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Twitch potentiation by organophosphate anticholinesterases in rat phrenic nerve diaphragm preparations

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1 Twitch potentiation produced by anticholinesterases has been variously attributed to the prolonged postjunctional action of acetylcholine (ACh), a prejunctional action of ACh involving the initiation of antidromic firing (ADF) in the nerve or a direct action of the anticholinesterases on nerve terminals initiating ADF.

2 The organophosphate anticholinesterases, paraoxon (diethyl-4-nitrophenylphosphate) and DFP (di*iso*propyl fluorophosphate), when applied to rat isolated diaphragm preparations for 30 min, produced twitch potentiation which subsequently declined.

3 The rates of onset and decline of twitch potentiation were directly related to the concentration of the organophosphates and the reversibility of their effects was in line with the reactivation of the phosphorylated enzymes formed by them, whether reactivation was spontaneous or induced by the oxime, N,N'-trimethylene-1, 3-*bis* (pyridinium-4-aldoxime).

4 Reducing the output of ACh from nerve terminals (by reducing the ratio of calcium: magnesium ions in the bathing solution) or reducing the affinity of ACh for the nicotinic cholinoceptor (using the disulphide bond reducing agent, dithiothreitol) produced the same effects as did lowering the concentration of the organophosphates.

5 It is concluded that the twitch potentiation produced by paraoxon and DFP, and its failure to be maintained when the higher concentrations of the organophosphates were used, were the direct result of the excess of ACh in the synaptic cleft, following inhibition of acetylcholinesterase.

Introduction

In the presence of an anticholinesterase, the response of mammalian striated muscle to low frequency, supramaximal, indirect stimulation is potentiated because each nerve stimulus triggers repetitive muscle action potentials (muscle APs) (Brown, 1937). Studies of the molecular basis of this phenomenon, the so-called twitch potentiation, have led individual authors to different conclusions (see Hobbiger, 1976). Masland & Wigton (1940) observed that twitch potentiation was associated with repetitive antidromic action potentials (antidromic firing, ADF) in the motor nerve. Since an antidromic impulse originating in an individual nerve terminal will be conducted also to the other terminals of the same neurone, the initiation of ADF following a single orthodromic impulse could explain twitch potentiation. Masland & Wigton (1940) observed that when acetylcholine (ACh) was injected intra-arterially into cat hind limbs there followed a burst of antidromic action potentials recorded from the ventral root. They thus attributed anticholinesterase-induced ADF to an action of ACh on nerve terminals as the result of inhibition of acetylcholinesterase (AChE) which normally limits the diffusion of ACh and thus protects nerve terminals from its action. Eccles, Katz & Kuffler (1942) on the other hand observed that repetitive muscle APs could occur in the absence of recordable ADF and they concluded that the repetitive muscle response following a single nerve stimulus might be triggered by the anticholinesterase-induced prolongation of the end-plate potential (e.p.p.). Riker and his co-workers (Werner & Kuperman, 1963; Riker & Okamoto, 1969) questioned the role of ACh in twitch potentiation altogether and proposed that anticholinesterases produced twitch potentiation by a direct action on nerve terminals which in turn triggered ADF.

To obtain further information on the role of ACh

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in anticholinesterase-induced twitch potentiation, we have carried out a study of the twitch potentiation produced by the organophosphate anticholinesterases paraoxon (diethyl-4-nitrophenylphosphate) and DFP (di*iso*propyl fluorophosphate) in rat phrenic nerve diaphragm preparations. Since the diethylphosphoryl-AChE formed by paraoxon undergoes slow spontaneous reactivation, while the di*iso*propylphosphoryl-AChE formed by DFP does not (see Hobbiger, 1963), twitch potentiation, if a function of inhibition of AChE, should show corresponding characteristics.

In this paper, we report the results of such studies, together with observations on the consequences of modification of the stimulus-induced release of ACh from nerve terminals and of the reduction in the affinity of ACh for the nicotinic cholinoceptor. The former was accomplished by varying the ratio of calcium : magnesium ions in the bathing solution, which is known to alter the output of ACh from nerve terminals (Ginsborg & Jenkinson, 1976; Rahamimoff, 1976). The latter effect was achieved by use of the disulphide bond reducing agent, dithiothreitol (DTT), which has been shown to reduce the affinity of ACh for nicotinic cholinoceptors in the frog rectus abdominus (Bleehen, Clark & Hobbiger, 1979) and chick biventer (Rang & Ritter, 1971) preparations.

A preliminary account of this work has been presented to the British Pharmacological Society (Clark & Hobbiger, 1979).

Methods

Rat diaphragm preparations

Left or right hemidiaphragm preparations, obtained from male Sprague-Dawley rats, weighing 150–250 g, were suspended in an organ bath (40 ml capacity) filled with one of three types of Tyrode solution (Tyrode A, B or C). All solutions contained (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 12.0 and glucose 11, together with the following concentrations (mM) of calcium and magnesium ions: Tyrode A: $CaCl_2$ 2.0, $MgCl_2$ 0.1; Tyrode B: $CaCl_2$ 1.4, $MgCl_2$ 1.0; Tyrode C: $CaCl_2$ 1.0, $MgCl_2$ 1.0. The temperature of the organ bath was maintained at 37°C and the bathing solution gassed with O_2 containing 5% CO_2.

The central tendon of the muscle was connected by a thread either to a spring-loaded lever, using a 15 to 18 fold magnification and writing on a smoked kymograph drum, or to a Grass FT-10C force-displacement transducer, coupled to a Devices model M4 or a Grass model 7D polygraph. The latter type of recording was used in the experiments in which responses were recorded in the different Tyrode solutions (A, B and C). In all the other experiments, the former recording system only was used. All muscles were subjected to a resting tension of 10 g. Drug solutions were added in small volumes (0.1 to 1.0 ml) to the bathing solution for a period of 30 min unless otherwise stated and concentrations given in the text are final concentrations in the organ bath.

The phrenic nerve was stimulated via circular electrodes with a Farnell physiological stimulator (stimulation parameters: 0.2 Hz frequency, 50 or 200 μs pulse duration, supramaximal voltage).

Drugs

The following drugs were used: diethyl-4-nitrophenylphosphate (paraoxon, Koch-Light), di*iso*propyl fluorophosphate (DFP, Sigma), dithiothreitol (DTT, Sigma) and N,N′-trimethylene-1,3-*bis*(pyridinium-4-aldoxime) bromide (TMB-4, Flucka).

Statistical analysis

For comparing two groups of data, an unpaired two-tailed Student's *t* test was used and for more than two

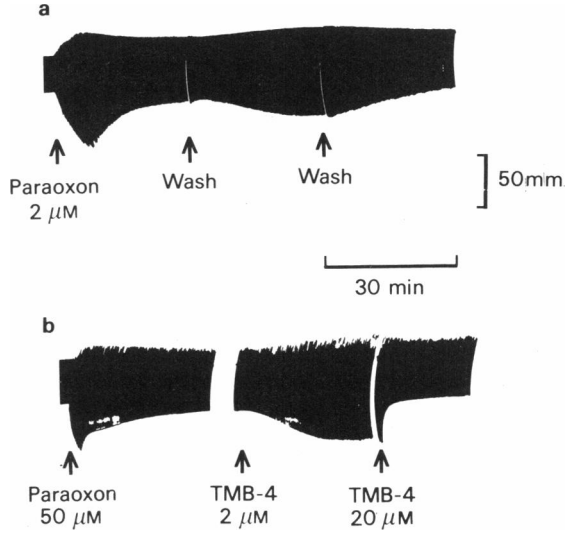

Figure 1 Typical effect of paraoxon on twitch height in rat diaphragm preparations. Contractions of the muscle were recorded with a spring-loaded lever with a downward sweep. (a) Paraoxon (2 μM) was applied for 30 min in Tyrode B solution. The preparation was then washed with fresh Tyrode B solution, and washed again 30 min later. (b) Paraoxon (50 μM) was applied for 30 min in Tyrode B solution. The preparation was then washed with fresh Tyrode B solution and TMB-4 (2 μM) was added to the organ bath; 30 min later the preparation was washed again and TMB-4 (20 μM) was added to the organ bath for a further 30 min period.

Table 1 Effect of paraoxon and di*iso*propyl fluorophosphate (DFP) on twitch height in the rat diaphragm preparation

Paraoxon conc (μM)	*Twitch height**				n
	Peak effect	*After 30 min*	*30 min after first wash*	*30 min after second wash*	
2	3.2±0.2	1.9±0.2	2.4±0.3	1.9±0.3	3
50	3.2±0.4	1.5±0.3	2.5±0.3	2.7±0.3	4
			30 min after TMB-4 (2 μM)	*30 min after TMB-4* (20 μM)	
2	3.8±0.2	2.5±0.2	1.9±0.1	0.7±0.1	3
50	3.3±0.6	1.2±0.2	2.5±0.2	1.4±0.2	3
DFP conc (μM)	*Twitch height**				n
	Peak effect	*After 30 min*	*30 min after first wash*	*30 min after second wash*	
10	3.9±0.2	2.3±0.2	2.3±0.3	2.1±0.3	4
250	4.9±0.4	2.2±0.2	2.0±0.2	2.0±0.1	4
			30 min after TMB-4 (2 μM)	*30 min after TMB-4* (20 μM)	
10	4.1±0.4	2.9±0.2	1.6±0.1	0 8±0.1	4
250	3.4±0.5	1.6±0.3	1.6±0.1	0.8±0.1	3

*Twitch height is expressed as a multiple or fraction of that recorded at the time of addition of paraoxon or DFP to the organ bath which is taken as 1. Paraoxon or DFP was added to the organ bath for 30 min, the bath fluid (Tyrode B) was then changed and changed again 30 min later.
Figures are mean ± s.e.mean, n = number of experiments.

groups of data, a one way analysis of variance was used. If the probability of the sample means belonging to the same population was less than 5% ($P < 0.05$) the difference between them was deemed to be statistically significant.

Results

Effect of paraoxon on twitch height

With preparations bathed in Tyrode B solution, the addition (for 30 min) of paraoxon (0.5 to 50 μM) to the organ bath increased the response of the muscle to indirect supramaximal stimulation at 0.2 Hz to 3 to 5 times control twitch height. The time taken for maximal twitch potentiation decreased as the concentration of paraoxon was increased. Subsequent to potentiation reaching a peak, it declined. The decline was most pronounced with the higher concentrations of paraoxon. Following removal of paraoxon from the organ bath, the decline in twitch potentiation was initially slowly reversed, the rate of this reversal being inversely related to the concentration of paraoxon. A further wash, 30 min later produced a steady decline in twitch height towards control levels (Figure 1).

Addition of TMB-4 (2 μM) to the organ bath accelerated the rate of recovery, which in the case of higher concentrations of paraoxon again included a transient increase in twitch potentiation.

When a concentration of 20 μM TMB-4 was used, twitch potentiation was very rapidly abolished, but this effect was not fully maintained when TMB-4 was added to the organ bath for only a few minutes (Figure 1).

These results are summarized in Table 1.

Effect of diisopropyl fluorophosphate on twitch height

With preparations bathed in Tyrode B solution, the addition (for 30 min) of DFP (10 and 250 μM) to the organ bath increased twitch height to 3 to 5 times control levels. As with paraoxon, the rate of onset of twitch potentiation was concentration-related, and having reached a peak, a decline of twitch potentiation occurred, which was more pronounced with the higher concentration of DFP.

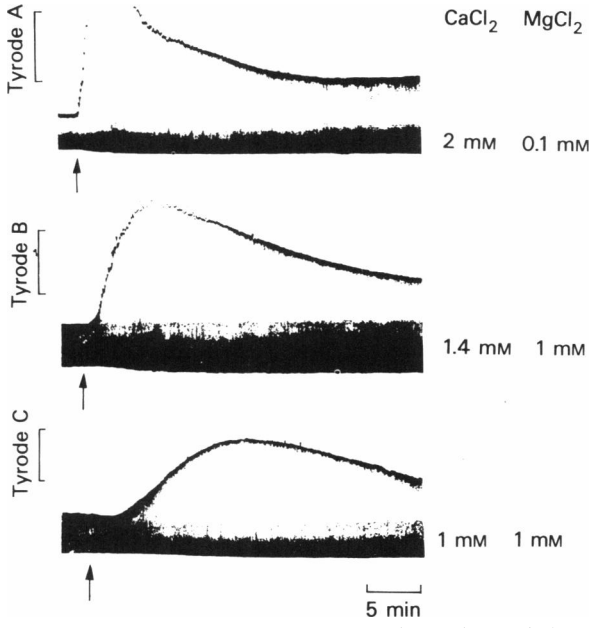

Figure 2 Typical effect of paraoxon (0.5 μM) on twitch tension in rat diaphragm preparations bathed in Tyrode solutions containing different concentrations of calcium and magnesium ions. Concentrations of $CaCl_2$ and $MgCl_2$ in the different Tyrode solutions (Tyrode A, B and C) are given on the right. Paraoxon was added where indicated by the arrow and left in contact with the preparation for 30 min. Contractions of the muscle were recorded with a force-displacement transducer producing an upward deflection of the pen. Vertical bars represent 20 g tensions.

In contrast to the results obtained in experiments with paraoxon, little or no recovery of twitch height was seen subsequent to removal of DFP from the organ bath. The oxime TMB-4 (2 and 20 μM) returned twitch height towards control levels and, as was the case in the experiments with paraoxon, the effect of the higher concentration of TMB-4 was not maintained if the oxime was removed from the organ bath a few minutes after its addition to it.

These results are also summarized in Table 1.

Effect of changing the ratio of calcium: magnesium ions in the bathing solution on the action of paraoxon

Changing the concentrations of calcium and magnesium ions in the bathing solution altered the response to paraoxon. For a given concentration of paraoxon (0.5 or 2 μM) increasing the ratio of calcium: magnesium ions relative to that in Tyrode B solution increased the rate at which twitch potentiation developed and enhanced its subsequent decline. Decreasing the ratio of calcium: magnesium ions had the reverse effect (Figure 2).

When the peak twitch potentiations produced by a given concentration of paraoxon (0.5 or 2 μM) were compared for preparations bathed in Tyrode solutions A, B and C, the difference was found not to be statistically significant ($P > 0.05$) (Table 2).

Effect of dithiothreitol treatment on the actions of paraoxon and diisopropyl fluorophosphate

Addition of DTT (1 mM) to the organ bath caused a gradual reduction in twitch height which, over 30 min, amounted to 18.5 ± 1.8% ($n = 33$). This reduction was found to be significantly greater ($P < 0.01$) than that observed in untreated preparations over the same period (13.0 ± 2.0%, $n = 25$).

In preparations bathed in Tyrode B solution and treated with DTT (1 mM) for 30 min, on subsequent addition of paraoxon (0.5 to 50 μM) the rate of onset of twitch potentiation was delayed and any subsequent decline of the twitch potentiation was abolished or greatly reduced (Figure 3).

Peak twitch potentiation produced by paraoxon (2

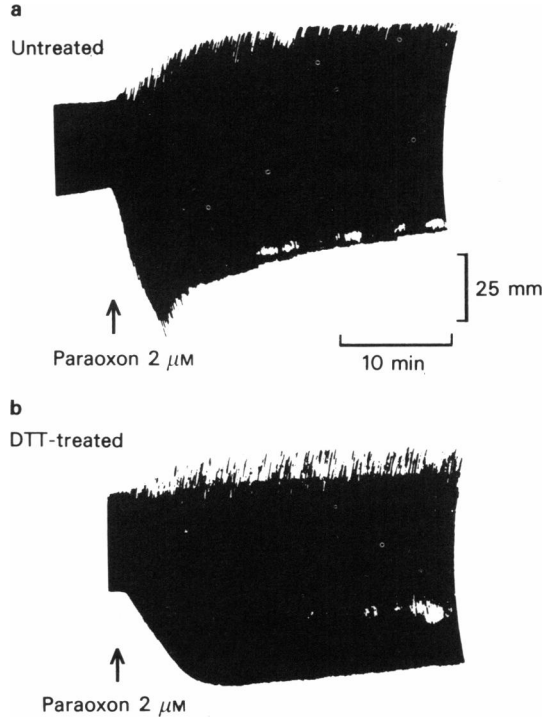

Figure 3 Typical effect of paraoxon on twitch height in rat diaphragm preparations. Contractions of the muscle were recorded with a spring-loaded lever with a downward sweep. (a) Paraoxon (2 μM) was applied for 30 min in Tyrode B solution. (b) The preparation was treated with dithiothreitol (DTT, 1 mM) for 30 min then washed with fresh Tyrode B solution and paraoxon (2 μM) was then applied for 30 min in Tyrode B solution.

Table 2 Effect of changing the ratio of calcium:magnesium ions in the bathing solution on the actions of paraoxon in rat diaphragm preparations, with and without dithiothreitol (DTT) treatment

Paraoxon conc (μM)		$CaCl_2$ $MgCl_2$	*Peak twitch height** *Tyrode A* 2 mM 0.1 mM	*Tyrode B* 1.4 mM 1 mM	*Tyrode C* 1 mM 1 mM	P†
0.5	Untreated		3.8±0.4(5)	4.1±0.4(3)	4.2±0.4(4)	>0.05(NS)
	DTT-treated		4.3±0.2(5)	2.9±0.3(3)	2.1±0.2(4)	<0.001
2	Untreated		4.0±0.4(3)	3.7±0.2(8)	3.7±0.1(3)	>0.05(NS)
	DTT-treated		4.4±0.2(3)	2.8±0.2(9)	2.2±0.2(3)	<0.001

*Peak twitch height produced by paraoxon (0.5 or 2 μM) was measured in untreated muscles and muscles incubated with DDT (1 mM) for 30 min prior to addition of paraoxon (DTT-treated), and bathed in one of three different Tyrode solutions (Tyrode A, B or C). Peak twitch height is expressed as a multiple of that observed at the time of addition of paraoxon to the organ bath, which is taken as 1.
Figures are mean ± s.e.mean (*n* experiments).
†*P* is the significance of the difference between peak twitch height obtained with a given concentration of paraoxon in the three different Tyrode solutions (Tyrode A, B and C) containing different concentrations of calcium and magnesium ions.

Table 3 Effect of dithiothreitol (DTT) (1 mM for 30 min) on the actions of paraoxon and di*iso*propyl fluorophosphate (DFP) in the rat diaphragm preparation

Paraoxon conc (μM)	*Twitch height** *Peak effect*	*After 30 min*	*30 min after first wash*	*30 min after second wash*	n
2	2.8±0.3	2.7±0.3	2.4±0.4	2.2±0.2	4
50	2.1±0.1	1.8±0.1	1.9±0.1	1.8±0.1	4
			30 min after TMB-4 (2 μM)	*30 min after TMB-4* (20 μM)	
2	2.6±0.3	2.5±0.3	1.5±0.3	0.9±0.1	3
50	2.6±0.1	2.2±0.1	2.1±0.1	1.0±0.1	4

DFP conc (μM)	*Twitch height** *Peak effect*	*After 30 min*	*30 min after first wash*	*30 min after second wash*	n
10	3.0±0.2	2.9±0.3	2.8±0.3	2.5±0.2	4
250	2.2±0.1	1.5±0.1	1.5±0.1	1.4±0.2	4
			30 min after TMB-4 (2 μM)	*30 min after TMB-4* (20 μM)	
10	2.8±0.3	2.8±0.4	1.0±0.1	0.9±0.1	4
250	2.6±0.3	1.8±0.2	1.2±0.2	0.9±0.1	4

*Twitch height is expressed as a multiple or fraction of that recorded at the time of addition of paraoxon or DFP to the organ bath, which is taken as 1.
DTT was removed from the organ bath before addition of the anticholinesterase. Paraoxon or DFP was then added to the organ bath for 30 min, the bath fluid (Tyrode B) was then changed and changed again 30 min later.
Figures are mean ± s.e.mean, *n* = number of experiments.

and 50 μM) was significantly reduced by DTT treatment ($P<0.005$ and $P<0.01$ for 2 and 50 μM, respectively) (see Tables 1 and 3).

On washing the preparation with fresh Tyrode B solution, there was no transient enhancement of twitch height as was seen in preparations not treated with DTT, but only a gradual decline towards pre-paraoxon levels. Addition of TMB-4 (2 and 20 μM), after removal of paraoxon from the organ bath, reversed the twitch potentiation more rapidly.

These results are summarized in Table 3.

In experiments with DFP, prior treatment of preparations with DTT (1 mM) for 30 min produced changes in the twitch potentiation which were comparable to those seen in the experiments with paraoxon.

These results are also summarized in Table 3.

Effect of changing the ratio of calcium: magnesium ions in the bathing solution on the action of paraoxon in dithiothreitol-treated preparations

In DTT-treated preparations, increasing and decreasing the ratio of calcium: magnesium ions relative to that in Tyrode B solution accelerated and reduced, respectively, the rate of onset of twitch potentiation produced by paraoxon. When the peak twitch potentiations produced by paraoxon (0.5 or 2 μM) were compared for preparations bathed in Tyrode solutions A, B, and C, the difference was found to be statistically significant ($P<0.001$ for 0.5 and 2 μM paraoxon). This is in contrast to the effect seen in preparations not treated with DTT.

These results are summarized in Table 2.

Discussion

The organophosphate anticholinesterases paraoxon and DFP, in the concentrations used, produced a biphasic response when added for 30 min to rat diaphragm preparations. The twitch response was initially enhanced and, having reached a peak, some decline towards control levels occurred. The rate of onset of twitch potentiation and its subsequent decline both increased with increasing concentrations of the anticholinesterases, i.e. as the rate and extent of inhibition of AChE increased.

The rate of spontaneous reactivation of the phosphorylated enzyme formed by paraoxon (diethylphosphoryl-AChE) is slow, in excess of 24 h being required for 50% reactivation (Burgen & Hobbiger, 1951; see Hobbiger, 1976). In spite of this, substantial reversal of the twitch potentiation occured within 1 h of removal of paraoxon from the organ bath, which, if a marked decline of twitch potentiation had occurred, involved a transient phase of further potentiation. Thus, as is the case with the tetanic fade produced by the organophosphate anticholinesterases (Berry & Lovatt-Evans, 1951; Barnes & Duff, 1953; Fleisher, Hansa, Killos & Harrison, 1960; Van der Meer & Wolthuis, 1965; Heffron & Hobbiger, 1979), small changes in the level of enzyme inhibition can produce marked changes in the muscle response to nerve stimulation.

No reversal of the effects of DFP on twitch height were seen following its removal from the organ bath. This is consistent with the lack of spontaneous reactivation of the phosphorylated enzyme formed by it (di*iso*propylphosphoryl-AChE) (see Hobbiger, 1976).

Reactivation of either type of phosphorylated enzyme with the oxime TMB-4 (2 μM) reversed the effects of both paraoxon and DFP and, when the decline of twitch potentiation was most pronounced (i.e. following 50 μM paraoxon), this reversal also included a transient period of twitch potentiation.

The higher concentration of TMB-4 (20 μM) produced a very rapid return of the twitch response to control height, which was not maintained if TMB-4 was added to the organ bath for less than 5 min. This probably is the consequence of a blocking action of the drug on postjunctional nicotinic cholinoceptors (Hobbiger & Sadler, 1959; Fleisher *et al.*, 1960) and comparable to the antagonism of twitch potentiation by tubocurarine (Brown, Dale & Feldberg, 1936).

Modification of the ACh output from nerve terminals by raising or lowering the ratio of calcium: magnesium ions in the bathing solution (Ginsborg & Jenkinson, 1976; Rahamimoff, 1976) produced effects corresponding to an increase or decrease, respectively in the concentration of the anticholinesterase.

These findings are consistent with the view that the actions of the organophosphate anticholinesterases in the rat diaphragm preparation are indeed a consequence of inhibition of AChE, and that the prolongation of the action of unhydrolysed ACh within the synaptic cleft is the primary event in the initiation of twitch potentiation and its subsequent decline.

To obtain information on how the prolonged action of ACh produces twitch potentiation, studies were carried out with DTT. DTT reduces the affinity of ACh for postjunctional nicotinic cholinoceptors to approximately one quarter (Rang & Ritter, 1971; Bleehen *et al.*, 1979). Consequently there is a reduction of the motor endplate response (the e.p.p.) both in the absence (Ben-Haim, Landau & Silman, 1973; Terrar, 1978) and presence (Terrar, 1976) of an anticholinesterase. The margin of safety for neuromuscular transmission in the rat diaphragm preparation is high and the e.p.p. can be reduced by 80 to 90% without impairment of the muscle response to single indirect stimuli (Boyd & Martin; 1956;

Galindo, 1972). This explains our finding that DTT (1 mM for 30 min) only marginally reduced the twitch response and also that of Mittag & Tormay (1970), who reported that DTT (2 mM) had no effect on twitch height.

The effect of DTT treatment on anticholinesterase-induced twitch potentiation and decline was similar to that expected from lowering the concentration of the organophosphate or from reducing the ACh output from nerve terminals, i.e. the rate of onset of twitch potentiation was delayed and the subsequent decline was reduced or abolished.

Spontaneous muscle fasciculations accompany twitch potentiation produced by paraoxon (Barnes & Duff, 1953) and DFP (Van der Meer & Meeter, 1956) in rat diaphragm preparations. These fasciculations are thought to be due to antidromic action potentials (ADF) being initiated at one nerve terminal and transmitted to other terminals of the same neurone, thus giving rise to repetitive action potentials in the muscle fibres contained within the same motor unit (Feng & Li, 1941; Webb & Bowman, 1974). We also observed fasciculations in our experiments, the time course of which varied according to the experimental conditions in line with the time course of the twitch potentiation. In preparations treated with DTT, fasciculations were reduced or absent. This indicates that in DTT-treated preparations no ADF is triggered. On the other hand, DTT only modified but did not abolish twitch potentiation. Thus a substantial degree of twitch potentiation appears to be possible in the absence of ADF. To substantiate this conclusion, measurements of ADF and the effects of DTT upon it are indicated.

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