THE TOXIC EFFECTS OF ENZYME INHIBITORS ON MUSCLE AND NERVE

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BY

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SUMMARY

Previous attempts have been made to determine the relationship between the reduction in activity of acetylcholinesterase (AChE) and the prolongation of extracellularly recorded miniature endplate potentials $(MEPPs)_o$. These attempts however have not been entirely successful and it was thought that this may be due to the biochemistry and the electrophysiology measuring different populations of AChE. In an attempt to avoid this the method of Younkin *et al* (1982) was used, by Mrs A. Rowbotham, to extract the different molecular isoforms of AChE and new correlations calculated. Using this technique a better correlation was arrived at and it has been suggested that it is the non extractable enzymes found specifically in the endplates which constitutes the functional enzyme, i.e. that responsible for the termination of transmitter action.

Increases in the variability of action potentials, "jitter", are seen with neuromuscular disease and also after intoxication with an anticholinesterase (anti-ChE), although the mechanism by which this jitter is caused are not entirely clear. An attempt has been made to clarify the origin of jitter by recording trains of action potentials at 1 and 30 Hz.. The data obtained suggests that jitter is a functional disorder caused by a postsynaptic mechanism.

The effect of multiple doses of the carbamates pyridostigmine and physostigmine, by implantated osmotic pump, on jitter, prolongation of the timecourse of $(MEPPs)_o$ and the deformation of the endplates have been measured. These results have then been compared with the activity of functional AChE. Pyridostigmine and physostigmine caused similar reductions in the activity of functional AChE resulting in similar prolongation of $(MEPPs)_o$ and deformation of the endplate. Both carbamates also caused an increase in jitter although the onset of this occurred sooner with physostigmine.

Single doses of carbamates have been shown to protect against poisoning with organophosphorus anti-ChEs. Here, the protective abilities of continuous administration of low doses of pyridostigmine and physostigmine against a dose of ecothiopate were tested. Complete protection from all of the effects of ecothiopate were not found with either pyridostigmine or physostigmine, although the protection found with physostigmine was considerably greater.

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DEDICATION

This work is dedicated to my father, without whose encouragement I would not have completed this research. He may no longer be with us, but his spirit lives on. Thank You, Dad, for everything.

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ABBREVIATIONS

Abbreviations used within the text

ACh	Acetylcholine
AChE	Acetylcholinesterase
Anti-ChE	Anticholinestetrase
EPP	Endplate potential
MEPP	Miniature endplate potential
$(MEPP)_o$	Extracellularly recorded miniature endplate potential
Trise	Rise time of extracellular miniature endplate potential
T50%	Time from peak to half amplitude of extracellular miniature endplate potential decay phase
W50%	Width of extracellular miniature endplate potential at 50% amplitude
20-80%R	Time from 20-80% amplitude of the rising phase of extracellular miniature endplate potentials
80-20%D	Time from 80-20% amplitude of the decay phase of extracellular miniature endplate potentials
Area	The area under the curve of extracellular miniature endplate potentials
Delay	Difference in latency between the 16th and 1st action potentials in a train
MCD	Mean consecutive difference of latencies of action potentials 11-30 in a train
W/L ratio	Width to length ratio of endplates
ECO	Ecothiopate
PYR	Pyridostigmine
PHY	Physostigmine

INTRODUCTION

CHAPTER 1

1.1 The aims of the investigation

All experiments contained within this study were carried out as part of a joint study with Mrs A. Rowbotham, whose experimental data has been compared with the electrophysiological findings.

The initial experiments within this study were an attempt to improve on the work of Ferry and Marshall (1971) and Bamforth (1989) who tried to determine the relationship between biochemical and electrophysiological methods of measuring the activity of AChE. It was hoped that a method for determining just functionally active AChE could be developed which would then allow comparisons of other effects with the activity of functional AChE.

Increases in neuromuscular jitter are seen with diseases, e.g. muscular dystrophy and also after single *in vivo* injections of anticholinesterases (anti-ChEs) (Kelly *et al*, 1990). The aim of this study was to determine if such inceases in jitter are found with continuous administration via an osmotic pump, and how any such increases are related to other changes in neuromuscular morphology or function after anti-ChE treatment, i.e. endplate shape, MEPP timecourse or AChE activity

A single dose of a carbamate prior to poisoning with an organophosphorus anti-ChE has been shown to protect against the toxic effects of the organophosphate (Kelly *et al*, 1992). Within this study an attempt has been made to determine whether a carbamate given by continuous administration is able to offer the same protection.

An attempt has also been made to clarify the mechanism by which increased neuromuscular jitter is caused.

1.2 Morphology of the synapse

The structure of the mammalian motor endplate can be seen in Figure 1.1. It shows a chemical synapse where there is no direct contact between the axolemma and sarcolemma. The width of this primary synaptic cleft varies between 20-60 nm depending on the muscle and the animal. The morphology of the muscle cell below the axon is specialised with increased numbers of mitochondria and nuclei within the cell and the sarcolemma is also folded forming secondary clefts which extend into the muscle. Heuser and Salpeter (1979) used electron microscopy to show that these secondary clefts contain a network of fibres, the basal lamina which Zacks and Blumberg (1961) compare to mucoprotein or mucopolysaccharide basement membranes.

Although the exact arrangement of these secondary folds differs they have been found in reptiles (Robertson, 1956), amphibians (Birks et al, 1960) and mammals (Zacks and Blumberg, 1961) where the width was 50-100 nm, the depth 500-1000 nm and the gap between them up to 2 μm.

The prejunctional membrane, i.e the axolemma, has also been shown to have areas of thickening which correspond with the peaks of the secondary clefts and Birks (1960) suggested that these may be the areas from which the vesicles are released.

The postjunctional membrane is further specialised by the presence of acetylcholine receptors which have been found to be unevenly distributed being more densely packed on the peaks of the secondary clefts, as many as 25000 per μm^2; but very few have been found in the secondary clefts at depths below 250 nm (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978).

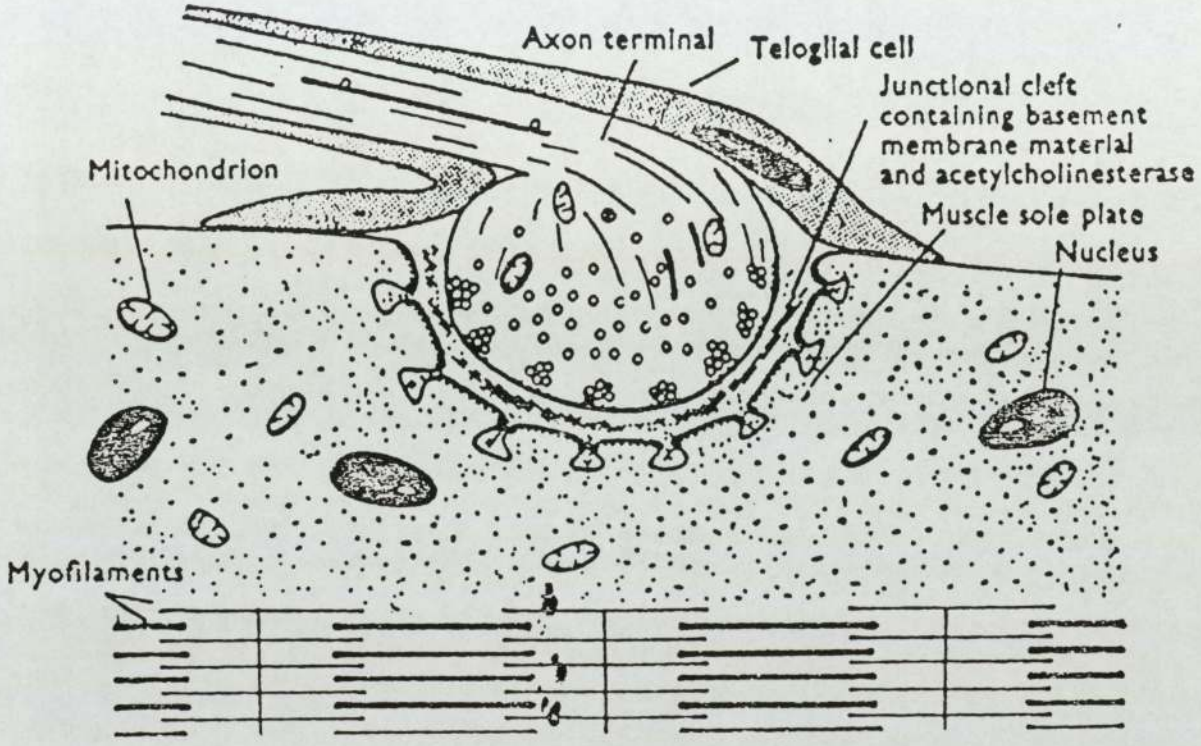


Figure 1.1 Diagrammatic representation of the morphology of the neuromuscular junction showing the presynaptic and postsynaptic regions of the synapse. (Bowman, 1980).

1.3 Transmission across the synapse

When an action potential arrives at the nerve terminal it opens voltage dependent calcium channels causing an influx of Ca^{2+} ions which triggers the release of pre-packed vesicles of transmitter, acetylcholine (ACh). The ACh crosses the synaptic cleft, which is very narrow and ACh can diffuse this short distance very rapidly, a few microseconds. Once it has crossed the cleft it combines with ACh receptors, 2 ACh molecules per receptor.

Any free ACh is rapidly hydrolysed by the AChE present in the cleft associated with the basement membrane. This rapid hydrolysis prevents ACh from diffusing very far laterally along the cleft so the action of a quantum is probably confined to a small area about $2\mu m^2$ (Hartzell *et al* 1975). The ACh molecules released in a quantum will be almost enough to saturate all the receptors in the small area meaning a substantial proportion of the molecules released bond, two molecules per receptor, whilst the rest diffuses or is hydrolysed by AChE. This binding causes most of the ion channels associated with the receptor to open and these remain open for a millisecond or so. The concentration of free ACh in the synaptic cleft falls much more rapidly than this however due to binding to receptors and the rapid hydrolysis of ACh, see Figure 1.2. An ACh molecule will not normally interact with the receptor more than once before it is hydrolysed or diffuses away.

The binding of ACh to the receptors opens associated ion channels allowing an influx of small cations into the muscle (Takeuchi and Takeuchi, 1959; 1960; Takeuchi, 1963). The ion movements resulting are an influx of sodium ions and efflux of potassium ions both through the same channel, which has a conductance of 50 -70 pS. Channel opening therefore produces a short circuit across the membrane reducing the membrane potential from rest, -70 μV, to a value near zero. A single channel when open causes a depolarisation of only $0.5\mu V$, which is too small to be seen directly. One quantum

however will open 1000 - 2000 channels almost simultaneously resulting in a total depolarisation of 0.5 to 1 mV, the miniature endplate potential.

The flow of ions produced by ACh depolarises the resting membrane potential (-70mV) primarily by an influx of Na^+ ions but with an associated efflux of K^+ which prevents the endplate potential (EPP) from depolarising beyond the zero potential level. The membrane actually depolarises to about -15mV (in frogs), the null potential, which is above the threshold of excitation meaning that the pre synaptic voltage gated Na^+ channels are opened as well as the transmitter mediated channels allowing an action potential to be propagated.

After the ACh receptor associated channels close the ACh is released back into the synaptic cleft where it is rapidly hydrolysed to choline and acetic acid (see section 1.3). Acetylcholinesterase (AChE) has one of the highest rates of activity for any enzyme (Silver, 1974). Berry (1951) and Wilson and Harrison (1961) estimated that each enzyme site can hydrolyse $1.6X10^5$ to $8.3X10^5$ molecules of ACh per minute. Hobbiger (1976) showed that there are at least ten ACh receptors and ten active enzyme sites per molecule of ACh released and he suggested that although the concentration of ACh in the cleft will partially inhibit enzyme activity there will still be enough activity to lower the ACh to inactive levels within 1ms, which is well within the refractory period of the muscle membrane (Wilson, 1971), and so prevents the accumulation of ACh from one action potential to the next.

Fatt and Katz (1952) found that as well as muscle membrane changes caused by nerve or muscle stimulation there are small, spontaneous depolarising potentials in the region of the frog motor endplate. The frequency of these events varied from 0.1-100 Hz and they had the same localisation, time course and pharmacological sensitivities as larger evoked endplate potentials (del Castillo and Katz, 1956; Liley, 1956a; Brooks, 1956; Thesleff,

1960). The size of the spontaneous events though was only about 1% of the EPP (Takeuchi and Takeuchi, 1960) so they are called miniature endplate potentials (MEPPs).

If ACh is applied ionophoretically to the post junctional membrane a discrete depolarisation is not seen, instead there is a graded depolarisation which indicates that a MEPP is produced not by a single molecule of ACh but rather by the synchronous release of 100 or more molecules (Fatt and Katz, 1952; Liley, 1956b; Katz and Miledi, 1972). When the bathing solution is manipulated to reduce EPP amplitude, i.e. raising Mg^{2+} and lowering Ca^{2+}, EPPs show stepped changes in amplitude where the size of the steps correspond to the size of a MEPP which sugests that the EPP is made up of a number of MEPP sized depolarisations, a suggestion confirmed by del Castillo and Katz (1954). They showed how the number of MEPPs making up an EPP conforms to the Poisson distribution for random events. Later researchers however have found that there may be deviations from the Poisson distribution, (Wernig 1975). This deviation is caused by the limited number of viscles available for release or by the limited number of release sites. Accounting for these limitations, the number of quanta released conforms more to a binomial distribution. However if the number of vesicles available is large the binomial distribution approaches the Poisson distrbution, (Stein 1980).

It has been shown therefore that the MEPP is the minimum functional unit of ACh release, with the exception of non-quantal release. The minimum unit is called the quantum and the post junctional potential it causes the quantum potential. The number of these quantum potentials which make up the EPP is the quantal content. There is some variation in the size of the MEPP from one muscle to the next but this can be mostly accounted for by the electrical properties of the muscle (Katz and Thesleff, 1957).

1.4 Principles of extracellular miniature endplate potential, and jitter recording

It is possible to record MEPPs externally using an electrode placed focally over the endplate region and this is the technique employed within this study. Using this technique what is actually recorded is a localised ion current caused by the different membrane potentials at the synapse where transmitter mediated channels are open allowing an ion flux and the adjacent membrane which is still near its resting potential. These are referred to as extracellular miniature endplate potentials [$(MEPPs)_o$].

Figure 1.2 illustrates the principle of (MEPP)o recording showing the current flow within the muscle fibre.

Although the shape of extracellular MEPPs and intracellular MEPPs is the same there are some differences, as the nature of the event is different. The polarity of the event is reversed as the microelectrode is negative with respect to the bath, and their time course is shorter, because with extracellular records the membrane capacitance has no effect. Care must be taken in positioning the electrode however as too much downwards pressure will result in an artificial lengthening of the $(MEPP)_o$ timecourse due to constriction of the diffusion path (Katz and Miledi, 1973).

(MEPPs)o have the same sensitivity as EPPs to the action of anti-ChEs due to their dependence on AChE activity and so have been used in these experiments as an indication of the activity of functional AChE, i.e. that AChE responsible for the termination of transmitter action. (MEPPs)o were used as there was no need to attempt voltage clamping which although giving an accurate record of endplate action is a difficult technique where 2 electrodes need to be placed in the same cell and a lot of current is needed to be passed through the clamping electrode. The experiments here were carried out at 37°C which further complicates voltage clamping.

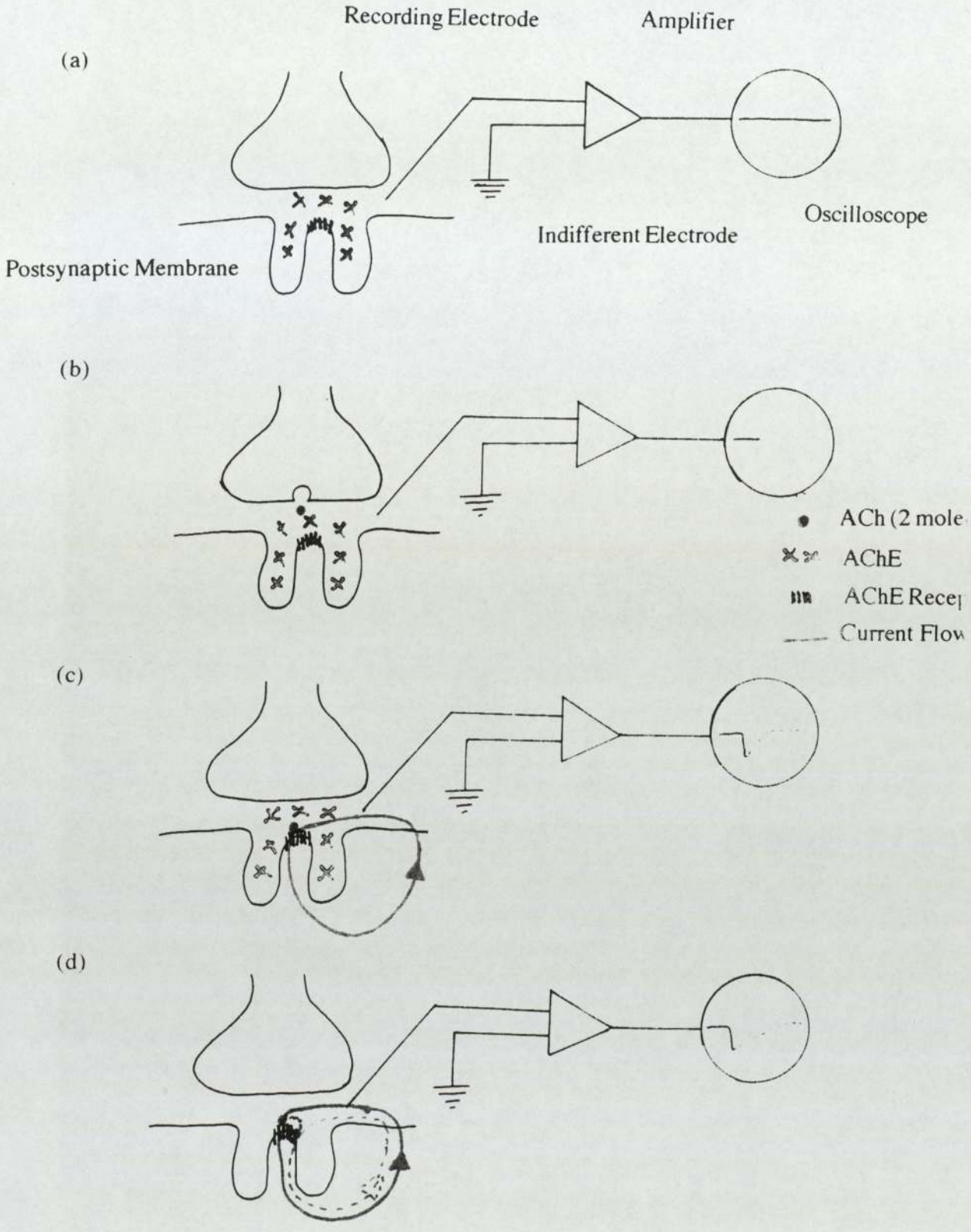


Figure 1.2 The principles of recording extracellular miniature endplate potentials (MEPPs)o showing the synapse a) at rest, b) upon the spontaneous release of a quantum of ACh, c) when there is no Anti-ChE present and d) in the presence of an anti-ChE - note the presence of the second current caused by the rebinding of the ACh molecules.

When recording the action potentials from two muscle fibres in the same motor unit there is variability in the time intervals between the two muscle fibre potentials at consecutive discharges. This "jitter" is due to variability in the transmission time from the point of stimulation to the area from which the recording is made. (Ekstedt, 1964)

Single fibre electromyography is a clinical technique developed by Stalberg and Trontelj (1979) which involves extracellular recording of action potentials in 2 single muscle fibres of the same motor unit. It is a technique which measures the variability of the latency of action potentials 'jitter', excessive amounts of which is a sensitive indicator of neuromuscular disease.

The variability will occur in different types of recordings. There may be variability in the time between the action potentials of two fibres in the same motor unit during voluntary activity (Stalberg and Trontelj, 1979), or in the time between stimuli and action potentials in a train of evoked responses (Trontelj et al, 1986). The variability is expressed as the standard deviation around the mean interpotential interval. Measurements of jitter gained however can be inaccurate due to the increased standard deviation which can be caused by slow trends superimposed on the short-term random variation. A more accurate way of expressing jitter is to measure the mean consecutive difference of a train of action potentials which measures short term variability but is relatively unaffected by slow trends (Ekstedt *et al* 1974, Stalberg and Trontelj, 1979).

Kelly *et al* (1990) modified the technique of single fibre electromyography to record stimulated action potentials from mouse diaphragm in vitro. In this technique the action potentials are recorded intracellularly as this ensures that succesive action potentials are recorded from the same part of the muscle fibre, and so eliminate any variability in delay caused by the recording of action potentials from different loci. They then went on to develop computer programs to analyse these action potential trains to investigate jitter after treatment with ecothiopate. The computer programs measure the delay for each of

the action potentials in the train and then calculates the mean consecutive difference of these.

It is this technique of Kelly *et al* (1990) which has been used in this study to determine the long term effects of low doses of anti-ChEs.

1.5 Acetylcholinesterase

Cholinesterases can be broadly divided into two categories true cholinesterase, which is found in nervous and striated muscle tissue, and pseudo cholinesterases, which are found in plasma, intestine, skin and other tissues. The role of pseudocholinesterases, the main one of which is butyrylcholinesterase, is not known but true cholinesterase is that which hydrolyses ACh.

Early attempts to isolate and find the structure of cholinesterase led to discrepancies in the molecular weight, the estimates differing from 30 million to 70,000 Daltons (Rosenberry *et al.*, 1972; Silver, 1974; Rosenberry, 1975). The discrepancies were due to the crudity of the techniques used and it was not until Bon (1979) studied this problem that AChE was properly classified when six molecular forms were found. Three of these forms were globular and three asymmetric (Figure 1.3).

The three globular forms were called G1, G2 and G4 as they were found to be made up of a number of identical chains each with a molecular weight of 71,000 Daltons where the number of chains is reflected in the name. The three asymmetric forms were called A4, A8 and A12 where the number again represents the number of the single chains. These forms are actually made up of one, two or three of the G4 forms connected to a collagen tail which is 50 nm long and has a molecular weight of 100,000. Massoulie and Bon (1982) suggested that the collagen tails hold the enzyme in place as they are a component of the basal lamina.

It is the asymmetric forms of the enzyme which are thought to be the functional enzyme and A12 in particular, where functional enzyme is defined as that isoform which is responsible for terminating transmitter action by hydrolysing ACh. It is thought that the synthesis of AChE is carried out inside the muscle cell and the asymmetrical forms are packaged into transport vesicles and are then transported to the cell surface where they are

incorporated into the basal lamina. Rotundo and Fambrough (1980b) suggested that AChE and the ACh receptor follow a single intracellular transport pathway as they are transported to the cell surface with similar kinetics.

The role of AChE at the neuromuscular junction is to hydrolyse ACh, which takes place in four stages. AChE consists of two binding sites an anionic site and an esteratic site (Figure 1.4a). The first stage of hydrolysis is the binding of ACh to AChE, the cationic head of ACh binds to the anionic site by its quarternary nitrogen ion and the ester group combines to a serine residue at the esteratic site. The bond at the anionic site is an ionic bond which is weaker than the covalent bond formed at the esteratic site, so the latter is more persistent. After attachment the enzyme-substrate compound undergoes hydrolysis and then the weaker ionic bond dissociates removing choline from the anionic site and leaving the enzyme acetylated. The final step is deacetylation which occurs rapidly. The final products of the hydrolysis then are choline and acetic acid, which are taken up by the nerve terminal to synthesise new transmitter, and regenerated enzyme (Figure 1.4b).

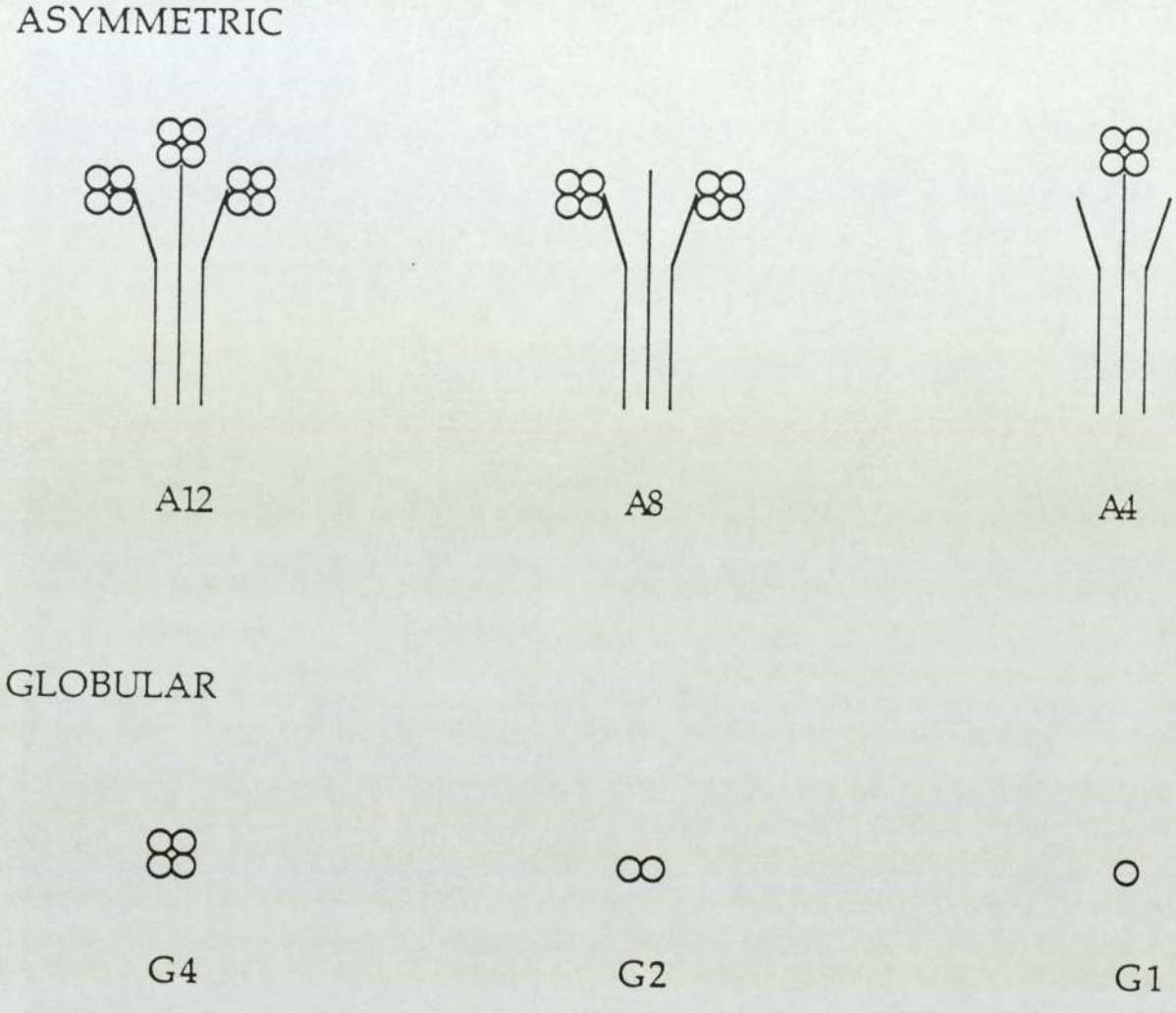
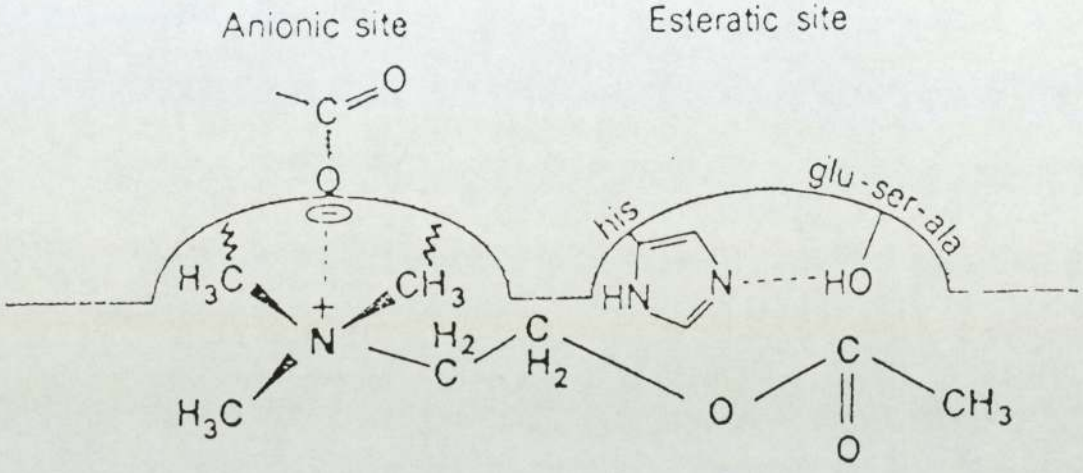


Figure 1.3 The molecular isoforms of acetylcholinesterase.

(a)



(b)

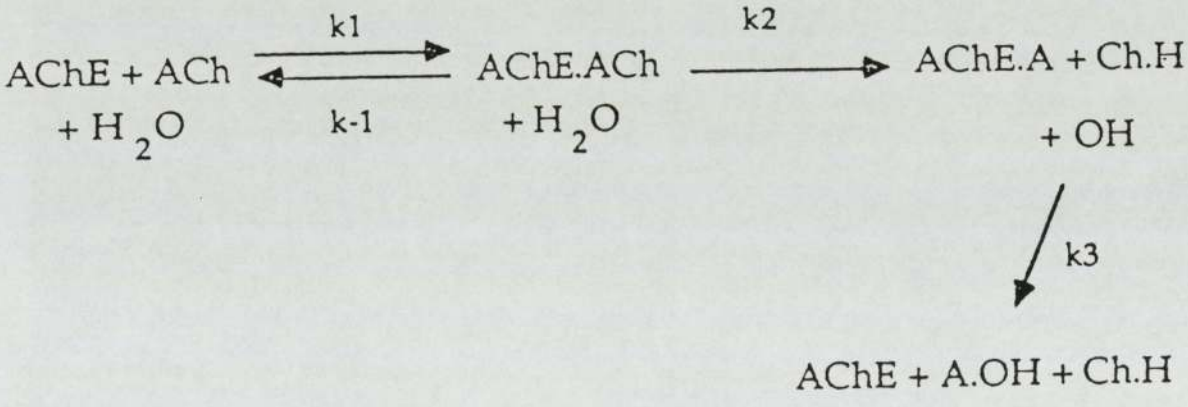


Figure 1.4 Diagramatic represntations of a) acetylcholinesterase structure and b) its mode of action. The structure illustrates the two binding sites and the mode of action shows the hydrolysis of ACh.

1.6 Anticholinesterases

1.6.1 Actions of Anticholinesterases

Drugs which have anticholinesterase (anti-ChE) properties can be divided broadly into two categories, those which dissociate quickly from the enzyme (e.g. edrophonium) and those which form a much longer lasting and stable enzyme complex. This second category can be further divided into two subgroups, carbamates which are reversible over tens of minutes and organophosphates some of which are reversible over tens of hours or days. In this study two carbamates and one organophosphate were used, pyridostigmine, physostigmine and ecothiopate respectively.

Figure 1.4a shows the enzyme sites and how ACh combines with it and is hydrolysed. Anti-ChE's intefere with one or more of the stages of hydrolysis and so allow the transmitter to exert a prolonged effect.

Pyridostigmine and physostigme are both carbamates and so attach to both the anionic and esteratic sites where there is hydrolysis of the enzyme-substrate compound. The bond with the anionic site is then dissociated leaving carbamylated enzyme. In order for the enzyme to be regenerated decarbamylation has to occur and this is a slow process compared to the deacylation carried out when ACh is being hydrolysed. The actions of carbamates such as pyridostigmine which have a quarternary nitrogen ion are further complicated by their direct nicotinic action at the skeletal neuromuscular junction, some, e.g. pyridostigmine interact with the ACh receptor and act as a weak agonist while others e.g. physostigmine interact directly with the ACh receptor associated channels. (Albuquerque *et al.*, 1984). There are some differences between pyridostigmine and physostigmine however, firstly their duration of action; pyridostigmine has a half life of approximately 30 minutes *in vivo* whereas that of physostigmine is shorter. Another

important difference is their area of action, pyridostigmine acts only on the periphery whereas physostigmine readily passes the blood/brain barrier (Koelle, 1963).

Ecothiopate is an organophosphate and attaches to the esteratic site of the enzyme. Organophosphates are non selective inhibitors, i.e. they don't only inhibit AChE but those which like ecothiopate have a quarternary nitrogen are thought to be more selective as there will be some interaction between the enzyme anionc site and the positively charged nitrogen ion. Once ecothiopate is bound, the enzyme-substrate compound is hydrolysed but unlike ACh and pyridostigmine there is no dissociation of the bonding which leaves the enzyme phosphorylated. To regenerate the enzyme it must be dephosphorylated but this is only one of the processes which can occur.

With organophosphates there is also the possibility of ageing, where one or more of the R groups is hydrolysed resulting in a highly stable form of AChE (Figure 1.5). Once the AChE-inhibitor complex has aged it can not be reactivated either spontaneously, or by the use of oximes. Fortunately ecothiopate shows slow spontaneous reactivation but ages slowly (Hobbiger, 1976) which means that if treatment with antidotes is rapid the enzyme can undergo some regeneration by the use of oximes.

Due to their reversible nature which results in a comparatively rapid enzyme reactivation carbamates can be used as a prophylactic treatment against organophosphate poisoning (Berry and Davis, 1970; Dirnhuber and Green, 1978; French *et al.*, 1979; Kelly *et al.*, 1992). Treatment with a carbamate such as pyridostigmine will cause some of the AChE to be inhibited, and after exposure to an organophosphate the remaining AChE will be inhibited. The carbamate inhibition will soon be dissociated though so that this enzyme will be regenerated, ready to carry out its function.

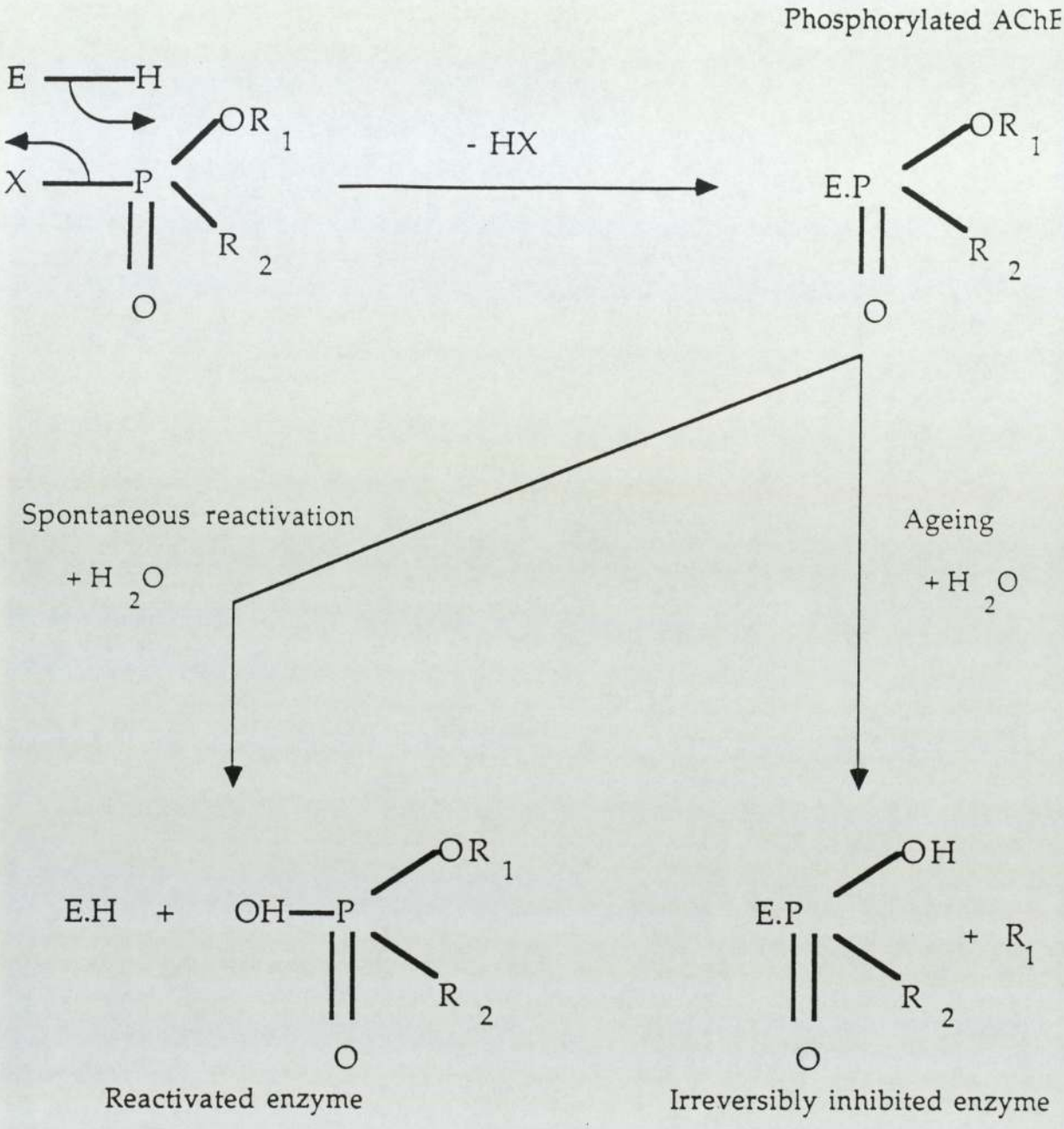


Figure 1.5 The inhibition of AChE by anti-ChEs, showing phosphorylation, spontaneous reactivation and ageing.

1.6.2 Effects of Anticholinesterases

Anti-ChE's have effects which are both muscarinic and nicotinic. The muscarinic effects e.g. nausea, diarrhoea, sweating, abdominal cramps, bradycardia and hypotension can all be alleviated by injection of atropine (Grob, 1956; Grob and Johns, 1958; Durham and Hayes, 1962). The nicotinic effects are seen as muscle fasciculations and cramps as well as fatigue and weakness but are not so easily alleviated (Durham and Hayes, 1962).

Along with the effects at the neuromuscular junction there can be resultant damage around the endplates seen in rat and mouse muscle (Wecker *et al.*, 1987; Gupta and Detbarn, 1987; Townsend, 1988; Das, 1989).

Anti-ChE's also have effects on single twitches where they potentiate the response to low frequency indirect stimulation in frog muscle (Brown *et al.*, 1936) and in the absence of any stimulation can produce spontaneous twitching of the muscle (Modell *et al.*, 1946; Meer and Meeter, 1956; Hobbiger, 1976).

The effects of carbamates on the neuromuscular junction have been studied extensively in both amphibian and mammalian muscle. As early as 1942 a prolongation of the EPPs at frog neuromuscular was seen after treatment with physostigmine (Eccles *et al*, 1942). Alerdice (1982) investigated the effects of physostigmine and neostigmine at the frog neuromuscular junction using intracellular recording, finding an increase in the amplitudes of MEPPs but no effect on MEPP frequency. Similar results were found by Fedorov (1976) when the effects of neostigmine were investigated at the frog neuromuscular junction, however an increase in the rise time and decay time of EPPs was also found. Further research was carried out by Kordas *et al* (1975) into the effects of physostigmine and prostigmine were the effect was found to depend upon the concentration of the drug at the frogs neuromuscular junction. At low concentrations the amplitude rise time and half time of the EPC were increased but at higher concentrations these were depressed.

Guinan (1980) used neostigmine on frog muscle and found results consistent with the previous studies. In the rat diaphragm Kuba and Tomita (1971) found that the decay of the EPP was increased by the addition of prostigmine, results which have been repeated by several investigators (Kordas, 1972; 1977) and also with other carbamates e.g. neostigmine (Blaber and Christ, 1967).

METHODS

CHAPTER 2

2.1 Animals

Male albino mice, purchased from Bantin and Kingman (Hull), were fed *ad libitum* on a breeding diet, with free access to water. They were ex-breeders aged 6-7 months weighing 40-50g. Animals of this age were used as Kelly *et al* (1978) and Banker *et al* (1983) showed that in CFHB rats and CBF-1 mice their neuromuscular properties such as resting membrane potential, frequency of miniature endplate potentials, numbers of acetylcholinesterase receptor and the safety factor of transmission are stable in young adult animals of this age. Stability is an important requirement in studies which last several weeks, so that developmental or aging changes are avoided.

2.2 Drugs

2.2.1 Pyridostigmine

2.2.1.1 Preparation of pyridostigmine for injection

Pyridostigmine bromide was made up from 1mg/ml ampoules of Mestinon (Roche) injection. This was diluted with distilled water to give a stock solution of 10^{-4}M. This stock solution was then diluted in physiological saline to give the required dose, for that particular experiment. For a "sign free" dose a 10^{-7}M solution was used. When a higher dose was administered, atropine (700 $nmolkg^{-1}$) was added to the injection to reduce the muscarinic effects, (Grob 1956, Grob and Johns 1958).

2.2.1.2 Preparation of pyridostigmine for infusion

Pyridostigmine bromide salt (Sigma) was dissolved into normal (0.9%) saline to give a dose of 11.4 nmolhr-^{1} when it was delivered by Alzet osmotic pump.

2.2.2 Physostigmine

2.2.2.1 Preparation of physostigmine for infusion

Physostigmine bromide salt (Sigma) was dissolved in normal (0.9%) saline to give a dose of 14 nmolhr^{-1} when it was delivered by Alzet osmotic pump.

2.2.3 Atropine sulphate

Atropine sulphate stock was a solution of 10 mg of atropine sulphate (Sigma) per ml of normal saline (0.9% NaCl). This was administered in a dose of 0.1 ml per 20g of mouse to give a final concentration of 700 nmolkg^{-1}.

2.2.4 Ecothiopate

Ecothiopate iodide was made up from Phospholine Iodide BNF eyedrops (Ayerst). The drops contain 12.5 mg ecothiopate iodide and 40mg potassium acetate in powder form and a diluent which contains 0.5% chlorobutanol, mannitol, boric acid and sodium phosphate. A 10^{-4}M stock solution was made by dissolving the dry powder in 3.26 ml of diluent which gives a 10^{-2} M solution which was further diluted 100 fold with atropine saline(2.2.3). This gave the solution for injection as 10^{-4} ecothiopate with atropine, which was injected at a dose of 0.1ml per 20g of mouse body weight to give a final concentration of 500 nmol kg^{-1} ecothiopate with 700 nmolkg^{-1} atropine.

All injections were given subcutaneously between the shoulder blades. The drug will then be absorbed by local capillaries and systemic administration will result.

2.3 Alzet Osmotic Pumps

The Alzet osmotic pumps used were models 2001 for 7 day delivery and 2002 for 14 day delivery. Both of these consist of an outer semipermeable membrane of cellulose ester blend and an internal drug reservoir made of thermoplastic hydrocarbon elastomer. Between the outer membrane and the drug reservoir is the osmotic sleeve which contains a high concentration of sodium chloride. They are 3cm in length, 0.7cm in diameter and have a total volume of 1ml, although both have a drug reservoir capacity of 200µl. They are fitted with a flow moderator which is comprised of a stainless steel tube (internal diameter 0.05cm) and an ethylene copolymer cap. Model 2001 has a drug delivery rate of 1µl per hour while that of model 2002 is 0.5µl per hour.

Figure 2.1 shows the cross section of an Alzet osmotic pump illustrating its components and mode of action.

It is the difference in osmotic pressure between the osmotic sleeve and the external environment of the implantation site that drives the delivery of the drug. The rate of delivery is kept constant by the presence of an osmotic driving agent that maintains constant osmotic activity.

The pump operates by water entering the osmotic sleeve via the semipermeable outer membrane so compressing the flexible drug reservoir and displacing the drug through the flow moderator into the external environment.

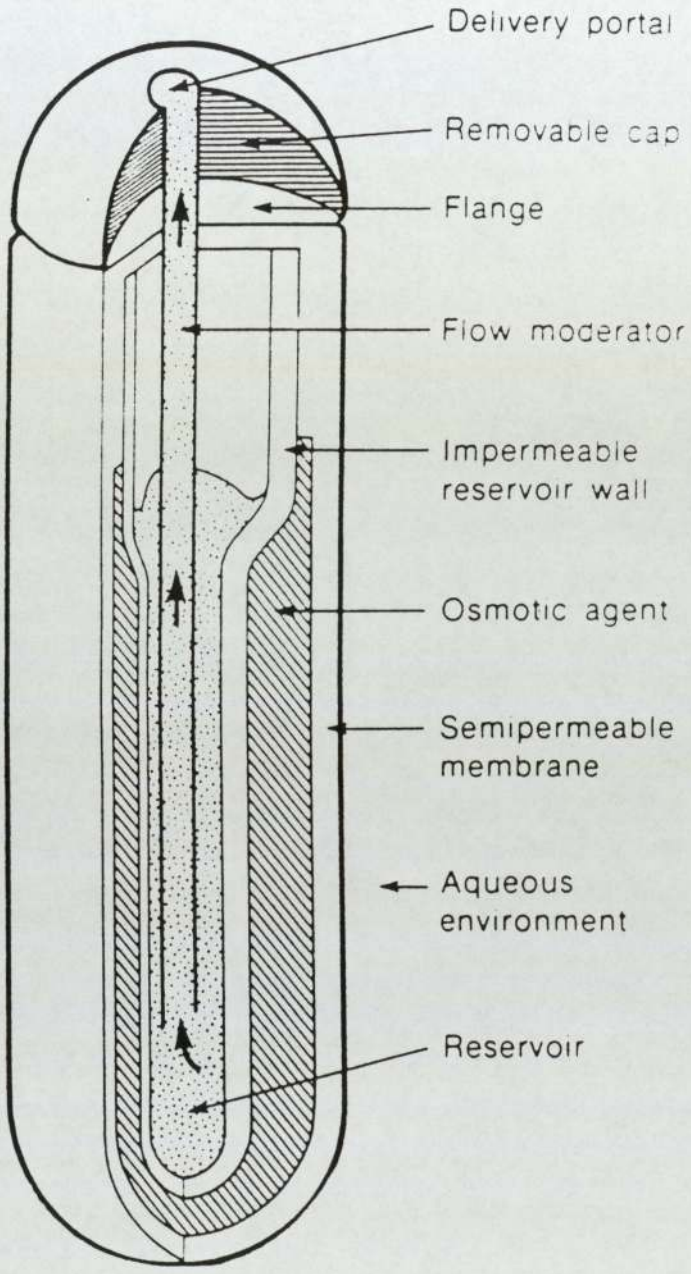


Figure 2.1 Diagram of the cross section of an Alzet osmotic pump showing its components and mechanism of operation.

2.3.1 Loading Alzet osmotic pumps

Throughout both loading and implantation the pumps were only handled with surgical gloves as skin oils may interfere with pump performance.

The pumps were loaded with the filling tube supplied. For both pump types this was a blunt ended tube of length 2.2cm, outside diameter 0.04cm and internal diameter 0.02cm. As sterility was of concern the sterile pumps were loaded through a bacterial 0.22μm syringe end filter (Millipore). The pumps must not contain any air bubbles as these may result in pumping rate fluctuations therefore it was important to ensure that the syringe containing the drug was free of air bubbles. With no flow moderator attached the pump was held in an upright position and the filling tube inserted into the opening until it would go no farther, this positions the tip of the tube at the bottom of the drug reservoir leaving no unfilled space as this would interfere with the pumping rate. With the pump still held upright the plunger of the syringe was slowly depressed until the drug solution appeared at the outlet, when loading was stopped and the filling tube carefully removed. The excess solution was then wiped off and the flow moderator inserted until the cap was flush with the top of the pump. Any drug solution which overflowed due to displacement was wiped off.

To ensure that that the pumps had been loaded properly, a selection were weighed before and after loading to obtain the net weight of the drug solution loaded. The volume of the drug solution could then be determined.

A pump loaded at room temperature with a drug solution also at room temperature placed in osmotic saline at 37°C will not reach a steady pumping rate for several hours. For the experiments it was essential that the pumps were pumping when they were implanted therefore it was necessary to "prime" the pumps before implanting. To achieve this the pumps were loaded and then placed in sterile normal saline in a sterile container, maintained at 37°C, for 4 hours before implanting.

2.3.2 Implanting Alzet osmotic pumps

The animals were anaesthetised using 3% halothane in 50% N_2O in O_2 and anaesthesia was maintained with 1.5% halothane. The scruff of the neck was shaved and a small midscapular incision made. A haemostat was inserted into the incision and the jaws opened, spreading the subcutaneous tissue, and so creating a pocket. The loaded pump was then inserted into the pocket delivery portal first in order to minimise any interaction between the drug delivered and the healing of the incision. The wound was then stitched up. The whole procedure lasted approximately 10 minutes and the animals recovered quickly from the anaesthesia showing no ill effects from either the operation or the presence of the pump.

A pump implanted subcutaneously will deliver the drug into the local subcutaneous space and absorption of the drug solution by local capillaries results in systemic administration.

2.4 Preparation of the diaphragm

The animals were killed by a blow on the head followed by cervical dislocation. The loose layer of skin and fur over the ventral surface of the thorax were lifted and an incision made from the region of the xiphisternum to the neck. This outer tissue was then removed to expose the thorax and upper abdomen.

The pectoral muscles and other underlying tissue were removed to completely expose the ribcage. The upper section of the sternum and most of the ribcage were then removed exposing the inner contents of the thorax.

The muscles of the abdomen were cut laterally dorsal to the last rib taking care not to damage the diaphragm.

When the experiment required indirect stimulation of the diaphragm the phrenic nerve was identified and tied off using surgical silk. The nerve was then carefully cut away from connective tissue so as to leave it attached solely by its insertion into the diaphragm. Typically it was the left hand hemidiaphragm which was used in these electrophysiological

experiments as the phrenic nerve on this side is more accessible. Experiments were carried out, however, to determine if there were any differences between the left and right hemidiaphragms in the parameters measured; none were found.

The pericardium and mesentery attached to the diaphragm on the thoracic and abdominal surfaces were removed and the posterior vena cava cut through so isolating the diaphragm and phrenic nerves from the surrounding tissue.

The diaphragm could then be completely removed from the animal along with the ribs and intercostal muscles still attached and immediately transferred to cold, gassed, Liley's physiological saline (Appendix 1).

The diaphragm could then be prepared by pinning it into a Sylgard covered petri dish which contained Liley's saline. The diaphragm was first cut into hemidiaphragms via the central tendon and then cleared of any blood clots, adipose or connective tissue, being careful not to damage the nerve. The ribs and abdominal muscle were then trimmed back and the hemidiaphragm was ready to be pinned out into the recording bath by the cut ribs and central tendon, or to be pinned out for histological staining.

All animals were dosed in groups of two and the hemidiaphragms divided between the different experimental methods. One from each animal was used for biochemical assay, and of the remainder one was used for electrophysiological recording and the other was stained for cholinesterase.

2.5 Electrophysiological methods

2.5.1 Measurement of Extracellular Miniature Endplate Potentials [$(MEPPs)_o$].

2.5.1.1 Recording $(MEPPs)_o$

The hemidiaphragm was dissected out and pinned in a water bath whose base was covered with Sylgard. The bath volume was 16ml and contained Liley's saline (Appendix 1) gassed with carbogen ($5\%CO_2$ in 0_2), constantly circulated and maintained at 37°C. The design of the bath was such that the flow of saline over the preparation caused a minimum amount of turbulence.

Recordings were made with capillary microelectrodes made from GC100TF-15 borosilicate glass (Clark Electromedical Instruments) with tip diameter 3-5μm pulled in two stages on a Kopf needle/pipette puller model 750 and filled with Liley's saline of the same composition as that in the bath.

The recordings were made using a WPI duo 773 electrometer and Devices AC preamp 3160, bandwidth 0.8Hz to 10KHz (-3db). The traces were displayed on a Tektronix dual beam oscilloscope D12 with a 5A81N dual trace amplifier and a 5B10N time base/amplifier. The events were recorded onto Maxell 25-120 magnetic tape using a RACAL store 4DS recorder and analysed off-line using Scope v3.2.1 on a Macintosh computer complete with a MacLab 4. Figure 2.2 shows a flow diagram of the equipment used when recording $(MEPPs)_o$.

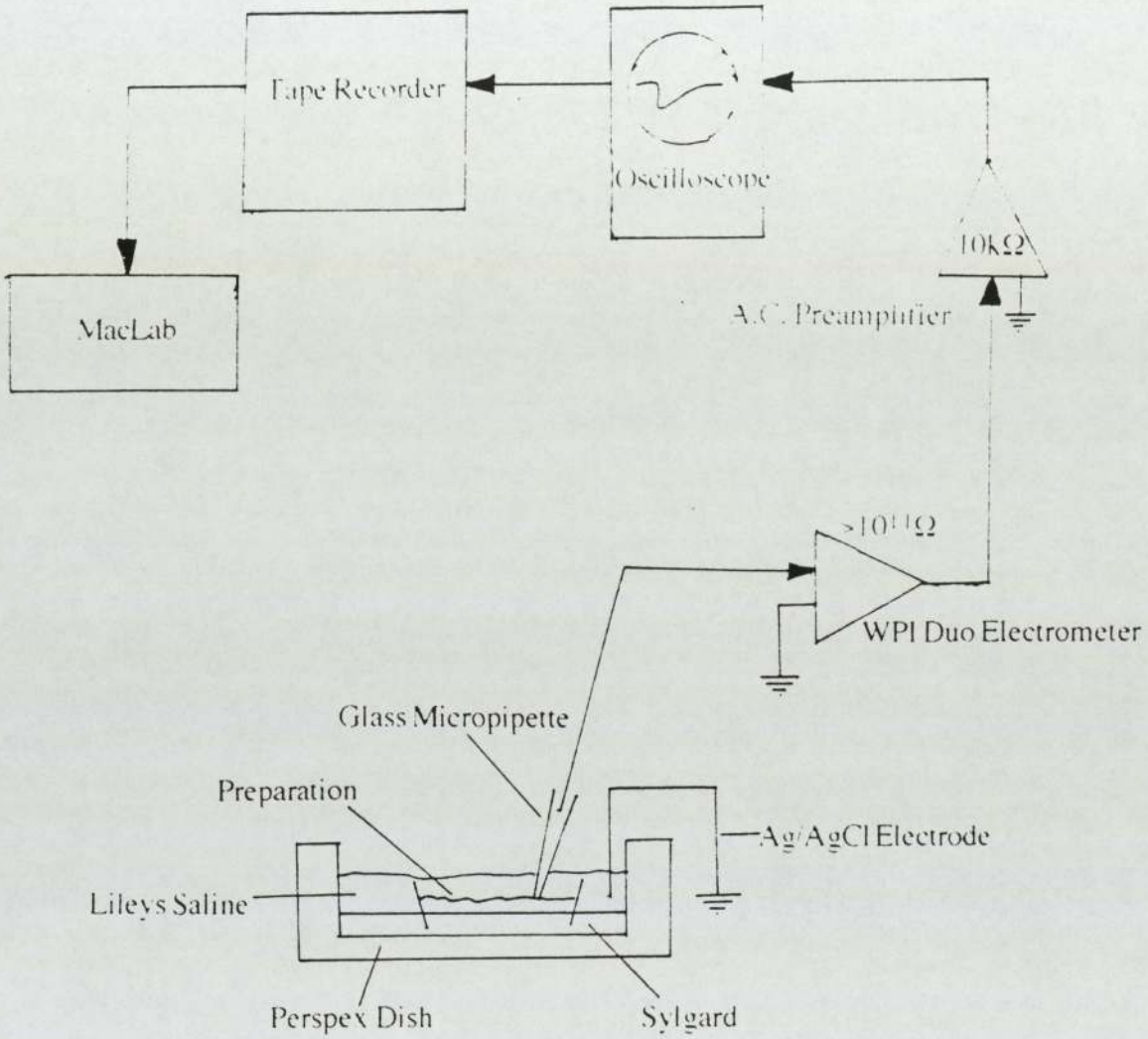


Figure 2.2 Flow diagram showing the equipment used during the recording of extracellular miniature endplate potentials. 10kΩ and >10^{11}Ω refer to the input resistance of the amplifiers.

The exact positioning of the microelectrode was crucial as a deviation of just a few microns could cause the $(MEPPs)_o$ to become unrecordable, they would decrease in amplitude to below the noise level.

The $(MEPPs)_o$ were found by placing the electrode over an area likely to contain endplates and then moving the electrode slowly across the sufrace of the preparation using a Leitz micromanipulator until $(MEPPs)_o$ appeared on the oscilloscope. Minimum downwards pressure was applied as this produced a distorted trace, with the timecourse of the $(MEPPs)_o$ being prolonged (Katz and Miledi, 1973). The amplitude of the $(MEPPs)_o$ recorded was typically 0.5 - 1.0 mV. Figure 2.3 shows the positioning of the electrode during recording of $(MEPPs)_o$.

For experiments where no drugs were added or the drugs were given *in vivo* this procedure was followed exactly. For experiments where drugs were introduced *in vitro*, prior to recording the hemidiaphragm was pinned on dental wax and incubated, in an organ bath, in Liley's saline containing the drug. The Liley's saline was maintained at 37°C and gassed with carbogen. The hemidiaphragm was then removed from this bath and transferred into the recording bath and the above procedure followed.

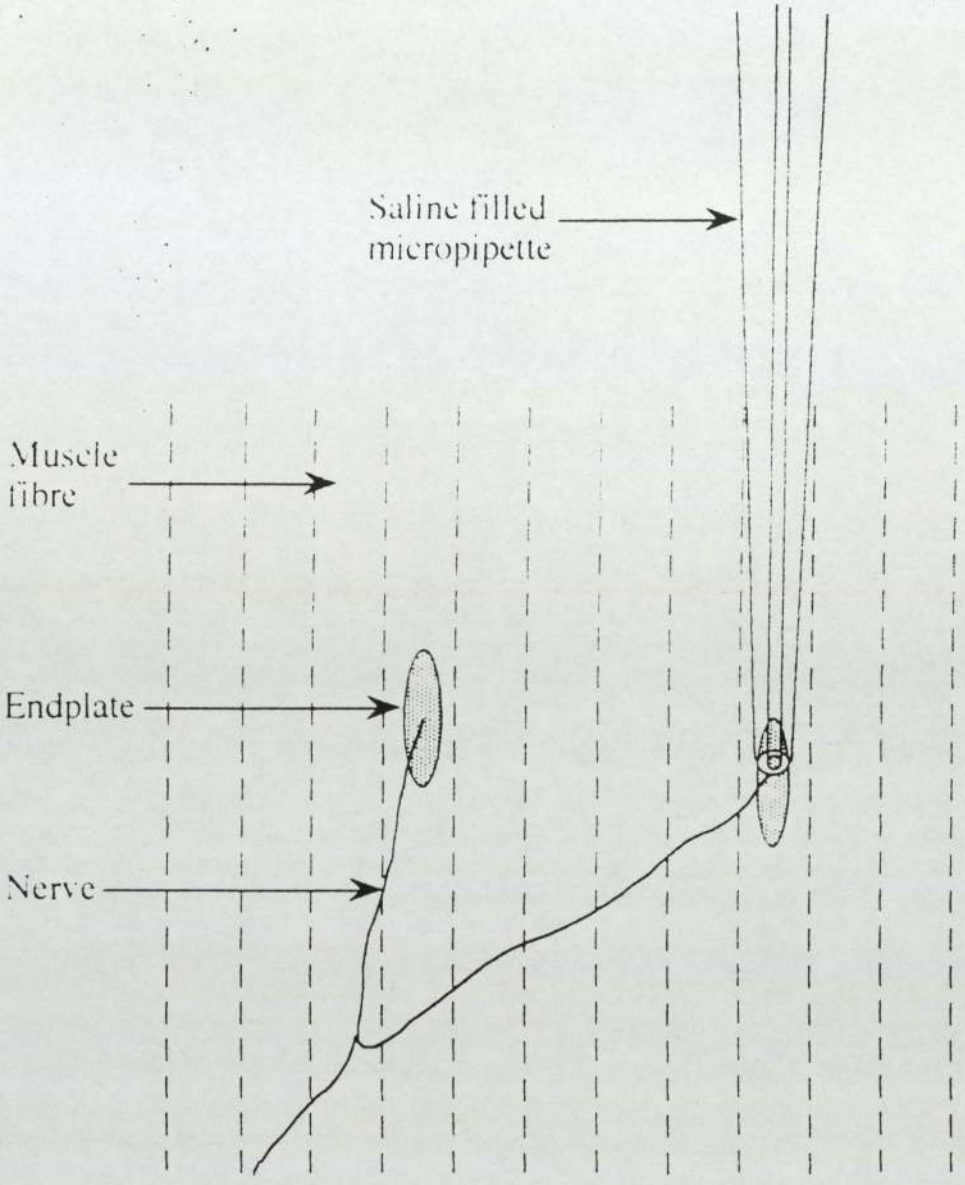


Figure 2.3 Schematic drawing showing the position of the Lileys saline filled glass micropipette during recording of extracellular miniature endplate potentials. Not to scale.

2.5.1.2 Analysis of $(MEPP)_o$ parameters

The analogue taped records of $(MEPPs)_o$ were digitised using the Scope program which allowed several parameters to be measured. Fig 2.4. shows examples of the typical traces recorded, with the measured parameters indicated.

The parameters measured were

a. Rise time of the event (Trise)

b. Time from peak amplitude to half amplitude of the decay phase (T50%)

c. Width of the event at half amplitude which is dependent on both the rising and decay phases (W50%)

d. Time from 20-80% amplitude of the rising phase (20-80%R)

e. Time from 80-20% amplitude of the decay phase (80-20%D)

f. The area under the curve which gives an indication of the total charge transferred. This was measured by integrating the trace produced using Scope v3.2.1 (Area)

The values used within this study are the averaged values from 5 cells within a diaphragm.

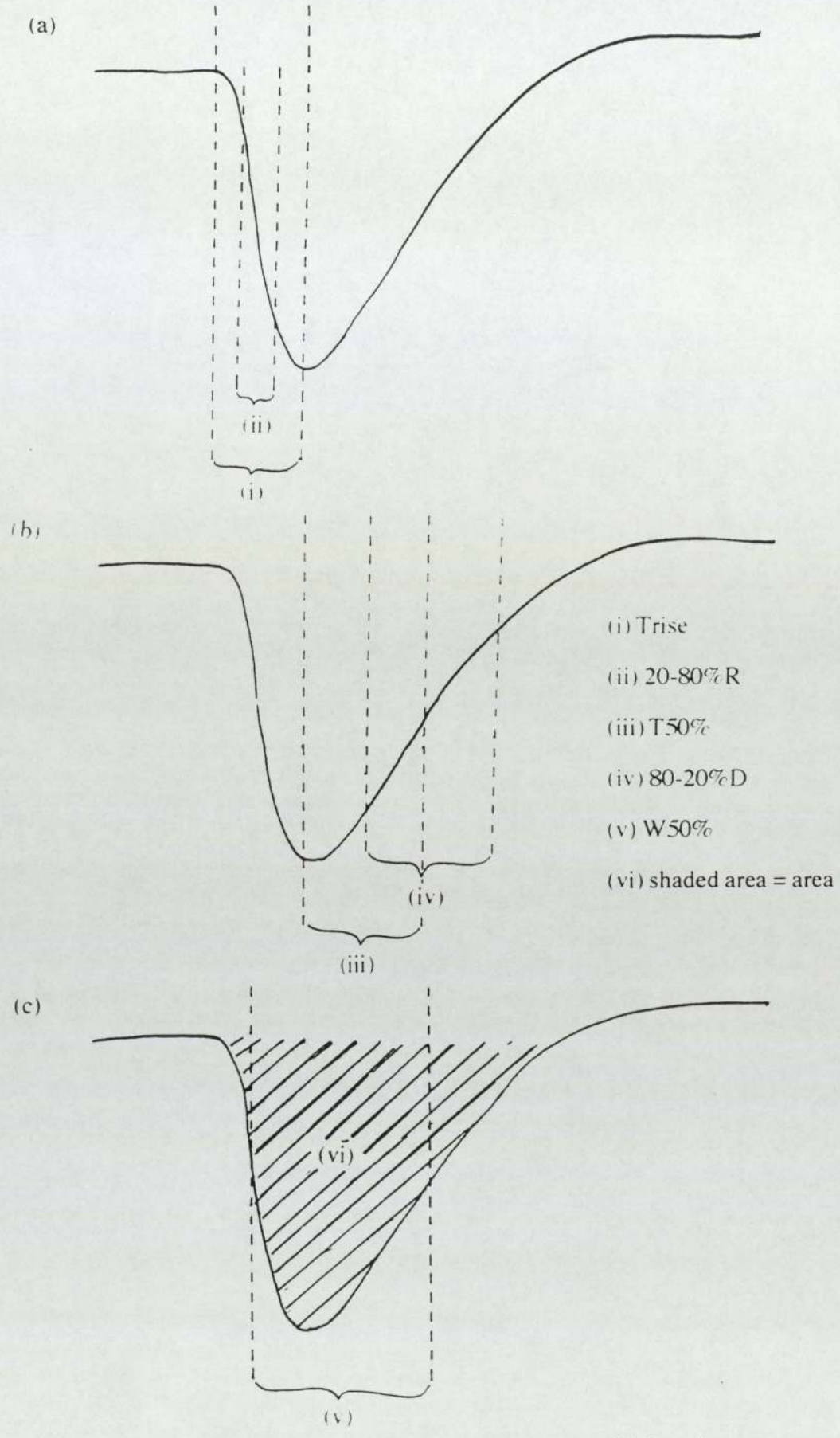


Figure 2.4 Drawing of extracellularly recorded miniature endplate potential showing the parameters which were measured during analysis. (a) shows those which measure the rising phase only. (b) shows those which measure the decay phase only and (c) shows those measuring both the rising and decay phases.

2.5.2 Measurement of "jitter" using intracellular single fibre electromyography

2.5.2.1 Recording intracellular action potentials

The hemidiaphragm was dissected out along with as much of its nerve as possible. It was then pinned out in a Sylgard coated perspex bath which was fitted with a circulating supply of Liley's saline constantly gassed with 95% O_2/5% CO_2 and maintained at 37°C. A Devices Digitimer type 3290 connected to a Digitimer DS2 isolated stimulator was used to stimulate the nerve via a tube or suction electrode with supramaximal pulses with a pulse width of 0.02msec. Excessive muscle movements which would pull the electrode out of the cell were prevented by pinning out the hemidiaphragm tightly using many pins.

Resting membrane potentials (RMP) and action potentials were recorded using an intracellular glass capillary microelectrode which was filled with 3M KCl. Recordings were made using a WPI duo 773 electrometer from which two outputs were taken . The first was to a Tektronix D12 dual beam oscilloscope with a 5A81N dual trace amplifier and a 5B10N time base/amplifier, allowing visualisation of the responses. The second output was to a Devices AC preamp 3160, bandwidth 0.8Hz to 10KHz (-3db), which amplified the signal 100x. This amplified signal was then recorded onto Maxell 25-120 magnetic tape using a RACAL store 4DS recorder at a tape speed of 30 inches per second. A trigger pulse was also recorded on this tape where the pulse occured 1msec before the stimulus pulse. Trains of 30 action potentials were recorded from each fibre with an average of 10 fibres being sampled from each hemidiaphragm. Fibres were rejected if (a) the RMP was more +ve than -65mV, (b) during the train of action potentials the RMP fell by more than 5mV, (c) the action potential amplitude was less than the RMP or (d) the train contained any failures.

For analysis of the action potentials the recordings were played back at 15/16 inches per second into an analogue-to-digital converter and PDP 11/03 computer. With a record/replay ratio of 32 and a sampling rate of 20KHz the effective sampling rate of the computer was 640KHz. The PDP 11/03 was triggered using a Devices Digitimer type

3290 which itself was triggered with the trigger pulse recorded during the experiment 1msec before the stimulus pulse. The taped trigger pulse is not used to trigger the PDP directly as this doesn't trigger reliably with minimum variability. Using the output pulse of the Digitimer to trigger the PDP gives much less intrinsic "jitter" especially if a large pulse is recorded on the tape. This allowed the correct sampling window to be determined and entered into the PDP 11/03.

Figure 2.5 shows a flow diagram of the equipment used when recording action potential trains and also the configuration of the equipment used for playing these back during analysis.

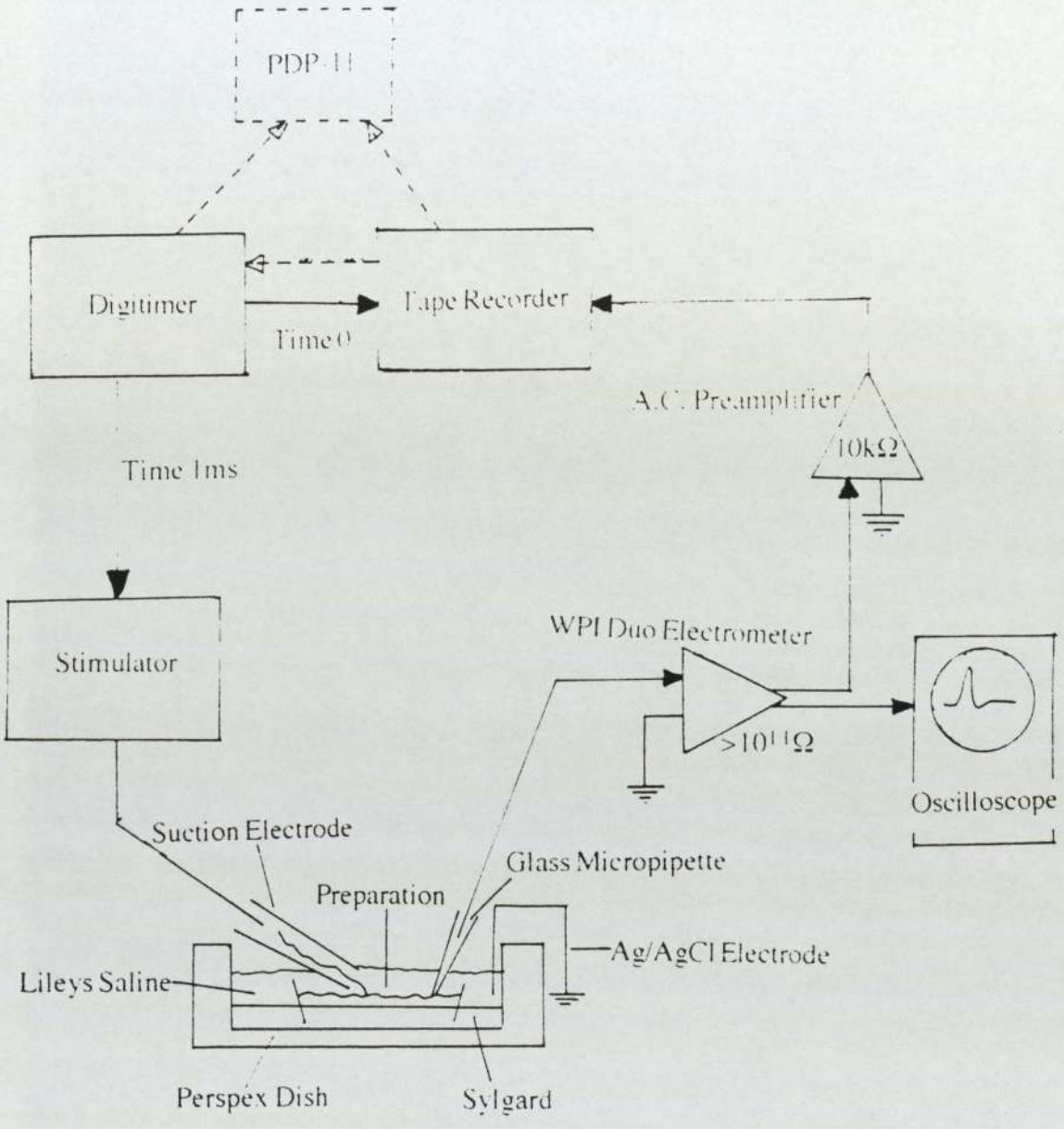


Figure 2.5 Flow diagram showing the equipment used during the recording of trains of action potentials. ---- indicates the configuration of the equipment during analysis when the recorded action potentials were played back. 10kΩ and >10^{11}Ω refer to the input resistance of the amplifiers.

2.5.2.2 Analysis of intracellular action potentials

Computer programs devised by Dr S.S. Kelly were used to analyse the action potentials. The amplitude, time course and latency of each action potential in the train was determined, where the latency was taken as the time interval between the stimulus and a point on the rising phase of the action potential at 10% of peak amplitude. From this data two parameters were calculated, the mean consecutive difference (MCD) of action potentials 11-30 and the difference in latency between the 16th and 1st action potentials (Delay). Figure 2.6 shows a typical train of action potentials with the Delay and MCD indicated.

The formula used for calculating the MCD of action potentials 11-30 was

$$MCD = \frac{[(L_{11}-L_{12})+(L_{12}-L_{13})+\ldots\ldots\ldots\ldots(L_{29}+L_{30})]}{19}$$

where L_X refers to the latency of action potential x

Action potentials 11-30 were used to calculate MCD as in control preparations the initial rundown of latency has reached a plateau by action potential 10.

The intrinsic error of the record/replay/analysis process was measured by evoking simulated action potentials using an accurate pulse generator and Digitimer to provide a train of pulses which were recorded and analysed as if they were action potentials. These trains of pulses gave an MCD of 2.4μs which means that the apparatus has a resolution limit of 2.4μs.

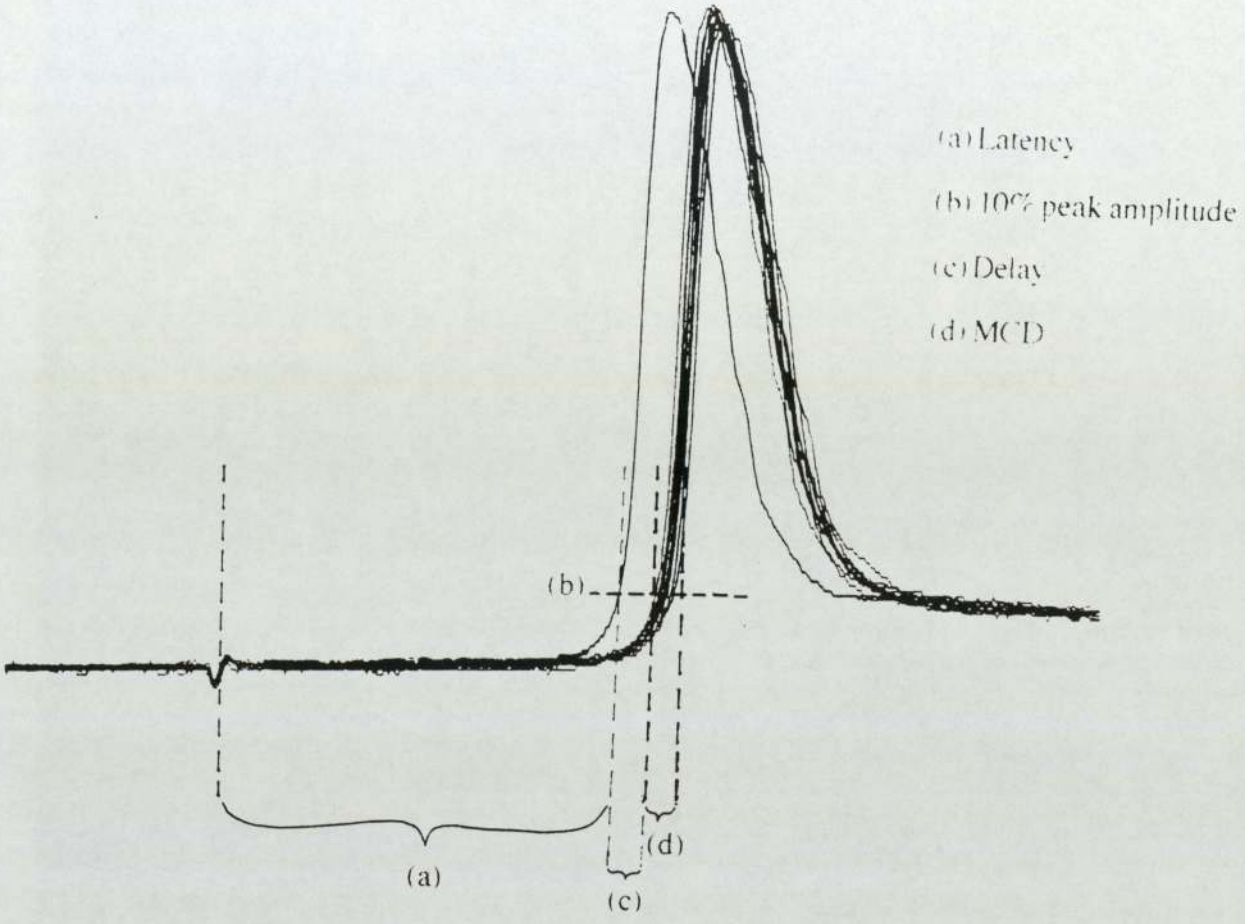


Figure 2.6 Typical train of action potentials showing the MCD and Delay. The point of 10% amplitude is marked as this is the position at which latency is read to determine the Delay and MCD.

2.6 Staining for Cholinesterase

Staining the diaphragm for cholinesterase is an easy way of visualising the endplates as it stains the dense concentration of cholinesterase which is at the endplate and therefore allows any changes in endplate numbers, size etc to be determined.

Diaphragms which had previously been fixed overnight in 4% formaldehyde were trimmed from the ribs and stained with the solution in the method of Karnovsky and Roots (1964) (Appendix 2). The process of staining was observed under a dissection microscope as the time at which optimum staining occurs differs according to the treatment the diaphragm has had. Control preparations stain in approximately 1 hour whereas those treated with an anticholinesterase need much longer to stain. The stained hemidiaphragms were then washed in distilled water and mounted in glycerol jelly (BDH). The preparations were viewed using a Zeiss microscope fitted with a x40 objective lens and a x10 eyepieces. An eyepiece graticule was fitted and the dimensions of the endplate i.e. length and width were measured. To give an easily comparable measure of endplate dimensions the width/length ratios were later calculated.

2.7 Statistical Tests

The details of all statistical tests used within this study can be found in appendix 3. When it was not shown that the data collected was normally distributed the statistical test utilised was the non-parametric Mann-Whitney test (Appendix 3.1), except for jitter which was analysed using the Kolmogorov-Smirnov test (Appendix 3.2). Spearmans Rank correlations were also calculated for some data (Appendix 3.3)

2.8 Biochemical Methods

The biochemical assay technique as used by A. Rowbotham is outlined in Appendix 4.

RESULTS

CHAPTER 3

The effect of ecothiopate on endplate function and shape

This investigation was carried out in two stages, the first was to determine the effect of dose of ECO on the timecourse of $(MEPPs)_o$, and the second was to determine how the timecourse was affected if a standard dose of ecothiopate was administered and $(MEPPs)_o$ recorded at different time intervals after dosing. The electrophysiological data gained from these studies has also been compared with biochemical and histological data to give a broader view of the effects of ecothiopate.

3.1 The effect of various doses of ecothiopate

Experimental Design

The mice used in this study were divided into groups, each treated with increasing doses of ecothiopate as outlined below.

700 $nmolkg^{-1}$ atropine treated controls

25 $nmolkg^{-1}$

50 $nmolkg^{-1}$

100 $nmolkg^{-1}$

300 $nmolkg^{-1}$

500 $nmolkg^{-1}$

For each experiment 2 animals were killed 3 hours after dosing and their diaphragms divided into hemidiaphragms. One hemidiaphragm from each animal was used for a biochemical determination of AChE activity and of the remaining 2 hemidiaphragms one was fixed and stained for cholinesterase while the final hemidiaphragm was transfered to

the recording set up for $(MEPP)_o$ measurement. When recording $(MEPPs)_o$ five cells were recorded from each hemidiaphragm.

Control preparations were taken from animals which had been given a dose of atropine saline and their diaphragms removed 3 hours later, although experiments showed no difference between these and untreated controls.

Results

Figure 3.1 shows typical records of $(MEPPs)_o$ recorded after different doses of ECO. For each single trace 25 $(MEPPs)_o$ were averaged in order to minimise the noise on the trace and it is from these averaged traces that all measurements were taken.

Table 3.1 shows the data for each parameter for this series of experiments including the control data. At no dose was there a significant difference in the rising phase of $(MEPPs)_o$ when this is compared to the rising phase of $(MEPPs)_o$ recorded from control prearations. The smallest dose of 25nmolkg^{-1} caused a change in $(MEPPs)_o$ timecourse, prolonging the decay phase as seen by the parameters T50% and 80-20%D which show a significant increase when compared to controls. W50% and Area however have values which although appearing to be greater than control $(MEPPs)_o$ are not significant meaning that it is not conclusive as to whether a 25nmolkg^{-1} dose of ecothiopate does cause a significant change in AChE activity. The two parameters which do not show significance however are those which while measuring the decay phase are also affected by the rising phase, and are maybe therefore not the best measure of prolongation of decay phase . At all of the other doses however all of the parameters which measure prolongation of decay phase showed a significant difference from controls.

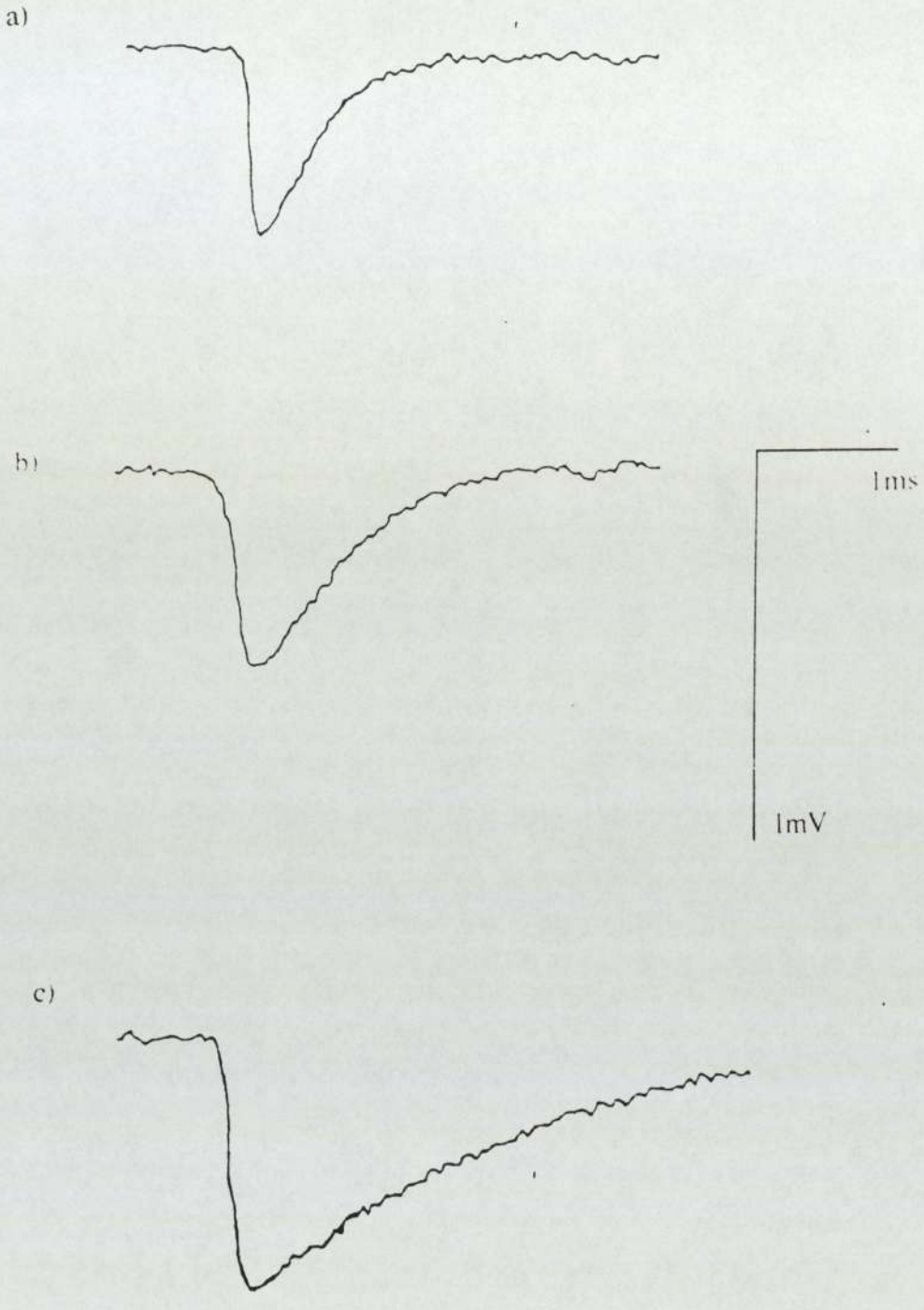


Figure 3.1 Typical examples of extracellular miniature endplate potentials recorded after various doses of ecothiopate. a) untreated b) 300nmolkg^{-1} c) 500nmolkg^{-1}

Dose ($nmolkg^{-1}$)	Trise (ms)	T50% (ms)	W50% (ms)	20-80%R (ms)	80-20%D (ms)	Area (ms.mV)
0	0.26	0.44	0.54	0.11	0.55	1.01
(10)	±0.07	±0.05	±0.05	±0.02	±0.05	±0.38
25	0.27	0.49*	0.64	0.10	0.65*	1.14
(5)	±0.03	±0.03	±0.05	±0.02	±0.05	±0.19
50	0.28	0.57*	0.72*	0.09	0.72*	1.28*
(5)	±0.02	±0.07	±0.06	±0.02	±0.09	±0.24
100	0.27	0.76*	0.91*	0.10	0.96*	1.57*
(5)	±0.02	±0.05	±0.05	±0.01	±0.07	±0.42
300	0.28	0.82*	0.94*	0.10	1.15*	1.90*
(5)	±0.02	±0.05	±0.06	±0.02	±0.06	±0.38
500	0.24	0.90*	1.06*	0.07	1.27*	2.09*
(8)	±0.06	±0.15	±0.17	±0.02	±0.12	±0.24

Table 3.1 Effect of ecothiopate at various doses on the timecourse of extracellularly recorded miniature endplate potentials. All values are shown as the mean ± s.d. The number beneath the dose in parentheses is the number of animals tested therefore the number of cells recorded from is 5 times this number. Those values which differ significantly from controls are indicated by * (Mann - Whitney $p<0.05$)

Figure 3.2 shows the values for the parameters which measure the rising phase only. It can be seen that at no dose does ecothiopate have an effect on $(MEPP)_o$ rising phase, the correlation coefficients being 0.03 and 0.06 for Trise and 20-80%R respectively which when tested were non significant.

Figure 3.3 shows the data for W50% and Area which are affected by both the rising and decay phases. Both of these show a non significant increase with a 25nmolkg^{-1} dose but significant increases for all of the higher doses, as well as significant differences between doses. The correlation coefficients for these parameters were 0.776 and 0.899 which were both significant.

The data for the parameters which measure the decay phase only can be found in Figure 3.4. These parameters both show a significant increase at and between each dose. There is significant correlation between the dose and values of these parameters the correlation coefficients being 0.767 and 0.874 for T50% and 80-20%D respectively.

Figure 3.5 shows photographs indicating the effect of ecothiopate in increasing doses on the shape of endplates. This effect can be measured as the ratio of endplate width to endplate length (W/L ratio) and the effect of ecothiopate on these can be seen in Figure 3.6. It can be seen that after dosing with 25nmolkg-^{1} ecothiopate there is already an increase in the W/L ratio, as the endplates become more rounded. This increase proves to be significant if the W/L ratio values are compared to those of controls (Mann-Whitney $p<0.05$). As the dose of ecothiopate is increased the increase becomes more extreme as the endplates become more and more distorted, at 500 nmolkg^{-1} ECO approximately 40% of endplates are so distorted they are unmeasurable. These unmeasurable endplates will have a significant effect on the results found with 500 nmolkg^{-1} ECO. As the width and length of these will have not been measured they do not contribute to the W/L ratio, meaning that the ratio for this dose is likely to be inaccurate. It is however not possible to prevent this distortion and so the W/L ratio has to be calculated as accurately as possible

with the number of unmeasurable endplates being noted. It is also not known if it is possible to record $(MEPPs)_O$ from these endplates and so there may an added effect on the electrophysiological data.

Appendix 5 contains the assay data for the AChE at the same doses. The changes in endplate activity show a similar pattern with more isoforms becoming affected with increasing dose as well as the reduction in enzyme activity becoming larger with increasing doses.

Ecothiopate therefore has no effect on the rising phase of $(MEPPs)_O$ and so does not affect any of the numerous steps in the production of $(MEPPs)_O$. Ecothiopate does however cause a prolongation of the decay phase by presumably causing a prolongation of transmitter action. While the data for the very lowest dose is not clear it can be said that even a small dose of ecothiopate when administered acutely causes significant inhibition of AChE. This very small dose also causes a significant distortion in the shape of endplates when this is measured as a change in the ratio of their width to lengths. The changes in AChE activity are very complex as seen in Appendix 5 but as a whole ecothiopate causes a reduction in the activity of all enzyme fractions measured, the reduction in activity increasing as the dose increases.

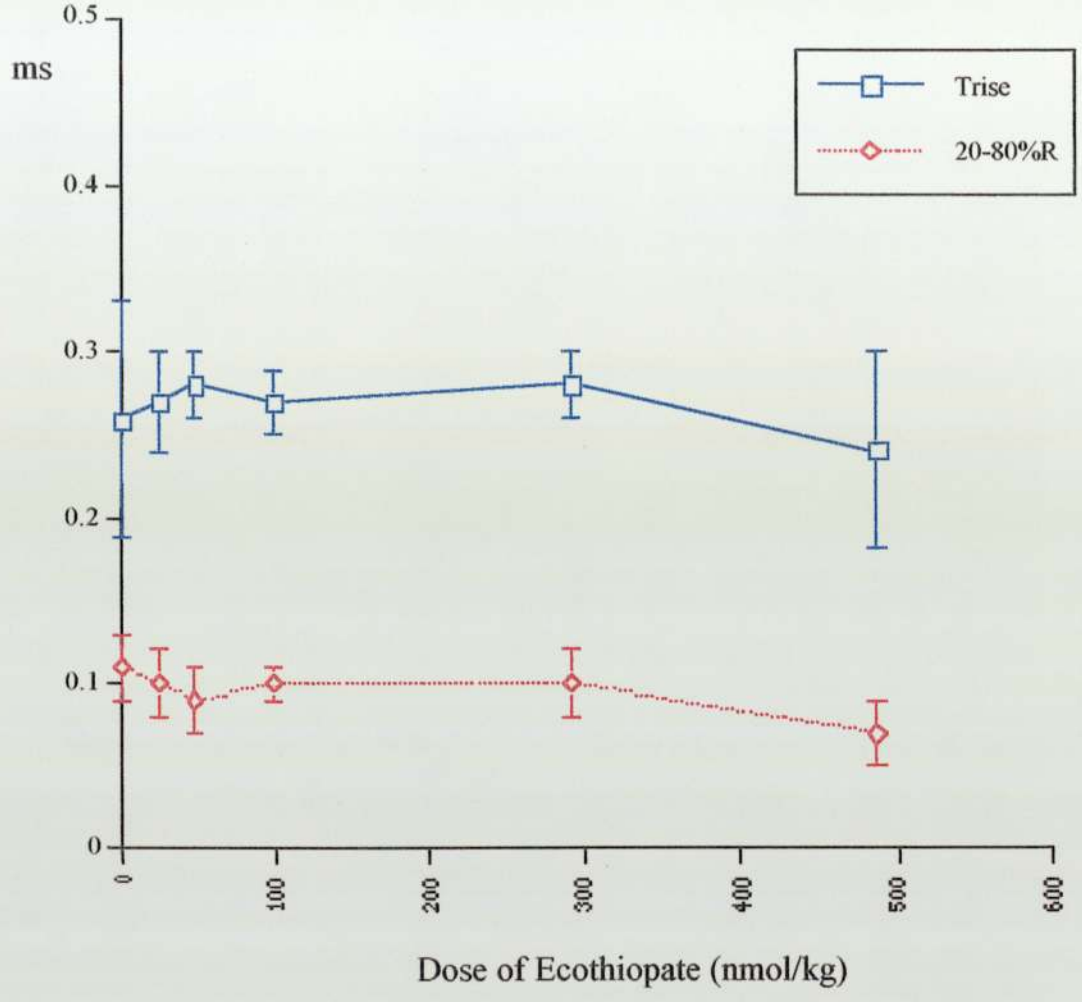


Figure 3.2 The effect of increasing doses of ecothiopate on Trise and 20-80%R, the parameters which measure the rising phase of extracellularly recorded miniature endplate potentials. All of the records from which this data was obtained were recorded 3 hours after the dose of ecothiopate. All of the values shown are the mean ± s.d. These were calculated from 5 animals for each dose apart from 500nmolkg^{-1} which was calculated from 8 animals, and controls which was calculated from 10. * indicates a significant difference from control data and + indicates a significant difference between doses.

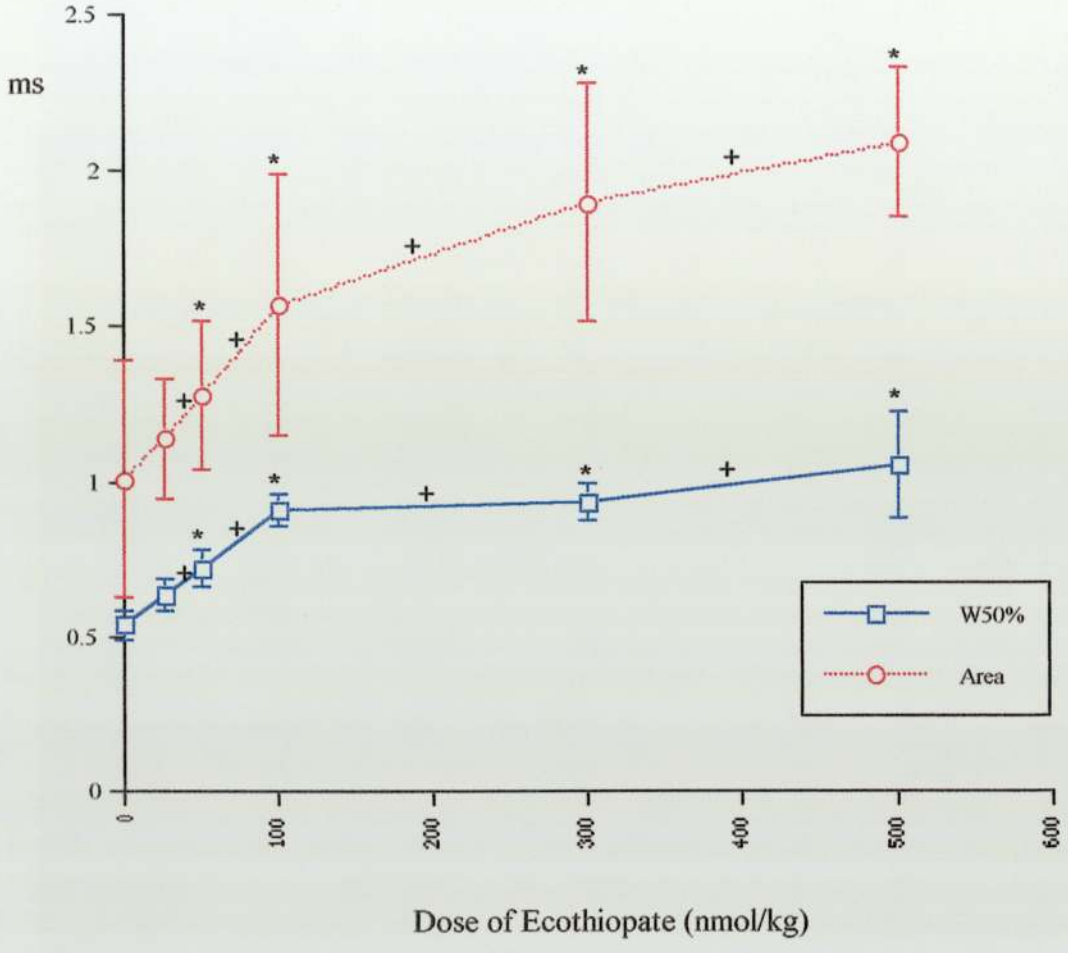


Figure 3.3 The effect of increasing doses of ecothiopate on W50% and Area, the parameters which measure both the rising and decay phase of extracellularly recorded miniature endplate potentials. All of the records from which this data was obtained were recorded 3 hours after the dose of ecothiopate. All of the values shown are the mean ± s.d. . These were calculated from 5 animals for each dose apart from 500nmolkg^{-1} which was calculated from 8 animals, and controls which was calculated from 10. * indicates a significant difference from control data and + indicates a significant difference between doses.

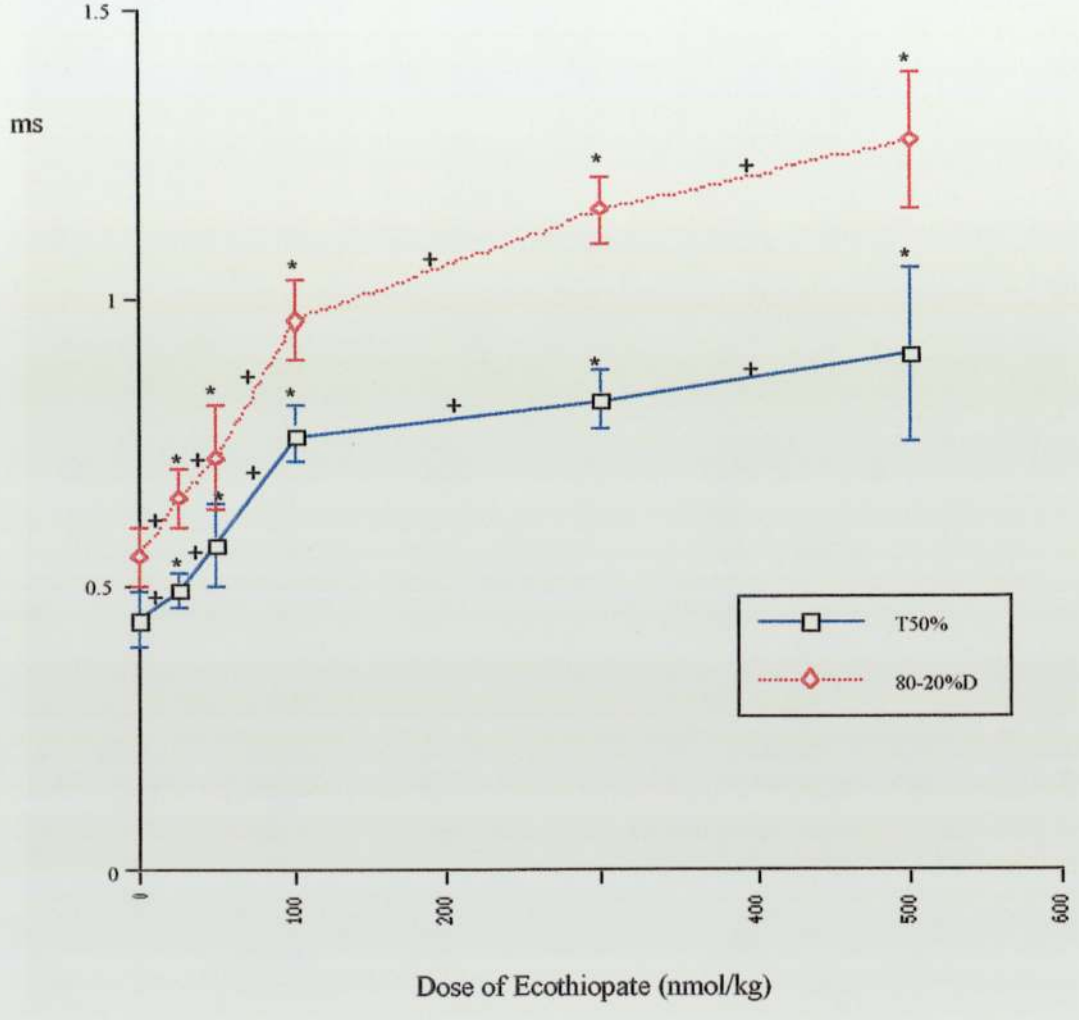


Figure 3.4 The effect of increasing doses of ecothiopate on T50% and 80-20%D, the parameters which measure the decay phase of extracellularly recorded miniature endplate potentials. All of the records from which this data was obtained were recorded 3 hours after the dose of ecothiopate. All of the values shown are the mean ± s.d. . These were calculated from 5 animals for each dose apart from 500nmolkg^{-1} which was calculated from 8 animals, and controls which was calculated from 10. * indicates a significant difference from control data and + indicates a significant difference between doses.

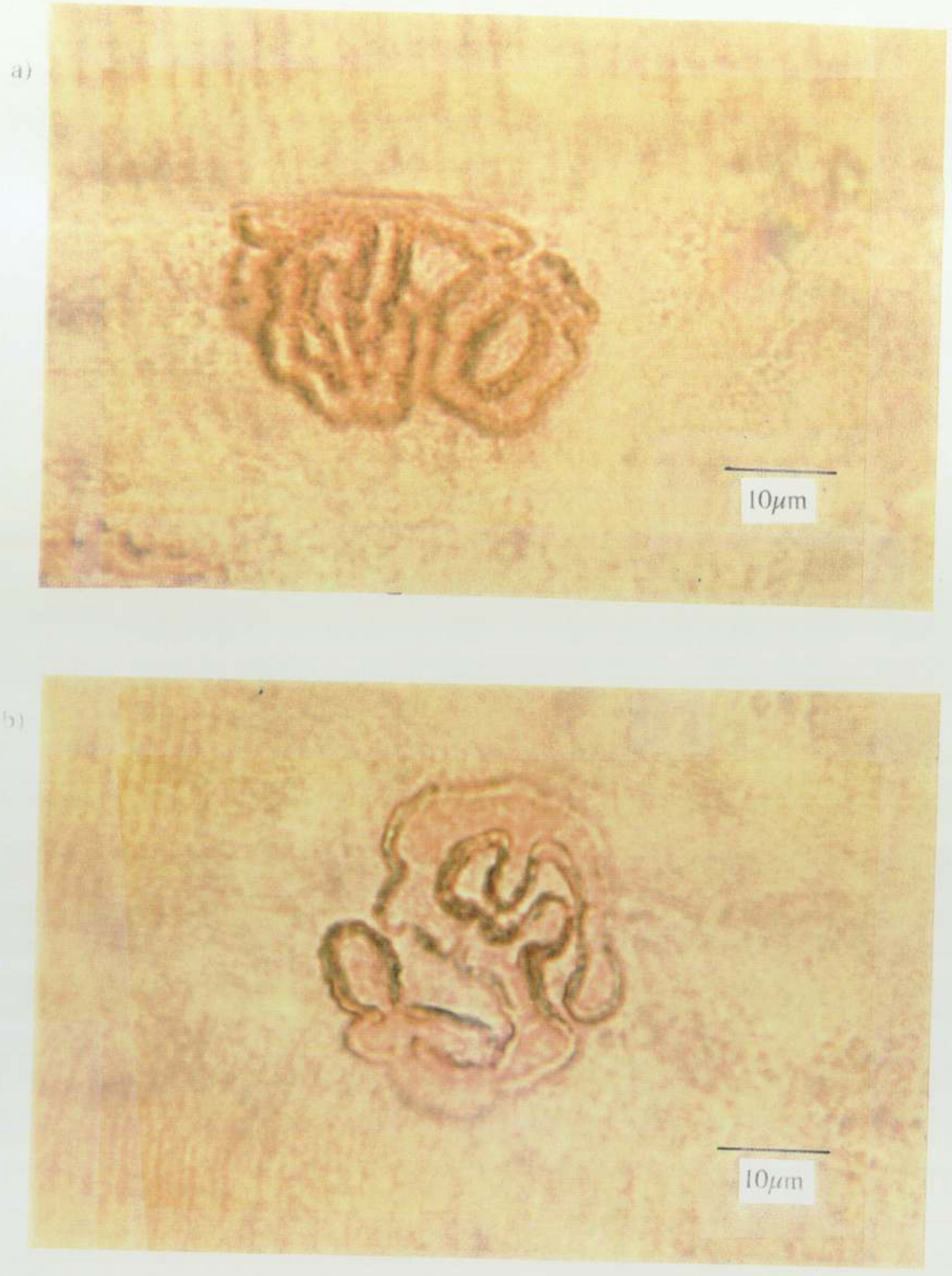


Figure 3.5 Photomicrographs of endplates showing the effect of increasing doses of ecothiopate. a) untreated b) 500nmolkg^{-1}.

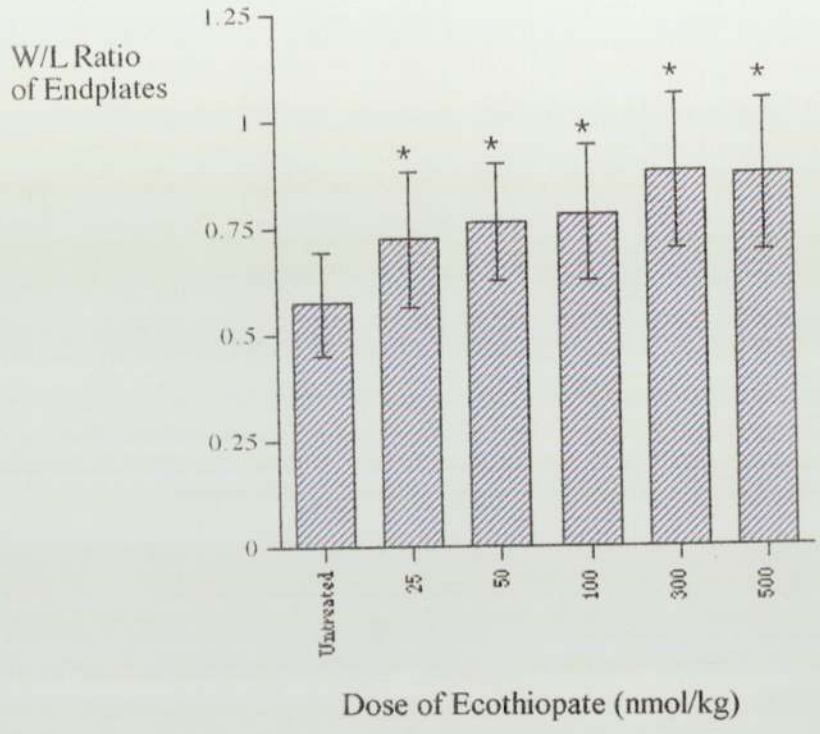


Figure 3.6 The effect of increasing doses of ecothiopate on endplate shape. This is shown as the width to length ratio of endplate size. All of the endplates measured were from diaphragms fixed 3 hours after dosing. The values shown are means ± s.d. These were calculated from 5 animals for each dose apart from 500nmolkg^{-1} which was calculated from 8 animals, and controls which was calculated from 10. * indicates a significant difference from the untreated value (Mann- Whitney $p<0.05$).

3.2 The effect of time after a standard dose of ecothiopate

Experimental Design

The mice used in this study were divided into 6 series treated with a standard dose of ecothiopate 500 $nmolkg^{-1}$ + 700 $nmolkg^{-1}$ atropine but increasing time intervals between dosing and killing as outlined below.

1 hour

3 hours

6 hours

1 day

5 days

7 days

For each experiment 2 animals were killed and their diaphragms divided into hemidiaphragms and the hemidiaphragms were shared between the biochemical, histological and electrophysiological investigations in the same way as with the previous studies. When recording $(MEPPs)_o$ five cells were recorded from each hemidiaphragm in the same way as before.

Control preparations were taken from animals who had been given a dose of atropine saline and their diaphragms removed at corresponding time intervals.

Results

Figure 3.7 shows typical records of $(MEPPs)_o$ at the various time intervals where each trace is an average of 25 individual events. It can be seen that 1 hour after the dose of ecothiopate there is a large prolongation of the decay phase which becomes less as the interval between dosing and killing increases.

Table 3.2 contains the data for these experiments.

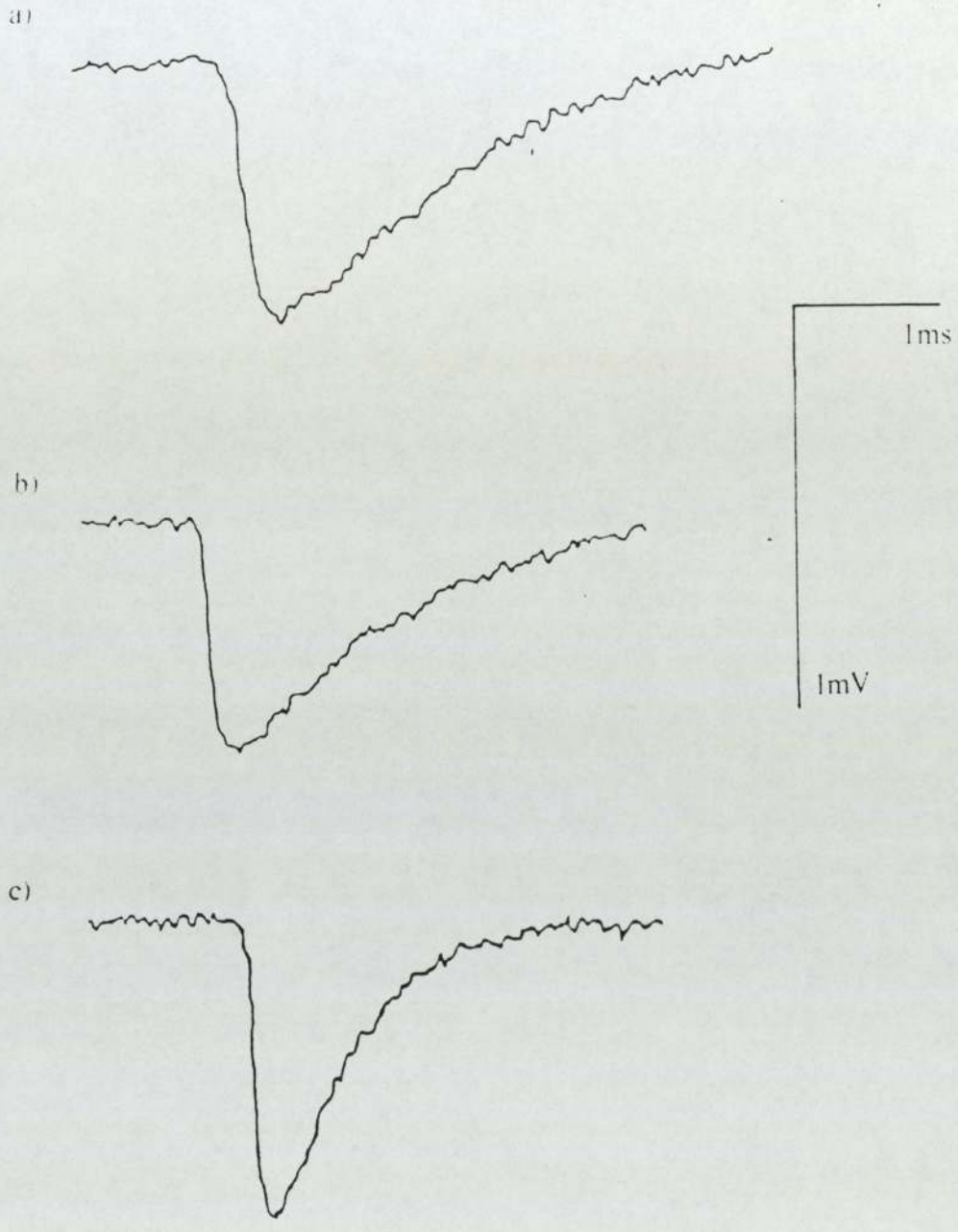


Figure 3.7 Typical examples of extracellular miniature endplate potentials recorded after increasing time interval between dosing with 500nmolkg^{-1} ecothiopate and killing. a) 3 hours b) 24 hours and c) 5 days.

Time	Trise (ms)	T50% (ms)	W50% (ms)	20-80%R (ms)	80-20%D (ms)	Area (ms.mV)
Controls (10)	0.26 ±0.07	0.44 ±0.05	0.54 ±0.05	0.11 ±0.02	0.55 ±0.05	1.01 ±0.38
1 hour (5)	0.27 ±0.03	1.26* ±0.05	1.39* ±0.09	0.11 ±0.03	1.50* ±0.11	2.31* ±0.36
3 hours (8)	0.24 ±0.06	0.90* ±0.15	1.06* ±0.17	0.08 ±0.02	1.27* ±0.12	2.09* ±0.24
6 hours (5)	0.23 ±0.03	0.82* ±0.07	0.91* ±0.06	0.10 ±0.04	0.94* ±0.05	1.83* ±0.08
24 hours (6)	0.27 ±0.02	0.66* ±0.08	0.80* ±0.10	0.10 ±0.02	0.91* ±0.19	1.63* ±0.51
5 days (6)	0.28 ±0.06	0.58* ±0.09	0.68* ±0.10	0.09 ±0.02	0.66* ±0.12	1.31* ±0.12
7 days (9)	0.23 ±0.05	0.43 ±0.06	0.52 ±0.07	0.08 ±0.02	0.54 ±0.09	1.01 ±0.10

Table 3.2 Effect of ecothiopate (500 $nmolkg^{-1}$) on the timecourse of extracellularly recorded miniature endplate potentials. All values shown are the mean ± s.d. The number below the time in parentheses is the number of animals tested therefore the number of cells recorded from is 5 times this number. Those values which differ significantly from controls are indicated by * (Mann - Whitney $p<0.05$)

The control preparations were unchanged over the range of time intervals and so the data has been pooled and just the average of all the preparations is shown. The parameters which measure rise time only show no significant difference from controls at any time. The parameters which measure decay phase are significantly different from controls at all time intervals up to 5 days where T50% and 80-20%D are not significantly different. At 7 days all parameters are not significantly different fom controls.

Figure 3.8 shows the data for the rising phase parameters only which show no change at any time point . The correlation coefficients for Trise and 20-80%R are 0.009 and 0.003 respectively, both of which prove to be non significant.

Figure 3.9 shows the parameters which measure both the rising and decay phases. Both show a decrease in prolongation as the interval between dosing and killing increases, the values being significantly different between every time interval except between Area at 6 and 24 hours. The correlation coefficients for W50% and Area were significant, 0.865 and 0.865 respectively.

Figure 3.10 shows the parameters measuring only the decay phase of the $(MEPP)_o$ which show an initial prolongation after ecothiopate which decreases as the interval between dosing and killing increases until at 7 days there is no significant difference between the decay of an ecothiopate treated prearation and a control preparation. All of the values are significantly different from the previous time point except for 6 hours and 24 hours for both parameters. The correlation coefficients are 0.859 and 0.802 for T50% and 80-20%D respectively, both of which were significant.

Figure 3.11 shows the effect of increasing the time before killing after a single 500 $nmolkg^{-1}$ dose of ecothiopate on endplate shape. After 3 hours there is a large significant increase in the W/L ratios of endplates as they are very distorted. As the time interval increases however the W/L ratio decreases as the distortion reduces until at 7 days there is

no significant difference between the ratios of an ecothiopate treated diaphragm and those of the control diaphragm.

Appendix 5 contains the assay data for the AChE fractions at the same time intervals. The changes in the enzyme activities of the various fractions are complex but as a whole they too show recovery over the 7 day period.

Therefore the animal shows recovery over a period of days, until at 5 days there is no difference between a dosed and a control animal, the effect ecothiopate has upon the timecourse of extracellularly recorded miniature endplate potentials, endplate shape and AChE while appearing to be quite drastic is reversible after a single acute dose.

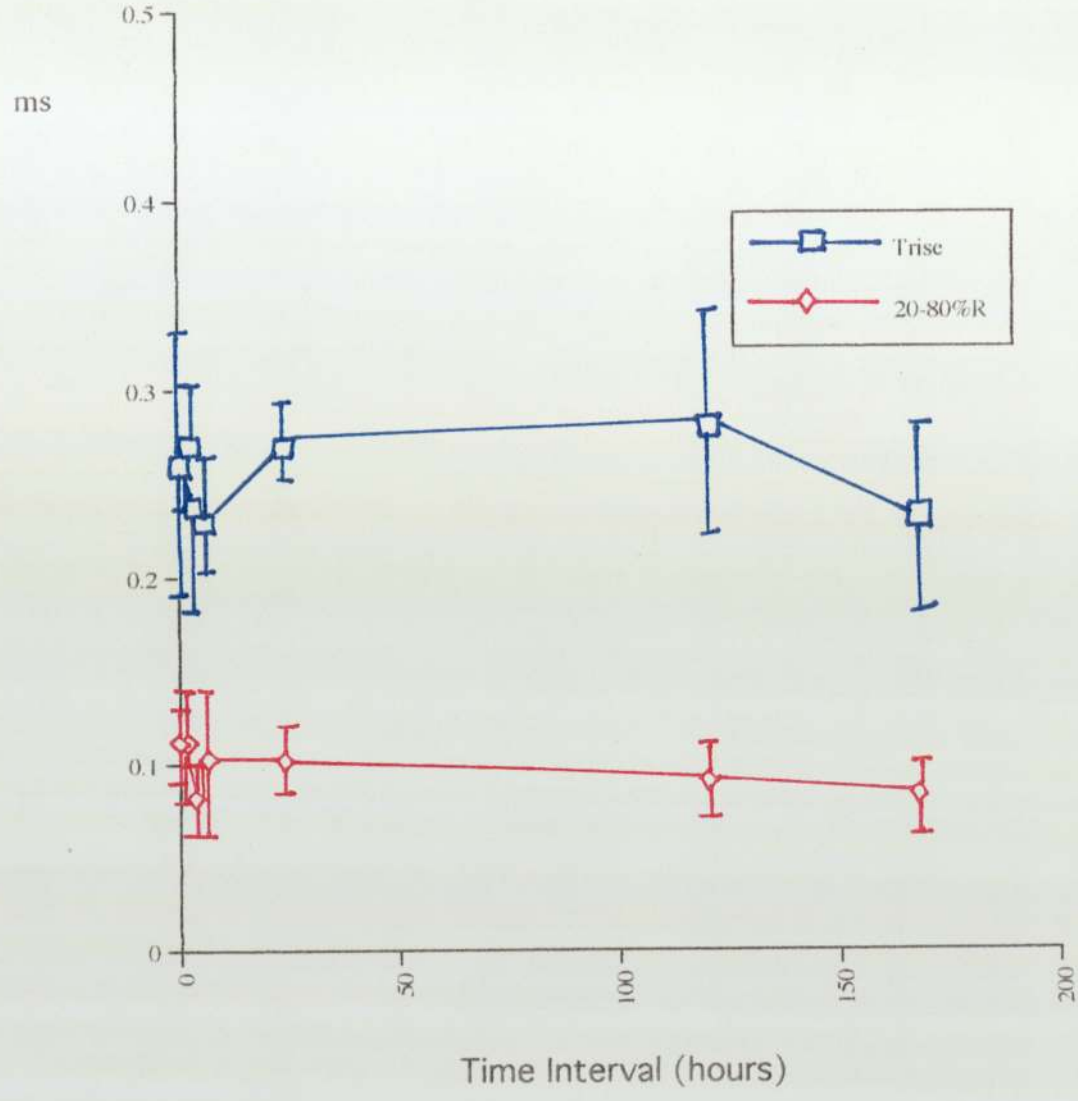


Figure 3.8 The effect of increasing time interval between dosing with 500 nmolkg-[1] ecothiopate and killing on Trise and 20-80%R, the parameters which measure the rising phase of extracellularly recorded miniature endplate potentials. All values shown are the mean ± s.d. These were calculated from 5 animals for 1 hour and 6 hours, 6 animals for 24 hours and 5 days, 8 animals for 3 hours, 9 animals for 7 days and 10 animals for controls. * indicates a significant difference from control data and + indicates a significant difference between time intervals.

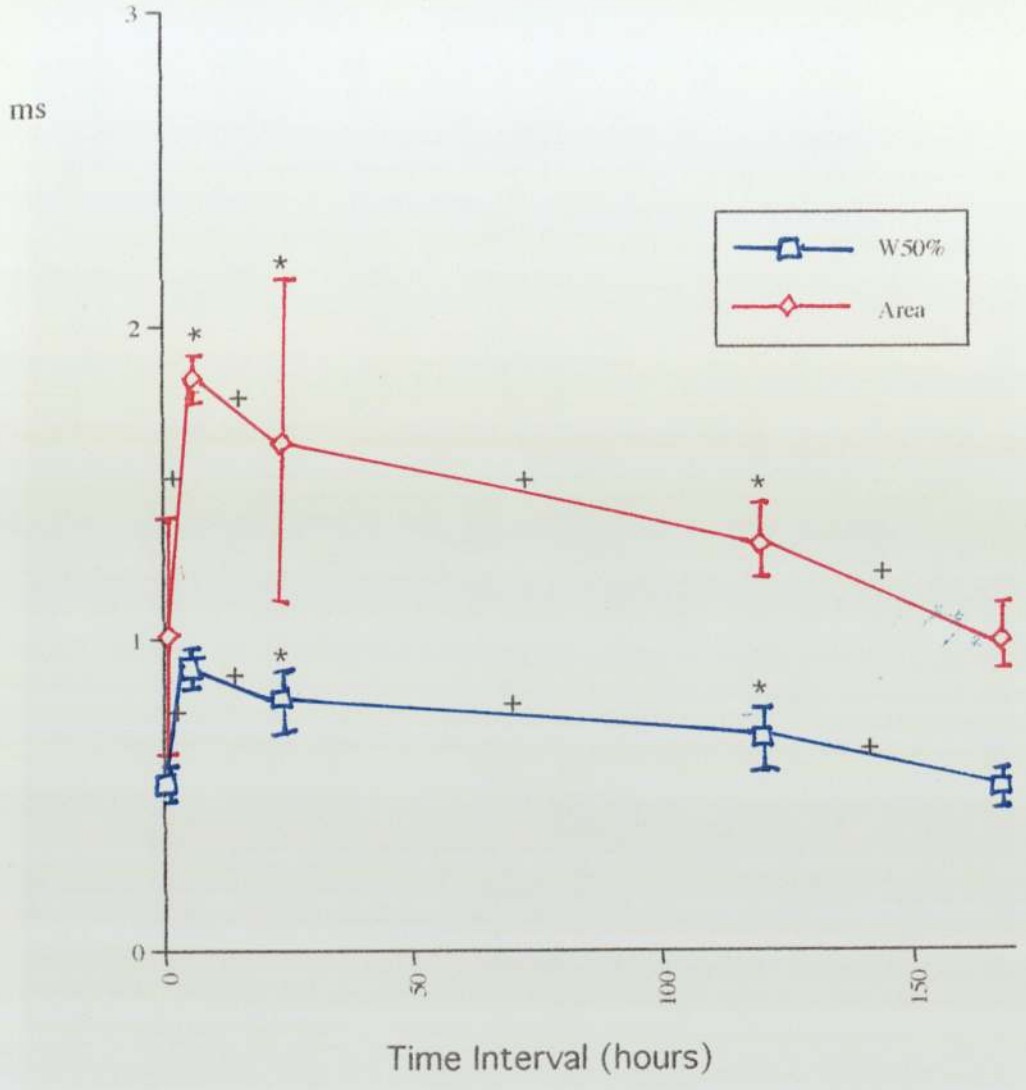


Figure 3.9 The effect of increasing time interval between dosing with 500 nmolkg^{-1} ecothiopate and killing on W50% and Area, the parameters which measure both the rising and decay phases of extracellularly recorded miniature endplate potentials. All values shown are the mean ± s.d. These were calculated from 5 animals for 1 hour and 6 hours, 6 animals for 24 hours and 5 days, 8 animals for 3 hours, 9 animals for 7 days and 10 animals for controls. * indicates a significant difference from control data and + indicates a significant difference between time intervals.

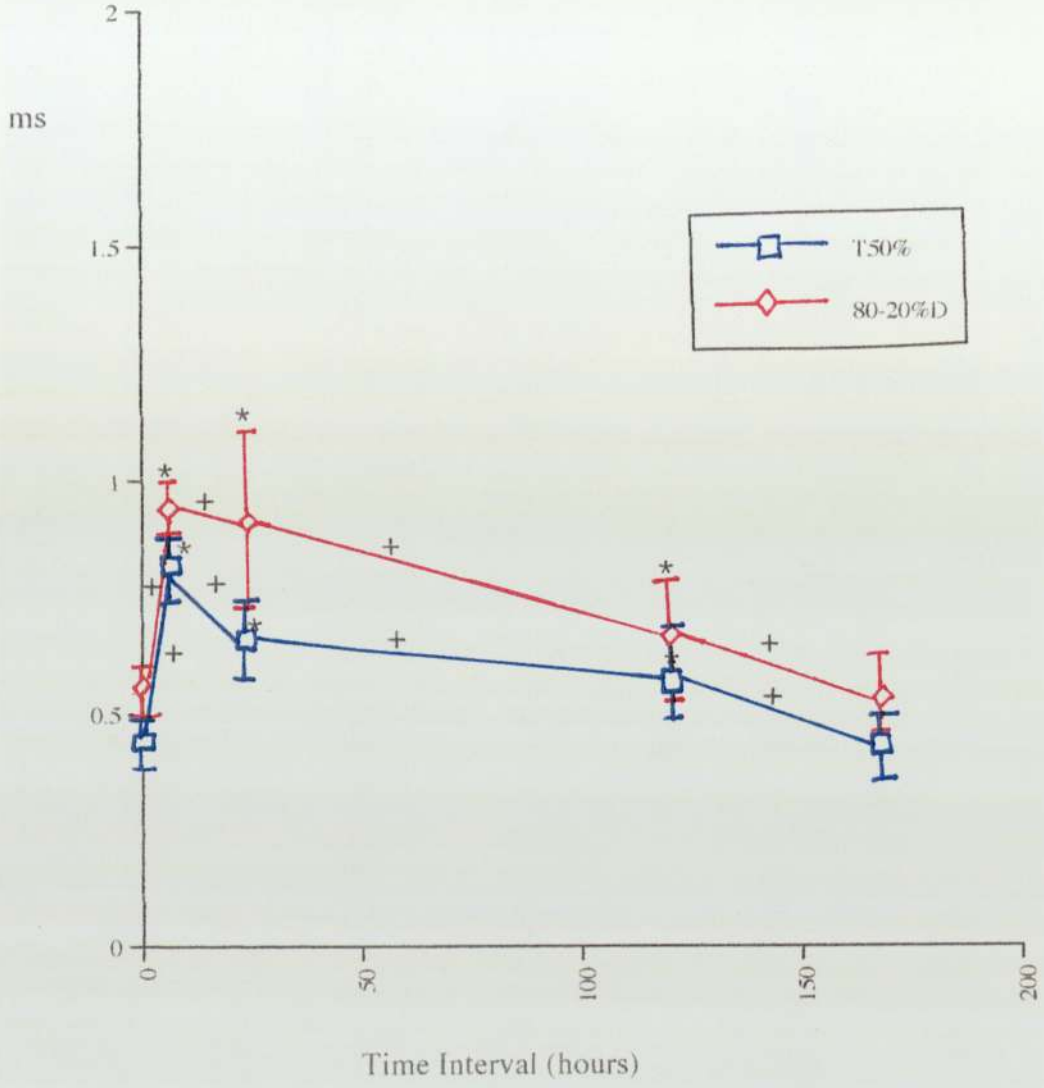


Figure 3.10 The effect of increasing time interval between dosing with 500 nmolkg^{-1} ecothiopate and killing on T50% and 80-20%D, the parameters which measure the decay phase of extracellularly recorded miniature endplate potentials. All values shown are the mean ± s.d. These were calculated from 5 animals for 1 hour and 6 hours, 6 animals for 24 hours and 5 days, 8 animals for 3 hours, 9 animals for 7 days and 10 animals for controls. * indicates a significant difference from control data and + indicates a significant difference between time intervals.

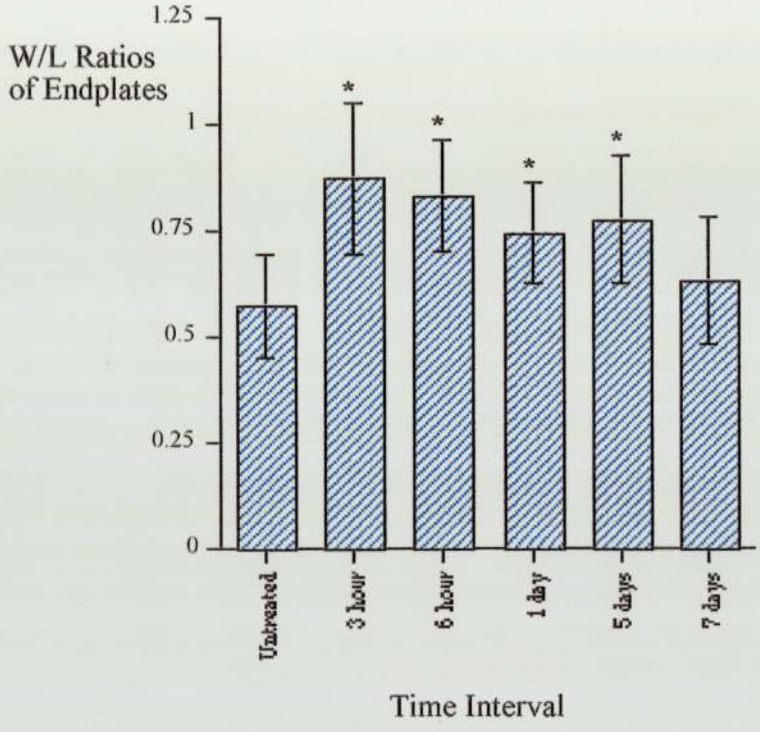


Figure 3.11 The effect of increasing time interval between dosing with 500 nmolkg^{-1} ecothiopate and killing on endplate shape. This is shown as the width to length ratio of endplate size. All of the endplates measured were from diaphragms fixed 3 hours after dosing. The values shown are means ± s.d. These were calculated from 5 animals for 1 hour and 6 hours, 6 animals for 24 hours and 5 days, 8 animals for 3 hours, 9 animals for 7 days and 10 animals for controls. * indicates a significant difference from the untreated value (Mann- Whitney $p<0.05$).

3.3 Correlation of $(MEPP)_o$ timecourse and AChE activity measured biochemically

The prolongation of $(MEPPs)_o$ after ecothiopate dosing shows either a prolongation in the open time of endplate channels or a reduction in the activity of that AChE which is responsible for the termination of transmitter action i.e. functional enzyme. Tattershall (1988) showed that ecothiopate does not have an effect on the open times of endplate channels at the doses used in these experiments so it is possible to assume that a reduction in enzyme activity is the major cause of $(MEPP)_o$ timecourse prolongation. It is not possible however to determine the exact relationship between AChE activity and prolongation of a $(MEPP)_o$. AChE inhibition can be assessed by biochemical assay techniques but this measures all extractable AChE not just the functional portion. It was hoped therefore that by using a sequential extraction technique which allows separation of the molecular forms of AChE and comparing the inhibitions of these various forms with the prolongation of $(MEPPs)_o$ a biochemical method of determining functional AChE activity could be developed.

Experimental Design

$(MEPPs)_o$ were recorded after increasing doses of ecothiopate and after increasing time intervals between dosing and killing. AChE activities were also assessed at the same doses and time intervals by separating the molecular forms by the method of sequential extraction Younkin *et al* (1982) and assaying these using the Ellman *et al* (1961) spectrophotometric method. All of these data are presented by courtesy of Mrs Anna Rowbotham and are detailed in appendices 5 and 6.

When functional AChE is inhibited or physiologically reduced the action of ACh at the post - synaptic membrane is prolonged because there is insufficient enzyme to hydrolyse it. This is seen as a prolongation of the decay phase of $(MEPPs)_o$ due to the rebinding of ACh molecules. Only one of the many parameters measured from the $(MEPPs)_o$ was

used in these comparison studies. T50% was chosen as this is a parameter measuring only the decay phase and as previously shown there is no effect on the rising phase.

Each of the various fractions of AChE extracted from the diaphragm of control and ecothiopate treated mice was correlated with the prolongation of the $(MEPP)_o$ timecourse. The Spearman's Rank Correlation Coefficient was calculated for each set of data, the best correlation determined and an equation calculated for the relationship between functional AChE activity and the prolongation of the $(MEPP)_o$ timecourse.

Results

The sequential extraction technique gave a series of fractions each containing various molecular forms of AChE. Velocity sedimentation on sucrose density gradients showed the non extractable fraction to be the richest in the A12 functional molecular form. (A. Rowbotham pers comm). Addition of ecothiopate indicated that the endplate specific non extractable (see Appendix 4) most likely represented the activity of the functional AChE within the synaptic cleft, as this was the fraction most affected by ecothiopate. If this truly represents functional enzyme then it should correlate well with the time course of $(MEPPs)_o$ after addition of ecothiopate, any reduction of functional AChE would be reflected by a corresponding increase in T50%.

The level of A12 however was calculated not measured directly and so depended on the activity of other enzymes. If any metabolic changes occurred then the calculated value would not give a true indication of functionally active AChE and the correlation with $(MEPPs)_o$ would be affected.

The calculated Spearman Rank correlation coeficients for the dose and time interval studies are shown in Table 3.3. "All points" indicates that the correlation coefficient was calculated using each individual experiment whereas "means" indicates that the correlation

coefficient was calculated from the mean values of each experiment at a particular dose or time interval.

In the series with increasing doses of ecothiopate, and measurements taken 3 hours later, the Spearman's Rank correletion coefficients for all of the enzyme fractions are higher than when time intervals between dosing with 500 nmolkg-^{1} ecothiopate and killing were longer. Concentrating initially on just the coefficients obtained from these studies where increasing doses of ecothiopate were administered it appears that using the mean values gives a set of coefficients which are higher. Within these experiments the best correlations are found with the junctional set of enzymes the non-junctional fraction and also with the endplate specific non-extractable enzyme, however the location of these enzymes in the junctional or non-junctional regions and the molecular isoforms which make up these fractions should be considered when deciding which fraction best represents functional enzyme.

Considering the correlation coefficients, those fractions which show the highest correlations are the endplate specific non extractable, junctional globular and junctional non extractable. Considering the composition of each of these fractions it suggests that the endplate specific non extractable fraction which comes from the A12 rich pellet and has been corrected for any non-junctional, therefore non functional, activity is the best availale measure of functional enzyme.

AChE Enzyme Fraction	Time (All points)	Time (Means)	Dose (All points)	Dose (Means)
Junctional Globular	-0.58	-1.00	-0.77	-0.94
Junctional Asymmetric	-0.57	-0.80	-0.72	-0.71
Junctional Non Extractable	-0.57	-1.00	-0.77	-0.89
Non-Junctional Globular	-0.64	-0.90	-0.71	-0.83
Non-Junctional Asymmetric	-0.30	-0.80	-0.37	-0.26
Non-Junctional Non Extractable	-0.18	-0.40	-0.34	-0.09
Endplate Specific Globular	-0.38	-0.90	-0.52	-0.66
Endplate Specific Asymmetric	-0.49	-0.90	-0.52	-0.66
Endplate Specific Non Extractable	-0.42	-1.00	-0.77	-1.00

Table 3.3 Spearman's Rank correlation coefficients for the relationship between prolongation of $(MEPPs)_o$ timecourse and the activity of the various AChE fractions. All points indicates that the correlation coefficient was calculated using each individual value from individual experiments whereas means indicates that the correlation coefficient was calculated from the mean values from each group of experiments at a particular dose or time interval.

The correlation coefficients for the experiments where the time between dosing and killing was increased do not indicate any particular molecular form or group of molecular forms which correlate best with the prolongation of $(MEPPs)_o$ and therefore one molecular form does not seem to best represent functional enzyme. This does not mean that when experiments are carried out at different time intervals after dosing it is not possible to correlate the electrophysiological and biochemical methods of measuring AChE activity. In the study when all experiments were carried out 3 hours after dosing there may have been insufficient time for major metabolic changes in enzyme metabolism to occur. When the time between dosing and killing the animal is increased however any metabolic changes can have profound effects on all of the molecular forms which will affect any correlation made with electrophysiology.

The fraction which best represents functional enzyme therefore is the endplate specific non extractable but the exact nature of the relationship between this functionally important enzyme and the prolongation of $(MEPPs)_o$ is not known. In an attempt to specify this relationship the data obtained from the experiments with increasing doses of ecothiopate were plotted as a scattergraph and several models were applied to find the line of best fit. Figure 3.12 shows the exponential line of best fit with the equation was $y=3.736*10^{-1.789x}$ and the correlation coefficient was 0.833, which proved to be statistically significant (t-test $p<0.05$). It is not clear why the relationship between enzyme and $(MEPPs)_o$ should be exponential but statistically this seems to be the best representation.

From the measurement of $(MEPPs)_o$ therefore it is possible to assess how much of the AChE activity that is assayed biochemically is present in amounts different from that attributable to functional AChE in untreated preparations. This has then been used by Mrs A. Rowbotham to study time dependent trophic changes in AChE activities after treatment with an anti-cholinesterase. This "functional" fraction is also that chosen to compare with electrophysiology in later experiments.

There will however be differences in the A12 functional enzyme measured biochemically and electrophysiologically due to differences in the location of the enzyme measured. Figure 1.2 shows the location of the A12 AChE measured using these different methods Electrophysiology measures only that shown in red, only the enzyme at the top of the secondary folds, whereas biochemistry measures all of the functional AChE, even that deep in the secondary folds as this will be released during extraction . This difference will lead to some variance in the correlation .

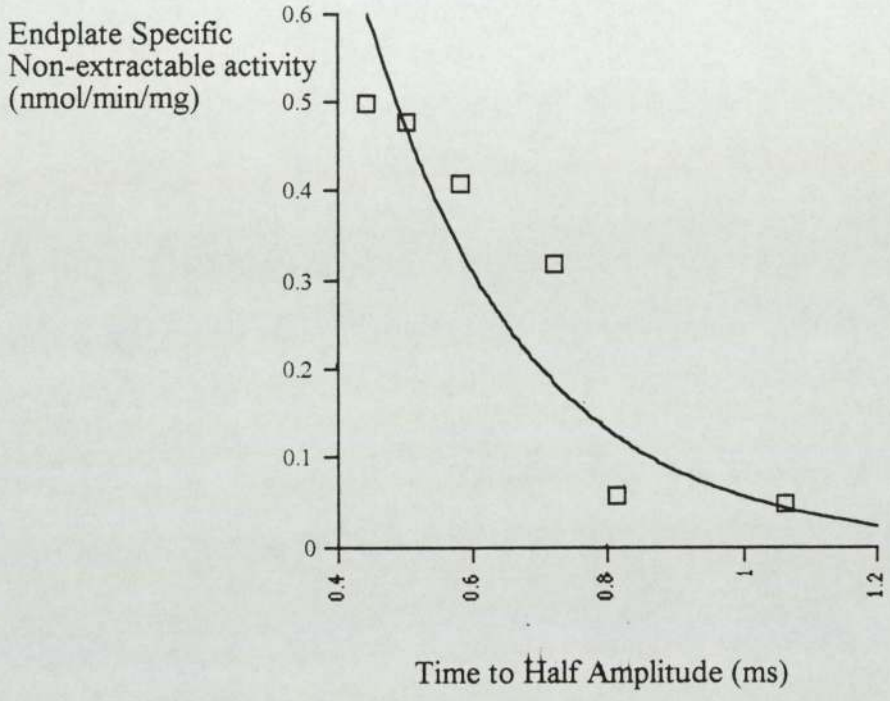


Figure 3.12 Relationship betweeen endplate specific non-extractable enzyme activity with the time to half amplitudes of extracellular miniature endplate potentials after increasing doses of ecothiopate (25nmolkg^{-1} to 500 nmolkg^{-1}). The line fitted is the exponential line of best fit.

CHAPTER 4

The effect of stimulation frequency on mouse diaphragm muscle

These studies were carried out in an attempt to come closer to understanding what causes the increase in neuromuscular jitter after treatment with an anticholinesterase. The effect of stimulation frequency on the latencies of action potentials was investigated to determine if increased jitter is a result of some permanent change at the neuromuscular junction or if it is a functional phenomenon occuring only under certain experimental conditions, e.g. stimulation frequencies. If jitter was due to a permanent deficit it was expected that it would occur at all stimulation frequencies in a diaphragm treated with an anticholinesterase at a dose sufficient to elicit increased jitter. The effect stimulation frequency has on the latency of the endplate potential (EPP) was also investigated.

4.1 The effect of stimulation frequency on the latencies of action potentials

Experimental Design

The anticholinesterase chosen was ecothiopate at a dose of 500 $nmolkg^{-1}$ with atropine 700 $nmolkg^{-1}$, which has previously been shown to cause increased jitter 5 days after dosing when the fibres were stimulated at 30Hz (Table 4.1)

Time after injection	Delay (μs)	MCD (μs)
Untreated	32±22 (42)	9.9±3.6 (42)
5 days	119±104 (40) *	25.6±28.0 (39) *

Table 4.1 The MCDs and Delays after injection of ecothiopate 500 nmolkg-^{1}. Values shown are means ± standard deviation with the number of muscle fibres shown in parantheses. * indicates a significant difference from untreated values. (Kolmogorov - Smirnov $p<0.05$). (Data from Kelly *et al* 1990)

Trains of action potentials were recorded as described in 2.4.2 from ecothiopate treated and from untreated preparations at 1 and 30Hz in the same cells. These frequencies were chosen as 30Hz, as this is the usual stimulation frequency in jitter studies, and 1Hz is a low frequency providing a distinct contrast. The action potentials recorded were then analysed to find the values for mean consecutive difference (MCD) and Delay and so determine if jitter was increased.

Results

Figures 4.1 and 4.2 show typical traces recorded during this series of experiments. Marked on these records is the point at 10% of amplitude where latency was measured. Figure 4.1 shows records from an untreated fibre, where it can be seen that the action potentials lie almost on top of one another at the 10% level and so have a small MCD. If this is compared with Figure 4.2 the increase in jitter can be clearly seen as the action potentials are all shifted to the right with reference to the first.

Table 4.2 shows the values for Delay and MCD for the 4 experimental conditions, and these are also shown graphically in Figure 4.3

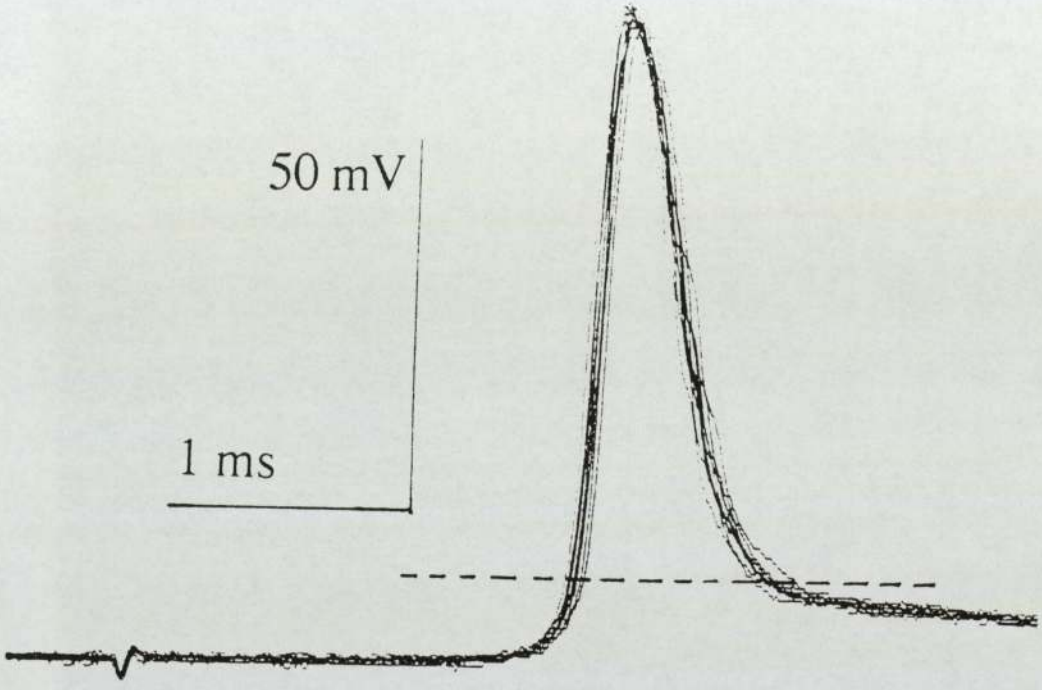


Figure 4.1 Typical intracellular action potentials recorded at 30Hz from the tendon end of a muscle fibre from an untreated animal. The broken line indicates 10% peak amplitude, the point at which the latency of each of each action potential was measured.

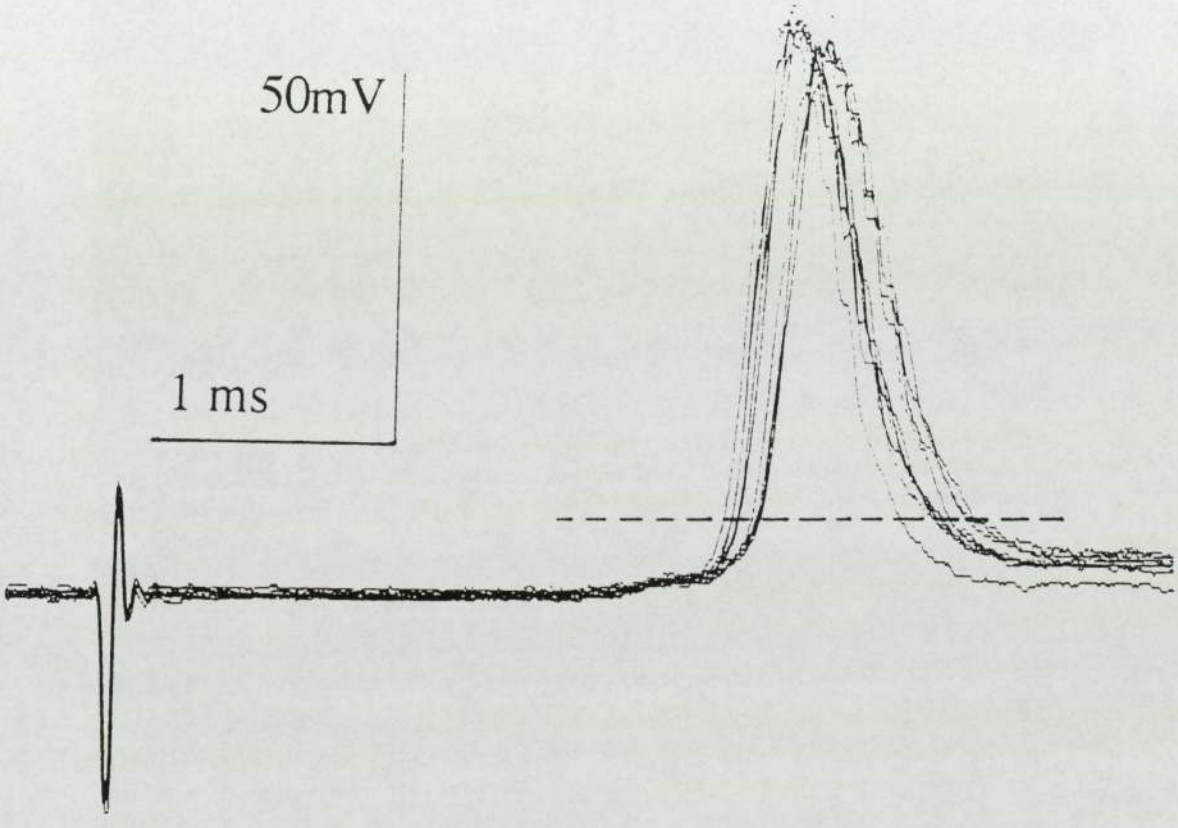


Figure 4.2 Typical intracellular action potentials recorded at 30Hz from the tendon end of a muscle fibre from an animal treated with 500nmol/kg ecothiopate 5 days previously. The broken line indicates 10% peak amplitude, the point at which the latency of each of each action potential was measured.

Condition	Delay (μs)	MCD (μs)
Untreated 1Hz	-9±28 (24)	9.5±4.7 (24)
	*	
Untreated 30hz	23±26 (24)	11.4±5.3 (24)
	*	*
Ecothiopate 30Hz	43±34 (31)	22.3±20.2 (31)
	*	*
Ecothiopate 1Hz	-4±18 (31)	9.3±5.1 (31)

Table 4.2 The MCDs of action potentials 11-30, and Delays of 16th action potential in trains recorded from untreated and ecothiopate treated diaphragms at 1 and 30Hz, in the same cell. The values shown are the mean ± standard deviation with the number of fibres shown in parentheses. * indicates a significant difference between groups. (Kolmogorov - Smirnov $p<0.05$)

Kelly et al (1990) determined the Delay and MCD of untreated fibres at 1, 10 and 30Hz. They found that Delay was increased when the stimulation frequency was increased from 1 to 10Hz, but no further increase when frequency was increased to 30Hz. They found a similar pattern in the increase of MCD with stimulation frequency which does not agree with the data presented here.

There was a significant increase in the Delay of untreated fibres when the stimulation frequency was increased from 1 to 30 Hz, however there was no significant difference between the Delay of treated and untreated fibres when they were stimulated at 1Hz. Increasing the stimulation frequency in treated fibres caused a significant increase in the Delay and this was also found to be significantly higher than the Delay of untreated fibres stimulated at 30Hz.

The frequency distributions for Delay are shown in Figure 4.4 where it can be seen that the distributions of Delay change at the different stimulation frequencies. However even with the conditions which show significant changes from the untreated 30Hz Delays some fibres were unaffected, and could therefore be assumed to be "normal".

The reason for the increase in Delay can be seen if the latencies of the first 16 action potentials are plotted against the action potential number (Figure 4.5).
Both the untreated and ecothiopate treated fibres stimulated at 1Hz show very little change in the latencies of the first 16 action potentials, the lines on the graph are relatively flat. If an untreated fibre is stimulated at 30Hz however the action potentials show a steady increase in latency. The latency increases over the first 10 action potentials, reaches a plateau and then shows only a slight variation, the MCD. This increase in latency occurs to a greater extent in treated fibres and also seems to occur earlier. The latency increases over the first 5 action potentials, reaches a plateau and then also shows some variation. The initial increase in latency which occurs when the fibres are stimulated at 30Hz would account for the increased Delay seen in both treated and untreated fibres, but it seems as if ecothiopate affects the fibres causing the increase to be enhanced in treated fibres.

In figure 4.5 the variation in latency seen after the plateau has been reached shows graphically what can be seen in Figures 4.1 and 4.2, i.e. the increase in MCD. Table 4.2 shows that there was no significant increase in MCD when the stimulation frequency in an untreated fibre was increased from 1 to 30Hz. There was also no significant difference in MCD between an ecothiopate treated and an untreated fibre when they were both stimulated at 1Hz. If the MCD of treated and untreated fibres are compared however it can be seen that there was a significant increase in MCD in the treated fibres. This increased MCD seen in treated fibres stimulated at 30Hz was also significantly different from the MCD in the same fibres stimulated at 1Hz.

The frequency distributions for the MCDs are shown in Figure 4.6 where it can be seen that untreated fibres stimulated at 1 or 30Hz show a similar distribution as do treated fibres at 1Hz. Stimulating treated fibres at 30Hz however causes a shift in the distribution to the right due to fibres showing higher values of MCD, however in the same way as Delay some fibres stay within the "normal" range and therefore could be unaffected.

Although both Delay and MCD show fibres which are unaffected and therefore "normal" no significant correlation and therefore no association was found between increases of Delay and MCD, suggesting that "normal" Delays and "normal" MCDS do not occur in the same cells.

In conclusion stimulation frequency does play an important role in the phenomenon of increased jitter. The Delay is increased by stimulating at 30Hz and is further increased by pre-treatment with ecothiopate, whereas the MCD is independent of stimulation frequency in untreated fibres but is increased in ecothiopate treated fibres only if they are stimulated at 30Hz. Increased jitter then seems to be a functional phenomenon, treatment with an anticholinesterase does not increase jitter in all situations.

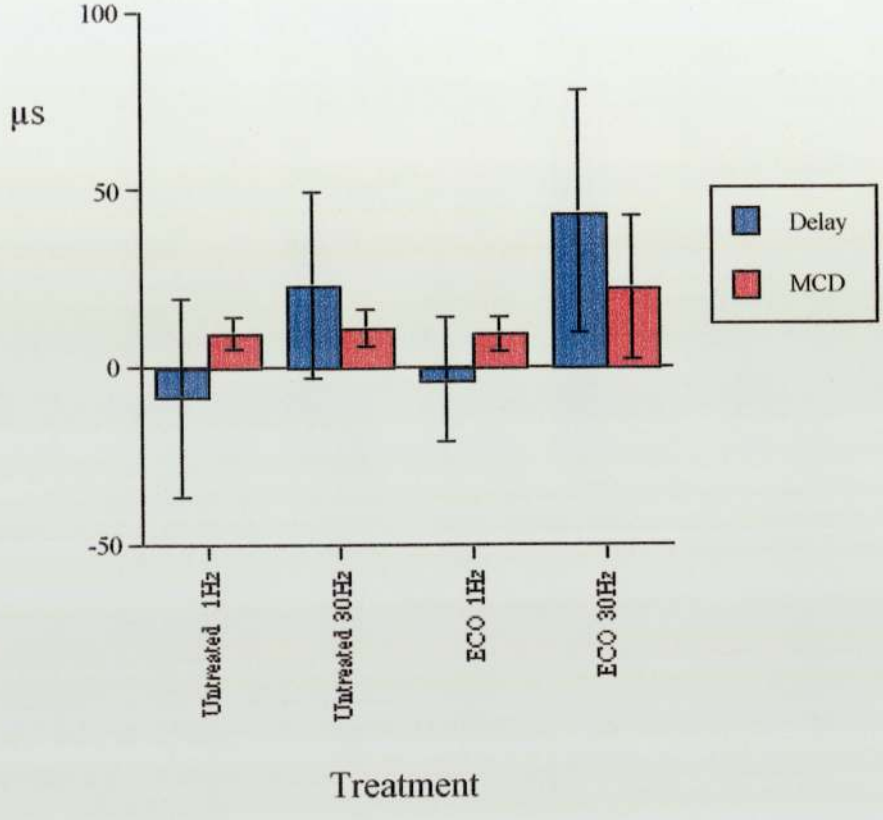


Figure 4.3 The MCDs and Delays of action potentials redorded from untreated fibres and preparations treated with 500 nmolkg-^{1} ecothiopate with 700 nmolkg-^{1} atropine at 1 and 30Hz. The columns show the mean ± s.d. The number of fibres tested were 24 for untreated preparations and 31 for treated preparations.

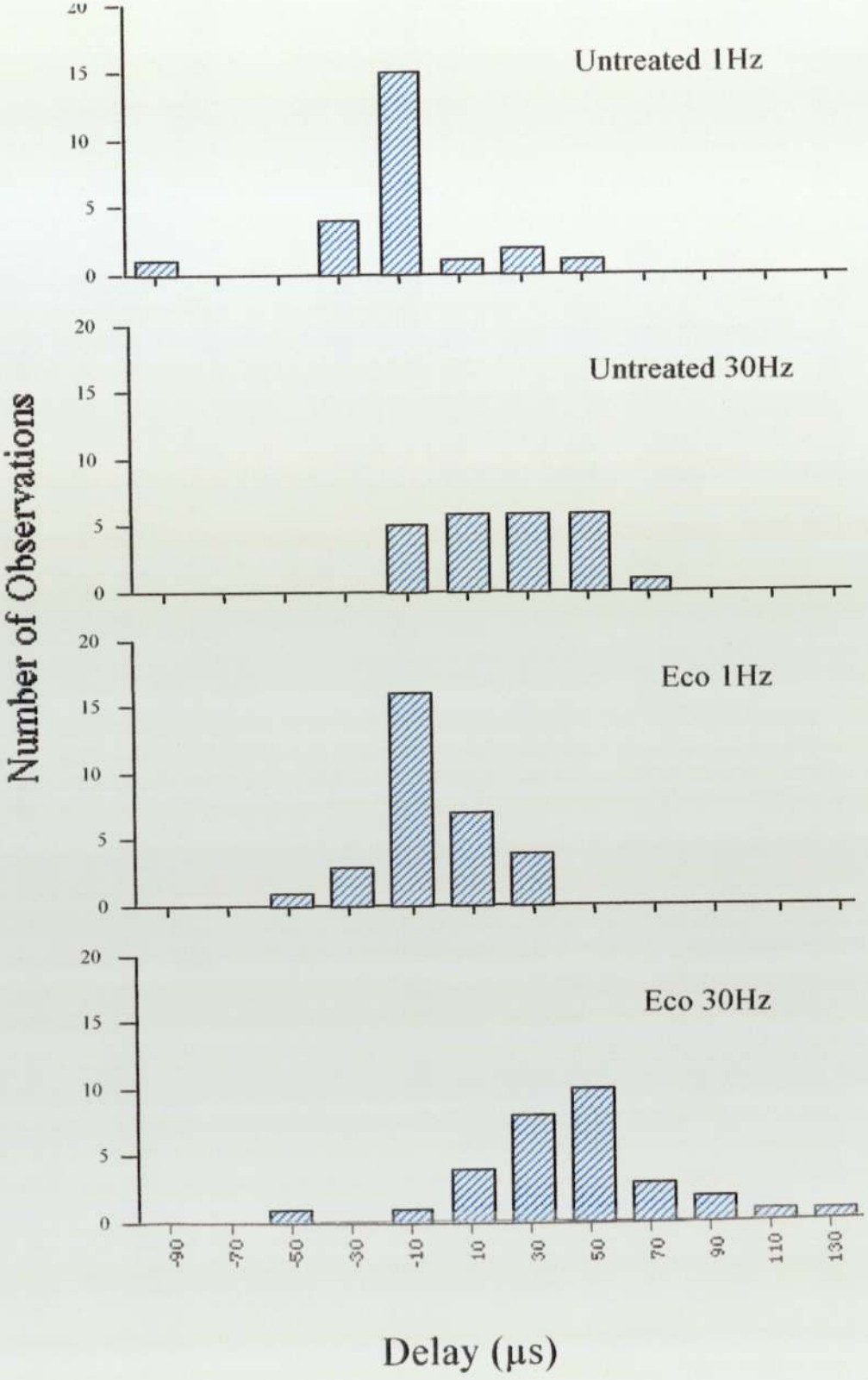


Figure 4.4 Frequency distributions of Delays from untreated preparations and preparations 5 days after ecothiopate 500 nmolkg^{-1} with atropine 700 nmolkg^{-1} stimulated at 1 and 30Hz. The number of fibres tested were 24 for untreated preparations and 31 for treated preparations.

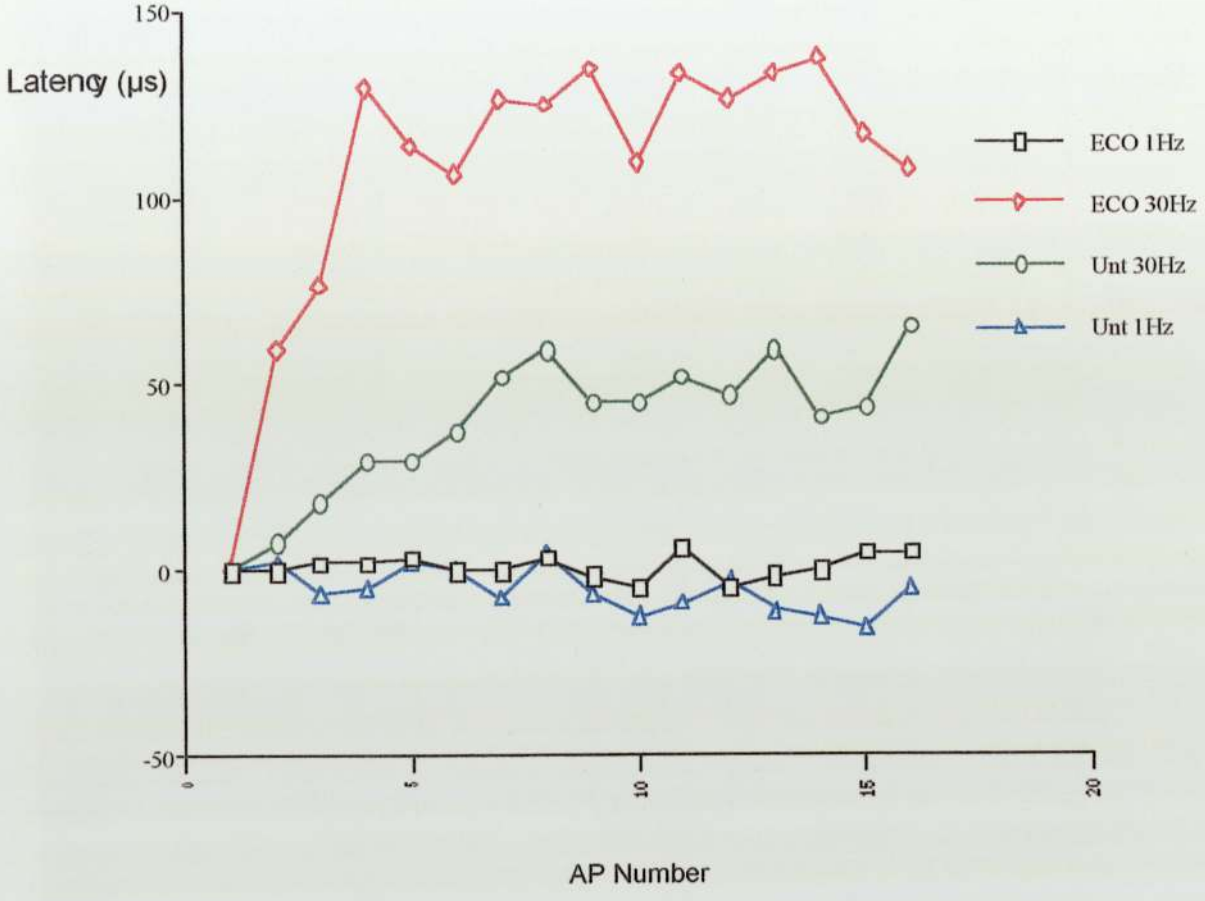


Figure 4.5 Latencies of action potentials 1 to 16 in fibres of untreated prerarations and preparations dosed 5 days earlier with 500 nmolkg^{-1} ecothiopate and 700 nmolkg^{-1} atropine stimulated at 1 and 30 Hz in the same cells.

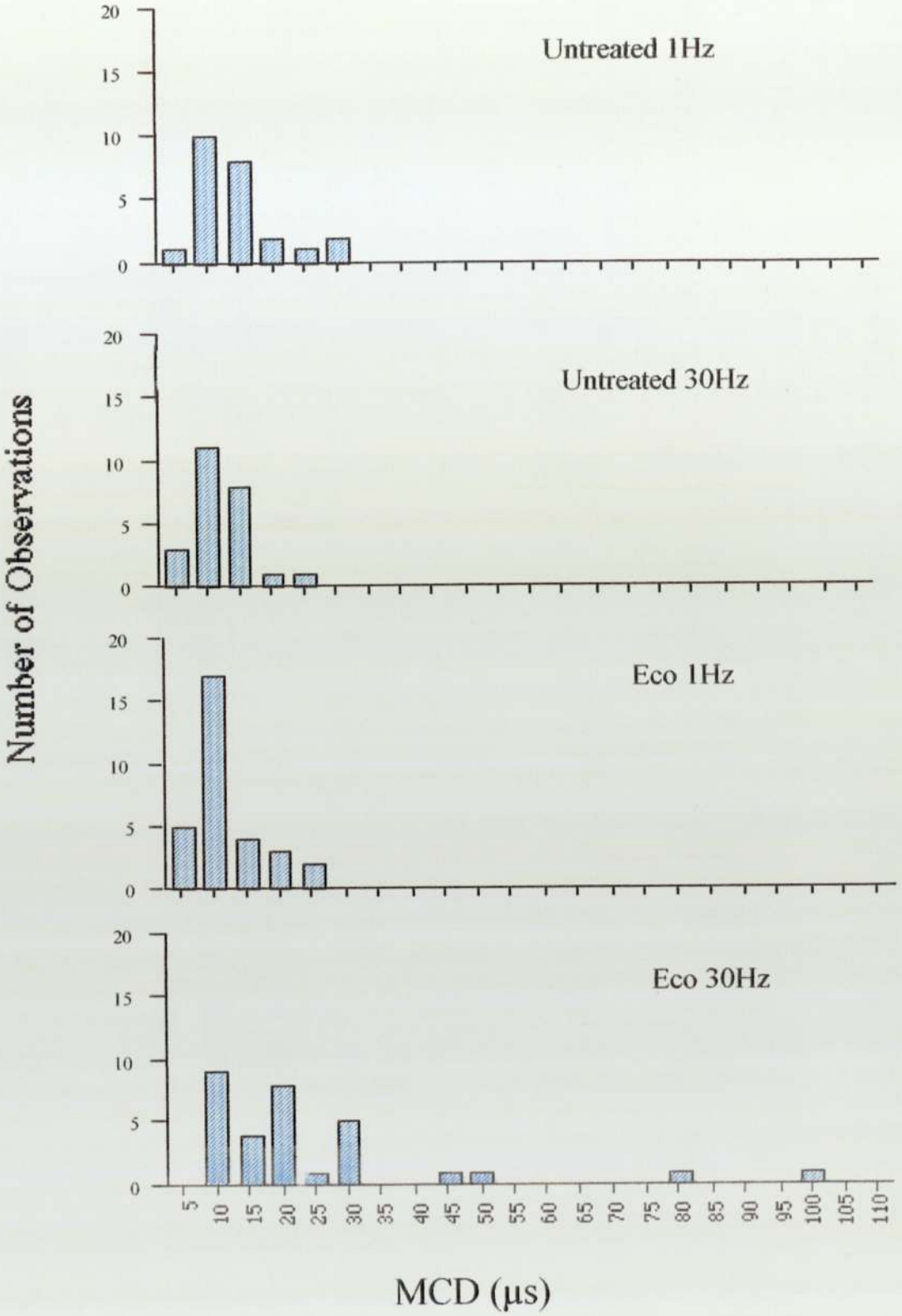


Figure 4.6 Frequency distributions of MCDs from untreated preparations and preparations 5 days after ecothiopate 500 nmolkg^{-1} with atrropine 700 nmolkg^{-1} stimulated at 1 and 30Hz. The number of fibres tested were 24 for untreated preparations and 31 for treated preparations.

CHAPTER 5

The electrophysiological effects of pyridostigmine

This chapter contains a description of the results from various series of experiments.

It is known that a "sign-free" dose of pyridostigmine (100 μgkg^{-1}) does not cause any increase in neuromuscular jitter, where a "sign-free" dose is defined as that which does not cause diarrhoea, bradycardia, pupillary constriction, asthma etc., the symptoms associated with about 30% inhibition of erythrocyte ChE. To determine if PYR was capable of producing jitter a larger dose of pyridostigmine (500 μgkg^{-1} + atropine 700 $nmolkg^{-1}$) was administered and it's effect on neuromuscular jitter and $(MEPP)_o$ timecourse measured. $500\mu kg^{-1}$ was used as the larger dose as in a series of experiments where increasing doses of PYR were administered this dose was found to cause fasciculation after approximately 30 mins and so was a comparable dose to 500 nmolkg-^{1} ECO.

After completion of the first set of experiments some unexpected results were found, AChE still seemed to be inhibited 1 day after dosing which is in contrast to the accepted half time of PYR (30 minutes, Koelle, 1963). If AChE was not still inhibited by PYR why were the $(MEPPs)_o$ prolonged? Could there be a reduction in the amount of enzyme there? This led to an investigation of how long pyridostigmine remains attached to, and so is inhibiting, AChE. This was done by recording $(MEPPs)_o$ after adding pyridostigmine *in vitro* and immediately after the diaphragm was removed after pyridostigmine administration via an Alzet osmotic pump.

In the final set of experiments a "sign-free" dose of pyridostigmine was given twice a day for up to 14 days and any changes in neuromuscular jitter and $(MEPP)_o$ timecourse measured to determine if there was any cumulative effect. The electrophysiological results obtained during these experiments are compared to biochemically determined AChE activity.

5.1 The effect of a 500 μgkg^{-1} dose of pyridostigmine

Experimental Design

To determine the effect of a 500 μgkg^{-1} dose of pyridostigmine animals were dosed with 500 μgkg^{-1} pyridostigmine with atropine (700 $nmolkg^{-1}$), which caused fasciculation after approximately 20 minutes and so was classed as a myopathic dose (Das, 1989). The diaphragms were then removed 1 or 5 days later and action potential trains for jitter analysis at 30Hz were recorded first then $(MEPPs)_0$ from the same diaphragms.

Results

Figure 5.1 shows the time to half amplitude of $(MEPPs)_0$ recorded 1 and 5 days after the pyridostigmine dose. At 1 day after dosing there is a significant increase in the time to half amplitude compared with the untreated control value. By 5 days after dosing however the time to half amplitude of these treated and the untreated controls were not significantly different.

Table 5.1 contains the values for Delay and MCD of action potential trains recorded at 30Hz after pyridostigmine dosing, and these are also shown graphically in Figure 5.2.

The Delay is significantly decreased 1 day after the dose of pyridostigmine but not 5 days after. Figure 5.3 shows the distributions of the Delay values after pyridostigmine. 1 day after dosing the distribution is much wider but it is skewed to the left, when compared to the untreated fibres, by the presence of many fibres with smaller values of Delay, so shifting the mean. Five days after dosing the distribution is still wide showing the presence of fibres with small Delays but is not skewed to the left and so the mean is close to that of untreated fibres.

Treatment	Delay (μs)	MCD (μs)
Untreated (43)	23±26	11.4±5.3
Pyridostigmine 1 Day (44)	12±41 *	16.5±6.6 *
Pyridostigmine 5 Days (41)	26±51	17.6±8.4*

Table 5.1 Effect of Pyridostigmine 500 μgkg^{-1} on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

The MCD is significantly increased both 1 and 5 days after pyridostigmine and Figure 5.4 shows the distributions of the MCD values at these times. At both times after pyridostigmine there are fibres which show large values of MCD, but there are also those fibres which have MCDs within the "normal" range and so are unaffected.

In the same way as with the ECO studies there was no significant correlation between the values for Delay and MCD suggesting that "normal" Delays did not occur in the same cells as "normal" MCDs.

In conclusion, a single myopathic dose of pyridostigmine prolongs the timecourse of extracellular miniature endplate potentials 1 day after dosing, these show recovery and by 5 days after dosing are not different from control $(MEPPs)_O$. It also causes changes in neuromuscular jittter seen as an increase in MCD and a decrease in Delay. The change in Delay is reversed 5 days after dosing but the MCD is still increased.

It was not expected that a dose of pyridostigmine would cause inhibition of AChE which would still be significant 1 day after the dose was given. To determine if it would be possible for pyridostigmine to still be attached to the AChE for such a long time experiments were then carried out to determine the timecourse of pyridostigmine inhibition and the experiments to test this are described in the next two sections.

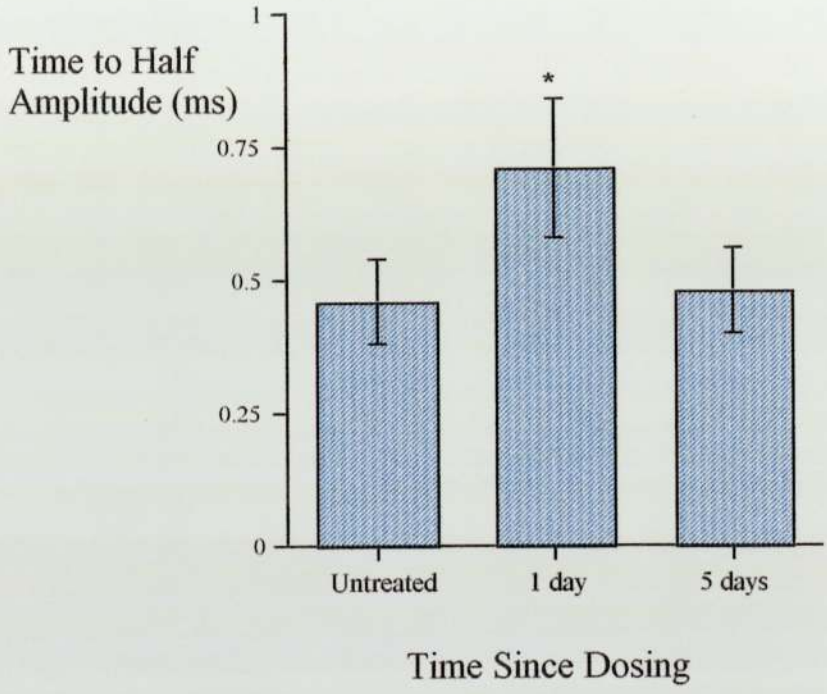


Figure 5.1 The effect of a single dose of pyridostigmine 500 μgkg^{-1} with atropine on the time to half amplitude of extracellular miniature endplate potentials recorded 1 and 5 days after dosing. 5 animals were tested for each condition. * indicates a significant difference from the untreated control value.

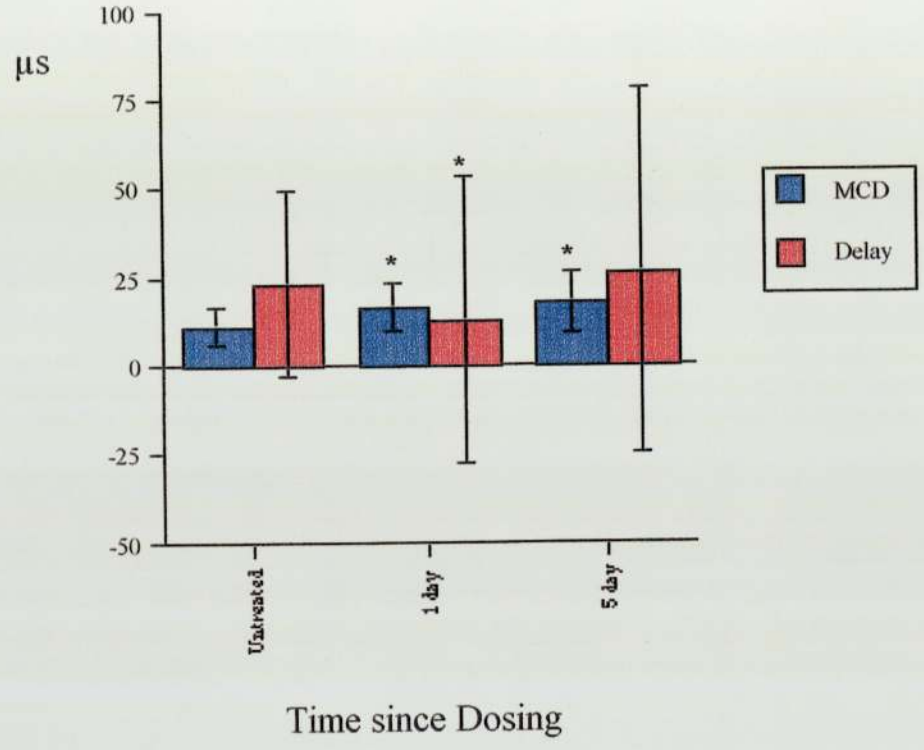


Figure 5.2 The effect of a single dose of pyridostigmine 500 μgkg^{-1} with atropine on the Delays and MCDs of action potential trains recorded from prerarations 1 and 5 days after dosing. The number of fibres tested were 43 for untreated preparations, 44 for 1 day and 41 for 5 days. * indicates a significant difference from the untreated control value.

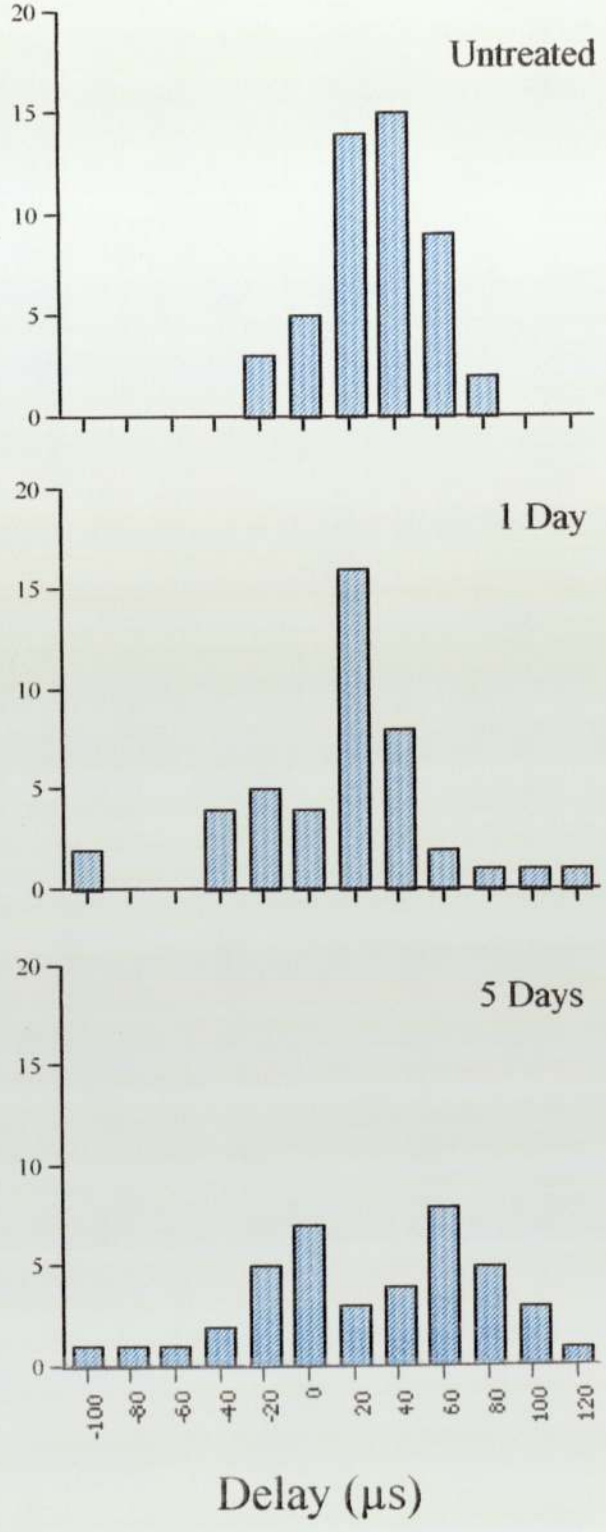


Figure 5.3 The distributions of Delays after pyridostigmine 500 μgkg^{-1} with atropine. *The Y axis indicates the number of fibres having a particular value of Delay.* The number of fibres tested were 43 for untreated preparations, 44 for 1 day and 41 for 5 days

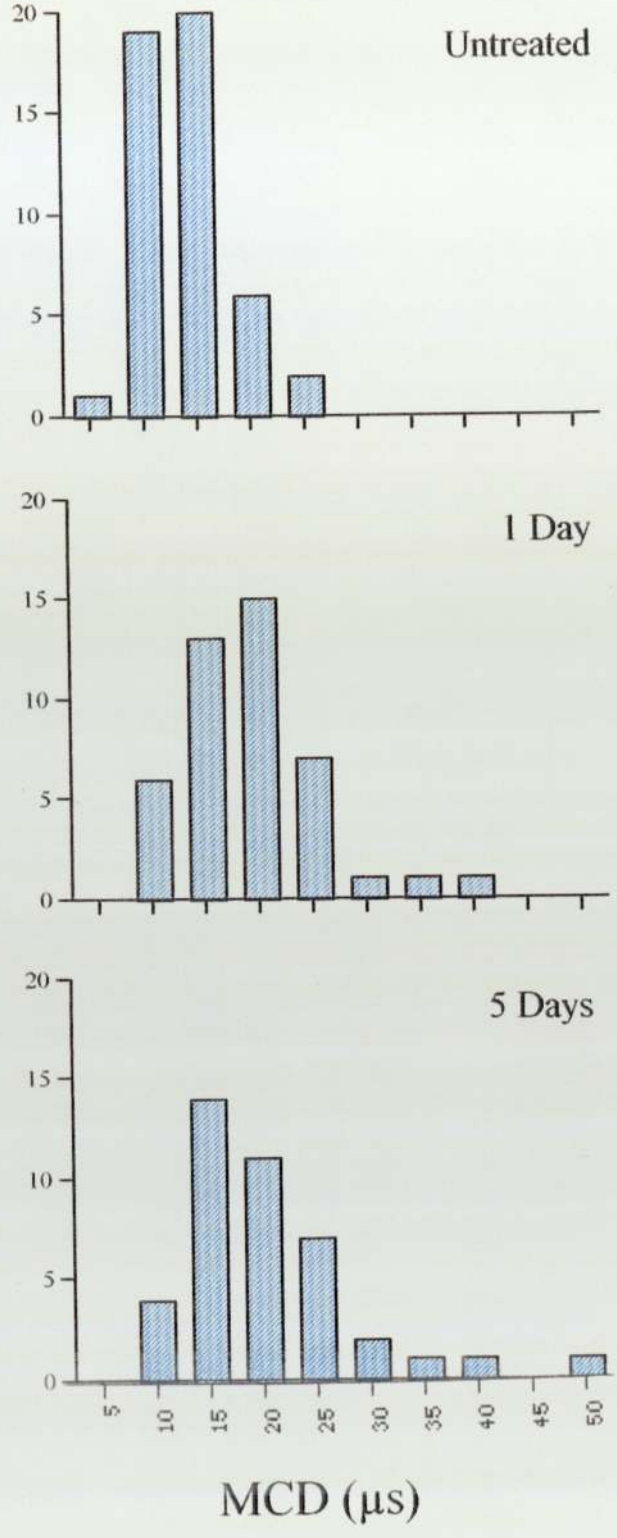


Figure 5.4 The distributions of MCDs after pyridostigmine 500 μgkg^{-1} with atropine. The *Y axis indicates the number of fibres having a particular value of MCD.* The number of fibres tested were 43 for untreated preparations, 44 for 1 day and 41 for 5 days.

5.2 The timecourse of pyridostigmine inhibition

5.2.1 The effect of temperature on $(MEPPs)_o$

Experimental Design

Initially a concentration response curve for pyridostigmine was determined by incubating a hemidiaphragm, while in the recording bath, in a particular concentration of pyridostigmine for 20 minutes and $(MEPPs)_o$ recorded thereafter.

To determine the timecourse of PYR inhibition diaphragms were removed and then incubated with 10^{-6} pyridostigmine at 37°C for 20 minutes. At the end of this 20 minutes the hemi-diaphragms were transfered to the recording bath and $(MEPPs)_o$ recorded. As during biochemical assay it was necessary to keep the diaphragm on ice for 3 hrs there was additional concern as to whether PYR was dissociating over this time. To determine if the relaxation of enzyme inhibition was temperature dependent, during recording the diaphragm was maintained at either 37°C or 30°C and recordings taken every 15 minutes. The temperature could not be dropped too drastically as it needed to be kept at a point at which $(MEPPs)_o$ would still occur. To make a fair comparison control $(MEPPs)_o$ were also recorded from untreated diaphragms at 30□C and 37□C.

Results

Figure 5.5 shows the concentration response curve for pyridostigmine over the concentration range 10^{-9} - 10^{-6}. It can be seen that there is no difference in the time to half amplitude of $(MEPPs)_o$ at 10^{-9} and 10^{-8} pyridostigmine, these concentrations also show no significant difference from controls. At 10^{-7} there is a significant increase in time to half amplitude which is increased further at 10^{-6}. It is not until a concentration greater than 10^{-8} therefore that there is any significant inhibition of AChE when this is assessed by the prolongation of $(MEPPs)_o$. In order to get a significant prolongation in

$(MEPP)_o$ timecourse therefore for the following experiments a concentration of 10^{-6} was chosen.

Figure 5.6 shows the effect of temperature on the measured parameters of $(MEPPs)_o$ from untreated hemi-diaphragms when these were maintained at 30°C and 37°C. All of the parameters measured showed a significant difference between the two temperatures except for 20-80% of the rise time. At 30°C they were all significantly longer.

The values for the parameters after incubating the hemi-diaphragms in 10^{-6} pyridostigmine for 20 minutes are seen in Figure 5.7. The presence of pyridostigmine caused a prolongation of all parameters which measure the decay phase of the $(MEPPs)_o$ but not the two which measure the rising phase only, in the same way as ecothiopate (see chapter 3). This is the case for the $(MEPPs)_o$ recorded from hemi-diaphragms maintained at both temperatures, comparing each to the relevant control. The $(MEPPs)_o$ recorded at 30°C however show parameters which are also significantly longer than those recorded at 37°C except for 20-80% rise time.

To determine the time taken for pyridostigmine to dissociate from the enzyme recordings were taken every 15 minutes and Figure 5.8 shows the effect of time on the time to half amplitude of $(MEPPs)_o$ recorded at both 37°C and 30°C. The change seen over time was the same for all of the parameters which measure the decay phase of the $(MEPPs)_o$ so time to half amplitude is shown only as an example. It can be seen that at 37°C the time to half amplitude is significantly prolonged for up to 60 minutes after the hemi-diaphragm was transfered to the recording bath. At 30°C however the prolongation of time to half amplitude is maintained for much longer, it is not until 180 minutes after washout is begun that there is no significant difference between the time to half amplitude and that of untreated controls.

In conclusion, lowering the temperature at which $(MEPPs)_o$ are recorded causes a prolongation of both the rising and decay phases even in untreated control preparations. The addition of pyridostigmine causes a further prolongation of the decay phase and the time taken for this to be reversed increases by 200% if the recording temperature is decreased to 30°C. As a reduction of 7°C causes such an increase in the time taken for relaxation of inhibition it is likely that at 0°C there is minimal dissociation of PYR, and so during biochemical assay the enzyme and PYR will still be associated.

The important point however is that pyridostigmine does dissociate from the enzyme within a relatively short time at 37°C and so suggests that in the experiments where 500 μgkg^{-1} pyridostigmine was administered subcutaneously to a mouse and the diaphragm removed 1 day later there would be no pyridostigmine still associated with the enzyme, and therefore the prolongation of $(MEPPs)_o$ timecourse may be due to a physical decrease in the amount of enzyme.

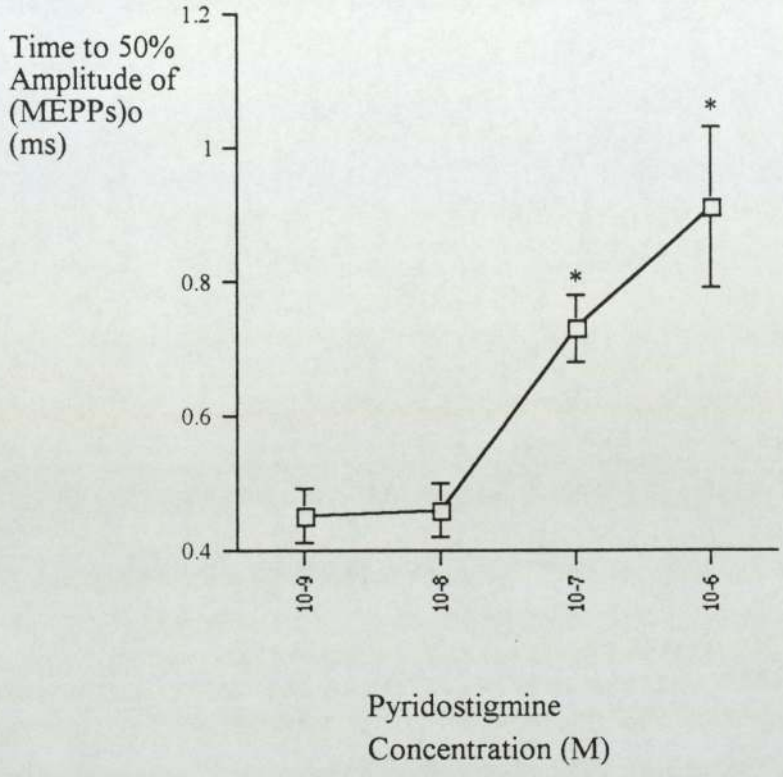


Figure 5.5 Concentration response curve for pyridostigmine. The response being the increase in the decay phase of extracellular miniature endplate potentials. * indicates a significant difference from controls.

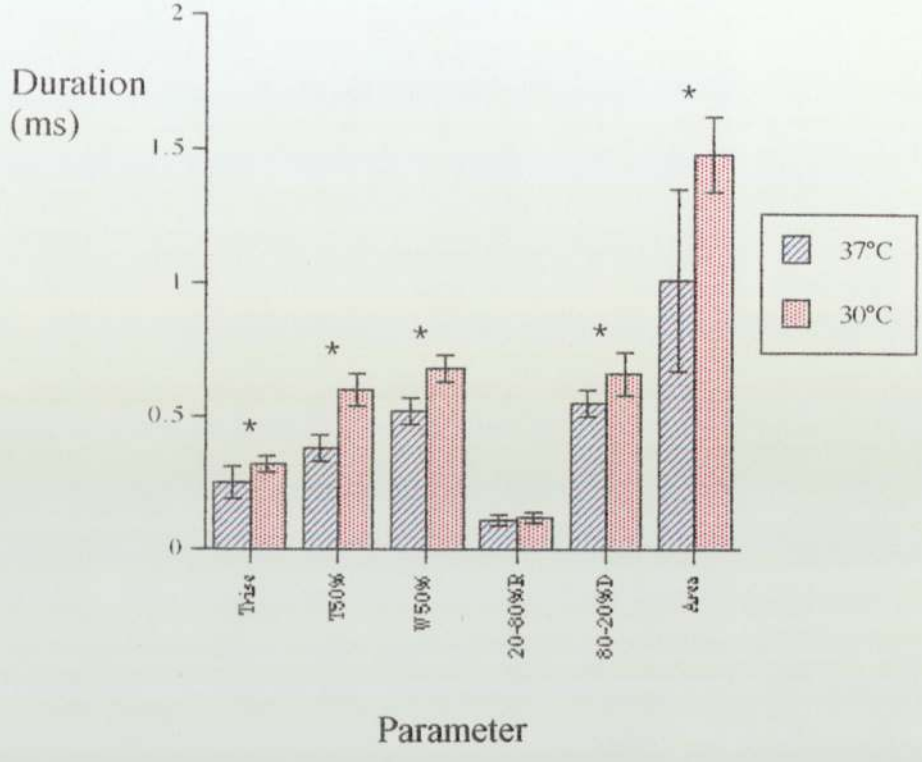


Figure 5.6 The effect of temperature on the measured parameters of extracellular miniature endplate potentials recorded from untreated control preparations maintained at either 37°C or 30°C. For each temperature 5 animals were tested. * indicates a significant difference between the values at the two temperatures.

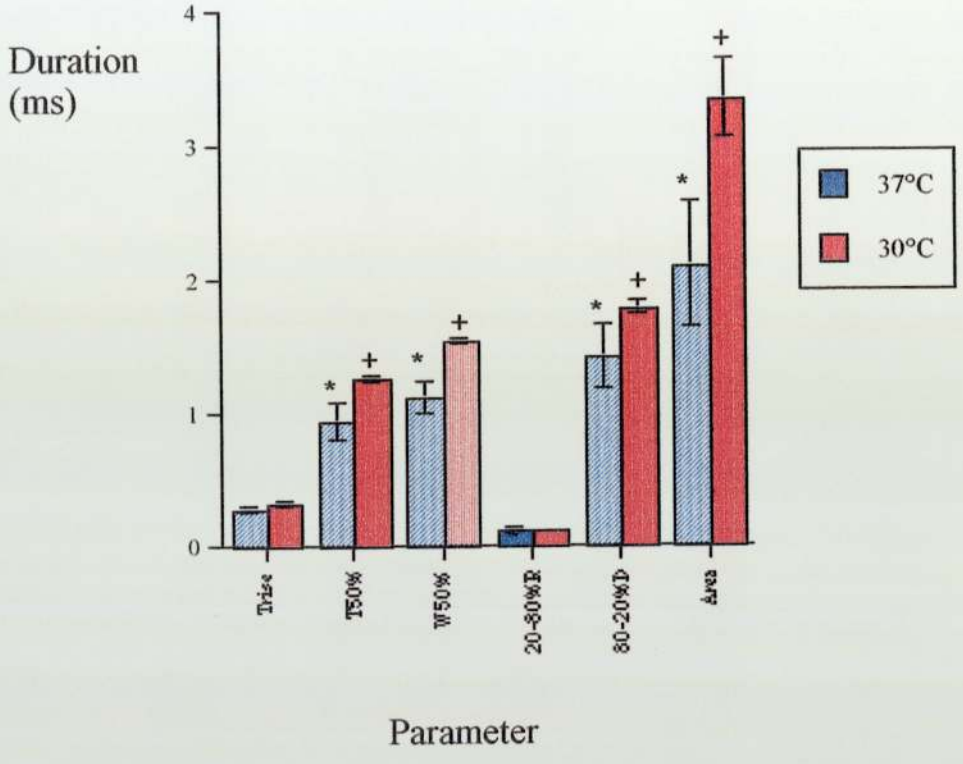


Figure 5.7 The effect of temperature on the measured parameters of extracellular miniature endplate potentials recorded from preparations incubated in 10^{-6}M pyridostigmine for 20 minutes, and then maintained at either 37°C or 30°C. For each temperature 5 animals were tested. * indicates a significant differnce from untreated controls at 37°C and + indicates a significant difference from controls at 30°C.

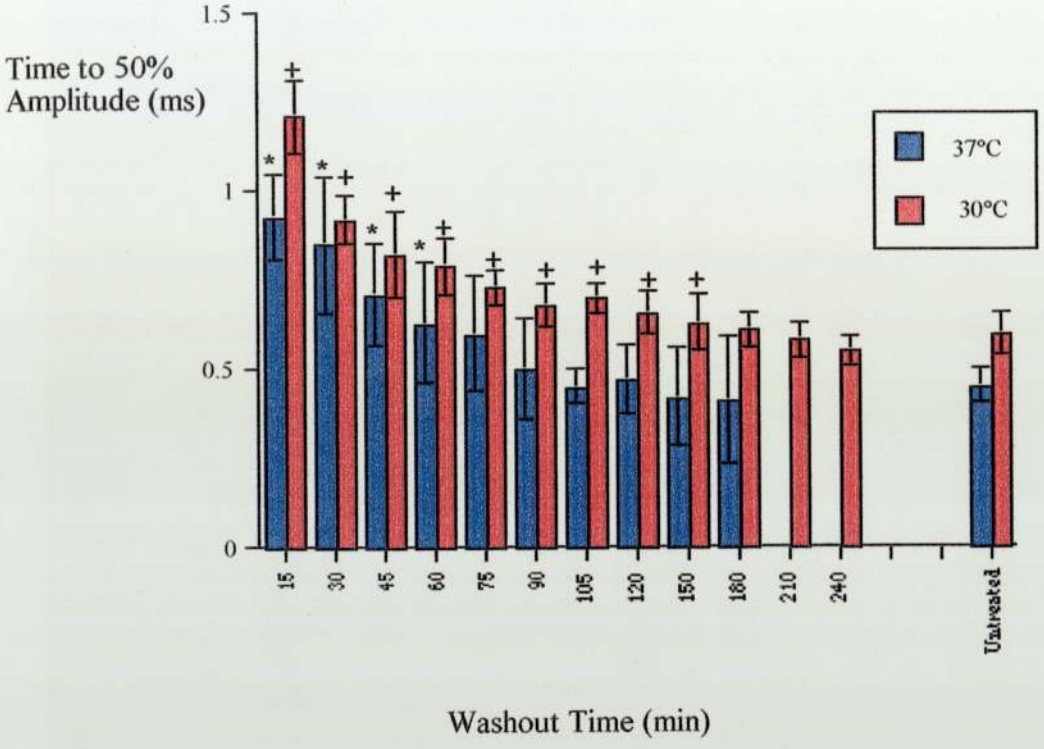


Figure 5.8 The effect of temperature on the time needed for washout of pyridostigmine. The time to half amplitude of extracellular miniature endplate potentials recorded from preparations incubated in 10^{-6}M pyridostigmine for 20 minutes was measured where these preperations were maintained at either 37°C or 30°C. For each temperature 5 animals were tested. * indicates a significant differnce from untreated controls at 37°C and + indicates a significant difference from untreated controls at 30°C.

5.2.2 The effect of time after diaphragm removal on T50% measured following anti-ChE in vivo.

Experimental Design

In the experiments where diaphragms were removed and action potentials recorded prior to recording $(MEPPs)_o$ it would seem from the *in vitro* experiments that there would be no pyridostigmine associated with the enzyme as it was approximately 1 hour after recording was begun that $(MEPPs)_o$ were recorded. Therefore experiments were carried out in which Alzet osmotic pumps were implanted to give a dose of pyridostigmine at a rate of $3\mu ghr^{-1}$, for 1, 2 and 7 days. After killing the animals and preparing the diaphragm, in some of these experiments $(MEPPs)_o$ were recorded immediately and in others they were recorded 1 hour later.

Results

Figure 5.9 shows the values for T50% from those $(MEPPs)_o$ recorded immediately and those recorded 1 hour later. It can be seen that at no time point was there a significant difference in the values of T50% for these 2 conditions. This would suggest that either pyridostigmine did not dissociate from the enzyme during this extra hour after dissection or that the pyridostigmine had already dissociated prior to the $(MEPPs)_o$ being recorded even when they were being recorded immediately.

The *in vitro* experiments showed that pyridostigmine does dissociate from the enzyme irrespective of temperature or drug concentration so the first explanation cannot be true therefore the pyridostigmine must have dissociated prior to recording. As soon as the diaphragm is removed it is placed in saline and from this time to when the first $(MEPP)_o$ is recorded takes approximately 45 minutes; 20 minutes for the dissection, 10 minutes to trim the hemidiaphragm and pin out in the recording bath, and then anything from 10

minutes upwards to find a recordable cell. Therefore the *in vitro* experiments show that 45 minutes is long enough for the majority of the pyridostigmine to dissociate.

The values of T50% are large enough to suggest that inhibition may be present but previous experiments have shown that after 1 hour at 37°C no significant inhibition is present. If prolongation of $(MEPPs)_o$ is apparent but there is no significant inhibition T50%. After anti-ChE does prolong $(MEPPs)_o$ not represent inhibited enzyme but must be prolonged due to some other factor or factors. Possible explanations are a depression in the level of functional AChE or a change in the characteristics of the ion channels.

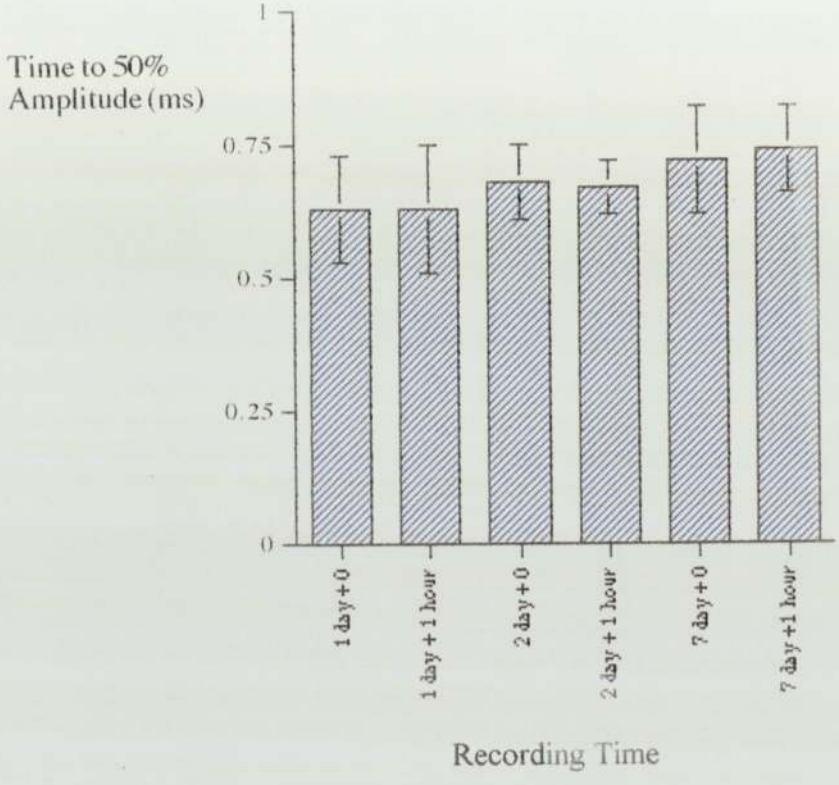


Figure 5.9 The time to half amplitudes of extracellular miniature endplate potentials recorded from the diaphragm of an animal fitted with an Alzet osmotic pump delivering pyridostigmine at a rate of 11.4 molhr^{-1} for up to 7 days. The (MEPPs)o were recorded either immediately after removal (+0) or 1 hour after removal (+1 hour). For each condition 5 animals were tested. At no time interval was there a significant difference between those recorded immediately and those recorded 1 hour later.

5.3 The effect of repetitive dosing with pyridostigmine

5.3.1 Effects seen during dosing

Experimental Design

During this series of experiments animals were dosed twice daily with 100 μgkg^{-1} pyridostigmine. The diaphragms were then removed for electrophysiological recording at the times shown below.

1 day

2 days

4 days

7 days

14 days

Results

Figure 5.10 shows the time to half amplitude of $(MEPPs)_o$ after repetitive pyridostigmine dosing. All of the $(MEPPs)_o$ were recorded 3 hours after the final dose except one set of recordings which were made only 1 hour after the dose. All of the $(MEPPs)_o$ recorded 3 hours after the final dose are not significantly different from untreated control $(MEPPs)_o$. Those recorded just 1 hour after the dose however show a significant prolongation, thus prolongation of $(MEPP)_o$ timecourse is only seen acutely. It was suggested in the previous section that pyridostigmine has dissociated from the enzyme after 45 minutes which disagrees with the data presented here. The 1 hour in these experiments however was measured from the time of injection so the time taken by the drug to travel from the point of injection to the endplates has not been accounted for and may have an effect on these results. The *in vitro* experiments meant that the drug could reach the endplates quicker as it was applied directly to the diaphragm and the endplates from which

(MEPPs)o were recorded were those on the surface. Functional enzyme activity i.e. the activity of the endplate specific non extractable fraction agrees with the electrophysiological data in that at no time point was there a significant difference between this activity and that of control preparations. (Data courtesy of A. Rowbotham)

Table 5.2 contains the values of Delay and MCD of action potential trains recorded 3 hours after the final dose of pyridostigmine. These are also shown graphically in Figure 5.11.

The Delay was decreased every day after dosing was begun when compared to the untreated control. This decrease is not significant 1 day after dosing was begun but every subsequent time point shows a significant difference from untreated controls. Figure 5.12 shows the distributions of Delays during the period of dosing. All of the Delays during the period show a wider distribution, having fibres with Delays both larger and smaller than the untreated controls, but the majority are smaller so reducing the mean value.

Treatment	Delay (μs)	MCD (μs)
Untreated (43)	23±26	11.3±5.3
1 Day (35)	16±37	12.4±2.9
2 Days (33)	9±43 *	12.5±3.6
4 Days (36)	8±41 *	16.2±9.8 *
7 Days (35)	11±50 *	19.9±15.4 *
14 Days (37)	7±47 *	26.7±18.2 *

Table 5.2 Effect of Pyridostigmine 100 μgkg^{-1} twice daily on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

The MCD was initially unaffected by the pyridostigmine dosing. It was not significantly different from untreated controls 1 and 2 days after dosing was begun. Four days after dosing was begun however the MCD was significantly increased with respect to the untreated controls, as was the MCD at 7 and 14 days into dosing. The distributions of MCD can be found in Figure 5.13. The distributions are shifted to the right after dosing is begun and shift further and further to the right as dosing continues and more and more fibres are affected and show larger MCD values. The distributions get wider as responses show more variability perhaps with only part of the population being affected, some fibres remaining "normal".

Figure 5.14 shows the effect of repetitive dosing on the shape of endplates as indicated by their width/length ratios. After just 3 hours there was a significant distortion of the endplates when compared to untreated controls, the width/length ratio is increased as the endplates become rounder. This deformation continues throughout the period of dosing.

In conclusion, repetitive administration with pyridostigmine caused a prolongation of the decay phase of $(MEPPs)_O$ immediately after dosing but this recovered rapidly so 3 hours after the dose no prolongation is seen. This is as expected if the half life of pyridostigmine is considered. After 3 hours it would have dissociated from the AChE and so would no longer be inhibiting it.

An increase in neuromuscular jitter is also seen as an increase in MCD and a decrease in Delay. The rapidity at which pyridostigmine dissociates therefore is not preventing whatever mechanism causes increased neuromuscular jitter. The decrease in Delay is contrary to what is observed after dosing with ecothiopate when an increase in Delay is seen. A decrease in Delay was also seen when 500 μgkg^{-1} pyridostigmine was administered acutely, suggesting a drug difference but why this is so is not clear.

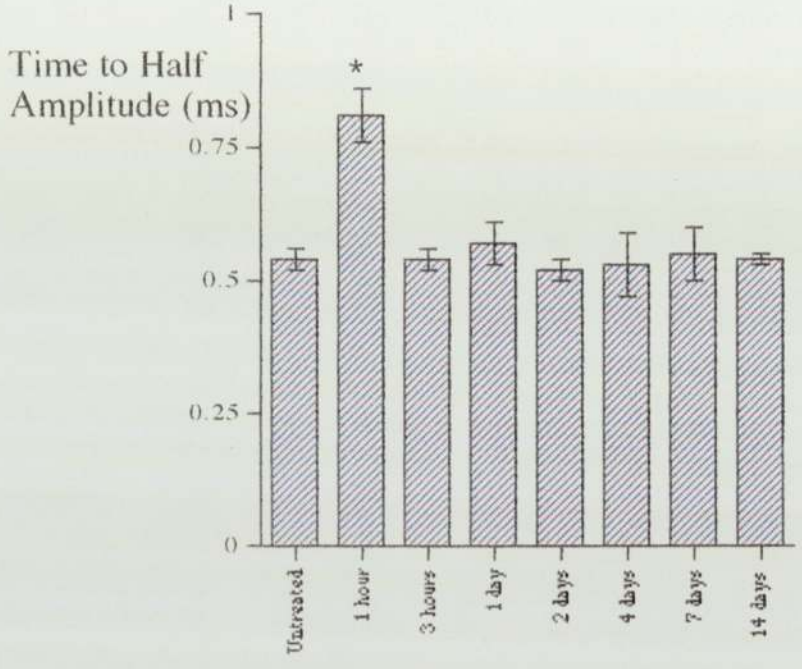


Figure 5.10 The effect of pyridostigmine 100 μgkg^{-1} twice daily on the time to half amplitude of extracellular miniature endplate potentials. All recordings were made 3 hours after the final dose except for the 1 hour value. For each condition 5 animals were tested. * indicates a significant difference from the untreated control value.

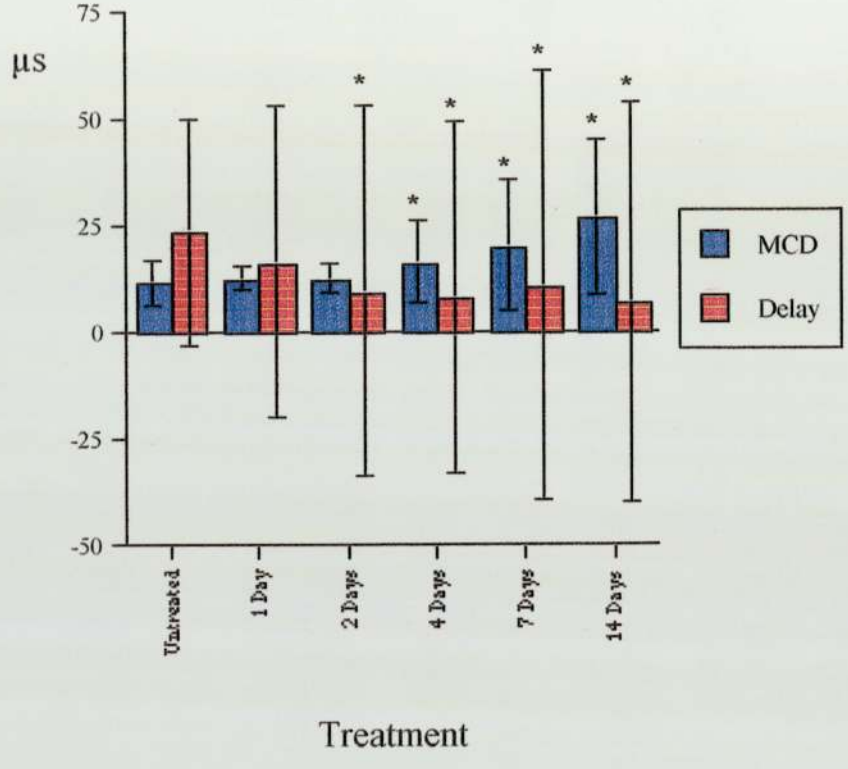


Figure 5.11 The effect of pyridostigmine 100 μgkg^{-1} twice daily on the Delays and MCDs of action potential trains. All recordings were made 3 hours after the final dose. The number of fibres tested were 43 for untreated preparations, 35 for 1 day, 33 for 2 days, 36 for 4 days, 35 for 7 days and 37 for 14 days. * indicates a significant difference from the untreated control value.

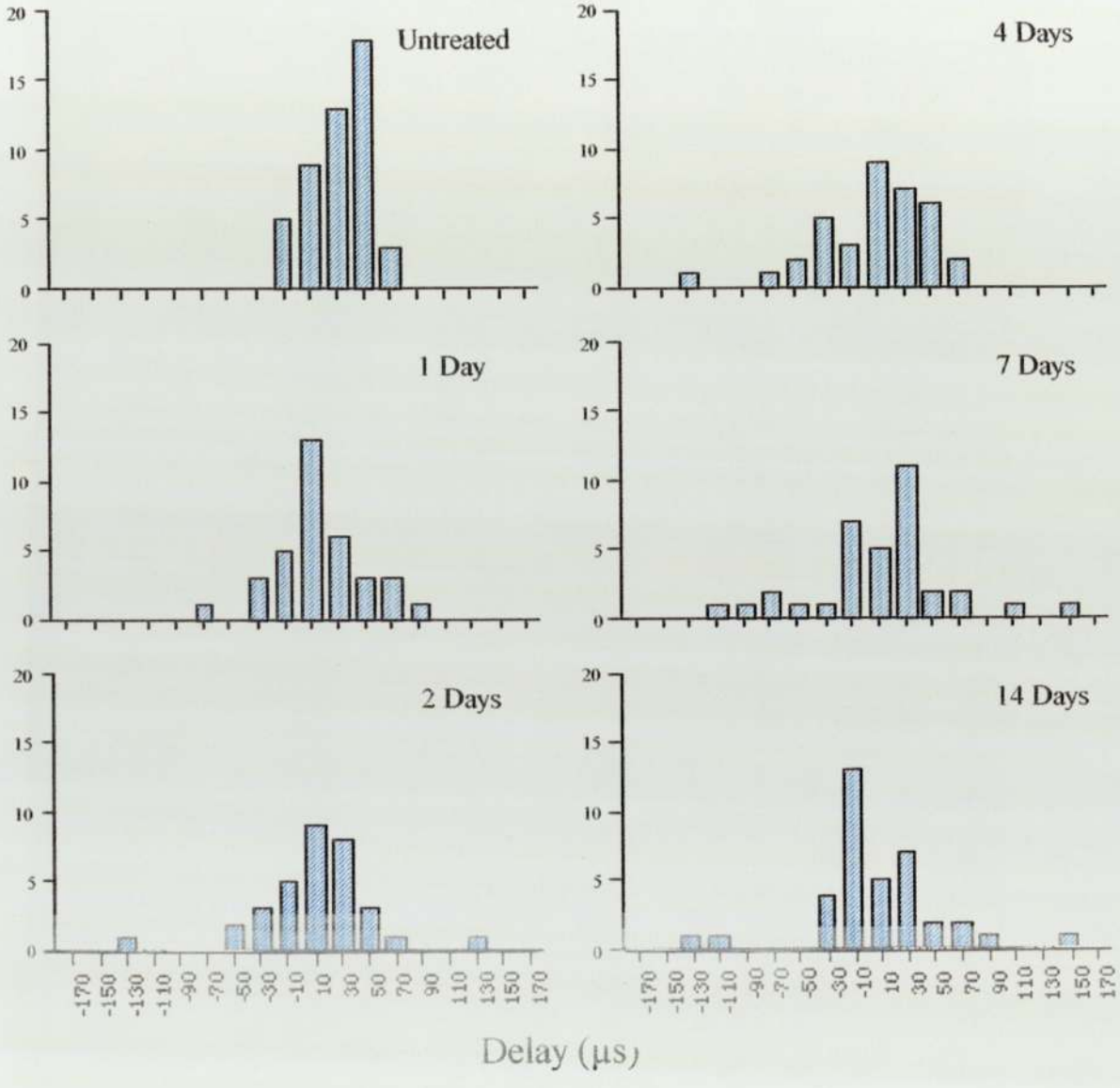


Figure 5.12 The distributions of Delays after pyridostigmine 100 μgkg^{-1} twice daily. *The Y axis indicates the number of fibres having a particular value of Delay.* . The number of fibres tested were 43 for untreated preparations, 35 for 1 day, 33 for 2 days, 36 for 4 days, 35 for 7 days and 37 for 14 days.

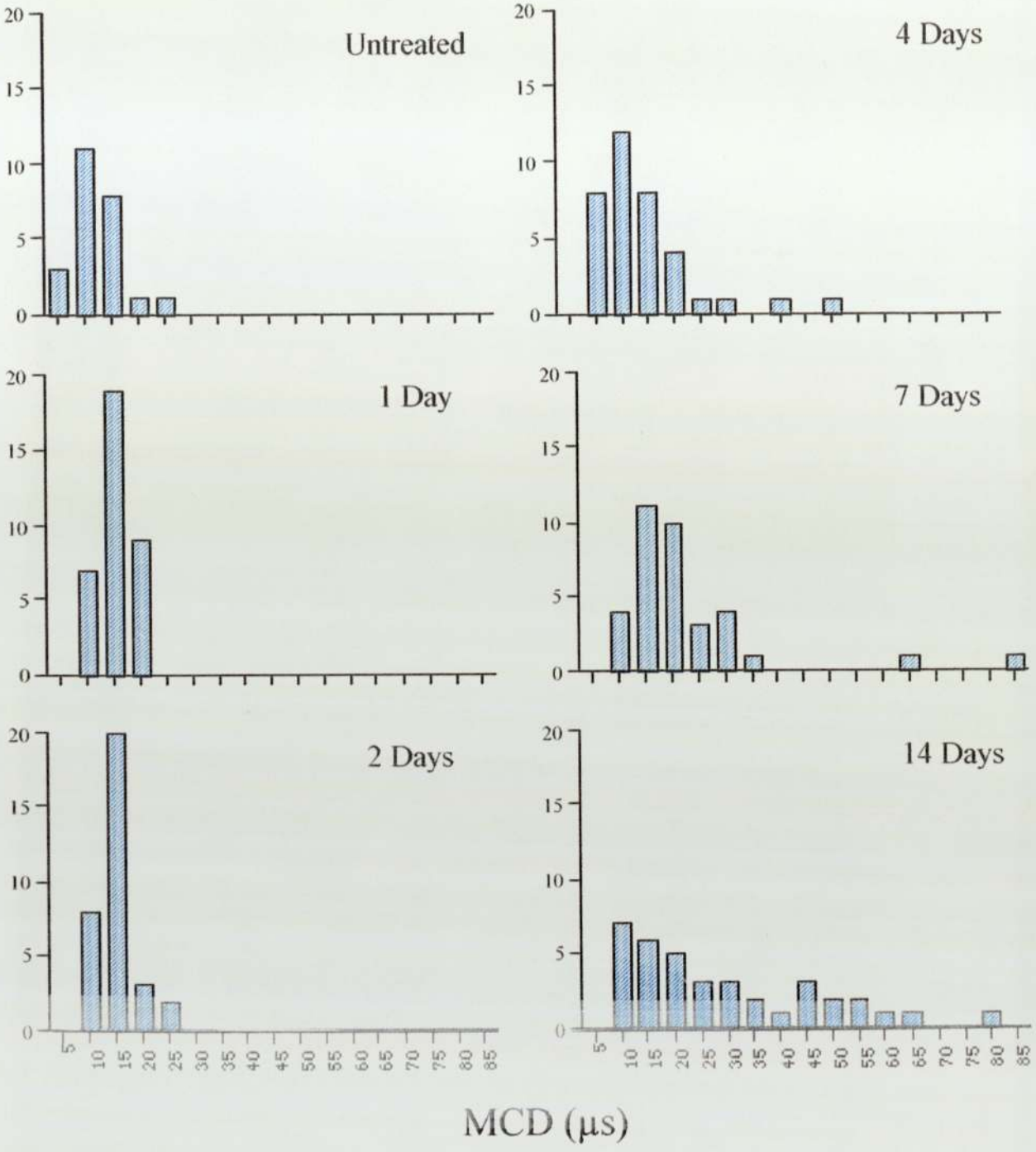


Figure 5.13 The distributions of MCDs after pyridostigmine 100 μgkg^{-1} twice daily. *The Y axis indicates the number of fibres having a particular value of MCD.* . The number of fibres tested were 43 for untreated preparations, 35 for 1 day, 33 for 2 days, 36 for 4 days, 35 for 7 days and 37 for 14 days.

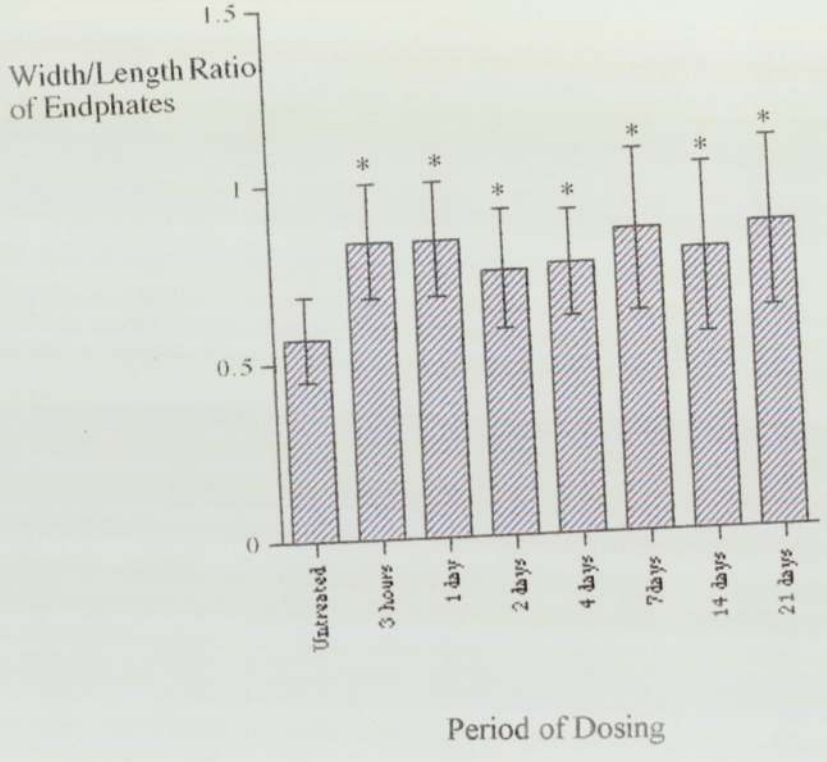


Figure 5.14 The effect of pyridostigmine 100 μgkg^{-1} twice daily on the shape of endplates, shown as the width/length ratio of the endplate. The values are shown as the mean ± s.d. For each condition 5 animals were tested. * indicates a significant difference from untreated controls (Mann-Whitney $p<0.05$)

5.3.2 Recovery from the effects of repetitive pyridostigmine administration

Experimental Design

To determine if there was any recovery from the effects of repetitive pyridostigmine, dosing was stopped after 7 days and electrophysiological recordings made over the following days at the times indicated below.

2 days

7 days

14 days

Results

Figure 5.15 shows the time to half amplitude of (MEPPs)o recorded during this recovery period. At no time point was there a significant difference between the time to half amplitude of the test group and that of the untreated controls. Once again this is consistent with the activities of the functional enzyme after dosing was ceased which also showed no significant difference from controls at any of the time points. (Data courtesy of A. Rowbotham)

Table 5.3 contains the values for Delay and MCD of action potential trains recorded during the recovery period, and these are also shown graphically in Figure 5.16.

Treatment	Delay (μs)	MCD (μs)
Untreated (43)	23±26	11.4±5.3
7 Days Dosing (35)	11±50 *	19.9±15.4 *
2 Days Recovery (34)	14±36 *	18.8±10.6 *
7 Days Recovery (35)	15±25 *	14.6±4.3 *
14 Days Recovery (35)	21±23	11.0±2.7

Table 5.3 Effect of discontinuing pyridostigmine 100 μgkg^{-1} twice daily on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

Pyridostigmine administered repetitively caused a decrease in the Delay of action potential trains. This decrease was still evident 2 days after dosing ceased and Delay continued to be significantly reduced with respect to untreated controls 7 days after dosing was stopped. 14 days after dosing was stopped however there is no significant difference between the value of Delay for these fibres and untreated controls. The distributions of the Delays during this recovery period are seen in Figure 5.17. It can be seen that as recovery time continues there is a shift in the distributions to the right as the number of affected fibres, which show smaller Delays, is reduced.

During the period of dosing the MCD of action potential trains was increased with respect to the untreated controls. Two days into the recovery period there is still an increase in

the value of the MCD and this increase continues until 7 days after dosing was stopped. The MCDs at these time points are significantly different from that of untreated controls. 14 days after dosing was stopped the MCD had recovered and shows no significant difference from untreated controls. Figure 5.18 shows the distributions of MCDs during the recovery period. The distribution 2 days into the recovery period is shifted to the right due to the presence of a number of fibres with large MCDs, which are therefore still affected by pyridostigmine. Five days later the distribution has shifted back to the left and looks more like that of the untreated fibres as the number of affected fibres is reduced, and 14 days after dosing was stopped the distribution has completely shifted back to the left.

In conclusion, discontinuing repetitive pyridostigmine administration does not affect the timecourse of $(MEPPs)_o$ or the activity of functional enzyme but allows recovery from increased neuromuscular jitter, and endplate deformation. The recovery from neuromuscular jitter occurs slowly. It is not until 14 days after dosing was stopped that both Delay and MCD returned to "normal".

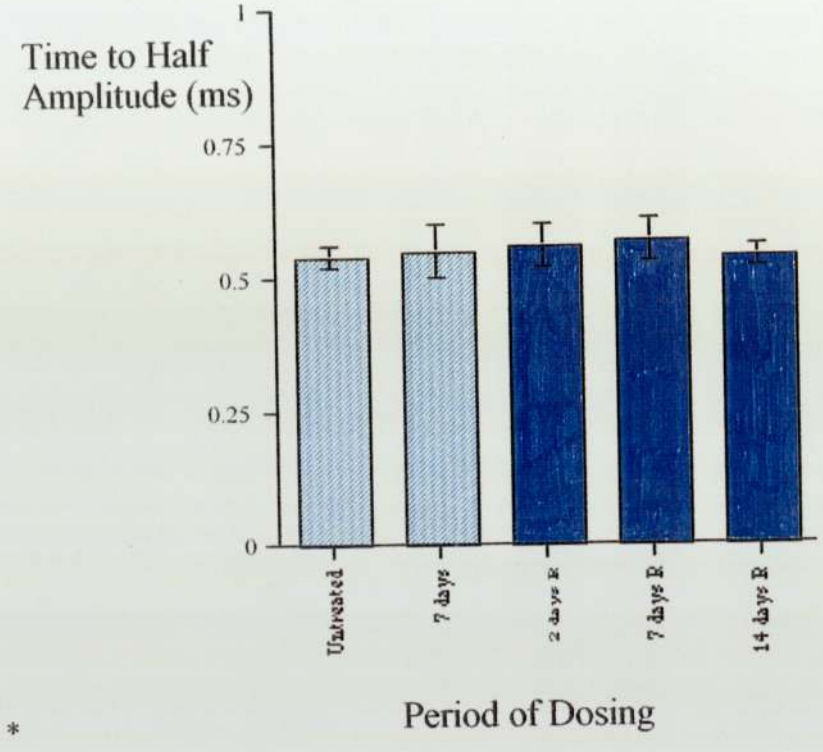


Figure 5.15 The effect of discontinuing pyridostigmine 100 μgkg^{-1} twice daily on the time to half amplitude of extracellular miniature endplate potentials. For each condition 5 animals were tested. * indicates a significant difference from the untreated control value.

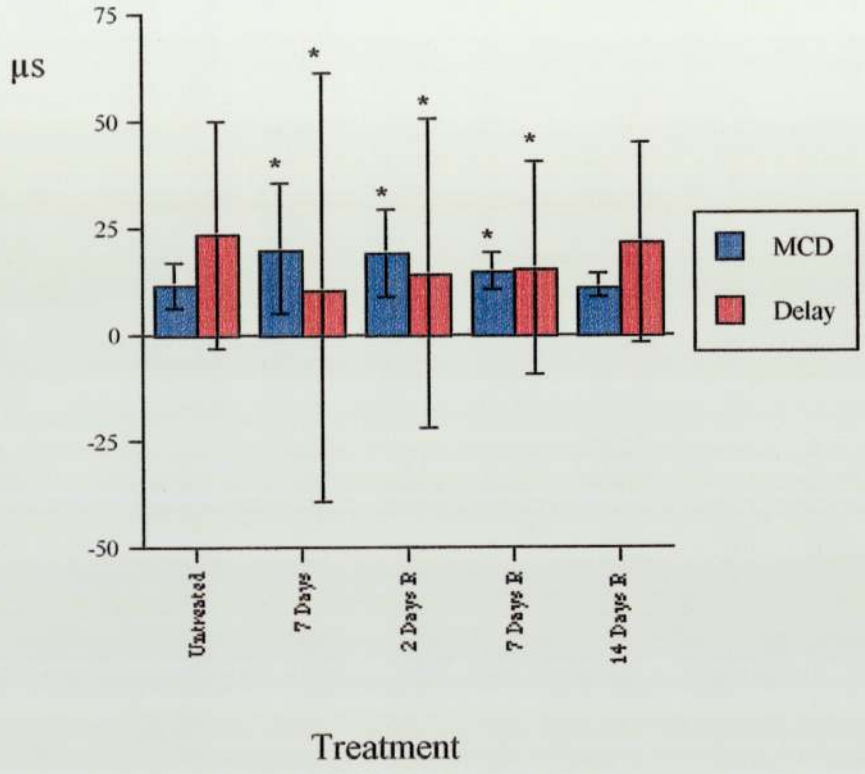


Figure 5.16 The effect of discontinuing pyridostigmine 100 μgkg^{-1} twice daily on the Delays and MCDs of action potential trains. . The number of fibres tested were 43 for untreated preparations, 35 for 7 days dosing, 34 for 2 days recovery, 35 for 7 days recovery and 35 for 14 days recovery. * indicates a significant difference from the untreated control value.

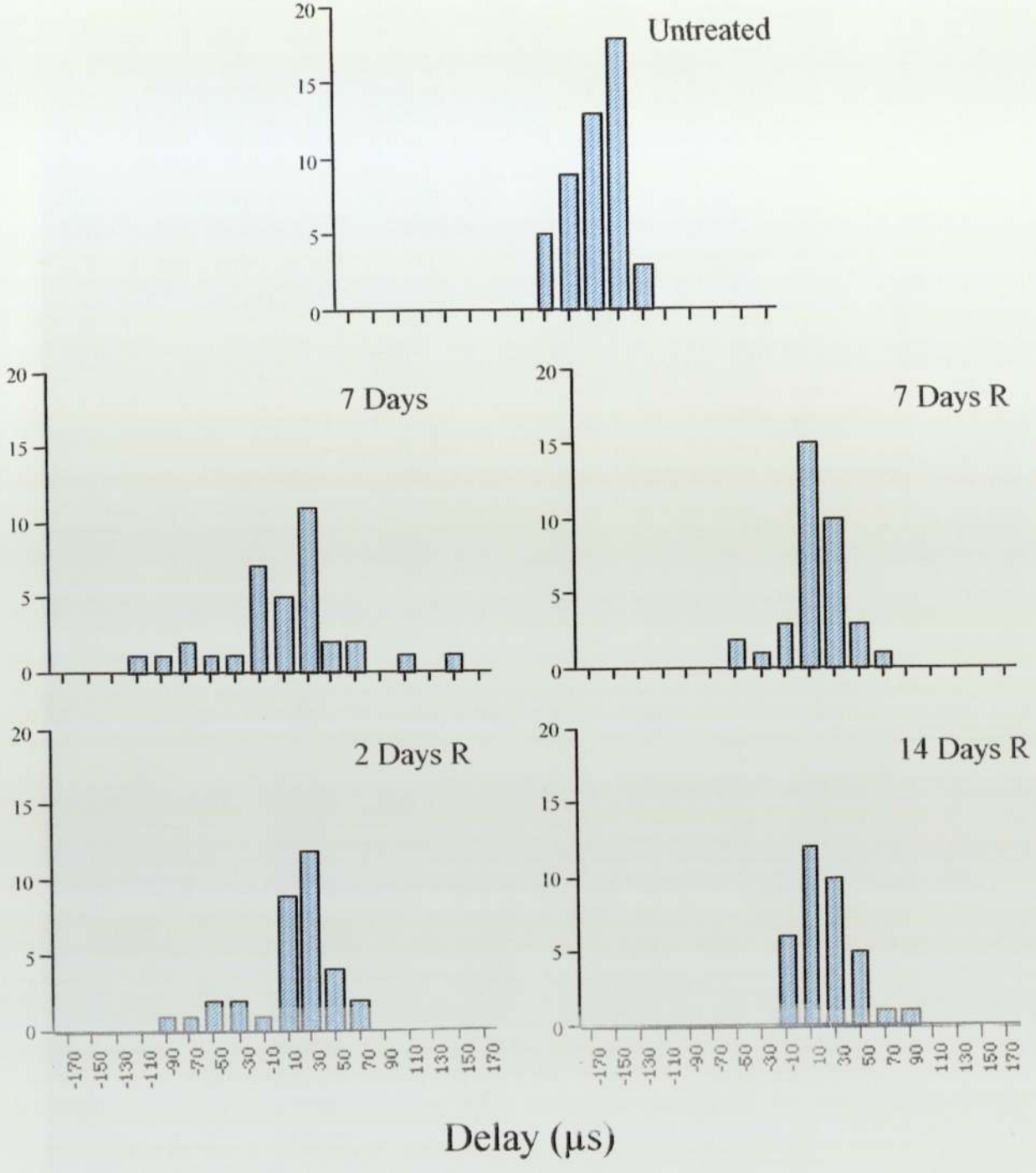


Figure 5.17 The distributions of Delays after discontinuing pyridostigmine 100 μgkg^{-1} twice daily. *R indicates the number of days recovery. The Y axis indicates the number of fibres having a particular value of Delay.* The number of fibres tested were 43 for untreated preparations, 35 for 7 days dosing, 34 for 2 days recovery, 35 for 7 days recovery and 35 for 14 days recovery.

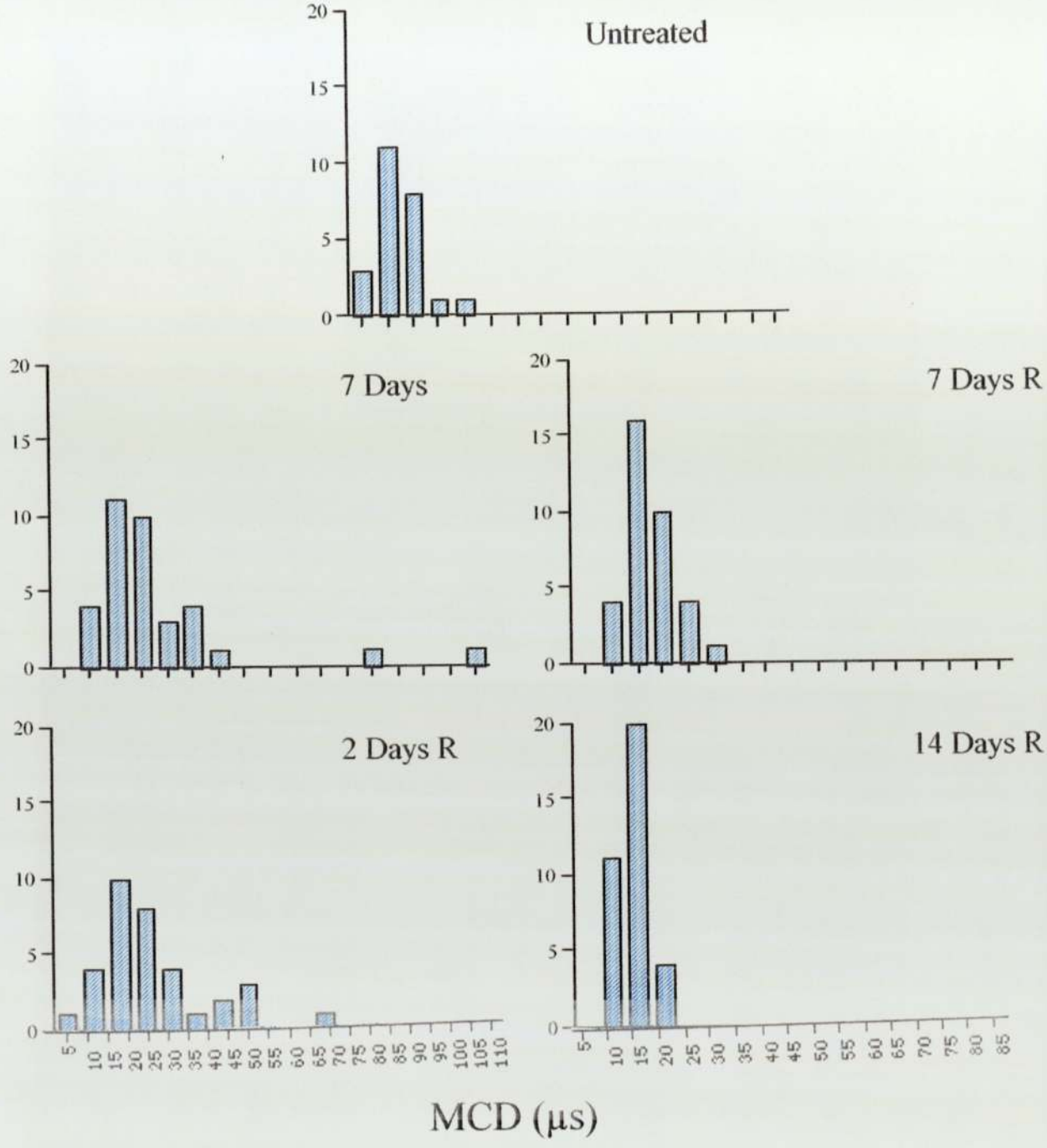


Figure 5.18 The distributions of MCDs after discontinuing pyridostigmine 100 μgkg^{-1} twice daily. *R indicates the number of days recovery. The Y axis indicates the number of fibres having a particular value of MCD.* The number of fibres tested were 43 for untreated preparations, 35 for 7 days dosing, 34 for 2 days recovery, 35 for 7 days recovery and 35 for 14 days recovery.

CHAPTER 6

The effect of continuous administration of pyridostigmine

This series of experiments was made to determine the effect of pyridostigmine administered continuously via an Alzet osmotic pump at a rate of 11.4 $nmolhr^{-1}$, which was a dose resulting an inhibition of erythrocyte ChE of approximately 30% which is equivalent to the dose given in the injections previously. The effect of this type of administration on jitter and the time course of extracellular miniature endplate potentials $(MEPPs)_o$ was investigated and these results are then compared with the acetylcholinesterase inhibition and the deformation of the endplates. It has previously been shown that a single dose of a carbamate will protect against the effects of a myopathic dose of ecothiopate (Kelly *et al* 1992). In another series of experiments the protective effects of continuous pyridostigmine administration were also investigated.

The series of experiments were divided into two parts, the first dealing with the effects of the drug during the dosing period i.e. when the pump was still delivering the drug, and the second investigating recovery from the drug, by removing the diaphragms after dosing was discontinued.

6.1 The effect of continuous administration of pyridostigmine.

Experimental Design

The mice used in this study were divided into 5 groups implanted with Alzet osmotic pumps and their diaphragms removed at the timepoints shown below.

1 day

2 days

4 days

7 days

14 days

For each experiment the diaphragm was divided into 2 hemidiaphragms. The first was fixed and stained for cholinesterase while the other was transfered to the recording set up for electrophysiological measurements. After the hemidiaphragm was transfered to the recording set up the first recordings made were of trains of 30 action potentials stimulated indirectly at 30Hz. After approximately 10 cells had been recorded $(MEPPs)_O$ were recorded, an average of five cells per hemidiaphragm.

Control preparations were taken from animals which had been implanted with Alzet osmotic pumps delivering physiological saline only and their diaphragms removed 1, 2, 4 and 7 days later. It was found that there was no significant difference between these saline treated preparations at any timepoint for either the jitter parameters or the time to 50% amplitude of the $(MEPPs)_O$ (see table 6.1) and so for later statistical testing these data have been pooled.

Results

Figure 6.1 shows the effect of pyridostigmine on the time course of $(MEPPs)_O$. It shows the time to 50% amplitude of the decay phase increasing as the time between pump implantation and removal of the diaphragm increases. The increase in time to half amplitude seen after 1 day of pumping is significant as is the difference between the time to half amplitude for each consecutive timepoint against the control value. When the time to half amplitude for each timepoint is tested against the previous one the increases are not significant, except between day 7 and day 14.

Appendix 7 contains the biochemical assay data for the functional enzyme (i.e. the endplate specific non extractable fraction) during dosing with pyridostigmine via Alzet osmotic pump. It can be seen that although there appears to be a reduction in the

functional enzyme activity during the dosing period it is only days 4 and 7 which show a significant decrease when compared to control values. The values for enzyme activity do not agree with the electrophysiological data, which shows a significant difference at all time points. One, 2 and 14 days after pyridostigmine there is no significant inhibition of AchE however the standard deviation is very large at these time points and for days 1 and 2 it is even bigger than the value of the mean which will greatly affect calculations of significance. The biochemistry therefore should really be repeated to obtain reasonable data which can then undergo statistical testing.

	Time to half amplitude (ms)	Mean Consecutive Difference (μs)	Delay (μs)
1 day	0.52±0.10 (6)	11.4±3.2 (10)	32±23 (10)
2 days	0.48±0.06 (8)	11.2±4.0 (10)	31±33 (10)
4 days	0.43±0.08 (7)	11.7±3.2 (10)	25±35 (10)
7 days	0.43±0.10 (5)	10.8±3.5 (10)	25±22 (10)

Table 6.1 Effect of physiological saline on the time to half amplitude of extracellularly recorded miniature endplate potentials, and the Delay and MCD of action potential trains from diaphragm muscle fibres, from the same preparations. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested.

Table 6.2 contains the values for the MCD and Delay of action potentials recorded from fibres of diaphragms removed after 1 to 14 days of dosing, and these are shown graphically in Figure 6.2.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28	11.7±3.3
1day (40)	9±35	12.0±4.4
2 days (41)	19±19 *	12.0±6.3
4 days (40)	11±23 *	12.6±8.6
7 days (44)	13±43	18.0±13.3 *
14 days (42)	5±64 *	33.7±27 *

Table 6.2 Effect of Pyridostigmine 11.4 $nmolhr^{-1}$ on the Delay and MCD of diaphragm muscle fibres. All values shown are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

The Delay appears to be decreased at every timepoint after dosing is begun however it is only days 2, 4 and 14 which show a significant decrease. Figure 6.3 shows the frequency distributions for the values of Delay. The means are reduced after drug administration but the distributions are wider at most of the timepoints showing increased variances which prevents significance from being shown. Pyridostigmine administered continuously

therefore causes the same decrease in Delay, as opposed to the increase seen with ecothiopate, which is seen when pyridostigmine is administered repetitively or acutely in a myopathic dose.

The MCD however is not affected initially, but remains constant throughout the first few days of dosing. It is only day 7 and day 14 which show a significant increase in the value of MCD. There is also significant difference between the MCDs of day 7 and day 14. Figure 6.4 shows the frequency distributions for the MCD values. It can be seen that there is a trend for the distributions to move further to the right as time since osmotic pump implant increases, more and more fibres are affected and so show increased values of MCD. Throughout all of the time points though there are some cells which are unaffected, their MCDs staying within the normal range.

Figure 6.5 shows the change in endplate shape after continuous dosing with pyridostigmine. The first day after dosing is started there is a significant increase in the width/length ratio of the endplates indicating a rounding up of the endplates which continues throughout the period of dosing with every timepoint showing a significant difference from control values. There is no significant difference bvetween timepoints however.

Pyridostigmine administered continuously therefore causes a prolongation of $(MEPP)_o$ timecourse over the entire period of dosing, as well as a deformation in endplate shape. Neuromuscular jitter increase is also seen as a decrease in the Delay which occurs after the second day of dosing and an increase in MCD which is seen only after the seventh day. There is a reduction in the activity of functional enzyme but this is also not seen initially, occuring only on days 4 and 7.

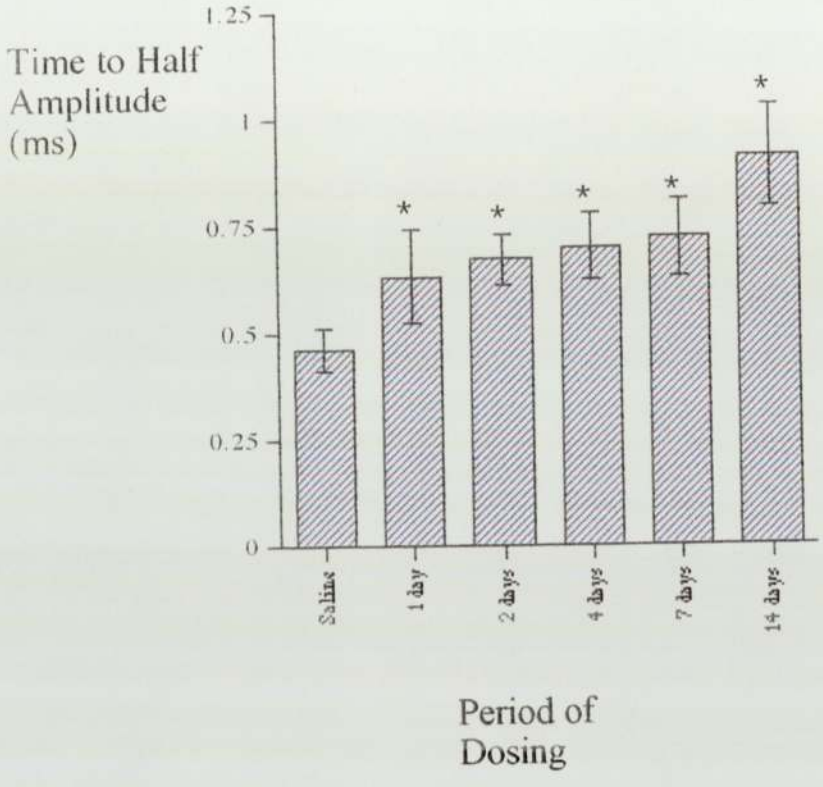


Figure 6.1 The effect of continuous administration of pyridostigmine (11.4 $nmolhr^{-1}$) on the time to half amplitude of extracellular miniature endplate potentials. The values shown are means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

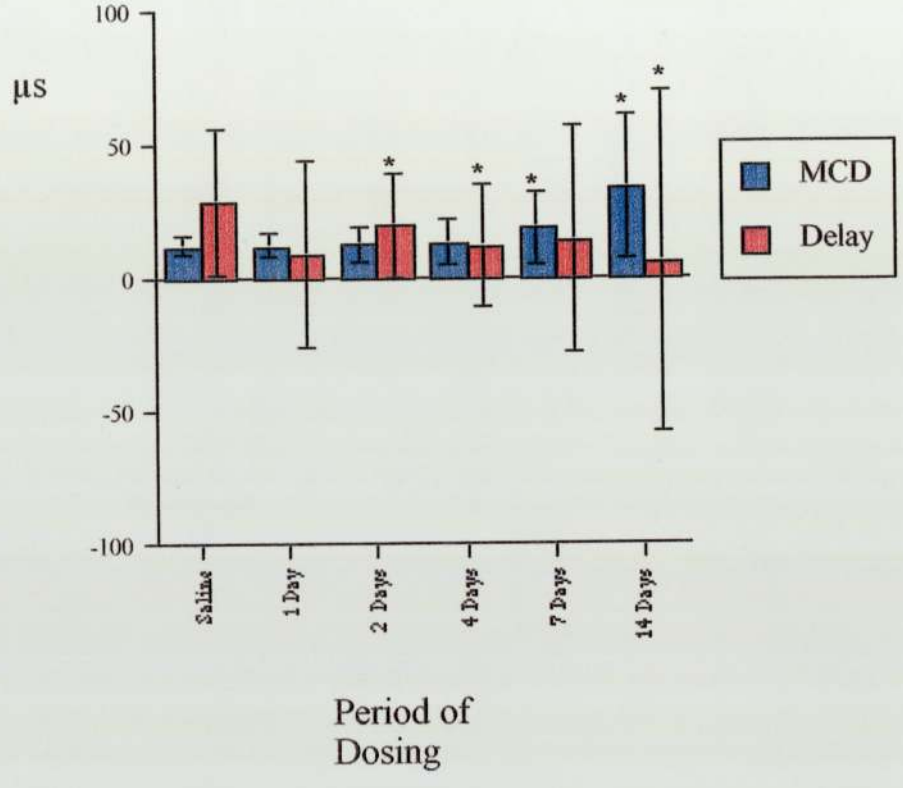


Figure 6.2 The effect of continuous administration of pyridostigmine (11.4 nmol hr^{-1}) on the Delay and MCD of action potential trains. The values shown are means ± s.d. The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days and 5 for 7 days and 42 for 14 days. * indicates a significant difference from saline controls (Kolomogorov-Smirnov $p<0.05$)

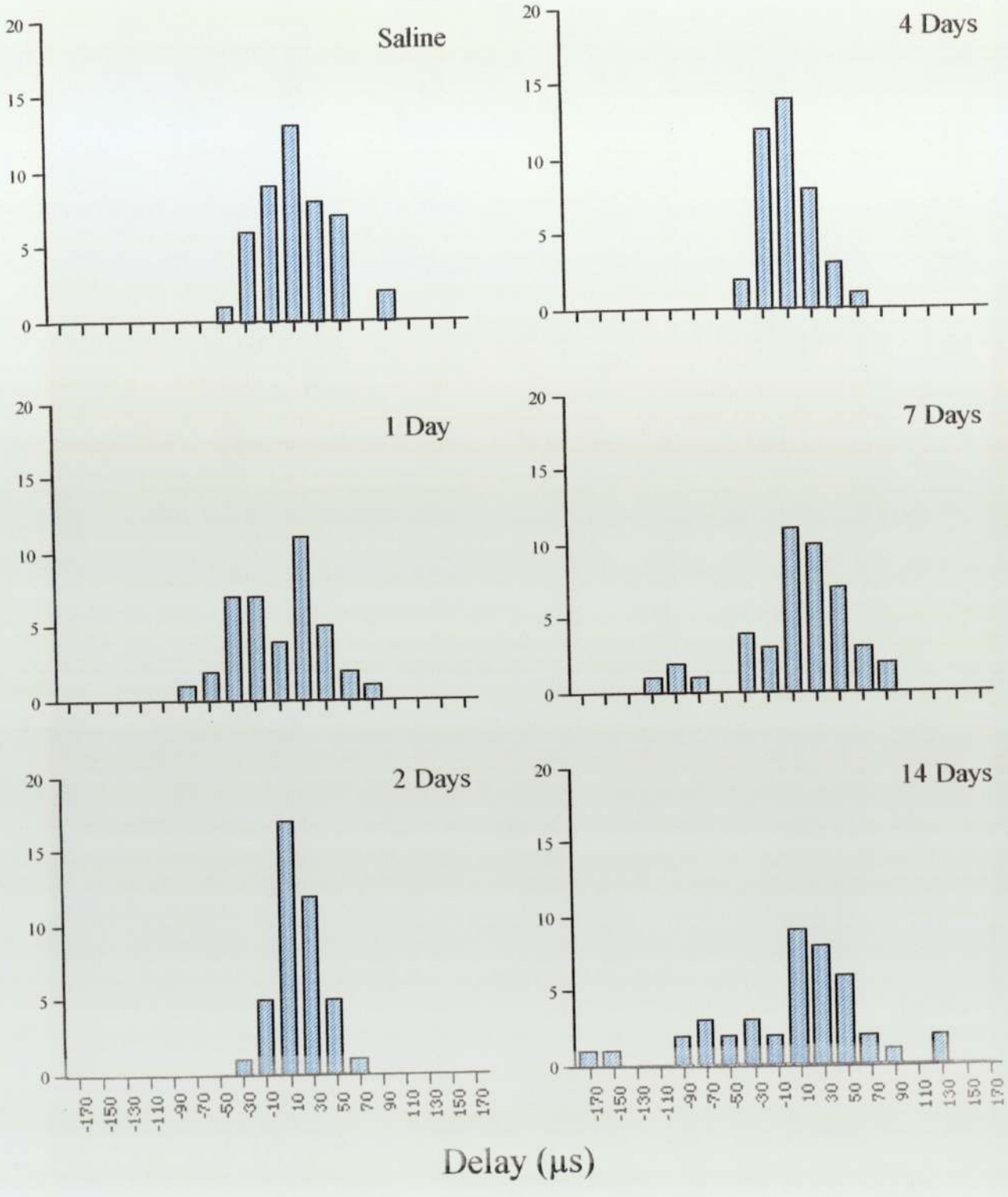


Figure 6.3 The effect of continuous pyridostigmine administration (11.4 nmol hr-1) on the distribution of action potential Delays. *The Y axis shows the number of fibres exhibiting a particular Delay.* The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days and 44 for 7 days and 42 for 14 days

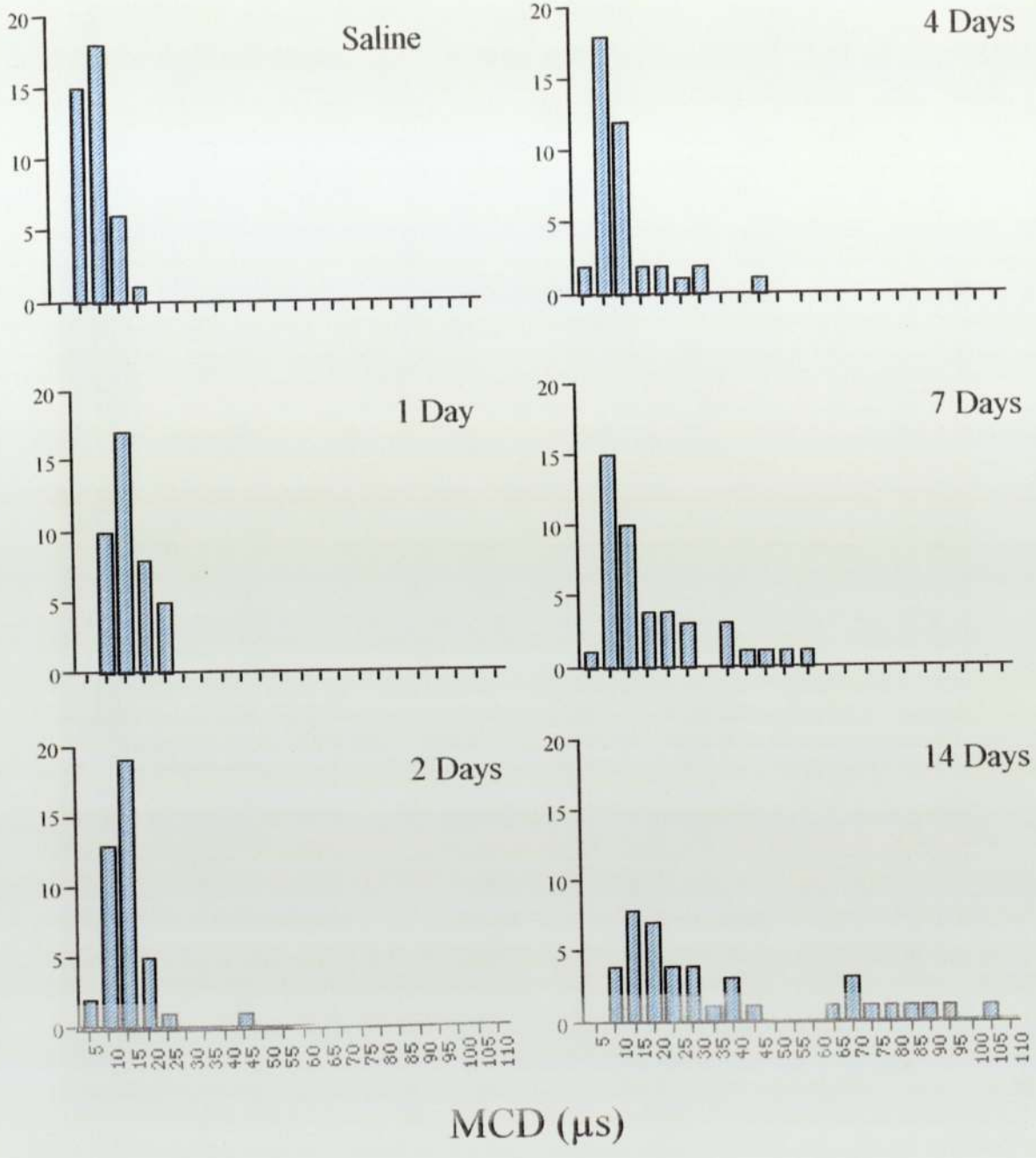


Figure 6.4 The effect of continuous pyridostigmine administration (11.4 nmol hr^{-1}) on the distribution of action potential MCDs. *The Y axis shows the number of fibres exhibiting a particular MCD.* The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days and 44 for 7 days and 42 for 14 days

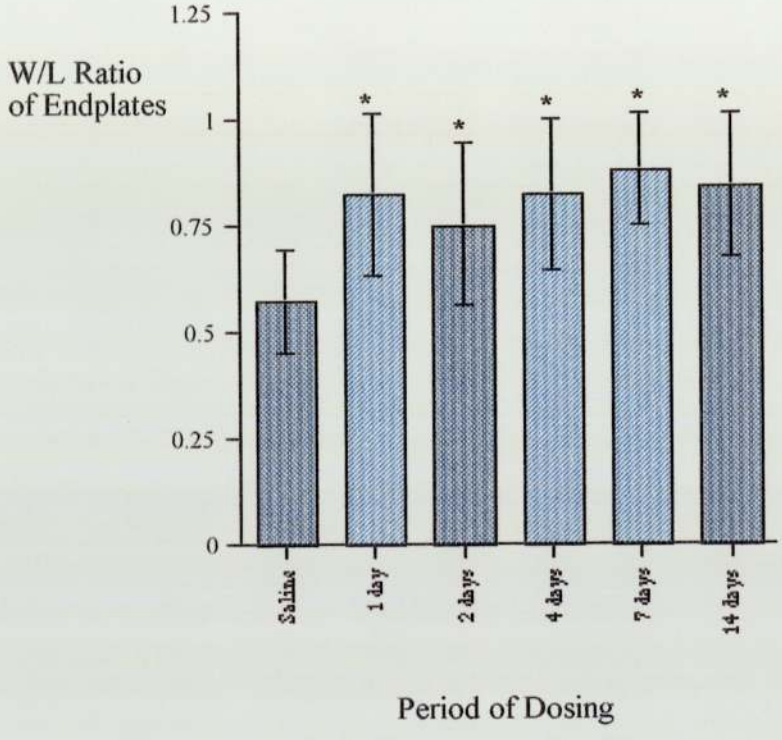


Figure 6.5 The effect of continuous administration of pyridostigmine (11.4 nmol hr^{-1}) on endplate shape shown as the width/length ratio of the endplate. The values shown are the mean ± s.d. For each condition 5 animals were tested. * indicates a significant difference from control preparartions (Mann - Whitney $p<0.05$)

6.2 Recovery from the effects of continuous pyridostigmine administration

Experimental Design

The mice used in these series of experiments were all implanted with an Alzet osmotic pump delivering pyridostigmine at a rate of 11.4 nmolhr-^{1}. The pumps were removed 7 days later and the animals then killed at the time points shown below

2 days

7 days

14 days

The diaphragms were then removed and the usual electrophysiological, biochemical and histological measurements made.

Results

Figure 6.6 shows the recovery of $(MEPP)_o$ time course after removal of the Alzet osmotic pump. After the osmotic pump had been removed for 2 days there was no significant change in $(MEPP)_o$ timecourse from the final treated timepoint at 7 days. However 7 days after removal $(MEPP)_o$ timecourse was reduced and shows no significant difference from control preparations. The timecourse of $(MEPPs)_o$ 14 days after pump removal show no significant difference from control or 7 day preparations.

At no timepoint after the pump was removed did the activity of the functional enzyme show a significant difference from control values (see appendix 7), although the electrophysiological data does show recovery. The data for the activity may present some problems however due to the large standard deviations which will affect the statistical tests which have been carried out on this data.

Table 6.3 contains the values for the Delay and MCD of action potentials recorded from fibres after removal of the osmotic pump and these are also shown graphically in Figure 6.7.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28	11.7±3.3
7 Days Pump in (44)	13±43	18.0±13.3 *
2 Days Removal (39)	9±39 *	20.8±15.5 *
7 Days Removal (39)	33±54	20.7±6.5 *
14 Days Removal (35)	17±33	16.4±6.9

Table 6.3 Effect of Alzet osmotic pump removal after continuous dosing for 7 days with 11.4 $nmolhr^{-1}$ pyridostigmine on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

During the period of osmotic pump implantation the Delay of muscle fibres was reduced. Two days after removal of the osmotic pump the Delay was still smaller showing significant difference from saline controls. At 7 days and 14 days after osmotic pump removal the Delay was increased, and there is no significant difference between the Delays 7 and 14 days after osmotic pump removal and saline control preperations. Figure 6.8 shows the frequency distributions for Delay of the action potentials recorded during the

period after osmotic pump removal. The distributions are still wide showing the presence of affected fibres even in the preparations which show no significant difference from the saline control.

MCD is significantly increased during the dosing period. Two days after the pump had been removed there was some reduction in the MCD but this is still significantly different from the saline control. At 7 days after osmotic pump removal there was no significant decrease in MCD, it is still significantly larger than that of saline control preparations. At 14 days after osmotic pump removal however there was a further reduction in MCD which at this point does not show any significant difference from the saline control preparations. Figure 6.9 shows the MCD frequency distributions for the preparations during this recovery period. It can be seen that as the mean value returns to normal there is a shift to the left as the number of fibres showing large MCDs is reduced.

Figure 6.10 shows the change in endplate shape after removal of the Alzet osmotic pump. It can be seen that there was no change over the period studied as the width/length ratios proved to be significantly different from saline controls at all timepoints.

In conclusion, there is recovery from the effects of continuous pyridostigmine administration after the osmotic pump is removed. $(MEPPs)_o$ have not returned to normal 2 days after the pump was removed which is not what is expected when the half life of pyridostigmine is considered, however this is consistent with what happens after a myopathic dose of pyridostigmine when the recovery is also slow. There is recovery by 7 days however. Neuromuscular jitter recovers slower than $(MEPPs)_o$, Delay returning to normal 7 days after the osmotic pump was removed and MCD returning to normal by 14 days after. The deformation of the endplates caused by pyridostigmine did not recover over the period studied, and there was no change seen in the functional enzyme levels.

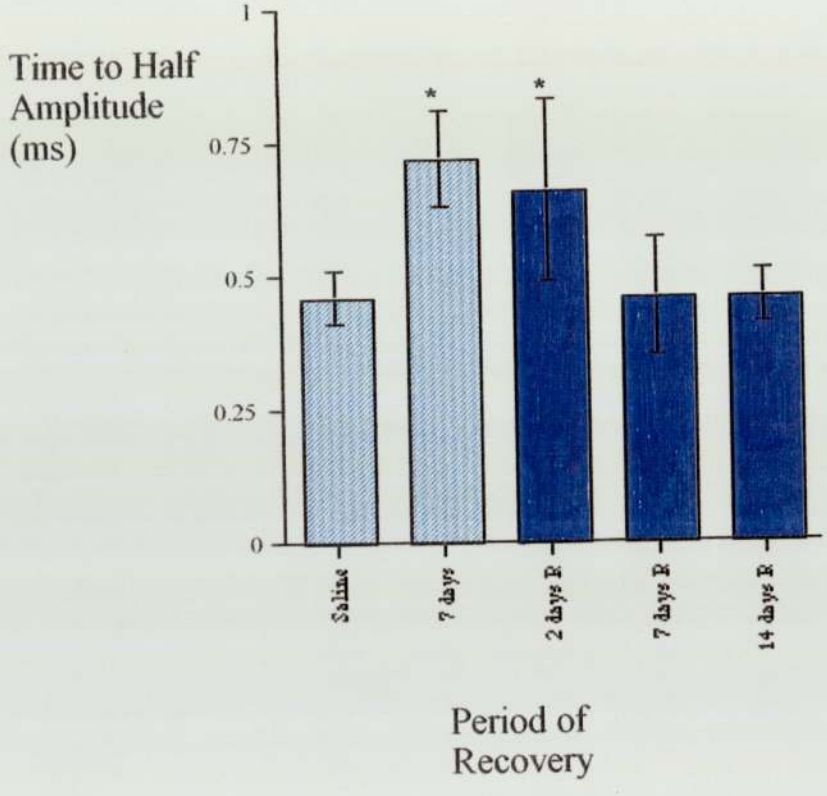


Figure 6.6 The effect of discontinuing pyridostigmine administration on the time to half amplitudes of extracellular miniature endplate potentials. R indicates the number of days recovery. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

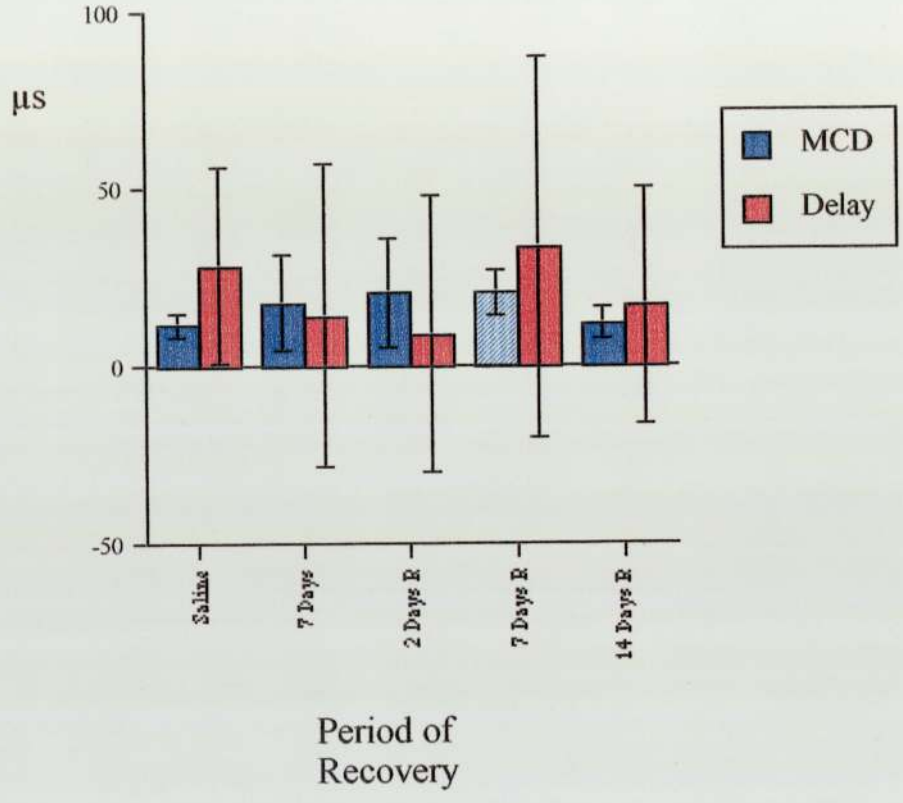


Figure 6.7 The effect of discontinuing pyridostigmine administration on the Delays and MCDs of action potential trains. R indicates the days of recovery. The values are shown as means ± s.d. The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

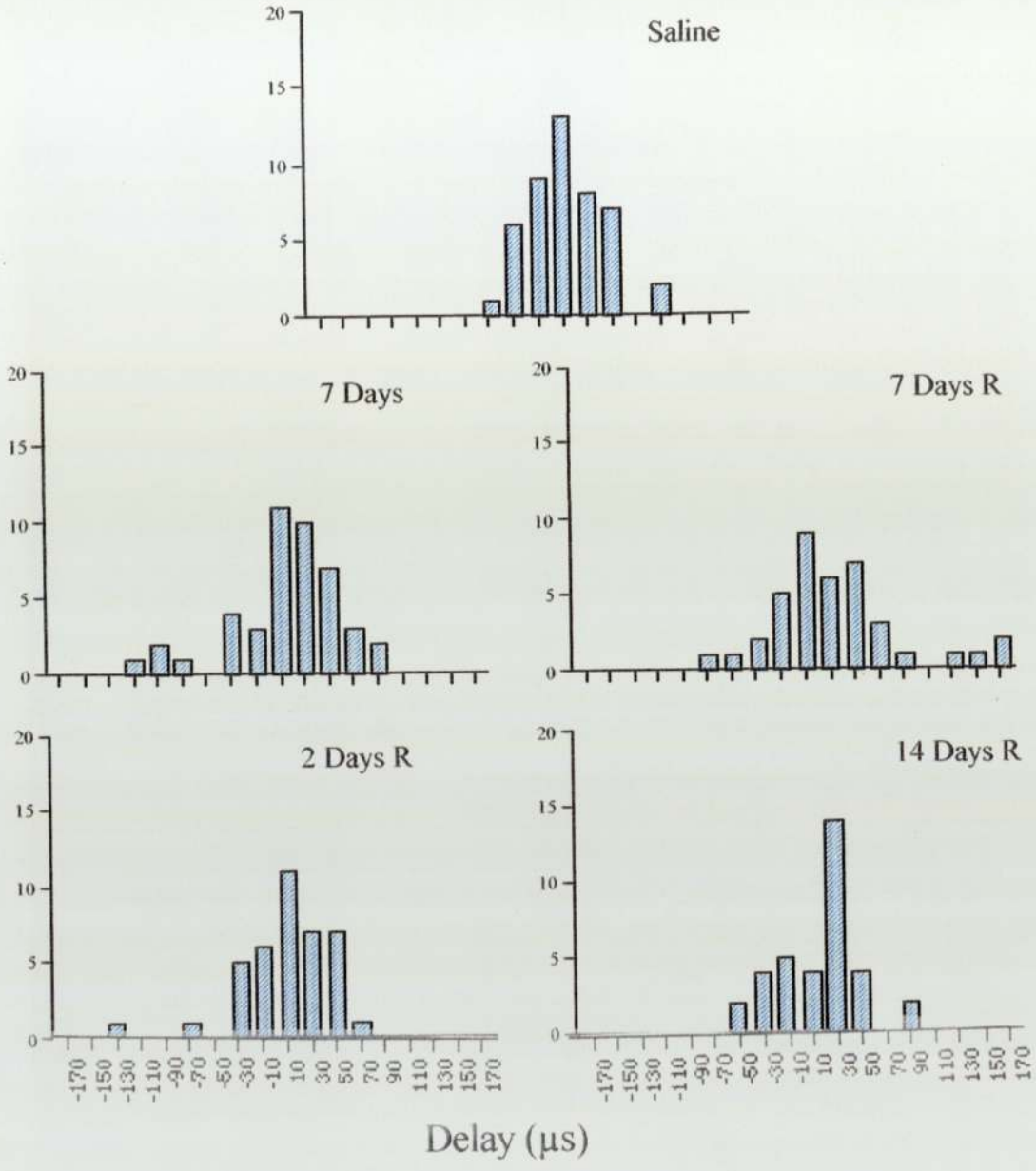


Figure 6.8 The effect of discontinuing pyridostigmine administration on the distribution of action potential train Delays. R indicates the days of recovery. *The Y axis shows the number of fibres exhibiting a particular Delay.* The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery.

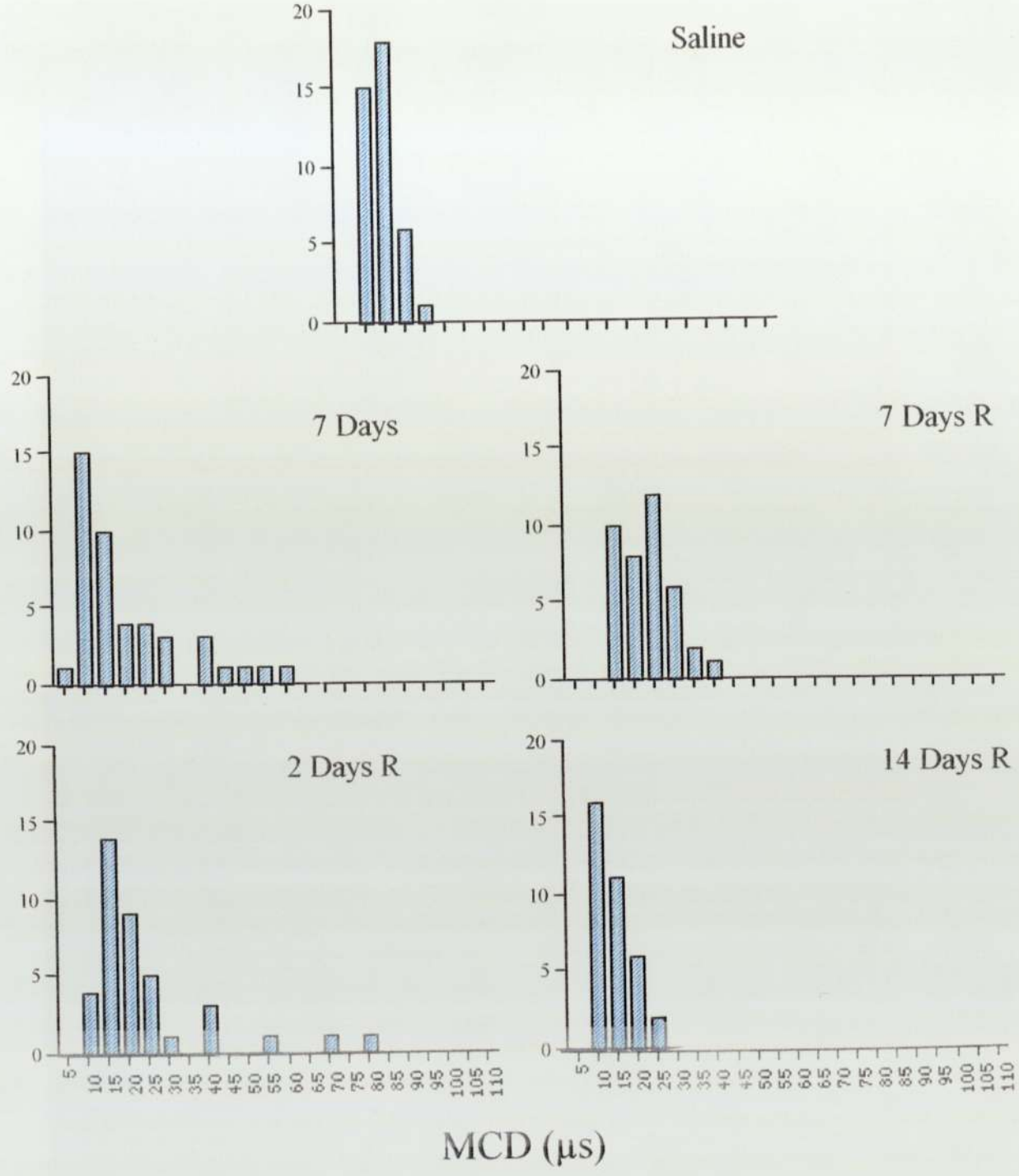


Figure 6.9 The effect of discontinuing pyridostigmine administration on the distribution of action potential MCDs. R indicates the days of recovery. *The Y axis shows the number of fibres exhibiting a particular MCD.* The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery.

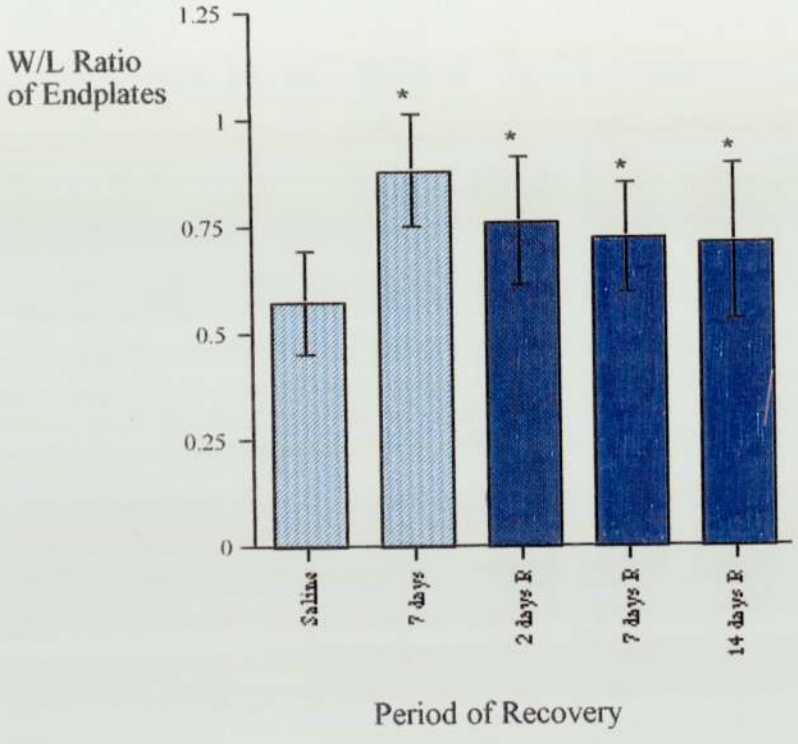


Figure 6.10 The effect of discontinuing pyridostigmine administration on the shape of endplates, shown as the width/length ratio of the endplate. R indicates the days of recovery. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

6.3 Continuous pyridostigmine administration as a protective agent

Experimental Design

A single dose of pyridostigmine has a protective effect against a dose of organophosphate, but does continuous administration also have this protective effect. It is not anticipated that continuous administration will produce more of a protective effect but repeated exposure could prove only to be detrimental to the animal, causing changes in enzyme activity and electrophysiological properties of the endplate. To investigate the protective ability Alzet osmotic pumps were implanted to deliver pyridostigmine at a dose of 11.4 $nmolhr^{-1}$. It was necessary to remove the osmotic pump before the onset of increased jitter so they were removed 4 days later when it has already been established that there is no increase in MCD. Immediately after removal the animals were given a subcutaneous injection of ecothiopate 500 $nmolkg^{-1}$ with atropine. The animals were then killed 5 days later, a time at which we know ecothiopate at this dose will cause increased neuromuscular jitter (see Chapter 4),and the diaphragms removed. Trains of action potentials were then recorded and analysed as normal. Miniature endplate potentials were also recorded. The term PYR + ECO will be used to denote all animals given both drugs in this manner. ECO denotes animals given only a myopathic dose of ecothiopate.

Results

Figure 6.11 shows the effect of continuous pyridostigmine followed by a myopathic dose of ecothiopate on the time course of $(MEPPs)_o$. The time to half amplitudes have been compared between saline control, ECO and PYR + ECO preparations. It can be seen that pretreatment with pyridostigmine does not change the effect of the single myopathic dose of ecothiopate. Both the ECO treated and PYR + ECO treated preparations show significant increases when compared to the saline control data, but there is no significant

difference between the time to half amplitudes of the ECO and PYR + ECO treated preparations.

After a 500 nmolkg^{-1} dose of ecothiopate there is a significant reduction in the activity of functional enzyme as seen in Appendix 4. Pretreatment for 4 days with pyridostigmine (11.4 nmolhr^{-1}) prior to the dose of ecothiopate also causes a significant reduction in the activity of functional enzyme when compared to untreated preparations, and there is no significant difference between the activity of functional enzyme after a myopathic dose of ecothiopate with or without pretreatment with pyridostigmine.

Table 6.4 contains the values of Delay and MCD for saline control, ECO and PYR + ECO preparations, and these are shown graphically in Figure 6.12.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28 +	11.7±3.3 +
ECO (31)	43±34 *	22.3±20.2 *
PYR + ECO (37)	52±39 * +	16.4±6.9 * +

Table 6.4 The effect of pyridostigmine (11.4 nmolhr-^{1}) pretreatment for 4 days on the Delay and MCD changes induced by ecothiopate (500 nmolkg^{-1} + atropine), given immediately after pump removal and recordings were made 5 days layter. All values are shown as the mean ± s.d. The number in parentheses is the number of fibres tested. * indicates a significant difference from saline control data and + indicates a significant difference from ECO data.

A myopathic dose of ecothiopate causes a significant increase in Delay. Continuous administration of pyridostigmine prior to the ecothiopate injection also causes a significant increase in Delay when compared to saline controls, but this increase is also significantly more than that caused by ecothiopate alone. Figure 6.13 shows the frequency distributions for Delays under these conditions. After ECO there is a slight shift to the right in the distribution sufficient to increase the mean signigficantly, but after PYR + ECO the shift is much greater with many more fibres having large Delays, although many are still normal.

After a myopathic dose of ecothiopate the MCD is significantly increased. Pretreatment with pyridostigmine also causes a significant increase in the MCD of the fibres, but this increase is significantly less than that caused by ecothiopate alone. Figure 6.14 shows the frequency distributions for the MCDs of the preparations. After ECO there is a shift to the right, a few fibres having very large MCDs and so shifting the mean. After PYR + ECO however the distribution looks more normal and the mean has been shifted due to a large number of fibres showing a small increase in MCD.

Figure 6.15 shows the effect ecothiopate (500 nmolkg-^{1}) has on endplate shape with and without pretreatment with pyridostigmine. Ecothiopate alone causes a significant increase in the width/length ratio of endplates when compared to untreated values. A similar significant increase is seen after pretreatment with pyridostigmine, there being no significant difference between the increase in width/length ratio of endplates with or without pyridostigmine pretreatment.

In conclusion, continuous pyridostigmine administration does not completely protect against the effects of a myopathic dose of ecothiopate. It does not affect the prolongation of $(MEPPs)_O$, the reduction in activity of functional enzyme or the deformation of endplates. It does reduce the amount of neuromuscular jitter however, the MCD not showing as great an increase after pretreatment with pyridostigmine.

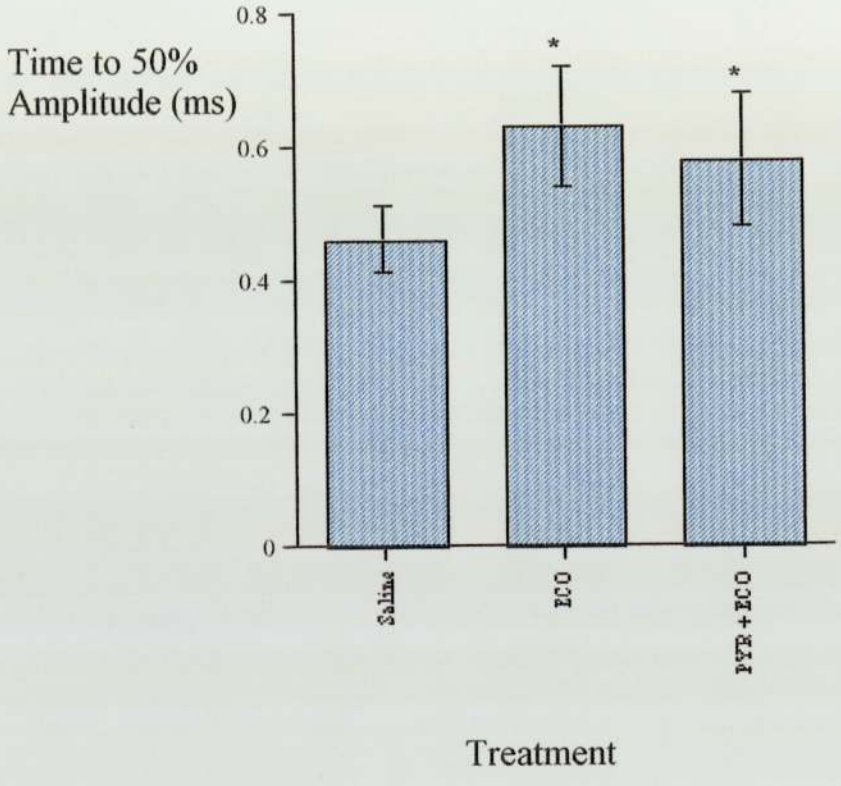


Figure 6.11 The effect of pyridostigmine pretreatment (11.4 nmol hr^{-1}) on the prolongation of extracellular miniature endplate potentials due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PYR + ECO indicates 4 days pretreatment with pyridostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$).

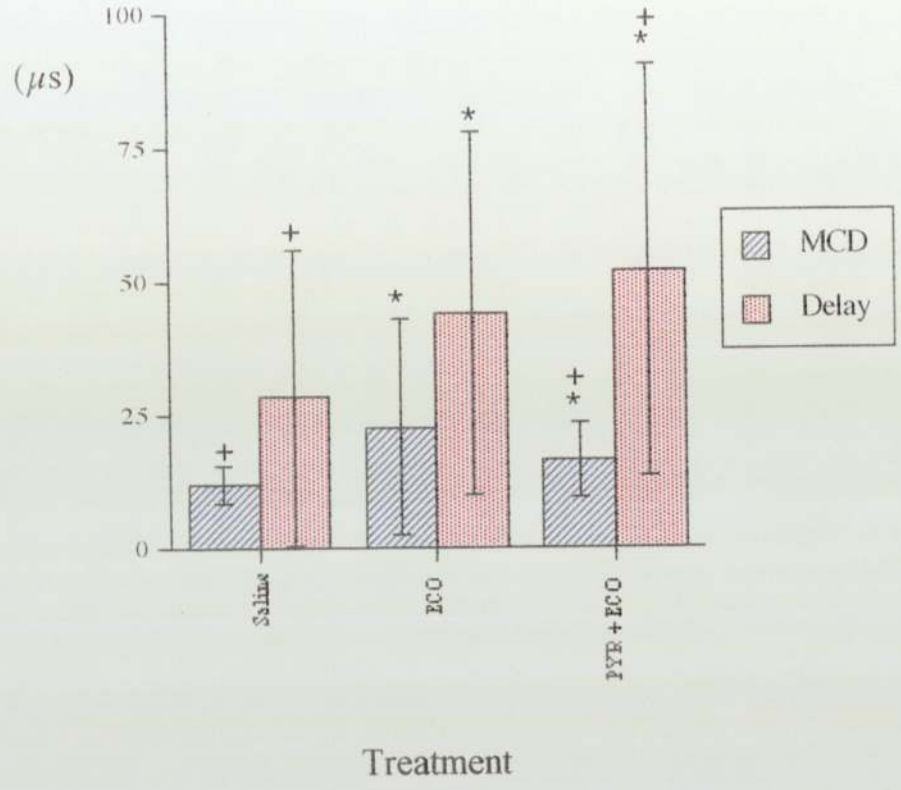


Figure 6.12 The effect of pyridostigmine pretreatment (11.4 nmol hr^{-1}) on the changes in action potential train Delays and MCDs due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PYR + ECO indicates 4 days pretreatment with pyridostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. The number of fibres tested were 40 for saline preparations, 31 for ECO and 37 for ECO + PYR. * indicates a significant difference from saline controls and + indicates a significant difference from the ECO value (Kolmogorov - Smirnov $p<0.05$)

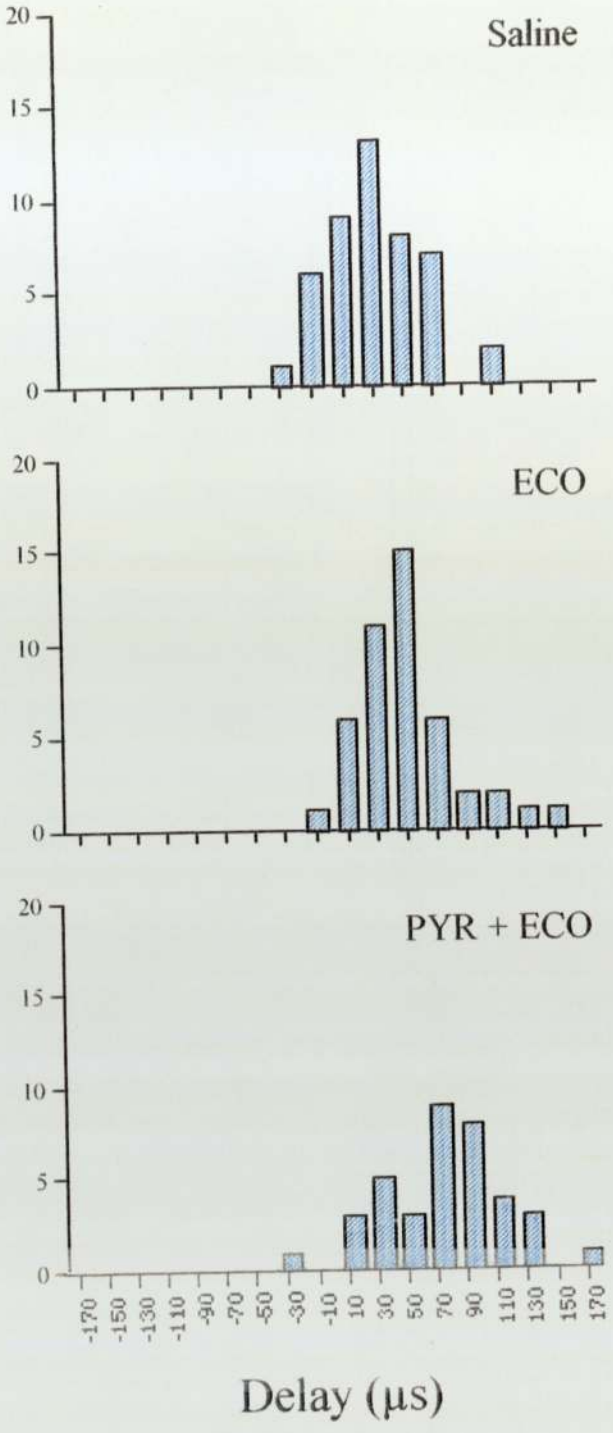


Figure 6.13 The distributions of Delays after ecothiopate 500 nmolkg^{-1} with and without pyridostigmine (11.4 nmol hr^{-1}) pretreatment. ECO indicates a single dose of ecothiopate alone, and PYR + ECO indicates 4 days pretreatment with pyridostigmine followed by a single dose of ecothiopate. *The Y axis indicates the number of fibres having a particular value of Delay.* The number of fibres tested were 40 for saline preparations, 31 for ECO and 37 for ECO + PYR.

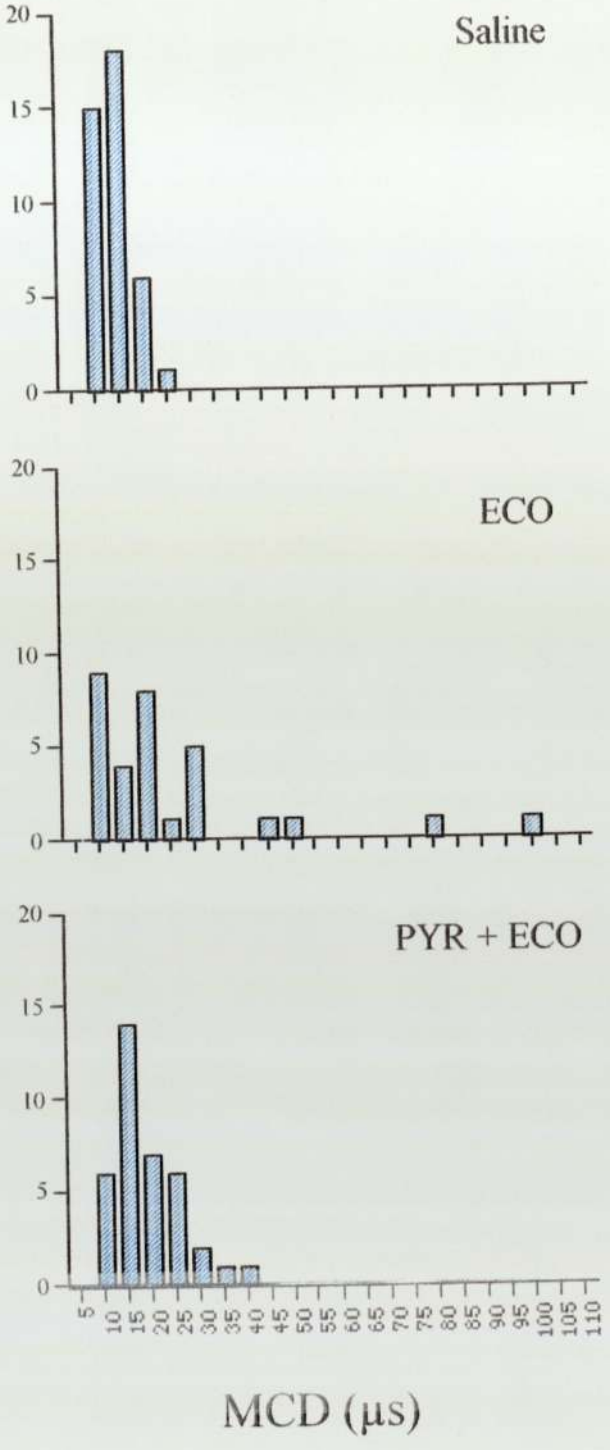


Figure 6.14 The distributions of MCDs after ecothiopate 500 nmolkg^{-1} with and without pyridostigmine (11.4 nmol hr^{-1}) pretreatment. ECO indicates a single dose of ecothiopate alone, and PYR + ECO indicates 4 days pretreatment with pyridostigmine followed by a single dose of ecothiopate. *The Y axis indicates the number of fibres having a particular value of MCD.* The number of fibres tested were 40 for saline preparations, 31 for ECO and 37 for ECO + PYR.

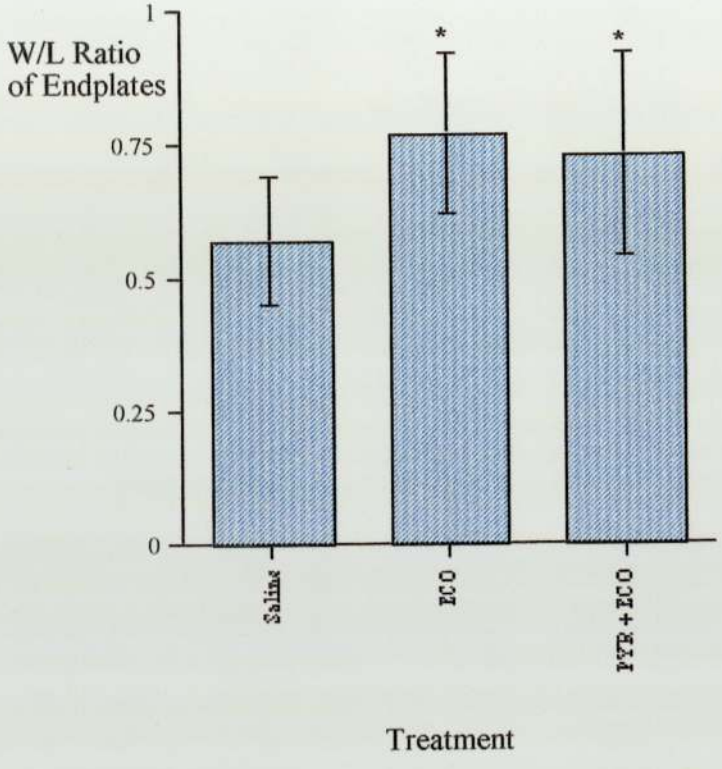


Figure 6.15 The effect of pyridostigmine pretreatment (11.4 nmol hr^{-1}) on the changes in endplate shape shown as the width/length ratio of the endplate due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PYR + ECO indicates 4 days pretreatment with pyridostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls and + indicates a significant difference from the ECO value (Mann-Whitney p.0.05)

CHAPTER 7

The effect of continuous administration of physostigmine

The experiments described in this chapter were carried out to determine the effect of physostigmine delivered at a constant rate of 14 nmol hr^{-1} by Alzet osmotic pump, which preliminary experiments by Mrs A. Rowbotham had showed to produce an approximate 30% inhibition of erythrocyte ChE, which makes the dose comparable to that of pyridostigmine. The effect of physostigmine, delivered in this manner, on neuromuscular jitter and the time course of extracellularly recorded miniature endplate potentials was investigated. The effectiveness of physostigmine administered constantly as a protective agent was also investigated. These electrophysiological results are then compared with functional AChE activity and the deformation of endplates.

As with pyridostigmine the initial set of experiments determining the effect of continuous dosing with physostigmine was divided into two series. The first dealing with the effects of the drug during the dosing period and the second series dealing with the effects after the Alzet osmotic pump was removed and the mouse was allowed to recover from the drug.

7.1 The effect of continuous administration of physostigmine.

Experimental Design

Alzet osmotic pumps, filled with physostigmine so as to deliver a dose of 14 nmol hr-1 over either a 1 week or 2 week period, were implanted into a series of mice and the animals killed at the times listed below.

1 day

2 days

4 days

7 days

14 days

The diaphragms were then removed, cut into hemidiaphragms and used for several different experiments. Some hemdiaphragms were used to determine the activity of AChE by biochemical assay, while others were fixed and stained with Karnovsky and Roots stain. The remaining hemidiaphragms were used for electrophysiological recording. After the hemidiaphragms were pinned out in the recording bath trains of action potentials were recorded first and approximately 1 hour later $(MEPPs)_o$ were recorded. Trains of action potentials were recorded from an average of 10 cells and $(MEPPs)_o$ were recorded from another 10.

The control preparations for these experiments were the same as for the experiments dealing with the effects of continuous dosing with pyridostigmine, see Table 6.1.

Results

Figure 7.1 shows the prolongation of extracellular miniature endplate potentials due to physostigmine. The time to half amplitude of the decay phase of $(MEPPs)_o$ increases as the period of dosing increases. The increase seen at 1 day is significant when compared to the control value as is the increase seen at every timepoint thereafter. The increase seen at each timepoint however is not significant when compared to the previous timepoint.

Appendix 8 contains the data for the biochemical assay of functional enzyme, i.e the endplate specific non-extractable fraction. It can be seeen that during the dosing period it is only at day 4 that there is any significant reduction in the activity of functional enzyme when compared to control levels, although it appears to be reduced at every timepoint measured. In the same way as with pyridostigmine however there is little agreement with the electrophysiological results and the biochemical data again shows very large standard

deviations which will have affected the statistical tests carried out on this data. Ideally the experiments should be repeated to reduce the standard deviation.

Table 7.1 contains the values of Delay and MCD of action potential trains recorded at various times during the 14 day period of dosing. These are also shown graphically in Figure 7.2.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28	11.7±3.3
1day (40)	16±30	11.3±5.1
2 days (41)	7±24 *	12.5±4.9
4 days (40)	10±23 *	14.3±9.0 *
7 days (44)	13±49 *	18.2±10.8 *
14 days (42)	5±40 *	22.9±14.6 *

Table 7.1 Effect of physostigmine 14 nmol hr^{-1} on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

The Delay is decreased every day during the period of dosing when compared to the saline control value. This decrease is not significant 1 day into dosing but every subsequent

timepoint shows a significant decrease when compared to the saline controls. Figure 7.3 shows the distributions of the Delays during the period of dosing. The distributions are not greatly affected but do show a slight shift to the left due to the emergence of fibres with shorter values of Delay. All of the distributions however have fibres which show Delays within the "normal" range and are therefore presumably unaffected. The decrease seen in Delay is in contrast to the increase seen with ecothiopate but is consistent with the effect of pyridostigmine on the delays of diaphragm muscle action potentials. It appears therefore to be a definite drug difference as both carbamates caused a decrease in Delay but the organophosphate caused an increase in Delay.

The MCD is initially unaffected by the physostigmine administration. One and two days into the period of dosing the MCD is not significantly different from that of the saline control. Four days after dosing is begun however the MCD shows a significant increase when compared to the saline control, and a significant increase is seen at every timepoint thereafter. The distributions of MCDs during the period of dosing can be found in Figure 7.4. The distributions show a shift to the right as fibres showing large values of MCD are found after dosing is begun. There are a number of fibres at each time point however which are unaffected by physostigmine and show MCDs within the "normal" range.

Figure 7.5 shows the effect of continuous physostigmine administration on the shape of endplates as illustrated by their width/length ratios. After 1 day of dosing there is a distortion of the endplates, they become rounder, as seen by the significant increase in the width/lemgth ratio when compared to saline controls. This increase in width/length ratio continues to be significant throughout the period of dosing, with no significant difference between timepoints.

In conclusion continuous administration of physostigmine causes prolongation of the decay phase of $(MEPPs)_o$ and deformation of the endplates over the entire dosing period in the same way as pyridostigmine administered continuously. There is also an increase in

neuromuscular jitter seen as a decrease in Delay and an increase in MCD. The decrese in Delay is seen after the second day as with pyridostigmine but it is not until 4 days into the dosing that any change in MCD is seen which is much earlier than with pyridostigmine when no change in MCD is seen until 7 days after dosing was begun. There is also an apparent reduction in functional enzyme activity which is again not significant until 4 days after dosing is begun. There is not a significant decrese at the later timepoints however this lack of significance could be due to the small n number and the large variation seen in the data.

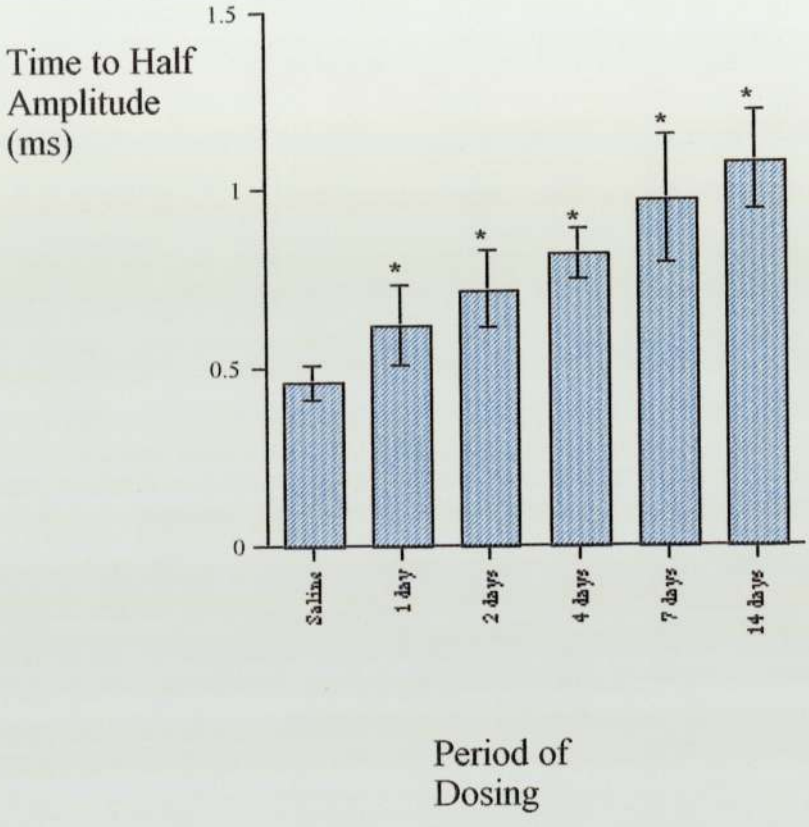


Figure 7.1 The effect of continuous administration of physostigmine (14 nmol hr^{-1}) on the time to half amplitude of extracellular miniature endplate potentials. The values shown are means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

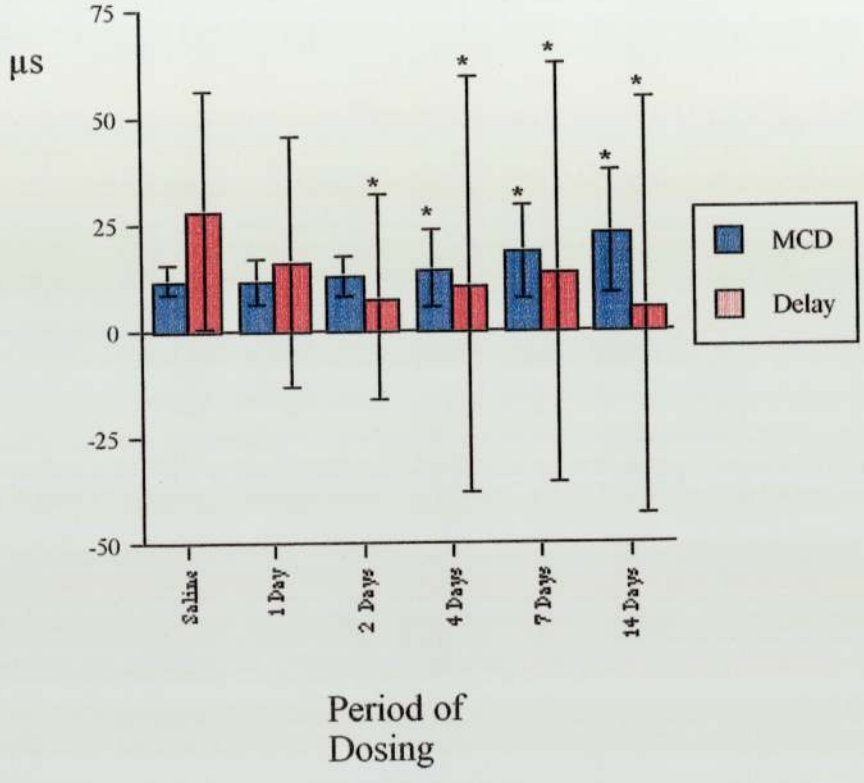


Figure 7.2 The effect of continuous administration of physostigmine (14 nmol hr^{-1}) on the Delay and MCD of action potential trains. The values shown are means ± s.d. The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days, 44 for 7 days and 42 for 14 days. * indicates a significant difference from saline controls (Kolomogorov-Smirnov $p<0.05$)

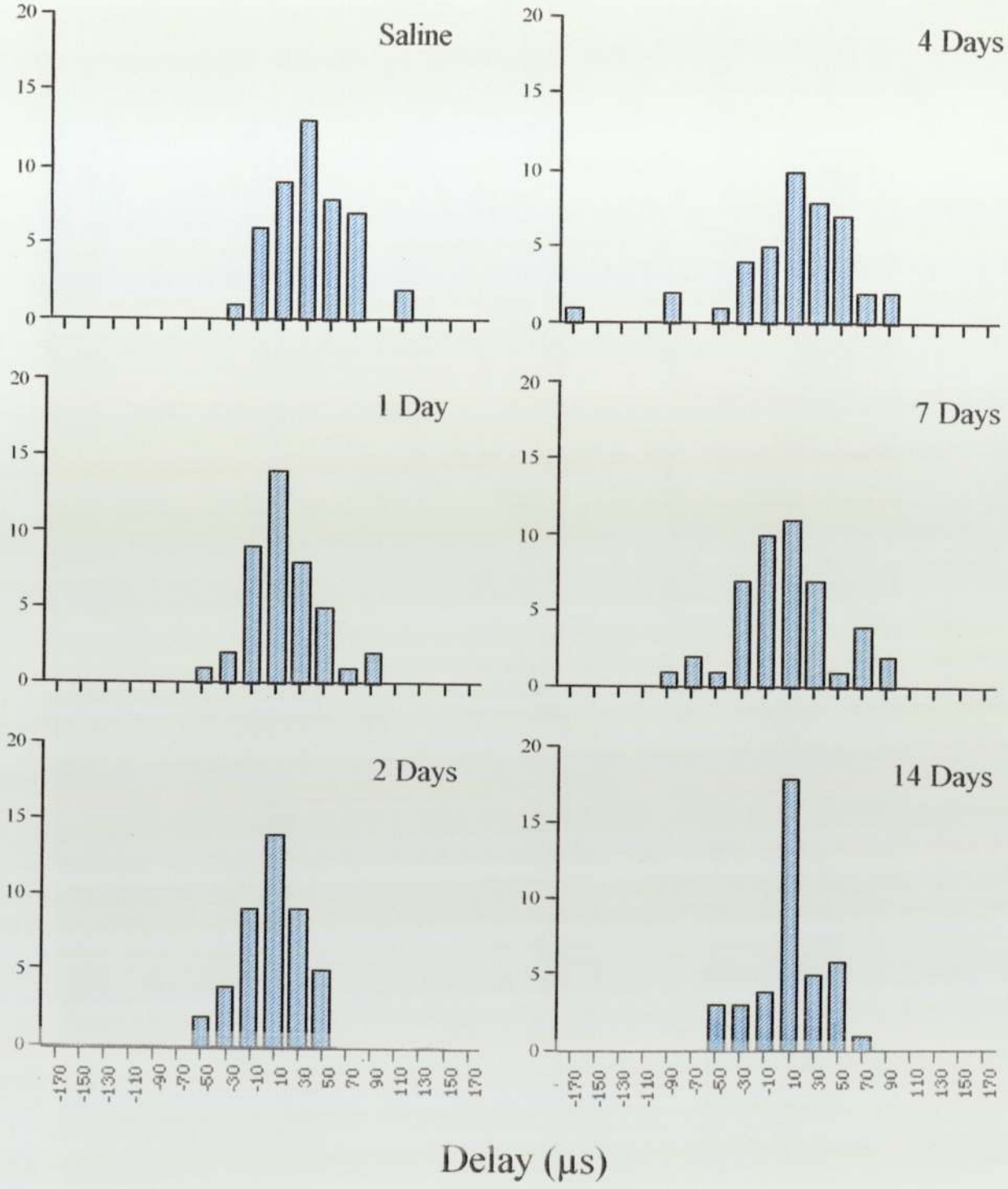


Figure 7.3 The effect of continuous administration of physostigmine (14 nmol hr^{-1}) on the distribution of action potential Delays. *The Y axis shows the number of fibres exhibiting a particular Delay.* The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days, 44 for 7 days and 42 for 14 days.

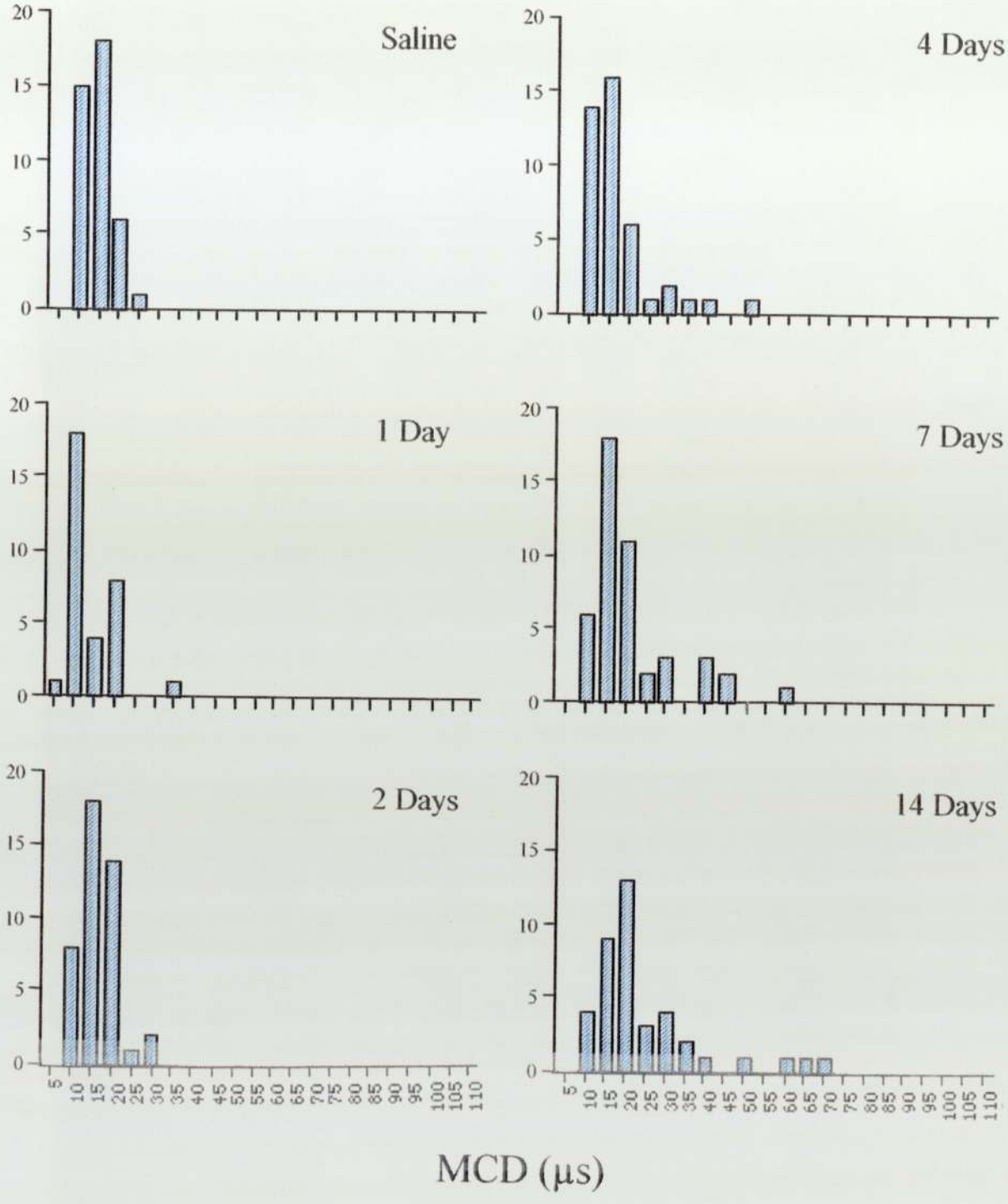


Figure 7.4 The effect of continuous physostigmine administration (14 nmol hr^{-1}) on the distribution of action potential MCDs. *The Y axis shows the number of fibres exhibiting a particular MCD.* The number of fibres tested were 40 for saline preparations, 40 for 1 day, 41 for 2 days, 40 for 4 days, 44 for 7 days and 42 for 14 days.

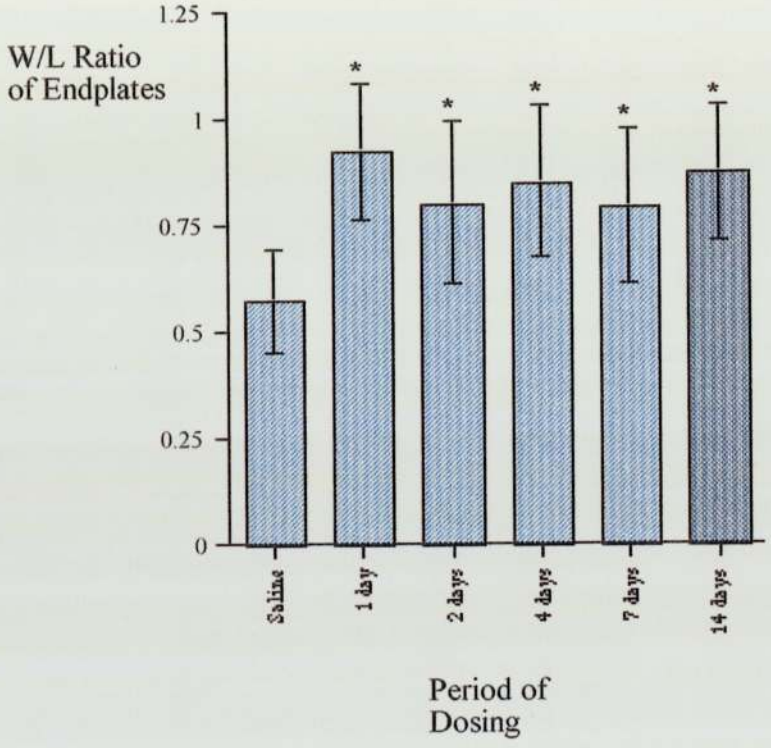


Figure 7.5 The effect of continuous administration of physostigmine (14 nmol hr-1) on endplate shape as shown by the width/length ratio of the endplate. The values shown are the mean ± s.d. For each condition 5 animals were tested. * indicates a significant difference from control preparartions (Mann - Whitney $p<0.05$)

7.2 Recovery from the effects of continuous administration of physostigmine

Experimental Design

All of the mice usd in this series of experiments were implanted with an Alzet osmotic pump delivering physostigmine at a rate of 14 nmol hr^{-1} for a period of 7 days after which the pumps were removed and the animals killed on the days after removal as shown below.

2 days

7 days

14 days

The diaphragms removed were then used for biochemical, histological and electrophysiological measurements.

Results

Figure 7.6 shows the recovery of $(MEPP)_o$ timecourse after removal of the Alzet osmotic pump. Two days after the osmotic pump was removed the time to half amplitude of the decay phase was significantly reduced however it is still significantly longer than that of the saline control. On the seventh day after the pump was removed the time to half amplitude is further decreased and shows no significant difference from the saline control value, neither does that 14 days after osmotic pump removal.

The data for the biochemical assay of endplate specific non-extractable enzyme, i.e. that presumed to assess functional enzyme, are contained in appendix 8. There is a significant reduction in the activity of the functional enzyme when compared to control values 2 days after the pump was removed but on the seventh and fourteenth day after removal no significant reduction in activity was seen. These data agree with the electrophysiological results with a recovery from the effects of physostigmine becoming apparent on days 7 and 14 of the recovery period.

Table 7.2 contains the values of Delay and MCD for action potential trains recorded from diaphragms up to 14 days after the removal of the osmotic pump. These are also shown graphically in Figure 7.7.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28	11.7±3.3
7 Days Pump in (44)	13±49 *	18.2±10.8 *
2 Days Removal (39)	11±28 *	21.1±12.3 *
7 Days Removal (39)	20±35	20.6±9.1 *
14 Days Removal (35)	26±30	11.0±5.9

Table 7.2 Effect of Alzet osmotic pump removal after continuous dosing for 7 days with 11 nmol hr^{-1} physostigmine on the Delay and MCD of diaphragm muscle fibres. All values are the mean ± s.d. The number in parentheses indicates the number of fibres tested. * indicates a significant difference from control (saline treated) fibres (Kolmogorov-Smirnov $p<0.05$).

During the period of dosing the Delay of action potential trains was significantly decreased, and 2 days after the osmotic pump was removed the Delay is still significantly decreased when compared to that of the saline control. However 7 and 14 days after the pump was removed the Delays increased and show no significant difference compared to the saline control. The distributions of these delays are seen in Figure 7.8. During the

period of dosing the distribution of Delays widened due to the presence of affected fibres and this increase in width is still seen after the osmotic pump was removed indicating the presence of affected fibres. The width of the distribution decreases after the removal of the pump as the fibres recover and their Delays return to normal.

The MCD of action potentials trains was also affected by physostigmine, they were significantly increased. The MCD is still significantly increased with respect to the control MCD 2 days after the osmotic pump was removed, this is also the case 7 days after osmotic pump removal. Fourteen days after the pump was removed however the MCD was reduced and shows no significant difference from saline controls. Figure 7.9 shows the distributions of the MCD values for up to 14 days after osmotic pump removal. The distribution of MCDs 2 and 7 days after osmotic pump removal are skewed to the right due to the presence of fibres which have large MCDs and are therefore still affected by the physostigmine. Fourteen days after osmotic pump removal however the number of affected fibres is greatly reduced so the distribution shifts back to the left and so resembles the distribution of saline controls.

Figure 7.10 show the change in endplate shape after the removal of the Alzet osmotic pump. There is still a significant increase in the width/length ratio of the endplates when compared to saline controls at every timepoint after pump removal indicating that the deformation of the endplates caused by physostigmine showed no recovery over the period studied.

In conclusion, discontinuing continuous physostigmine administration allows recovery from the effects of the drug. The timecourse of $(MEPPs)_o$ returns to "normal" but this recovery takes longer than 2 days which is longer than would be expected when the half life of physostigmine is considered however it is consistent with the slow recovery seen with pyridostigmine. Neuromuscular jitter also shows recovery but this occurs much slower taking 7 days for Delay to return to normal and up to 14 days for MCD to return

to normal, recovery therfore occuring on the same timescale as with pyridostigmine. Functional enzyme showed recovery, the activity returning to normal 7 days after the pump was removed, in contrast to the behaviour of the enzyme after pyridostigmine when no change was seen As with pyridostigmine the deformation of the endplates however did not recover after physostigmine over the period studied.

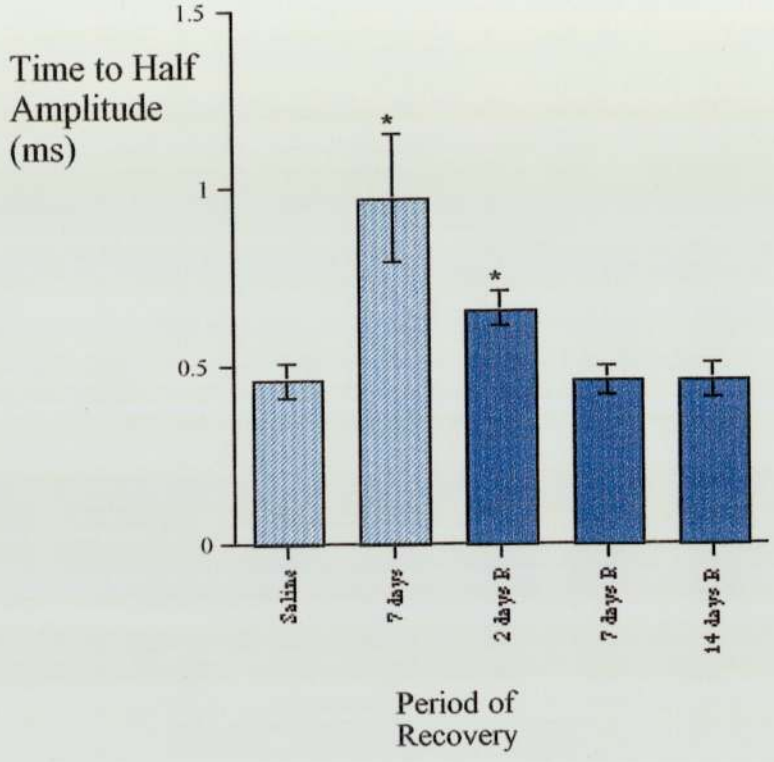


Figure 7.6 The effect of discontinuing physostigmine administration on the time to half amplitudes of extracellular miniature endplate potentials. R indicates the number of days recovery. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

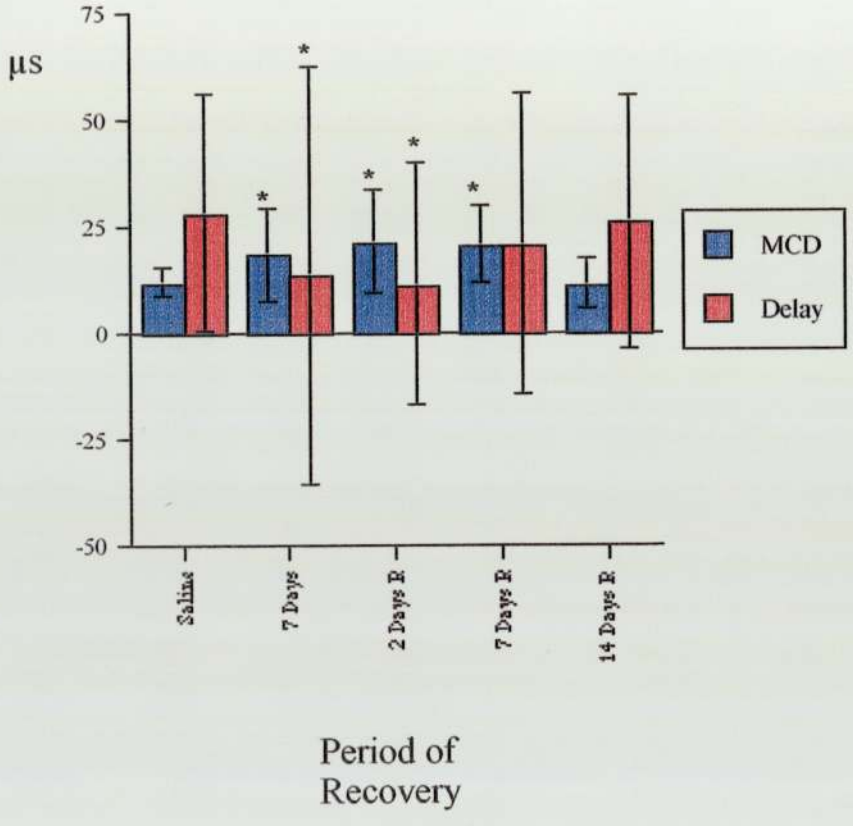


Figure 7.7 The effect of discontinuing physostigmine administration on the Delays and MCDs of action potential trains. R indicates the number of days recovery. The values are shown as means ± s.d. The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

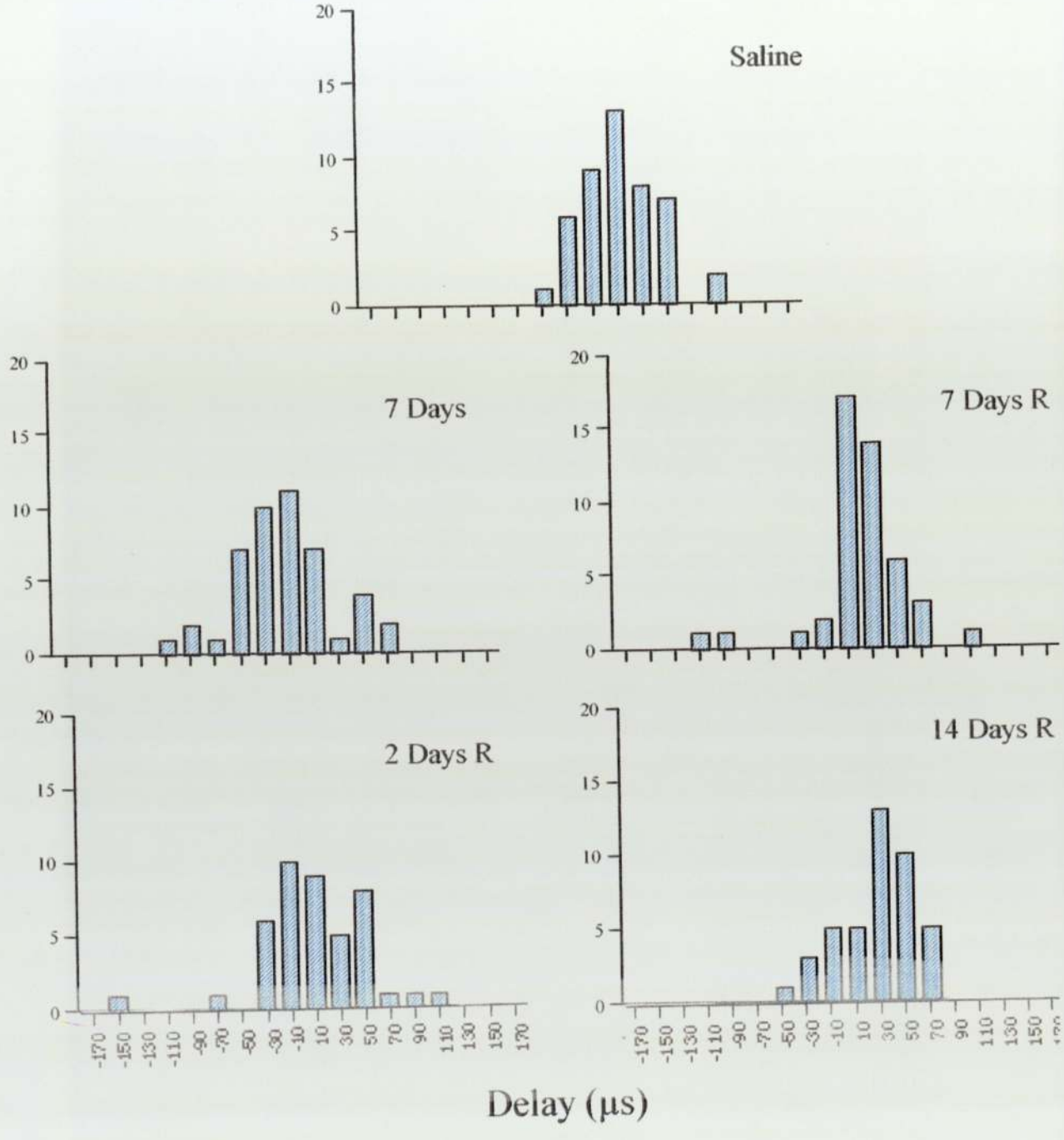


Figure 7.8 The effect of discontinuing physostigmine administration on the distribution of action potential Delays. R indicates the number of days recovery. *The Y axis shows the number of fibres exhibiting a particular Delay.* The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery.

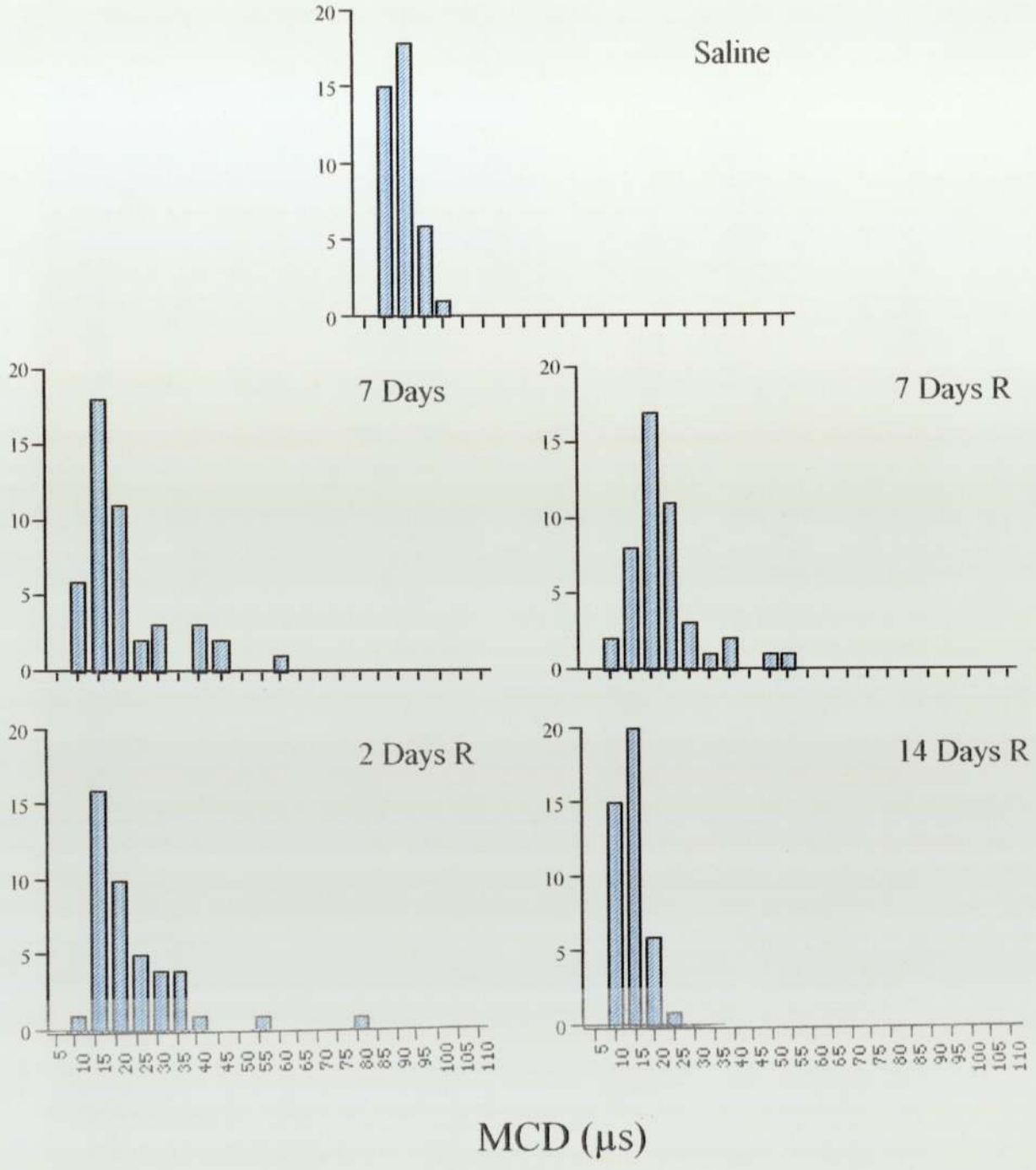


Figure 7.9 The effect of discontinuing physostigmine administration on the distribution of action potential MCDs. R indicates the number of days recovery. *The Y axis shows the number of fibres exhibiting a particular MCD.* The number of fibres tested were 40 for saline preparations, 44 for 7 days pump in, 39 for 2 days recovery, 39 for 7 days recovery and 35 for 14 days recovery.

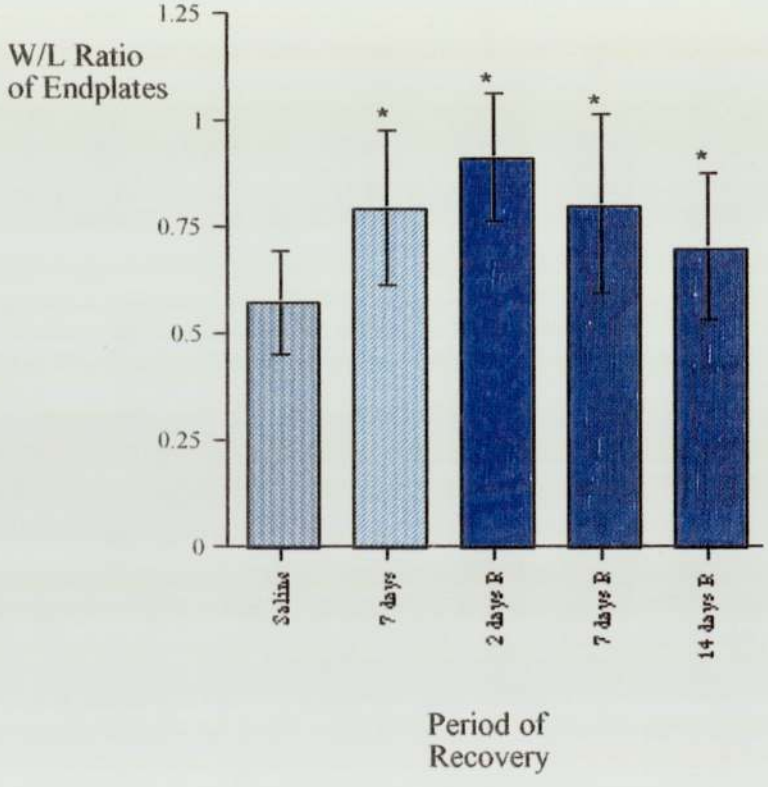


Figure 7.10 The effect of discontinuing physostigmine administration on the shape of endplates, shown as the width/length ratio of the endplate. R indicates the number of days recovery. The values are shown as means ± s.d.For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$)

7.3 Continuous administration of physostigmine as a protective agent

Experimental Design

Determination of the effectiveness of physostigmine as protection against a single myopathic dose of ecothiopate was investigated in the same way as with pyridostigmine. Physostigmine was administered continuously by Alzet osmotic pump for a period of 2 days and then the pump was removed and a injection of 500 $nmolkg^{-1}$ ecothiopate with atropine was given. Physostigmine was only administered for 2 days due to the fact that it was necessary to cease carbamate administration prior to the onset of increased neuromuscular jitter, which with physostigmine occured at 4 days. The animal was then killed 5 days later, the diaphragm removed and action potentials recorded and analysed as normal. Miniature endplate potentials were also recorded to determine if there was any change in the levels of functional AChE. The term PHY + ECO will be used to indicate any value from fibres treated with both physostigmine and ecothiopate and ECO will be used to denote any value from a fibre treated with myopathic ecothiopate with atropine alone.

Results

Figure 7.11 shows the effect of continuous physostigmine followed by a myopathic dose of ecothiopate on the time course of $(MEPPs)_O$. The time to half amplitudes have been compared between saline control, ECO and PHY + ECO preparations. Treatment with ecothiopate alone causes a prolongation in the decay phase of $(MEPPs)_O$ which is significant when compared to saline controls. Giving a myopathic dose of ecothiopate after pretreatment with physostigmine does not cause a significant prolongation of $(MEPP)_O$ timecourse.

There was a significant decrease in the activity of functional enzyme after a myopathic dose of ecothiopate alone (see appendix 4), but after pretreatment with physostigmine giving a myopathic dose of ecothiopate does not cause any significant decrease in the activity of functional enzyme when compared to control values.

Table 7.3 contains the values of Delay and MCD for saline control, ECO and PHY + ECO preparations, and these are shown graphically in Figure 7.12.

Treatment	Delay (μs)	MCD (μs)
Saline (40)	28±28 +	11.7±3.3 +
ECO (31)	43±34 *	22.3±20.2 *
PHY + ECO (34)	15±31 +	13.2±5.3 +

Table 7.3 The effect of physostigmine (14 nmol hr^{-1}) pretreatment for 2 days on the Delay and MCD changes induced by ecothiopate (500 $nmolkg^{-1}$ + atropine). Ecothiopate was given immediately after pump removal where the recordings were made 5 days later. All values are shown as the mean ± s.d. The number in parentheses is the number of fibres tested. * indicates a significant difference from saline control data and + indicates a significant difference from ECO data (Kolmogorov-Smirnov $p<0.05$).

A myopathic dose of ecothiopate with atropine causes a significant increase in Delay. Pretreatment with physostigmine prior to the ecothiopate injection appears to cause a decrease in the value of Delay but this is not significant when compared to the control value. Figure 7.13 shows the distributions for Delays of action poential trains recorded from diaphragms dosed with myopathic ecothiopate with and without physostigmine

pretreatment. After ECO the distribution shifts to the right due to the presence of affected fibres, but after PHY + ECO there is no shift in the distribution so it resembles that of the saline treated fibres.

After a myopathic dose of ecothiopate the MCD is significantly increased. Pretreatment with physostigmine prior to myopathic ecothiopate prevents this increase in MCD. There is no significant difference between the values of MCD for PHY + ECO and the saline controls. Figure 7.14 shows the distributions of MCDs for action potential trains recorded from diaphragms dosed with myopathic ecothiopate with and without physostigmine pretreatment. After ECO there is an increase in the number of fibres showing large MCDs which causes a shift to the right. After PHY + ECO the distribution shows no shift to the right as there are no fibres with large MCDs.

Figure 7.15 shows the changes in endplate shape after a myopathic dose of ecothiopate alone and a myopathic dose of ecothiopate given after 2 days pretreatment with physostigmine. Ecothiopate alone causes a significant deformation of the endplate seen as an increase in the width/length ratio of the endplates. Pretreatment with physostigmine followed by a dose of ecothiopate also causes a significant deformation of the endplates there being no significant difference in the width/length ratio of endplates after ecothiopate with or without physostigmine pretreatment.

In conclusion, 2 days continuous physostigmine administration does not completely protect against the effects of a myopathic dose of ecothiopate. It prevents the prolongation of $(MEPPs)_o$, the increase in myopathic jitter and the reduction in functional enzyme activity, but it does not prevent the deformation of the endplates, Physostigmine is a better protective agent than pyridostigmine however as 4 days pretreatment with pyridostigmine is only able to reduce the increase in neuromuscular jitter, having no effect on the timecourse of (MEPPs)o, the activity of functional enzyme and the deformation of the endplates.

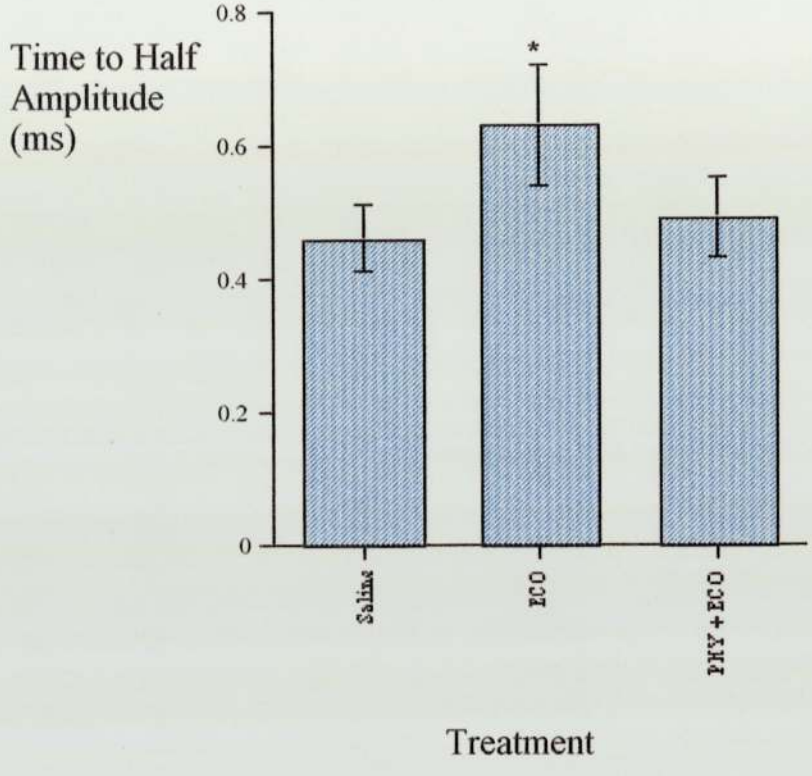


Figure 7.11 The effect of physostigmine pretreatment (14 nmol hr^{-1}) on the prolongation of extracellular miniature endplate potentials due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PHY + ECO indicates 2 days pretreatment with physostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls (Mann - Whitney $p<0.05$).

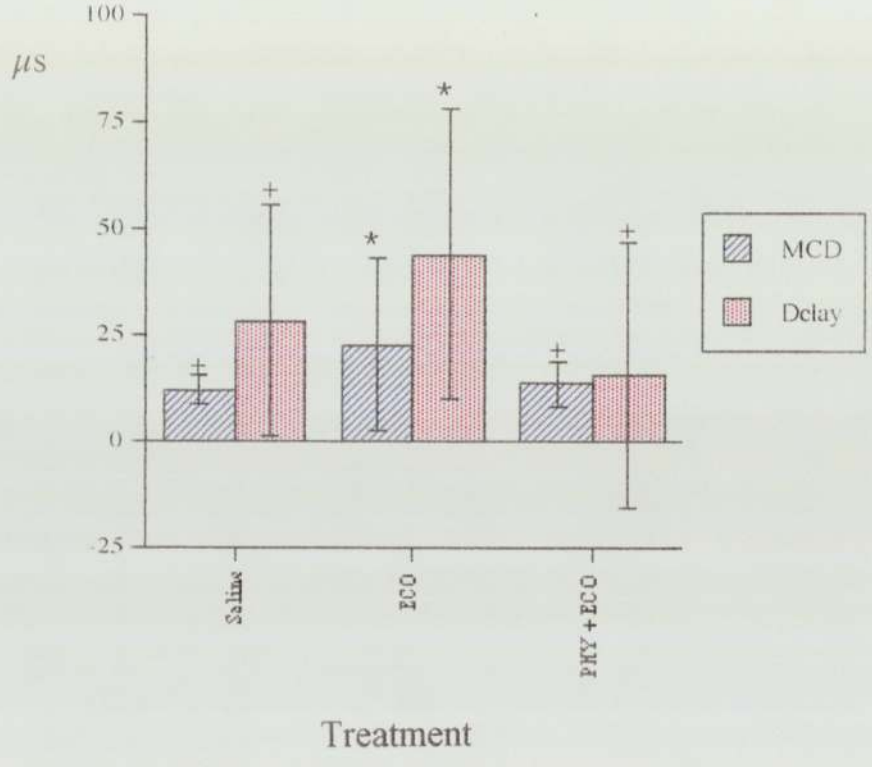


Figure 7.12 The effect of physostigmine pretreatment (14 nmol hr^{-1}) on the changes in action potential train Delays and MCDs due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PHY + ECO indicates 2 days pretreatment with physostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. The number of fibres tested were 40 for saline preparations, 31 for ECO and 34 for PHY + ECO. * indicates a significant difference from saline controls and + indicates a significant difference from the ECO value (Kolmogorov - Smirnov $p<0.05$).

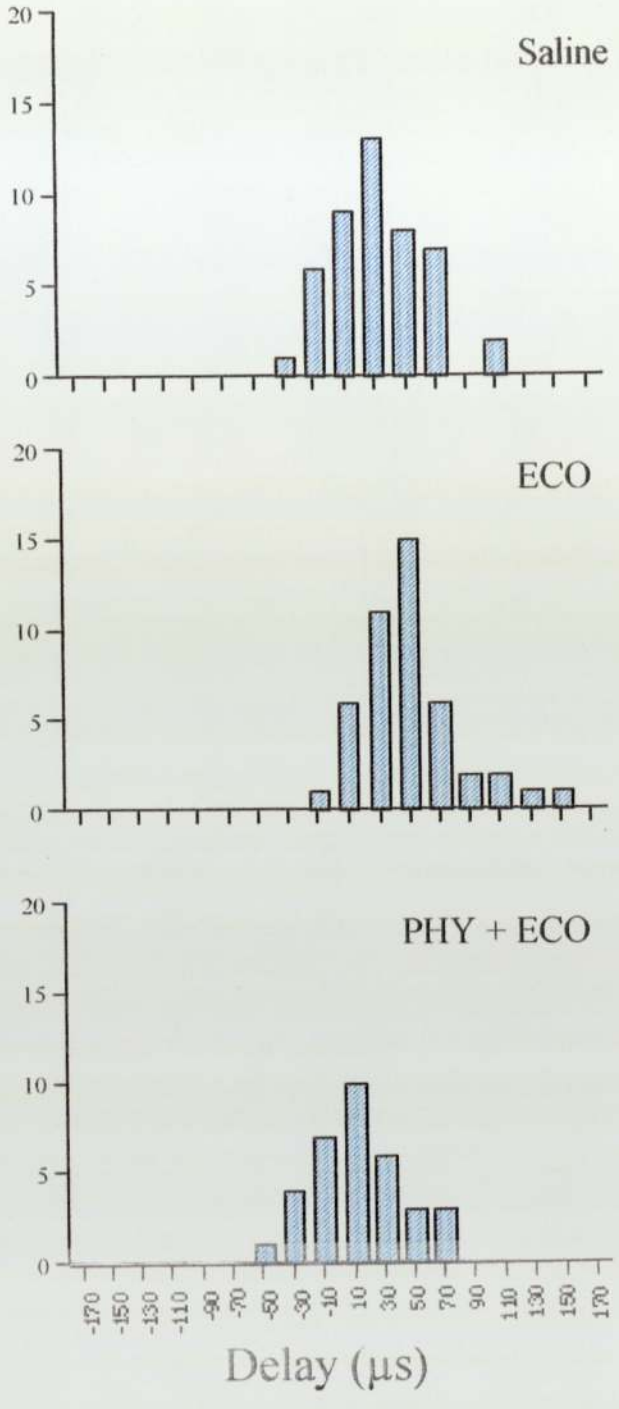


Figure 7.13 The distributions of Delays after ecothiopate 500 nmolkg^{-1} with and without physostigmine (14 nmol hr^{-1}) pretreatment. ECO indicates a single dose of ecothiopate alone, and PHY + ECO indicates 2 days pretreatment with physostigmine followed by a single dose of ecothiopate. *The Y axis indicates the number of fibres having a particular value of Delay*. The number of fibres tested were 40 for saline preparations, 31 for ECO and 34 for PHY + ECO.

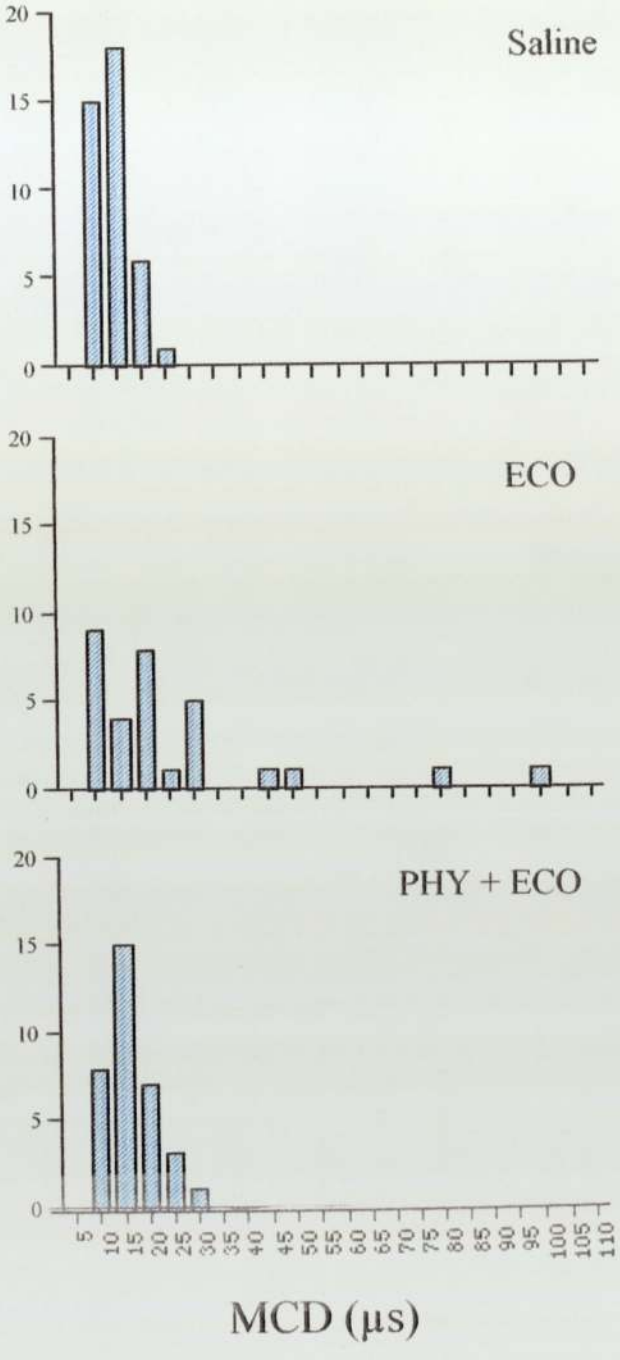


Figure 7.14 The distributions of MCDs after ecothiopate 500 nmolkg^{-1} with and without physostigmine (14 nmol hr^{-1}) pretreatment. ECO indicates a single dose of ecothiopate alone, and PHY + ECO indicates 2 days pretreatment with physostigmine followed by a single dose of ecothiopate. *The Y axis indicates the number of fibres having a particular value of MCD.* The number of fibres tested were 40 for saline preparations, 31 for ECO and 34 for PHY + ECO.

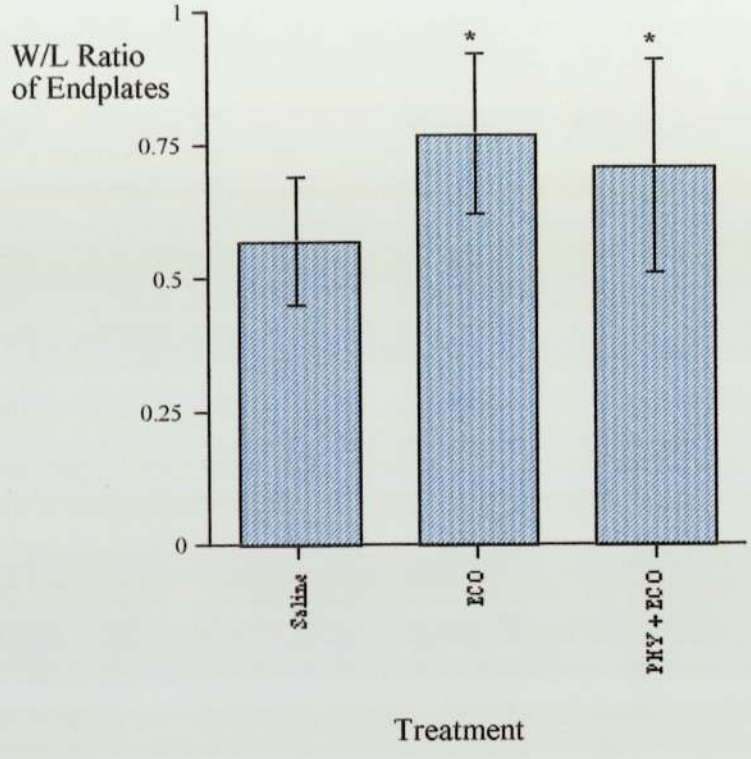


Figure 7.15 The effect of physostigmine pretreatment (14 nmol hr^{-1}) on the changes in endplate shape shown as the width/length ratio of the endplate due to ecothiopate 500 $nmolkg^{-1}$. ECO indicates a single dose of ecothiopate alone, and PHY + ECO indicates 2 days pretreatment with physostigmine followed by a single dose of ecothiopate. The values are shown as means ± s.d. For each condition 5 animals were tested. * indicates a significant difference from saline controls and + indicates a significant difference from the ECO value (Mann-Whitney p.0.05)

DISCUSSION

CHAPTER 8

Throughout the investigations presented here the same techniques have been used to determine the effects of different drugs, doses and delivery methods of these drugs. To allow for comparison of these drugs and delivery methods the results will be discussd not by drug but by technique. The recording of $(MEPPs)_o$ will be discused first followed by the deformation of the endplate and the variability in the latencies of action potentials will be discussed last. Finally the effectiveness of continuous pyridostigmine or physostigmine administration as a protection against all these effects will be discussed.

8.1 The prolongation of $(MEPP)_o$ timecourse after AChE inhibition.

It was found that the addition of any anticholinesterase caused an increase in the timecourse of the $(MEPP)_o$. Increasing doses of ecothiopate caused an increase in the timecourse of $(MEPPs)_o$ seen as an increase in the decay phase of these events. The T50% increasing from 0.49ms with a dose of 25nmolkg^{-1} to 1.00ms with a 500 nmolkg^{-1} dose. This was also seen with the other parameters which measure the decay phase but not those which measure the rising phase. A similar pattern was also seen after the addition of pyridostigmine in vitro where a dose of 10^{-6} M caused the decay phase to be prolonged, the T50% increasing from 0.46ms to 0.93ms but no change in the rising phase. With continuous administration of both pyridostigmine and physostigmine there is a significant prolongation of the $(MEPP)_o$ after only 1 day of dosing and this prolongation is maintained throughout the whole period of dosing.

Other cholinesterase inhibitors have been used in experiments where the EPP or MEPP have been recorded, where similar results were found. Kordas *et al* (1975) used the irreversible inhibitor methanesulphonyl fluoride, in frogs, and found that it caused a slight increase in the rise time of EPPs but a large increase in the decay phase the half time values increasing from 0.8ms to 2.6ms. Diisopropylfluorophosphate (DFP) was used by

Kuba and his co-workers (1974) and they again found an increase in the decay phase of the EPP in amphibian muscle. Gage and McBurney (1975) investigated the action of neostigmine on frogs and found an increase in total decay time of MEPPs to 1.43ms from control values of 0.88ms, an increase of 63%, but found no significant increase in the time course of the rising phase, and the same effect is also seen in the larger evoked endplate potentials (Guinan, 1980; Cherki-Vakil *et al* 1995). Other studies have also found the same effect on amphibian muscle with other reversible inhibitors, e.g. prostigmine (Kuba and Tomita, 1971; Magleby and Stevens, 1972; Kordas, 1972a; Katz and Miledi , 1973).

Why was the rising phase of the $(MEPPs)_O$ not affected by inhibition with ecothiopate whereas the decay phase was? Firstly consider the rising phase of the $(MEPP)_O$. For this to remain unchanged it must mean that the factors which determine it's time course are unaffected by anti-AChE's. Dwyer (1981) carried out a detailed investigation into the rising phase of the MEPP at the frog neuromuscular junction. He investigated the possibilities which had been previously suggested i.e that it was dependent on the time taken for ACh to diffuse across the cleft or it was dependent on the membrane potential of the post synaptic membrane. The first of these seemed unlikely as Eccles and Jaeger (1958) calculated that the diffusion of ACh was complete early in the rising phase. Dwyer slowed the diffusion of ACh, by replacing the Ringer solution in the recording bath with a high viscosity solution, which slowed the diffusion coefficient of Ach by 38%. This had no effect on the rising phase although it did increase the decay time, but depolarising the membrane slowed the rising phase, and therefore changes within the membrane must be the limiting step, the most likely factor being the binding constant of the receptor for the acetylcholine molecule.

Slowing the ACh diffusion did increase the decay time of the MEPPs which indicates that this is a limiting factor in the decay phase. It was felt however that free diffusion from the cleft was too rapid to account for the prolongation and so it was suggested that the open times of the ACh associated channels were increased by anti-ChE's (Kuba and Tomita,

1971: Magleby and Stevens, 1972). Katz and Miledi (1973) dismissed this idea however when using a spectral analysis of voltage-noise accompanying the action of ACh which showed that anti-ChE's didn't affect the open time of these channels. Their hypothesis was that the prolongation of the decay phase was due to the diffusion of the excessive amounts of transmitter out of the cleft. While diffusing the molecules would repeatedly bind to receptors in their path, being removed and then rebinding, so prolonging the potential by causing opening and reopening of the ACh receptor associated channels. If there were no anti-ChE activity though this repetitive binding would not occur as the ACh would be hydrolysed rapidly after dissociation from the receptor. Since it was first suggested several other investigators have backed up the hypothesis of Katz and Miledi (1973) by suggesting similar mechanisms (Hartzell *et al.*, 1975; Magleby and Terrar, 1975; Linder *et al.*, 1984).

Some of the investigations into the effects of anti-ChE's showed an increase in the amplitude of the MEPPs or EPPs recorded from the neuromuscular junctions of frogs (e.g. Goldner and Narahashi, 1974; Gage and McBurney, 1975; Kordas *et al.*, 1975; Fedorov, 1976) the amplitudes of those recorded in these experiments have not been detailed however as in extracellular recording the position of the electrode will greatly affect the amplitude, so any change in amplitude seen could not necessarily be attributed to the action of the anti-ChE.

8.2 Recovery of (MEPP)o timecourse after inhibition of AChE.

If a 500 nmolkg^{-1} dose of ecothiopate is given and the period of time before the diaphragm is removed increased, the time course of the $(MEPP)_o$, which is initially prolonged begins to get shorter, until at 7 days there is no difference between the $(MEPPs)_o$ from a treated preparation and those from an untreated preparation. Similar recovery is seen after a single dose of pyridostigmine, and contiuous dosing with pyridostigmine and physostigmine. After addition of pyridostigmine 10^{-6} M *in vitro* the time taken for the timecourse of the $(MEPPs)_o$ to return to normal was 75 minutes. After a large myopathic dose *in vivo* however the $(MEPPs)_o$ are still prolonged 24 hours after dosing. With continuous administration of pyridostigmine the $(MEPPs)_o$ have a prolonged timecourse for the period of dosing which lasts for 2 days after dosing is stopped.

Repetitive dosing with pyridostigmine shows a different pattern of inhibition. During the dosing period no prolongation of $(MEPPs)_o$ was seen when the diaphragms were removed 3 hours after the final dose, however there was a prolongation of $(MEPP)_o$ decay if the diaphragm was removed 1 hour after dosing. Once the dosing was stopped there was no prolongation of the $(MEPPs)_o$. The lack of any reduction in AChE activity even after 2 weeks of repetitive dosing does not suggest any cumulative effect on AChE activity after dosing in this manner, although there could be a cumulative effect on some other system wilthin the synaptic cleft.

The results of the *in vitro* dosing with pyridostigmine show us that the drug initially inhibits the AChE in the cleft but that this is reversed within a matter of minutes as the enzyme-drug complex dissociates, complete recovery being seen in 75 minutes at 37°C. This would suggest that when an in vivo dose of pyridostigmine is given it should dissociate from the enzyme within approximately the same time frame. This is what

happens when a 100 μgkg^{-1} was given twice daily as indicated by the prolongation of the $(MEPPs)_o$ 1 hour after dosing but not 3 hours later.

Ecothiopate causes prolongation of $(MEPPs)_o$ for long periods as it is an organophosphorous compound which attaches only to the esteratic site of the enzyme. After hydrolysis the enzyme is left phosphorylated therefore for the enzyme to be regenerated dephosphorylation must occur. This process can occur spontaneously but the extent is negligible. With ecothiopate there will be some ageing (see figure 1.5) of the enzyme-drug complex which makes the drug irreversible as after it has aged the bond is irreversible either spontaneously or by use of an antidote e.g. pralidoxime. The half time for ageing of ecothiopate given by Hobbiger (1976) was 41 hours. Although this is a relatively slow rate of ageing when compared to other irreversible inhibitors e.g. soman which has a half time of 2.4 minutes (Harris *et al* 1971), the time course of these experiments is such that some ageing will have occured and some enzyme will never be reactivated. The recovery in enzyme activity therefore must be due to some spontaneous reactivation, but the majority will probably be due to the synthesis of new enzyme.

AChE biosynthesis is a complex process which occurs in several stages. If a blocker of protein synthesis is added to a muscle culture AChE levels continue to increase which indicates that the complex form may first be synthesised as an active precursor (Walker and Wilson, 1976). After organophosphate treatment increased levels of the globular forms of AChE have been found in the endoplasmic reticulum, where active synthesis of the new enzyme is taking place (Brzin *et al,* 1980). AChE has been found to be released mainly in the G4 form at the endplate of the rat, originating from both the nerve terminal (Skau and Brimijoin, 1978) and the muscle itself (Carter and Brimijoin, 1980). In rat diaphragm it was found that the G1 and G4 forms recovered much faster the A8 and A12 forms (Massoulie and Bon, 1982), suggesting that cells incorporate the catalytic subunits into the G4 form and then into the collagen tailed asymmetric forms. Lazar *et al* (1984) and Massoulie *et al* (1985) used heavy isotopes to investigate the biosynthesis of AChE in

cultured muscle cells. Initially there was a delay in the appearance of labelled enzyme indicating the existence of inactive precursors whose half life was approximately 30 minutes. G1 and G4 showed much longer half times with mean values of 5 and 40 hours respectively, followed by a further 3 hour delay before secretion of the G4. Fernandez and Stiles (1984) inhibited AChE with diisopropyl fluorophosphate (DFP) and found that the A12 form of the enzyme was newly synthesised at the endplates of rat gracilis muscle and showed recovery within 75 hours.

It is possible therefore that within the time course of these experiments there would have been synthesis of entirely new enzyme which would show increased enzyme activity levels as were seen.

After carbamate inhibition the period over which $(MEPP)_o$ prolongation lasts is shorter due to the lack of ageing with this class of anti-cholinesterases. Carbamates attach to both the esteratic and anionic sites of the enzyme after which hydrolysis occurs leaving carbamylated enzyme, which needs to undergo spontaneous decarbamylation before it can be regenerated. Decarbamylation occurs very much slower than deacetylation and it is this which prevents the acetylcholine from being hydrolysed and allows it to exert prolonged action at the endplate until the enzyme is regenerated which in these experiments was shown to be 75 minutes.

If the prolongation of the $(MEPPs)_o$ is due to inhibition of the enzyme however why is there prolongation of the $(MEPPs)_o$ for so long after a myopathic dose of pyridostigmine or after continuous administration of pyridostigmine and physostigmine has stopped? Further evidence that it is not inhibition which is causing the prolongation of the $(MEPPs)_o$ comes from the experiments in section 5.2.2 where $(MEPPs)_o$ were recorded immediately upon removal of the diaphragm after continuous administration of pyridostigmine, or recorded 1 hour after removal. There was no difference in the time to half amplitudes of $(MEPPs)_o$ under these two conditions, the $(MEPPs)_o$ were equally

prolonged, which suggests that there is no inhibited enzyme at this time as if there was inhibition of any enzyme this extra hour would have allowed for some reactivation which would have been seen in the shortening of the time to half amplitude of the $(MEPPs)_O$.

There are a number of possibilities which could cause the prolongation of the $(MEPPs)_O$. The first is the lack of enzyme within the cleft, not through inhibition but because of some mechanism which reduces the amount of functional enzyme within the synaptic cleft. There could also be changes in the AChE receptor linked ion channels prolonging their open time, or the excess ACh could diffuse to extrajunctional receptors with different gating properties, or there could be a reduction in the population of the receptors at the endplate as the system works to nullify the effects of excess ACh within the cleft.

There is evidence to support the first hypothesis in the results of Mrs A. Rowbotham (pers. comm.) who measured the activity of AChE during and after the period of dosing. She found that the activity of the AchE was reduced for up to 2 days after dosing had stopped, a reduction which could not be accounted for by inhibition. This downregulation of AChE may be due to some other effect of Anti-ChE action possibly affecting the gene responsible for production of AChE. Further experimentation needs to be done into the expression of the AChE gene before any conclusions can be drawn.

There have been several investigations into the actions of carbamates on the receptor linked ion channels themselves and these show that they act differently depending on the dose. At low doses their effects reflect inhibition of cholinesterase, i.e. potentiation of nerve elicited muscle twitches, increased amplitude and timecourse of EPPs and MEPPs. At higher doses however these effects are reversed (Pascuzzo *et al*, 1984). Single channel studies have shown that at higher doses the carbamates can cause shortened channel lifetime and decreased channel conductance (Akaike *et al*, 1984; Shaw *et al*, 1985). To increase the timecourse of $(MEPPs)_O$ the mean channel lifetime would need to be increased not decreased therefore it is probable that changes in channel open time are not

the cause of the prolongation of $(MEPPs)_o$. The other factor which makes changes in ion channel properties an unlikely reason for the prolongation of $(MEPPs)_o$ are prolonged is the doses at which this direct action on the ACh receptor-channel complex occur. Pyridostigmine and physostigmine both only exert a direct effect at doses within the μM range (Albuquerque et al,1984; Fiekers 1985a; Bradley *et al*, 1986; Oyama *et al*, 1989), as does neostigmine (Fiekers, 1985b). The doses used within these experiments are very much smaller and could only reach these levels if there was a significant accumulation of drug in the synaptic cleft which has not been shown to occur.

Whether there has been any changes in the ACh receptor itself has not been investigated during these studies but it seems unlikely that diffusion to extrajunctional receptors causes any additional prolongation of the $(MEPPs)_o$ other than by normal binding as Brehm and Kullberg (1987) found no differnce in the properties of junctional and extrajunctional receptors.

Of the various hypotheses suggested for the prolongation of (MEPPs)o beyond the period of enzyme inhibition it seems likely that there may be down regultion of the AChE in the cleft. To determine if this is correct or if there is some other explanation further experimentation is needed.

8.3 The effect of temperature on the prolongation of $(MEPPs)_o$

To determine the effect of temperature on the timecourse of pyridostigmine inhibition initially $(MEPPs)_o$ were recorded from untreated diaphragms at 30°C and 37°C, followed by recording $(MEPPs)_o$ at these temperatures after addition of pyridostigmine *in vitro* at 10^{-6}M. Reducing the bath temperature to 30°C caused a prolongation of $(MEPP)_o$ timecourse in untreated preparations in both the rising and decay phases. The rise time was increased from 0.25ms to 0.33ms and the decay phase was prolonged to a greater extent the increase being from 0.45ms to 0.65ms. After the addition of pyridostigmine the decay phase was prolonged at both temperatures with respect to their relevant controls. The $(MEPPs)_o$ recorded at 30°C however also show a greater prolongation when compared to those recorded at 37°C, which agrees with earlier findings that the decay phase of EPPs and MEPPs in amphibians is affected both by junctional AChE activity and the temperature of the bath (Kordas, 1972).

The temperature dependence of the rise time agrees with that found by Dwyer (1981) and Head (1983) who measured the rise time at various temperatures and found that it was sensitive to temperature but thought that diffusion did not contribute to this greatly, but that binding and isomerisation were more likely to be the rate limiting factors.

Several studies have investigated the role of temperature in the timecourse of EPPs and MEPPs. Many of these have found the Q_{10} for the decay phase to be approximately 3 (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972; Kordas, 1972; Anderson and Stevens, 1973; Gage and McBurney, 1975; Head, 1983; Kordas and Zorec, 1984).

The value of 3 for Q_{10} suggests that diffusional dilution of ACh is not the rate limiting factor for the decay (Magleby and Stevens, 1972). Another argument against diffusion being the rate limiting factor is the effect membrane potential has on the decay (Takeuchi and Takeuchi, 1959; Gage andArmstrong, 1968; Kordas, 1969; Gage and McBurney,

1972; Magleby and Stevens, 1972; Anderson and Stevens, 1973), as it is hard to explain how membrane potential affects diffusion of AChE (Gage and McBurney, 1975). The activity of the AChE could be affected by post-synaptic membrane potential but the voltage sensitivity of decay is unaltered if the AChE is inhibited (Gage and McBurney, 1975). Bamforth (1989) found no difference in the activity of AChE at 30°C and 37°C as measured using the technique of Ellman *et al* (1961) keeping the diaphragm samples at the respective temperatures. This, combined with the other evidence suggests that the rate limiting step in MEPP decay is not solely AChE activity.

The other possibility is that the rate limiting step occurs within the post synaptic membrane and this is supported by the voltage sensitivity (Magleby and Stevens, 1972a and b; Kordas, 1972 a and b; Gage and McBurney, 1975). Kordas suggests that the rate limiting step is the dissociation of ACh from the receptors, but Magleby and Stevens argue that it involves a conformational change in the ACh-receptor complex.

Whichever hypothesis is true there is no doubt that temperature affects the open time of the ion channels associated with the ACh receptor prolonging the timecourse of $(MEPPs)_o$ and the experiments presented here show that it also affects the time taken for a reversible inhibitor to dissociate from the AChE.

8.4 The deformation of endplates caused by inhibition of AChE

The experiments in this study show that addition of an anticholinesterase causes a significant change in the shape of endplates, from oval in shape to round. When ecothiopate is added in increasing doses a deformation of the endplate is seen even at the very lowest dose of 25nmolkg^{-1} where the width/length ratio has increased from 0.57 to 0.72. This deformation gets progressively worse with increasing doses until it reaches 0.88 with a 500 nmolkg^{-1} dose. Continuous administration of pyridostigmine and physostigmine also caused rounding up of the endplate. At day 1 after dosing had begun with pyridostigmine the width/length ratio had increased to 0.82 and this same amount of deformation was seen throughout the period of dosing. A similar pattern was seen with physostigmine where the width/length ratio was increased to 0.92. Repetitive dosing with pyridostigmine caused a deformation of the endplate, the width/length ratio increasing to 0.84 at 1 day and this deformation being maintained throughout the period of dosing.

Why was such a deformation of the endplate seen? A possible explanation is a localised contraction underlying the region of the endplate.

The action of anticholinesterases cause an accumulation of transmitter present in the synaptic cleft (Katz and Miledi, 1973) and this results in an influx of Ca^{2+} into the muscle cell (Ashley and Ridgeway, 1968; Evans, 1974; Miledi *et al*, 1977) thus increasing the levels of intracellular Ca^{2+}. An increase in intracellular Ca^{2+} has been shown to be a cause of myopathy after anticholinesterase treatment via a variety of processes (Leonard and Salpeter, 1979; Kawabuchi, 1982; Toth *et al*, 1988; Kar and Pearson 1978, Gupta and Dettbarn, 1987; Hudson *et al*, 1985) but it has also been shown that small non necrotising doses can cause an increase in intracellular Ca^{2+} (Das, 1989). An influx of Ca^{2+} may not be the only method by which intracellular Ca^{2+} is raised. The prolonged action of ACh within the synaptic cleft will cause a prolonged depolarisation of the post synaptic membrane and this can cause the release of Ca^{2+} from the sarcoplasmic reticulum (SR)

(Endo, 1977). There may also be release of Ca^{2+} from intracelullar Ca^{2+} -buffers within the mitochondria (Oberc and Engel, 1977). The presence of elevated intracellular Ca^{2+} can in itself induce release of Ca^{2+} from the SR (Ford and Podolski, 1970; Endo, 1977; Fabiato, 1982). There is a further mechanism by which intracellular Ca^{2+} may be elevated by its release from mitochondria and this is via the Na^{+}/Ca^{2+} transporter which is stimulated by increases in Na^{+} which would be caused by a prolonged depolarisation (Affolter and Carafoli, 1980; Carafoli and Zurini, 1982).

The increases in intracellular calcium could activate the local contractile apparatus and cause a contraction of the muscle fibre underlying the endplate region. This would be a localised effect as the depolarisation caused by prolonged action of ACh is itself localised at the endplate. Ferry and Cullen (1991) found that diaphragms which had been exposed to ecothiopate *in vitro* after stimulation changed appearance and underwent ultrastructural changes in the endplate region. There was a swelling of the cell at the endplate region and this was associated with a closing up of the cross-striations and an increase in the diameter of the cell. Upon stimulation fibres exposed to ecothiopate exhibited a prolonged localised contraction at the endplate which was felt to be due to an elevation in Ca^{2+} (Burd and Ferry, 1987).

The prolonged localised contraction seen by Burd and Ferry (1987) lasted only for about 30 minutes whereas the deformation of the endplates seen in the experiments here lasted not only for the duration of dosing but also throughout the whole of the recovery period studied. With ecothiopate the deformation lasted for up to 5 days after a single dose of 500 $nmolkg^{-1}$, and after continuous administration of pyridostigmine and physostigmine the deformation persisted up to 14 days after dosing was stopped. For 2 of these 14 days there may still a reduction in the activity of the AChE in the cleft as shown by the prolongation of the $(MEPPs)_o$ and the reduction in activity seen by Mrs. A. Rowbotham as discussed previously. After this period however when the other techniques indicate that

AChE activity has returned to normal the deformation of the endplate cannot be due to the continued influx of Ca^{2+}.

The lack of correlation between electrophysiological data and endplate deformation could be due to the time course of the different recovery processes. AChE activity recovers relatively quickly allowing ACh to be rapidly hydrolysed after it becomes dissociated from the receptors. One possible reason for the continued deformation of the endplate could be the slow rate at which Ca^{2+} is removed from the endplate, either by mobilisation out of the endplate region or by reabsorption into the mitochondria or SR. As there was a deformation of the endplate after repetitive dosing even though no significant reduction in AChE activity persisted up to 3 hours after the dose was given this supports the hypothesis that Ca^{2+} when it is accumulated intracellularly takes a considerable time to be removed.

Another possibility is that the sarcomeres in the endplate region have become locked into rigor conformation due to the lack of ATP and are unable to relax, so returning the endplates to normal dimensions. The lack of ATP within the region could also be due to the action of excess intracellular Ca^{2+} disrupting the mitochondria for which there is some evidence. After exposure to pyridostigmine Hudson *et al* (1985) found the most common morphological change was alterations to the mitochondria, both pre- and post-synaptically. Bowman *et al* (1989) used light and electron microscopy to investigate ultrastructural changes in the rat diaphragm after pyridostigmine treatment, one of which was disruption of the mitochondria consistent with an accumulation of calcium. Further ultrastructural evidence comes from Meshul *et al* (1985) who found swelling of the mitochondria in the endplate region of rat soleus and extensor muscles as soon as 30 minutes after exposure to sarin. (Laskowski, 1975; Hudson et al, 1985;).

To determine if either of these hypotheses is correct further investigation into the ultrastructure of the endplate after this type of anticholinesterase inhibition needs to be

carried out, maybe by use of electronmicroscopy. Investigations could also be carried out into the distribution and concentration of Ca^{2+} in the endplate region during anti-ChE treatment. Calcium blockers could also be added to determine if they exert any protective effect.

8.5 The variability in the latencies of action potentials caused by inhibition of AChE

Inhibition of AChE causes an increase in neuromuscular jittter seen as changes in MCD and Delay. A myopathic dose of pyridostigmine increased the MCD of the action potential trains both 1 day and 5 days after the dose was given, whereas the Delay was decreased on the first day only. When a much smaller dose of pyridostigmine was given twice daily however no increase in MCD was seen until day 4 although Delay was decreased on day 2. After dosing was stopped the increase in jitter persisted for 7 days. Continuous administration of pyridostigmine and physostigmine induced the onset of increased jitter on different days. Pyridostigmine induced an increase in Delay on day 2 and MCD on day 7 whereas for physostigmine although the Delay showed a significant effect on the same day the MCD was increased on day 4. Both drugs showed a persistance of Delay decrease until 2 days after dosing had stopped and an increase in MCD for 7 days after the end of dosing.

In an attempt to determine the cause of increased neuromuscular jitter this has been compared with the other consequences of AChE inhibition. As continuous administration of pyridostigmine and physostigmine are directly comparable as the same timepoints have been measured a summary of their effects is shown in Table 8.1. Comparison with repetitive dosing is not easy as this dosing allowed reactivation of the enzyme prior to any measurements being made as discussed earlier.

a)

Period of Pyridostigmine Dosing	T50% (ms)	MCD (μs)	Delay (μs)	AChE activity (nmol/min/mg)	Width/length Ratio of Endplates
1 day	0.63±0.11 *	12.0±4.4	9±35	0.11±0.48	0.82±0.19 *
2 days	0.67±0.06 *	12.0±6.3	19±19 *	0.03±0.40	0.75±0.19 *
4 days	0.70±0.08 *	12.6±8.6	11±23 *	0.11±0.07 *	0.82±0.18 *
7 days	0.72±0.09 *	18.0±13.3 *	13±43	0.30±0.17 *	0.88±0.13 *
14 days	0.91±0.12 *	33.7±27 *	5±64 *	0.47±0.26	0.84±0.17 *
2 days R	0.66±0.17 *	20.8±15.5 *	9±39 *	0.39±0.44	0.76±0.15 *
7 days R	0.46±0.11	20.7±6.5 *	33±54	0.16±0.40	0.72±0.13 *
14 days R	0.46±0.05	16.4±6.9	17±33	0.91±0.46	0.71±0.18 *

b)

Period of Physostigmine Dosing	T50% (ms)	MCD (μs)	Delay (μs)	AChE activity (nmol/min/mg)	Width/length Ratio of Endplates
1 day	0.62±0.11 *	11.3±5.1	16±30	0.40±0.14	0.92±0.16 *
2 days	0.72±0.11 *	12.5±4.9	7±24 *	0.32±0.11	0.80±0.19 *
4 days	0.82±0.07*	14.3±9.0 *	10±23 *	0.21±0.19 *	0.85±0.18 *
7 days	0.97±0.18 *	18.2±10.8 *	13±49 *	0.38±0.31	0.79±0.18 *
14 days	1.08±0.14 *	22.9±14.6 *	5±40	0.60±0.60	0.87±0.16 *
2 days R	0.66±0.05 *	21.1±12.3 *	11±28 *	0.14±0.24 *	0.91±0.15 *
7 days R	0.46±0.04	20.6±9.1 *	20±35	0.26±0.15	0.80±0.21 *
14 days R	0.46±0.05	11.0±5.9	26±30	0.69±0.38	0.70±0.17 *

Table 8.1 Summary of the effects of a) pyridostigmine (11.4 $nmolhr^{-1}$) and b) physostigmine(14 $nmolhr^{-1}$). R indicates the days of recovery. Values shown are the mean ± s.d. * indicates a significant difference from saline treated preparations.

Kelly *et al* (1990) determined that although jitter was a consequence of AChE inhibition the relationship between these two was unclear, and Smith (1993) also found no clear relationship between jitter and AChE activity. The data here agrees with this as the patterns seen in increased jitter after the various drugs at the different doses is not the same as that seen for the reduction in activity of AChE, there was still increased jitter long after AChE activity had returned to normal. This suggests that jitter and AChE activity are not related and indeed no correlation exists between the degree of jitter and the activity of functional AChE as measured by the method outlined in 3.3.

It has been suggested that it is the degree of AChE inhibition or the rate of onset of inhibition which determines the onset and extent of jitter however the data here does not support this. If this was true physostigmine, which when administered continuously causes an increase in jitter before pyridostigmine administered in the same manner, would cause a change in AChE activity sooner or greater, and this does not happen. Repetitive dosing also caused a decrease in Delay and an increase in MCD even though the inhibition was short lived and at no point was there any significant reduction in the activity of AChE measured electrophysiologically or biochemically 3 hours after dosing, the time action potentials were recorded.

Although the data presented here does not support this theory it does not mean that no relationship between the two exist it could just be that the relationship is complex and may rely on other factiors, as it is clear that increased jitter does not occur without there first being significant inhibition of AChE.

When the period over which MCD and Delay are increased is compared to that over which the timecourse of $(MEPPs)_o$ are prolonged some similarities are seen. When the time to half amplitude of $(MEPPs)_o$ and the Delay of the action potential trains are compared a significant correlation is found. No such correlation, however, is found with MCD. The relationship between $(MEPPs)_o$ and Delay is negative indicating that as one

increases there is a corresponding decrease in the other. Why such a relationship should exist and why it is only seen with pyridostigmine administered continuously is unclear and it may be just a fluke of the data, more experiments will need to be carried out to determine if this relationship is true.

There was no significant correlation between the MCD and the Delay for any of the data presented and this is also consistent with what has been previously found (Kelly *et al* 1990). Delay and MCD do not become affected at the same time and do not recover over the same time period suggesting that they may have different causes, which may also be indicated by the lack of correlation for MCD with $(MEPPs)_o$ where one is found with Delay. It is interesting to note that with ecothiopate there was an increase in Delay yet with pyridostigmine and physostigmine there was a decrease in Delay. This difference is a split between reversible and irreversible inhibitors, although why such a difference should occur is not clear. Smith (1993) when looking at the irreversible inhibitors sarin and soman found an increase in Delay consistent with that seen here and by Kelly *et al* (1990) with ecothiopate. It has been suggested that Delay is affected by the tension at which the hemidiaphragm is pinned out while recording. However it does not seem that this is the case as it is unlikely that there would have been a consistent difference in tension for the experiments testing the different drugs.

There are some possibilities as to why Delay is increased with ecothiopate but decreased with the carbamates. Firstly, it could be that in the carbamate experiments there are 2 populations of muscle fibres. Some are affected by the carbamate and show ecothiopate like effects but these are masked by "unaffected" fibres which respond more quickly to stimulus and fire earlier giving a short Delay . This hypothesis is supported by the distributions of Delay which show a shift in both directions ie. widening of the distribution, however this does not happen in all cases. Secondly, it could be that the effect of carbamates are much smaller than the effects of ecothiopate such that once an action potential is established the 16th is quicker than the first just as in a normal fibre ie. there are two effects which co-exist; a) Delay in excitation

b) Speeding up of action potential once excited.

Ecothiopate may have a greater effect because it causes myopathy at the dosage used whilst the carbamates do not. Myopathy occurs at the endplate and also along the length of the fibre were contraction clumps can be seen (Ferry and Cullen 1991). If this damage slows down conduction, which is possible, ecothiopate in a myopathic dose would cause a delay in excitation and the slowing of action potentials resulting in an increase in MCD and Delay . Carbamates on the other hand cause a delay in excitation and because the rest of the fibre is normal a speeding up of action potentials resulting in an increased MCD, although this would not cause the decreased Delay.

Smith (1993) raises the possibility that the increase in jitter could be related to the accumulation of calcium within the post synaptic membrane. He provides no evidence for this and the data presented here does not make the relationship clear. The increase in intracellular Ca^{2+} has already been discussed and the consequence of it is seen as a deformation of the endplate. When the deformation of the endplate is compared to the onset of jitter the result is inconclusive. A significant relationship is seen only with continuous administration of pyridostigmine. The relationship is positive MCD increases as the width/length ratio increases. The presence of increased jitter after repetitive dosing with pyridostigmine supports the involvement of Ca^{2+} in the onset of jitter as it has been shown that after this type of dosing the only significant effect is on jitter and endplate shape.

Continued increases in intracellular Ca^{2+} will cause myopathy and eventually cell death, necrosis. These necrotic cells will be electrically silent and will not be recorded however it could be that the cells which show increased MCD are those who are suffering from some sort of myopathy in a pre necrotic state. However this is only speculation as no relationship berween Ca^{2+} accumulation and jitter has been proved, but this could be investigated by looking at Ca^{2+} concentrations in fibres treated with anticholinesterases and recording action potentials before determinig if any relationship did exist.

Further attempts have been made to discover the origin of neuromuscular jitter after cholinesterase treatment firstly by looking at the effect of stimulaton frequency and then by looking at the action potentials in conjunction with the endplate potential which gives rise to them.

It has been shown that inhibition of AChE causes an increase in the variabilities of action potentials, but data exhibited here shows that this is not always the case. When an ecothiopate treated fibre is stimulated at 1Hz no increase in jitter is seen however the same fibres will show an increase if they are stimulated at 30Hz. There was no difference seen in the jitter of untreated fibres stimulated at 1 or 30Hz.

The lack of an increase in jitter when the fibres are stimulated at 1Hz suggests that increased MCD and Delay is not caused by any structural change at the endplate, but rather is due to some functional change in the process of neuromuscular transmission, either presynaptically or postsynaptically.

If the change was presynaptic i.e. the release of transmitter there would be a corresponding increase in the MCD and Delay of the EPP and indeed Stalberg and Trontelj (1979) suggested that there was increased variability in the rise times of EPPs. It has been shown however that this is not the case with ecothiopate (Kelly *et al*, 1990), and so the most likely possibility is that the increase in jitter is due to some postsynaptic change, either in the generation of the action potential or its propogation along the muscle fibre.

The possibility is that after treatment with an anticholinesterase there is some change in the membrane properties which alters the generation of the action potential. It could be that there is a change in the site of action potential generation because of the depolarisation of the membrane around the endplate, after an action potential.

In untreated preparations the first action potential is generated at some point at the endplate and successive action potentials are generated at the same point or at some point very close to it. When the preparation has previously been treated with an anticholinesterase the first action potential is generated at a point at the endplate as in untreated preparations. The membrane however does not recover sufficiently before the next action potential needs to be generated and so it must be generated at some point futher away where the membrane is suitable. This would account for the stimulation frequency difference, seen as the increased time between action potentials. When they are stimulated at 1 Hz it leaves greater time for the membrane to recover, allowing subsequent action potentials to be generated at the same site or very close to the site of the first generation.

Whether there is any difference in the membrane potential between the action potentials of untreated and anticholinesterase treated fibres could be determined by electrophysiological recording. There may also be some change in the propagation of the action potential along the muscle fibre and it may be possible to investigate this by using a two electrode recording technique which would allow the measurement of action potential propogation before and after anticholinesterase treatment.

Whatever the mechanism of jitter onset it seems clear that it is a complex process which has been shown here to be due to a functional change not a structural one and a change probably in some postsynaptic mechanism. It is clear however that more investigation into the onset of jitter needs to be carried out. The onset of jitter is further complicated by the type of anticholinesterase used as shown by de Blaquiere et al (1994) who found that mipafox also caused increase jitter of EPPs suggesting a presynaptic role as well as any possible postsynaptic one.

8.6 Continuous carbamate administration as protection against the effects of myopathic ecothiopate

A single dose of carbamate has been shown to protect against the effects of organophosphate poisoning (Dirnhuber and Green, 1978; Gordon *et al*,1978; Wecker *et al*, 1978; Dirnhuber *et al*, 1979; French *et al*, 1979; Kelly *et al*, 1992)). The exact dose of carbamate given does not seem important (Gordon *et al*, 1978) and so the question was raised as to whether continuous dosing was also able to protect against organophosphate poisoning. Wetherall (1994) found that continuous administration of physostigmine and hyosine prior to a dose of soman prevented the behavioural signs of toxicity and incapacitation, as well as reducing the inhibition of AChE. Experiments were therfore carried out to detemine if continuous carbamate administration would protect against the effects of anticholinesterases.

There was a distinct difference in the protective abilities of the two carbamates used. Continuous administration of physostigmine prior to a 500 $nmolkg^{-1}$ dose of ecothiopate was found to have no beneficial effect on $(MEPP)_o$ prolongation, reduction in AChE activity or deformation of endplates, the increase in Delay seen after a single dose of ecothiopate was actually made worse. The only beneficial effect was seen with MCD where the increase in this was not so great after pyridostigmine pretreatment. Physostigmine showed much better protective abilities. It successfully prevented an increase in $(MEPP)_o$ timecourse, a reduction in the activity of AChE and an increase in either MCD or Delay. It did not protect against the deformation of endplates caused by ecothiopate however.

The extent of the protection offered by carbamates has also been shown to exhibit variability by Smith (1993) who gave a single dose of pyridostigmine ($0.38\mu molkg^{-1}$) 30 minutes before a dose of sarin or soman and found that it offered complete protection

against the increase in jitter seen with sarin but only reduced the increase in jitter after soman.

Carbamates are thought to protect against organophosphate intoxication due to the reversibility of their action. They will inhibit some of the functional AChE within the synapse and so prevent this AChE from being irreversibly inhibited by the organophosphate. Decarbamylation will then occur and along with the removal or destruction of the carbamate which frees the enzyme to restore normal neuromuscular function (Berry and Davies, 1970; Dirnhuber and Green, 1978; Dirnhuber *et al*, 1979; Green and Smith, 1983)

The results presented here indicate that the extent to which protection is offered is not solely due to the inhibition of the AChE as the doses of pyridostigmine and physostigmine administered caused similar reductions in the activity of functional AChE, while giving very different protection. Why physostigmine should give complete protection when pyridostigmine does not however is not known but Harris *et al* (1984) found physostigmine to be superior to pyridostigmine for protection against soman.

There is the possibility that it is due to some direct action of the carbamate on the ACh receptor - channel complex itself. While there is evidence that pyridostigmine and physostigmine do interact directly the concentrations required for this to occur need to be considered. While the exact concentration of the carbamate in the synaptic cleft at any one time is unknown it seems unlikely that a small dose of 11.4 or 14 $nmolhr^{-1}$ could accumulate to μM range which is the dose range showing direct interactions (Albuquerque et al,1984; Fiekers 1985a; Bradley *et al*, 1986; Oyama *et al*, 1989).

Neither of the carbamates protect against the deformation of the endplate caused by ecothiopate. This may be due to the increase in Ca^{2+} following the dose of carbamate, which would have a slow rate of extrusion out of the endplate region meaning that

intracellular Ca^{2+} is still elevated when the dose of ecothiopate is given. Or it could be due to the underlying sarcomeres being maintained in rigor formation as discussed earlier.

Whatever the mechanism by which protection is offered it seems clear that pretreatment with a carbamate can give protection from the effects of an organophosphate although the degree of protection differs with the carbamate and organophosphate used. Added to this, the effects of the carbamates alone when administered for long periods suggests that further research is needed into the effects of long term dosing with carbamates.

8.7 Conclusions

1 By using a sequential extraction method it was possible to find a highly significant relationship between the prolongation of $(MEPP)_O$ decay and endplate specific non extractable AChE suggesting that it is this fraction which best biochemically represents functional enzyme.

2 Addition of anti-ChEs causes a prolongation in the timecourse of $(MEPPs)_O$. This was seen specifically in the decay phase. A postsynaptic cause seems most likely, probably the binding and rebinding of excess ACh within the cleft, which has not been hydrolysed due to the inhibition of the AChE.

3 The prolongation of the $(MEPPs)_O$ persists for longer than the inhibitory action of the anti-ChE suggesting the presence of some other cause of prolonged ACh action. This may have been caused by the downregulation of AChE within the synaptic cleft as a consequence of anti-ChE action.

4 Reducing the temperature at which $(MEPPs)_O$ were recorded caused an increase in the rising and decay phases of the $(MEPP)_O$. Addition of an anti-ChE made the increase in the decay phase more prolonged and a reduction in the temperature also increased the time taken for the anti-ChE to dissociate from the enzyme.

5 Addition of an anti-Che even at very small doses caused a deformation of the endplate. This deformation was possibly due to accumulation of Ca^{2+} in the fibre underlying the endplate causing a localised contraction. The deformation persists for a long time after anti-ChE inhibition has been removed possibly due to the time taken for Ca^{2+} to be removed from the fibre.

6 Neuromuscular jitter was increased after dosing with pyridostigmine at a myopathic dose, repetitive dosing of a "sign-free" dose, and continuous dosing of a very small dose. It was also seen after continuous administration of a very small dose of physostigmine. It was not thought that this increase in jitter was directly related to the extent of AChE inhibition. If it is not a directly related to AChE inhibition it must be due to some other change in the synapse caused as a consequence of AChE inhibition.

7 Jitter was found to occur only in fibres treated with an anti-ChE when these were stimulated at 30Hz suggesting a functional cause of jitter onset

8 Continuous administration of physostigmine was found to give better protection from the effects of ecothiopsate than did pyridostigmine even though these caused a similar amount of AChE inhibition. This suggested that the protective abilites of carbamates may not only be due to their capabilities as AChE inhibitors.

APPENDICES

Appendix 1

Composition of Lileys physiological saline (pH 7.4) modified by Krnjevic and Miledi (1958)

Substance	Concentration (nM)
NaCl	137
KCl	5
$CaCl_2$	2
$MgCl_2$	1
NaH_2PO_4	1
$NaHCO_3$	12
Glucose	25

Gassed with 5% CO_2 in O_2.

$CaCl_2$ was added as 1.0M $CaCl_2$ solution (2ml per litre). This solution is referred to as Lileys saline throughout the text.

Appendix 2

Preparation of the cholinesterase stain pH6.0 (Karnovsky and Roots, 1964)

1. Mix:-	0.06N	Sodium acetate	15.8ml
	0.1N	Acetic Acid	0.5ml
	0.1N	Sodium Citrate	3.6ml

2. Add 12.5mg acetylthiocholine iodide and dissolve.

3. Add:-	30mM	Copper Sulphate	2.5ml
	5mM	Potassium Ferricyanide	2.5ml

Appendix 3

3.1 Mann - Whitney test

This test is used when the data has not been shown to be normal i.e. it is said to be non parametric. The test assumes that the samples tested are independent, the scores in each group are drawn at random and that they are rankable.

The null hypothesis is that the two samples are drawn from two populations with the same distribution characteristics. This is tested by using the equation below;

$$R' = n1(n1+n2+1)-R$$

where n1 is the number of values in group 1, n2 is the number of values in group 2 and R is the sum of the ranks in the smaller group, which is always group 1.

To determine the significance of the difference between the two populations under consideration the smaller value R or R' can be compared to the values of R given in the tables for the appropriate number of values in each group. If the calculated level of R or R' is equal or less than the value of R given in the table at the 5% confidence level then it is unlikely that the null hypothesis would be true, i.e. the two populations from which the samples were drawn have significantly different distribution characteristics.

A significant result indicates a difference in the medians of the populations sampled.

If the sample is large the equation below must be used

$$z = \frac{n1(n1+n2+1)-2R}{\sqrt{\frac{n1n2\,(n1+n2+1)}{3}}}$$

The significance of z can be assessed with reference to the appropriate statistical table. If the value of z is equal to or greater than that in the table at the 5% confidence level it suggests that the null hypothesis is untrue.

3.2 Kolmogorov - Smirnov (K-S) two sample test.

This test is used for data when there appears to be a number of distinct populations from which a particular sample can be drawn. The test assumes that the two samples are independent, the scores within each sample are drawn at random and that the ordering of the samples is not arbitrary.

The null hypothesis is that the two samples have the same distribution characteristics.A number of ranked categories are asssigned within which a particular score may fall and the two samples are then summarised in terms of the cumulative frequencies (C) in each of these categories. Each C is converted to a cumulative proportion (CP) by dividing by the appropriate sample size, n1 or n2. The sum of the CPs for both samples should be 1.0. The difference between the CPs for both samples in each category is calculated and the category with the largest difference, irrespective of sign, is assigned the letter D. K is calculated using the equation below

$$K = D\sqrt{\frac{n1n2}{n1 + n2}}$$

The significance of K can be assessed with reference to the appropriate table. If the value of K is equal to or greater than that found in the table at the 5% confidence level it suggests that the null hypothesis is untrue.

3.3 Spearman's Rank Correlation Coefficient

This test is used to measure the relationship between two samples when it has not been shown that the data has a normal distribution, i.e. it is non parametric. It assumes that the samples are independent, drawn at random and rankable.

The two samples are first ranked and then the difference in the ranking for each pair calculted. For tied ranks, both places are given the average rank of the equal values. The null hypothesis is that the correlation between the samples is zero. The equation used to calculate r_s is shown below

$$r_s = 1 - \frac{6\Sigma d^2}{n(n^2-1)}$$

where d^2 is the square of the difference in the rankings for each pair and n is the number of items in the samples.

$r_s = 1$ for perfect positive correlation

$r_s = 0$ for no correlation

$r_s = -1$ for perfect negative correlation

The significance of r_s can be tested by substituting the value of r_s into the equation below

$$t = \frac{r_s\sqrt{n-2}}{\sqrt{1-r_s^2}}$$

The significance of t can be assessed with reference to the appropriate table. If the value of t is equal to or greater than that found in the table at the 5% confidence level it suggests that the null hypothesis is untrue.

Appendix 4

4.1 Preparation of diaphragm for ChE activity determination

The hemidiaphragm was pinned out onto Sylgard by the ribs and central tendon. Connective and adipose tissue was cut away, the phrenic nerve was trimmed close to the muscle and excess blood was removed by gently squeezing the blood vessels. The central tendon and ribs were removed and the central strip, which contained virtually all the endplates, was cut 3mm wide and was designated the junctional region (J). The remainder of the hemidiaphragm which consisted of 2 strips adjacent to the central region was designated the non - junctional region (NJ).

In the following appendices any enzyme extracted from the central region is referred to as junctional, any extracted from the remainder of the hemidiaphragm is referred to as non - junctional and endplate specific refers to the activity of the junctional region minus that of the non - junctional region as this difference in activity is attributed to the endplates themselves which are not present in the non junctional region.

4.2 Extraction of AChE isoforms

The sequential extraction method of Younkin *et al* 1982 is summarised in Figure A1, and was the chosen method chosen by A. Rowbotham for the extraction of the AChE isoforms. In the following appendices globular refers to the activity of S1 and S2 added together, asymmetric refers to the S3 and S4 fraction activities added together and non - extractable refers to the activity of the H5 fraction

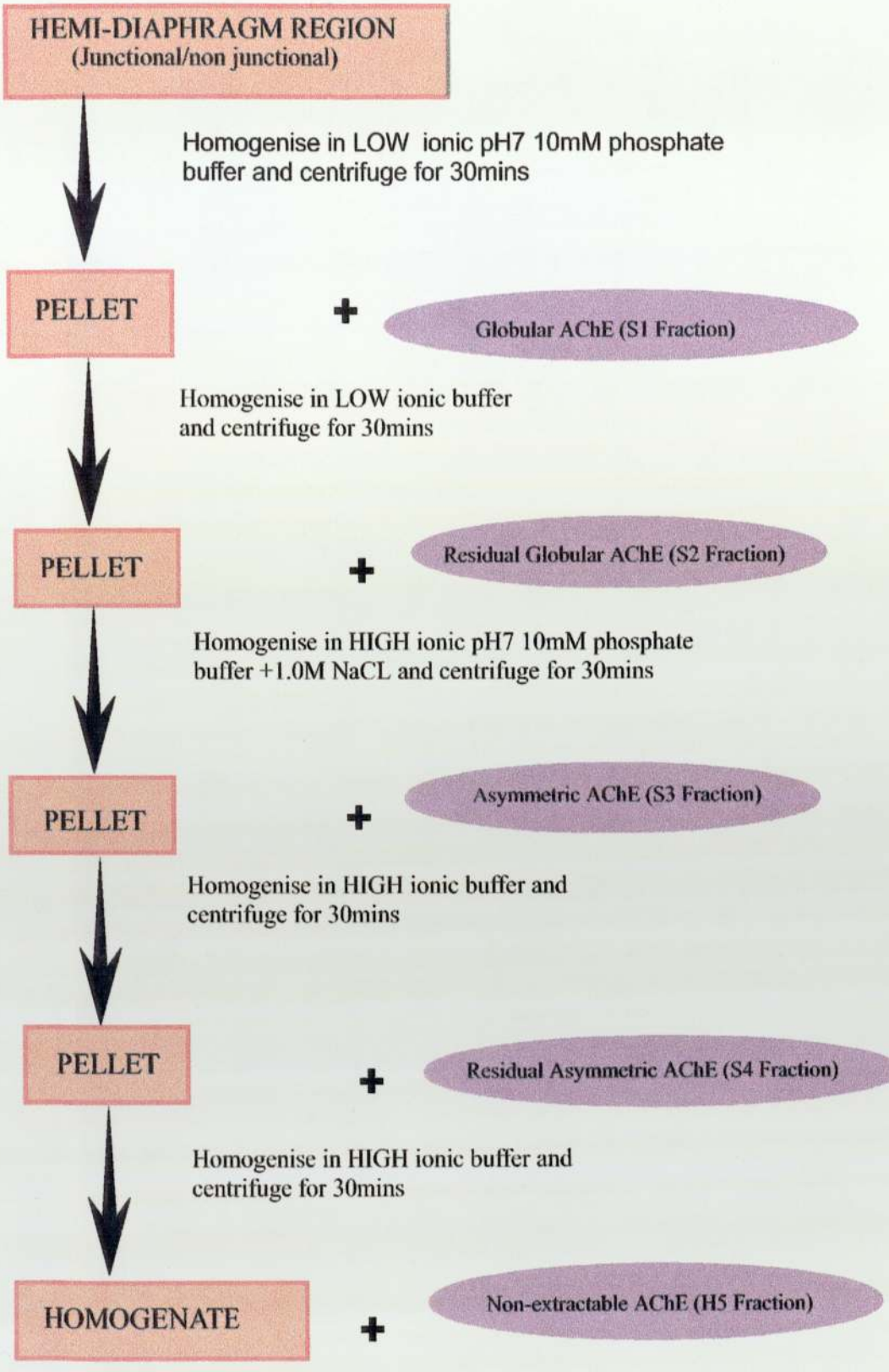


Figure A.1: Sequential extraction of the molecular forms of AChE.
Schematic representation of the sequential extraction technique for the separation of low and high molecular weight forms of AChE on the basis of their differential solubility in low ionic pH7.0, 10mM phosphate buffer and high pH7.0 10mM phosphate buffer + 1.0M NaCl based on the method of Younkin et al., 1982.

4.3 Determination of AChE activity

The actual activity of the AChE isoform was determined using the spectrophotometric enzyme assay technique of Ellman *et al* (1961). This method follows the rate of production of thiocholine, a product of AChE hydrolysis, as it reacts with 5,5,-dithiobis-2-nitrobenzoate (DTNB) giving yellow coloured 5-thiol-2-nitrobenzoic acid. This product absorbs UV light at 412nm, therefore the rate of change of absorbance at this wavelength can be linked to enzyme activity.

ACETYLTHIOCHOLINE ----------< THIOCHOLINE + ACETATE

THIOCOLINE +DTNB ----------< YELLOW COLOUR

Appendix 5

	Control	25 nmol	50 nmol	100 nmol	300 nmol	500 nmol
Junctional Globular	3.46 ±0.68	2.71 ±0.63	2.82* ±0.28	2.41* ±0.27	2.06* ±0.36	1.67* ±0.83
Junctional Asymmetric	1.32 ±0.25	0.86* ±0.26	1.05* ±0.21	0.69* ±0.09	0.61* ±0.12	0.70* ±0.25
Junctional Non Extractable	0.80 ±0.20	0.49 ±0.31	0.52 ±0.25	0.39* ±0.10	0.11* ±0.08	0.18* ±0.11
Non-Junctional Globular	1.96 ±0.40	1.54* ±0.27	1.50* ±0.31	1.56* ±0.26	1.28* ±0.24	1.04* ±0.04
Non-Junctional Asymmetric	0.77 ±0.26	0.22 ±0.12	0.39 ±0.19	0.33* ±0.34	0.15* ±0.11	0.40* ±0.21
Non-Junctional Non Extractable	0.29 ±0.15	0.01 ±0.01	0.12 ±0.16	0.08* ±0.09	0.05* ±0.07	0.14 ±0.14
Endplate Specific Globular	1.57 ±0.61	1.17 ±0.63	0.32 ±0.18	0.85 ±0.48	0.78* ±0.16	0.63* ±0.50
Endplate Specific Asymmetric	0.66 ±0.26	0.64 ±0.14	0.67 ±0.14	0.57 ±0.12	0.45 ±0.22	0.31* ±0.17
Endplate Specific Non Extractable	0.50 ±0.20	0.48 ±0.31	0.41 ±0.20	0.32 ±0.15	0.09* ±0.26	0.05* ±0.04

The effect of ecothiopate ($nmolkg^{-1}$) on AChE molecular isoforms extracted from different regions of the diaphragm. The values shown are the activity measured in nmol/min/mg ± s.d. Those which differ significantly from controls are shown by * (Mann-Whitney $p<0.05$) (Data Courtesy of A. Rowbotham)

Appendix 6

	Control	3 hours	1 day	5 days	7 days
Junctional Globular	3.46 ±0.68	1.67* ±0.83	1.73* ±0.31	3.30 ±0.61	3.34 ±0.55
Junctional Asymmetric	1.32 ±0.25	0.70* ±0.25	0.63* ±0.19	1.05 ±0.37	0.97 ±0.34
Junctional Non Extractable	0.80 ±0.20	0.18* ±0.11	0.27* ±0.13	0.51* ±0.20	0.61 ±0.27
Non-Junctional Globular	1.96 ±0.40	1.04* ±0.41	1.23* ±0.31	1.95 ±0.26	1.94 ±0.37
Non-Junctional Asymmetric	0.77 ±0.26	0.40* ±0.21	0.39* ±0.17	0.65 ±0.21	0.42* ±0.12
Non-Junctional Non Extractable	0.29 ±0.15	0.14 ±0.14	0.16 ±0.06	0.25 ±0.12	0.10 ±0.17
Endplate Specific Globular	1.57 ±0.61	0.63* ±0.50	0.50* ±0.21	1.36 ±0.70	1.52 ±0.34
Endplate Specific Asymmetric	0.66 ±0.26	0.51* ±0.17	0.24* ±0.20	0.40 ±0.26	0.54 ±0.28
Endplate Specific Non Extractable	0.50 ±0.20	0.05* ±0.04	0.12* ±0.12	0.26* ±0.14	0.48 ±0.23

The effect of ecothiopate (500 $nmolkg^{-1}$) on AChE molecular isoforms extracted from different regions of the diaphragm at various times after dosing. The values shown are the activity measured in nmol/min/mg ± s.d. Those which differ significantly from controls are shown by * (Mann-Whitney $p<0.05$) (Data Courtesy of A. Rowbotham)

Appendix 7

Period of Dosing	Activity (nmol/min/mg)
Saline	0.50±0.20
1 day	0.11±0.48
2 days	0.03±0.40
4 days	0.11±0.07 *
7 days	0.30±0.17 *
14 days	0.47±0.26
2 days recovery	0.39±0.44
7 days recovery	0.16±0.40
14 days recovery	0.91±0.46

The effect of continuous administration of pyridostigmine (11.4 nmol hr-1) on the activity of endplate specific non-extractable, i.e. functional enzyme. The values shown are the mean ± s.d. * indicates a significant difference from saline treated preparations. Data courtesy of Mrs A. Rowbotham.

Appendix 8

Period of Dosing	Activity (nmol/min/mg)
Saline	0.50±0.20
1 day	0.40±0.14
2 days	0.32±0.11
4 days	0.21±0.19 *
7 days	0.38±0.31
14 days	0.60±0.60
2 days recovery	0.14±0.24 *
7 days recovery	0.26±0.15
14 days recovery	0.69±0.38

The effect of continuous administration of physostigmine (14 nmol hr-1) on the activity of endplate specific non-extractable, i.e. functional enzyme. The values shown are the mean ± s.d. * indicates a significant difference from saline treated preparations. Data courtesy of Mrs A. Rowbotham.

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