Br. J. Pharmac. (1983), **80**, 489–496

Effects of pancuronium and hexamethonium on paraoxon-induced twitch potentiation and antidromic firing in rat phrenic nerve diaphragm preparations

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1 The actions of pancuronium, a selective antagonist of acetylcholine (ACh) at nicotinic cholinoceptors at motor endplates, and hexamethonium, a selective antagonist of ACh at nicotinic cholinoceptors in autonomic ganglia, have been studied in rat phrenic nerve diaphragm preparations. The effects on paraoxon-induced twitch potentiation and antidromic firing (ADF) in the phrenic nerve, were compared with the effects on normal twitch tension and intracellularly recorded miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps.)

2 In preparations exposed to paraoxon, pancuronium was found to be approximately 10 times more effective in reducing the potentiated component of the twitch than the component which corresponded to the pre-paraoxon twitch. A similar result was obtained with hexamethonium.

3 Pancuronium and hexamethonium, in concentrations which reduced paraoxon-induced twitch potentiation but had no effect on the twitch tension of preparations not treated with paraoxon, reduced paraoxon-induced ADF. The lowest concentrations of pancuronium and hexamethonium required for this also reduced the amplitude of m.e.p.ps and e.p.ps.

4 Dithiothreitol, a disulphide bond reducing agent which reduces the affinity of ACh for nicotinic cholinoceptors, enhanced the potency of pancuronium 2 to 3 fold. The same also applied for hexamethonium.

5 It is concluded that the experiments failed to provide evidence for an action of ACh on prejunctional nicotinic cholinoceptors of the ganglionic-type being involved in the initiation by paraoxon of twitch potentiation and ADF. Furthermore, the results obtained can be explained by pancuronium and hexamethonium reducing the action of ACh at the postjunctional membrane.

Introduction

Masland & Wigton (1940) and Feng & Li (1941) suggested that antidromic firing (ADF), which is associated with twitch potentiation by anticholinesterases, arises from an action of acetylcholine (ACh) on nerve terminals. It was subsequently proposed (Blaber & Karczmar, 1967; Webb & Bowman, 1974; Bowman, 1980a, b) that this action of ACh was mediated by cholinoceptors located either at the nerve terminal or at the first node of Ranvier. Binding studies in rats (Lentz, Mazurkiewicz & Rosenthal, 1977) and mice (Daniels & Vogel, 1975) have provided direct evidence for the existence of prejunctional nicotinic cholinoceptors at motor endplates.

Webb & Bowman (1974) suggested that the prejunctional cholinoceptors were of the ganglionic nicotinic type since in cat hind limb muscles exposed to neostigmine, hexamethonium, a selective antagonist of ACh in autonomic ganglia, could suppress ADF without affecting twitch potentiation or the normal twitch response. They also observed that pancuronium, a selective antagonist of ACh at nicotinic cholinoceptors at motor endplates, showed no such effects.

In an attempt to separate pre- and postjunctionally mediated actions of ACh, we have compared the effects of pancuronium and hexamethonium in rat phrenic nerve diaphragm preparations on (1) twitch tension, endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps) in the absence of paraoxon and (2) paraoxon-induced twitch potentiation, ADF and m.e.p.p.s in paraoxon-treated preparations. It has previously been observed (Clark, Hobbiger & Terrar, 1979; 1983) that dithiothreitol (DTT), a disulphide bond reducing agent which re-

duces the affinity of ACh for nicotinic cholinoceptors at motor endplates (Terrar, 1978), abolishes paraoxon-induced ADF but not twitch potentiation. This paper also includes data on the effects of pancuronium and hexamethonium in DTT-treated preparations.

A preliminary account of this work has been presented to the British Pharmacological Society (Clark & Hobbiger, 1981).

Methods

Left hemidiaphragms from adult, male, Sprague–Dawley rats were suspended in an organ bath containing Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 12, $CaCl_2$ 2, $MgCl_2$ 0.1, and glucose 11. The solution was gassed with oxygen containing 5% CO_2 and the temperature maintained at 37 ± 2°C.

Twitch tension

The phrenic nerve was stimulated via circular electrodes (stimulation parameters: 0.2 Hz frequency, 200 μs pulse duration, supramaximal voltage) and twitch responses of the muscle were recorded isometrically with a Grass FT-10C force-displacement transducer, coupled to a Grass model 7D polygraph.

For treatment of preparations with DTT, DTT (1 mM) was added to the organ bath for 15 min.

Twitch potentiation was produced by the anticholinesterase paraoxon (0.5 μM), added to the organ bath for 5 min (in preparations not treated with DTT) or 10 min (in DTT-treated preparations). Following removal of paraoxon from the organ bath a steady level of twitch potentiation was usually seen. In some cases a small decline of twitch potentiation preceded the steady level.

To assess the effects of pancuronium or hexamethonium on twitch tension, either in the absence of or following exposure to paraoxon, their concentrations in the organ bath were increased stepwise at 10 min intervals. This was sufficient for twitch tension to reach a steady level in the presence of an individual concentration of pancuronium or hexamethonium.

Antidromic firing

Rat hemidiaphragms were set up in a perspex bath with 2 chambers. One chamber contained the muscle for tension recording and the other contained the phrenic nerve for recording ADF (Clark *et al.*, 1979; 1983).

In the absence of paraoxon, a single stimulus applied to the nerve produced a single muscle twitch and a single antidromically conducted action potential (AP). In preparations exposed to paraoxon this AP was followed within 2 to 5 ms by a series of repetitive antidromic APs (ADF) which reached a peak within 10 to 15 ms after stimulation of the nerve. This peak voltage was used for quantitative assessment of ADF. In experiments in which, after removal of paraoxon from the organ bath, twitch potentiation was maintained at a steady level, the same applied to ADF. In experiments in which twitch potentiation showed some initial decline before reaching a steady level, ADF also showed the same behaviour.

Recordings from motor endplates

Rat diaphragm strip preparations, consisting of 2 to 6 mm wide strips of intact muscle fibres and their nerve supply, were set up in a perspex chamber and immobilised by stretch. Conventional intracellular microelectrodes were used to investigate postjunctional effects of pancuronium and hexamethonium (see Clark, Hobbiger & Terrar, 1980).

A reduction in the amplitude of m.e.p.ps was taken as the most straightforward indication of a postjunctional action; the mean amplitude was determined either from measurements by hand of 40 or more individual m.e.p.ps, or from the output of a Neurolog NL 750 averager after sampling 16 or more m.e.p.ps. Giant m.e.p.ps (Liley, 1957) were excluded from this analysis.

E.p.ps were recorded under two conditions. In some experiments a second current-passing microelectrode was inserted into the muscle fibre to depolarize the membrane sufficiently to abolish muscle APs, which would not be expected to occur at membrane potentials less negative than −55 mV where sodium currents would be inactivated (Adrian & Marshall, 1977; Pappone, 1980). In some cases the e.p.ps were recorded using a single electrode only if the muscle membrane potential was already in the depolarized range where e.p.ps could be observed without muscle APs. The most likely cause of depolarization is the formation of a low resistance pathway around the electrode and not a general deterioration of the preparation.

Two to 3 min exposures of preparations to pancuronium or hexamethonium were sufficient for a maximal effect on m.e.p.p. and e.p.p. amplitude to be reached, and the effects of these drugs were fully reversed within 3–4 min after their removal from the Tyrode solution.

Drugs

The following drugs were used: diethyl-4-nitrophenylphosphate (paraoxon, Koch-Light),

dithiothreitol (DTT, Sigma), hexamethonium bromide (Koch-Light), pancuronium bromide (Organon).

Statistical analysis

For comparing two groups of data, a paired or unpaired two-tailed Students *t* test was used. The probability level chosen was 5%.

Results

Effects of pancuronium and hexamethonium on normal twitch tension

In the absence of paraoxon, a stepwise increase in the concentration of pancuronium (2 to 8 μM) in the organ bath produced a concentration-related reduction in twitch tension, with a steep concentration-effect relationship. The effect of pancuronium was rapidly reversed on its removal from the organ bath. Comparable results were obtained with concentrations of hexamethonium between 2 and 10 mM.

In preparations treated with DTT (1 mM for 15 min) twitch tension was reduced to 82.9 ± 1.4% ($n = 16$) of its pre-DTT level. In these preparations, the effectiveness of pancuronium and hexamethonium in reducing twitch tension was increased as compared to that in preparations not treated with DTT.

Figure 1 shows the relationship between twitch tension (as a % of control) and log concentration of pancuronium or hexamethonium in control and DTT-treated preparations, and Table 1 gives IC_{50} values, i.e. the molar concentrations of pancuronium or hexamethonium which reduced twitch tension by 50%, derived from these data.

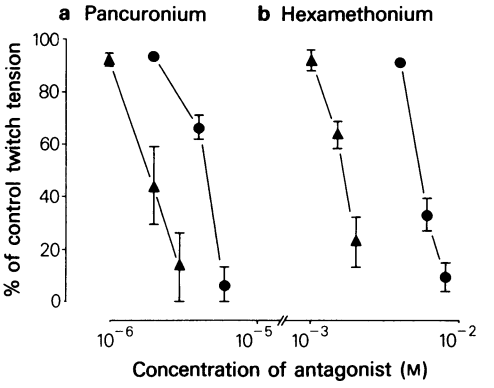

Figure 1 Effects of pancuronium and hexamethonium on twitch tension in rat diaphragm preparations not treated with dithiothreitol (DTT) (●) and pre-treated with DTT (1 mM for 15 min), (▲). Twitch tension, in the presence of a given concentration of (a) pancuronium or (b) hexamethonium is expressed as a % of the twitch tension prior to the addition of the drugs. Results are presented as means ± s.e.mean ($n = 4$).

Effects of pancuronium and hexamethonium on paraoxon-induced twitch potentiation

The addition of paraoxon (0.5 μM) to the organ bath for 5 min, produced a steady level of twitch potentiation which amounted to an increase in tension to 4.0 ± 0.1 ($n = 12$) times control (pre-paraoxon) tension. Pancuronium produced a concentration-related reduction of the potentiated twitch in concentrations below those which affected normal twitch tension. In preparations in which, following removal of paraoxon from the organ bath, twitch potentiation showed some decline before reaching a steady level, pancuronium initially caused a small increase in twitch

Table 1 Effects of pancuronium and hexamethonium on normal twitch tension and on paraoxon-induced twitch potentiation in rat diaphragm preparations, without or with initial dithiothreitol (DTT) treatment (1 mM for 15 min)

		Control twitch IC_{50}	*Paraoxon-potentiated twitch* IC_{50a}	IC_{50b}
Pancuronium	Untreated	4.2 ± 0.2 μM	1.4 ± 0.4 μM	12.3 ± 0.8 μM
	DTT-treated	1.9 ± 0.2 μM	0.6 ± 0.1 μM	6.1 ± 1.1 μM
Hexamethonium	Untreated	5.4 ± 0.2 mM	0.6 ± 0.1 mM	5.8 ± 0.3 mM
	DTT-treated	1.7 ± 0.1 mM	0.3 ± 0.1 mM	2.2 ± 0.3 mM

IC_{50} = molar concentration which reduced normal twitch tension by 50%.
IC_{50a} = molar concentration which, in paraoxon-treated preparations, reduced the potentiated component of the twitch tension by 50%.
IC_{50b} = molar concentration which, in paraoxon-treated preparations, reduced twitch tension to 50% of its pre-paraoxon level.
Individual values were obtained by plotting twitch tension (as a % of control) against log concentration of pancuronium or hexamethonium. The values are mean ± s.e.mean ($n = 4$).

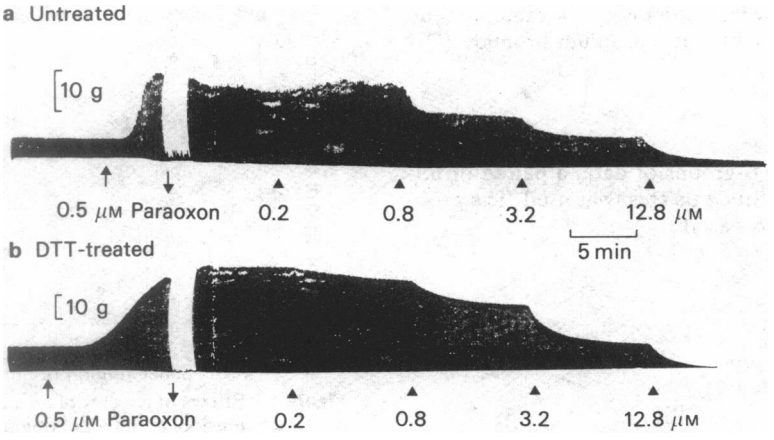

Figure 2 Effects of pancuronium on paraoxon-induced twitch potentiation in rat diaphragm preparations. Arrows indicate addition (↑) and removal (↓) of paraoxon (0.5 μM). Concentrations shown are the final concentrations in the organ bath. The effect of cumulative concentrations of pancuronium (▲) in a preparation not treated with dithiothreitol (DTT) is shown in (a). Whereas (b) shows the effect of cumulative concentrations of pancuronium (▲) in a preparation treated with DTT (1 mM for 15 min).

tension. Raising the concentration of pancuronium then again produced a concentration-related reduction in twitch tension. (Figure 2a).

The effects of hexamethonium were comparable to those of pancuronium (Figure 3a).

In preparations treated with DTT (1 mM for 15 min) addition of paraoxon (0.5 μM for 10 min) to the organ bath produced a peak twitch potentiation comparable to that seen in preparations not pretreated with DTT i.e. an increase in twitch tension to 4.1 ± 0.1 times control (pre–paraoxon) tension ($n = 12$). Following removal of paraoxon from the organ bath, the effectiveness of both pancuronium and hexamethonium in reducing paraoxon-induced twitch potentiation was enhanced relative to that obtained in preparations not treated with DTT. Again the reduction of twitch potentiation was concentration-related (Figures 2b and 3b).

For comparing the effects of pancuronium and hexamethonium, twitch tension (as a % of control) was plotted against log concentration of the drugs. From these plots IC_{50a} and IC_{50b} values were derived, where: IC_{50a} = the molar concentration which in paraoxon-treated preparations reduced the potentiated component of twitch tension by 50%; IC_{50b} = the molar concentration which, in paraoxon-treated preparations, reduced twitch tension to 50% of its control (pre-paraoxon) level. In other words, if

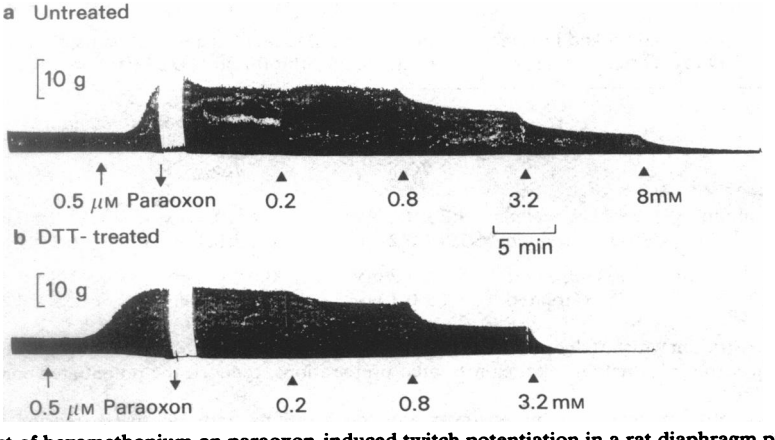

Figure 3 Effect of hexamethonium on paraoxon-induced twitch potentiation in a rat diaphragm preparation not treated with dithiothreitol (DTT) (a) and in preparation treated with DTT (1 mM for 15 min) prior to the addition of paraoxon to the organ bath (b). Other details as in Figure 2.

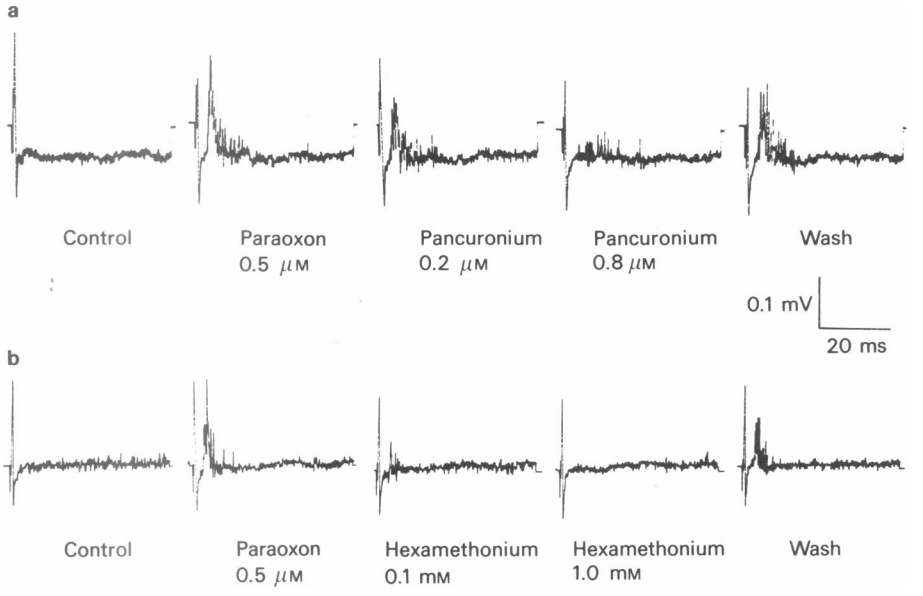

Figure 4 Effects of pancuronium and hexamethonium on paraoxon-induced ADF (recorded from the phrenic nerve) in rat diaphragm preparations. Records of electrical activity were obtained in the absence of drugs (control), following the addition of paraoxon (0.5 μM) for 5 min to the Tyrode solution, following the subsequent addition of two concentrations of pancuronium (a) or hexamethonium (b) and after removal of pancuronium or hexamethonium from the Tyrode solution (Wash). In all the traces, the large upward deflection at the beginning of the trace represents the antidromically conducted AP initiated by the stimulus applied to the nerve. This AP was too large to be accurately recorded at the level of amplification used.

twitch tension before paraoxon is x units, and that after is y units, then at IC_{50a} the tension is reduced to $x+(y-x)/2$, and at IC_{50b} it is reduced to x/2. The values obtained are shown in Table 1.

Effects of pancuronium and hexamethonium on paraoxon-induced ADF

Following removal of paraoxon (0.5 μM) from the Tyrode solution, preparations not treated with DTT and with a steady level of twitch potentiation also had a steady level of ADF with a mean peak amplitude of 218 ± 28 μV ($n=20$). Under these conditions pancuronium and hexamethonium produced a concentration-related reduction in ADF in the same range of concentrations as those which affected paraoxon-induced twitch potentiation (Figure 4).

In experiments in which, following removal of paraoxon from the Tyrode solution, twitch potentiation showed some transient decline before reaching a steady level, ADF behaved in the same way. In such cases, addition of pancuronium or hexamethonium to the Tyrode solution caused a slight enhancement of the twitch response, as described before, but consistently reduced ADF. In all cases ADF was restored by removal of pancuronium or hexamethonium from the Tyrode solution.

Effects of hexamethonium and pancuronium on endplate responses

When diaphragm strip preparations were exposed to hexamethonium or pancuronium, in concentrations which reduced paraoxon-induced ADF but had no effect on the twitch tension of preparations not treated with paraoxon, the mean amplitude of m.e.p.ps was reduced. In six muscle fibres exposed to hexamethonium (three in the absence of paraoxon, three treated with paraoxon) the mean amplitude was reduced to $65\pm2\%$ of the value before hexamethonium (2×10^{-4} M). Pancuronium (2×10^{-7} M) reduced the mean amplitude to $71\pm2\%$ in four muscle fibres (one in the absence of paraoxon, three treated with paraoxon). This observation is illustrated in Figure 5 which shows the average of 16 m.e.p.ps in the presence and absence of either hexamethonium or pancuronium.

In preparations in which the membrane potential at the motor endplate region was less negative than −55 mV (see Methods), a single stimulus to the

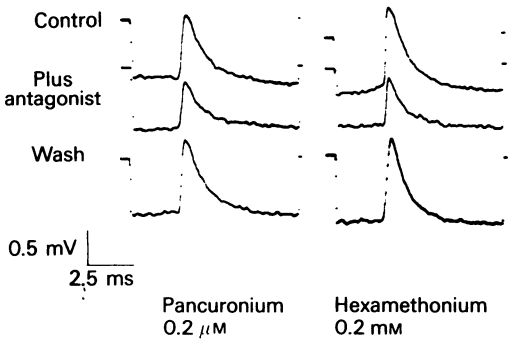

Figure 5 Effects of pancuronium and hexamethonium on intracellularly recorded m.e.p.ps in rat diaphragm strip preparations. Each record represents the average of 16 m.e.p.ps obtained using a Neurolog Averager (one sample point per 40 μs) in the absence of any drugs (Control), in the presence of pancuronium or hexamethonium and following their removal (Wash). The steady membrane potential was approximately − 55 mV and did not vary by more than 2 mV when pancuronium or hexamethonium were added to the Tyrode solution.

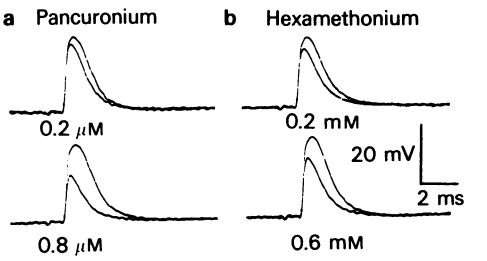

Figure 6 Effects of (a) pancuronium and (b) hexamethonium on intracellularly recorded e.p.ps in rat diaphragm strip preparations. In each panel, the upper trace is the control e.p.p. recorded in the absence of any drugs, and the lower trace is the e.p.p. recorded following addition of pancuronium or hexamethonium to the Tyrode solution. The steady membrane potential was approximately − 55 mV and did not vary by more than 2 mV when pancuronium or hexamethonium were added to the Tyrode solution.

nerve elicited an e.p.p. which no longer triggered a propagated muscle AP. Hexamethonium and pancuronium, again in concentrations which reduced ADF but had no effect on the twitch tension of preparations not treated with paraoxon, reduced the amplitude of the e.p.p. (Figure 6). This effect was concentration-related and was rapidly reversed following removal of the hexamethonium and pancuronium from the Tyrode solution.

Discussion

The observation that in cat hind limb muscles hexamethonium, a selective antagonist of ACh in autonomic ganglia, can suppress neostigmine-induced ADF without affecting its associated twitch potentiation, led Webb & Bowman (1974) to the conclusion that ganglionic-type nicotinic cholinoceptors at nerve terminals were involved in anticholinesterase-induced ADF. If anticholinesterase-induced ADF was indeed mediated by an action of ACh at prejunctional ganglionic-type nicotinic cholinoceptors, one would expect that hexamethonium would selectively antagonize the ADF without affecting the postjunctionally mediated actions of ACh. The results reported in this paper have shown that this was not the case in rat phrenic nerve diaphragm preparations exposed to paraoxon. Furthermore, pancuronium, a selective antagonist of ACh at nicotinic cholinoceptors at motor endplates, behaved in the same way but at lower concentrations. The ratio of the concentration of hexamethonium which in paroxon-treated preparations reduced twitch tension to 50% of its pre-paraoxon level (IC_{50b}) to that which reduced the potentiated component of twitch tension to 50% (IC_{50a}) was 9.8 ± 1.2 ($n = 4$). For pancuronium the ratio $IC_{50b}:IC_{50a}$ was 10.5 ± 2.0 ($n = 4$), which does not differ from the ratio for experiments with hexamethonium.

In DTT-treated preparations exposed to paraoxon in which ADF was absent, the potency of hexamethonium was not reduced, but like that of pancuronium was enhanced, and the values of the ratio $IC_{50b}:IC_{50a}$ were not significantly different from those obtained in preparations not treated with DTT. For hexamethonium the value was 7.8 ± 0.8, $n = 4$, and for pancuronium 10.5 ± 0.8, $n = 4$.

These findings suggest that, under the conditions of our experiments, paraoxon-induced twitch potentiation and ADF did not involve an action of ACh on ganglionic-type nicotinic cholinoceptors at nerve terminals. The observation that hexamethonium and pancuronium, in concentrations which reduced paraoxon-induced ADF, also reduced the amplitude of m.e.p.ps and e.p.ps, suggests that competition between the two drugs and ACh for the nicotinic cholinoceptor at the postsynaptic membrane could be entirely responsible for their effects. However, our experiments do not rule out the possibility that ACh induces ADF by acting on prejunctional nicotinic cholinoceptors of the motor endplate type. Even if this were the case, at least part of the reduction in ADF by pancuronium and hexamethonium could arise from an increase in the rate of diffusion of ACh from the synaptic cleft as the result of receptor occupation by the drugs (Katz & Miledi, 1973; Magleby & Terrar, 1975).

The e.p.p. in rat diaphragm preparations has a wide safety margin (Boyd & Martin, 1956). When a prolonged e.p.p. gives rise to repetitive muscle APs

and thus twitch potentiation, there is no comparable safety margin for the additional muscle action potentials and accompanying twitch potentiation. Consequently, twitch potentiation is reduced by lower concentrations of hexamethonium or pancuronium than is the normal twitch. In paraoxon treated preparations the concentration of pancuronium required for reducing twitch tension to 50% of the value before paraoxon treatment (IC_{50b}) is higher than that required for an equal effect on preparations not treated with paraoxon (IC_{50}). This is not unexpected since in preparations exposed to paraoxon, the safety margin for the initiation of the first muscle AP will be increased as a result of the enhancement of the postsynaptic action of ACh. In the case of hexamethonium the difference, however, is not significant. Ascher, Large & Rang (1979) observed that in rat parasympathetic ganglia hexamethonium, in addition to being a competitive antagonist of ACh, had channel blocking properties. Channel block is favoured by the existence of open ion channels and their number is increased when acetylcholinesterase is inhibited, e.g. by paraoxon. If hexamethonium also has channel blocking properties at the neuromuscular junction this can explain why its IC_{50b} and IC_{50} values do not differ significantly.

DTT treatment of preparations reduces the safety margin of the e.p.p. (Ben-Haim, Landau & Silman, 1973; Terrar, 1978). This should reduce the IC_{50} value if it arises from an antagonism between pancuronium or hexamethonium and ACh at the postjunctional membrane. As Figure 1 shows this is indeed the case.

The reason why Webb & Bowman (1974) were able to abolish ADF without affecting twitch potentiation in neostigmine-treated cat muscles is not clear. It could arise from species differences between neuromuscular junctions and/or experimental conditions. Webb & Bowman made their observations *in vivo*, giving single i.v. injections, whereas our experiments were carried out on an isolated preparation under equilibrium conditions. As shown by Lancaster (1972; 1973) the distribution of drugs under the two conditions can vary considerably. In some of our experiments removal of paraoxon from the organ bath was followed by a transient decline of ADF and twitch potentiation before both settled at a steady level. Under these conditions the lowest concentrations of pancuronium and hexamethonium used restored twitch potentiation to the level present at the time of removal of paraoxon from the organ bath but reduced ADF. It is thus possible that a fortuitous choice of doses and timing of their administration might also account at least in part for the observations of Webb & Bowman (1974). We have also shown previously (Clark, Hobbiger & Terrar, 1979, 1983) that, under certain conditions, twitch potentiation can be sustained in the absence of ADF. Reduction in ADF by hexamethonium therefore does not need to be associated with any reduction in twitch potentiation.

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