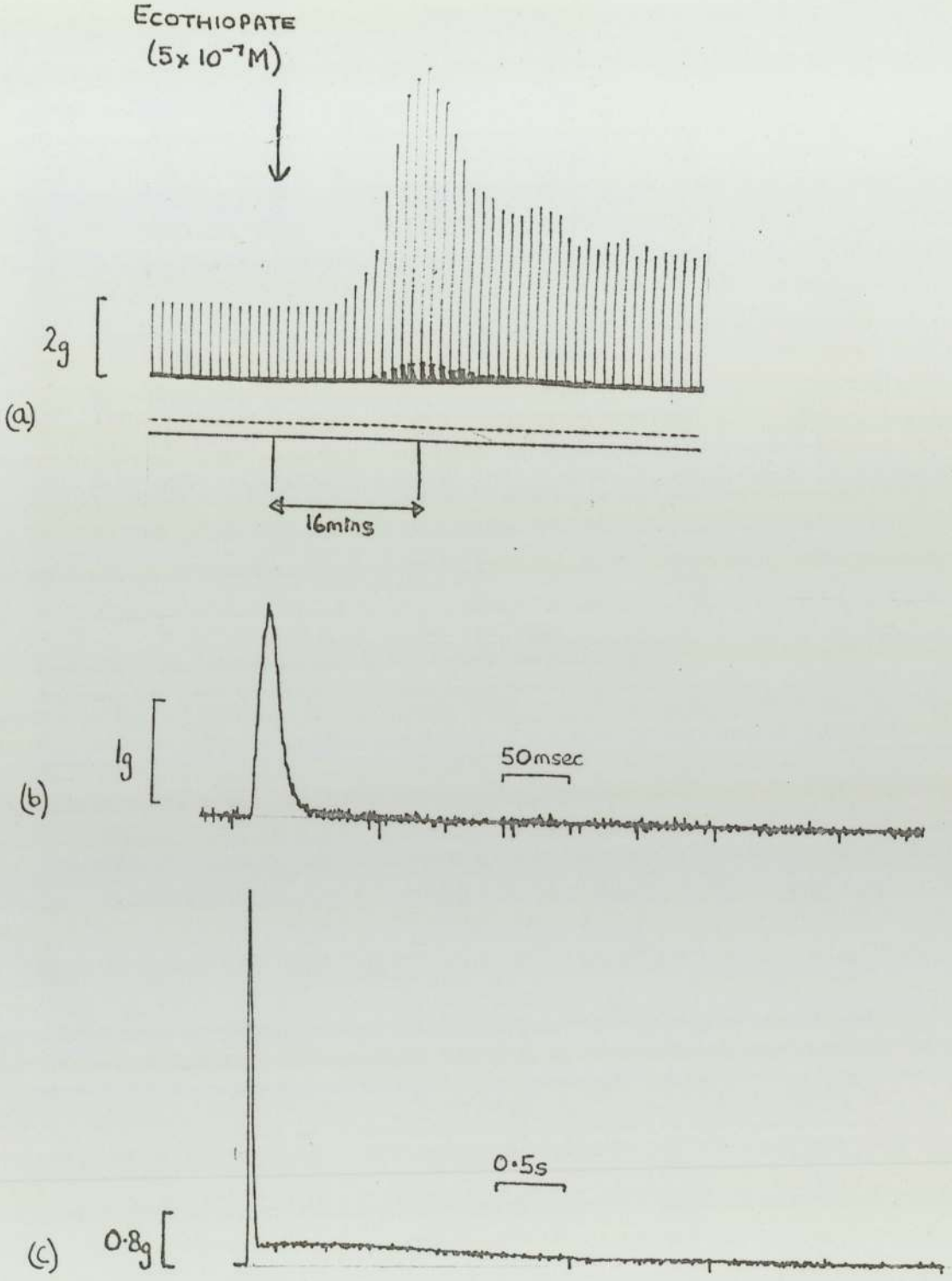
Fig. 23

(a) Mouse hemi - diaphragm. 37°C. Physiological saline. Record of contraction to nerve stimulation (0.017Hz). Initial tension 2g. Ecothiopate added as shown. Note the increase in tension of contraction, the development and waning of prolonged localised contractions and the presence of a few spontaneous contractions.

Below are records made using a storage oscilloscope and an XY Plotter.

(b) Control response. Note the time course of contraction and the tension achieved.

(c) Response 15 mins after ecothiopate (5×10^{-7}M.) Note the increase in tension achieved followed by the prolongation of the late part of the contraction lasting about 4.25 sec.


ECOTHIOPATE
(5x 10⁻⁷M)
2g
(a)
16mins
1g
(b)
50msec
0·8g
(c)
0·5s

Calcium accumulation in the absence of drugs

The procedures used to calculate accumulation in the different pieces of muscle and any stasticial treatment of the results are detailed in Methods (pp 36-37 / 46-47). It is important to note that the results indicate the total calcium accumulated and not just ^{45}Ca (see Methods p. 37). For reasons to be discussed later (p. 122), where stimulation apparently made little difference to calcium accumulation, these experiments were made on unstimulated muscles. Whole diaphragms were incubated in physiological saline, oxygenated and maintained at 37°C, containing $^{45}Ca\,Cl_2$ tracer for periods to 90 mins.

The results (Table 4 and Fig. 24) indicate that there was no calcium accumulation at the endplates. However, in the non-junctional pieces of muscle, a progressive increase in calcium accumulation had apparently occurred (Fig. 25) which was significant between 18 and 30 mins and between 30 and 60 mins (Table 5).

Table 6 is presented to show all the relevant data provided by the experiments used in the compilation of Table 4. All other experiments gave similar raw data.

Table 4 Calcium accumulation in unstimulated mouse hemi - diaphragms (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C followed by washout for 30 mins in tracer - free physiological saline before immersion in acetone. (The letters A_1 to H_1 are for future reference)

Time of incubation in ^{45}Ca - physiological saline (minutes).	n - moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
18	A_1 - 0.016 ± 0.020(9)	B_1 0.166 ± 0.024(9)
30	C_1 0.004 ± 0.031(10)	D_1 0.321 ± 0.042(10)
60	E_1 -0.038 ± 0.041(6)	F_1 0.493 ± 0.054(6)
90	G_1 -0.077 ± 0.098(6)	H_1 0.660 ± 0.068(6)

Table 5 Independent t values and probabilities between the unpaired sets of data in Table 4.

Unpaired sets of data	t value	degrees of freedom	Probability	statistically significant / insignificant
A_1 v C_1	0.5463	17	0.600	insignificant
C_1 v E_1	0.8624	14	0.593	insignificant
E_1 v G_1	0.4062	10	0.694	insignificant
B_1 v D_1	3.2822	17	0.005	significant
D_1 v F_1	2.6516	14	0.018	significant
F_1 v H_1	2.1200	10	0.058	insignificant

Table 6 The raw data provided by one experiment used to compile Table 4.
(cpm j = counts per minute in the junctional or endplate region of the hemi - diaphragm
cpm nj = counts per minute in the non-junctional region of the hemi - diaphragm
wt. = dry weight of muscle, mg.)

Experimental Conditions	Hemi - diaphragm	cpm j(wt)	cpm nj(wt)	calcium accumulated n-moles mg^{-1} muscle	
				endplate	non-junctional
(1) Unstimulated Physiological Saline ^{45}Ca(30 mins)	1 L	245.8(2.2)	263.8(2.6)	0.021	0.255
	R	348.0(2.9)	458.8(3.1)	- 0.062	0.351
	2 L	269.7(2.4)	252.8(2.6)	0.034	0.234
	R	219.2(1.9)	222.8(2.3)	0.038	0.246
	3 L	252.2(2.6)	203.2(2.0)	- 0.013	0.204
	R	219.8(2.9)	208.9(1.8)	- 0.115	0.203
	4 L	271.9(1.8)	391.7(2.7)	0.013	0.522
	R	344.5(1.8)	357.3(2.7)	0.142	0.462
	5 L	193.4(1.8)	318.0(2.0)	- 0.147	0.500
	R	329.0(2.7)	204.5(2.5)	0.125	0.236
			mean :	0.004	0.321 (10 expts.)
			S.D. :	0.093	0.127
			S.E.m :	0.031	0.042

Discussion

The results, showing the absence of endplate calcium accumulation, are in agreement with those of Evans (1974) who found that even stimulating muscles at high frequencies (50Hz for 15 mins) failed to result in any endplate calcium accumulation. In other words, when neuromuscular transmission is apparently normal, there is no extra uptake of Ca^{2+} at the endplate.

The accumulation of calcium in the rest of the muscle (i.e. non-junctional) is presumably the result of calcium exchange mechanisms between the inside of the muscle fibres and the extracellular space associated with muscle activity (Bianchi and Shanes 1959).

Since it has been shown that there is no calcium accumulation at the endplates in physiological saline only, any subsequent accumulation must be due to any alterations in the experimental conditions as, for example, the presence of the drug ecothiopate.

Effect of ecothiopate on calcium accumulation

Whole mouse diaphragms were incubated in physiological saline containing $^{45}CaCl_2$ and ecothiopate (5×10^{-7}M) for periods ranging from 15 to 90 mins.

The results (Table 7 and Fig. 24) show that there was a progressive increase in calcium accumulation at the endplates. This increase was significant between 15 and 30 mins and between 30 and 60 mins but there was no significant difference between the calcium that accumulated in 60 and 90 mins (Table 8). The latter suggests that endplate calcium accumulation had reached a maximum. Endplate calcium accumulation in the presence of ecothiopate was significantly higher than that accumulated in the absence of ecothiopate (Table 9).

Calcium accumulation in the non-junctional parts of fibres showed a progressive increase which was significant between 18 and 30 mins and between 30 and 60 mins (Tables 7 & 8). The results were not significantly different to those obtained in the absence of ecothiopate except for the period between 60 and 90 mins (Table 9 and Fig. 25).

Table 7 Calcium accumulation in unstimulated mouse hemi-diaphragms (mean ± S.E. of mean, number of hemi-diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca-physiological saline at 37°C in the presence of ecothiopate (5×10^{-7}M) followed by washout for 30 mins in tracer-free physiological saline before immersion in acetone. (The letters A_2 to H_2 are for future reference).

Time of incubation in ^{45}Ca-physiological saline and ecothiopate (minutes)	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
15	A_2 0.162 ± 0.017(8)	B_2 0.160 ± 0.012(8)
30	C_2 0.531 ± 0.077(15)	D_2 0.419 ± 0.076(15)
60	E_2 1.169 ± 0.156(7)	F_2 0.440 ± 0.090(7)
90	G_2 0.836 ± 0.212(7)	H_2 1.114 ± 0.084 (7)

Table 8 Independent t values and probabilities between the unpaired sets of data in Table 7 .

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ insignificant
A_2 v C_2	3.5586	21	0.0022	significant
C_2 v E_2	4.3564	20	0.000	significant
E_2 v G_2	1.3660	12	0.195	insignificant
B_2 v D_2	2.5250	21	0.0187	significant
D_2 v F_2	0.1765	20	0.856	insignificant
F_2 v H_2	5.9225	12	0.000	significant

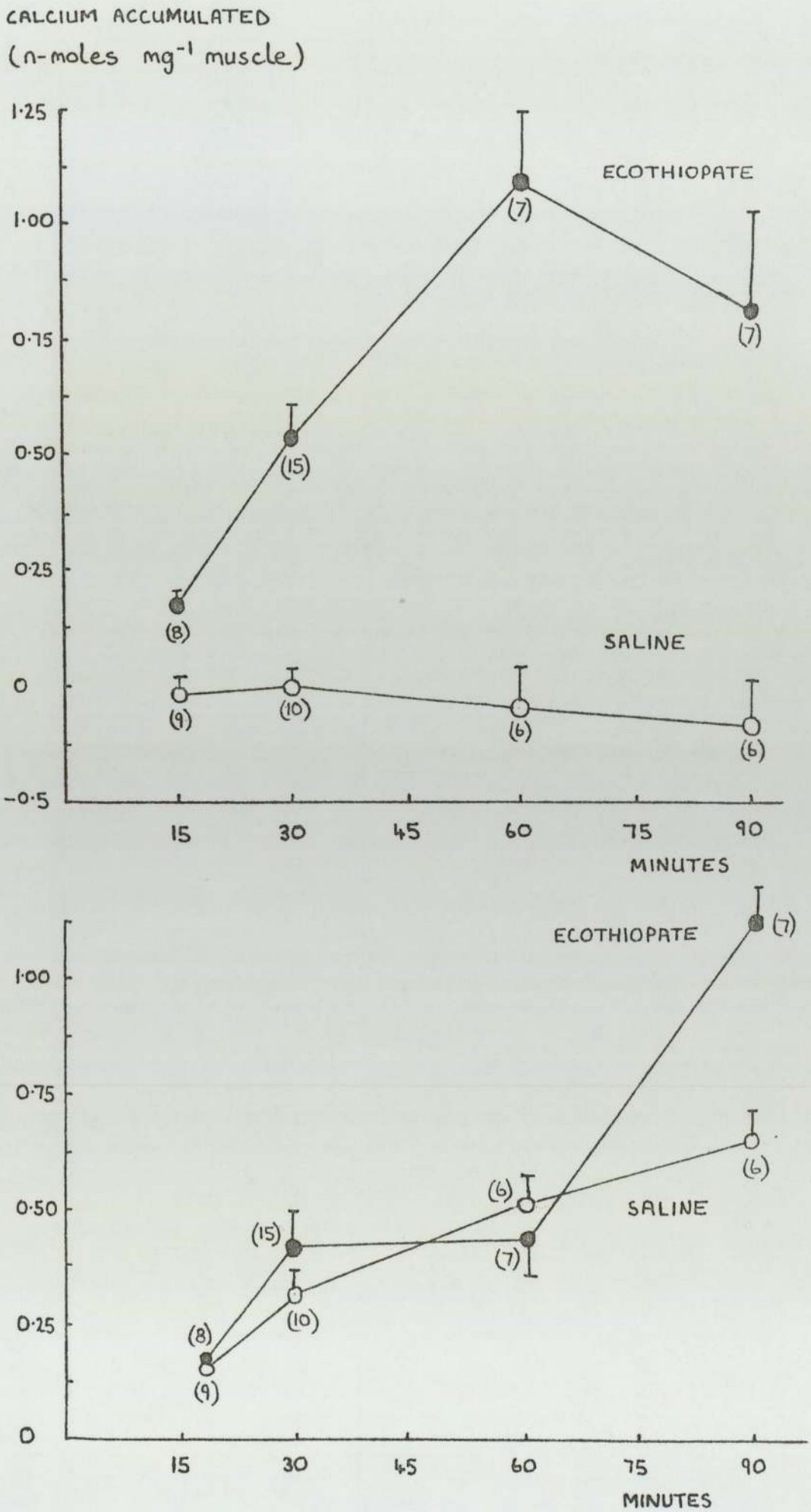
Table 9 Independent t values and probabilities between unpaired sets of data comparing calcium accumulation in the presence and absence of ecothiopate.

Unpaired sets of data	t value	degrees of freedom	Probability	significant/ insignificant
A_1 v A_2	7.0330	15	0.000	significant
C_1 v C_2	5.5579	23	0.000	significant
E_1 v E_2	7.4982	11	0.000	significant
G_1 v G_2	3.9873	11	0.002	significant
B_1 v B_2	0.2193	15	0.824	insignificant
D_1 v D_2	1.0079	23	0.325	insignificant
F_1 v F_2	0.5175	11	0.620	insignificant
H_1 v H_2	4.4513	11	0.001	significant

Fig. 24. Time course of endplate calcium accumulation in unstimulated muscles. Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C in the presence (●——●) and absence (○——○) of ecothiopate (5×10^{-7} M), followed by washout for 30 mins in tracer - free physiological saline before immersion in acetone. Each point is the mean of a number of hemi - diaphragms (parentheses) ± S.E. of mean. Note standard error bars.

Fig. 25. Time course of non - junctional calcium accumulation in unstimulated muscles. Diaphragms were incubated as above.

Each point is the mean of a number of hemi - diaphragms (parentheses) ± S.E. of mean. Note standard error bars.


CALCIUM ACCUMULATED
(n-moles mg⁻¹ muscle)
1·25
1·00
0·75
0·50
0·25
0
-0·5
ECOTHIOPATE
SALINE
(8)
(15)
(7)
(7)
(9)
(10)
(6)
(6)
15
30
45
60
75
90
MINUTES
ECOTHIOPATE
SALINE
(7)
(6)
(6)
(15)
(7)
(8)
(10)
(9)
1·00
0·75
0·50
0·25
0
15
30
45
60
75
90
MINUTES

Discussion

Ecothiopate apparently causes a progressive and significant increase in calcium accumulation at the endplate regions of mouse diaphragm. The evidence suggests that this increase reaches a maximum at approximately 60 mins after the addition of ecothiopate to the muscles. This endplate calcium accumulation is believed to reflect post-junctional Ca^{2+} entry resulting from ecothiopate action. This is because calcium has been shown not to accumulate at the endplates in the absence of ecothiopate (Table 4 and Fig.24)

That accumulation of calcium was mainly in the muscle fibres and not due to a significant accumulation in the nerve cells was shown by experiments made by Evans (1974) on denervated muscles.

That ecothiopate does not significantly change calcium accumulation in non-junctional parts of fibres for periods up to 60 mins suggests that the action of ecothiopate is limited to the endplates (see also pp.176-). However, between 60 and 90 mins, non-junctional calcium accumulation in the presence of ecothiopate shows, apparently, a large significant increase compared to the absence of ecothiopate (Fig. 25). This increase of 0.674 (mean 7 muscles) n-moles mg^{-1} total dry muscle, coincides with a decrease in endplate calcium accumulation in the presence of ecothiopate of 0.333 (mean 7 muscles) n-moles mg^{-1} total dry muscle. This latter decrease was not significant. Since it appears that endplate calcium accumulation in the presence of ecothiopate has reached maximum (saturation), it is possible to speculate that if post-junctional Ca^{2+} entry persists, then calcium might start to spread outwards from the endplates into the non-junctional parts of the muscle fibres. This would effectively increase non-junctional calcium accumulation. This hypothesis would be more plausible if the apparent gain in

non - junctional calcium was more or less the same as the apparent loss from the endplate.

Alternatively, the large increase in non - junctional calcium accumulation between 60 and 90 mins may be due to some deterioration of the muscle tissue. This deterioration might involve cell membrane disruption somehow effectively resulting in an inward leakage of Ca^{2+}. If this were to be so then the effect must be associated with the presence of ecothiopate since a similar increase in non - junctional calcium accumulation did not occur in the absence of ecothiopate.

Effect of stimulation on calcium accumulation

Whole diaphragms were placed in ^{45}Ca - physiological saline and stimulation of the nerve (0.02Hz) to one half of the diaphragm was started approximately 5 mins later (see Methods p. 34). The other halves of the diaphragms were used as unstimulated controls. Ecothiopate (5×10^{-7}M) was added to the physiological saline 10 mins after the start of stimulation in order to allow the muscles time to reach a steady - state condition. After incubation with ecothiopate for 15 mins, the muscles were placed in tracer - free physiological saline for 30 mins and then immersed in acetone.

The results (Table 10 and Fig. 26) show that the endplate calcium accumulation in muscles stimulated (0.02Hz) indirectly for 15 mins in the presence of ecothiopate was 0.334 ± 0.034 (mean of 6 muscles ± S.E. of mean) n-moles mg^{-1} total dry muscle. Endplate calcium accumulation in the unstimulated control muscles was 0.213 ± 0.048 n-moles mg^{-1} total dry muscle. Subsequent statistical analysis showed that the difference in Ca accumulation between stimulated and unstimulated muscles was just significant (Table 11) Further experiments made on unstimulated muscles incubated for 15 mins in ^{45}Ca - physiological saline before the addition of ecothiopate for 15 mins gave results for endplate Ca accumulation (Table 10) which, when treated statistically along with the previous control unstimulated muscles, were found not to be significantly different from the results with stimulated muscles (Table 11).

Stimulation did not result in any significant changes in non - junctional calcium accumulation (Tables 10 and 11).

An interesting observation that may prove to be important is the difference in calcium accumulation between unstimulated muscles pre - incubated in ^{45}Ca - physiological saline for 15 mins before the

addition of ecothiopate (Table 10) and those unstimulated muscles which were not pre-incubated (Table 7). The muscles which were pre-incubated showed a significantly higher accumulation in both the endplate and the non-junctional regions of the muscle. The difference was, presumably, due to the greater equilibration of the ^{45}Ca in the extracellular space of the pre-incubated muscles.

Table 10 Ca accumulation in mouse hemi - diaphragms (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C in the presence of ecothiopate (5×10^{-7}M), one half being stimulated via the phrenic nerve (0.02Hz)(s) and the other half acting as the unstimulated controls (usc). After 15 mins in ecothiopate the diaphragms were placed in tracer - free physiological saline before immersion in acetone. (The letters A_3 to F_3 are for reference in conjunction with Table 11).

S = stimulated muscles

USC = unstimulated control muscles

	n - moles of Ca accumulated mg^{-1} total dry muscle	
	endplates	non-junctional
S	A_3 0.334 ± 0.034(6)	B_3 0.378 ± 0.029(6)
USC	C_3 0.213 ± 0.048(6)	D_3 0.516 ± 0.073(6)
Total USC (all experiments)	E_3 0.273 ± 0.027(21)	F_3 0.433 0.037(21)

Table 11 Independent t values and probabilities between the unpaired sets of data comparing Ca accumulation in stimulated and unstimulated muscles.

Unpaired sets of data	t value	degrees of freedom	Probability	significant/ insignifcant
A_3 v C_3	2.2741	10	0.044	significant
A_3 v E_3	1.1597	25	0.256	insignificant
B_3 v D_3	1.9361	10	0.079	insignificant
B_3 v F_3	0.7807	25	0.552	insignificant

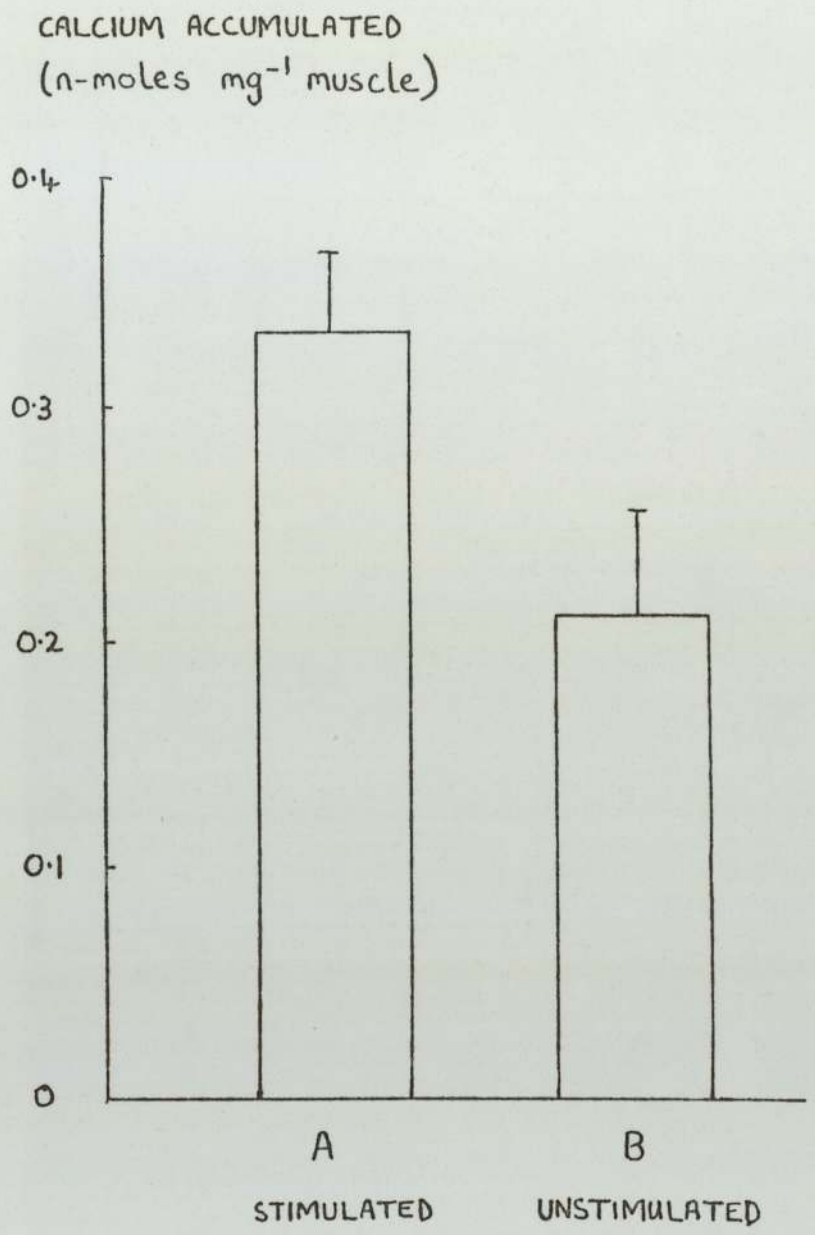


Fig. 26. Endplate calcium accumulation (n-moles mg^{-1} total dry muscle) in muscles incubated with ecothiopate (15 mins).

A. stimulated (0.02Hz).

B. unstimulated.

Note the standard error bars.

Discussion

It appears that indirect stimulation (0.02Hz) increases endplate calcium accumulation in muscles incubated with ecothiopate for 15 mins (Fig. 26). The difference in accumulation between stimulated and unstimulated muscles was, however, subsequently shown to be of doubtful significance (Table 11). Any increase in accumulation in stimulated muscles is, presumably, due to the greater transmitter action resulting from evoked release. It is, therefore, concluded that stimulation (0.02Hz) made only a slight, if any, difference to the amount of endplate calcium accumulation over 15 mins, and it is assumed that stimulation would not, therefore, alter the time course of accumulation over 60 mins. Unfortunately, experiments to confirm this assumption were not made because it was believed that the procedure for stimulated muscles might cause greater variation, as it had done in previous experiments on rat diaphragms, resulting in statistical insignificance.

My original intention to compare endplate calcium accumulation with plc development was frustrated because, apparently, endplate calcium accumulation in stimulated muscles was similar to that in unstimulated muscles. It was, therefore, decided to interpolate an investigation into the nature of the Ca^{2+} uptake at the endplate.

Summary

An accumulation of calcium has been found at the endplates only after ecothiopate. It has been suggested that this accumulation reflects an increased post-junctional entry of Ca^{2+}. The results implied that stimulation, at low frequency (0.02Hz), made little difference to the accumulation of calcium.

SECTION VI

What Causes the Accumulation of Calcium at the Endplate ?

Introduction

The post-junctional entry of Ca^{2+} during normal neuromuscular transmission has been shown not to result in an accumulation of calcium at the endplate region. Ca^{2+} entry is, apparently, more substantial when depolarisation at the endplate is prolonged, for example, when ecothiopate inhibits ChE thus prolonging and intensifying transmitter action. Endplate calcium accumulation in stimulated muscles was believed to result from the prolonged depolarisation developed after each indirect stimulus and is thought to be a possible cause of plcs. Whatever the cause of plcs, it is apparent that endplate calcium accumulation in stimulated muscles is not totally associated with plc development (plc accumulation) since calcium, apparently, continues to accumulate after plcs have waned. Therefore, there must be additional, if not alternative, factors responsible. It seems probable that these factors would have to act either by effectively prolonging endplate depolarisation or by directly effecting Ca^{2+} entry through the post-junctional membrane.

That similar endplate calcium accumulation occurred in unstimulated muscles suggested that other factors were also responsible since there obviously could be no plc-accumulation in the absence of nerve stimulation. It was possible that those factors responsible for endplate calcium accumulation in unstimulated muscles were also responsible, perhaps in conjunction with plc-accumulation, for the total uptake in stimulated muscles.

i.e. Ca accumulation in stimulated muscles with eocthiopate

= plc + A + B etc.

Ca accumulation in unstimulated muscles with ecothiopate

= A + B + etc.

Since calcium accumulation in stimulated muscles is approximately equal to that in unstimulated muscles, it was thought that one or more of the factors A, B, etc must make a greater contribution in unstimulated muscles compared to stimulated muscles.

Calcium accumulation is believed to result from prolonged transmitter action. Therefore, in the absence of indirect stimulation, the increase in transmitter action must, presumably, result from that transmitter spontaneously released and that released as a consequence of spontaneous antidromic firing. The latter has been suggested, by axon reflex action, to result in spontaneous contractile activity (p 65). Firstly, it is possible that a prolongation of the endplate depolarisation, associated with the SCs, might contribute to the endplate calcium accumulation (SC accumulation). Secondly, a prolongation of the post-junctional action of the spontaneously released miniature endplate potentials (mepps) by ecothiopate might also significantly contribute to endplate calcium accumulation (mepp accumulation). Finally, as has already been suggested, endplate calcium accumulation might be due to a direct action of ecothiopate.

In this section, the results of experiments made to determine the significance of SC-accumulation and mepp-accumulation to overall endplate calcium are presented and discussed.

Results

(i) The development of spontaneous contractions (SCs) after ecothiopate.

The development of SCs in rat hemi-diaphragms stimulated via the nerve at low frequency (e.g. 0.017 - 0.01Hz) has been described (see Section 1, pp 50-). Experiments made using mouse hemi-diaphragms have shown a similar development of SCs (e.g. Fig. 23) It is thought, however, that the variable but usually small number of SCs observed in stimulated muscles would make only a minor contribution to the total calcium accumulated. If SCs were to make a more significant contribution in unstimulated muscles then it was believed that they would need to develop to a much greater extent. It is interesting to note that the unstimulated diaphragms incubated in ^{45}Ca - physiological saline and ecothiopate were observed to contract spontaneously for periods of, at least, 60 mins.

Effect of ecothiopate on unstimulated muscles

Experiments were made to record the development of spontaneous contractions in unstimulated rat hemi-diaphragm. Ecothiopate (5×10^{-7}M) clearly provokes the development of spontaneous contractions (Fig. 27), the latter starting approximately 6 mins (mean of 6 muscles) after ecothiopate addition. There was an initial surge of activity lasting approximately 10 - 15 mins, whereafter, the contractions were observed to be reduced in both frequency and amplitude. Spontaneous contractions were still being recorded in some preparations $2\frac{1}{2}$ hours after ecothiopate addition. In unstimulated mouse diaphragms, SCs persist for several hours after ecothiopate (Chahal, personal communication, 1980).

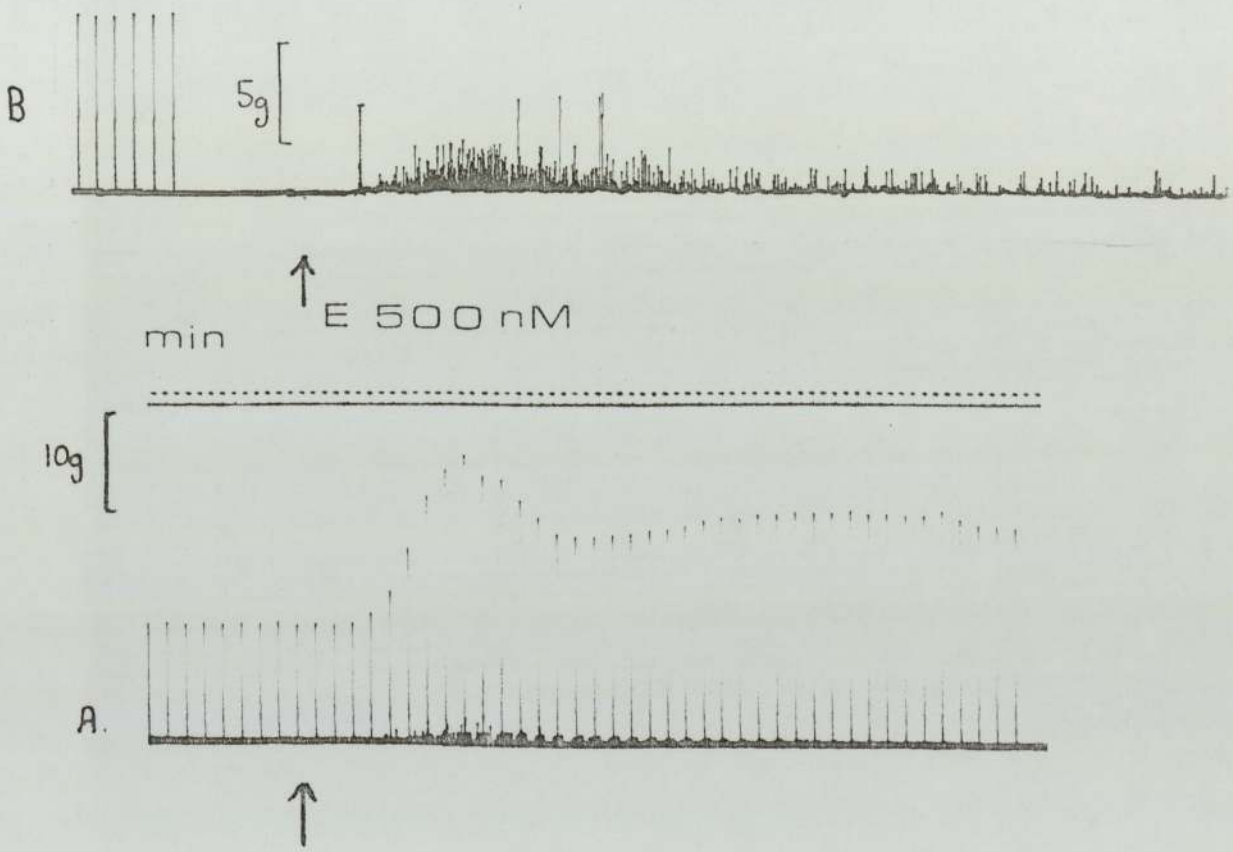


Fig. 27

Rat hemi - diaphragm. Physiological saline ($[Mg^{2+}]$ 10^{-3}M) at 37°C. Records of contraction. Ecothiopate (5×10^{-7}M) added at arrows.

(A) Muscle stimulated via the phrenic nerve (0.01Hz). Note the development of spontaneous contractions approximately 7 mins after ecothiopate disappearing completely approximately 18 mins later.

(B) Unstimulated muscle. Note the surge of spontaneous contractile activity beginning 7 mins after ecothiopate and lasting approximately 10 - 15 mins.

Discussion

There was, thus, a marked difference in the amount of spontaneous contractile activity between stimulated and unstimulated preparations. The greater development of spontaneous contractions in unstimulated muscles suggests that if SC accumulation is a contributory factor then the latter would play a more significant role in unstimulated muscles compared with stimulated muscles.

(ii) Effect of [Mg^{2+}] (3.5×10^{-3}M) on the contractile response of rat hemi - diaphragm in the presence of ecothiopate

Ferry and Ward (unpublished) found that with a [Mg^{2+}] (3.5×10^{-3}M), ecothiopate did not result in the enhancement of the early contraction and the development of SCs and plcs. Experiments were first made to confirm this observation in both stimulated and unstimulated muscles and then to determine the effect this [Mg^{2+}] has on endplate calcium accumulation in order to confirm plc- and sc- linked contributions to the latter.

a) In stimulated muscles

After establishing control contractile responses to nerve stimulation (0.01Hz) in physiological saline ([Mg^{2+}] 10^{-3}M), the latter was increased to 3.5×10^{-3}M. There was no apparent effect on the contractions which suggested that any depression of ACh release was insufficient to cause failure at the junctions, the reduction apparently being within the safety factor for transmission. The addition of ecothiopate about 25 mins later also had no further effect on the contractions, the higher [Mg^{2+}] apparently preventing any enhancement of the early contraction and the development of SCs and plcs (Fig. 28).

b) In unstimulated muscles

After establishing an initial development of spontaneous contractions by adding ecothiopate (5×10^{-7}M), the [Mg^{2+}] of the physiological saline was increased to 3.5×10^{-3}M. The results (Fig. 28) clearly demonstrate that the development of the spontaneous contractions was quickly and completely blocked within 6 mins.

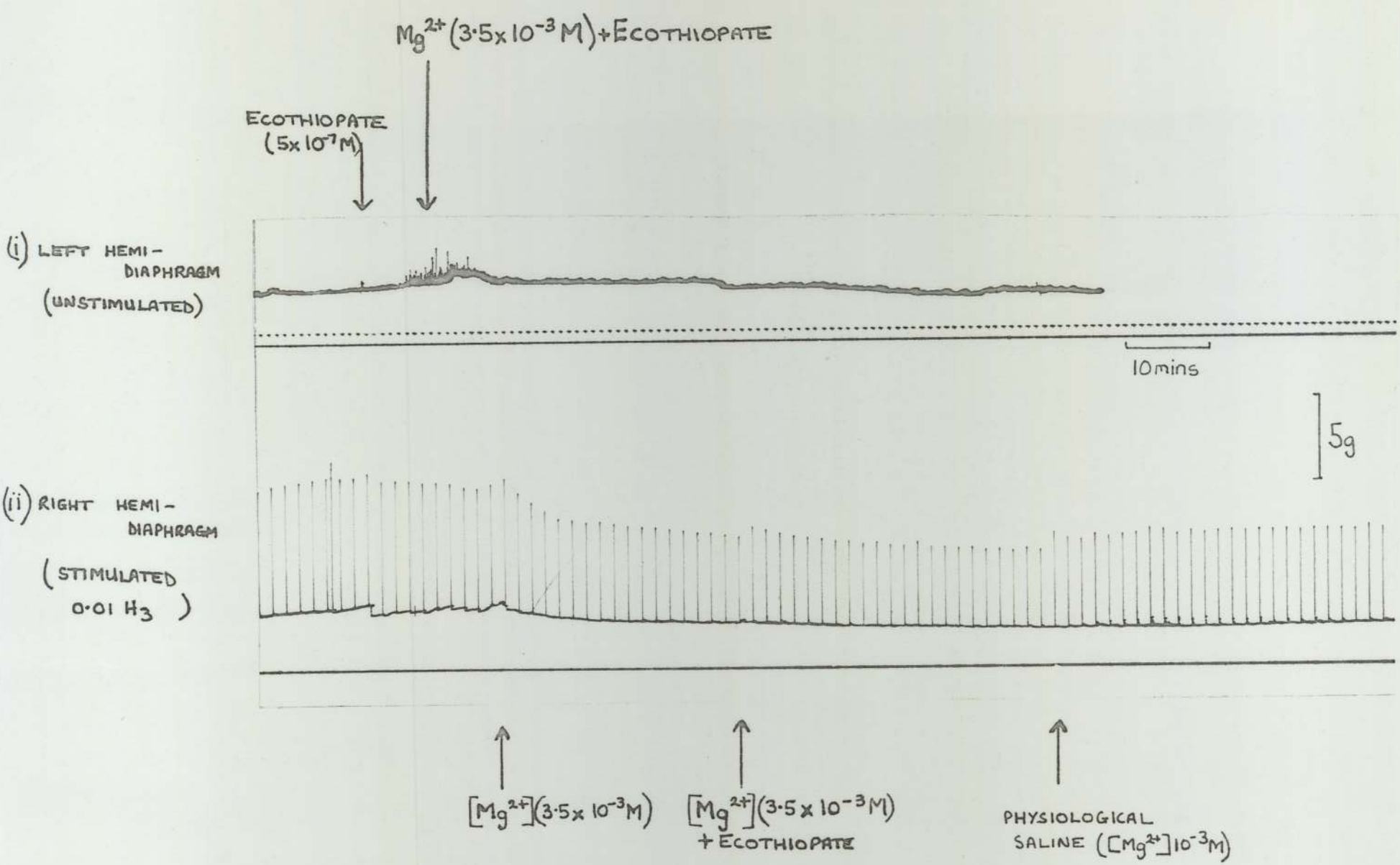

$Mg^{2+}(3{\cdot}5\times10^{-3}M)$+ECOTHIOPATE
ECOTHIOPATE ($5\times10^{-7}M$)
(i) LEFT HEMI-DIAPHRAGM (UNSTIMULATED)
10mins
5g
(ii) RIGHT HEMI-DIAPHRAGM (STIMULATED 0·01 Hz)
$[Mg^{2+}](3{\cdot}5\times10^{-3}M)$
$[Mg^{2+}](3{\cdot}5\times10^{-3}M)$ + ECOTHIOPATE
PHYSIOLOGICAL SALINE ($[Mg^{2+}]10^{-3}M$)

Fig. 28 Rat hemi - diaphragm. 37^{oC}.
Contraction records.

(i) Unstimulated hemi - diaphragm (Left)
Ecothiopate (5×10^{-7}M) added as shown. Note the development of considerable spontaneous activity
$[Mg^{2+}]$ of physiological saline increased to 3.5×10^{-3}M as shown. Note the rapid termination of any spontaneous activity.

(ii) Stimulated hemi - diaphragm (Right) (via the nerve 0.01Hz).
$[Mg^{2+}]$ of physiological saline increased to 3.5×10^{-3}M as shown. Note the slight reduction in twitch amplitude. Ecothiopate (5×10^{-7}M) added as shown. Note that there is no change in the contraction record. Note also that washing with physiological saline ($[Mg^{2+}]$ 10^{-3}M) leads to a small increase in the amplitude of the early contraction and to the development of plcs.

Discussion

It has been shown that $[Mg^{2+}]$ (3.5×10^{-3}M) blocks the enhancement of the early contraction and the development of SCs and plcs in stimulated muscles and the development of SCs in unstimulated muscles. If endplate calcium accumulation is linked to the development of plcs and SCs then it would be expected that $[Mg^{2+}]$ (3.5×10^{-3}M) would also prevent the accumulation of calcium at the endplates.

(iii) Effect of $[Mg^{2+}]$ (3.5×10^{-3}M) on calcium accumulation

Experiments were made on unstimulated mouse diaphragms incubated in ^{45}Ca - physiological saline containing $[Mg^{2+}]$ (3.5×10^{-3}M) and ecothiopate (5×10^{-7}M) for a period of 30 mins. The results (Table 12 and Fig. 29) show a mean endplate calcium accumulation of - 0.003 ± 0.066 n-moles mg^{-1} dry muscle (mean ± S.E. of mean, 5 hemi - diaphragms). This was significantly lower than the endplate calcium accumulation in muscles incubated in normal physiological saline (i.e. $[Mg^{2+}]$ 10^{-3}M) and ecothiopate and was not significantly different to the value obtained for muscles incubated in normal physiological saline only (Table 13). However, non - junctional calcium accumulation was significantly greater in muscles incubated with $[Mg^{2+}]$ (3.5×10^{-3}M) than with $[Mg^{2+}]$ (10^{-3}M), both with ecothiopate.

Table 12. Calcium accumulation in unstimulated mouse hemi-diaphragms (mean ± S.E. of mean, number of hemi-diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca-physiological saline at 37°C containing various $[Mg^{2+}]$ and ecothiopate (5×10^{-7}M) for 30 mins, followed by washout for 30 mins in tracer-free physiological saline before immersion in acetone. The letters A_4 to F_4 are for future reference).

$[Mg^{2+}]$	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional
3.5×10^{-3}M	A_4 -0.003 ± 0.066(5)	B_4 0.708 ± 0.082(5)
10^{-3}M	C_4 0.531 ± 0.077(15)	D_4 0.419 ± 0.076(15)
10^{-3}M (No ecothiopate)	E_4 0.004 ± 0.031(10)	F_4 0.321 ± 0.042(10)

Table 13 Independent t values and probabilities between the unpaired sets of data in Table 12.

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
A_4 v C_4	3.9480	18	0.0012	Significant
B_4 v D_4	2.124	18	0.0451	Significant
A_4 v E_4	0.1139	13	0.9071	Insignificant
B_4 v F_4	5.0606	13	0.0004	Significant

Fig. 29(a). Endplate calcium accumulation in unstimulated mouse diaphragm in the presence of ecothiopate (30 mins)

A. $[Mg^{2+}]$ 3.5×10^{-3}M

B. $[Mg^{2+}]$ 10^{-3}M

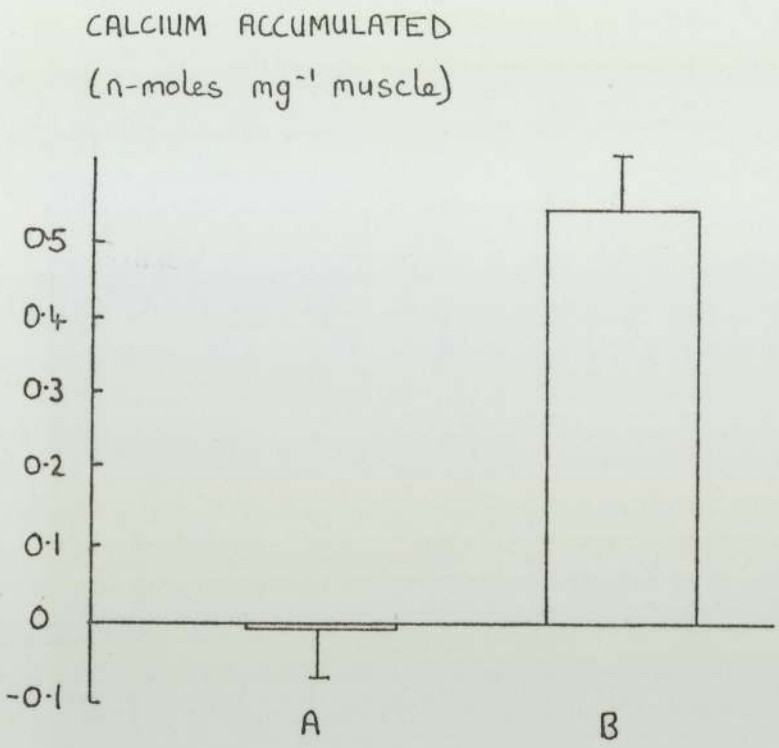
Note standard error bars.

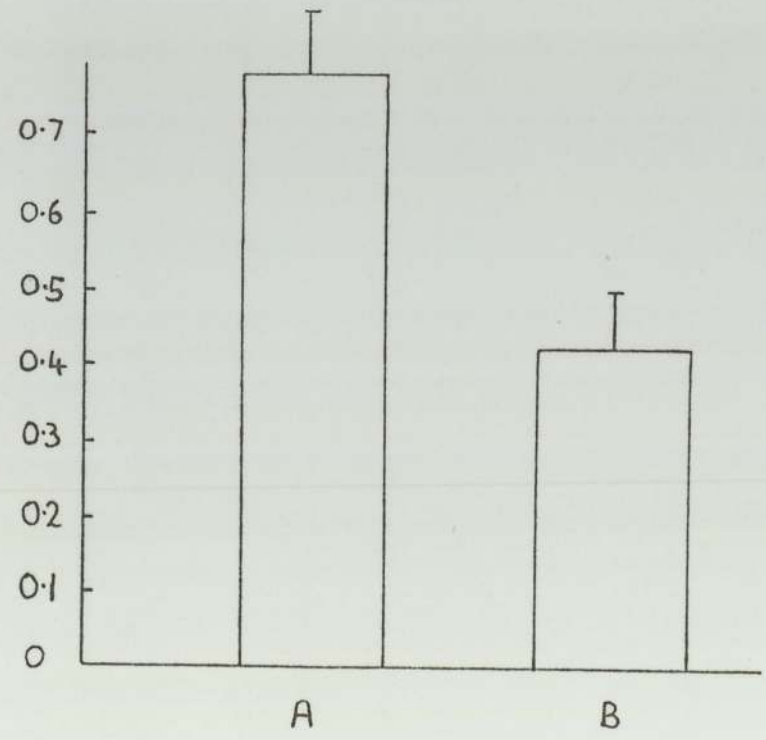
Fig. 29(b). Non-junctional calcium accumulation in unstimulated mouse diaphragm in the presence of ecothiopate (30 mins)

A. $[Mg^{2+}]$ 3.5×10^{-3}M

B. $[Mg^{2+}]$ 10^{-3}M

Note standard error bars.


CALCIUM ACCUMULATED
(n-moles mg⁻¹ muscle)
0·5
0·4
0·3
0·2
0·1
0
-0·1
A
B


0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
A
B

Discussion

The results have shown that $[Mg^{2+}]$ (3.5×10^{-3}M) apparently prevents the endplate calcium accumulation usually caused by ecothiopate. This appeared to confirm, therefore, that the development of plcs and SCs is associated with endplate calcium accumulation.

It may be that $[Mg^{2+}]$ (3.5×10^{-3}M) prevents post-junctional Ca^{2+} entry either by modifying transmitter action, perhaps by reducing release, or by directly antagonising post-junctional Ca^{2+} entry. Hubbard (1961) found that $[Mg^{2+}]$ (10^{-3}M) depressed spontaneous release in rat hemi - diaphragm and calculations based on the equation formulated by Hubbard, Jones and Landau (1968) for evoked quantal release, apparently show a 60% reduction in transmitter release when $[Mg^{2+}]$ is changed from 10^{-3}M to 3.5×10^{-3}M (Ferry, personal communication). An increase in $[Mg^{2+}]$ is also believed to decrease the sensitivity of the muscle membrane by lowering the threshold membrane potential and thus increasing the depolarisation necessary to activate the specific increase in sodium permeability (Engbaek, 1972). However, the main action of $[Mg^{2+}]$ (3.5×10^{-3}M) is believed to be pre-junctional. It is therefore thought that $[Mg^{2+}]$ (3.5×10^{-3}M) reduces post-junctional Ca^{2+} entry by substantially reducing the amount of transmitter released and thus the extent of the subsequent endplate depolarisation. Presumably any prolonged endplate depolarisation is below the mechanically effective threshold and, consequently, plc development does not occur. Extracellular endplate current records might have helped to elucidate this problem but these experiments were unfortunately not made. A possible post-junctional antagonistic by Mg on Ca^{2+} entry cannot, however, be discounted.

The reduced transmitter action at the neuromuscular junction, following a reduction in release, presumably is sufficient not to initiate antidromic firing and consequently, axon reflex excitation of motor units. This would effectively explain the absence of SCs. Several findings have indicated that Mg also causes a reduction in excitability in the terminal nerve branches (Engbaek, 1972). Lastly, the absence of any enhancement of the early part of contraction can be effectively explained if muscle repetitive activity is not initiated. Since the latter is thought to be partly attributable to the endplate depolarisation and partly to antidromic firing in the motor nerve terminals (Morrison, 1977), the reduction of both presumably leads to the reduction of repetitive firing in the muscle. Further experiments made with dTC, β-Bungarotoxin(β-Butx) and on denervated muscles, in which transmitter action is reduced, also prevented any change in the contractile response due to ecothiopate action (see pp. 176-).

I find that I am unable to explain why $[Mg^{2+}]$ (3.5×10^{-3}M) significantly increases non-junctional calcium accumulation in the presence of ecothiopate above that found with $[Mg^{2+}]$ (3.5×10^{-3}M).

(iv) Do SCs have a late prolonged component ?

SCs generated pre-synaptically might be expected to have a similar time course to those contractile responses following single maximal stimulation of the nerve, that is, to exhibit prolongation. If spontaneous contractions are prolonged, then the preceding prolonged endplate currents would help to explain the post-junctional Ca^{2+} entry and subsequent accumulation in unstimulated muscles. Experiments were, therefore, made to find out whether (a) SCs had a prolonged component and (b) were preceded by prolonged endplate currents.

(a) Experiments were made on unstimulated rat hemi-diaphragms in order to record SCs elicited by ecothiopate (5×10^{-7}M). The contractions were recovered using a Signal Recovery System (Datalab 102A) in the pre-trigger mode and the averaged responses ($\bar{x} = 8$) were displayed on paper using an XY Plotter (see Methods p.26).

The results show clearly that the late part of the SCs becomes prolonged as a result of ecothiopate action (Fig. 30). This prolongation was observed to last, at least, approximately 2 sec. Prolongation was observed to begin at approximately 13 mins reaching a maximum at approximately 22 mins before disappearing approximately 30 to 35 mins after ecothiopate addition. These times were similar to those of plc development and waning (Table 1).

(b) Experiments were made to record the spontaneous action potentials associated with SCs. These records were made with intracellular electrodes (see Methods p.20,25).

The intracellular records (e.g.Fig. 31) show the endplate potentials associated with SCs to be prolonged, presumably due to prolonged endplate currents.

(c) Visual examination of diaphragms also showed that SCs were associated with prolonged contraction at the endplate region.

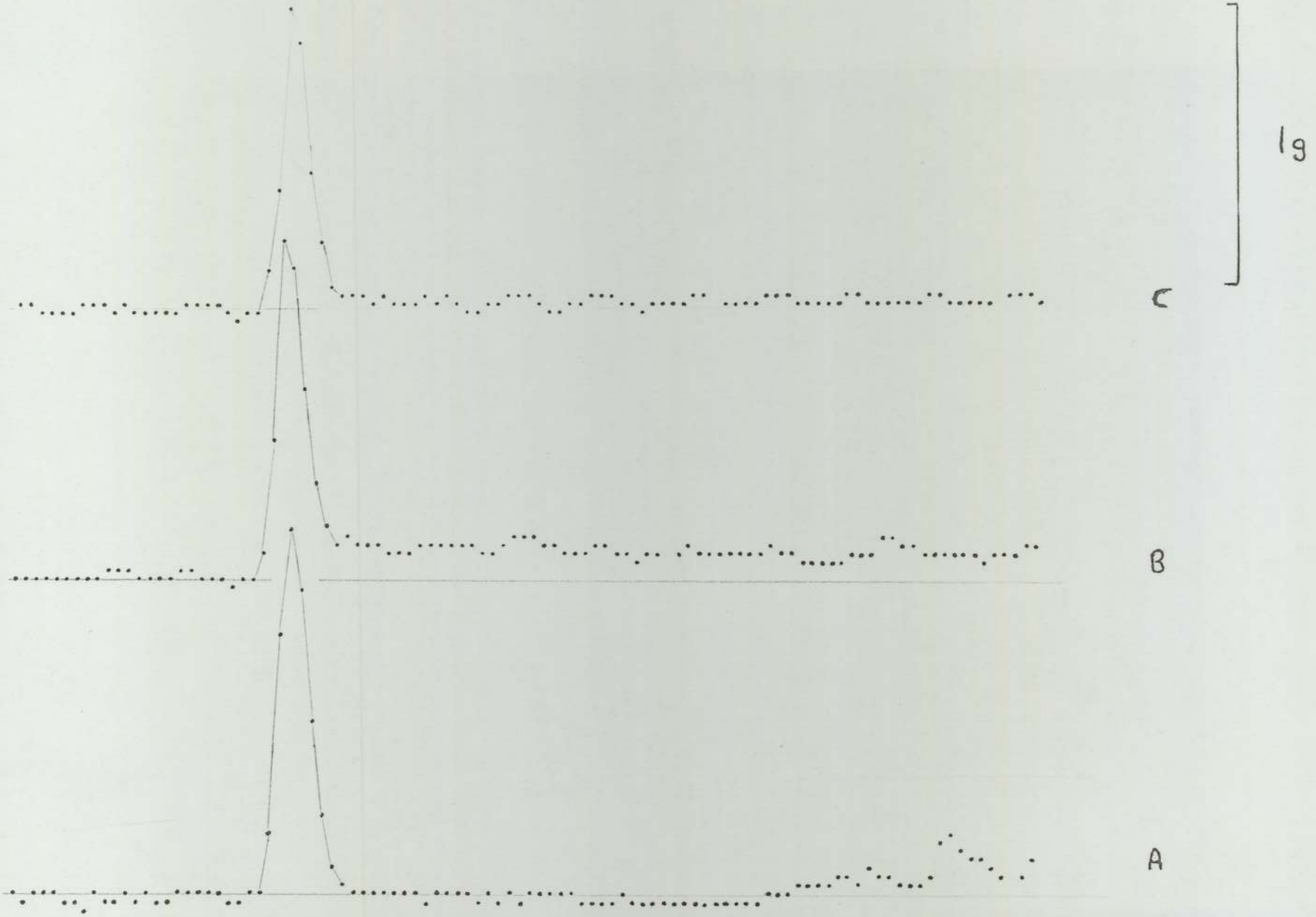

I_g
C
B
A

Fig. 30 Rat hemi - diaphragm. 37°C. Physiological Saline. Unstimulated.

Each trace shows an average of 8 responses in the time intervals stated.

A	2s sweep	10 - 13 mins after + Ecothiopate
B	2s sweep	22 - 24 mins after + Ecothiopate
C	2s sweep	29 - 31 mins after + Ecothiopate

Note the prolongation of the late part of the contraction in B.

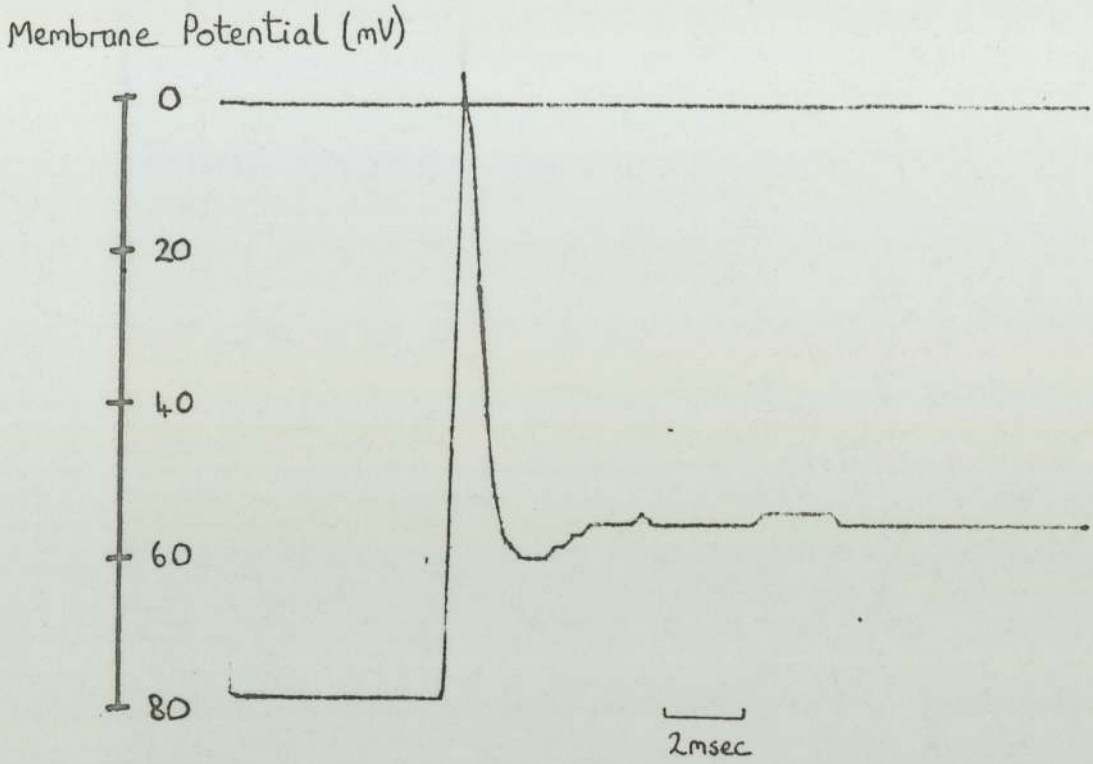


Fig. 31. Rat hemi - diaphragm 37°C.

Physiological saline.

Intracellular record of spontaneous action potential after ecothiopate (5×10^{-7}M) at an endplate.
Microelectrode filled with 3M KCl.
Note the prolonged endplate potential (20 msec +)

(Records recovered using a Signal Recovery System in the pre - trigger mode, see Methods p. 26).

Discussion

It has previously been suggested that SC - accumulation might make an important contribution to the total endplate accumulation of calcium. It was also suggested that, in the absence of any plc - accumulation, SC - accumulation would need to make a greater contribution in unstimulated muscles. The possibility that SC - accumulation might be significant was confirmed by the observations that $[Mg^{2+}]$ (3.5×10^{-3}M) rapidly diminishes spontaneous contractile activity and also prevents any endplate calcium accumulation. The observation that ecothiopate action leads to a much greater development of SCs, both in number and time course, in unstimulated muscles confirms the greater contribution from SC - accumulation that was thought to be needed in unstimulated muscles.

The results of the latest experiments have shown that SCs become prolonged in a similar way to plcs and that SCs are associated with prolonged endplate currents. It is believed that this SC - related prolonged endplate depolarisation is responsible for the post - junctional entry of Ca^{2+} and subsequent accumulation thought to comprise SC - accumulation. The above observations also appear to confirm previous conclusions that SCs are generated pre - synaptically.

(v) The effect of ecothiopate on mepps

The observation that endplate calcium accumulation progressively increases at an approximately constant rate (see Fig.24) up to, at least, 60 mins whereas the frequency and amplitude of spontaneous contractions does not remain constant in both stimulated and unstimulated muscles would suggest that there is, at least, another factor responsible for endplate calcium accumulation. As has already been suggested in the Introduction to this section (pp.123-) this factor might be the prolongation of mepps (i.e. mepp - accumulation).

Experiments were made on an unstimulated muscle in physiological saline to determine the duration at half - maximum amplitude of intracellularly recorded mepps. Ecothiopate (5×10^{-7}M) was then added to the physiological saline and mepps were continuously recorded and subsequently measured in order to compare with the above control values. After a number of attempts, these experiments were not continued since the SCs, developed as a result of ecothiopate action, invariably pushed the recording electrode out of the cells. For details of recording procedures see Methods (pp.20,25).

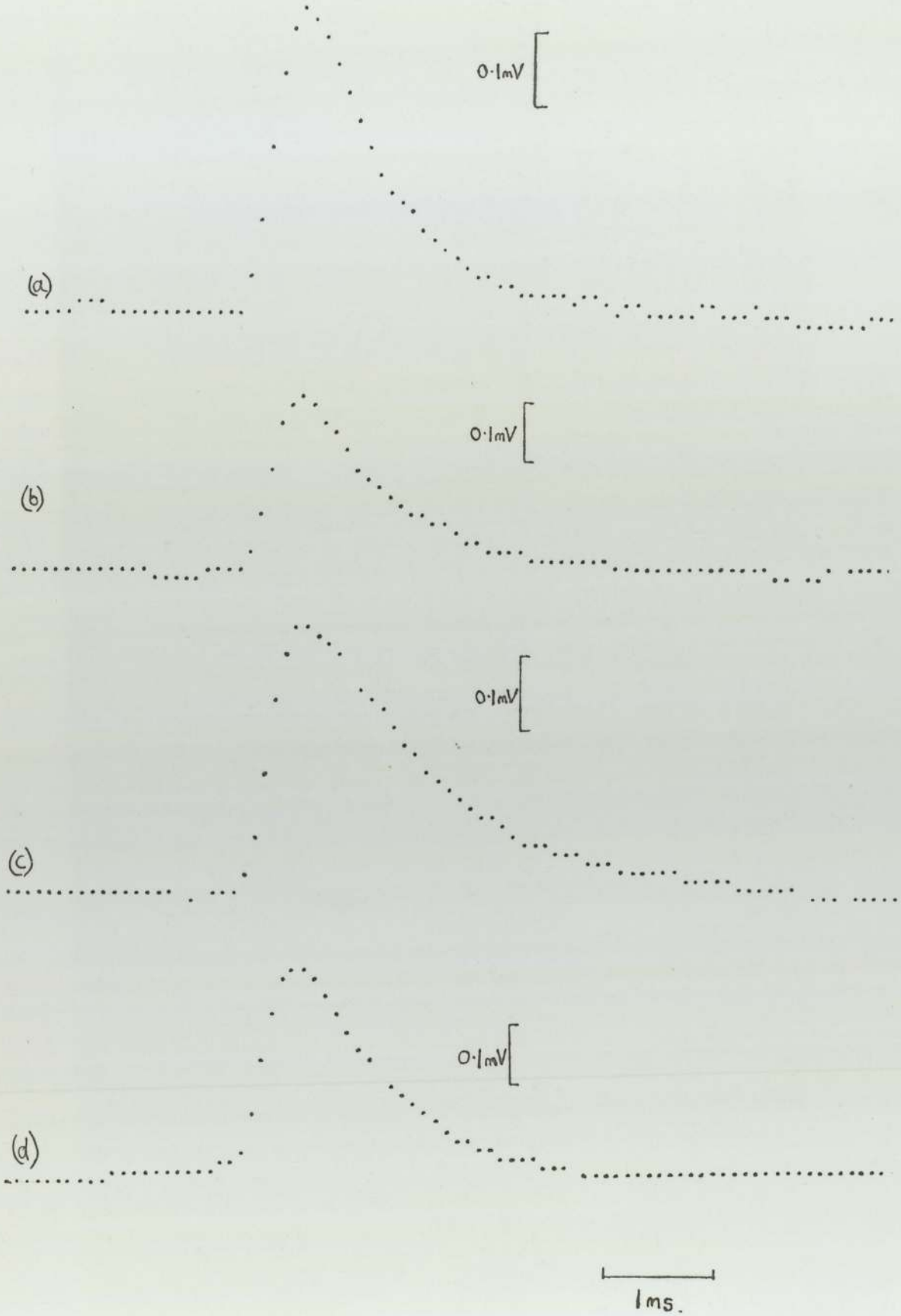

0·1mV
(a)
0·1mV
(b)
0·1mV
(c)
0·1mV
(d)
1ms.

Fig. 32 Rat hemi - diaphragm. Unstimulated. Physiological saline. Examples of intracellularly recorded mepps as recorded with glass microelectrodes filled with 3MKCl through a system of bandwidth 0.8Hz - 10KHz and displayed on a Bryans XY Plotter. Each trace is the average of 32 responses.

(a) Control (i.e. in saline containing $[Mg^{2+}]$ 10^{-3}M)

(b) In saline containing $[Mg^{2+}]$ 3.5×10^{-3}M.

(c) In saline containing $[Mg^{2+}]$ 10^{-3}M and ecothiopate (5×10^{-7}M) 8 minutes after its addition to the superfusate.

(d) In saline containing $[Mg^{2+}]$ 3.5×10^{-3}M and ecothiopate (5×10^{-7}M) also 8 minutes after its addition to the superfusate.

All responses shown here have a rise time of 600×10^{-6}s.

	Rise time (10^{-6}s)	Time after ecothiopate(mins)	Mean Amplitude(mV)	Mean duration at half-maximum amplitude(10^{-3}s) (figures in parentheses indicate number of fibres)
(a)	600	-	0.54	1.35 (10)
(b)	600	-	0.3	1.35 (2)
(c)	600	+5	0.55	1.42 (1)
		+8	0.37	1.78 (1)
		+10	0.39	2.05 (1)
		+11	0.28	1.58 (1)
		+12	0.38	2.29 (1)
(d)	600	+8	0.36	1.72 (1)
(e)	600	-	0.53	1.67 (1)

Table 14.

Rat hemi - diaphragm. Unstimulated. Physiological saline at 37°C. Rise times, amplitude and times to half - maximum amplitude of Intracellular mepps recorded with glass microelectrodes filled with 3MKCl. Bandwidth of recording system 0.8Hz - 10KHz.

(a) Control Values (saline only). $[Mg^{2+}]$ (10^{-3}M).

(b) Values with saline ($[Mg^{2+}]$ 3.5×10^{-3}M)

(c) Values with saline ($[Mg^{2+}]$ 10^{-3}M) and ecothiopate (5×10^{-7}M)

(d) Values with saline ($[Mg^{2+}]$ 3.5×10^{-3}M) and ecothiopate (5×10^{-7}M).

(e) Values with saline ($[Mg^{2+}]$ 10^{-3}M) after washing out ecothiopate.

Each endplate value is the mean of 32 'averaged' responses.

Analysis of the results (see Fig. 32 and Table 14) showed that mepps, with rise times of 600ms or less, were recorded up to 12 minutes after ecothiopate addition. These results apparently showed that ecothiopate prolonged the duration at half-maximum amplitude by up to approximately 75% of control values. There were not sufficient records made in order to apply any statistical tests.

Accompanying this apparent prolongation of mepps was an apparent reduction in amplitude from 0.54mV (mean of 10 endplates) to approximately 0.38mV (mean of 3 endplates) between 8 and 12 mins after ecothiopate.

Discussion

The results, although inconclusive, suggest, however, that ecothiopate prolongs mepps. These results are confirmed by later experiments in which extracellular miniature endplate currents (mepcs) were recorded from 'slack' muscles (pp. 211 and Fig. 42). This prolongation reflects the prolongation of the action of spontaneously released transmitter resulting from ChE inhibition. The question now arises as to whether this prolongation of mepps is sufficient to make any significant contribution to endplate calcium accumulation. Presumably, prolonged spontaneously released transmitter action must be sufficient to excite motor nerve terminals to produce antidromic firing and the subsequent axon reflex excitation of motor nerve terminals resulting in the development of SCs.

Effect of Mg^{2+} (3.5×10^{-3}M) on Mepps.

It has already been established that $[Mg^{2+}]$ (3.5×10^{-3}M) blocks (i) the ecothiopate - mediated effects on the contractile responses of rat hemi - diaphragms and (ii) endplate Ca accumulation.

Since the aim of recording mepps was to determine any mepp contribution to calcium accumulation (mepp accumulation), it was now decided to investigate the effect of Mg^{2+} (3.5×10^{-3}M) on mepps both in the presence and absence of ecothiopate (5×10^{-7}M). If $[Mg^{2+}]$ (3.5×10^{-3}M) reduced the prolongation of mepps in the presence of ecothiopate, ecothiopate - prolonged mepps might make some contribution to calcium accumulation.

(a) In the absence of ecothiopate

One experiment was made on an unstimulated muscle in which mepps were recorded and measured as before. $[Mg^{2+}]$(3.5×10^{-3}M) appeared to have no effect on the duration at half - maximum amplitude (Table 14 and Fig. 33).

(b) In the presence of ecothiopate

Mg^{2+} (3.5×10^{-3}M) was found not to alter the duration at half - maximum amplitude from that recorded in ecothiopate only ($[Mg^{2+}]$ 10^{-3}M) (Table 14 and Fig. 33). It must be stressed that only one averaged response was recorded.

Discussion

Since (i) mepps recorded in the presence of ecothiopate had similar durations at half - maximum amplitude regardless of $[Mg^{2+}]$ and which were greater than control values (indicating that ecothiopate is working) and

(ii) $[Mg^{2+}]$ (3.5×10^{-3}M) prevented any significant endplate calcium accumulation, it was tentatively concluded that any mepp contribution to calcium accumulation was negligible provided that there was no post - junctional antagonistic action of magnesium on calcium entry which might be independent of mepc prolongation. It would have been interesting to have performed some experiments investigating the effect of $[Mg^{2+}]$ (3.5×10^{-3}M) on calcium accumulation in the presence of carbachol.

(vi) The effect of ecothiopate on membrane potential.

Endplate calcium accumulation has been concluded to be due to plc- and sc- accumulation and not to mepp - accumulation. It is also thought that the two former factors are not the only factors responsible for calcium accumulation because of inconsistencies in their respective time courses. Therefore, it was possible that other contributory factors were involved.

Besides a direct effect of ecothiopate, another possibility may be the effect on post - junctional Ca^{2+} entry of a persistent depolarisation due to an increased persistent transmitter action following neurally evoked and/or spontaneous release when ChE is inhibited.

Experiments were made on rat hemi - diaphragms to determine any effect of ecothiopate on the membrane potential of muscle fibres both at the endplate and in the rest of the muscle fibre. For details of methods used see pp. 22,25 The proximity of the electrode to the endplate was confirmed by the presence of mepps of amplitude greater than 0.5mV and rise times of less than 0.6msecs. Membrane potentials were measured with the hemi - diaphragms in physiological saline (i.e. controls), then after a minimum exposure to ecothiopate (5×10^{-7}M) of 15 mins and, finally, in some experiments after removing ecothiopate from the saline solution. The results (Tables 15 and 16) show that there was a significant reduction in the membrane potential of 6 - 10mV both in the presence of ecothiopate (5×10^{-7}M) and after any excess of the drug had been washed out. In one of the two experiments where non - junctional recordings were made there was a significant reduction shown in the value of the membrane potential in the presence of ecothiopate as compared with control values.

Experiment No.	Endplate Membrane Potentials (mV)			Non-Junctional Membrane Potentials (mV)		
	Physiological Saline	Ecothiopate	After Washing	Physiological Saline	Ecothiopate	After Washing
1	A -79.39 ± 0.76(15)	B -72.75 ± 3.22(4)	C -73.30 ± 1.42(11)	- - - -	- - - -	- - - -
2	D -81.34 ± 0.56(18)	E -74.83 ± 2.31(12)	- - - -	F -74.05 ± 1.71(9)	G -70.63 ± 1.64(9)	
3	H -76.52 ± 1.60(5)	- - - -	I -65.43 ± 1.29(11)	- - - -	- - - -	- - - -
4.	J -67.90 ± 1.50(30)	- - - -	K -62.0 ± 1.30(30)	L -71.90 ± 1.00)8)	- - - -	M -62.60 ± 1.70(8)

Table 15 Rat hemi - diaphragm. 37°C. Membrane Potentials (means ± S.E.M)
Control readings were made in physiological saline over periods of up to 2 hours. Readings were then made in the presence of ecothiopate(5×10^{-7}M) and in two experiments the drug was washed out and further measurements were made. Figures in parentheses represent numbers of fibres from which measurements were made.

Table 16 Independent t values and probabilities between the unpaired sets of data specified in Table 15.

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
A v B	3.396	17	< 0.01	Significant
A v C	4.665	24	< 0.001	Significant
D v E	3.760	28	< 0.01	Significant
F v G	-	16	> 0.05	Insignificant
H v I	5.332	14	< 0.001	Significant
J v K	3.068	58	< 0.001	Significant
L v M	5.1003	14	< 0.001	Significant

Discussion

The reduction in membrane potential apparently caused by ecothiopate action might have been due to the persistent presence of a slightly higher concentration of transmitter in the synapse as a result of cholinesterase inhibition giving rise to a persistent depolarisation of the endplate membrane of a few millivolts. It was not thought that a direct post-junctional effect of excess ecothiopate was involved since the reduction in membrane potential continued even when the drug had been washed out. That this persistent depolarisation might make a contribution to endplate calcium accumulation is suggested (persistent-accumulation).

Since ecothiopate appeared to significantly reduce the membrane potential in one of two experiments made to measure non-junctional membrane potentials, the possibility exists of a direct effect of ecothiopate on muscle fibres.

It has already been established that prolonged mepps are unlikely to make any significant contribution to endplate calcium accumulation. In addition, it is believed that, in the presence of an antiChE, transmitter release associated with action potentials in the nerve eventually diffuses from the synapse even if it does not clear the synapse for a matter of seconds. Hence, what could cause the apparent reduction in membrane potential and is a permanent component of transmission in these conditions ?

In 1963, Mitchell and Silver suggested that in the rat diaphragm preparation only a very small proportion (1 - 3%) of the ACh spontaneously liberated can be associated with mepps which represent the quantal release of transmitter. Similarly, Fletcher and Forrester (1975) calculated that a maximum of 2% only of the resting release of ACh could be responsible for the production of

mepps, also in rat diaphragm. This suggests that the majority of ACh release is non-quantal arising from preterminal branches of the motor nerve (Mitchell and Silver, 1963; Fletcher and Forrester, 1975) or the result of a steady leakage from nerve terminals (Katz and Miledi, 1965, 1977; Vyskočil and Illés, 1977), In addition, depolarisation of the motor nerve terminals is thought to increase both quantal and non-quantal release in mouse diaphragm (Vizi and Vyskočil, 1979). Katz and Miledi (1977) found that non-quantal release of ACh could depolarise the post-junctional membrane by about 40μV in the frog. In the mouse diaphragm, this depolarisation reached the order of millivolts when ChE was inhibited by reversible inhibitors in low concentration (Vyskočil and Illés, 1977) or by pre-treatment of diaphragm with organophosphate inhibitors (Vyskočil and Illés, 1978). It is, therefore, suggested that the persistent depolarisation observed in the presence of ecothiopate (5×10^{-7}M) might be caused by non-quantal transmitter release.

It is, therefore, suggested that quantal release (i.e. miniatures and stimulus-evoked) not only cannot explain the persistent depolarisation observed but also cannot explain the apparently constant accumulation of calcium at the endplates for periods of up to 60 mins. However, sufficient non-quantal release, which may be the cause of the persistent low level depolarisation, might make a significant contribution to endplate calcium accumulation and may explain why such accumulation appears to go on at an approximately constant rate up to 60 mins.

Non-quantal release appears to be, at least, partly controlled by membrane $Na^+ - K^+$ - activated ATPase and that inhibition of this enzyme has been found to enhance non-quantal release (Vizi and Vyskočil, 1979). Calcium is an effective inhibitor of this enzyme

(Skou, 1957; Somogyi, 1964) and it has been suggested that under physiological conditions when calcium entry is expected to occur, that the transient inhibition of membrane ATPase caused by pre-synaptic entry of Ca^{2+} might release ACh from the cytoplasm (Vizi and Vyskočil, 1979).

Since pre-synaptic receptors are believed to be further excited in the presence of an antiChE, presumably resulting in further Ca^{2+} entry, it is suggested that ecothiopate may increase non-quantal release. It can be speculated that, since Mg^{2+} apparently antagonises Ca^{2+} action pre-synaptically, a higher $[\ Mg^{2+}\ ]$ would inhibit non-quantal release. Hence, if this were to be true, Mg^{2+} (3.5×10^{-3}M) would be expected to reduce non-quantal release. This may explain why calcium does not accumulate at the endplates in $[\ Mg^{2+}\ ]$ (3.5×10^{-3}M).

Finally, it may be that some of the SCs, at least, are due to the normally subthreshold non-quantal release becoming suprathreshold in certain groups of endplates.

(vii) The effect of stimulation on the development of spontaneous contractions

That there are considerably more SCs in unstimulated muscles compared with stimulated muscles, as a result of ecothiopate action (e.g. Fig. 27) suggests that stimulation, itself, has an inhibiting effect on the development of SCs.

Experiments were made on pairs of rat hemi - diaphragms set up in the usual way to make records of their contractions (see Methods p. 14). Control responses to nerve stimulation (0.01Hz) (Fig.33(i)) and in the absence of stimulation (Fig.33(ii)) of both muscles were established. These records did not show any spontaneous contraction. Ecothiopate (5×10^{-7}M) was then added to both muscles. Eight minutes after ecothiopate addition, spontaneous contractions had developed in both muscles (Fig.33(iii)). Stimulation of the right hemi - diaphragm was now re-started (0.01Hz). Fig.33(iv) shows quite conclusively that stimulation appears to have abolished all the spontaneous contractions within the 20 sec post - stimulus period recorded. However, the unstimulated left hemi - diaphragm continued to contract spontaneously. On stopping stimulation of the right hemi - diaphragm, spontaneous contractions were observed to return approximately 3 mins later (Fig. 33 (v)).

Further experiments were made in which all contractile activity (both stimulated and spontaneous) over the whole time course of the experiments was recorded. This was done by photographing records stored on a storage oscilloscope. After ecothiopate had caused extensive spontaneous contraction development in unstimulated muscles, the latter were stimulated (0.01Hz) for periods of approximately 15 mins after which stimulation was again stopped.

Fig. 34 shows the complete records of one such experiment made by triggering numbers of successive 95 sec sweeps at the same horizontal level on the oscilloscope screen.

The records (i) show that stimulation inhibits the development of spontaneous contraction and (ii) suggest that this stimulation block of SCs was more pronounced immediately following the stimulus.

Discussion

These observations suggest that the subsequent intensification and prolongation of transmitter action following nerve stimulation in the presence of ecothiopate inhibits the development of spontaneous contractions and that this inhibition is greatest when, presumably, the action of transmitter is greatest i.e. immediately following nerve stimulation. Since ecothiopate (5×10^{-7}M) does not apparently block the development of spontaneous contractions in unstimulated muscles, at least not to the same extent, it is suggested that the inhibition of SCs is the result of a reduced activity of transmitter on the pre-synaptic membrane. This may be due to desensitisation of pre-synaptic receptors brought about by the prolongation of transmitter action following nerve stimulation.

It is, however, possible that desensitisation of post-synaptic receptors is responsible for the waning of the SCs in stimulated muscles and that this desensitisation is further responsible for the waning of the enhanced early contraction and subsequently, the waning of the plcs (see also Section XII).

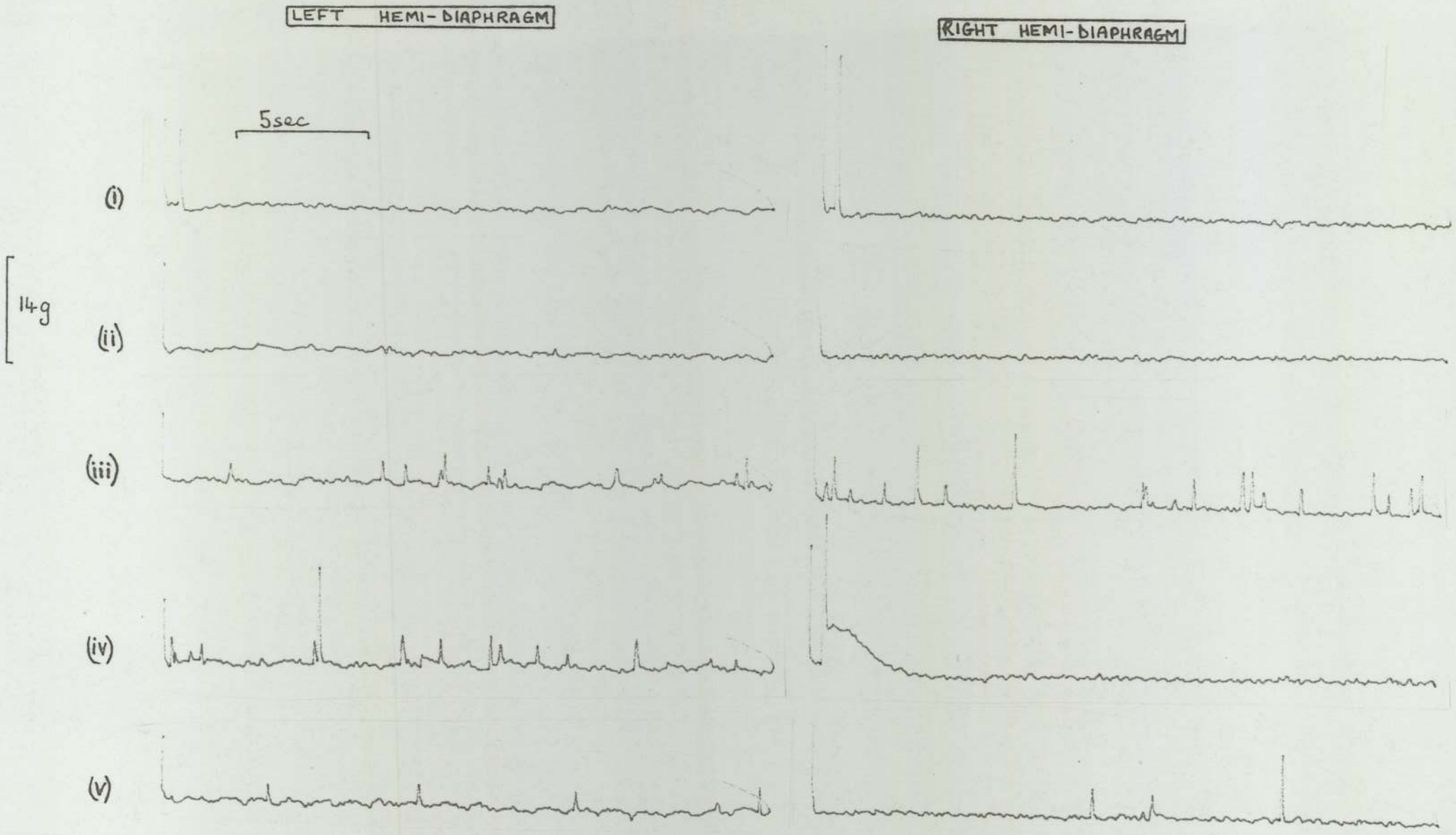

LEFT HEMI-DIAPHRAGM
RIGHT HEMI-DIAPHRAGM
5sec
14g
(i)
(ii)
(iii)
(iv)
(v)

Fig. 33 Rat hemi - diaphragm. Physiological saline at $37^{o}C$.

Effect of stimulation on the development of spontaneous contractions caused by ecothiopate ($5 \times 10^{-7}M$).

(i) The contractile responses of both left and right hemi - diaphragms in response to nerve stimulation (0.01Hz). (Stimulation stopped)

(ii) The traces showing the stability of both hemi - diaphragms in the absence of any stimulation. (Ecothiopate added ≡ 0 mins)

(iii) The spontaneous contractile activity of both hemi - diaphragms still in the absence of stimulation 8 mins after Ecothiopate added. (Stimulation of right hemi - diaphragm re-started (0.01Hz) 9 mins after ecothiopate added).

(iv) The contractile response of both hemi - diaphragms 16 mins after Ecothiopate added. Left - unstimulated
Right - stimulated
Note the presence of the plc and the absence of scs in the right hemi - diaphragm.
(Stimulation of right hemi - diaphragm stopped 24 mins after ecothiopate).

(v) The contractile responses of both hemi - diaphragms in the absence of stimulation 27 mins after the addition of Ecothiopate.

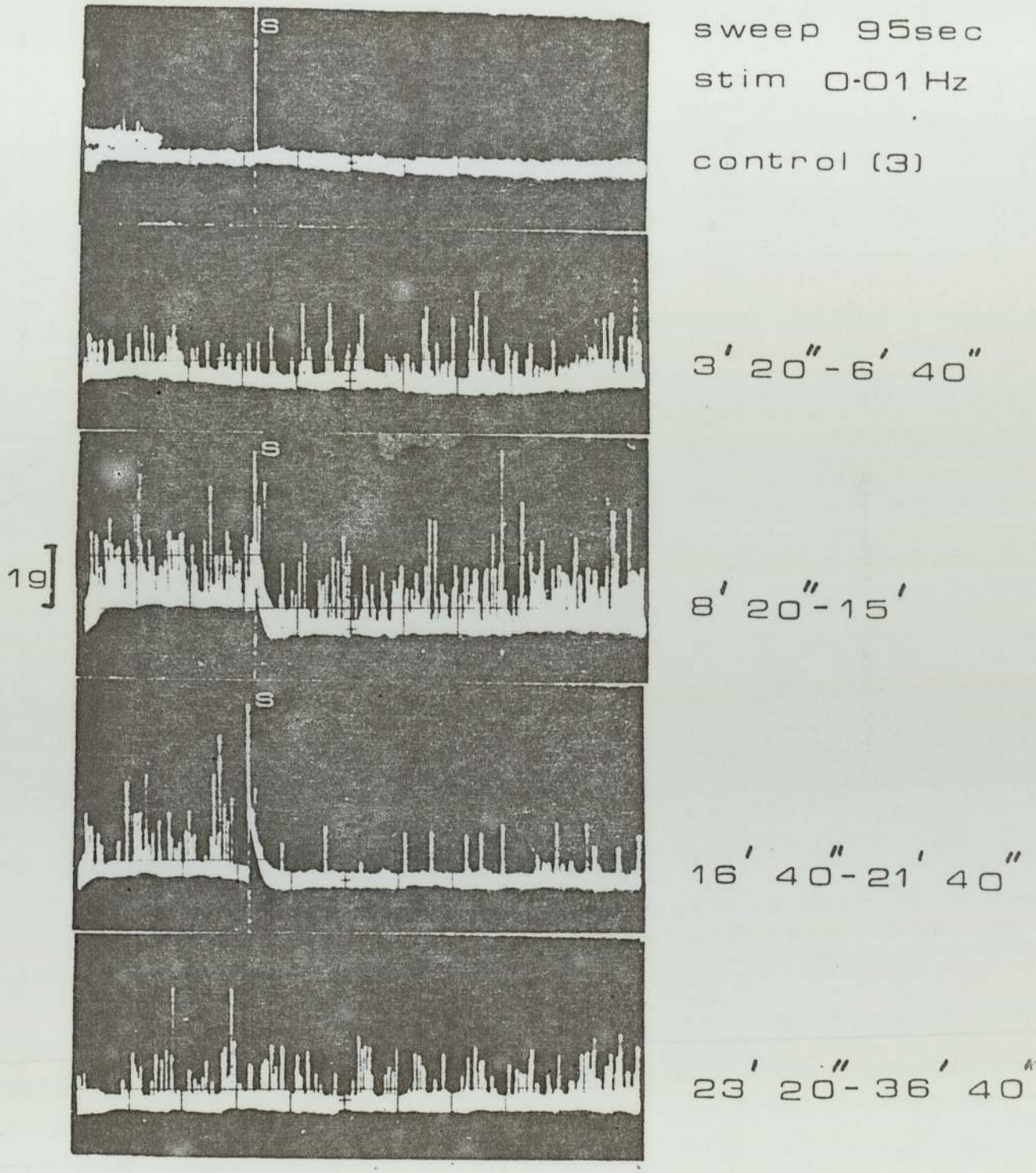


Fig. 34. Photographic records showing the effect of nerve stimulation (0.01Hz) on the development of SCs in the presence of ecothiopate.

S = stimulus

Comparison of the time course of endplate calcium accumulation and plcs development and waning.

That the reasons for the apparent similarity in endplate calcium accumulation in stimulated and unstimulated muscles have now been elucidated (summarised on pp. 139), I can now proceed with my original intention to compare endplate calcium accumulation in unstimulated muscle with plc development in stimulated muscles.

Maximum prolongation of the late contractile response after nerve stimulation (0.01 - 0.017Hz) has been shown to occur at approximately 16 mins after ecothiopate in both rat and mouse diaphragms. It is assumed that plcs in both diaphragms are due to prolonged Ca^{2+} action in the endplate regions only, as a result of prolonged transmitter action. It has previously been suggested that post-junctional Ca^{2+} entry is directly or indirectly responsible for the plcs. If this were so then it might have been expected that the progressive development of plcs would have accompanied a progressive post-junctional entry of Ca^{2+} resulting in a progressive accumulation of calcium at the endplate. In addition, it might have been expected that the maximum prolongation of the endplate-localised contraction would have coincided with maximum post-junctional entry of Ca^{2+}. Finally, the waning of the plcs (17 - 35 mins approximately, see Table 1) might have been expected to coincide with either the waning of the post-junctional entry of Ca^{2+} which, presumably, would have resulted in a reduction in the rate of increase of endplate calcium accumulation or, alternatively, a reduction in the total endplate accumulation of calcium. Either reduction is not observed (Fig.24) and, moreover, the results indicate an, almost constant post-junctional entry of Ca^{2+} up

to, at least, 60 mins. Therefore, while plc development might still be related to post-junctional Ca^{2+} entry and subsequent progressive accumulation at the endplate, some other mechanism must be responsible for plc waning (see later discussion).

Further Discussion

These experiments have shown that ecothiopate action leads to an accumulation of calcium at the endplate, presumably resulting from a post-junctional entry of Ca^{2+}. However, whether or not this post-junctional entry of Ca^{2+} is subsequently responsible for the plcs has not been determined. Plcs, perhaps may be better explained as resulting from prolonged endplate depolarisation directly delaying the local sequestration of Ca^{2+} by the SR (as suggested in Section III, p. 78). Waning of plcs might then be explained by either the waning of the prolonged endplate depolarisation below the so-called mechanically effective threshold or by some depolarisation block or desensitisation at the post-junctional membrane (see Section XII). Endplate calcium accumulation would then merely be a side-effect of prolonged depolarisation, playing no part in plcs and, presumably, being unaffected by any desensitisation.

The initial development of spontaneous contractions in stimulated muscles approximately coincided with the increase in amplitude of the contractions. The latter and, therefore by implication, possibly the former are believed to be associated with the development of repetitive action potentials in both the nerve and muscle fibres, found to be between 4 - 8 mins by Morrison (1977). If the disappearance of the spontaneous contractions were to coincide with the disappearance of the repetitive firing in the nerve terminals, not only would this confirm the previous conclusion that spontaneous contractions are pre-synaptically generated but also that their waning is also associated with pre-synaptic mechanisms, for example, the abolition of axon reflex action.

Morrison (1977) found that repetitive activity in the motor nerve terminals was abolished approximately 15 - 20 mins after ecothiopate addition which coincides well with the disappearance of spontaneous activity in most experiments (see p. 51). It may be that a reduced excitability of the motor nerve terminals in the continued presence of ecothiopate is responsible for the waning of the spontaneous activity and results from a reduced activity of the pre - synaptic receptors as a consequence of prolonged transmitter action and/or from a direct pre - synaptic blocking effect by ecothiopate. Morrison (1977) suggested a pre - synaptic inhibiting effect of ecothiopate (5×10^{-7}M) since he found it suppressed previously initiated repetitive firing in the nerve terminals. Also, experiments made on stimulated muscles involving short exposures to ecothiopate (5×10^{-7}M) showed that the removal of ecothiopate from the physiological saline 5 - 8 mins after its addition, resulted in a large increase in the number and extent of spontaneous contractions (see Fig. 44) which was also, apparently, coincident with an increase in the amplitude of the contractile response (also see Section XII).

Further experiments made to determine the effect of low concentrations of dTC ($5 - 15 \times 10^{-9}$M) (pp. 213), which were believed to act primarily pre - synaptically, on the action of ecothiopate showed that SCs were abolished.

This also confirms the conclusion that SCs are generated pre - synaptically. The effect of both ecothiopate and dTC may involve competitive blocking actions preventing transmitter action at the pre - synaptic membrane.

General Discussion

Evidence has been presented which suggests that ecothiopate action leads to the accumulation of calcium at the endplates. It is assumed that this accumulation is either the result of prolonged transmitter action at the post-junctional membrane or a direct effect of ecothiopate.

Takeuchi (1963) has suggested that the post-junctional muscle fibre membrane becomes slightly permeable to Ca^{2+} during ACh action because she observed a localised shortening at the endplate region of frog muscle fibres treated with ACh in high calcium Ringer solution. ACh and other depolarising drugs have been shown to increase the uptake of labelled Ca into frog sartorius muscle (Ahmad and Lewis, 1961; 1962) and in depolarised rat diaphragm rendered supersensitive by previous denervation (Jenkinson and Nicholls, 1961. Evans (1974) found that calcium accumulates in the junctional regions of mouse diaphragm in the presence of ACh or carbachol. In addition, the calcium-sensitive dyes Alizarin Red S (Csillik and Savay, 1963; Lièvrement et al., 1968) and glyoxal-bis-(2-hydroxyanil) (Meunier, 1972; Evans, 1974) have shown that ACh facilitates staining of motor endplates of skeletal muscle. Lièvrement et al. (1968) also reported staining obtained by stimulation of the phrenic nerve in the presence of neostigmine. These results were interpreted as an indication of a calcium entry due to ACh action.

It is, therefore, thought that the endplate calcium accumulation in my experiments reflects post-junctional Ca^{2+} entry resulting from a prolonged ACh action caused by the inhibition of ChE by ecothiopate. A direct action of ecothiopate on calcium accumulation is considered in Chapter XIII of the Results (pp. 176-).

The prolonged transmitter action associated with (i) quantal release, that is, mepps, spontaneous epps and stimulated epps and (ii) non-quantal release have been considered to be responsible for the endplate calcium accumulation.

Prolonged mepcs are not thought to make any significant contribution since the experiments made with $[Mg^{2+}]$ (3.5×10^{-3}M), although preventing endplate calcium accumulation, did not appear to shorten the mepcs prolonged by ecothiopate. However, further experiments would need to be made to confirm previous observations.

Non-quantal release may make the most significant contribution to endplate calcium accumulation since it may be a permanent component of neuromuscular transmission and also depolarises the post-junctional membrane in the presence of organophosphate inhibitors. Whether such depolarisation is sufficient to allow post-junctional Ca^{2+} entry is not known. It has been speculated that $[Mg^{2+}]$ (3.5×10^{-3}M) reduces non-quantal release and that this might well be associated with the observation that this $[Mg^{2+}]$ prevents the accumulation of calcium at the endplates. However, experiments have not been made to investigate the potential importance of non-quantal release and it is therefore not considered further in the present attempt to explain the endplate accumulation of calcium.

Endplate calcium accumulation associated with spontaneous epps and stimulated epps is believed to be the most significant since experiments made with $[Mg^{2+}]$ (3.5×10^{-3}M) abolish SCs and plcs. However, since experiments were not made to record endplate currents in the presence of $[Mg^{2+}]$ (3.5×10^{-3}M) it is not known whether such currents are still prolonged in the presence of ecothiopate and, hence, whether post-junctional Ca^{2+}

entry is associated with prolonged transmitter action.

Calcium accumulation associated with spontaneous epps has been suggested to make a more significant accumulation in unstimulated muscles because (i) SC development in unstimulated muscles is greater and (ii) in the absence of stimulation there can be no calcium accumulation associated with stimulated and, subsequently, prolonged epps. It would appear that for muscles incubated with ecothiopate for 15 mins the endplate calcium accumulation associated with the greater development of SCs in unstimulated muscles approximately balances the calcium accumulated associated with the small number of spontaneous epps together with the stimulated epps in stimulated muscles. It is not known whether such a balance is maintained for periods up to 60 mins with ecothiopate.

Summary

Endplate calcium accumulation has been considered to result from the prolonged transmitter action associated with (i) stimulus - evoked epps, (ii) spontaneous epps, (iii) mepps and (iv) non - quantal release after ecothiopate. Evidence has been presented which indicates that endplate calcium accumulation may be associated with (i) and (ii) but not with (iii). The importance of (iv) has been speculated only.

SC - accumulation has been suggested to make a more significant contribution in unstimulated muscles compared to stimulated muscles and evidence has been presented which indicates that stimulation (0.01Hz) inhibits the development of SCs. The latter inhibition has been suggested to be due to receptor desensitisation resulting from the prolonged transmitter action following indirect stimulation in the presence of ecothiopate.

SECTION VII

Miscellaneous Radiochemistry

Results

(i) Effect of 30 minute wash-out period on Ca accumulation

These experiments were made in order to determine any possible efflux of calcium from hemi - diaphragms during the usual 30 mins wash-out period in physiological saline following incubation in ^{45}Ca and ecothiopate (5×10^{-7}M) for 30 mins. After rinsing very briefly in the tracer-free physiological saline for approximately 3 - 5 secs the muscles were immediately placed in acetone (see p.34 Methods). Measurement of the calcium remaining in the muscles (see Table 17 and Fig 35) showed no significant difference in calcium accumulation at the endplates when compared with that from muscles washed for the usual 30 mins period. However, there was a very significant difference in calcium accumulation in the rest of the muscle. The increase, approximately 0.65 n-moles mg^{-1}, was assumed to be due to that present in the extracellular space during incubation plus, perhaps, that Ca^{2+} lost from the muscle fibres during wash-out period. It was calculated that 0.5 n-moles mg^{-1} dry weight would represent the $[Ca^{2+}]$ in the extracellular space during incubation.

Table 17 Calcium accumulation in unstimulated mouse hemi-diaphragms (mean ± S.E. of mean, number of hemi-diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca-physiological saline at 37°C in the presence of ecothiopate (5×10^{-7} M) and were then only briefly rinsed in tracer-free physiological saline. These results are compared with those muscles treated similarly with ecothiopate but having undergone the usual 30 mins wash-out period.

Wash-out Period	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
brief	A_5 0.675 ± 0.169(7)	B_5 1.074 ± 0.165(7)
30 mins	C_5 0.531 ± 0.077(15)	D_5 0.419 ± 0.076(15)

Table 18 Independent t values and probabilities between the unpaired sets of data in Table 17.

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
A_5 v C_5	0.9437	20	0.641	Insignificant
B_5 v D_5	4.3962	20	0.000	Significant

Fig. 35. Calcium accumulation in mouse diaphragm after ecothiopate

(i) Endplate calcium accumulation

A. after brief wash

B. after 30 min wash

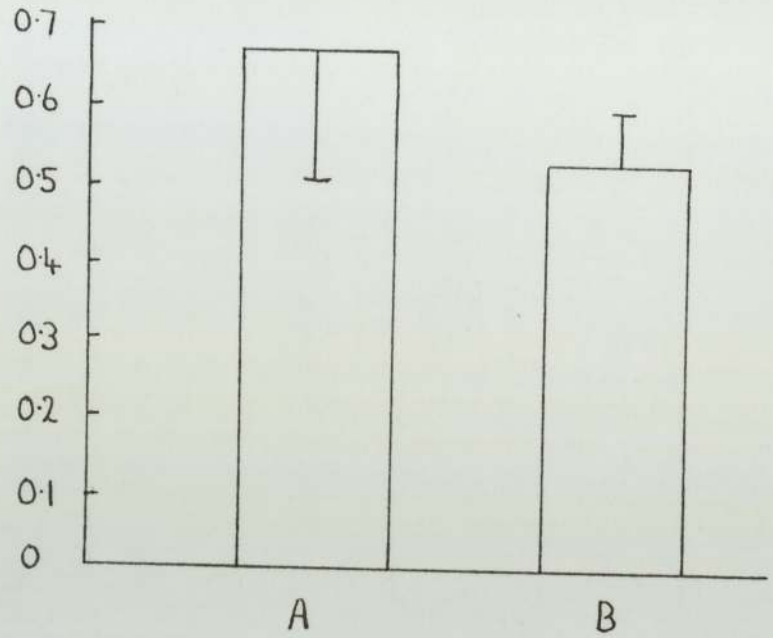
(ii) Non - junctional calcium accumulation

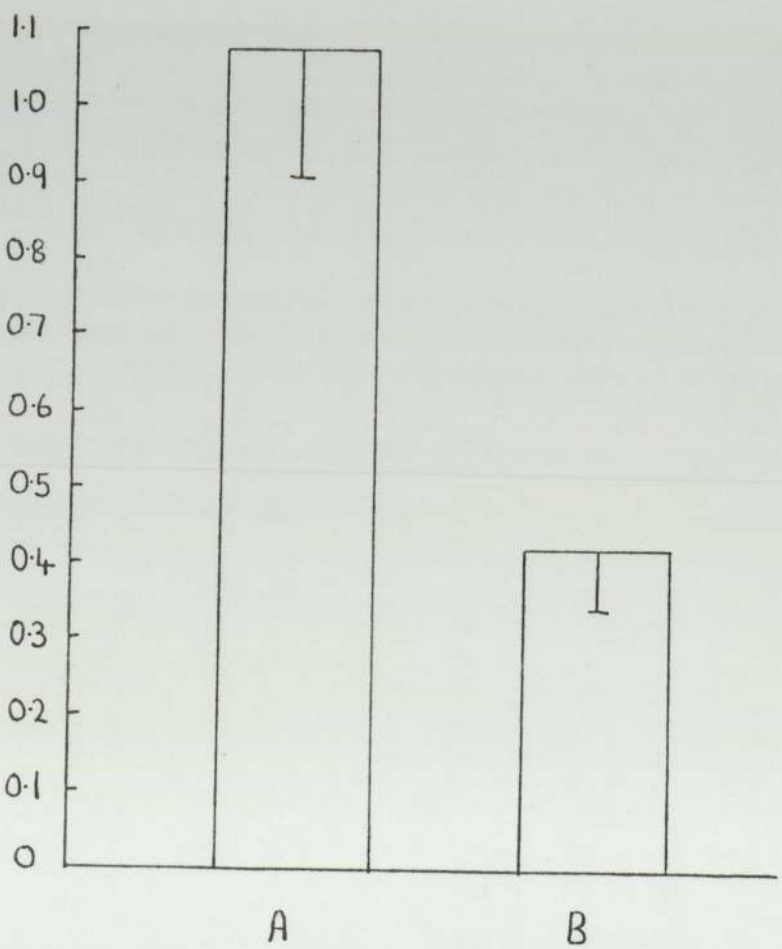
A. after brief wash

B. after 30 min wash

Note standard error bars.

CALCIUM ACCUMULATED
(n-moles mg^{-1} muscle)





Discussion

It was concluded that the usual 30 mins wash-out period in tracer-free saline had no effect on calcium accumulation at the endplates . This suggests that the calculations of calcium accumulated at the endplates were not modified by the washing-out technique and, perhaps, more importantly that the calcium accumulated at the endplates had either passed into the muscle fibres or was bound to the endplates in some way. Evans (1974) also suggested that any calcium that was found in the endplate regions as a result of depolarisation must be held at or in the postsynaptic membrane or area respectively which would suggest that the Ca^{2+} become quickly 'bound' in some way on entering the muscle fibre. This would help to explain the unsuccessful attempts to locate accumulated calcium by using histochemical methods. However, this did not prevent the calcium accumulated due to carbachol action from being stained.

(ii) Effect of Carbachol on calcium accumulation

Since the histochemical results (pp.93-104) showed a greater accumulation of Ca^{2+} at the endplates in unstimulated muscles in the presence of carbachol (10^{-4}M) in physiological saline containing $[Ca^{2+}]$ (5×10^{-3}M) compared with the presence of ecothiopate (5×10^{-7}M) in physiological saline containing $[Ca^{2+}]$ (2×10^{-3}M), it was expected that the tracer experiments would also show a greater accumulation of calcium with carbachol.

Experiments were made to confirm this observation. Muscles were incubated with carbachol (10^{-4}M) in physiological saline containing $[Ca^{2+}]$ (2×10^{-3}M) for 30 mins and were then treated as in the Methods (pp. 34-37) and measured for calcium accumulation. The values obtained were compared with those previously obtained for muscles incubated with ecothiopate (5×10^{-7}M) for 30 mins. Carbachol - treated muscles showed a significant increase in endplate calcium accumulation (0.649 n-moles mg^{-1}, mean of 5 muscles) compared to the absence of the drug (0.004 n-moles mg^{-1}, mean of 10 muscles) but did not significantly increase accumulation in the rest of the muscle. However, although carbachol appeared to increase endplate calcium accumulation compared with ecothiopate (0.53 n-moles mg^{-1}, mean of 15 muscles), the difference was not significant (Tables 19 and 20, Fig. 36).

Table 19 Ca accumulation in instimulated mouse hemi - diaphragms (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca-physiological saline at 37°C in the presence of carbachol ($10^{-4}M$) followed by wash-out for 30 mins in tracer-free physiological saline before immersion in acetone. These results ae compared with those muscles treated similarly but with ecothiopate ($5 \times 10^{-7}M$).

Drug	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
Carbachol ($10^{-4}M$)	A_6 0.649 ± 0.017(5)	B_6 0.406 ± 0.039(5)
Ecothiopate($5 \times 10^{-7}M$)	C_6 0.531 ± 0.077(15)	D_6 0.419 ± 0.076(15)
No drug (i.e. physiological saline only for 30 mins)	E_6 0.004 ± 0.031(10)	F_6 0.321 ± 0.042(10)

Table 20 Independent t values and probabilities between the unpaired sets of data in Table 19.

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
A_6 v C_6	0.8644	18	0.5971	Insignificant
B_6 v D_6	0.0978	18	0.9201	Insignificant
A_6 v E_6	10.6747	13	0.000	Significant
B_6 v F_6	1.3484	13	0.198	Insignificant

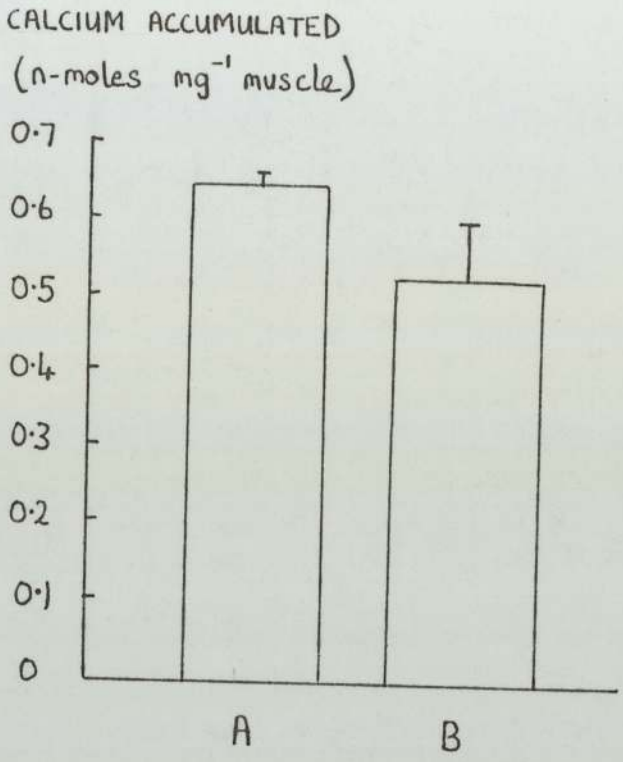


Fig. 36. Endplate calcium accumulation after

A. Carbachol (10^{-4}M) ($[Ca^{2+}]$ 2 x 10^{-3}M)

B. Ecothiopate (5 x 10^{-7}M) ($[Ca^{2+}]$ 2 x 10^{-3}M)

both for 30 mins.

Note standard error bars.

Discussion

It is difficult to explain why calcium accumulation is similar with both ecothiopate and carbachol in the tracer experiments whereas in the histochemical experiments Ca^{2+} accumulation is apparently much greater in the presence of carbachol, the only variant in the conditions being that physiological saline containing $[Ca^{2+}]$ (5×10^{-3}M) was used in the carbachol histochemical experiments. Although the fact that carbachol was being used in a much higher concentration might have accounted for the apparently higher accumulation of Ca^{2+} in the histochemical experiments, this is not confirmed in the tracer experiments.

It was believed by Evans (1974) that bath application of Carbachol (10^{-4}M) rapidly depolarised the post-junctional membrane for not more than a few milliseconds before rapid depolarisation took place. The rapid depolarisation was thought to be due to an equally rapid and complete desensitisation of the receptors thus effectively producing neuromuscular block. That the period of depolarisation produced by a persistent concentration of a depolarising drug was brief and the motor endplate region was rapidly repolarised was also observed by Thesleff (1955a and b, 1956, 1958), Katz and Thesleff (1957b) and Axelsson and Thesleff (1958). They also found that despite the repolarisation of the endplate region it remained refractory (i.e. desensitised) to nerve impulses or to ACh during the application of the depolarising drug.

Therefore, if carbachol (10^{-4}M) effectively desensitises the post-junctional membrane in my experiments, the desensitisation process does not prevent post-junctional Ca^{2+} entry. It can further

be speculated that Ca^{2+} entry is, perhaps, the result and, indeed, may even be the cause, of desensitised receptors (see Final Discussion). It is possible to explain the greater endplate accumulation of Ca^{2+} due to carbachol in the histochemical results on the basis that ecothiopate (5×10^{-7}M) action results in only a partial desensitisation of the receptors. This is confirmed by the observation that indirect contractions can still be elicited throughout the experiments. The endplate accumulation due to ecothiopate resulting from a reduced post-junctional entry of Ca^{2+} might then reflect sub-maximal desensitisation. (This sub-maximal desensitisation might be responsible for the waning of the various ecothiopate-mediated changes in the contraction - see Section XII). The similar endplate calcium accumulations observed in the radioisotope experiments with both ecothiopate and carbachol could then be explained if such accumulation with carbachol had reached a maximum, perhaps, by saturation of the uptake mechanism. This saturation might then have allowed for the accumulation of free Ca^{2+} which was then detected histochemically. That maximum endplate accumulation occurs with this concentration of carbachol (i.e. 10^{-4}M) was shown by Evans (1974).

That carbachol does not have any significant effect on calcium accumulation away from the endplate regions confirms the belief that carbachol action is also restricted to the endplates under these experimental conditions.

(iii) Effect of Ecothiopate on Ca^{2+} efflux

Experiments were made to determine whether ecothiopate promotes Ca^{2+} efflux as well as the apparent uptake and subsequent accumulation as previously shown.

Muscles were loaded with ^{45}Ca by incubating them in carbachol (10^{-4}M) for 30 mins. They were then transferred to tracer-free physiological saline containing ecothiopate (5×10^{-7}M) for 30 mins before finally immersing them in acetone. The results were then compared with those obtained previously (see p. 168) with carbachol but in the absence of ecothiopate in the tracer-free physiological saline. If ecothiopate did promote Ca^{2+} efflux then a lower Ca accumulation would have been expected.

The results (see Table 21 and Fig.37) show that ecothiopate (5×10^{-7}M) does not promote Ca^{2+} efflux since there is no significant difference in Ca accumulation at either the endplates or in the rest of the muscle as compared with the absence of ecothiopate from the tracer-free physiological saline (Table 22).

Discussion

It was concluded that the effect of ecothiopate action was to induce post-junctional Ca^{2+} entry. It is possible, assuming Ca^{2+} efflux uses the same ion channels as Ca^{2+} entry, that the latter presumably continues in the presence of ecothiopate, and prevents any efflux.

Table 21 Ca accumulation in unstimulated mouse hemi - diaphragm (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C in the presence of carbachol ($10^{-4}M$) for 30 mins followed by wash-out in tracer-free physiological saline containing ecothiopate ($5 \times 10^{-7}M$) for 30 mins before finally immersing in acetone. These results are compared with those muscles treated similarly but without ecothiopate in the tracer-free physiological saline.

Tracer-free physiological saline	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
With Ecothiopate	A_7 0.664 0.127(8)	B_7 0.442 0.091(8)
Without Ecothiopate	C_7 0.649 0.071(5)	D_7 0.406 0.039(8)

Table 22 Independent t values and probabilities between the unpaired sets of data in Table 21.

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
A_7 v C_7	0.0904	11	0.937	Insignificant
B_7 v D_7	0.3206	11	0.752	Insignificant

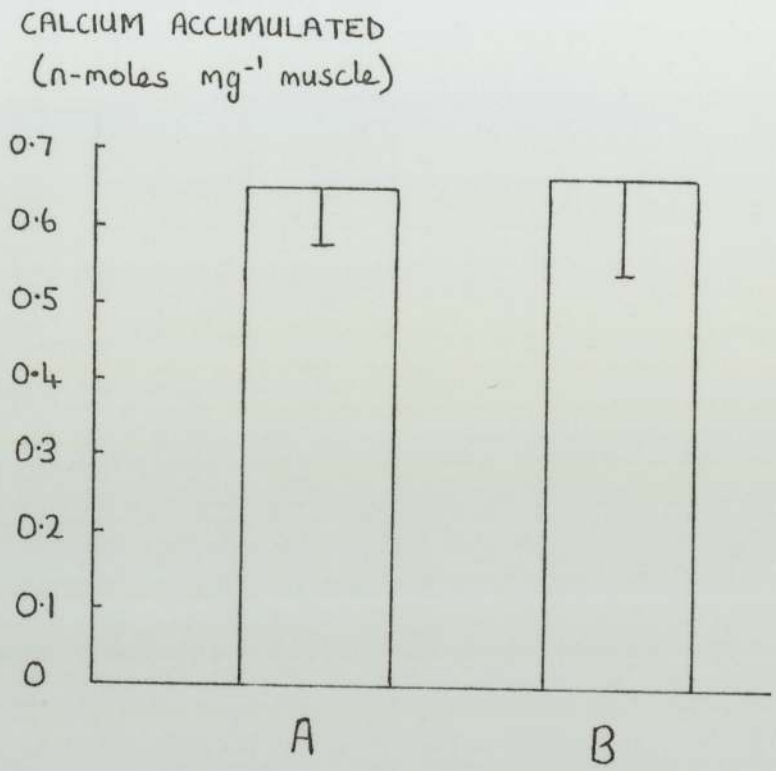


Fig. 37. Endplate calcium accumulation after carbachol (10^{-4}M) for 30 mins.

A. after tracer-free (30 mins)

B. after tracer-free and ecothiopate (30 mins).

Note standard error bars.

Summary

Evidence has been presented which suggests that the calcium accumulated at the endplates has either passed into the muscle fibres or is bound to the endplates in some way. It has been speculated that Ca^{2+} entry is associated with receptor desensitisation. In addition, evidence has been presented which suggests that ecothiopate promotes Ca^{2+} entry only.

SECTION VIII

Does Ecothiopate (5×10^{-7}M) Have A Direct Effect On Muscle Fibres?

Introduction

Previous experiments have shown that ecothiopate action causes (i) certain changes in the contractile response of hemi-diaphragms in response to single maximal stimuli of the nerve at low frequencies (Section I),

(ii) the firing of repetitive action potentials and the prolongation of endplate currents (Section III) and

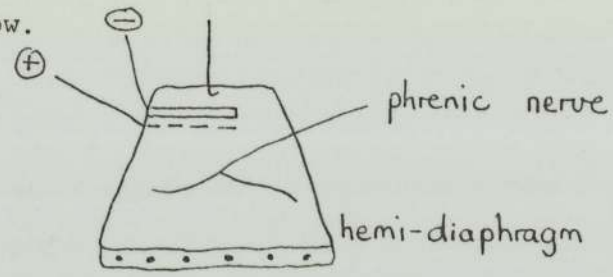
(iii) post-junctional Ca^{2+} entry resulting in accumulation of calcium at the endplate (Section V). It has been suggested that the likely cause of these changes is the antiChE action of ecothiopate resulting in an intensification and prolongation of transmitter action. An alternative hypothesis, that the above effects are due to a direct action of ecothiopate at the neuromuscular junction, has been mentioned previously in this work on several occasions.

To exclude a direct effect of ecothiopate on muscle fibres in causing muscle repetitive firing and the associated enhancement of the early contraction, SCs and plcs, experiments were made to record the contractile response of rat hemi-diaphragms in which neuromuscular transmission has been blocked by various means. The muscles, therefore, had to be stimulated directly.

Results

Effect of direct muscle stimulation

An experiment was made to determine the effect of direct muscle stimulation on the contractile response of a rat hemi - diaphragm in the presence of ecothiopate. This was an essential control experiment which could then be used to compare with subsequent experiments in which neuromuscular transmission was to be disrupted. The hemi - diaphragm was directly stimulated by means of a pair of electrodes arranged near the tendon end as shown below.



Maximal pulses of 50v amplitude and 10^{-3} sec in duration at a frequency of 0.01Hz were applied to the hemi - diaphragm. The results (see Fig. 38 and Table 23) were similar to those obtained from indirect nerve stimulation, that is, enhancement of the early part and prolongation of the late part of the contractions were observed. However, spontaneous contractions were not observed in this experiment.

Table 23 The time course of the changes in the contractile response brought about by ecothiopate(5×10^{-7}M) due to stimulation of the nerve and direct stimulation of the muscle (0.01Hz). Drugs added at 0 mins. Physiological saline ($[Mg^{2+}]$ 10^{-4}M). The data exhibited for nerve stimulation is the same as that used in Table 1.

Experimental Conditions		Latency of Twitch Enhancement (mins)	Latency of maximum amplitude of contraction (mins)	% increase in amplitude of contraction	Late Prolongation of Contractions		Latency of maximum development of plcs	Time Course of occurence of SCs. (x) indicates the approx. number observed
					START (mins)	FINISH (mins)		
Indirect Stimulation (3 expts.)	mean	1.5	11	99	10	79	18	None Visible None Visible
	range	1 - 2	9 - 6	92 - 108	8 - 12	70 - 90	18 - 19	7 - 26 (19)
Direct Stimulation (1 expt.)		4	12	96	8	82	15	None Visible

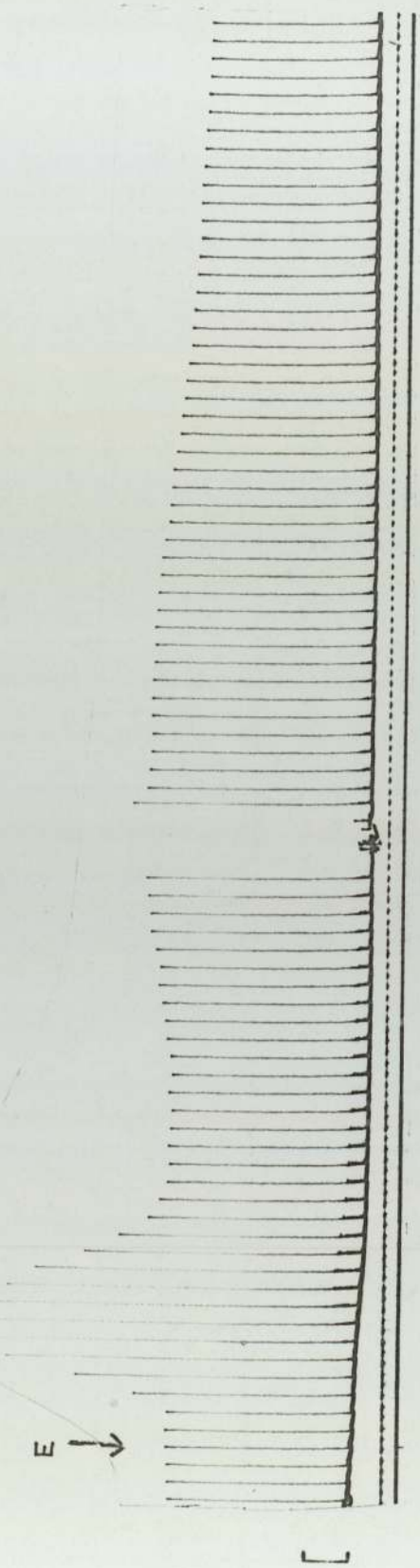


Fig. 38 Rat hemi - diaphragm. Physiological saline containing $[Mg^{2+}]$ (10^{-4}M) at 37°C.
Effect of direct muscle stimulation (0.01Hz). Ecothiopate added at E.
Record of contraction made on a Devices pen recorder (0.025 x 10^{-3} ms^{-1})

Discussion

It was considered that the large stimulating pulses used might, in fact, be stimulating the nerves so as to release transmitter.

A difference from nerve stimulation was that the amplitudes of the contractile responses were generally larger when the hemi-diaphragms were stimulated directly. This effect was perhaps explained by Merton (1954) as being due to the more synchronous response of the muscle when stimulated directly compared to the more asynchronous response of the muscle when stimulated via the nerve.

This method of stimulation was used subsequently after transmitter release and/or action had been reduced or blocked by high concentrations of dTC, by denervation or by β-Bungarotoxin in experiments made to determine whether ecothiopate (5×10^{-7}M) had a direct effect on the muscle fibres.

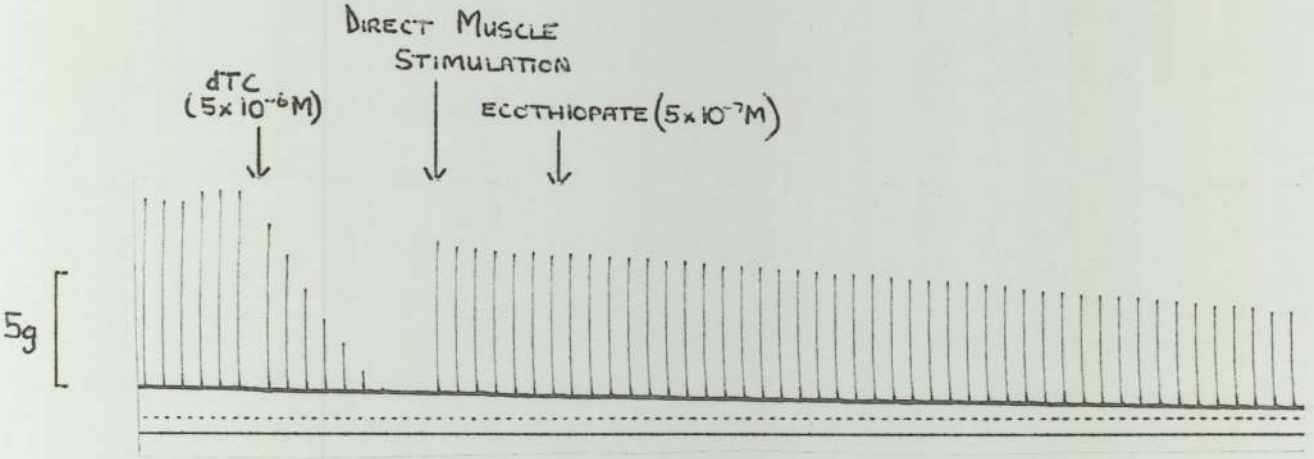


Fig. 39 Rat hemi - diaphragm in physiological saline at 37°C. The effect of ecothiopate (5×10^{-7}M) on the contractile response to direct muscle stimulation (0.01Hz) following the abolishment of response to nerve stimulation by dTC (5×10^{-6}M).

Effect of dTC (5×10^{-6}M)

dTC (5×10^{-6}M) was added to the physiological saline (Fig. 39). This had the effect of quickly abolishing the contractile response to indirect stimulation. Stimulation was now switched to direct, as described previously, and ecothiopate (5×10^{-7}M) was added in the continued presence of dTC (Fig. 39). Ecothiopate was observed to have no apparent effect on the contractile response.

Discussion

Regardless of its precise actions, it is not thought that dTC has any action outside the neuromuscular junction in mammalian skeletal muscle (Cheymol and Bourillet, 1972). It would, therefore, appear that ecothiopate does not have a direct action on muscle.

Effect of β - Bungarotoxin (β - Butx)

β - Butx is believed to block neuromuscular transmission by exhausting pre - synaptic stores of transmitter (Chen and Lee,1970).

β - Butx (117×10^{-9}M); 3.1/3 μg cm^{-3}) added to the physiological saline took an average of approximately 135 minutes (4 expts) to abolish the contractile response to nerve stimulation(0.01Hz) which agrees reasonably well with the results of Chang and Huang (1974). In 3 out of 5 experiments, after the response to nerve stimulation had been abolished and the muscles were stimulated directly, the addition of ecothiopate (5×10^{-7}M) had no effect on the contractile response (Fig. 40). In 2 experiments, enhancement of the contraction only was observed.

Discussion

These results might have implied a direct effect of ecothiopate on the muscle fibres but more probably suggests an incomplete block of ACh release by β - Butx in these particular experiments. Also, in some of the experiments, the amplitude of contraction continued to wane in response to direct muscle stimulation suggesting a direct effect of β - Butx on muscle fibres. Alternatively, the β - Butx was not as pure as claimed by the manufacturers.

It can, therefore, be tentatively concluded that ecothiopate has no direct effect on muscle fibres, after disruption of neuromuscular transmission, in causing repetitive firing and enhancement of the early contraction, SCs and plcs.

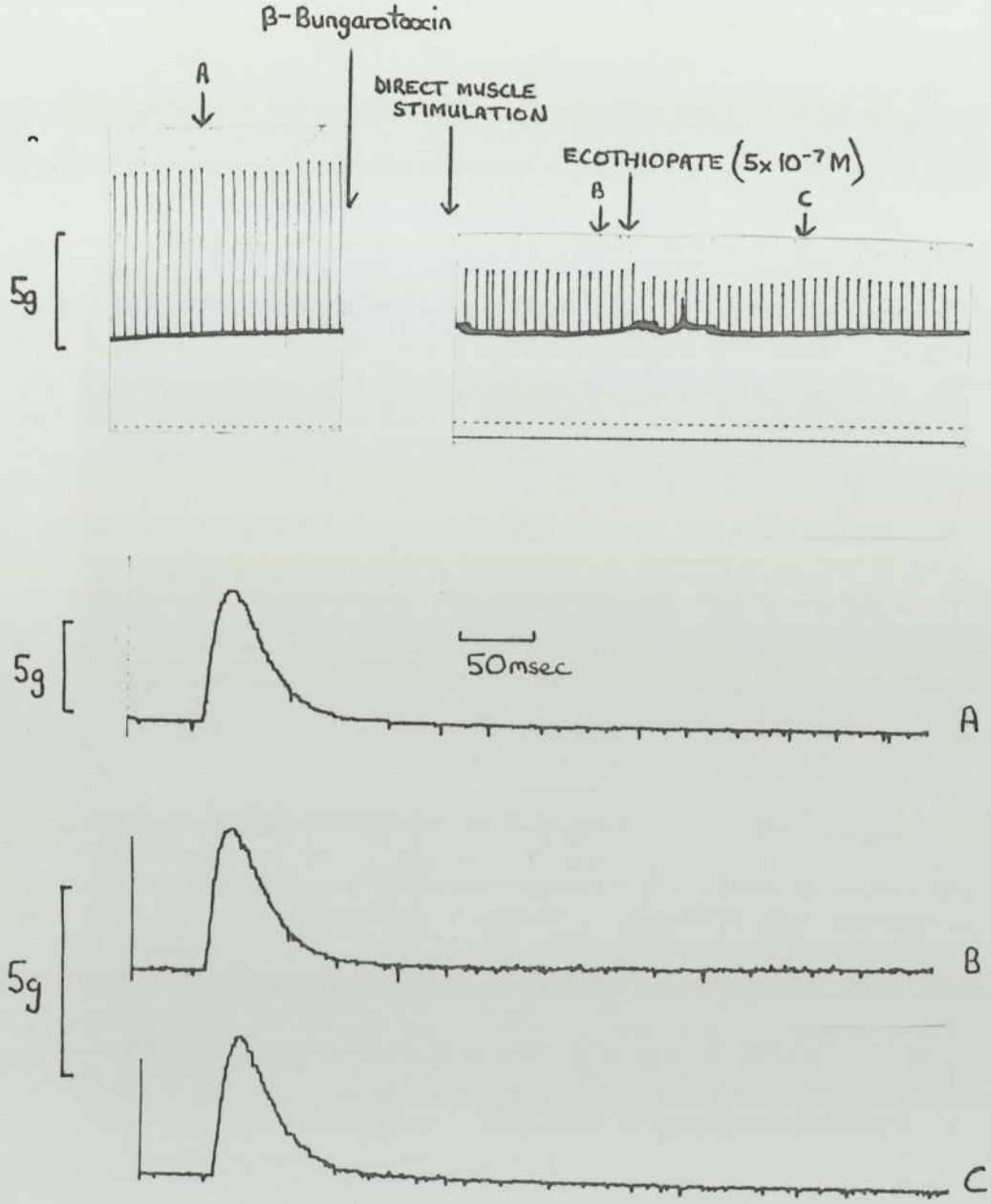


Fig. 40 The effect of ecothiopate (5×10^{-7}M) on a hemi-diaphragm previously treated with β-Bungarotoxin (1.17×10^{-7}M) to "block" ACh release. Direct muscle stimulation (74v) at a frequency of 0.01Hz). 37°C. Physiological saline.

Upper record: Devices trace (0.025×10^{-3} mms^{-1})

Lower records: Contractile responses displayed on X-Y plotter.

A. Control response to direct stimulation before β-Butx.

B. 4 hours after β-Butx.

C. 18 mins after the addition of ecothiopate.

Effect of Denervation

Denervation results in the degeneration of the nerve terminals within 3 days (Guth, 1968). This, presumably, effectively abolishes any transmitter - mediated effects at the neuromuscular junction.

Hemi - diaphragms from 4 rats, previously denervated for a minimum of 3 days by section of the left phrenic nerve, were set up to record their contractile responses as previously described. In all four experiments, ecothiopate (5×10^{-7}M) did not change the response at all (see Fig. [41]). In order to check the muscles for effective denervation, at the end of each experiment, ACh (10^{-4}M) was added to the superfusate. This produced a contracture in all four diaphragms. The four right innervated hemi - diaphragms all responded to ecothiopate (5×10^{-7}M) as described previously (Section 1).

Discussion

It can be concluded that ecothiopate has no direct effect on muscle fibres. However, it is important to remember that denervated muscle is different from normal muscle. The wide spectrum of changes in muscle fibres following nerve section has been reviewed on a number of occasions (Guth,1968; Harris,1974 and Guttman, 1976). The most important changes being the degeneration of the nerve terminals, the decrease by 50% of the junctional ChE activity (Guth, 1968), both within 3 days, and the centrifugal spread of ACh sensitivity from the junctional region (Axellson and Thesleff,1959; Miledi,1960b). The importance of denervation experiments is also limited because nerve section implies not only the interruption of nerve impulse activity but

also the 'deprivation' of trophic agents released from the motor nerve terminals. These trophic agents are believed to regulate and maintain many of the intrinsic membrane and intracellular properties of muscle fibres, (Gutmann, 1977).

Accepting this, since denervation effectively abolished transmitter-mediated effects, always assuming that muscle is not an important site of release, the action of ecothiopate (5×10^{-7}M) at the neuromuscular junction appears to be confined to its effect in prolonging transmitter action by inhibiting ChE.

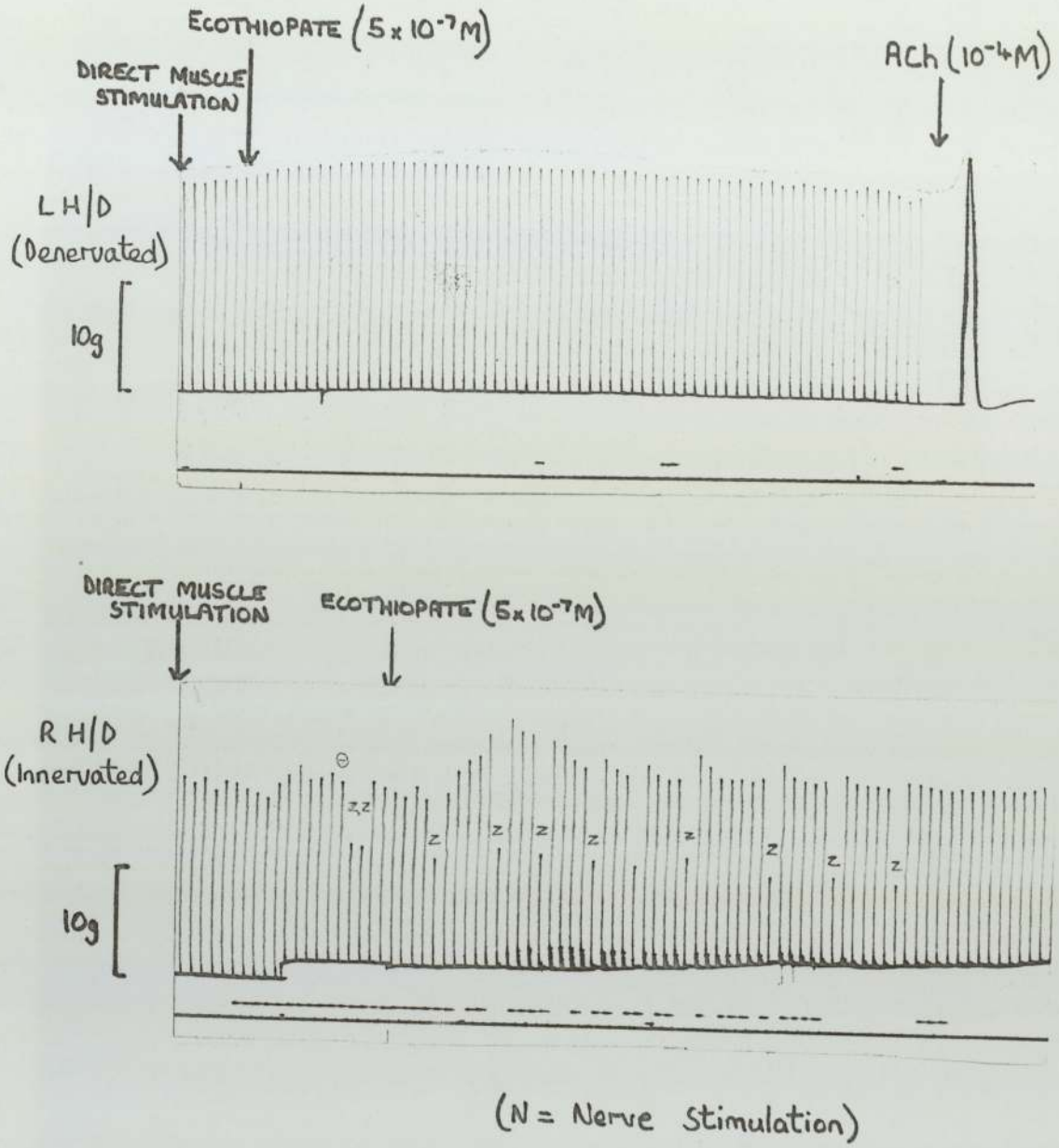


Fig. 41. The effect of ecothiopate on denervated (left) and innervated (right) hemi-diaphragms in response to direct muscle stimulation (0.017Hz). 37°C. Physiological saline.

N = responses to nerve stimulation.

General Discussion

Together with the observation from experiments made where Mg^{2+} (3.5×10^{-3}M) also blocks ecothiopate action (pp.128-), it would appear that the changes in the contractile response caused by ecothiopate are seen only if neuromuscular transmission is left intact.

Since dTC, β-Butx, Mg^{2+} and denervation all effectively reduce transmitter action, the enhancement of the early contraction (reflecting muscle repetitive firing) and the development of SCs and plcs would appear to be associated with a sufficiently prolonged and intensified action of transmitter.

It can be tentatively concluded that ecothiopate (5×10^{-7}M) has no direct effect on muscle when transmitter action is reduced. This, however, does not discount the possibility that ecothiopate has a direct effect on open ion channels when neuromuscular transmission is intact (see Final Discussion).

Summary

Disruption of neuromuscular transmission with dTC, β-Butx or denervation abolished the ecothiopate-mediated changes in the contractile response previously recorded. This evidence suggested that ecothiopate (5×10^{-7}M) had no direct action on muscle.

SECTION IX

The Effect Of Ecothiopate On Endplate Cholinesterase Activity

Introduction

In this section, experiments are made to determine the time course of inhibition of functional endplate ChE by ecothiopate in order to relate (i) the apparently time - dependent changes in the contractile response and (ii) the prolongation of endplate currents, with the degree of ChE inhibition.

Results

Experiments were made on perfused rat hemi - diaphragms in order to minimise any temporal effects of ChE inhibition caused by the diffusion of ecothiopate throughout the extracellular space. It was thought that this diffusion might be partly responsible for the apparent time - dependent changes in contraction recorded from non - perfused muscles (see Section I). The irreversible nature of ecothiopate allowed for this biochemical determination of ChE activity. For details of methods and treatment of results see Methods (pp. 38-45).

Perfused preparations were incubated with ecothiopate (5×10^{-7}M) for varying periods up to 40 mins. Some muscles were initially stimulated indirectly (0.01Hz). Control preparations that were not incubated with ecothiopate were assumed to demonstrate 100% ChE activity. The results of the assays, that is, the remaining ChE activity following ecothiopate action, are shown in Table 24 and Fig. 42 . In these experiments, the estimated value of 332 ± 18 (mean ± S.D.) muscle fibres (i.e. endplates) per mm^2 of diaphragm was used to calculate the amount of radioactivity per endplate (see Methods p. 45).

Stimulation apparently made no difference in the assays and, therefore, all results were pooled. These results (Table 24 and Fig. 42) show that in perfused muscles, treatment with ecothiopate (5×10^{-7}M) brings about the inhibition of 70% of the functional endplate ChE within 2½ mins, 80% within 5 mins and reaches a maximum of 90% 20 mins after ecothiopate addition.

Incubation Time with ecothiopate (mins)	ENDPLATE CHOLINESTERASE ACTIVITY		
	cpm/endplate	% Activity	% Inhibition
0 (control)	17.49 ± 0.82 (21 expts.)	100	0
2.5	5.09 ± 0.76 (8 expts.)	29.1	70.9
5.0	3.34 ± (S.D.= 0.77) (3 expts.)	19.1	80.9
10.0	2.55 ± 0.35 (4 expts.)	14.6	85.4
20.0	1.79 (2 expts.)	10.1	89.9
40.0	2.66 (2 expts.)	15.2	84.8

Table 24

Rat hemi - diaphragms perfused with physiological saline at 37°C. Assayed for Cholinesterase Activity following incubation with ecothiopate (5×10^{-7}M) for varying times up to 40 mins. Radioactive counts expressed per minute ± S.E.M.

Fig. 42. Time courses of

(i) % ChE activity after ecothiopate (5×10^{-7}M) (○—○)

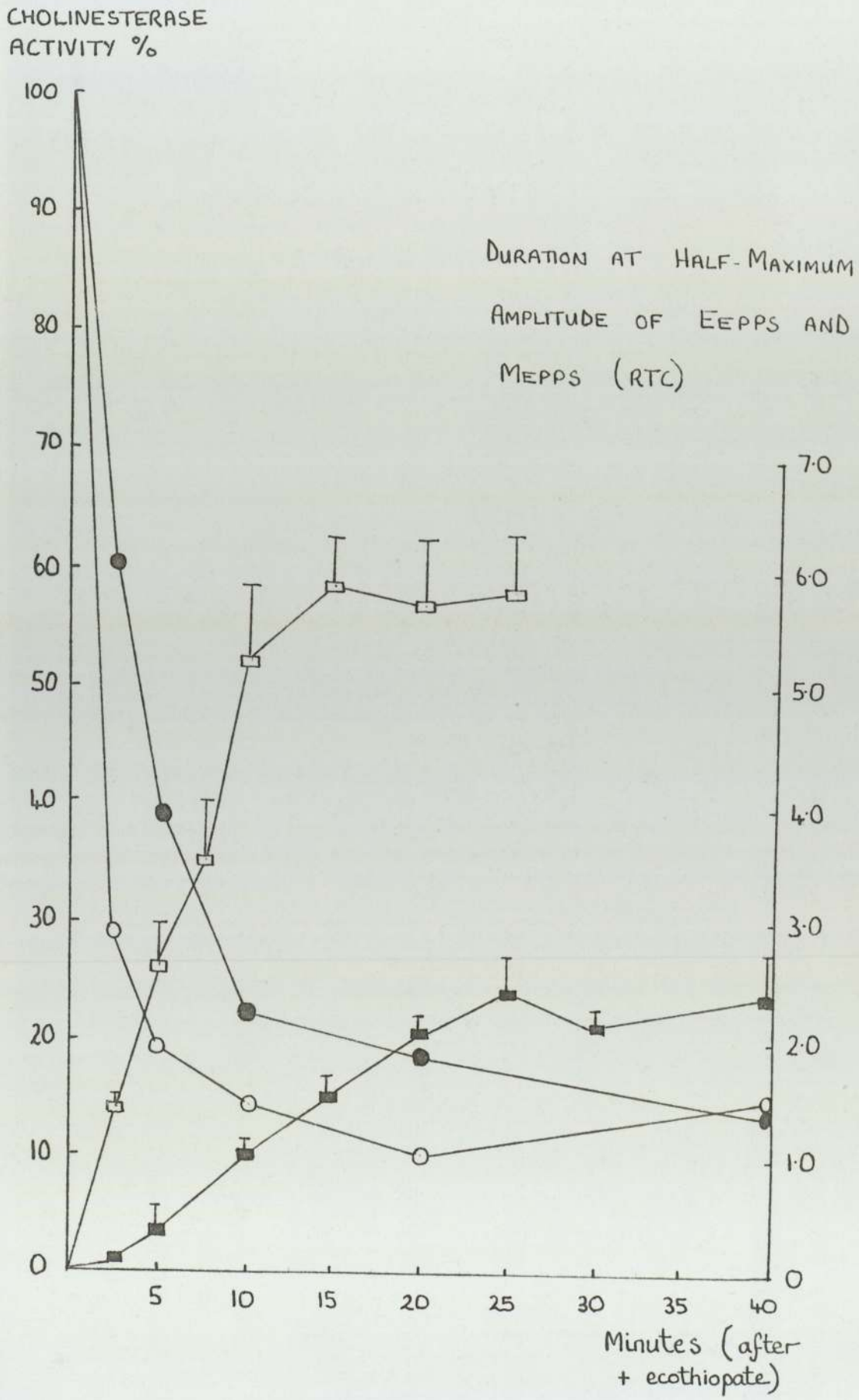
(ii) % ChE activity after ecothiopate in the presence of dTC (2×10^{-6}M). (●—●)

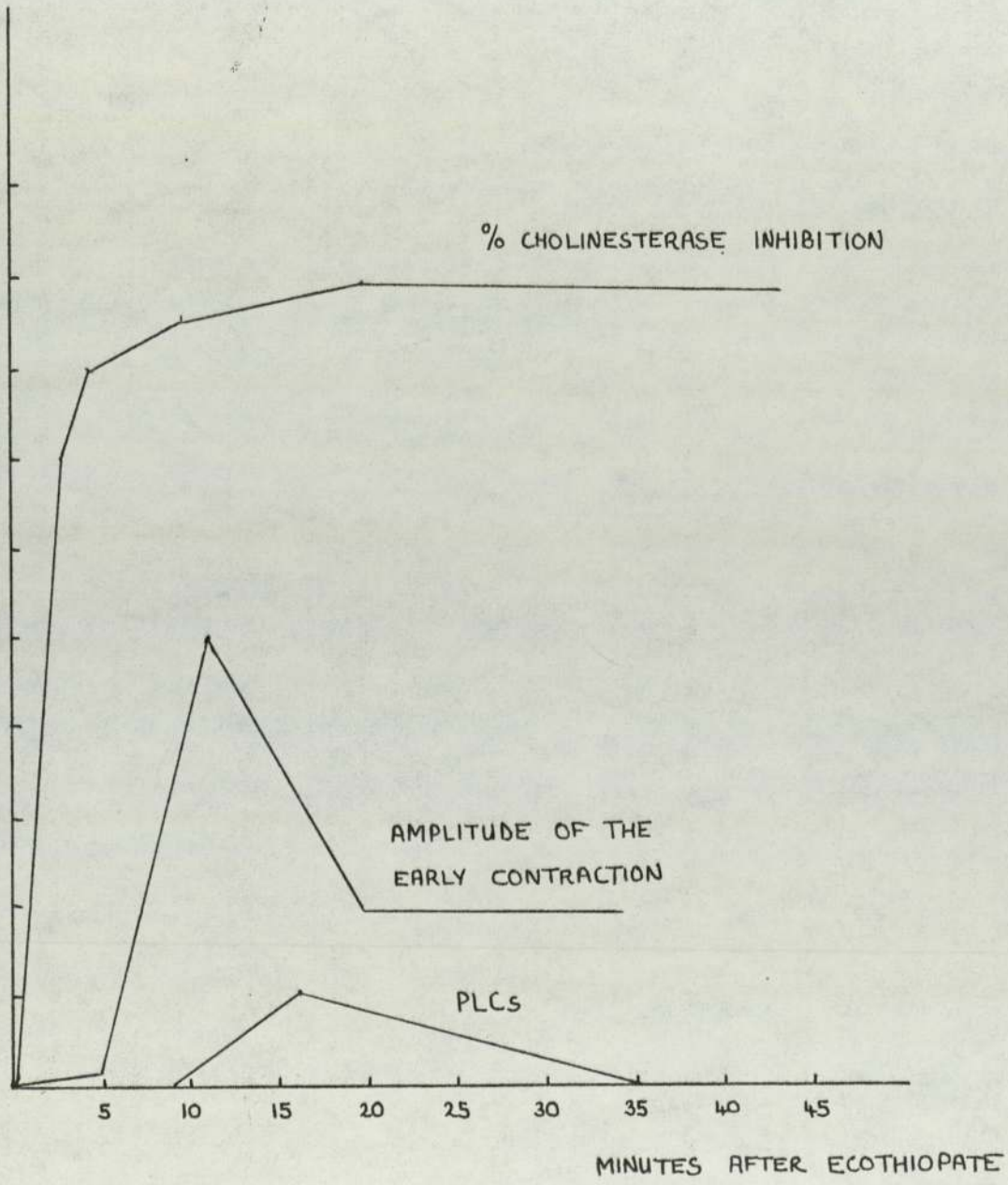
(iii) Relative duration at half - maximum amplitude of extracellular epps (Eepps) in the presence of dTC (2×10^{-6}M). (■—■)

(iv) Relative duration at half - maximum amplitude of extracellular mepps recorded from slack hemi - diaphragms. (□—□)

Note standard error bars.

Note also that ecothiopate added at 0 mins.


CHOLINESTERASE ACTIVITY %
100
90
80
70
60
50
40
30
20
10
0
DURATION AT HALF-MAXIMUM AMPLITUDE OF EEPPS AND MEPPS (RTC)
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
5
10
15
20
25
30
35
40
Minutes (after + ecothiopate)


% CHOLINESTERASE INHIBITION
AMPLITUDE OF THE
EARLY CONTRACTION
PLCs
5
10
15
20
25
30
35
40
45
MINUTES AFTER ECOTHIOPATE

Discussion

The earliest observable effect of ecothiopate on the contractile response of rat hemi - diaphragms was the enhancement of the early contraction which started, in most experiments, approximately 5 mins after drug addition. This time was, however, very variable being dependent on the rate and method of addition of the drug. It has been demonstrated in my experiments that substantial inhibition of functional endplate ChE (80%) has been achieved within 5 mins of exposure to ecothiopate in perfused muscles. It might be that the time - dependent enhancement of the early contraction and the development of SCs and plcs are the result of an effectively maximal, even if not complete, inhibition of ChE of rapid onset (i.e. time - independent inhibition).

Alternatively, the changes in the contractile response are brought about by a progressive (i.e. time - dependent) inhibition of ChE. Unless intensified and prolonged transmitter action is not mechanically effective until 80% of the ChE is inhibited and the time - dependent changes in contraction are a reflection of a progressive inhibition from 80% towards, presumably, maximal inhibition, then the time - dependent changes in contraction more likely reflect a time - independent inhibition. The observation, from my experiments, that the time courses of the progress of ChE inhibition and the progress of the changes in the contractile response are different (Fig. opp.) would suggest that the latter changes are the result of a time - independent ChE inhibition.

Barnes and Duff (1953), investigating the effect of paraoxon on the isolated rat diaphragm preparation, estimated that as ChE activity diminishes from 50% to 10% the response to single

stimuli becomes enhanced and is accompanied by irregular twitching. Van der Meer and Meeter (1956), investigating the effect of DFP on the isolated rat diaphragm, found that enhanced contractions start when about 20% of the ChE activity is left. This agrees well with my results. Mittag, Ehrenpreis and Hehir (1971), who investigated the effect of ecothiopate (4×10^{-7}M) on the same preparation, found that the external AChE (i.e. that located on the muscle fibre surface) is 91% inhibited after 7 mins (30^{o}C).

Mittag, Ehrenpreis and Hehir (1971) used a method which, apparently, allowed the inhibition of ChE in the intact rat diaphragm muscle in vitro. This has the advantage that the ChE enzymes remain in their native state bound to membrane sites. However, their results do not take into account the problem of diffusion of drugs and substrates within the tissue. Consequently, their results may not represent the whole muscle. Hence, it is doubtful whether their results could be correlated successfully with the changes in contraction brought about by ecothiopate action.

On the other hand, the methods used by Van der Meer and Meeter and myself minimise the problem of diffusion and therefore, the results of ChE activity may represent the whole of the muscle. However, these methods involve homogenisation and/or freezing which may introduce artefactual changes and also liberate internal ChE. This internal ChE, if uninhibited, may give misleadingly low results for ChE inhibition. Mittag, Ehrenpreis and Hehir (1971) apparently found that the external endplate - localised AChE accounted for only approximately 20% of the total AChE sites in muscle homogenates. Namba and Grob (1968) also found that the ChE activity of motor endplates in rat tibialis anterior muscle accounted for about 20% of the total ChE activity of the muscle.

Unlike the experiments made by Van der Meer and Meeter (1956), my experiments made allowance for any internal ChE activity (see Methods p. 42) so that any uninhibited ChE liberated by homogenisation and/or freezing would not be reflected in the final calculations of endplate ChE activity. The observation that a maximal inhibition of only 90% was achieved indicates that there was some active ChE which ecothiopate could not reach. It was possible that this ChE was located inside the muscle, perhaps, in close proximity to the endplate membrane or, alternatively, in the nerve terminals. However, Mittag et al. (1971) suggested that ecothiopate could penetrate the post-junctional membrane even though it had a low lipid solubility. Presumably, insufficent could penetrate to inhibit all functional ChE.

It would appear, therefore, that my results for endplate ChE inhibition were slightly underestimated assuming that only external enzyme is functionally effective in terminating transmitter action. It is, therefore, possible that maximal ChE inhibition occurs within 5 mins of adding ecothiopate to vascularly-perfused muscles.

However, the results from two different procedures for applying physiological saline and drugs are being compared. It is reasonable to assume that equilibration of any drug in the extracellular space of the muscle is faster in those muscles perfused compared to those that are not. Indeed, there is evidence from my ^{45}Ca experiments (p. 119) that complete equilibration might take, at least, 10 mins. since calcium accumulation in the muscles varied with the pre-incubation time in the tracer-physiological saline solution before the addition of the drug. If diffusion is an important factor then it is possible that the ecothiopate-

mediated changes in the contractile response of non - perfused muscles are, at least, partly due to the progressive spread of an effective inhibition of ChE from the outer muscle endplates to the inner endplates, as the ChE comes into contact with ecothiopate as the latter diffuses inwards (i.e. time - dependent inhibition)

It was now decided to investigate the effect of ecothiopate on the contractile response of perfused muscles. A comparison of the time courses of the various changes with those from non - perfused muscles might afford information as to the importance of diffusion on the time - dependent changes of the contractile response.

The effect of ecothiopate on the contractile response of the perfused rat phrenic nerve hemi - diaphragm preparation to single nerve stimulation

An experiment was made on a hemi - diaphragm preparation maintained in vitro and perfused with physiological saline at 37°C. The perfused preparation was more difficult to set up in the tissue bath but all other experimental conditions were identical (see Methods pp. 14-18).

The resultant record of contraction (Fig. 43) shows that ecothiopate produces enhancement of the early contraction and the development of SCs and plcs in a similar way to non - perfused muscles. Although not at all conclusive, the time course of effects are, apparently, different in that the onset of SCs and plcs is earlier compared to muscles that were not perfused.

The enhancement of contraction is also maintained for a longer period than in non - perfused muscles.

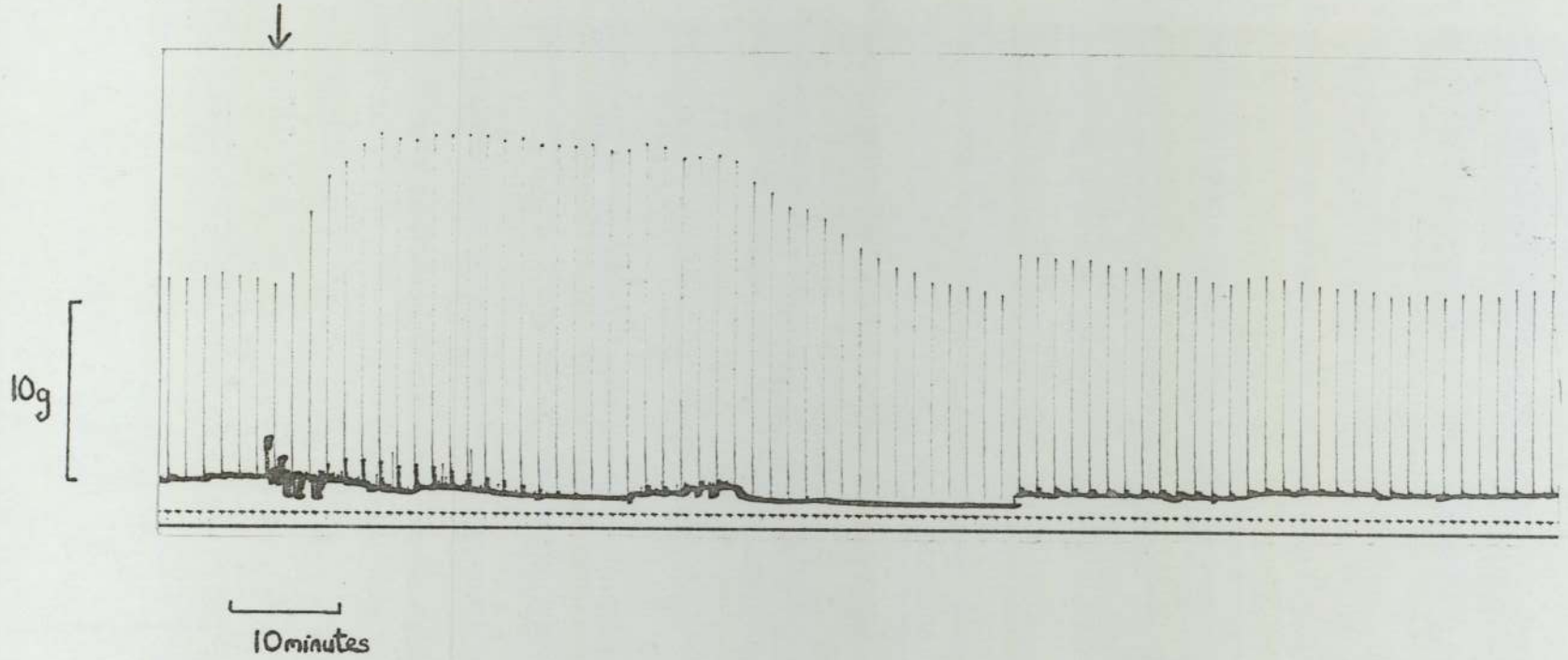


Fig. 43 Perfused rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Record of contraction to nerve stimulation (0.01Hz). Ecothiopate (5×10^{-7} M) added as indicated by the arrow.

Discussion

The above differences in the two techniques would be consistent with a faster drug equilibration time achieved in the extracellular space in the perfused hemi - diaphragms.

The results indicate that the effects of ecothiopate action on the contractile response of non - perfused muscles are not entirely the result of a time - dependent inhibition of ChE since the contraction record of a perfused muscle is not all that different from a muscle not perfused.

If time had allowed, it would have been interesting to determine the cholinesterase activity of non - perfused muscle treated with ecothiopate since this would have allowed a direct comparison between the two procedures for adding ecothiopate.

On the basis of previous discussion it is assumed that substantial ChE inhibition (i.e. 80%) is achieved relatively fast (i.e. within 5 mins) in perfused muscles and it is possible that such inhibition in non - perfused muscles is only marginally slower, and is accounted for by the slower equilibration time of ecothiopate in the extracellular space. However, such an assumption does not really allow speculation as to whether the effects of ecothiopate in changing the contractile response of non - perfused muscles is the result of a time - independent inhibition of ChE.

Previous observations of endplate currents prolonged by ecothiopate action, presumably reflecting prolonged transmitter action, suggest that they are progressively prolonged apparently achieving maximum prolongation in non - perfused muscles between 15 and 20 mins. The time to maximum prolongation appears to coincide with the time to maximum inhibition of ChE which was also, apparently, at 20 mins. This observation suggests that,

if the enhancement of the early contraction and the development of SCs and plcs are associated with the prolongation of transmitter action resulting from ecothiopate action, then they are due to a progressive (i.e. time - dependent) inhibition of ChE. The latter, presumably, is from about 80% ChE inhibition towards maximum inhibition. It was decided, therefore, to compare the time course of the prolongation of endplate currents with the degree of ChE inhibition.

The effect of ecothiopate on endplate potentials

All previous electrophysiological recording was of necessity from surface muscle fibres and, therefore, did not necessarily reflect the electrical events occurring in the inner fibres particularly if ecothiopate does take time to equilibrate with the extracellular space. In perfused muscles, however, it is reasonable to assume, that since ecothiopate equilibrates much more quickly, electrical recording of the surface fibres can be taken to represent the electrical activity of all the muscle fibres.

Experiments were made on vascularly perfused rat hemi - diaphragms to record extracellular endplate potentials (eepps) following treatment with ecothiopate for periods up to 25 mins before washing out the drug. The aim of the experiments was to correlate the duration of the eepps at half - maximum amplitude with the degree of ChE inhibition due to ecothiopate as determined radioisotopically. For details of the recording of eepps (see Methods p. 25). Stimulation of the perfused hemi - diaphragms via the phrenic nerve was as previously described (p. 17) and usually at a frequency of 0.1Hz. In order to record eepps it was necessary to treat the muscles with dTC(2×10^{-6}M)

to block the formation of action potentials. This concentration was chosen since it allowed control eepps that were large enough to be measured accurately whilst it was sufficient to prevent the restoration of neuromuscular transmission when the ecothiopate was added. After perfusion with dTC until a steady size of eepps in response to nerve stimulation was obtained, which was usually within 30 minutes, the fluid entering the bath was changed to include ecothiopate (5×10^{-7}M). The ecothiopate was applied for a set time and then washed out with the dTC - physiological saline for 30 mins. Eepps were recorded during the control period, throughout the duration of the applied ecothiopate and finally after washing. The recorded responses were averaged ($\bar{x} = 8$) (see Methods p. 26).

The possibility existed that this concentration of dTC might affect the inhibition of ChE by ecothiopate. Experiments were, therefore, made on a series of muscles to determine whether or not dTC affected ChE inhibition. These experiments were carried out in a similar way to the previous assayed muscles except for the presence of dTC. The prolongation of the eepps, relative to controls, is shown in Fig. 42 and the time course of ChE inhibition in the presence of dTC is shown in Fig. 42 . Both sets of data are detailed in Tables 26 and 25 respectively. The relative durations at half - maximum amplitude of the eepps are the means of, at least, 4 experiments (Table 26).

Incubation Time with ecothiopate (mins)	ENDPLATE CHOLINESTERASE ACTIVITY		
	cpm endplate	% Activity	% Inhibition
0 (control)	12.76 ± 1.26 (7 expts.)	100	0
2.5	7.70 ± 0.37 (6 expts.)	60.4	39.6
5.0	5.00 ± 0.45 (10 expts.)	39.2	60.8
10.0	2.91 ± 0.65 (7 expts.)	22.8	77.2
20.0	2.37 ± 0.36 (4 expts.)	18.6	81.4
40.0	1.88 ± 0.38 (5 expts.)	13.9	86.1

Table 25 Rat hemi - diaphragms perfused with physiological saline containing dTC (2×10^{-6}M) at 37°C. Assayed for ChE activity following incubation with ecothiopate (5×10^{-7}M) for varying times up to 40 mins. Radioactive counts expressed per minute ± S.E.M.

Table 26 Rat hemi - diaphragms perfused with physiological saline containing dTC (2×10^{-6}M) at 37°C. Time course of the relative time to half - maximum amplitude of eepps (means ± S.E.M.) in the presence of ecothiopate (5×10^{-7}M).

Time with Ecothiopate (x mins)	Relative time to half - maximum amplitude (x) represents the number of experiments
2.5	1.40 ± 0.16 (6)
5.0	2.59 ± 0.35 (6)
7.5	4.01 ± 0.51 (6)
10.0	5.24 ± 0.68 (6)
15.0	5.89 ± 0.41 (6)
20.0	5.71 ± 0.56 (6)
25.0	5.80 ± 0.57 (4)

The results show that the relative duration at half-maximum amplitude has increased after only $2\frac{1}{2}$ minutes of ecothiopate application. This corresponds to approximately 30% inhibition of ChE. The maximum prolongation of eepps (almost a 6-fold increase) occurs at approximately 15 minutes after ecothiopate which corresponds to an inhibition of ChE of about 75 - 80%. This latter value is practically the maximum inhibition achieved in the presence of dTC.

Discussion

The time courses of eepp prolongation and ChE inhibition appear to be similar, both achieving maximal or near-maximal values approximately 15 mins after ecothiopate (5×10^{-7}M).

Comparison of the time courses of ChE inhibition with and without dTC (2×10^{-6}M) (Fig. 42) shows that dTC, although not apparently affecting maximal inhibition, apparently reduces the rate of such inhibition. For example, after 5 mins exposure to ecothiopate, dTC-treated muscles show that only 50% of cholinesterase is inhibited whereas in normal muscles, approximately 80% of cholinesterase is inhibited. Therefore, it is possible to speculate that dTC, at this concentration, appears to interfere with the inhibition of cholinesterase by ecothiopate. This 'protecting' effect might be due to the close proximity of cholinesterase with the AChRs at the post-synaptic membrane, the believed site of action of dTC.

The observation that dTC appears to reduce the control counts (Tables 24 and 25) would suggest that dTC is interfering in some way with ChE activity.

It is not possible to speculate further as to whether the ecothiopate - mediated effects in changing the contractile response of hemi - diaphragms is due to a time - independent or time - dependent inhibition of ChE since with dTC, the change in eepp prolongation would appear to be due to time - dependent inhibition. However, it is possible that the presence of dTC not only interferes with transmitter action but may also, perhaps, delay the onset of the prolongation of epps. This would effectively lead to maximal prolongation being achieved at a later time than in the absence of dTC.

Finally, these extracellularly recorded currents are believed to reflect the time course of transmitter action (Fatt and Katz, 1951; Maeno, 1966). Therefore, accepting the limitations of dTC, the maximum prolongation of transmitter action is achieved at approximately 15 mins which coincides almost exactly with the maximum prolongation of the late part of the contraction. Therefore, on the basis of these results, the progressive prolongation of transmitter action parallels the progressive development of plcs. However, the observation that the eepps remain maximally prolonged for, at least, a further 10 mins whilst plcs are observed to wane, would suggest that the latter is not due to a reduction in transmitter action at the post - junctional membrane by, for example, receptor desensitisation. Either some other mechanism is responsible for the waning of plcs or dTC, in some way, prevents the receptors from becoming desensitised.

Effect of short exposures of ecothiopate on the contractile response

It has previously been suggested that the apparently time-dependent changes in contraction result from a constant degree (80%+) of ChE inhibition. In order to substantiate this hypothesis, experiments were made on non-perfused rat diaphragms to record the contractile response to indirect stimulation (0.01Hz) for varying periods with ecothiopate (5×10^{-7}M) from 2 - 16 mins. After these times the drug was washed out. It was hoped that the contraction records resulting from short exposures to ecothiopate, which could be correlated with the degree of inhibition of ChE, would show the changes in contraction, that is, the enhancement of the early contraction and the development of SCs and plcs to be time-dependent. It was assumed that any changes following removal of ecothiopate were due to a constant inhibition of ChE. Some of the record traces are shown in Fig. 44 . The contraction records were measured in the same way as before (p. 54) and the results were tabulated (Table 27).

In the two experiments where the muscles had been exposed to ecothiopate for two minutes only, there appeared to be no change in response compared with controls. In the two experiments where the muscles had been exposed for three minutes, enhancement of the early contraction and a small number (i.e. about 5) of SCs were observed. In the two experiments where the muscles had been exposed for 4 minutes, a substantial enhancement of the early contraction and a considerable number of SCs (i.e. approx. 45) were observed. The latter might have masked a slight development of the plcs. In the two experiments where the muscles had been exposed for 5 minutes, there was also a substantial enhancement

of the early contraction and even more development of SCs (approx. 75+). In addition, there was considerable development of plcs appearing approximately 12 minutes after initial exposure to ecothiopate and disappearing approximately 45 mins later, achieving a maximum prolongation approximately 14 minutes after initial drug exposure. In the two experiments where the muscles had been exposed for 8 mins, all the effects observed with 5 mins exposure were seen differing mainly in their time courses (see Table 27).

Finally, in the two experiments where the muscles had been exposed for 16 mins before washing, the enhancement of the early contraction, the presence of a small number of SCs and the development of plcs were all observed and subsequent measurement found their time courses and other parameters to be quite similar to the effects observed in muscles that were continually exposed to ecothiopate (5×10^{-7}M). It was also observed that, in the muscles exposed for 8 and 16 minutes, the reduction in the enhanced early contraction normally observed in the continuous presence of ecothiopate is reversed shortly after washing out the drug. Indeed, analysis of these results must take into account the possible modification of the results that the washing process might produce. The most striking difference between those muscles exposed continuously to ecothiopate and those exposed for 8 mins and 16 mins was the much longer time course of occurrence of plcs in the latter two pairs of muscles. The results, apparently, show that the longer the ecothiopate remains in the physiological saline, the shorter is the time course of occurrence of plcs.

Fig. 44

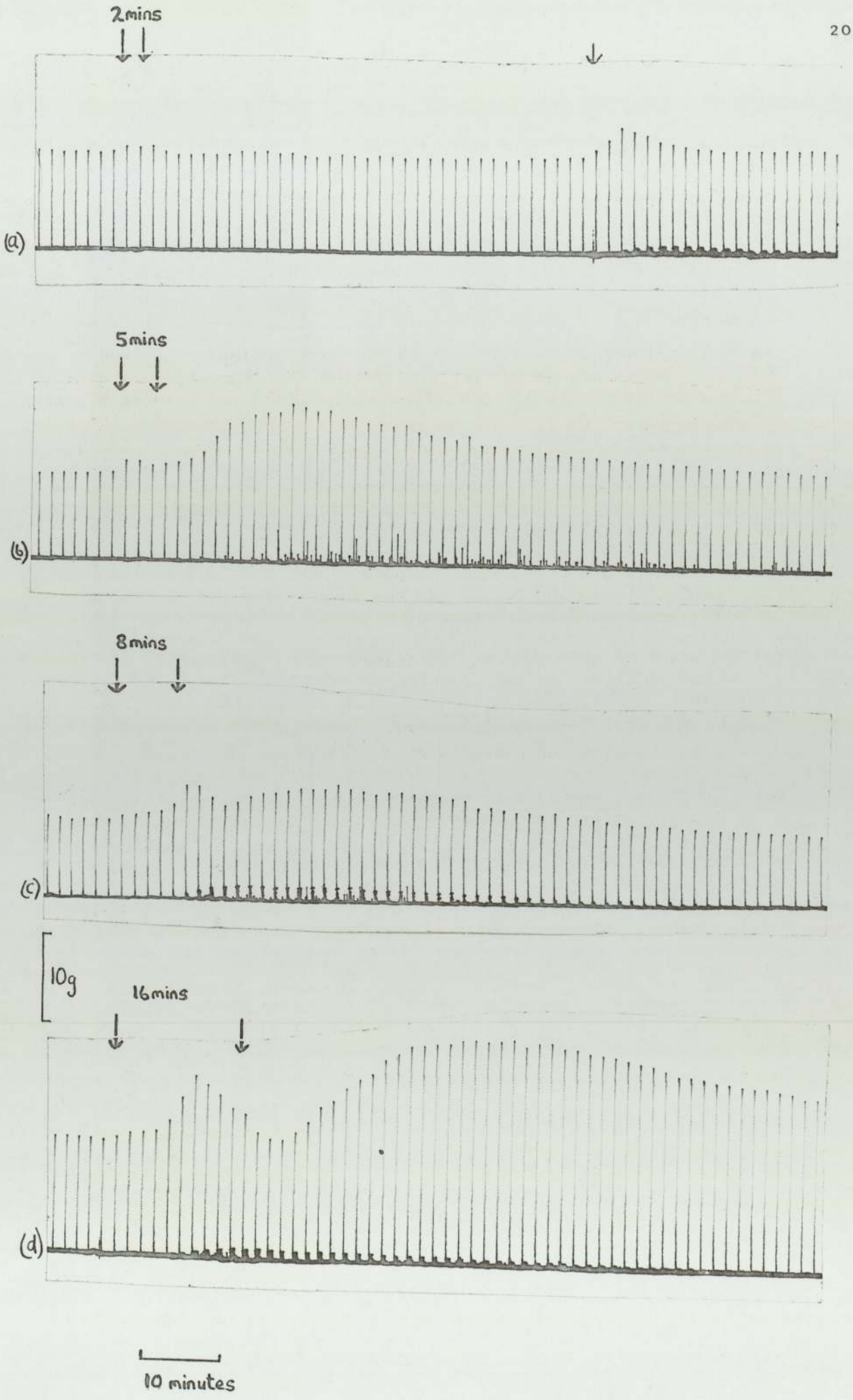
Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Record of contraction in response to nerve stimulation (0.01Hz) in the presence of ecothiopate (5×10^{-7}M) (added at arrows) for varying periods.

(a) 2 minutes exposure to ecothiopate. Note the absence of any observable effects. Also note the further addition of ecothiopate (arrowed) results in some of the changes in the contractile response usually observed.

(b) 5 minutes exposure to ecothiopate. Note the increase in amplitude of contraction and the development and persistence of spontaneous contractions.

(c) 8 minutes exposure to ecothiopate. Note, in addition to (b), the prolongation of the contractions and the reversal of the decrease in amplitude of contraction (following the wash-out of ecothiopate) normally seen in the continued presence of ecothiopate (5×10^{-7}M).

(d) 16 minutes exposure to ecothiopate. A response similar to that normally found in the continued presence of ecothiopate (5×10^{-7}M) except for the secondary increase in amplitude of contraction following the washing of the drug from the preparation.


2mins
(a)
5mins
(b)
8mins
(c)
10g
16mins
(d)
10 minutes

Experimental Conditions (Exposure times to ecothiopate)		Latency of Maximum Amplitude of Contraction (mins)	% Increase in the Amplitude of Contraction at Peak (cf control)	Late Prolongation of Contractions		Latency of maximum development of Prolonged Contractions)	Presence of Spontaneous Contractions (mins) (x) indicates the approx.no.observed
				START (mins)	FINISH (mins)		
2 mins (2 expts.)		No observed effect					
3 mins (2 expts.)		∿3 ∿4	∿21%	∿30 ∿13	None observed		18 - 30(5) 8 - 31(5)
4 mins (2 expts.)		∿18 ∿13	185% 55%		None observed		9 - 45(∿50) 4 - 50(40)
5 mins (2 expts.)		19 23	88% 82%	12.5 17	58 46	26 27	10 - 60+(75+) 11 - 60+(75+)
8 mins (2 expts.)		9.5 9.5	43% 32%	8 8	∿80 ∿80	17 - 30 14 - 25	16 - 15(30) 8 - 59(22)
16 mins (2 expts.)		13 11.5	77% 59%	11 9	55 70	22 17	12 - 17(5) 50+ (10) 12 - 17(5) 33+ (10)
Continuous (10 expts.)	mean range	13 9 - 20	76 30 - 136	9 7 - 12	35 28 - 55	16 13 - 19	Variable (see Table 2

(∿ = approximately)

Table 27

The time course of the changes in the contractile response brought about by exposure to ecothiopate (5×10^{-7}M) for varying times before washing. Nerve stimulation (0.01Hz). Physiological saline at 37°C. Drugs added at 0 mins. Note data from all experiments included.

Discussion

Assuming that there is no time-dependent inhibition in non-perfused muscles, it can be estimated (Fig. 42) that 2, 3, 4, 5, 8 and 16 mins exposures to ecothiopate corresponds to % inhibitions of endplate ChE of approximately 56, 73, 77, 81, 84 and 88% respectively. From the results (Table 27), the development of (i) the enhancement of the early contraction and (ii) SCs and (iii) plcs requires a minimum exposure time to ecothiopate of approximately 5 mins. For all 3 changes in response to occur, therefore, an inhibition of ChE of about 80% would appear to be required. If this analysis were to be correct then the results would imply that the time-dependent ecothiopate-mediated changes in contraction result from a time-independent inhibition of ChE.

However, that there is a diffusion component to the time course of inhibition is almost certain in non-perfused muscles. The inner muscle fibres would, presumably, then be reflecting varying degrees of ChE inhibition below 80% at a time when the ecothiopate-mediated changes in contraction have been developing and, possibly, also during their waning.

It is, therefore, tentatively concluded that the time-dependent changes in the contractile response due to ecothiopate result largely from a time-dependent inhibition of ChE. In addition, it appears quite possible that the changes in the contraction of the whole muscle, are partly the result of a change in the mix of responses made by individual muscle fibres, as ecothiopate penetrates the muscle.

Finally, it would appear from the results that a 5 min. exposure to ecothiopate results in substantial development of all the changes in the contraction of non-perfused muscles. Further exposure to ecothiopate is observed to result in the reduction of the extent and time course of these changes (Table 27), in particular, the time course of occurrence of plcs. This, together with the observation that the usual reduction in the enhancement of the early contraction is rapidly reversed on washing, would suggest that the continued presence of ecothiopate is either directly or indirectly (via transmitter action) responsible for the eventual waning of the changes in the contractile response.

Effect of ecothiopate on Mepps

Previous experiments made to record intracellular mepps (p. 140) proved not to be very successful. It was decided to make further attempts to record mepps, so as to determine the time course of any change in mepp parameters. This information could then be correlated with the time course of ChE inhibition previously charted. Since the mepps were to be extracellularly recorded from surface fibres then the results could be compared with the ChE assay results using perfused muscles.

Previous attempts to record intracellularly were unsuccessful mainly due to the spontaneous activity of the muscles, brought on by ecothiopate action, ejecting recording micropipettes after only short times of penetration into a cell. This spontaneous activity also distorts extracellular recording by moving the endplate away from the recording electrode. In order to overcome this difficulty the 'slack' preparation was used in which the 'tendon end' of the hemi - diaphragm was left free. This had the effect of abolising any spontaneous activity when ecothiopate was applied to the muscle.

Extracellular mepps were recorded and averaged ($\bar{x}$ = 32) from 4 muscles exposed to ecothiopate for a period of 40 minutes. The duration of the mepps of half - maximum amplitude was measured and was subsequently correlated with the degree of ChE inhibition due to ecothiopate as determined radioisotopically. The results are expressed graphically in Fig. 42. The results show that the duration of the mepps at half maximum amplitude increased above control values almost immediately (and certainly within 2½ mins) after the application of ecothiopate to the muscle. At this time approximately 70% of the endplate cholinesterase has been determined to be inhibited. The maximum prolongation of the mepps is reached at approximately 25 mins after ecothiopate

application which is only 5 minutes after maximal inhibition of cholinesterase (approximately 90%) has been achieved.

Discussion

The observations that the time course of the prolongation of endplate currents appear to parallel the time course of ChE inhibition would tend to support the hypothesis that, in perfused muscles at least, the effects of ecothiopate are the results of a time - dependent inhibition of ChE.

Summary

A method to determine ChE activity has been used which is believed to give results which represent endplate ChE activity only. ChE activity in perfused muscles is reduced to 20% 5 mins after ecothiopate and is reduced maximally (to approximately 10%) after 20 mins. An attempt has been made to correlate these changes in ChE activity with the ecothiopate - mediated changes in contraction of perfused muscles.

The time courses of the progressive prolongation of extracellular epps and mepps and the loss of ChE activity have been shown to be similar.

It was concluded that, on balance, the time - dependent changes in contraction after ecothiopate were largely due to a time - dependent inhibition of ChE.

SECTION X

Effect Of dTC On Ecothiopate Action

Results

(i) On the changes in the contractile response

Experiments were made on rat hemi - diaphragms to determine the effect of dTC on the changes in the contractile response, brought about by ecothiopate (5×10^{-7}M), in response to nerve stimulation (0.01Hz). dTC ($5 - 15 \times 10^{-8}$M) was added to the physiological saline ($[Mg^{2+}]$ 10^{-4}M) before and at the same time as ecothiopate and, in some experiments, at a point coincident with the maximum enhancement of the early contraction following ecothiopate.

(a) addition after ecothiopate

The addition of dTC ($5 - 15 \times 10^{-8}$M) at approximately 6 - 7 mins after ecothiopate apparently resulted in a progressive shortening of the time course of occurrence of plcs. Plcs had completely waned by approximately 84 mins (mean 3 expts.) in the absence of any dTC (5×10^{-8}M), 27 mins (mean 3 expts.) in dTC (10×10^{-8}M) and by 18.5 mins (mean of 3 expts.) in dTC (15×10^{-8}M) (Fig. 45 and Table 28). The reduction in the enhanced early contraction was also observed to be less severe compared to that observed in the absence of dTC. There did not appear to be much change in the overall number and time course of SCs.

(b) addition before ecothiopate

dTC ($10 - 15 \times 10^{-8}$M) added before ecothiopate did not prevent enhancement of the early contraction but, apparently, prevented the development of SCs and plcs. Again, the reduction in the enhanced early contraction was observed to be less severe.

In both (a) and (b) the absence of both ecothiopate and dTC from the physiological saline after times much longer than previously found necessary to achieve maximal cholinesterase inhibition resulted in (i) an immediate and progressive enhancement of the early contraction, (ii) the development of a considerable number of SCs and (iii) the development of slightly prolonged localised contractions. This recovery was found to be most effective after treatment with dTC (5×10^{-8}M) and least effective with dTC (15×10^{-8}M).

(c) addition of dTC (5×10^{-8}M) and ecothiopate together.

This resulted in only a considerable enhancement of the early contraction which was maintained even when the drugs were washed from the physiological saline (Fig. 46).

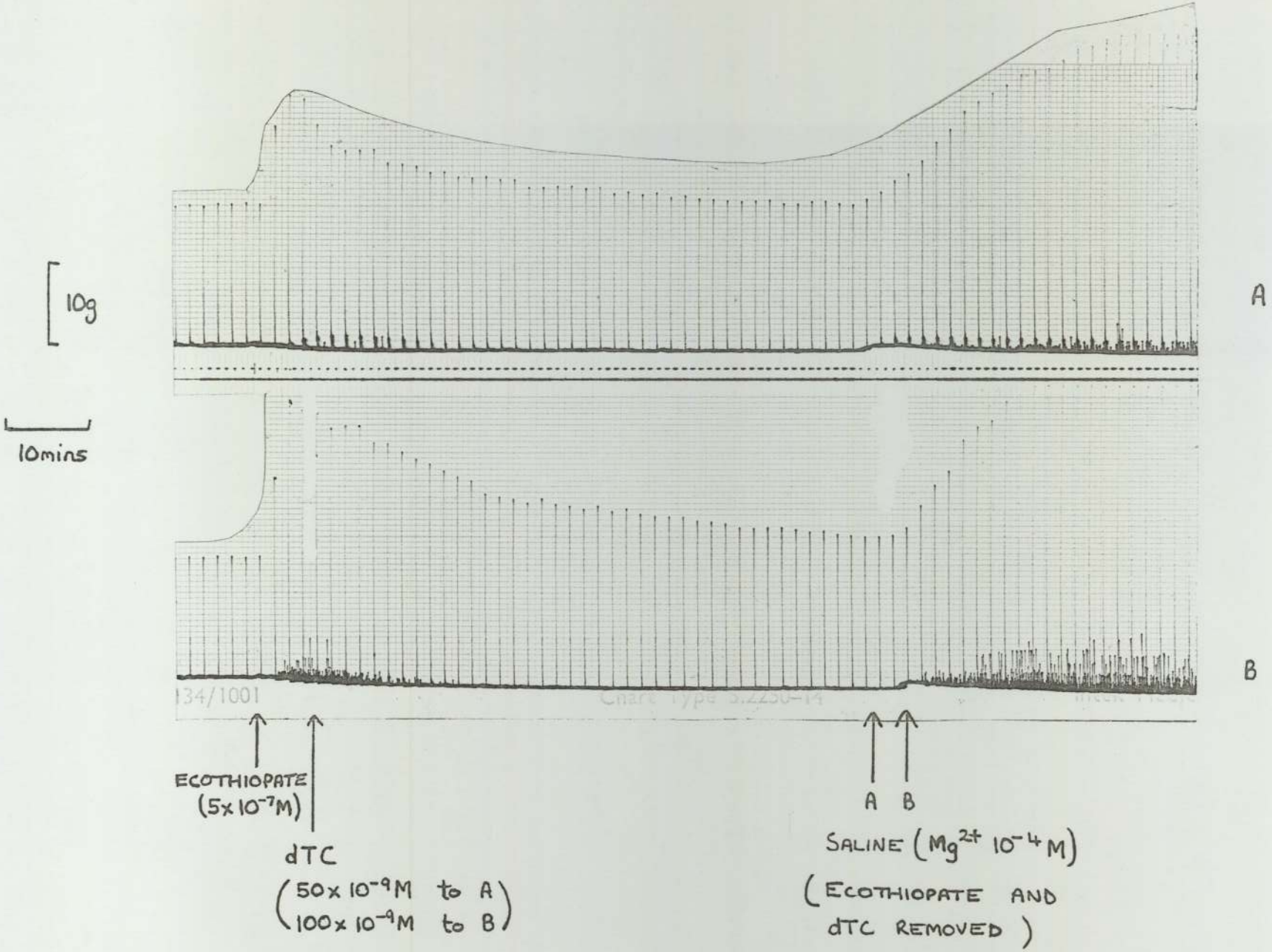

10g
10mins
A
B
ECOTHIOPATE
(5x10⁻⁷M)
dTC
(50x10⁻⁹M to A
100x10⁻⁹M to B)
A B
SALINE (Mg²⁺ 10⁻⁴M)
(ECOTHIOPATE AND
dTC REMOVED)

Fig. 45

Rat hemi - diaphragm in physiological saline ($[Mg^{2+}]$ 10^{-4}M) at 37°C. Records of contraction in response to nerve stimulation (0.01Hz) in the presence of ecothiopate (5×10^{-7}M).

A. dTC (50×10^{-9}M) and B. dTC (100×10^{-9}M) were added to the saline 6 minutes after ecothiopate addition.

Note the recovery of ecothiopate effects on removal of both drugs from the saline solution.

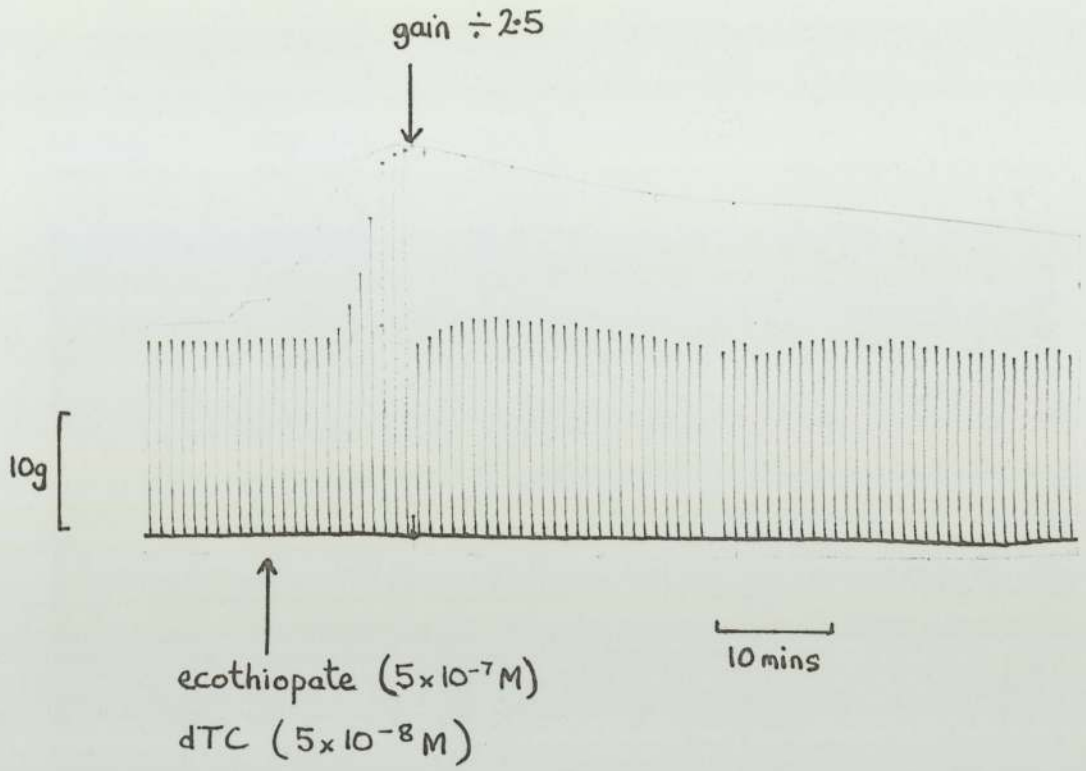


Fig. 46

Rat hemi - diaphragm in physiological saline ($[Mg^{2+}]$ 10^{-4}M) at 37°C. Record of contraction in response to nerve stimulation (0.017Hz). Ecothiopate (5×10^{-7}M) and dTC (50×10^{-9}M) added as indicated.

Note the change in gain of the pen recorder.

Experimental Conditions		Latency of maximum amplitude of contraction (mins)	% Increase in amplitude of contraction (mins)	Late Prolongation of contractions		Latency of maximum development of plcs (mins)
				START (mins)	FINISH (mins)	
Ecothiopate only	mean	5	59	5	84	13
(3 expts.)	range	4 - 6	45 - 69	3 - 7	60 - 100	12 - 14
Ecothiopate + dTC(5×10^{-8}M) at + 6 mins	mean	3.75	63	3.75	35	10.5
(2 expts.)	range	3.5 - 4	48 - 78	3.5 - 4	31 - 34	10 - 11
Ecothiopate + dTC(10^{-7}M) at + 6 mins	mean	4	47	4	27	11
(3 expts.)	range	-	33 - 60	3 - 5.5	22 - 36	-
Ecothiopate + dTC(15×10^{-3}M) at + 7 mins)	mean	6.5	86	8	18	10.5
(3 expts.)	range	5.5 - 7	84 - 90	5 - 10	16 - 21	10 - 11
*Ecothiopate + dTC(5×10^{-8}M) at 0 mins		20	179	-	-	-
(1 expt.)				(Enhanced amplitude of contraction maintained)		

Table 28

The time courses of the changes in the contractile response brought about by ecothiopate in the presence of dTC ($5 - 15 \times 10^{-8}$M). Frequency of nerve stimulation 0.01Hz. Except * = 0.017Hz.

Physiological saline ($[Mg^{2+}]$ 10^{-4}M).

Ecothiopate (5×10^{-7}M) added at 0 mins. dTC added as indicated.

The data for ecothiopate only the same as in Table 1.

Note: Physiological saline and drug solutions not continuously added to tissue bath except (*)

Discussion

dTC ($5 - 15 \times 10^{-8}$M) has been found to interfere with the development of plcs and SCs but did not appear to affect the enhancement of the early contraction brought about by ecothiopate action. Since the latter is believed to be associated with muscle repetitive firing and SCs are believed to be generated pre-synaptically, it is suggested that these [dTC]s (i.e. $5 - 15 \times 10^{-8}$M) are only effective pre-synaptically. It is probable that dTC abolishes the antidromic firing in the nerve terminals and thus prevents the axon reflex excitation of motor units. Morrison (1977) found that dTC (10^{-7}M) abolished repetitive firing in the nerve. That these concentrations are not as effective post-synaptically is, perhaps, due to the lower density of receptors thought to be associated with the pre-synaptic membrane. However, that plc development, which is believed to be associated with prolonged post-junctional transmitter action, is inhibited or prevented by dTC ($5 - 15 \times 10^{-8}$M) is more difficult to explain. It may be that a significant factor in prolonging transmitter action is the secondary release resulting from the antidromic firing - axon reflex excitation event. Since dTC has effectively abolished this source of transmitter, the subsequent intensification and prolongation of post-junctional transmitter action is, perhaps, sufficient to fire repetitive action potentials and thus enhance concentration, but is insufficient in initiating plcs. Morrison (1977) also found that dTC (10^{-7}M) only diminished muscle repetitive firing and the prolonged endplate currents.

The observation that the removal of both dTC and ecothiopate from the preparation is followed by the progressive enhancement of

the early contraction and a progressive development of SCs and plcs suggests that the dTC block, primarily at the pre-synaptic membrane, is only slowly and progressively abolished due to the known difficulty in removing dTC by washing. The considerable enhancement of the early contraction following dTC removal might also imply a post-junctional blocking action of dTC in the nano-molar range of concentration (e.g. $5-15 \times 10^{-8}$M) which reduces some inhibitory process on repetitive firing and allows the latter to develop to a greater extent than normal.

This recovery, following the removal of both dTC and ecothiopate, also suggests that dTC ($5-15 \times 10^{-8}$M) has a partial ChE-protecting effect against inhibition by ecothiopate. This is because by the time the drugs were removed, ChE inhibition by ecothiopate should have been maximal. Maximal inhibition has been suggested to eventually lead to the waning of the enhanced early contraction, the SCs and the plcs. However, the observation that the 'recovery' of effects is maintained for, at least, 20 mins suggests that maximal ChE inhibition has not been achieved. It has also been previously established (pp. 200-) that dTC (2×10^{-6}M) apparently partially protects against ChE inhibition by ecothiopate. Ehrenpreis, Hehir and Mittag (1971) also found dTC to activate membrane-bound AChE. However, whether dTC (10^{-7}M) can protect endplate ChE from inhibition is not known. Alternatively since the waning of the ecothiopate-mediated effects is thought, perhaps, to be due to receptor inactivation resulting from prolonged transmitter action, it is possible that the slow removal of dTC from the preparation prevents effective receptor inactivation in the time course observed.

Finally, the possibility remains that ecothiopate, perhaps the excess ecothiopate not involved in inhibiting ChE, has a direct blocking action at either the pre- or the post-junctional membrane or both, thus interfering with transmitter action. It is possible that this blocking action has only become important because transmitter action has been reduced by dTC. Previous work has suggested that the continued presence of ecothiopate is responsible for the reduced enhancement of the early contraction (Section IX , pp. 205—). Ecothiopate (5×10^{-7}M) has also been suggested to inhibit the development of nerve repetitive firing under certain conditions (Morrison, 1977).

(ii) On the extracellular action currents recorded at the endplate.

Since it has been established that dTC (5×10^{-8}M), added to a rat hemi - diaphragm preparation in physiological saline ($[Mg^{2+}]$ 10^{-4}M) at the same time as ecothiopate (5×10^{-7}M), inhibits the development of both the spontaneous and prolonged localised contractions, experiments were made to determine whether dTC (5×10^{-8}M) abolished the prolongation of the extracellularly recorded endplate currents. For details of methods used see Methods p. 24).

The results (Section III. Fig. 17) show that in response to nerve stimulation (0.017Hz) the endplate currents still become prolonged. However, the associated contractions, although enhanced, do not show any late prolongation. Experiments made by adding dTC (5×10^{-8}M) 7 minutes after ecothiopate application did not appear to show any reduction in the prolongation of the epcs (unpublished).

Discussion

These results appear to confirm previous conclusions in that, although plcs are apparently associated with prolonged endplate currents, the latter can still be recorded in the absence of plcs. It is possible that dTC (5×10^{-8}M) reduces the prolonged depolarisation below the mechanically effective threshold for plc development (Section III). However, the above results appear to make this suggestion unlikely. Assuming that endplate calcium accumulation reflects prolonged transmitter action and since dTC ($5 - 15 \times 10^{-8}$M) prevents the development of both SCs and plcs (Fig. 46), it was decided to investigate the effect of dTC (10^{-7}M) on calcium accumulation.

(iii) Effect of dTC on calcium accumulation

Experiments to determine the effect of dTC (10^{-7}M) on calcium accumulation in the presence of ecothiopate (5×10^{-7}M) were made on mouse hemi - diaphragms as described previously (Methods pp. 34-37). In these experiments dTC (10^{-7}M) was added to the physiological saline 10 mins before ecothiopate and $^{45}CaCl_2$ were added. After 15 mins incubation in ecothiopate the muscles were transferred to tracer - free physiological saline.

The results (Table 29 and Fig. 47) apparently show that the presence of dTC (10^{-7}M) does not significantly alter the endplate accumulation of calcium due to ecothiopate. In addition, experiments made with dTC (10^{-7}M) only, gave results of endplate calcium accumulation that were not significantly different to those obtained either with ecothiopate or with both ecothiopate and dTC (Table 30).

Calcium accumulation in the rest of the muscle showed a significantly greater accumulation with ecothiopate and dTC. dTC, on its own, apparently caused an even greater accumulation which was also found to be significant (Fig. 48).

Discussion

It has, apparently, been established that dTC (10^{-7}M) does not prevent the endplate calcium accumulation in mouse diaphragm due to ecothiopate. This [dTC] has, however, prevented the development of SCs and plcs in rat diaphragm. This evidence, therefore, suggests that the presumably greater prolongation of endplate depolarisation associated with SCs and plcs do not make an effective contribution to calcium accumulation. However,

Table 29 Calcium accumulation in mouse hemi - diaphragms (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C in the presence of dTC (10^{-7}M) only and dTC (10^{-7}M) and ecothiopate (5×10^{-7}M), both for 15 mins, followed by washout for 30 mins in tracer - free physiological saline before immersion in acetone. (The letters A_8 to D_8 are for reference in Table 30).

	n-moles of calcium accumulated mg^{-1} dry muscle	
	endplates	non-junctional
dTC and ecothiopate	A_8 0.144 ± 0.053(8)	B_8 0.561 ± 0.117(8)
dTC only	A_8 0.167 ± 0.038(7)	D_8 0.865 ± 0.066(7)
ecothiopate only (15 mins, Table 7)	A_2 0.162 ± 0.017(8)	B_2 0.160 ± 0.012(8)
physiological saline only (18 mins, Table 4)	A_1 -0.016 ± 0.02(9)	B_1 0.166 ± 0.024(9)

Table 30

Independent t values and probabilities between unpaired sets of data in Table 29 .

Unpaired sets of data	t value	degrees of freedom	Probability	Statistically Significant/ Insignificant
A_8 v A_2	0.3464	14	0.7332	Insignificant
A_8 v A_1	3.1614	15	0.0065	Significant
A_8 v C_8	0.3664	13	0.7198	Insignifcant
C_8 v A_2	0.1305	13	0.8935	Insignificant
C_8 v A_1	4.8574	14	0.0004	Significant
B_8 v B_2	3.6447	14	0.0029	Significant
B_8 v B_1	3.7481	15	0.0022	Significant
B_8 v D_8	2.3298	13	0.0349	Significant
D_8 v B_2	12.1032	13	0.0000	Significant
D_8 v B_1	11.8050	14	0.0000	Significant

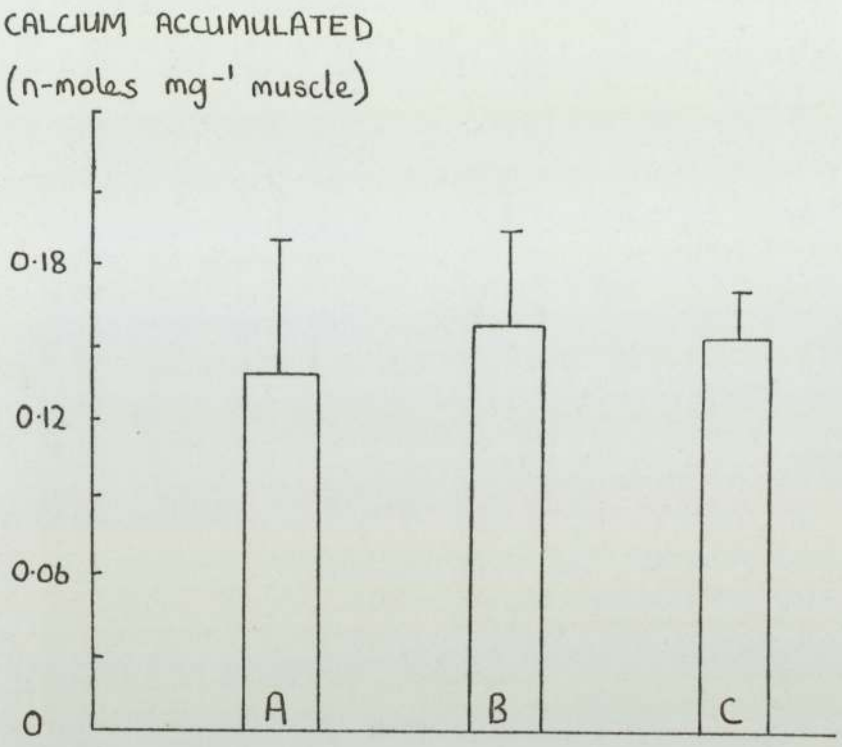


Fig. 47. Endplate calcium accumulation (n-moles mg^{-1} total dry muscle) in muscle incubated for 15 mins with

A. dTC (10^{-7}M) and ecothiopate (5×10^{-7}M)

B. dTC (10^{-7}M) only

C. ecothiopate (5×10^{-7}M) only.

Note standard error bars.

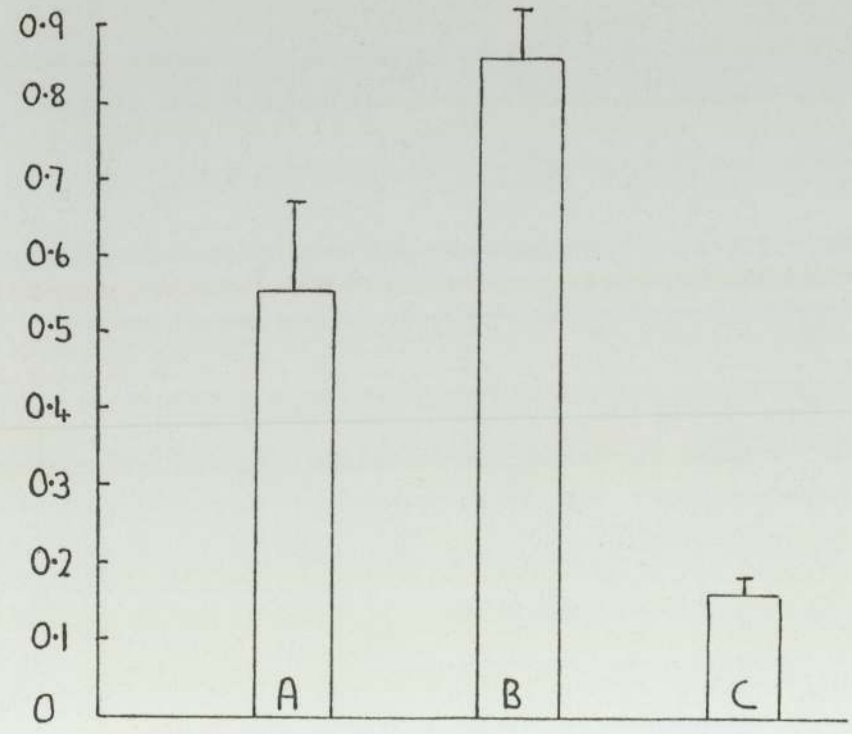


Fig. 48. Non - junctional calcium accumulation.

A, B and C as above.

since depolarisation is still, apparently, prolonged, even in the presence of dTC, it might still be responsible for the calcium accumulated. This suggests that if dTC does act to reduce endplate depolarisation below a mechanically effective threshold, then resultant mechanically ineffective depolarisation is still sufficient to cause post-junctional Ca^{2+} entry. This would support the conclusion, made in Section VIII, that the persistent and more intense transmitter action, in the presence of ecothiopate, might be sufficient to cause post-junctional Ca^{2+} entry.

The observation that dTC, itself, results in endplate calcium accumulation suggests that dTC (10^{-7}M) also has a post-junctional effect. It is difficult to envisage any pre-synaptic action of dTC (10^{-7}M) altering the Ca^{2+} permeability of the post-junctional membrane. Presumably, dTC, although blocking transmitter receptor interaction and subsequent depolarisation, induces ion channels to open thus allowing entry of Ca^{2+}.

Finally, the observation that dTC, either with ecothiopate or on its own, significantly increases non-junctional calcium accumulation above that observed in the absence of both drugs, suggests that dTC (10^{-7}M) directly affects the muscle membrane. Apart from accepting that these calcium accumulation results might be artefactual, I am unable to elucidate further.

Further discussion

dTC (10^{-7} to 10^{-8}M) has been observed to interfere with the development of SCs and plcs but not the enhancement of the early contraction. Indeed, this enhancement of the early contraction appears to be maintained in the presence of dTC. Since the enhancement of the early contraction was presumably due to repetitive firing in the muscle, it is concluded that dTC (10^{-7} to 10^{-8}M) is not greatly effective post - synaptically. Additionally, the abolition of SCs, previously believed to be generated pre - synaptically, further suggested that dTC (10^{-7}M to 10^{-8}M) was primarily effective pre - synaptically. Furthermore, the hypothesis put forward to explain why plcs did not develop in muscles pre - treated with dTC before ecothiopate addition was based on a pre - synaptic action of dTC.

However, the interference in plc development may be associated with a post - synaptic action of dTC (10^{-7} to 10^{-8}M). The experiments where dTC was added to the physiological saline at a time coinciding with the maximum enhancement of the early contraction following ecothiopate treatment showed the concentration - dependent progressively earlier cessation of plcs was not accompanied by the usual reduction in enhancement of the early contraction. A progressive post - synaptic action of dTC in reducing the time course of occurrence of plcs would surely also result in a further reduction in the enhancement of the early contraction. This would be because dTC is presumably reducing transmitter action by competitively blocking the receptors. Hence, it would appear that dTC (10^{-7} to 10^{-8}M) is working primarily pre - synaptically. But what if dTC was working in some other way at the post - junctional membrane ?

For example, if instead of dTC blocking closed ion channels what if it was blocking receptors while the ion channels were open? Katz and Miledi (1978) and Colquhoun, Dreyer and Sheridan (1979) have found that dTC may partially act by blocking open ion channels. It might, therefore, be speculated that dTC does not affect the enhancement of the early contraction since this possibly involves a period of channel opening when insufficient dTC has penetrated the ion channels. Plcs, which normally develop after the enhancement of the early contraction, are now prevented from developing as sufficient dTC has now penetrated and blocked ion channels in their open state. A partial block of receptor - activated open ion channels may explain the endplate accumulation of calcium with dTC only. One disadvantage of this hypothesis is that Colquhoun et al. (1979) used [dTC]s in the micromolar range or higher. Therefore it is likely that [dTC]s in the nanomolar range are even less effective.

Summary

dTC ($5 - 15 \times 10^{-8}$M) has been shown to inhibit the development of SCs and plcs but not the enhancement of the early contraction. It has been suggested that dTC ($5 - 15 \times 10^{-7}$M) does not significantly depress the prolonged endplate currents observed after ecothiopate. dTC (10^{-7}M) does not, apparently, alter the endplate calcium accumulation obtained with ecothiopate and, furthermore, dTC itself has been discussed in terms of possible pre- and/or post- synaptic effects of dTC.

SECTION XI

The Effect Of Temperature On Ecothiopate Action

Introduction

Experiments were made on rat hemi - diaphragms maintained in vitro in physiological saline to determine the effect of lowering the temperature of the physiological saline (from 37 to 30°C) on the ecothiopate - mediated changes in the contractile response to nerve stimulation (0.01Hz). These experiments were made in order to investigate the effect of further prolonging and intensifying transmitter action known to be caused by cooling. It was hoped that these experiments would afford further information regarding the antiChE action of ecothiopate.

Results

The muscles were set up for recording as detailed in Methods (pp. 14-18). After a stable control response had been obtained at 37°C the temperature of the physiological saline was lowered to 30°C before adding ecothiopate (5×10^{-7}M). The contraction record (Fig. 50) was supplemented by recording the responses using faster time bases (50msec to 1 sec per cm) (Figs 49 and 51).

Lowering the temperature from 37°C to 30°C of the control solution had the effect of prolonging the time to half-maximum amplitude of each contraction from 36.5 to 59.5 msecs (mean 2 expts.) and of enhancing the amplitude of the early contraction by approximately 20% (Figs. 49 and 50). The addition of ecothiopate at this lower temperature produced a further but usual enhancement of the early contraction followed by the usual reduction in enhancement. This reduction was accompanied by the development of plcs and a small number of SCs, the latter appearing at about 5 mins and had waned completely by 17 mins approximately after ecothiopate. The most striking difference observed between 37°C and 30°C was that the plcs at 30°C were still being observed 100 mins after ecothiopate. Although maximum prolongation of the endplate-localised contraction was, apparently, achieved 15 mins after ecothiopate, which agrees well with the time observed at 37°C, the prolongation, approximately 9 secs (Fig. 51) was twice that obtained at 37°C. The plcs were even observed after removing ecothiopate from the physiological saline (see also Table 31).

Raising the temperature back to 37°C resulted in the virtual disappearance of the previously well-established plcs accompanied by further enhancement of the early contraction together with the development of a few more SCs.

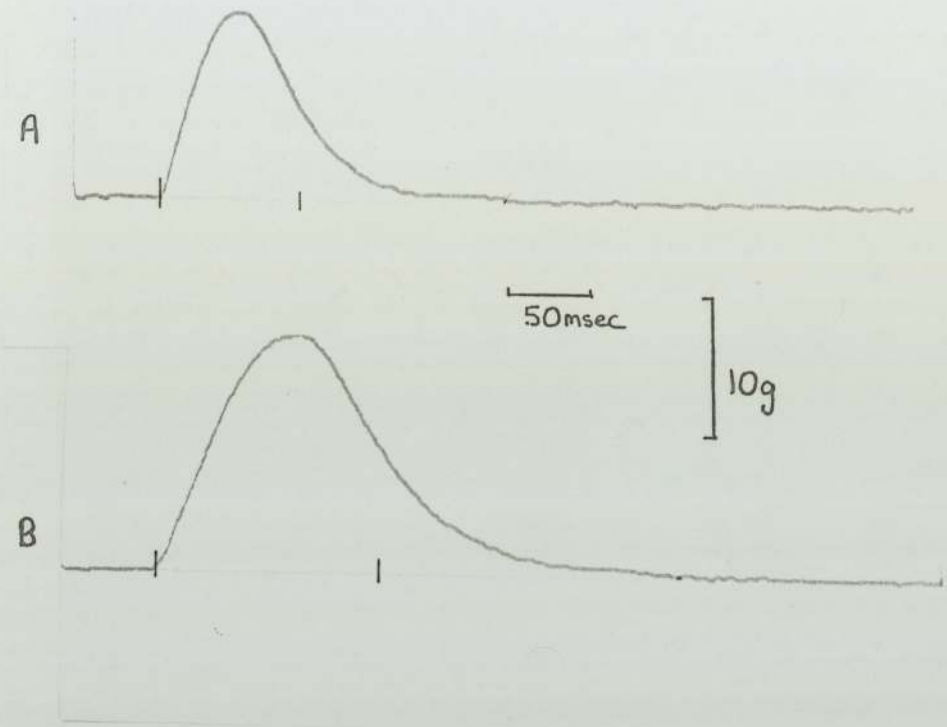


Fig. 49 Rat hemi - diaphragm. Physiological saline. Effect of lowering the temperature from 37°C to 30°C on the amplitude and time course of the contractile response. Frequency of nerve stimulation 0.01Hz.

A. Response at 37°C.

B. Response at 30°C.
Note the increases in amplitudes and time course.
Markers indicate the start of the contraction and the time to half - maximum amplitude respectively.

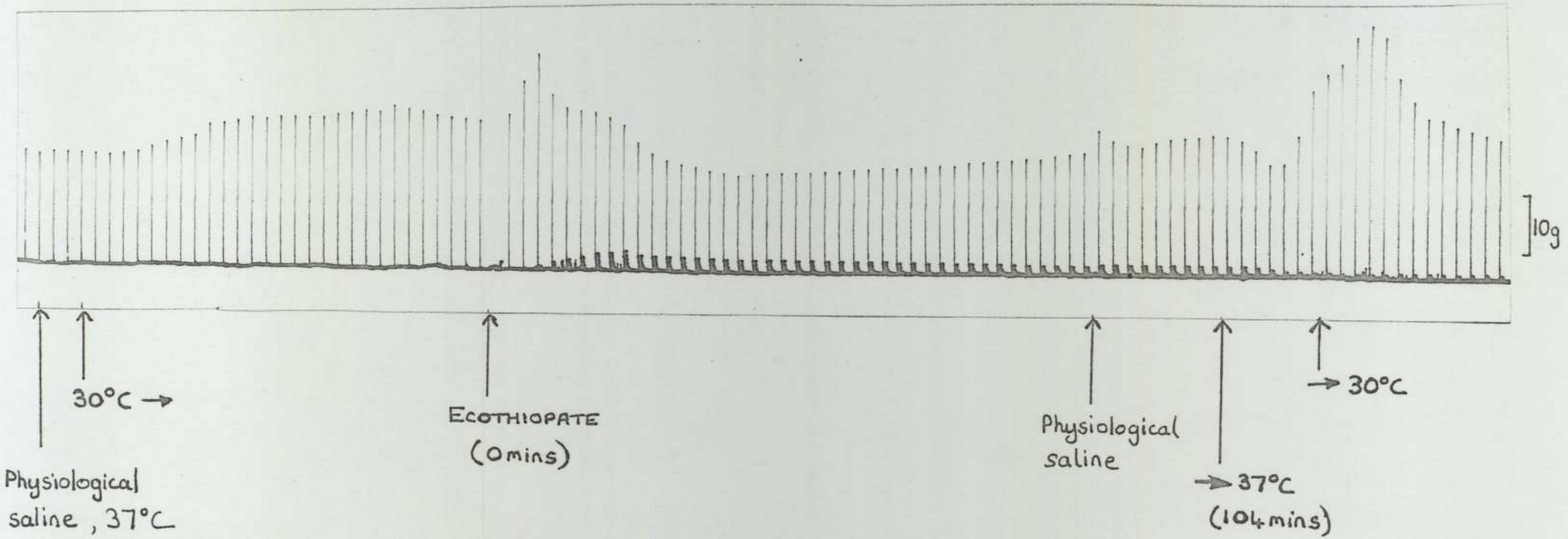

10g
30°C →
ECOTHIOPATE
(0 mins)
Physiological
saline
→ 30°C
Physiological
saline, 37°C
→ 37°C
(104 mins)

Fig. 50. Effect of temperature on the contractile response of rat hemi-diaphragm in physiological saline containing ecothiopate (5×10^{-7}M). Frequency of nerve stimulation, 0.01Hz.

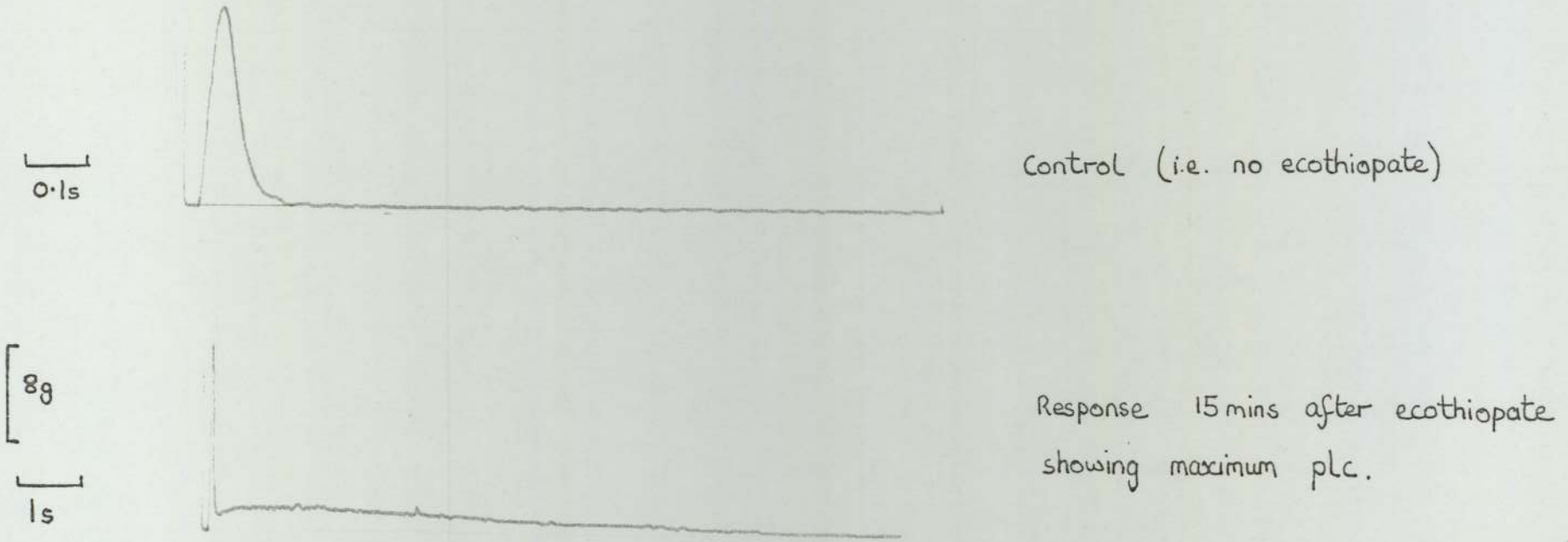


Fig. 51 The effect of temperature (30°C) on the maximum prolongation of the late part of the contractile response of a hemi - diaphragm in the presence of ecothiopate (5×10^{-7}M)

Table 31 The time courses of the changes in the contractile response brought about by ecothiopate at 37°C and 30°C. Drugs added at 0 mins.
Frequency of nerve stimulation, 0.01 or 0.017Hz.
The data for 37°C is the same as that exhibited in Table 1.

Temperature		Latency of twitch enhancement (mins)	Latency of maximum amplitude of contraction (mins)	% increase in amplitude of contraction	Late prolongation of contractions		Latency of maximum development of prolonged contractions (mins)
					START (mins)	FINISH (mins)	
37°C	mean	5.6	13	76	9.2	35	16.2
	S.D.	2.2	2.9	38.8	1.7	8.6	2.1
(10 expts.)	range	1 - 7	9 - 20	30 - 136	7 - 12	28 - 55	13 - 19
30°C	mean	3	6	45	5	104+	15
(2 expts.)	range	2 - 4	5 - 7	43 - 47	4 - 6	—	—

Discussion

Transmitter action has been shown to be prolonged on cooling (Liley, 1956a; Boyd and Martin, 1956b; Li, 1958; Harris and Leach, 1968). Lowering the temperature by 10°C lengthens the average channel duration at frog neuromuscular junctions by 2.5 - 3 times (Anderson and Stevens, 1973). A prolongation of the action potential and/or a slowing of the contraction would effectively explain the initial prolongation of the contraction. The slowing of the contraction may be due to the prolongation of the active state, perhaps, due to a reduction in the rate of Ca^{2+} removal back to the SR. A prolongation of the active state might explain the enhanced amplitude of the early contraction.

It has already been established that the intensification and prolongation of transmitter action as a result of ecothiopate action somehow leads to the development of plcs. The observation by Harris and Leach (1968), that cooling further prolongs transmitter action in the presence of an antiChE, would suggest that further prolongation of the plcs at 30°C in my experiments is due to the further prolongation of transmitter action which presumably occurs at this temperature. However, experiments were not made to determine if there was further prolongation of the endplate currents on cooling in the presence of ecothiopate. Alternatively, the further prolongation of plcs may be due to a temperature - dependent delay in the removal of Ca^{2+} from the sarcoplasm in the endplate region.

The results also show (Fig. 50) that raising the temperature to 37°C abolishes the plcs and that lowering it back down to 30°C brings them back. It would appear that raising the temperature to 37°C has some inhibitory action on the generation of plcs.

This might be related to a temperature - dependent re-activation of ChE activity or, alternatively, a temperature - dependent re-activation of inactivated receptors. The latter would also explain the concurrent enhancement of the early contraction.

The observation that plc development, apparently, coincides with a reduction in the enhanced early contraction and the waning of plcs coincides with a further enhancement of the early contraction, confirms earlier similar observations (Section 1).

It is tentatively suggested that whatever mechanisms are responsible for the development of plcs, they somehow interfere with the enhancement of the early contraction.

Summary

Lowering the temperature from $37^{o}C$ to $30^{o}C$ enhances and prolongs contractions both in the presence and absence of ecothiopate ($5 \times 10^{-7}M$). Plcs have been observed to be prolonged up to 9 secs. These results have been discussed in terms of a prolongation of both transmitter action and Ca^{2+} - action.

SECTION X

Does Ecothiopate Action Lead To A Reduction In Transmitter Activity ?

Introduction

In rat hemi - diaphragms maintained in vitro in physiological saline and treated with ecothiopate (5×10^{-7}M), the enhancement of the amplitude of the early part of the contractile response is not maintained and the amplitude subsequently declines. Furthermore, the SCs wane quite quickly and the plcs are also not maintained.

The waning of a contractile response may be due to internal mechanisms in the muscle fibres or may be due to a modification of the external influences on muscle fibre contraction, the latter effectively resulting in neuromuscular block.

A possible reduction in transmitter action has been suggested on several occasions to explain the waning of the various ecothiopate - mediated changes in the contractile response. This reduction may be associated with pre - synaptic and/or post - synaptic receptors.

A reduction in transmitter action may result from desensitisation of receptors (Thesleff, 1955) or from depolarisation block (Burns and Paton, 1951). Both result in a loss of response of a receptive membrane following the continuous application of a receptor - activating drug. Desensitisation is associated with a rapid repolarisation of the post - junctional membrane following a brief but persistent depolarising action, the receptors remaining refractory to further action (Thesleff, 1955a and b; Katz and Thesleff, 1957b; Axelsson and Thesleff, 1958).

It has been suggested in this work that, following inhibition of ChE by ecothiopate, transmitter action is both intensified and prolonged.

In this section investigations are made to determine whether ecothiopate action results in the loss of subsequent electrical and contractile responses.

Results

Experiments were made on rat hemi - diaphragms maintained in vitro in physiological saline to record the contractions and extracellular endplate currents following various trains of stimuli (Methods pp. 14-). The first stimulus acted as the conditioning pulse. The subsequent responses were analysed for any evidence of neuromuscular block.

In the first experiment, the muscle was stimulated via the nerve at 200Hz for 11msec (i.e. 3 pulses 5msec apart) every 60 secs. After control responses were obtained, ecothiopate (5×10^{-7}M) was added to the physiological saline. The results (Fig. 52) show that, in the absence of ecothiopate, the 3 muscle action currents were similar in amplitude, shape and time course suggesting that they were apparently independent of one another. The associated contraction was, in fact, a brief tetanus developing 57g of tension. 17 mins after ecothiopate, the results clearly show a marked reduction in amplitude of the second and third action currents compared with the first. The endplate currents associated with the first action currents appear to be prolonged. The associated contraction shows maximal development of the plc.

A similar reduction in the amplitude of the muscle action currents is observed (Fig. 53) when 2 pulses 20msec apart at 0.017Hz are applied to a muscle.

In another experiment, 3 pulses 0.1 sec apart (i.e. 10Hz for 0.21 sec) were applied to a muscle. The first muscle action currents and the three associated contractile responses were recorded. The results (Fig. 54) show clearly that, after 16 mins exposure to ecothiopate, while the first contractile response shows the usual increase in amplitude of the early contraction the subsequent

responses show a marked reduction in comparison even beyond control values. Only the first contraction appears to be prolonged.

In another experiment where the record of contraction in response to stimulation of the nerve 5 times, each 0.5 sec apart (2Hz for 2.6 secs) at a frequency of 0.017Hz was made, the first response (Fig. 55) 17 mins after ecothiopate showed the usual increase in amplitude of the early contraction while the subsequent four responses showed a marked reduction in comparison. This time, however, all responses increased above control values. In addition, the most marked reduction in amplitude was seen in those responses which appeared to fall within the time course of the plc apparently developed from the first response. The responses, apparently, outside the time course of the plc showed an increased amplitude although still reduced compared to the first response. Similar results were obtained when 5 pulses, 0.2 and 1.0 sec (Figs.56 and 57) apart were applied to the muscle. All three previous experiments showed a degree of recovery of equality of amplitude of contraction, although still increased above control values. This recovery appeared to coincide with the waning of the plcs.

This apparent loss of response is observed in muscles when stimulus pulses are as much as 4 secs apart. The greatest loss of response apparently coincides approximately with the maximum prolongation of the endplate localised contractions.

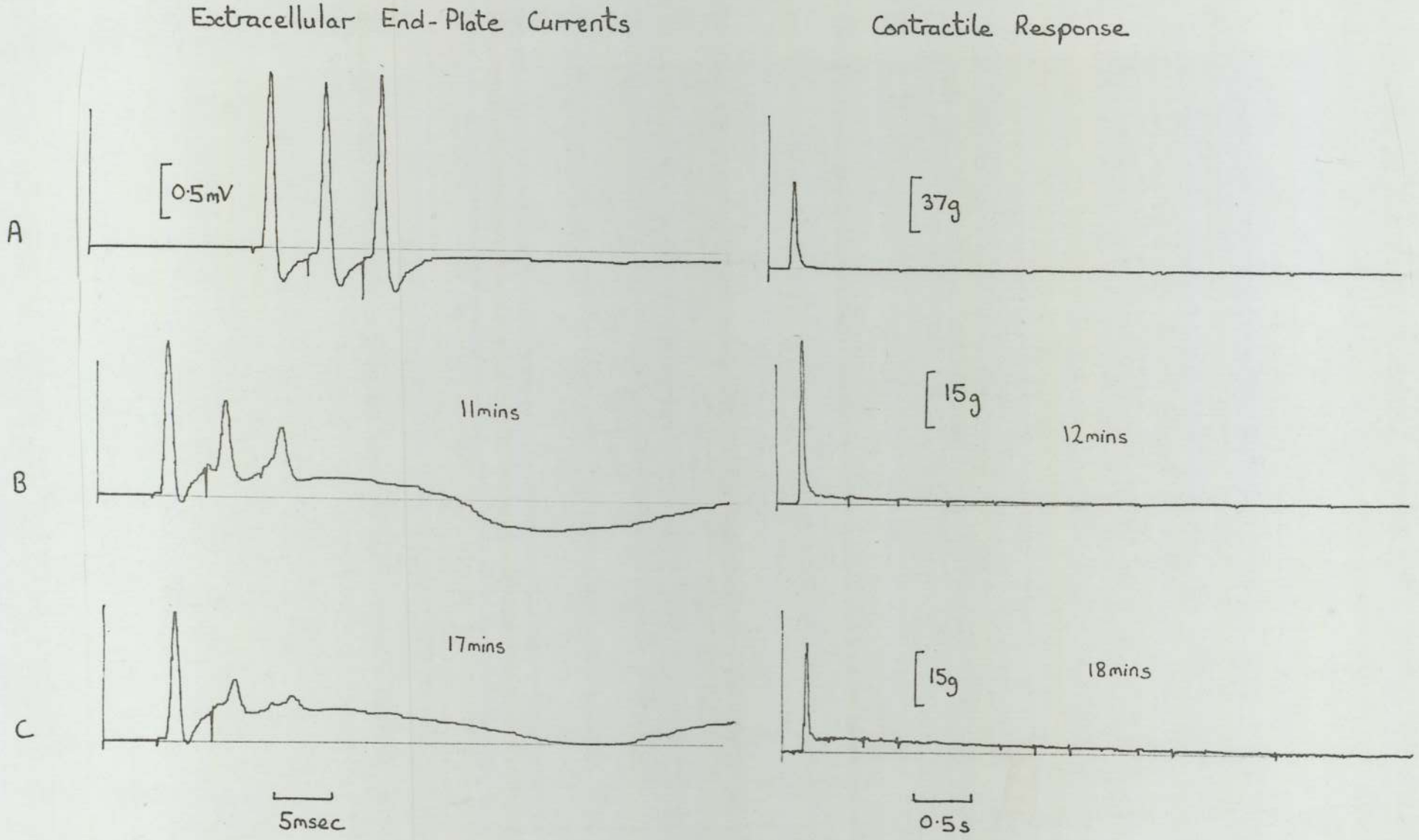

Extracellular End-Plate Currents
Contractile Response
A
B
C
0·5mV
37g
11mins
15g
12mins
17mins
15g
18mins
5msec
0·5s

Fig. 52

Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Alternate records of Contraction and AC extracellular endplate currents in response to nerve stimulation (200Hz for 11msecs at 0.017Hz)

A. Control records. Note the similar responses of the extracellular currents.

B. 11 and 12 minutes after the addition of Ecothiopate (5×10^{-7}M). Note the progressive reduction in amplitude of the second and third extracellular responses and the development of a plc.

C 17 and 18 minutes after ecothiopate. Note maximum plc development.

Note also the progressive reduction in amplitude of both sets of records.

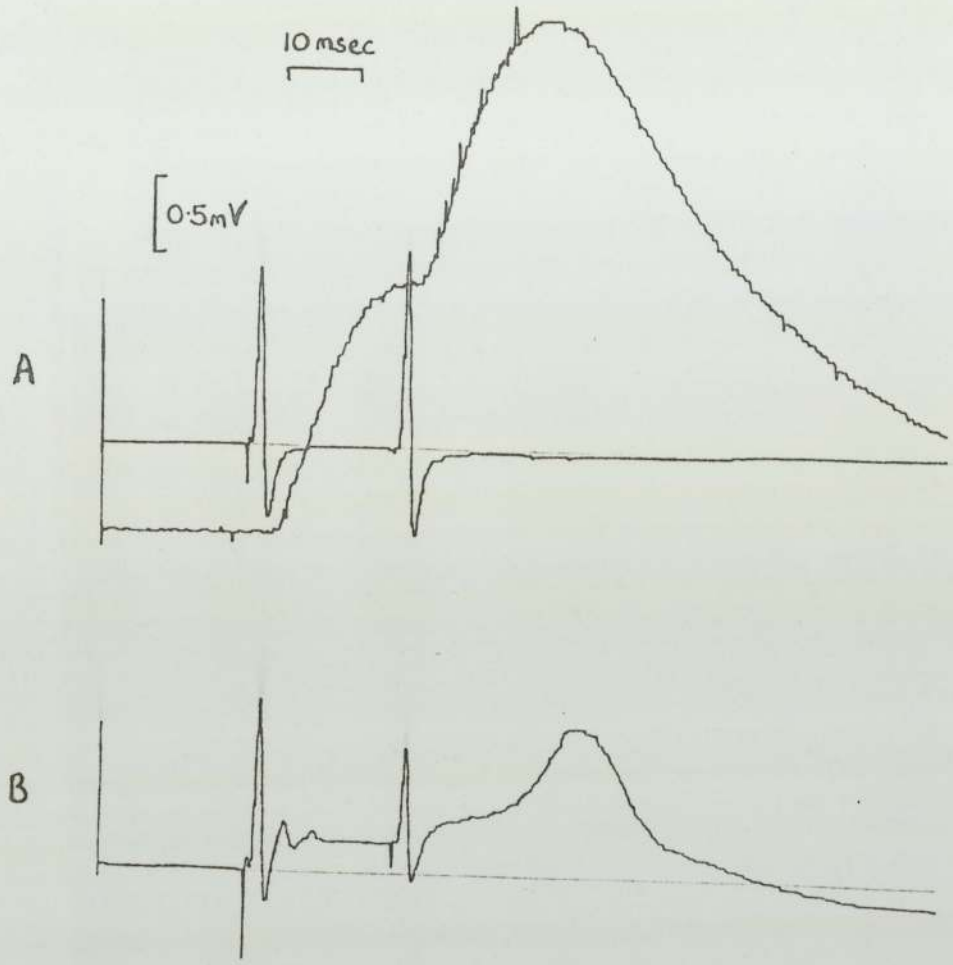


Fig. 53. Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Records of AC Extracellular endplate currents in response to 2 stimuli of the nerve 20 msecs apart (0.01Hz).

A. Control Records. Note the superimposed muscle contractions.

B. Record 19 mins after ecothiopate (5×10^{-7}M)

Note the movement artifact which does not interfere with the records with this time course.

End-Plate Currents

Contractile Responses

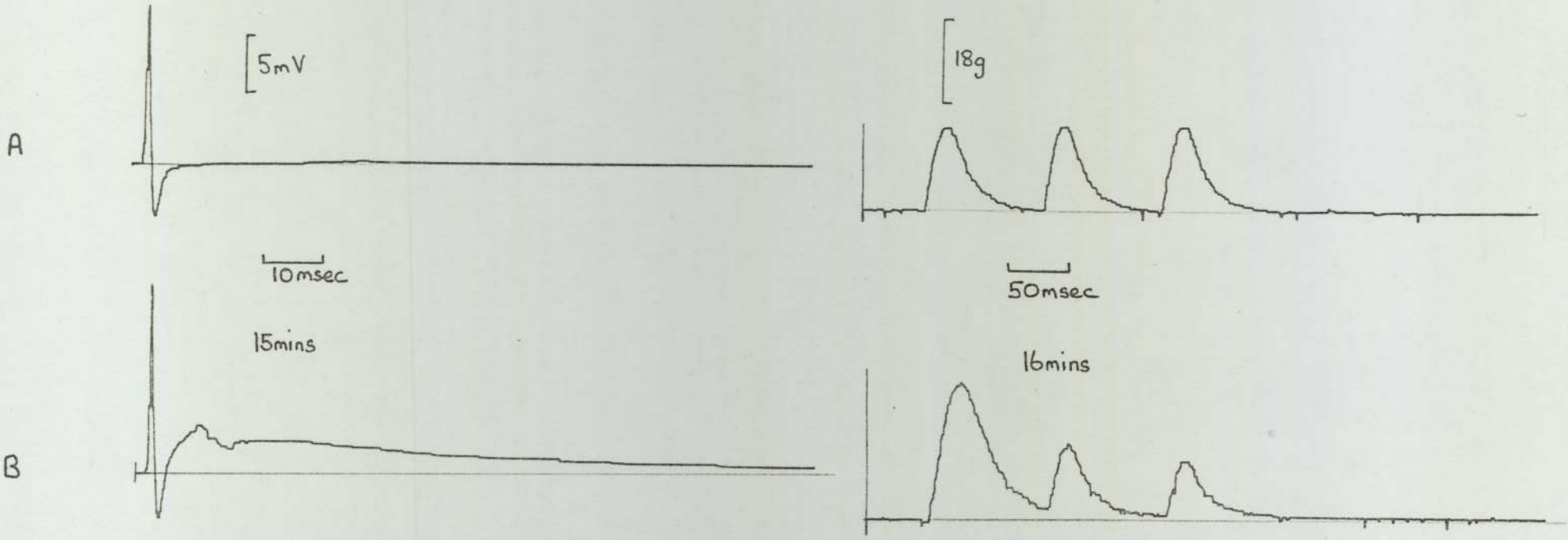


Fig. 54.

Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Refords of AC extracellular endplate currents and contraction in response to 3 stimuli 100 msecs apart (i.e. 10Hz) at 0.017Hz . Only the extracellular response to the first stimulus was recorded.

A. Control Records. Note the similar contractile responses.

B. Records 15 and 16 mins after ecothiopate (5×10^{-7}M) Note the prolongation of the e.p. currents and the progressive reduction in amplitude of the second and third contractile responses. Note also the plc development of the first response.

Fig. 55.

Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Records of contractions in response to 5 stimuli 0.5 secs apart (2Hz for 2.6 secs) at 0.017 Hz.

A. Control records. Note the similar responses.

B. Records 17 mins after ecothiopate (5×10^{-7}M).

Note the plc development of the first response and the maximum reduction of amplitude of subsequent responses within the time course of the plc.

C. Records 40 mins after ecothiopate.

Note the disappearance of any plc development and the almost complete recovery of the contractile response (although amplitudes higher than controls).

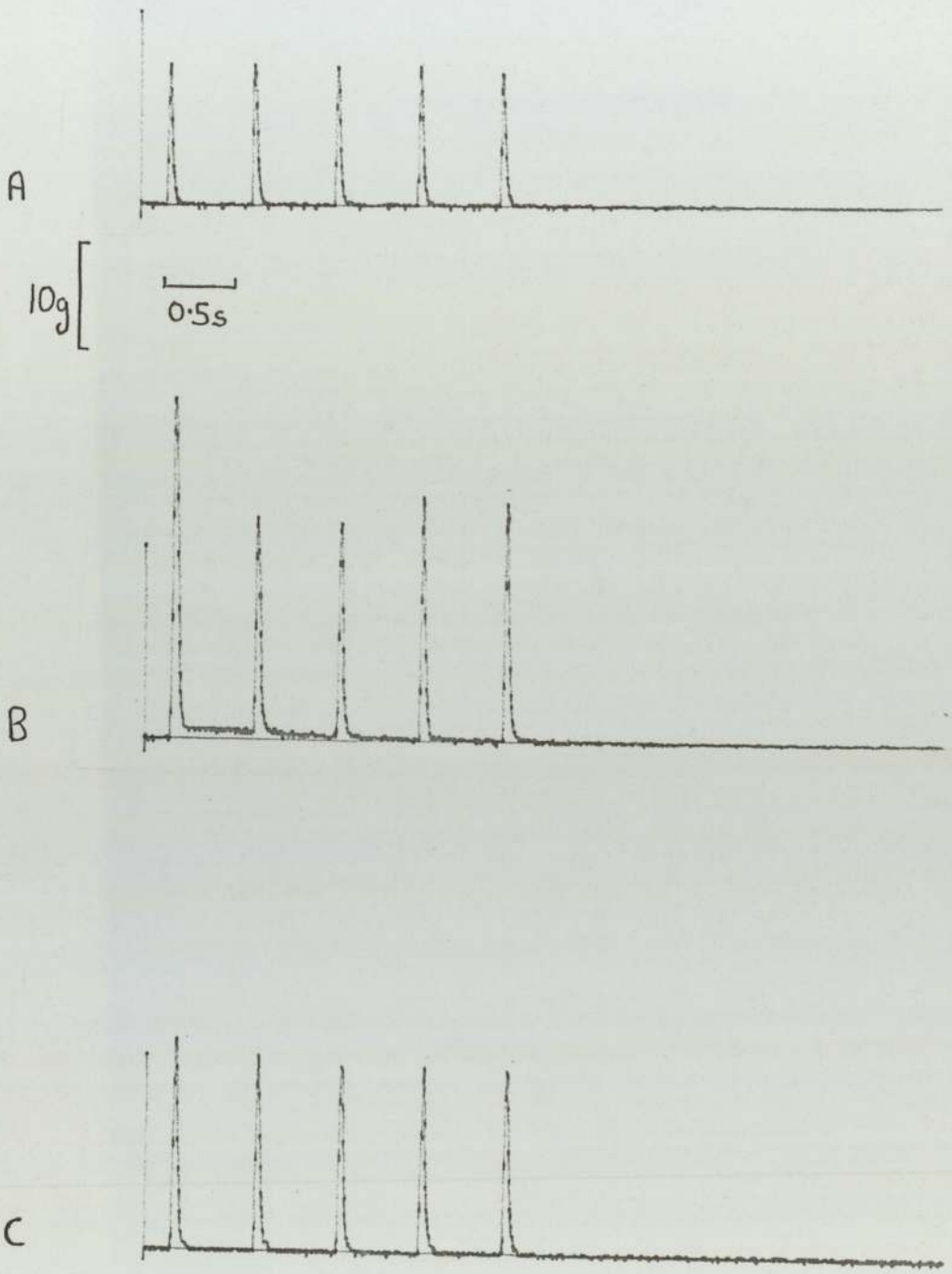

A
10g
0·5s
B
C

Fig. 56

Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Records of contractions in response to 5 stimuli 0.2 secs apart (5Hz for 1 sec) at 0.017Hz.

A. Control records. Note the similar responses

B. Records 13 mins after ecothiopate (5×10^{-7}M)

C. Records 36 mins after ecothiopate.

Note the almost complete recovery of the contractile response to controls.

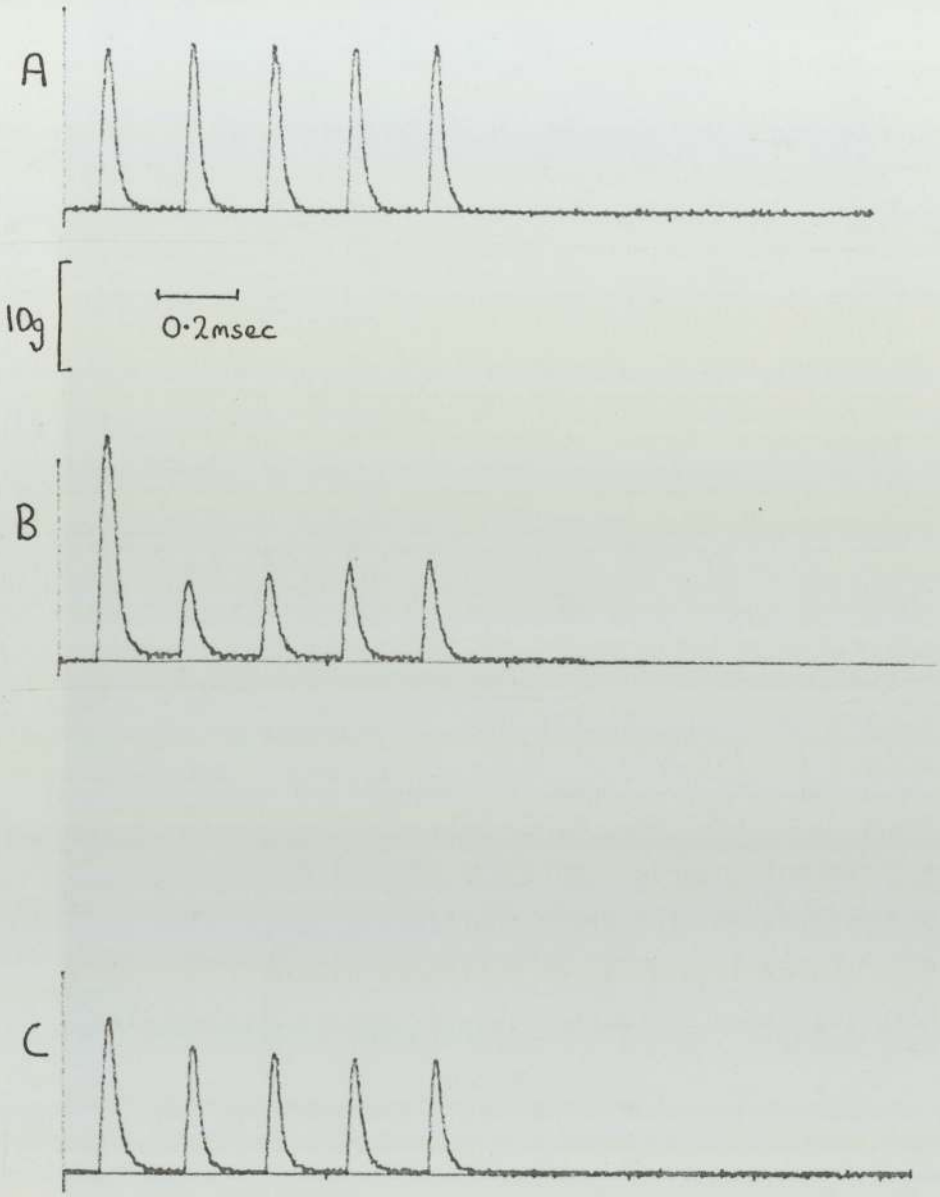

A
10g
0·2msec
B
C

Fig. 57.

Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C. Records of contractions in response to 5 stimuli 1 sec apart at 0.017Hz.

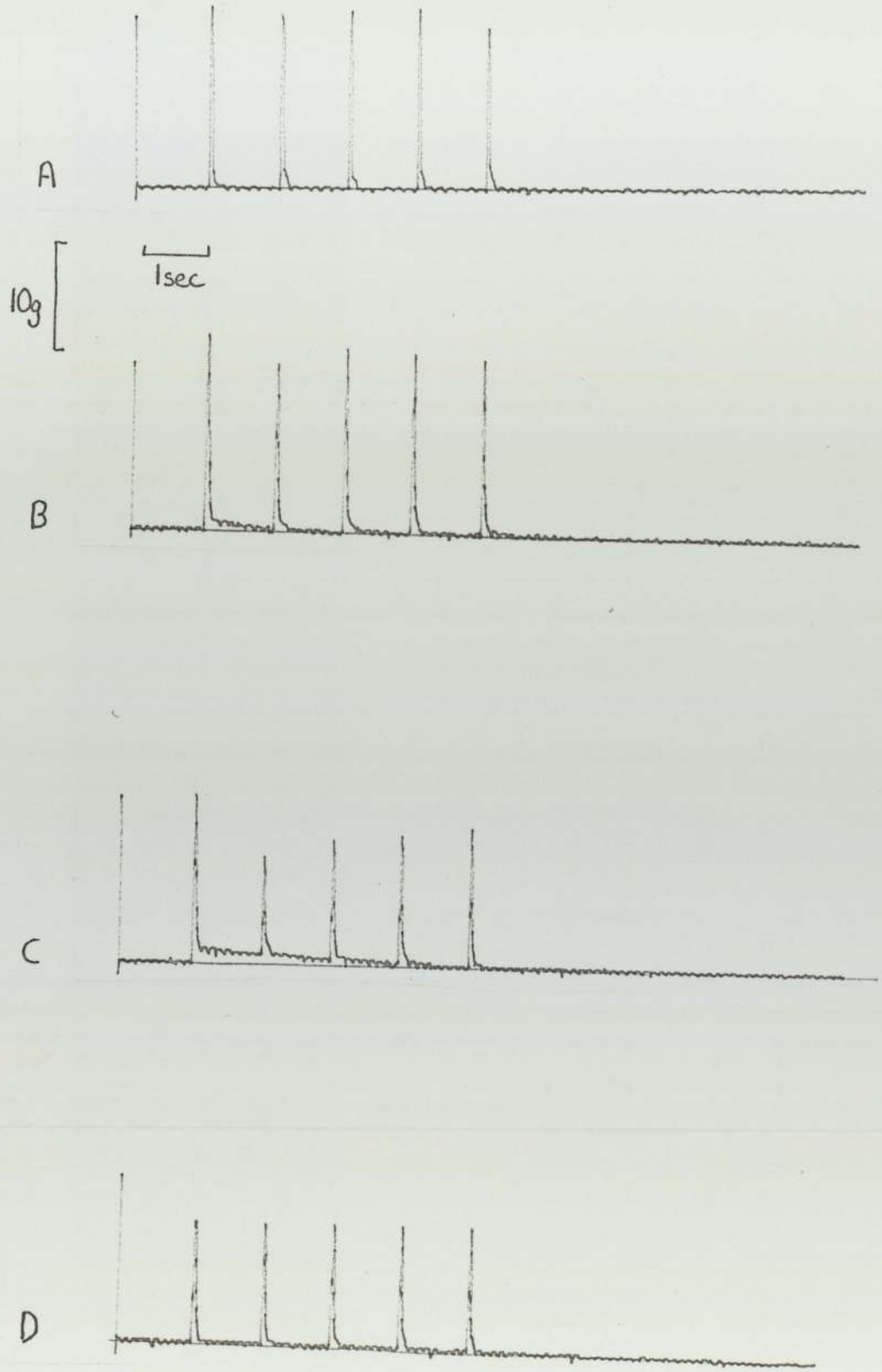
A. Control records. Note the similar responses

B. Records 14 mins after ecothiopate (5×10^{-7}M)

Note the plc development of the first response just beginning to effect the second response whose amplitude is reduced more than the last three responses.

C. Records 20 mins after ecothiopate. Note the maximum plc development of the first response. The greatest reduction in amplitude of the second response and the progressive recovery of amplitude of the last 3 responses as the plc disappears.

D. Records 35 mins after ecothiopate. Note the recovery of similar contractile responses.


A
1sec
10g
B
C
D

Discussion

It has been established that endplate action currents, generated as a consequence of maximal pulses to the nerve, are reduced in amplitude up to at least 20 msecs after initial single nerve stimulation. It has also been established that the amplitudes of the early parts of the contractile responses to trains of stimuli up to 4 secs apart are reduced in comparison to the first initiated response. The latter, in most experiments where stimuli were less than 1 sec apart, appeared to be the only response exhibiting plc development. In addition, the observed reduction in amplitude was more marked when the responses coincided with the time course of the plc of the first response. It was also observed that the first response, after developing a plc, appeared to prevent or reduce the subsequent responses from developing plcs. Finally, the waning of plcs of the first responses was accompanied by a recovery in the amplitudes of those responses which originally coincided with the time course of this plc. Experiments were not made to record endplate currents in response to stimuli greater than 20msecs apart.

This short-term block in electrical and contractile responses, described to persist for up to, at least, 4 secs, may be a consequence of the prolongation of transmitter action as a result of ChE inhibition. The neuromuscular block, which appears to be associated with plc development, may be due to (i) a pre-synaptic action of transmitter perhaps reducing release and/or (ii) a post-synaptic action of transmitter producing either partial depolarisation block or partial desensitisation.

Alternatively, a direct blocking effect by ecothiopate may explain some of the waning effects (see Section XV).

A depolarisation block at the endplate presumably inactivates the receptors whilst maintaining the membrane in a depolarised state. It has previously been established (pp. 145–) that ecothiopate (5×10^{-7}M) apparently leads to a persistent depolarisation of unstimulated muscles of 5 - 10mV. The significance of this persistent depolarisation in producing neuromuscular block is not known.

It might be expected that depolarisation block, in rendering the endplate membranes inexcitable, would prevent the propagation of action potentials, evoked by direct muscle stimulation, through the blocked endplate region (Burns and Paton, 1951). Perhaps, a partial depolarisation block might hinder the propagation of action potentials.

An experiment was made on a rat hemi - diaphragm where wire recording electrodes were placed in the endplate region and further along the same group of muscle fibres nearest the cut rib margin (Fig. 58). These were used to record the extracellular currents produced both at the endplate and further along the muscle as a result of both stimulation of the nerve (0.01Hz) followed, 60msecs later, by a further direct stimulus to the same group of muscle fibres (as above) near the tendon end of the hemi- diaphragm with a pulse sufficient only to excite this small group of muscle fibres. After control responses had been recorded, ecothiopate (5×10^{-7}M) was added to the physiological saline and recording was continued to see whether the prolongation of the epcs, due to prolonged transmitter action as a result of cholinesterase inhibition by ecothiopate, interfered with the conduction of the impulse through the endplate region as it travelled from its point of initiation to the site of recording along the length of the muscle.

The results (Fig. 58) appear to show that there is not any appreciable block in conduction of the impulse travelling from one

Fig. 58.

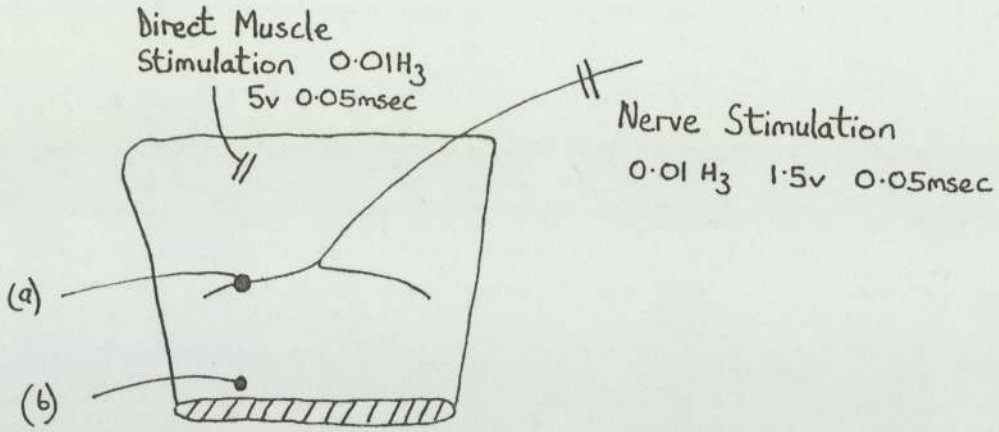
Rat hemi - diaphragm in saline at $37^{o}C$. Extracellularly recorded endplate currents at the endplate (a) further along the same group of muscle fibres (b) in response to nerve stimulation (0.01Hz) at 0 secs and direct muscle stimulation as shown at 60msecs.

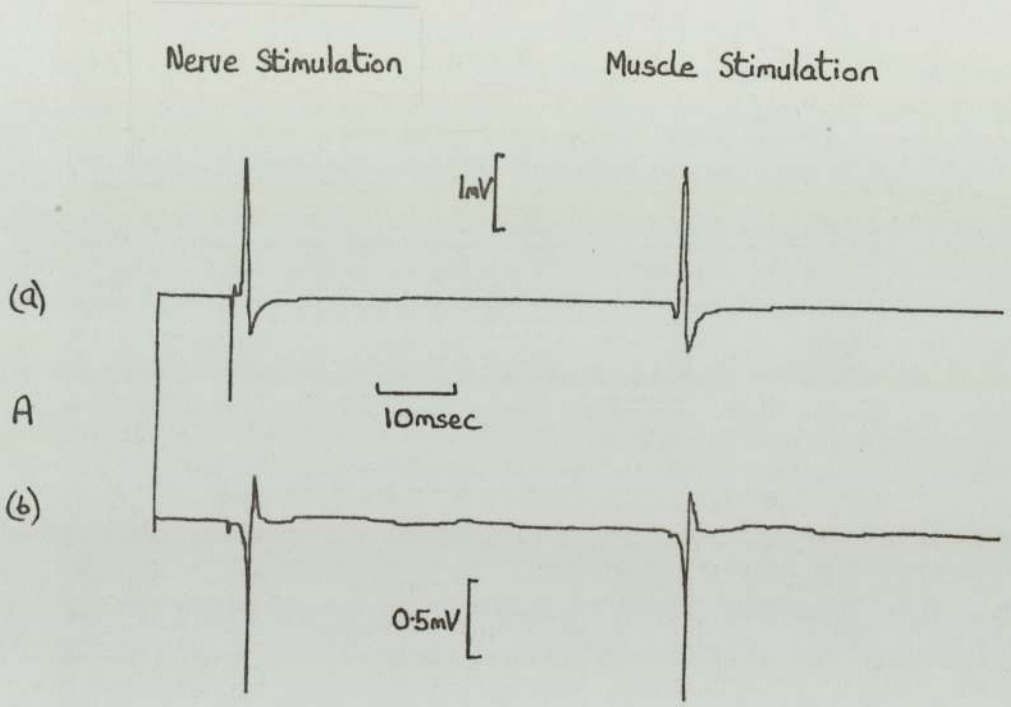
A. Control responses.

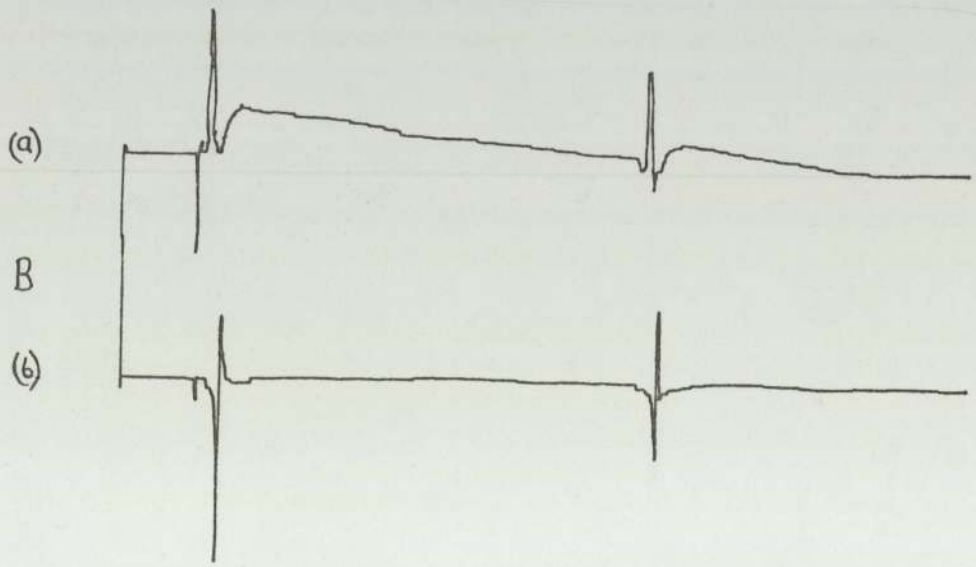
Note the negative - positive direction of the epcs to nerve stimulation and the positive - negative - positive direction of the epcs to muscle stimulation and to the currents recorded away from the endplate.

B. Responses 21 mins after ecothiopate ($5 \times 10^{-7}M$)

Note the prolongation of the currents as recorded at the endplate.


Direct Muscle
Stimulation 0·01H3
5v 0·05msec
Nerve Stimulation
0·01 H3 1·5v 0·05msec
(a)
(b)


Nerve Stimulation
Muscle Stimulation
1mV
(a)
A
10msec
(b)
0·5mV


(a)
B
(b)

end of the group of muscle fibres to the other 21 mins after ecothiopate application when the prolonged epcs are observed to be well established and persisting for longer than 60msecs. There does appear to be, however, a decrease in the amplitude of response at the endplate of the impulse initiated by direct muscle stimulation. It can be seen that this impulse also shows prolongation at the endplate. However, the currents recorded near the base of the muscle do not show any prolongation.

Although the results suggest that the propagation of action potentials might be hindered, the evidence is not sufficient to conclude that ecothiopate action produces partial depolarisation block. This assumes that desensitised receptors do not prevent the propagation of action potentials through the endplate regions.

It is possible that a partial block of pre-synaptic mechanisms may abolish antidromic firing. This would effectively lead to the waning of the SCs and reduce transmitter release. The latter, as has already been suggested (p. 135), may be sufficient to reduce prolongation of the endplate-localised contraction and, also, the enhancement of the early contraction.

Therefore, at the moment, the mechanism(s) responsible for the waning of the ecothiopate-mediated changes in contraction are not known. It may be speculated that, if the waning of the responses is caused by receptor desensitisation, then the mechanism of desensitisation might be associated with the endplate accumulation of calcium (see Final Discussion).

Effect of ACh on ecothiopate action. Is there any long-term reduction in transmitter action ?

It has previously been suggested that ecothiopate (5×10^{-7}M) action results in a short-term block (at least 4 secs) of neuromuscular transmission following stimulation (0.01Hz). However, in order to explain the waning of the ecothiopate - mediated changes in contraction, a longer-term block would be necessary. Neurally evoked transmitter action is thought to persist for, perhaps, 60 - 70 msecs only. Therefore, receptor inactivation arising from neurally evoked release is not thought to last for at least, the necessary 100 secs or so between stimuli. However, it would seem likely that the long - term reduction in transmitter action would arise from the persistent higher 'resting' concentration of transmitter, perhaps augmented by neurally evoked release.

Experiments were made on rat hemi - diaphragms maintained in vitro in physiological saline ($[Mg^{2+}]$ 10^{-4}M) where ACh (10^{-4}M) was added to the physiological saline both before and after ecothiopate (5×10^{-7}M). The records of contraction (Methods see p. 14) in response to nerve stimulation (0.01Hz) can be observed in Fig. 59. The results show

(i) When ACh is added before ecothiopate, (Fig. 59) a rapid but transient increase in contraction amplitude occurs. The addition of ecothiopate results in a reduction in the enhanced amplitude of the early contraction, a reduction in the development of plcs and approximately 25 mins after ecothiopate, the complete abolition of the contractile responses. The latter was, apparently, coincident with plc development. In addition, the removal of excess

ecothiopate only, resulted in the recovery of approximately 60% of the initial amplitude of the contractions.

(ii) When ACh is added after ecothiopate (75 mins) whether the drug has been washed out or not (Fig. 59) the complete abolition of the contractions occurs within approximately 3 mins. Removal of ecothiopate again results in the partial recovery of response (Fig. 59). This partial recovery of response even occurs when the ecothiopate was removed before the addition of ACh to the physiological saline.

Discussion

The reduction in the ecothiopate-mediated effects followed by the complete block of the contractile response is, perhaps, due to a desensitisation of receptors caused by prolonged and more intense transmitter (i.e. ACh) action. The initial application of ACh appeared not to have any long-term effects on the contractile response and that the 'critical' ACh responsible results from the inhibition of ChE. That the complete block of contractions took approximately 24 mins might give some indication as to the time course of ChE inhibition under the conditions of these experiments It is well known that ACh can protect ChE from irreversible inactivation by organophosphorus inhibitors (e.g. in rat diaphragm, Mittag, Ehrenpreis and Hehir, 1971). The time may also reflect a progressive desensitisation of receptors.

It has been suggested that the partial block, suggested as being responsible for the waning of the ecothiopate-mediated effects on the contractile response, is a result of the persistent transmitter action caused by the inhibition of cholinesterase. It is suggested that the persistent transmitter action resulting from

Fig. 59

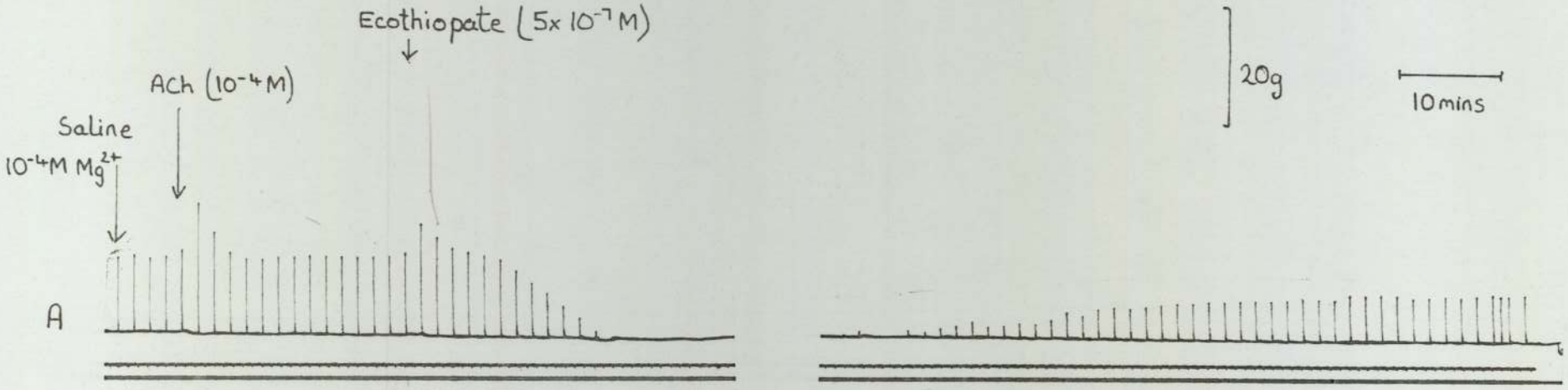
Rat hemi - diaphragm maintained in vitro in physiological saline ($[Mg^{2+}]10^{-4}M$) 37°C). Contraction records showing the effects of ACh ($10^{-4}M$) added before and after ecothiopate (10^{-7} x 5M).

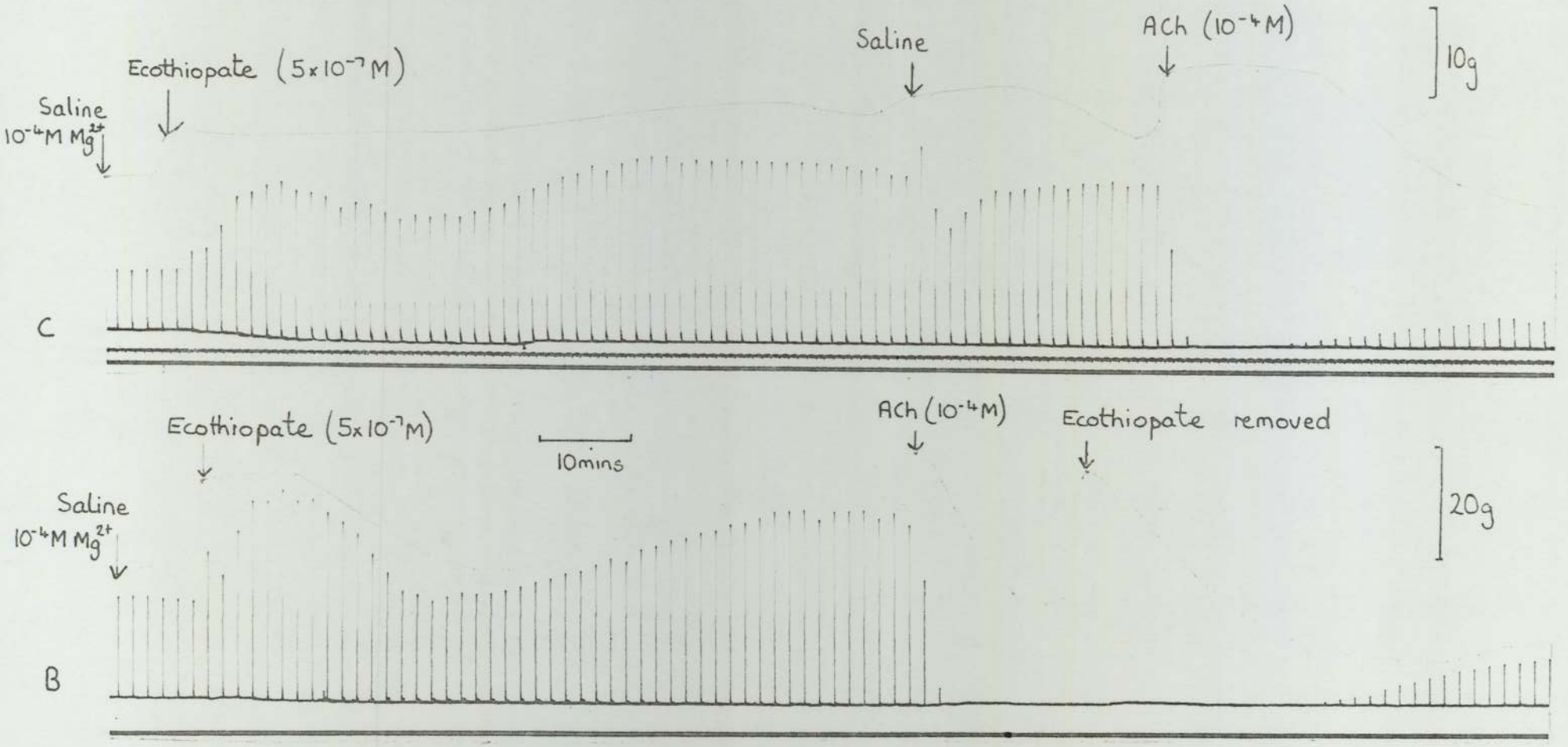
A. ACh added before. Note the transient increase in amplitude. Ecothiopate added as shown causes the complete abolishment of the response. The removal of ecothiopate allows a partial recovery of response.

B. ACh added after. Note the usual changes in the contraction record following ecothiopate addition.

The addition of ACh causes a very rapid and complete abolishment of the response. The removal of ecothiopate again allows a partial recovery of response.

C. Same as B but ecothiopate is removed from the saline before ACh addition.


Ecothiopate (5x 10⁻⁷M)
ACh (10⁻⁴M)
Saline
10⁻⁴M Mg²⁺
A
20g
10 mins


Ecothiopate (5×10^{-7} M)
Saline
ACh (10^{-4} M)
10g
Saline 10^{-4} M Mg^{2+}
C
Ecothiopate (5×10^{-7} M)
10mins
ACh (10^{-4} M)
Ecothiopate removed
20g
Saline 10^{-4} M Mg^{2+}
B

(i) spontaneous release is responsible for the long-term block, believed to result in the waning of the ecothiopate-mediated changes in contraction, and (ii) the relatively short-lasting evoked release is responsible for the short-term block previously described. It is probable that both types of release augment each other's effects.

The observation that a recovery of response occurred on removal of ecothiopate from the physiological saline would tend to suggest that the inhibition of ChE by ecothiopate is not as irreversible as is believed. Alternatively, ecothiopate has other actions on neuromuscular transmission which have been terminated by its removal, for example, a direct blocking action. However, the observation that recovery occurs even when ecothiopate is removed before ACh addition suggests that the muscles adapt in some other way to the complete neuromuscular block.

The observation that contractions were not blocked in muscles where ecothiopate was added after ACh (Fig.[59]) until plcs appeared confirms the previously suggested association that the mechanisms associated with plc development, in some way, block the early contraction and, perhaps, SCs.

Finally, since synaptic transmitter concentration following single nerve stimulation is thought to increase to approximately 10^{-5}M (Hubbard, 1974), and is only 10% of that already circulating in the tissue bath (10^{-4}M), the reduction in release by pre-synaptic transmitter action is not thought sufficient enough to be responsible for the waning of the ecothiopate-mediated changes in contraction in muscles treated with ecothiopate only. However, ACh in concentrations as low as 3.3×10^{-6}M in the presence of an anti ChE, have been found to reduce transmitter release (Hubbard, Schmidt and Yokota, 1965). The same authors, however, found no significant effect by the anti ChE (Neostigmine) itself (3.3×10^{-6}M).

Effect of Ecothiopate on brief tetanic contractions

Muscles do not normally work by responding to single nerve stimulation. Frequencies of nerve stimulation of the order of 30 - 40 Hz are more usual and so the response of the muscles is a tetanic one. Hence, it was thought useful to determine the effect of ecothiopate on muscles responding to tetanic stimulation.

However, in vitro rat hemi - diaphragms in physioiogical saline become quickly fatigued when stimulated at high frequencies (20 - 100 Hz) even for short periods of time with long recovery periods due to oxygen starvation or perhaps to a reduction in transmitter output.

Further experimentation enabled a compromise to be found in that a stable control record of contraction was found in response to brief tetanic trains of stimuli of 50Hz for 100msecs (5 pulses 20msecs apart) every 100 secs. Ecothiopate (5×10^{-7}M) was then added to the physiological saline.

The results (Fig. 60A - H) show that before the addition of ecothiopate, the response is an incomplete tetanus, the individual components resulting from each stimulus being observed. After 11 mins of ecothiopate exposure, the first component of the tetanus can be seen to increase in amplitude with respect to the four subsequent components and that the overall amplitude of contraction has decreased. As ecothiopate action continues, the overall amplitude of response continues to decrease while the first component of the response assumes the major component of the contraction, and eventually the tetanus becomes so truncated that the response fails to reach maximum summation. This truncated response takes on the appearance of a response to a single stimulus even to the extent of showing the development of a plc. The response to a single

Fig 60

Rat hemi - diaphragm maintained in vitro in saline at 37°C. Effect of ecothiopate (5×10^{-7}) on the contractile responses to nerve stimulation of 50Hz for 100msecs (i.e. 5 pulses 20msecs apart) at 0.017Hz.

A. Control record 1 stimulus.

B. Control record. Note the 5 components of the incomplete tetanic response.

C. Record 11 mins after ecothiopate.

D. Record 12 mins after ecothiopate.

E. Record 16 mins after ecothiopate.

F. Record 22 mins after ecothiopate.

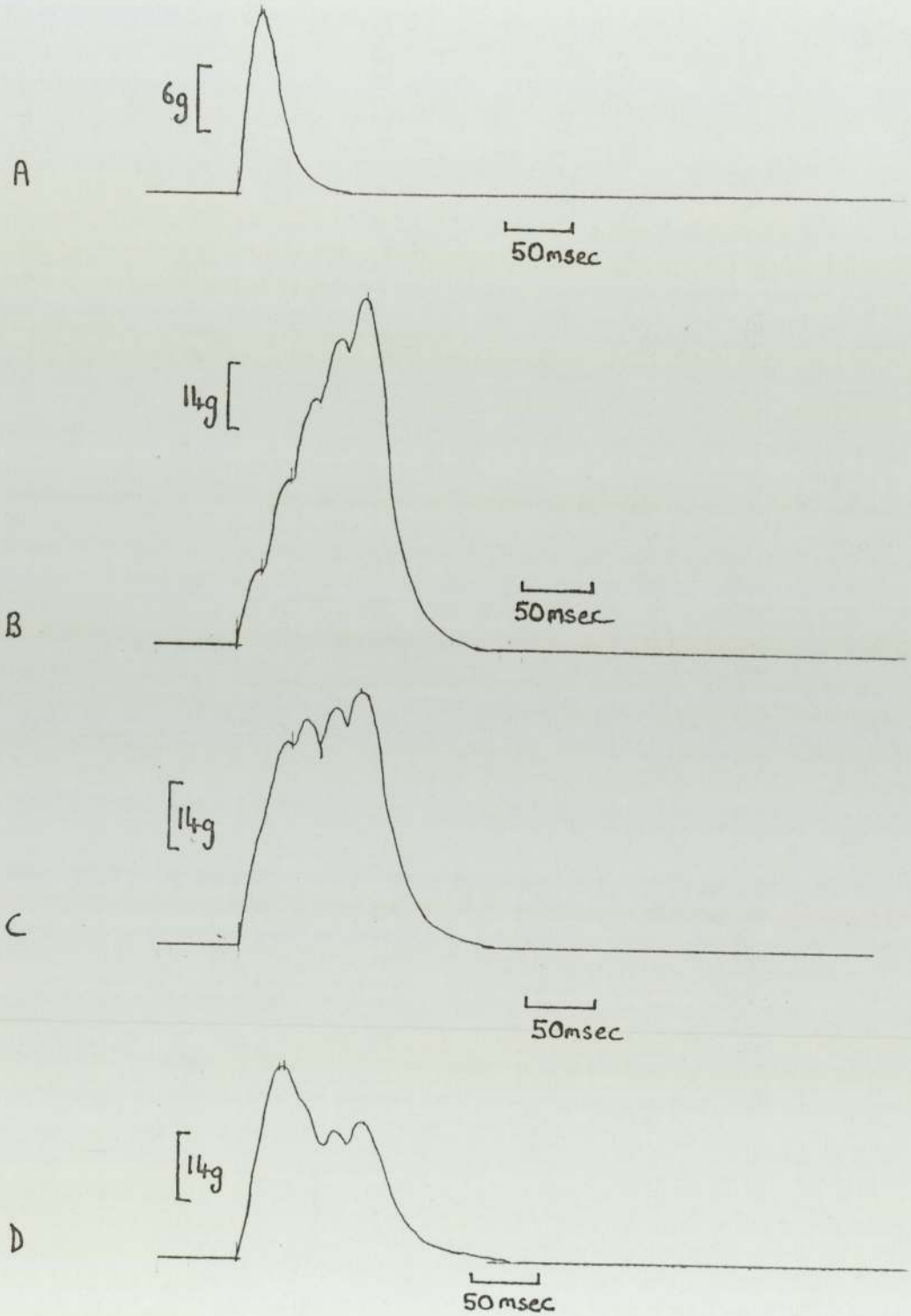
Note from B to E the progressive truncation of the incomplete tetanus, the first component gradually assuming the major part of the response.

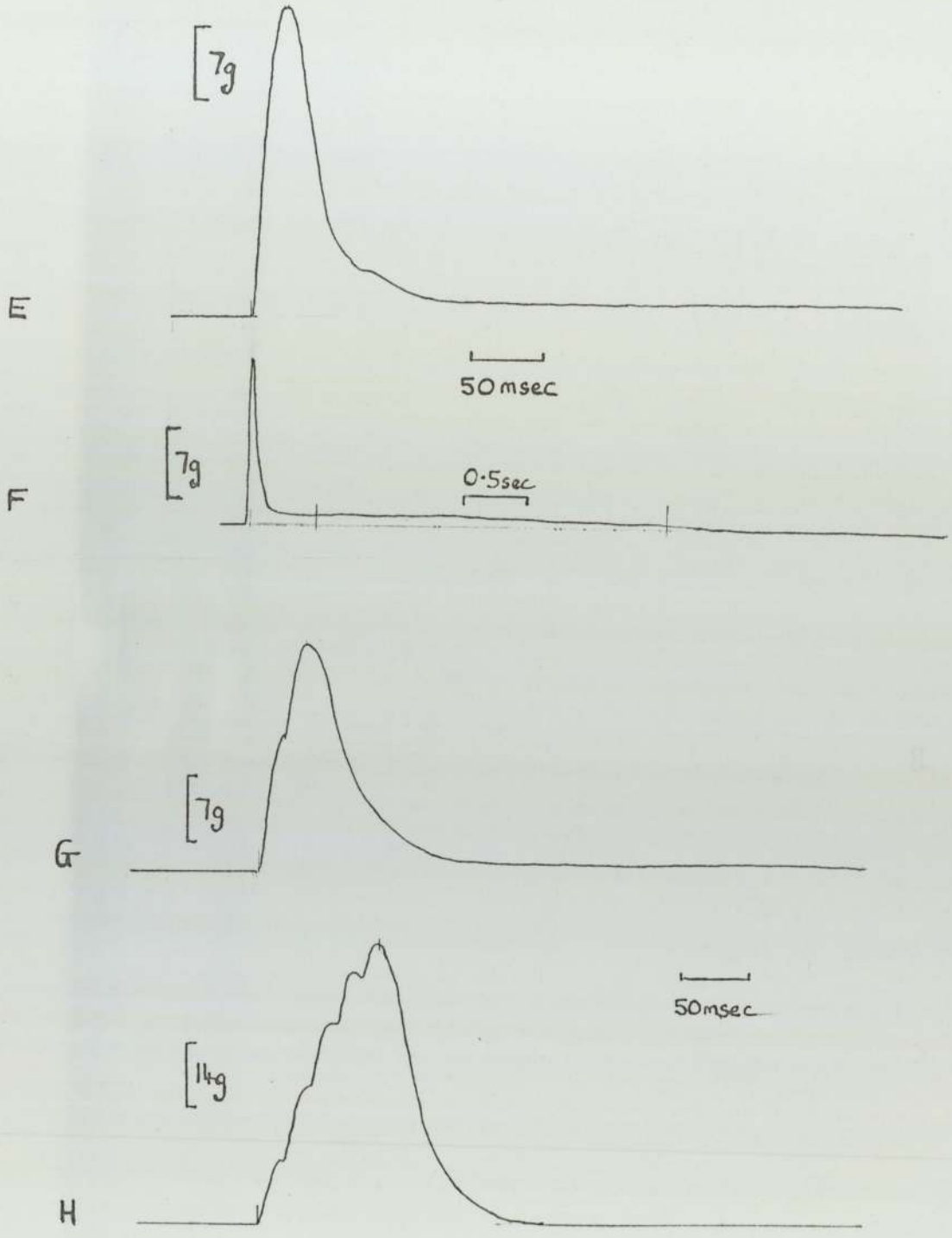
G. Record 38 mins after ecothiopate. Note the partial recovery of response.

H. Record 7 mins after the addition of 2 - PAM (1×10^{-6}M at 39 mins after ecothiopate)

Note the greater recovery of response.

The original traces have been touched up.


A
6g
50msec
B
14g
50msec
C
14g
50msec
D
14g
50 msec


E
7g
50 msec
F
7g
0·5sec
G
7g
50msec
H
14g

stimulus at this time develops a plc apparently similar in time course to previous single responses (see Fig. 6OF).

After a small degree of recovery of the tetanic response (Fig. 6OG), the addition of 2 - PAM (1×10^{-6}M) to the saline restores the incomplete tetanus within 7 mins (Fig. 6OH).

Discussion

The effect of ecothiopate on tetanic contractions is of additional interest since a tetanic contraction supposedly represents the maximum shortening of the particular muscle. This was confirmed to be the case in those experiments where maximum tetanic tension had developed since ecothiopate did not produce any further increase in tension.

However, apparently as ChE inhibition proceeds, the intensification and prolongation of transmitter action that apparently results after the first stimulus is sufficient to block the subsequent responses. The loss of the ability of the antiChE poisoned muscle to sustain tetanic contraction is believed to be caused by rapid desensitisation of endplate membrane due to the large amounts of ACh present after the arrival of the first nerve volley (Thesleff and Quastel, 1965).

The rapid recovery of the incomplete tetanus in the presence of the cholinesterase re-activator, 2 - PAM, confirms that the action of ecothiopate (5×10^{-7}M) is primarily that of inhibition of ChE. This evidence contributes further to the belief that the blocking effect of ecothiopate is more likely the result of desensitised receptors due to prolonged transmitter action.

Summary

Evidence has been presented which suggests that the prolongation of transmitter action after ecothiopate results in a loss of both electrical and contractile response. It has been suggested that this loss of response may be due to the desensitisation of receptors.

SECTION XIII

Does Ecothiopate Have A Direct Blocking Action ?

Introduction

It has previously been shown that when neuromuscular transmission has been impaired (Section VIII), ecothiopate (5×10^{-7}M) does not appear to have a direct action on muscle fibres. Consequently, it was thought that its action was indirect via ChE inhibition at this particular concentration of drug. The resulting prolongation of transmitter action was then thought to explain the onset of the ecothiopate - mediated changes in the contractile response. However, that these changes are not maintained has been suggested to be due to a reduction in transmitter action, perhaps, by desensitisation of receptors. Alternatively, that the waning of the responses might be due to a direct blocking effect by ecothiopate, has been suggested on several occasions in this thesis.

In order to investigate the latter possibility further, the results of previous experiments are reviewed and further experiments are made varying [ecothiopate] and using 2 - PAM. Ecothiopate (10^{-3}M) has been found to reversibly inhibit AChRs (Bartels and Nachmansohn, 1969).

Results

In rat hemi - diaphragms maintained in vitro in physiological saline and treated with ecothiopate (5×10^{-7}M), the removal of ecothiopate from the physiological saline 16 mins after its addition (Section IX, pp.206-7) results in further enhancement of the amplitude of the early contraction.

Discussion

Biochemical experiments, made to determine ChE activity in the presence of ecothiopate, suggest that the ChE is substantially inhibited by this time (i.e. 16 mins). If this is the case, the results of these experiments would suggest that ecothiopate (5×10^{-7}M) directly interferes with (i.e. partially blocks) neuromuscular transmission. It is not known whether such action might be pre - junctional or post - junctional or both. However, Morrison (1977) suggested a direct pre - junctional action of ecothiopate (5×10^{-7}M) in blocking nerve repetitive firing. It, also, may not necessarily be that ecothiopate not bound to ChE enzyme which is responsible for the partial block. Perhaps it is a consequence of its binding with ChE which subsequently leads to block.

It is suggested that this further enhancement of the early contraction is not due to a shortening of the prolonged transmitter action resulting from a recovery of ChE activity, since such inhibition is thought to be irreversible. Hence, washing ecothiopate from the preparation should not interfere with the degree of ChE inhibition. Alternatively, if the further enhancement reflects a recovery of ChE activity, thus shortening

transmitter action, then the binding of ecothiopate to ChE is not as irreversible as currently believed. If this was the case, the washing procedure must result in the removal of some of the 'bound' ecothiopate, resulting in a partial recovery of ChE activity and, subsequently, a partial recovery of response.

Effects of 2 - PAM

Experiments have been made on rat hemi - diaphragm to record the contractile response to nerve stimulation (0.017Hz) in the presence of ecothiopate (5×10^{-7}M) (see Methods pp. 14-18). Following the maximum prolongation of the endplate - localised contractions, at approximately 16 - 18 mins in these experiments, the ChE enzyme was reactivated by 2 - PAM (2×10^{-5}M). The record of contraction of one experiment is shown in Fig. 61 . Associated records of extracellular endplate currents are shown and discussed in Section III.

Within 5 mins plcs have waned completely. This compares with the usual 15 - 20 mins observed (Table 1, p. 54) in the absence of 2 - PAM. Within approximately 10 mins the amplitudes of the early contraction have returned to approximately control values.

Discussion

Assuming 2 - PAM acts only to re-activate ChE, the apparent reversal of the ecothiopate - mediated changes in contraction (except for the SCs) during the continued presence of ecothiopate

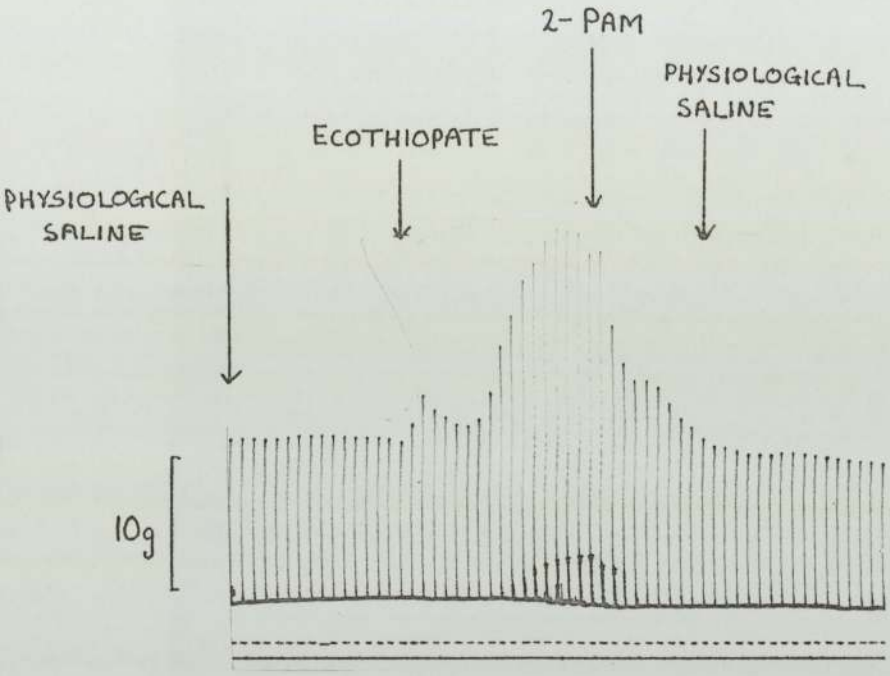


Fig. 61 Rat hemi - diaphragm maintained in vitro in physiological saline at 37°C.
Record of contraction in response to nerve stimulation (0.017Hz).
Note (i) the increase in contraction amplitude and the development of SCs and plcs as a result of ecothiopate (5×10^{-7}M) action and

(ii) the reversal of response as a result of ChE re-activation by 2 - PAM (2×10^{-5}M)

suggest the action of ecothiopate (5×10^{-7}M) in changing the contractile response results only from its antiChE action leading to prolongation of transmitter action.

Effect of ecothiopate (2×10^{-6}M) on the contractile response

An experiment was made on an isolated rat hemi - diaphragm preparation to determine the contractile response to indirect stimulation (0.01Hz) following addition of ecothiopate (2×10^{-6}M). The contraction record obtained (Fig. 62) was compared with those records obtained using ecothiopate (5×10^{-7}M) (Table 32).

It is apparent that the higher [ecothiopate] has a more rapid onset of action as indicated by the enhancement of the early part and the prolongation of the late part of the contractions. In addition, the waning of the enhanced early contraction and plcs appears to follow a more rapid time course. For example, the plcs in this one experiment have waned completely by approximately 25 mins compared to a mean of 35 mins (10 expts.) with ecothiopate (5×10^{-7}M). Finally, after the enhanced early contraction has waned almost back to control amplitudes, there appears to be a comparatively slower and progressive further enhancement of the early contraction. This further enhancement apparently coincides with the waning of the plcs.

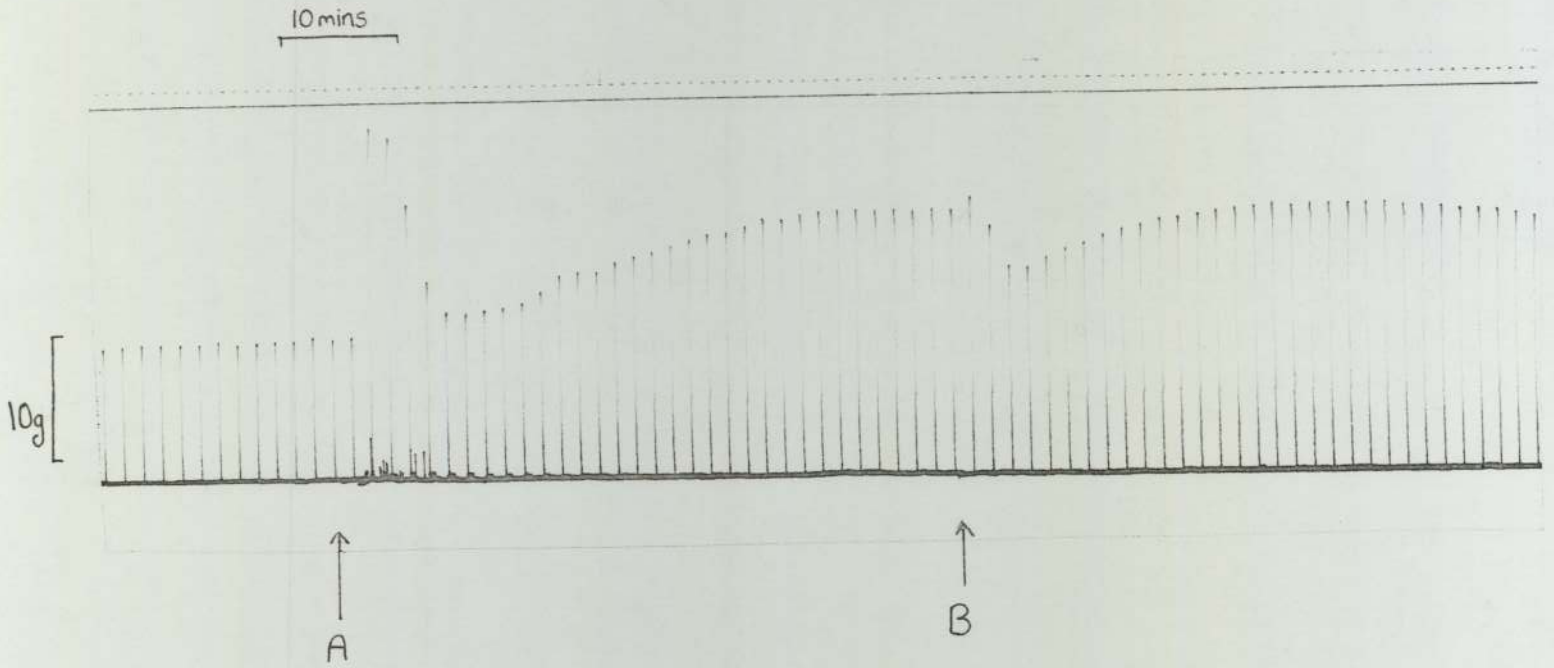


Fig. 62.

Rat hemi - diaphragm. Physiological saline. 37°C. Record of contraction to nerve stimulation (0.01Hz). Ecothiopate (10^{-6}M) added to physiological saline at A and removed at B.

Table 32 The time courses of the changes in the contractile response due to

(i) [ecothiopate] (5×10^{-7}M)

and (ii) [ecothiopate] (2×10^{-6}M)

Drugs added at 0 mins.

Physiological saline ([Mg^{2+}] 10^{-3}M). 37°C.

Frequency of nerve stimulation 0.01 or 0.017Hz.

The date for (i) is the same as that exhibited in Table 1.

		Latency of Twitch Enhancement (mins)	Latency of maximum amplitude of contraction (mins)	% Increase in amplitude of contraction (mins)	Late Prolongation of Contractions		Latency of maximum development of late prolonged contractions (mins)
					START (mins)	FINISH (mins)	
(i)	Mean	5.6	13	76	9.2	35	16.2
	S.D.	2.2	2.9	38.8	1.7	8.6	2.1
(10 expts.)	Range	1 - 7	9 - 20	30 - 136	7 - 12	28 - 55	13 - 19
(ii) (1 expt.)		2 - 3	3 - 4	151	3	25	8

Discussion

The more rapid onset of action can be, presumably, explained by the more rapid inhibiton of ChE resulting in a more rapid onset of prolonged transmitter action. This might imply that the waning of the enhanced early contraction and the plcs were due to a reduction in transmitter action resulting from receptor desensitisation. However, the alternative hypothesis that the waning is due to a direct effect of ecothiopate cannot be discounted since the latter is now present in a higher concentration.

The observation that the early contraction is further enhanced in the continued presence of ecothiopate (2×10^{-6}M) suggests that transmitter action is also further increased. It is difficult to speculate other than the latter being due to some recovery in either ChE activity and/or receptor activity. It is difficult to suggest why either should arise in the continued presence of ecothiopate. The presence of ecothiopate during the apparent reversal of this block in contraction would imply that ecothiopate (2×10^{-6}M) is not directly blocking receptors.

Effect of [Ecothiopate] on calcium accumulation

These experiments were made for two reasons:

1) If the proposed direct blocking action of ecothiopate (5×10^{-7}M) was competitive, thus preventing ion channels from opening), then an increase in [ecothiopate] above 5×10^{-7}M might have been expected to reduce endplate calcium accumulation because of an increase in block. Similarly, a reduction in concentration might have been expected to increase endplate calcium accumulation because of a decreased block.

2) If endplate calcium accumulation was a direct effect of ecothiopate, then it might have been expected that such accumulation was concentration - dependent.

Experiments were made on unstimulated mouse hemi - diaphragms incubated in physiological saline containing $^{45}CaCl_2$ and ecothiopate (2.5, 5 and 10 x 10^{-7}M). For details of Methods (see pp.34-). The results (Tables 33 and 34 , Fig. 63) show that the endplate accumulation of calcium in muscles incubated with ecothiopate (2.5 x 10^{-7}M) was not significantly different to that with ecothiopate (5 x 10^{-7}M). However, muscles incubated in ecothiopate (10 x 10^{-7}M) showed an increased accumulation of calcium at the endplates compared to ecothiopate (5 x 10^{-7}M). This increase was significant. The differences in accumulation of non - junctional calcium between the three concentrations of ecothiopate were insignificant.

Discussion

Increasing [ecothiopate] from 2.5 to 10 x 10^{-7}M did not have the effects on endplate calcium accumulation proposed if ecothiopate (5 x 10^{-7}) competitively blocked post - junctional receptors.

Therefore, it is concluded that, on the basis of these results, ecothiopate (5 x 10^{-7}) does not have an observable blocking action.

The difference between endplate calcium accumulation with ecothiopate concentrations of 2.5 and 5 x 10^{-7}M is only just statistically insignificant. The results, therefore, would imply a possible concentration - dependent accumulation of calcium. Ecothiopate might, therefore, be acting to directly increase post - junctional

Table 33 Calcium accumulation in unstimulated mouse muscles (mean ± S.E. of mean, number of hemi - diaphragms in parentheses). Diaphragms were incubated in ^{45}Ca - physiological saline at 37°C in the presence of ecothiopate (2.5, 5 and 10 x 10^{-7}M) for 30 mins followed by washout for 30 mins in tracer - free physiological saline before immersion in acetone. (The letters Aq to Fq are for future reference).

Concentration of Ecothiopate	n-moles of calcium accumulated mg^{-1} total dry muscle	
	endplates	non-junctional pieces
2.5 x 10^{-7}M	Aq 0.283 ± 0.059(5)	Bq 0.528 ± 0.05(5)
5 x 10^{-7}M	Cq 0.531 ± 0.077(15)	Dq 0.419 ± 0.076(15)
10 x 10^{-7}M	Eq 0.827 ± 0.043(7)	Fq 0.493 ± 0.016(7)

Table 34 Independent t values and probabilities between the unpaired sets of data in Table 33

Unpaired sets of data	t value	degrees of freedom	Probability	Significant/ Insignificant
Aq v Cq	1.8429	18	0.0788	Insignificant
Cq v Eq	2.6014	20	0.0163	Significant
Bq v Dq	0.8241	18	0.5743	Insignificant
Dq v Fq	0.6737	20	0.5147	Insignificant
Bq v Fq	0.8584	10	0.5850	Insignificant

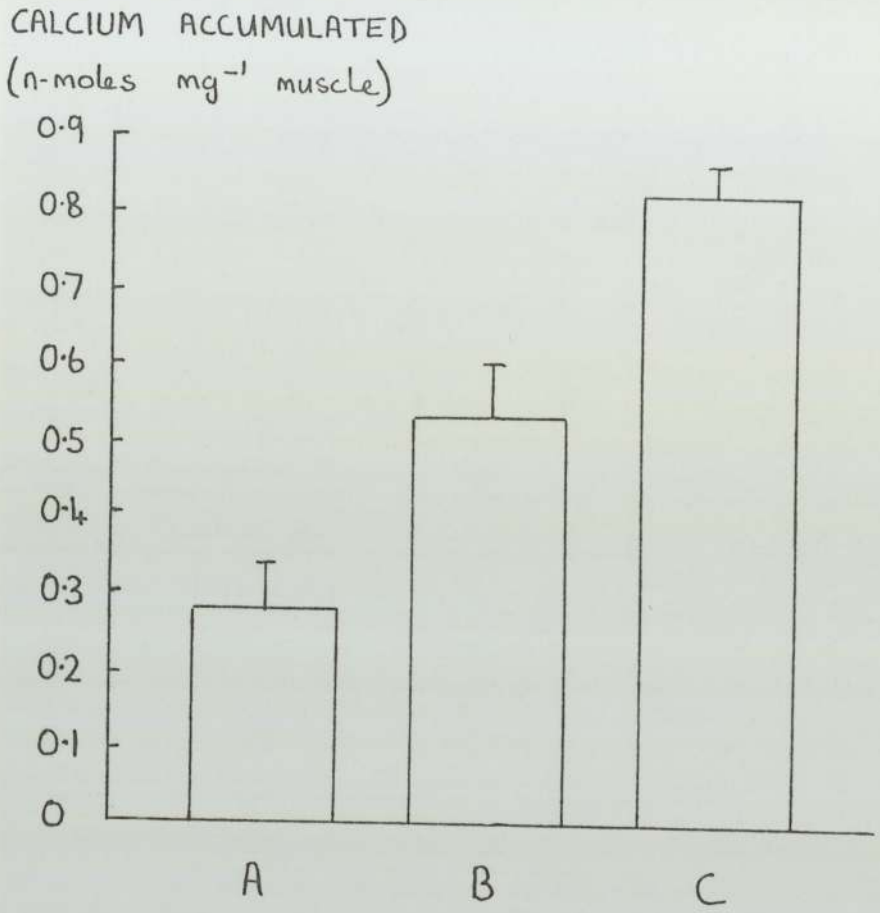


Fig. 63.

Endplate calcium accumulation in mouse diaphragm after 30 mins with ecothiopate at concentrations of:

A. 2.5×10^{-7}M

B. 5.0×10^{-7}M

C. 10×10^{-7}M

calcium permeability. If this is true, then any direct blocking action of ecothiopate (5×10^{-7}M) cannot be a competitive one. Alternatively, varying the concentration of ecothiopate of the order of 75×10^{-8}M alters the degree and rate of ChE inhibition thus changing the time course of the prolongation of transmitter action. Presumably, the higher the concentration of ecothiopate the more rapid the onset of prolonged transmitter action and, consequently, the greater accumulation of calcium.

General Discussion

The evidence presented in this section suggests that ecothiopate (10^{-7} to 10^{-6}M) might have a blocking action on neuromuscular transmission. However, these experiments cannot indicate whether this block is a direct effect or whether it is a consequence of prolonged transmitter action. It may be speculated that any direct blocking component might involve a 'freezing' of ion channels in their open states since endplate calcium accumulation appears to be enhanced rather than impeded with increasing [ecothiopate] (see Final Discussion).

FINAL DISCUSSION

Ecothiopate (5×10^{-7}M) has been shown to evoke a complex and, apparently, time - dependent series of changes in the contraction of rat and mouse hemi - diaphragm in response to low frequency (e.g. 0.01Hz) stimulation of the phrenic nerve. These changes were (i) a 50% increase in the amplitude of the early part and (ii) a prolongation of the late part of the contraction together with (iii) the development of a variable number of spontaneous contractions. These changes were suggested to be due to the antiChE action of the drug , resulting in a prolongation and intensification of transmitter action. It was, however, possible that a direct action of ecothiopate might be partly responsible (see later).

The enhancement of amplitude of contraction was believed to result from the repetitive muscle action potentials developed as a result of the increased transmitter action. An enhancement of contraction and the development of spontaneous muscle contractions after organophosphorus ChE inhibitors has also been observed in the isolated rat hemi - diaphragm by Burgen et al., (1949), Barnes and Duff (1953) and Van der Meer and Meeter (1956a) and many others. Van der Meer and Meeter (1956a) observed that the period of enhanced contractions coincided with the period of repetitive muscle firing. They also observed, however, that the contractions return to approximately control amplitudes following enhancement and that this coincided with the abolition of repetitive muscle firing. Barnes and Duff (1953) and Burgen et al. (1949) similarly observed that the enhancement of the contractions was not maintained with amplitude quickly diminishing to approximately

control size. In the majority of my experiments the diminishing contractions following enhancement rarely regained control heights (in physiological saline containing [Mg^{2+}] 10^{-3}M). This observation suggests that some repetitive muscle firing must be maintained throughout the experimental period in the presence of ecothiopate (5×10^{-7}M). That repetitive activity occurs in some muscle cells one hour after ecothiopate (5×10^{-7}M) has been observed by Ferry (unpublished).

A difference between my experiments and those made by Burgen et al., Barnes and Duff and Van der Meer and Meeter may be associated with the higher frequencies of stimulation used by the latter (i.e. 0.1 to 0.5Hz). My experiments have shown that after ecothiopate a single stimulus to the nerve results in effects at the neuromuscular junction lasting for about 4 sec. in the case of plcs, at least 2 sec. in the case of endplate currents and at least 10 secs for the inhibitory effect of nerve stimulation on the development of spontaneous contractile activity. Clearly frequencies of stimulation of 0.1 to 0.5Hz will involve a build-up of whatever causes the above effects and do not allow time for recovery between stimuli. My experiments in which the frequency of stimulation was usually 0.017 or 0.01Hz was thought to allow sufficient time for recovery from the causes of the above effects between stimuli. Indeed, 2 stimuli at a frequency of 0.25Hz, in the presence of ecothiopate, was found to depress the response made after the second stimulus.

It is thought likely that at frequencies of 0.1 to 0.5Hz the subsequent prolonged action of the transmitter liberated per stimulus progressively leads to the failure of more motor units or of ecothiopate - mediated facilitation of transmitter action

until some sort of equilibrium is reached at which time the amplitudes of contraction has returned to control size.

The spontaneous contractions have been suggested to be generated pre-synaptically by an axon reflex excitation of motor units resulting from prolonged transmitter action at the motor nerve terminals. Similar speculation was made by Masland and Wigton (1940), Eccles, Katz and Kuffler (1942) and Van der Meer and Meeter (1956a). Krnjević and Miledi (1958a) suggested that the majority of individual fibres of motor units of rat diaphragm are found within 1 cm of each other irrespective of depth. In my experiments, the spontaneous contractions appeared to represent a synchronised contraction of a number of fibres in close proximity rather than from a number of fibres scattered throughout the muscle.

The prolonged endplate-localised contractions (i.e. plcs), however, were phenomena which do not appear to have been previously recorded since I could find no mention of them in the literature. The obvious interpretations of this would be that plcs are specific to ecothiopate (10^{-7} - 10^{-6}M) and/or to low frequency stimulation (0.1 to 0.01Hz) and/or to hemi-diaphragm preparations. Plcs can be obtained, however, with both ambenonium and neostigmine ($4-5 \times 10^{-7}$M). In 1921, Riesser observed what he referred to as "after-contractures" which he believed to be characteristic of 'red' (or tonic or slow) striated muscles treated with the antiChE, physostigmine. This was because he could not find similar phenomena in the 'white' (or tetanic or fast) striated extensor communis muscle of the rabbit.

Gunther (1952) found red and white fibres in approximately equal numbers in the rat diaphragm. Gauthier and Padykula (1966) distinguished 3 types of fibre in rat diaphragm. Small red fibres (60%),

large white fibres (20%) and fibres with intermediate characteristics (20%). Werner and Kuperman (1963) speculated that any functional differences in the types of fibres may be connected to differences in synaptic morphology. The preterminal motor fibres in 'white' muscles apparently being more heavily myelinated than those in 'red' muscles. Bowmann and Webb (1972) speculated that any functional difference was connected with the different arrangements of the sarcoplasmic reticulum (Gauthier and Padykula, 1966; Hess, 1970).

'White' muscle fibres are believed to contract in response to propagating action potentials and red muscle fibres in response to prolonged membrane depolarisation, presumably as a result of repetitive stimulation (Bianchi, 1968). It is suggested that the contribution made by the 'white' and, possibly, the 'intermediate' muscle fibres to the contractile response of rat hemi - diaphragm to single maximal stimulation of the nerve might be greater than that suggested by the percentage of these fibres in the diaphragm. Thus the response is a twitch. It is assumed that the 'red' fibres do not respond appreciably to single action potentials. However, in the presence of an antiChE (e.g. ecothiopate), prolonged transmitter action results in both repetitive muscle action potentials (about 200 - 500Hz) and prolonged endplate depolarisation. It is speculated that following single indirect stimulation, the initial action potential and subsequent repetitive action potentials activate the white and, possibly, the intermediate muscle fibres, producing the observed enhanced early contraction which is accompanied by a relatively slow developing contracture mainly of the 'red' fibres caused by the prolonged depolarisation. That this slow contracture is only maintained in the endplate region is because these red

muscle fibres are only focally innervated.

If this were to be the case, it is difficult to explain what physiological importance the red muscle fibres have in diaphragm tissue. They might be a specialisation related to the relatively high frequency of contraction of diaphragms (e.g. in the rat, the frequency is about 100 per minute). It is interesting to note that as mammals increase in size, their breathing rates decrease and the proportion of 'red' muscle also decreases (Gauthier and Padykula, 1966).

Evidence has been presented which indicates that the prolonged late part of the contractile response (i.e. plc) is localised to approximately 1mm either side of the endplate region. It was subsequently suggested that the progressive prolongation of transmitter action, believed to be due to a progressive inhibition of ChE by ecothiopate, might involve the penetration of ACh to receptors not normally reached. Miledi (1960b) has shown that the acetylcholine receptor sites are not confined to the actual post-synaptic membrane of the endplate, but extend in progressively diminishing density for at least 0.3mm away from the endplates on muscle fibres of the rat diaphragm, where the actual endplate is less than 0.03mm in diameter (Cole, 1957). Furthermore, these sparsely distributed receptor sites were found to contribute to the depolarisation produced by relatively large injections of ACh at sites remote from the endplate. It is speculated that the localisation of the prolonged late contraction to within 1mm of the endplates might correspond to the spread of functional receptors (i.e. those receptors causing depolarisation) as observed by Miledi (1960b). It is envisaged that as the inhibition of ChE by ecothiopate progresses, the accumulating and unhydrolysed ACh

diffuses further away from the immediate endplate membrane and is able to activate those receptors it, presumably, does not usually reach during normal neuromuscular transmission.

It has been shown that the progressive prolongation of the endplate - localised contraction after ecothiopate is accompanied by a prolongation of endplate currents following single indirect stimulation at low frequency. Results similar to those of Feng (1940; 1941) and Eccles et al. (1942) who showed that physostigmine increased and prolonged extracellular endplate currents have been obtained with neostigmine and DFP in frog sartorius muscle (Eccles and Macfarlane, 1949), with neostigmine, edrophonium and ambenonium in cat tenuissimus muscle (Boyd and Martin, 1956b; Blaber and Christ, 1967) and with DFP in the isolated phrenic nerve - diaphragm preparation of the rat (Meeter, 1958).

Sandow et al., (1965) and Taylor et al., (1972) have suggested that the duration of the action potential and the threshold depolarisation for mechanical activity determine the subsequent duration and intensity of the mechanical activity. The plcs have been assumed to be due to a prolonged action of Ca^{2+} in the sarcoplasm of the endplates. Initiation of Ca^{2+} action is believed to be by depolarisation of the post - junctional membrane beyond a threshold. This threshold in frog skeletal muscle fibres has been found to be -50mV (Hodgkin and Horowicz, 1960). It was thought possible that the Ca^{2+} action in the endplate regions was prolonged by a mechanically effective and non - propagating endplate depolarisation. It is not known whether the prolonged endplate depolarisation recorded was mechanically effective at the diaphragm endplate. Records of prolonged

endplate accumulation in single cells after ecothiopate indicate that the peak depolarisation is to approximately -50mV which may be maintained for at least 10 - 50 msecs (Ferry, personal communication).

I have suggested that the minimum prolongation of endplate depolarisation (i.e. that observed with AC recording) was sufficient to cause the maximum prolongation of the endplate - localised contraction of approximately 4 - 5 secs according to Sandow's guidelines. Furthermore, it was tentatively concluded that the prolonged endplate depolarisation, as observed with DC recording, might last as long as the plcs.

Hodgkin and Horowicz (1960) have reported that, with sustained depolarisations produced by high K^+, the duration of contraction, and presumably of calcium activation is at least 1 - 2 secs with depolarisations to about OmV and 5 secs or more with smaller depolarisations. However, this work was with frog skeletal muscle and it is not known how applicable this might be to rat diaphragm. Furthermore, this work presumably involved the depolarisation of whole muscle fibres as opposed to the endplate regions in my experiments. Nevertheless, it remains feasible that the prolonged endplate depolarisation may cause the prolongation of the endplate - localised contractions after ecothiopate.

The next stage of the problem was to determine how the prolonged endplate depolarisation prolonged Ca^{2+} action in the endplate sarcoplasm. Evidence has been presented which suggests that ecothiopate action causes the accumulation of calcium at the endplates. Many authors (see Section VI) have interpreted observations of endplate calcium accumulation as indicating a calcium entry to ACh action. It was, therefore, thought that the endplate calcium accumulation in my experiments reflected a post - junctional Ca^{2+} entry resulting

from prolonged ACh action caused by the inhibition of ChE by ecothiopate. One can speculate that plcs were the result of a post-junctional entry of Ca^{2+} during prolonged endplate depolarisation. This Ca^{2+} perhaps prolonged the shortening of sarcomeres by a direct effect on troponin in the endplate region or perhaps was responsible for prolonging the release of Ca^{2+} from the SR or perhaps was responsible for delaying the return of Ca^{2+} to the local SR.

My results did not permit the calculation of the amount of Ca^{2+} which might have passed through the endplate membrane following single indirect stimulation because of the presence of spontaneous excitation of the nerve terminals but they do represent the amount of calcium that had accumulated from all causes at the endplate in a certain time. It was found that approximately 0.15 n-moles of calcium accumulated at the endplates per mg. of total dry muscle after 15 minutes incubation with ecothiopate, i.e. 1.5×10^{-7} moles of Ca g^{-1} dry weight. Evans (1974) calculated that the ratio of wet weight of tissue / dry weight for muscles dried in the same way as in my experiments to be 4. Therefore the amount of calcium at the endplates per gram of wet muscle is approximately 0.4×10^{-7} moles. Assuming that the volume of 1 g of the muscle fibres approximates to 1 cm^3 (i.e. density = 1), it can be calculated that the concentration of calcium at the endplates at a time coinciding approximately with the time at which maximum prolongation of the late contraction occurs (i.e. 16 mins) is 0.4×10^{-4} M. If only 10% of this was available in the free state following stimulation of the preparation, then it is thought that there would be sufficient Ca^{2+} to cause a contraction localised to the endplate region of the preparation.

However, free Ca^{2+} were not thought to accumulate in the endplate sarcoplasm in sufficient quantity to cause an increase in resting muscle tension, for changes in muscle tension were rarely observed

and even when they were the changes were not always increases. More significant was the failure of the histological techniques to detect free Ca^{2+}. It would appear, therefore, that the extra calcium found at the endplates after ecothiopate is bound in some way and thus unavailable for staining by GBHA. This would imply that if this extra uptake of extracellular calcium is the cause of plcs, some of it must become unbound and, subsequently, released into the sarcoplasm following stimulation.

However, the observation that plcs wane while endplate calcium apparently continues to accumulate leads to the suggestion that such accumulation is not the cause of plcs or, at best, that plc waning is caused by some other mechanism. This, therefore, allows the speculation that the prolonged endplate depolarisation directly prolongs Ca^{2+} action in the endplate sarcoplasm, perhaps, as has been suggested by delaying the sequestration of Ca^{2+} into the SR. Indeed the observation by Ferry (personal communication) in which epps are prolonged for at least 50 msec at depolarisations of approximately -50mV and that this depolarisation might be mechanically effective, might be the factor delaying the local sequestration of Ca^{2+} into the SR. However, the evidence provided by my experiments in which endplate currents remain prolonged after plcs have waned, does not support this proposed hypothesis. It all depends on what proportion, if any, of the prolonged endplate depolarisation is mechanically effective.

Evidence from my radioisotope experiments suggested approximately similar endplate accumulations of calcium with carbachol (10^{-4}M) and ecothiopate (5×10^{-7}M) while evidence from the histological experiments in which Ca^{2+} in solution were stained, suggested that only carbachol causes the accumulation of Ca^{2+} in solution. It has been speculated

that the desensitisation, thought to occur with carbachol (10^{-4}M) (Evans, 1974), is associated with the accumulation of such calcium ions. Presumably, this free calcium was insufficient to increase tension and maybe becomes sequestered into the SR. It has been argued that since ecothiopate action was not thought to desensitise the membrane to the same extent, if at all, accumulation of free Ca^{2+} (i.e. in solution) does not occur. That accumulation of such Ca^{2+} occurs with carbachol (10^{-4}M) but not with ecothiopate (5×10^{-7}M) has been shown. Since ecothiopate still causes the accumulation of calcium at the endplates, it was assumed that this calcium becomes unavailable for staining in some way. Evidence of some sort of binding was provided by the experiments which showed that washing the muscles did not result in a reduction in endplate calcium accumulation.

The obvious site of binding is the local SR. It is possible that the Ca^{2+} entering the endplate during the mechanically effective period of the prolonged depolarisation becomes sequestered into the SR when such depolarisation is no longer mechanically effective. Other sites of binding may be the mitochondria, which are well known to bind Ca^{2+}, and the endplate membrane itself. Eldefrawi, Eldefrawi, Penfold, O'Brien and Van Campen (1975) found that the AChR of Torpedo could bind with many Ca^{2+}. It is, therefore, possible that the staining and accumulation of endplate calcium shown by Ahmad and Lewis (1961, 1962), Jenkinson and Nicholls (1961), Csillik and Savay (1963), Lièvrement et al. (1968), Meunier (1972) and Evans (1974) was due to the binding of calcium to the receptors. However, even though Eldefrawi et al. (1975) claim a highly purified receptor preparation, it is not known how much of the endplate is actually in the preparation. Thus, it is still possible that Ca^{2+} binds at some other site at or near the endplate membrane.

Whatever the site of binding of the calcium following ecothiopate action, the observation that free Ca^{2+} could no longer be stained suggests that the binding process made the Ca^{2+} unavailable for staining. Lièvrement and Pascaud (1970, 1972) have isolated from the diaphragm endplate a lipoprotein fraction that is supposedly capable of binding Ca^{2+} and from which the Ca^{2+} can be displaced by ACh. According to these workers the ACh - sensitive Ca^{2+} binding lipoprotein is a component of the complete AChR. Lièvrement, Tazieff - Depierre and their colleagues (Tazieff - Depierre, Lièvrement and Szajka, 1968a, b ; Lièvremont et al., 1968, 1969; Lièveremont and Pascaud, 1970a, b, 1972, 1973) have shown that depolarising agents cause liberation of Ca^{2+} from the endplate and that non - depolarising antagonists prevent their mobilisation. Lièvremont and Pascaud (1970, 1972) suggest that the critical step in the mediation of the cholinergic response is suggested to be the displacement by the agonist, probably via an allosteric action, of Ca^{2+} bound to the lipoprotein and serving to maintain the ion channels in the closed state. Such displacement was believed to open the ion channels and, simultaneously, to activate the associated AChE to hydrolyse the transmitter. The rebinding of Ca^{2+}, following removal of the agonist, was thought to cause the ion channels to close so completing the excitability cycle.

Evans(1974) has suggested that endplate accumulation of calcium progresses in the presence of ACh (10^{-3}M) and carbachol (10^{-4}M) which are believed to desensitise the post - junctional membrane. Desensitisation is believed to be determined by increased Ca^{2+} binding at the internal membrane surface (Manthey, 1966, 1970; Nastuk and Parsons, 1970; Lambert and Parsons, 1971; Parsons, Johnson and Lambert, 1971, Parsons, Cochrane and Schnitzel ,1973).

Is desensitisation a necessary pre-requisite for post-junctional Ca^{2+} entry or is it caused by the accumulation of such an entry of Ca^{2+} at the endplate ? Manthey (1974) apparently found that during the development of carbachol-induced desensitisation there occurred a parallel decrease in the calcium permeability of the post-junctional membrane. Manthey suggested that this evidence implied that endplate calcium accumulation reduces the calcium permeability of the post-junctional membrane at the same time as causing desensitisation. That endplate calcium accumulation continues despite desensitisation of the endplate membrane (Evans, 1974) lends support to the suggestion that post-junctional Ca^{2+} entry follows endplate desensitisation. However, this does not exclude the possibility that desensitisation is caused by an accumulation of calcium at the endplate and it can be tentatively concluded that the staining and accumulation of calcium in the post-junctional region results from a modification of receptor activity (i.e. desensitisation) by Ca^{2+} without necessarily implying any change in calcium permeability. Kuba and Koketsu (1976) suggested that desensitisation was the result of an endplate accumulation of calcium effectively blocking sodium channels. It would appear that channels must remain open to allow, at least, calcium entry. Takeuchi (1963) suggested that increasing external [Ca] reduces sodium conductance of the endplate membrane produced by ACh action. Adams (1976) implies that intracellular calcium decreases the G_{Na} of the endplate membrane by blocking open sodium channels.

In my experiments, the waning of the enhanced early contraction, of the SCs and of the plcs, the inhibition of the development of SCs by stimulation and the inability to sustain tetanic contractions have been largely interpreted as being due to a reduction in transmitter action, perhaps by desensitisation of receptors. The evidence that

calcium accumulates at the endplates at the same time lends support to this hypothesis.

The possibility that ecothiopate (5×10^{-7}M) is directly involved in promoting the waxing and/or the waning of the ecothiopate - mediated changes in response and the endplate accumulation of calcium has been suggested. My experiments made with dTC, β - Butx and denervation show that ecothiopate (5×10^{-7}M) has no direct effect on muscle fibres, which suggests it may have no direct action on muscle in promoting the ecothiopate - mediated changes in response when neuromuscular transmission is intact. Experiments made where the removal of ecothiopate from the physiological saline following the waning of the changes has resulted in some recovery suggest that ecothiopate (5×10^{-7}M) might have some blocking effect. Finally, evidence has been presented which suggests that endplate calcium accumulation is [ecothiopate] - dependent although this may be due to a faster rate of ChE inhibition. In general, it was thought that ecothiopate at concentrations (10^{-6} to 10^{-7}M) has a negligible direct effect at the post - junctional membrane but which may be more significant at the pre - junctional membrane.

Higher concentrations of ecothiopate (e.g. 10^{-3}M) have been found to have a dual action, that is, the usual irreversible anti-ChE action and a reversible blocking action on the receptor (Bartels and Nachmansohn, 1969). The apparent reversal of the block of contraction observed in my experiments may be due to such a reversible block of receptors. However, Bartels and Nachmansohn, 1969) found no evidence of a reversible receptor block with ecothiopate (5×10^{-6}M).

The action of ChE inhibitors in causing an increase in the size and time course of the endplate potential has been attributed to the persistence of ACh molecules in the synaptic space, whereas, normally they are rapidly destroyed by enzymic hydrolysis (Katz and Miledi, 1973).

In addition, however, as recognised in the early studies of Feng (1941) and Eccles, Katz and Kuffler (1941, 1942), anti-esterases give rise to a cumulative slow depolarisation of the endplate region. This effect builds up to a high level during repetitive nerve stimulation and may take several seconds to decline after the tetanus. This phenomenon was shown by Eccles and MacFarlane (1949) to be closely associated with the degree of ChE inhibition. The nature of this cumulative slow depolarisation has been suggested by Kuba, Albuquerque, Daly and Barnard (1974), working with DFP (10^{-3}M), to be a direct effect of the drug, namely a 'freezing' of ion channels in their open state, long after the junction has been cleared by diffusion of ACh molecules. Thus it may be that ecothiopate, even at 5×10^{-7}M, also partly acts by 'freezing' ion channels in their open state. This might have helped, at least, to explain the accumulation of calcium in my expts. However, Katz and Miledi (1975) using noise analysis to investigate the life time of open channels, have shown that the slow wave of depolarisation caused by prostigmine (6×10^{-6}M) is largely due to 'transmitter noise' resembling ACh noise. This indicates that the time course of channel opening is unchanged. If ecothiopate (5×10^{-7}M) was acting similarly to neostigmine (6×10^{-6}M) then these latter results would indicate that ecothiopate (5×10^{-7}M) might only act to prolong ACh action. However, they do not completely refute the hypothesis proposed by Kuba et al. (1974) because of the higher concentration and irreversible nature of the drug used by the latter. The crucial experiments remain to be performed.

The speculation that a direct effect of organophosphorus ChE inhibitors 'freezes' ion channels in their open state leads to the question of whether these inhibitors can penetrate the endplate

membrane. If they can, they might also block the channels they traverse. Adams and Sakmann (1978) have shown that decamethonium both opens and blocks endplate channels. Creese and England (1970) found that decamethonium penetrates muscles in similar conditions to those needed to open endplate channels. Adams and Sakmann (1978) suggested that decamethonium could enter and traverse ion channels at concentrations comparable to those which also produced block. It, therefore, seemed probable that channel permeation and channel blockage were related. It is possible that ecothiopate, although supposedly a poor penetrator because of its low lipid solubility, could enter and block ion channels. This block may explain the waning of the enhanced early contraction and the SCs and finally the plcs. However, on this basis it is difficult to explain how post-junctional Ca^{2+} enters blocked channels. Mittag et al. (1970) demonstrated that ecothiopate could penetrate the endplate membrane since they observed that when inhibition of external AChE was 91%, the internal AChE was inhibited by 64%.

If ecothiopate is able to penetrate the endplate membrane in any significant amount then, perhaps, some of the ecothiopate-mediated changes of the contraction might be attributable to some mechanism or mechanisms in the muscle itself. For example, Binder, Landon, Wecker and Dettbarn (1975), working on isolated SR from rat diaphragm have reported that parathion (4×10^{-5}M) and, to a lesser extent, paraoxon (1.5×10^{-3}M) inhibits sarcoplasmic recticular calcium uptake activity. Such an action of ecothiopate (5×10^{-7}M) in the endplate region might explain the prolongation of the contraction in this region. Also, the action of Mg^{2+} (3.5×10^{-3}M) in preventing the development of plcs may be due to an antagonism of Ca^{2+} action at the site of activation of the contractile machinery. Ford and Podolsky (1972) suggested that magnesium may reduce the affinity of

the calcium binding sites on the myofilaments.

The waning of the ecothiopate - mediated changes of contraction have been discussed in terms of a reduction in transmitter action perhaps by desensitisation of receptors or of a direct action by ecothiopate itself. The waning may be, alternatively, due to a reduction in transmitter release. Experiments made to detect any effect of ecothiopate action on the frequency of mepps appeared not to show any change in frequency (unpublished). These results were not very reliable, however, since intracellular and extracellular recording was almost impossible for long periods of time due to the spontaneous movement of preparations in the presence of ecothiopate. The frequencies were also thought to be unreliable because of the reduction in the amplitude of mepps observed after ecothiopate (Table 14). However, Hubbard, Schmidt and Yokota (1965) found no significant effect on transmitter release by Neostigmine (3.3×10^{-6}M). Presumably, any long - term alteration in transmitter release results from the prolongation of synaptic transmitter action after ecothiopate. It has been shown that the post - junctional membrane is permanently depolarised by about 6 - 10mV in the presence of ecothiopate. It has been suggested that this is due to a higher and permanent concentration of transmitter in the synapse. It is believed that this persistent transmitter acts pre - synaptically to generate SCs. It can be speculated that it also may have some sort of negative feed-back on the motor nerve terminals modifying release. Miledi, Molenaar and Polak (1978) also suggest that the activation of pre - synaptic receptors could exert an inhibitory influence on transmitter release. This was because they found that α - Butx caused an increase in the amount of ACh collected after DFP. They suggested that α - Butx might have reduced an inhibiton of transmitter release by a pre - synaptic effect of ACh.

However, the waning of the ecothiopate - mediated changes in contraction might, at least, partly be due to internal mechanisms in the muscle fibres. It is thought that internal mechanisms would involve a reduction in Ca^{2+} action in the sarcoplasm. A possible effect on sarcoplasmic reticular Ca^{2+} uptake has already been mentioned. Perhaps the waning of the ecothiopate - mediated changes coincides with a reduction in the inhibition of such uptake. Alternatively, ecothiopate might directly or indirectly lead to some disruption of the SR or the myofibrils which would effectively disrupt the contractile process.

Hudson, Rash, Tiedt and Albuquerque (1978) have demonstrated disorganised cytoarchitecture at diaphragm endplates 30 mins after injection of paraoxon. Leonard and Saltpeter (1979) using DFP (1×10^{-3}M) for 1 - 2 hours on the isolated mouse EDL preparation stimulated at 2Hz found similar organisation, namely, an increase in large - diameter vesicles, the dissolution of Z - discs, the dilation of mitochondria, the destruction of SR, and often a highly specific contracture of the muscle under the endplate. Since a Ca^{2+} - activated protease which specifically removes Z - discs is known to exist in mammalian skeletal muscle (Leonard and Saltpeter, 1979) it was thought possible that the endplate accumulation of Ca^{2+} after ecothiopate could activate such proteases and, subsequently, lead to the disruption of, for example, the SR. Such a process in the endplate region might be associated with, at least, the waning of plcs. One may wonder whether the plcs themselves cause the local damage. Leonard and Saltpeter (1979) indicated that the early stages of disorganisation required agonist - receptor interaction and are mediated by Ca^{2+}, since they were prevented by both α - bungarotoxin and EGTA. They suggested that the Ca^{2+} influx in response to prolonged agonist - receptor interaction may overload the Ca^{2+} binding mechanisms or otherwise elevate intracellular Ca^{2+} sufficiently to activate the destructive proteases.

REFERENCES

Adams, P.R. (1976). Drug blockade of open endplate channels. J. Physiol. (Lond.) 260, 531 - 552.

Adams, P.R. and Sakmann, B. (1978). Decamethonium both opens and blocks endplate channels. Proc. Natn. Acad, Sci. U.S.A. 75, 2994 - 2998.

Adrian, E.D. and Lucas, K. (1912). On the summation of propagated disturbances in nerve and muscle. J. Physiol. (Lond.) 44, 68 - 124.

Ahmad, K. and Lewis, J.J. (1961). The effects of gallamine, carbachol, ryanodine and protoveratrine A and B upon flux of calcium - 47 in frog skeletal muscle. J. Pharm. Pharmac. 13, 383 - 384.

Ahmad, K. and Lewis, J.J. (1962). The influence of drugs which stimulate skeletal muscle and of their antagonists on flux of calcium, potassium and sodium ions. J. Pharmac. exp. Ther. 136, 298 - 304.

Anderson, C.R. and Stevens, C.F. (1973). Voltage clamp analysis of acetylcholine produced endplate current fluctuations at frog neuromuscular junction. J. Physiol. (Lond.) 235, 655 - 692.

Armstrong, C.M., Bezanilla, F.M. and Horowicz, P. (1972). Twitches in the presence of ethylene glycol - bis - (α - aminoethylether)- N, N' - tetraacetic acid. Biochim. Biophys. Acta. 267, 605 - 608.

Ashley, C.C. and Ridgeway, E.B. (1968). Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. Nature, Lond. 219, 1168 - 1169.

Axelsson, J. and Thesleff, S. (1958). The desensitising effect of acetylcholine on the mammalian motor endplate. Acta. Physiol. Scand. 43, 15 - 26.

Axelsson, J. and Thesleff, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. (Lond.) 149, 178 - 193.

Barnes, J.M. and Duff, J.I. (1953). The role of cholinesterase at the myoneural junction. Brit. J. Pharmacol. 8, 334 - 339.

Barrnett, R.J. and Palade, G.E. (1959). Enzymatic activity in the M band. J. Biophys. Biochem. Cytol. 6, 163 - 165.

Bartels, E. and Nachmansohn, D. (1969). Organophosphate inhibitors of acetylcholine - receptor and -esterase tested on the electroplax. Arch. Biochem. Biophys. 133, 1 - 10.

Bianchi, C.P. (1968). Cell calcium. Butterworth and Co.

Bianchi, C.P. and Shanes, M. (1959). Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. J. Gen. Physiol. 42, 803 - 815.

Bianchi, C.P. and Bolton, T.C. (1966). Effect of thiocyanate on radiocalcium uptake during potassium contracture of frog sartorius muscle. J. Pharmacol. Exptl. Therap. 151, 456 - 463.

Binder, N., Landon, E.J., Wecker, L. and Dettbarn, W-D. (1976). Effect of parathion and its metabolites on calcium uptake activity of rat skeletal muscle sarcoplasmic reticulum in vitro. Biochem. Pharmacol. 25/7, 835 - 839.

Birks, R., Huxley, H.E. and Katz, R. (1960). The fine structure of the neuromuscular junction of the frog. J. Physiol. (Lond.) 150, 134 - 143.

Blaber, L.C. and Christ, D.D. (1967). The action of facilitatory drugs on the isolated tenuissimus muscle of the cat. Int. J. Neuropharmacol. 6, 473 - 484.

Bowman, W.C. and Webb, S.N. (1972). Acetylcholine and anticholinesterase drugs. Chap. 16. Neuromuscular Blocking and Stimulating Agents . Vol. 2, Section XIV, International Encyclopedia of Pharmacology and Therapeutics. Pergamon Press.

Boyd, I.A. and Martin, A.R. (1956b). The endplate potential in mammalian muscle. J. Physiol. (Lond.) 132, 74 - 91.

Brown, G.L. (1937a). Action potentials of normal mammalian muscle. Effects of acetylcholine and eserine. J. Physiol. (Lond.) 89, 220 - 237.

Brown, G.L., Dale, H.H. and Feldberg, W. (1936). Reactions of the normal muscle to acetylcholine and to eserine. J. Physiol. (Lond.) 87, 394 - 424.

Brown, G.L. and Harvey, A.M. (1938). Neuromuscular conduction in the fowl. J. Phsyiol. (Lond.). 93, 285 - 300.

Bülbring, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. Brit. J. Pharmacol. 1, 38 - 61.

Burgen, A.S.V., Dickens, F. and Zatman, L.J. (1949). The action of botulinum toxin on the neuromuscular junction. J. Physiol. (Lond.) 109, 10 - 24.

Burgen, A.S.V., Keele, C.A. and Slome, D. (1949). Pharmacological actions of tetraethylpyrophosphate and hexaethyltetraphosphate. J. Pharmacol. expt.Ther. 96, 396 - 409.

Burns, B.D. and Paton, W.D.M. (1951). Depolarisation of the motor endplate by decamethonium and acetylcholine. J. Physiol. 115, 41 - 73.

Carslon, F.D. and Wilkie, D.R. (1974). Muscle Physiology. Prentice - Hall, New Jersey.

Chen, I.L. and Lee, C.Y. (1970). Ultrastructural changes in motor nerve terminals caused by β - Bungarotoxin. Virchows Arch. Abt. B. Zellpathol. 6, 318.

Cheymol, J. and Bourillet, F. (1972). Chapter 12, Neuromuscular Blocking and Stimulating Agents. Vol. 1, Section XIV, International Encyclopedia of Pharmacology and Therapeutics, Pergamon Press.

Chiarandini, D.J. and Stefani, E. (1973). Effects of manganese on the electrical and mechanical properties of frog skeletal muscle fibres. J. Physiol. (Lond.) 232, 129 - 147.

Cole, W.V. (1957). Structural variations of nerve endings in the striated muscles of the rat. J. Com. Neurol. 108, 445 - 463.

Colquhoun, D., Dreyer, F. and Sheridan, R.E. (1979). The actions of tubocurarine at the frog neuromuscular junction. J. Physiol. (Lond.) 293, 247 - 284.

Costantin, L.L. and Podolsky, R.J. (1966). Evidence for depolarisation of the internal membrane system in activation of frog semi - tendinosus muscle. Nature, Lond. 210, 483 - 486.

Costantin, L.L. and Podolsky, R.J. (1967). Depolarisation of the internal membrane system in the activation of frog skeletal muscle. J. Gen. Physiol. 50, 1101 - 1124.

Creese, R. and England, J.M. (1970). Decamethonium in depolarised muscle and the effects of tubocurarine. J. Physiol. (Lond.) 210, 345 - 362.

Csillik, B. and Savay, G. (1963). Release of calcium in the myoneural junction. Nature, Lond. 198, 399 - 400.

Dale, H.H. (1953). Adventures in Physiology. The Wellcome Trust, London.

Dale, H.H., Feldberg, W. and Vogt, M. (1936). Release of acetylcholine at voluntary motor nerve endings. J. Physiol. (Lond.) 86, 353 - 380.

Del Castillo, J. and Katz, B. (1954a). Quantal components of the endplate potential. J. Physiol. (Lond.) 124, 560 - 573.

Del Castillo, J. and Katz, B. (1954b). The membrane change produced by the neuromuscular transmitter. J. Physiol. (Lond.) 125, 546 - 565.

Del Castillo, J. and Katz, B. (1955b). Local activity of a depolarised nerve - muscle junction. J. Physiol. (Lond.) 128, 396 - 411.

Del Castillo, J. and Stark, L. (1952). The effect of calcium ions on the motor endplate potentials. J. Physiol. (Lond.) 116, 507 - 515.

Ebashi, S. (1976). Excitation - Contraction coupling. Ann. Rev. Physiol. 38, 293 - 313.

Eccles, J.C. (1964). The Physiology of Synapses. Springer - Verlag, Berlin.

Eccles, J.C., Katz, B. and Kuffler, S.W. (1942). Effect of eserine on neuromuscular transmission. J. Neurophysiol. 5, 211 - 230.

Eccles, J.C. and Macfarlane, W.V. (1949). Actions of anticholinesterases on endplate potential of frog muscle. J. Neurophysiol. 12, 59 - 80.

Ehrenpreis, S., Hehir, R.M. and Mittag, J.W. (1971). Assay and properties of essential (functional) cholinesterases of the rat diaphragm, in 'Cholinergic Ligand Interactions'. Academic Press, New York.

Eldefrawi, M.E., Eldefraw, A.D., Enfield, L.A., O'Brien, R.D. and Van Campen, D. (1975). Binding of calcium and zinc to the acetylcholine receptor purified from Torpedo californica. Life Sci. 16, 925 - 936.

Elmqvist, D. and Quastel, D.M.J. (1965a). Calcium dependence on spontaneous acetylcholine release at mammalian motor nerve terminals. J. Physiol. (Lond.) 181, 487 - 497.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 57, 71 - 108.

Engbaek, L. (1972). Inhibitor Ion: Magnesium. Chapter 14, Neuromuscular Blocking and Stimulating Agents, Vol. 1, Section XIV, International Encyclopedia of Pharmacology and Therapeutics, Pergamon Press.

Evans, R.H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm muscle. J. Physiol. (Lond.) 240, 517 - 533.

Fatt, P. and Katz, B. (1951). An analysis of the endplate potential recorded with an intracellular electrode. J. Physiol. (Lond.) 115, 320 - 370.

Fatt, P. and Katz, B. (1952). Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (Lond.) 117, 109 - 128.

Feng, T.P. (1940). Studies on the neuromuscular junction. XVIII. The local potentials around N - M junctions induced by single and multiple volleys. Chin. J. Physiol. 15, 367 - 404.

Feng, T.P. (1941). The local activity around the skeletal N - M junctions produced by nerve impulses. Biol. Sym. 3, 121 - 152.

Feng, T.P. and Li, T.H. (1941). Studies on the neuromuscular junction. XXIII. A new aspect of the phenomena of eserine potentiation and post tetanic facilitation in mammalian muscles. Chin. J. Physiol. 16, 37 - 54.

Fletcher, P. and Forrester, T. (1975). The effect of curare on the release of acetylcholine from mammalian motor nerve terminals and an estimate of quantum content. J. Physiol. (Lond.) 251, 131 - 143.

Ford, L.E. and Podolsky, R.J. (1972). Calcium uptake and force development in skinned muscle fibres. Federation Proc. 27, 375.

Ford, L.E. and Podolsky, R.J. (1972). Intracellular calcium movements in skinned muscle fibres. J. Physiol. (Lond.) 223, 21 - 33.

Fuchs, F. (1974). Striated muscle. Ann. Rev. Physiol. 36, 461 - 502.

Gauthier, G.F. and Padykula, H.A. (1966). Cytological studies of fiber types in skeletal muscle. J. Cell Biol. 28, 333 - 354.

Günther, P.G. (1952). Die morphologischen grundlagen der bewegungsund malteleistung (tetanus and tonus) des zwerchfelts. Acta. Anat. 14, 54 - 64.

Guth, L. (1968). Trophic influences of nerve on muscle. Physiol. Rev. 48, 645 - 687.

Gutmann, E. (1976). Neurotrophic relations. Ann. Rev. Physiol. 38, 56 - 95.

Gutmann, E. (1977). Trophic effects in nerve - muscle cell relations. Chap. 16. 'Synapses', Blackie and Son Ltd., Glasgow and London. (Eds., G.A. Cottrell and P.N.R. Usherwood).

Harris, A.J. (1974). "Inductive functions of the nervous system". Ann. Rev. Physiol. 36, 251 - 305.

Harris, J.B. and Leach, G.D.H. (1968). The effect of temperature on endplate depolarisation of the rat diaphragm produced by suxamethonium and acetylcholine. J. Pharm. Pharmac. 20, 194 - 198.

Hebb, C.O., Krnjević, K. and Silver, A. (1964). Acetylcholine and choline acetyltransferase in the diaphragm of the rat. J. Physiol. (Lond.) 171, 504 - 513.

Heilbrunn, L.V. and Wiercinski, F.V. (1947). The action of various cations on muscle protoplasm. J. Cell. Comp. Physiol. 29, 15 - 32.

Hill, A.V. (1948). On the time required for diffusion and its relaxation to processes in muscle. Proc. Roy. Soc. B. 135, 446 - 453.

Hodgkin, A.L. and Horowicz, P. (1960). Potassium contractures in single muscle fibres. J. Physiol. (Lond.) 153, 386 - 403.

Hubbard, J.I. (1961). The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. J. Physiol. (Lond.) 159, 507 - 517.

Hubbard, J.I. (1965). The origin and significance of antidromic activity in motor nerves. In 'Studies in Physiology' , pp. 85 - 92. Springer - Verlag, Berlin. (Eds., D.R. Curtis and A.K. McIntyre.

Hubbard, J.I. (1974). The Peripheral Nervous System. p. 185. Plenum Press, New York.

Hubbard, J.I., Jones, S.F. and Landau, E.M. (1968a). On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. J. Physiol. (Lond.) 194, 355 - 380.

Hubbard, J.I., Jones, S.F. and Landau, E.M. (1968b). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. J. Physiol. (Lond.) 196, 75 - 86.

Hubbard, J.I., Llinás, R. and Quastel, D.M.J. (1969). Electrophysiological Analysis of Synaptic Transmission. (Eds., H. Davson, A.D.M. Greenfield, R. Whittam and G.S. Brindley) Monograph of the Physiological Society, No. 19.

Hubbard, J.I., Schmidt, R.F. and Yokota, T. (1965). The effect of acetylcholine upon mammalian motor nerve terminals. J. Physiol. (Lond.) 181, 810 - 829.

Hudson, C.S., Rash, J.E., Tiedt, T.N. and Albuquerque, E.X. (1978). Neostigmine - induced alterations at the mammalian neuromuscular junction. II. Ultrastructure. J. Pharmacol. Exp. Ther. 205, 340 - 356.

Huxley, A.F. and Taylor, R.E. (1958). Local activation of striated muscle fibres. J. Physiol. (Lond.) 144, 426 - 441.

Jenkinson, D.H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. J. Physiol. (Lond.) 138, 438 - 444.

Jenkinson, D.H. and Nicholls, J.G. (1961). Contractures and permeability changes produced by acetylcholine in depolarised denervated muscle. J. Physiol. (Lond.) 159, 111 - 127.

Jöbsis, F.F. and O'Connor, M.J. (1966). Calcium release and reabsorption in the sartorius muscle of the toad. Biochem. Biophys. Res. Comm. 25, 246 - 252.

Karnovsky, M.J. (1964). The localisation of cholinesterase activity in rat cardiac muscle by electron microscopy. J. Cell Biol. 23, 217 - 232.

Karnovsky, M.J. and Roots, L. (1964). A "direct - colouring" thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219 - 221.

Kashiwa, H.K. and Atkinson, W.B. (1963). The applicability of a new Schiff base, glyoxal bis (2 - hydroxyanil), for the cytochemical localisation of ionic calcium. J. Histochem, Cytochem. 11, 258 - 264.

Katz, B. and Miledi, R. (1965). Release of acetylcholine from a nerve terminal by electric pulses of variable strength and duration. Nature, Lond. 207, 1097 - 1098.

Katz, B. and Miledi, R. (1967). The timing of calcium action during neuromuscular transmission. J. Physiol.(Lond.) 189, 535 - 544.

Katz, B. and Miledi, R. (1968). The role of calcium in neuro-muscular facilitation. J. Physiol. (Lond.) 195, 481 - 492.

Katz, B. and Miledi, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. J. Physiol. (Lond.) 231, 547 - 574.

Katz, B. and Miledi, R. (1975). The nature of the prolonged endplate depolarisation in anti-esterase treated muscle. Proc. Roy. Soc. London B. 192, 27 - 38.

Katz, B. and Miledi, R. (1977). Transmitter leakage from motor nerve endings. Proc. Roy. Soc. London B. 196, 59 - 72.

Katz, B. and Miledi, R. (1978). A re-examination of curare action at the motor endplate. Proc. Roy. Soc. London B. 203, 119 - 133.

Katz, B. and Thesleff, S. (1957b). A study of the "desensitisation" produced by acetlcholine at the motor endplate. J. Physiol. (Lond.) 138, 63 - 80.

Koelle, G.B. and Friedenwald, J.S. (1949). A histochemical method for localised cholinesterase activity. Proc. Soc. exp. Biol. Med. 70, 617 - 622.

Krnjević, K. and Miledi, R. (1958a). Motor units in the rat diaphragm. J. Physiol. (Lond.) 140, 427 - 439.

Krnjević, K. and Miledi, R. (1958b), Acetylcholine in mammalian neuromuscular transmission. Nature, Lond. 182, 805 - 806.

Kuba, K., Albuquerque, E.X., Daly, J. and Barnard, E.A. (1974). A study of the irreversible inhibitor Diisopropylfluorophosphate on the time course of endplate currents in frog sartorius muscle. J. Pharm. Exp. Ther. 189, 499 - 512.

Kuba, K. and Koketsu, K. (1976). Decrease of sodium conductance during desensitisation of frog endplate. Nature, Lond. 262, 504 - 505.

Kuperman, A.S. and Okamoto, M. (1964). The relationship between anticurare activity and time course of the endplate potential, a structure - activity approach. Brit. J. Pharmacol. 23, 575 - 591.

Lambert, D.H. and Parsons, R.L. (1970). Influence of polyvalent cations on the activation of muscle endplate receptors. J. Gen. Physiol. 56, 309 - 321.

Leonard, J.P. and Saltpeter, M.M.(1979). Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. J. Cell.Biol. 82, 811 - 819.

Li, C.H. (1958). Effect of cooling on neuromuscular transmission in the rat. Am. J. Physiol. 194, 200 - 206.

Lièvrement, M., Czajka, M. and Tazieff-Depierre, F. (1968). Etude in situ d'une fixation de calcium et de sa liberation à la jonction neuromusculaire. C. R. Acad. Sci. Paris, D. 267, 1988 - 1991.

Lièvrement, M., Czajka, M. and Tazieff-Depierre, F. (1969). Cycle du calcium à la jonction neuromusculaire. C.R. Acad. Sci. Paris, D. 268, 379 - 382.

Lièvrement, M. and Pascaud, M. (1970a). Bull. Soc. Chim. Biol. 52, 23.

Lièvrement, M. and Pascaud, M. (1970b). Intervention du calcium, des phospholipides et des cholinestérases dans la sensibilité cholinergique à la jonction neuromusculaire. C.R. Acad. Sci. Paris, D. 271, 1779 - 1782.

Lièvrement, M. and Pascaud, M. (1972). Etude de la sensibilité cholinergique à la jonction neuromusculaire, définition et rôle d'un operant calcique. C.R. Acad. Sci. Paris, D. 274, 1345 - 1348.

Lièvrement, M. and Pascaud, M. (1973). Venin de Bungarus Fasciatus et transmission neuromusculaire. Etude par la méthode de la réponse calcique cholinergique sur le diaphragme de souris. C.R. Acad. Sci. Paris, C. 277, 1045 - 1048.

Liley, A.W. (1956a). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. (Lond.) 132, 650 - 666.

Liley, A.W. (1956b). The quantal components of the mammalian endplate potential. J. Physiol. (Lond.) 133, 571 - 587.

Maeno, T. (1966). Analysis of sodium and potassium conductances in the procaine endplate potential. J. Physiol. (Lond.) 183, 592 - 606.

Manthey, A.A. (1966). The effect of calcium on the desensitisation of membrane receptors at the neuromuscular junction. J. Gen. Physiol. 49, 963 - 976.

Manthey, A.A. (1970). Further studies of the effect of calcium on the time course of action of carbamylcholine at the neuromuscular junction. J. Gen. Physiol. 56, 407 - 419.

Masland, R.L. and Wigton, R.S. (1940). Nerve activity accompanying fasciculation produced by prostigmine. J. Neurophysiol.3, 269 - 275.

Meeter, E. (1958). The relation between endplate depolarisation and the repetitive response elicited in the rat phrenic nerve diaphragm preparation by DFP. J. Physiol. (Lond.) 144, 38 - 51.

Merton, P.A. (1954). Interaction between muscle fibres in a twitch. J. Physiol. (Lond.) 124, 311 - 324.

Meunier, F. (1972). Localisation à l'appareil sous-neural du calcium apparaissant dans diverses conditions experimentales au niveau de la jonction neuromusculaire. C.R. Acad. Sci. Paris, D. 274, 1818 - 1821.

Miledi, R. (1960b). Junctional and extrajunctional acetylcholine receptors in skeletal muscle fibres. J. Physiol. (Lond.) 151, 24 - 30.

Miledi, R., Molenaar, P.C. and Polak, R.L. (1978). α - Bungarotoxin enhances transmitter 'released' at the neuromuscular junction. Nature, Lond. 272, 641 - 2.

Mitchell, J.F. and Silver, A. (1963). The spontaneous release of acetylcholine from the denervated hemi - diaphragm of the rat. J. Physiol. (Lond.) 165, 117 - 129.

Mittag, T.W., Ehrenpreis, S. and Hehir, R.M. (1971). Functional acetylcholinesterase of rat diaphragm muscle. Biochem. Pharmacol. 20, 2263 - 2273.

Miyamoto, M.D. (1978). The actions of cholinergic drugs on motor nerve terminals. Pharmac. Rev. 29, 221 - 247.

Morrison, J.D. (1977). The generation of nerve and muscle repetitive activity in the rat phrenic nerve - diaphragm preparation following inhibition of cholinesterase by ecothiopate. Brit. J. Pharmacol. 60, 45 - 54.

Namba, T. and Grob, D. (1968). Cholinesterase activity of the motor endplate in isolated muscle membrane. J. Neurochem. 15, 1445 - 1454.

Nastuk, W.L. and Gissen, A.J. (1965). Actions of acetylcholine and other quaternary ammonium compounds at the muscle post-junctional membrane. In: Muscle, pp. 389 - 402. Pergamon Press, Oxford.

Nastuk, W.L. and Parsons, R.L. (1970). Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. J. Gen. Physiol. 56, 218 - 249.

Pal, J. (1900). Physostigmin, ein gegengift des curare. Zbl. Physiol. 14, 255 - 258.

Parsons, R.L., Cochrane, D.E. and Schnitzel, R.M. (1973). Desensitisation of endplate receptors produced by carbachol in lithium Ringer solutions. J. Gen. Physiol. 61, 263.

Parsons, R.L., Johnson, E.W. and Lambert, D.H. (1971). Effects of lanthanum and calcium on chronically denervated muscle fibres. Amer. J. Physiol. 220, 401 - 405.

Paterson, G. (1965). Twitch responses with acetylcholine in the isolated innervated and chronically denervated rat diaphragm and their modification by neuromuscular blocking agents. J. Pharm. Pharmac. 17, 281 - 294.

Riesser, O. (1921). Untersuchungen an überlebenden roten und weissen Kaninchen muskeln. Pflügers Arch. ges. Physiol. 190, 137 - 157.

Robertson, J.D. (1956). The ultrastructure of reptilian myoneural junction. J. Biophys. Biochem. Cytol. 2, 381 - 394.

Sandow, A. (1970). Skeletal muscle. Ann. Rev. Physiol. 32, 87 - 138.

Sandow, A., Pagala, M.K.D. and Sphicas, E.C. (1975). Excitation - contraction coupling effects of 'zero' - Ca^{2+} medium. Biochim. Biophys. Acta. 404, 157 - 163.

Sandow, A., Taylor, S.R. and Preiser, H. (1965). Role of the action potential in excitation - contraction coupling. Fed. Proc. 24, 1116.

Siakotos, A.N., Filbert, M. and Hester, R. (1969). A specific radioisotopic assay for acetylcholinesterase and pseudo-cholinesterase in brain and plasma. Biochem. Med. 3, 1 - 12.

Silver, A.(1964). The Biology of Cholinesterases. North-Holland Research Monographs - Frontiers of Biology. Vol. 36. (eds. A. Neuberger and E.L. Tatum). North-Holland Publishing Co. Amsterdam, Oxford.

Skou, I.C. (1957). The Influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta. 23, 394 - 400.

Stefani, E. and Chiarandini, D.J. (1973). Skeletal muscle; dependence of potassium contractures on external calcium. Arch. Ges. Physiol. 343, 143 - 150.

Somogyi, J. (1964). Über die Wirkung der Ca - Ionen auf die durch Na^+ und K^+ aktivierbare Adenosintriphosphatase des Hirngewebes. Physiol. Chem. 336, 264 - 270.

Sulakhe, P.V. and St. Louis, P.J. (1980). Passive and active calcium fluxes across plasma membranes. Progr. Biophys. Molec. Biol. 35, 173 - 181.

Tada, M., Yamamoto, T. and Tonomuro, Y. (1979). Molecular mechanism of active calcium transport by sarcoplasmic reticulum. Physiol. Rev. 58, 1 - 79.

Takeuchi, A. and Takeuchi, N. (1960). On the permeability of endplate membrane during the action of the transmitter. J. Physiol. (Lond.) 154, 52 - 67.

Takeuchi, N. (1963). Effects of calcium on the conductance change of the endplate membrane during the action of transmitter. J. Physiol. (Lond.) 167, 141 - 155.

Taylor, S.R., Preiser, H. and Sandow, A. (1972). Action potential parameters affecting excitation - contraction coupling. J. Gen. Physiol. 59, 421 - 436.

Tazieff-Depierre, F., Lièvrement, M. and Czajka, M. (1968a). Mise en évidence de l'apparation de calcium dans des fibres musculaires par l'action du chlorure de potassium et des toxines du venin de scorpion. C.R. Acad. Sci. Paris, D. 267, 1477 - 1478.

Tazieff-Depierre, F., Lièvrement, M. and Czajka, M. (1968b). Nouvelle méthode d'étude des substances actives sur la transmission neuromusculaire. C.R. Acad. Sci. Paris, D. 267, 2383 - 2386.

Thesleff, S, (1955a). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. Acta. Physiol. Scand. 34, 386 - 92.

Thesleff, S. (1955b). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. Acta. Physiol. Scand. 34, 386 - 392.

Thesleff, S. (1956). A further analysis of the neuromuscular block caused by acetylcholine. Acta. Physiol. Scand. 37, 330 - 334.

Van der Meer, C. and Meeter, E. (1956a). The mechanism of action of anticholinesterases. II. The effect of diisopropylfluorophosphonate (DFP) on the isolated rat phrenic nerve - diaphragm preparation. A. Irreversible effects. Acta Physiol. Pharmacol. Neerl. 4, 454 - 471.

Varga, E., Konig, T., Kiss, E., Kovacs, T. and Hegedus, L. (1955). On the anticholinesterase activity of myosin. Acta Physiol. Acad. Sci. Hung. 7, 171 - 173.

Vizi, E.S. and Vyskočil, F. (1979). Changes in total and quantal release of acetylcholine in the mouse diaphragm during activation and inhibition of membrane ATPase. J. Physiol. (Lond.) 286, 1 - 14.

Vyskočil, F. and Illes, P. (1977). Non - quantal release of transmitter at mouse neuromuscular junction and its dependence on the activity of Na^+ - K^+ ATPase. Pflügers Arch. 370, 295 - 297.

Weiss, G.B.and Bianchi, C.P. (1965). The effect of potassium concentration on ^{45}Ca uptake in frog sartorius muscle. J. Cellular Comp. Physiol. 65, 385 - 392.

Werner, G. and Kuperman, A.S. (1963). Actions at the neuromuscular junction. Chap. 13. Cholinesterases and Anticholinesterase Agents, Handb. der Expt. Pharmak. Springer-Verlag, Berlin.