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Nicotinic antagonists produce differing amounts of tetanic fade in the isolated diaphragm of the rat

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1 The effects of nicotine antagonists on single twitches, trains of four twitches and tetanic contractions of the isolated diaphragm of the rat were examined.

2 Different drugs were found to produce different amounts of tetanic fade relative to depression of twitch tension.

3 The order of activity from most able, to least able to produce fade was: hexamethonium > trimetaphan = atracurium = tubocurarine > pancuronium > erabutoxin b.

4 The effect of erabutoxin b was distinctive for its almost complete lack of tetanic fade.

5 3, 4-Diaminopyridine increased tetanic fade produced by tubocurarine, atracurium and hexamethonium, but not that produced by erabutoxin b.

6 It is concluded that nicotinic antagonists act at more than one site at the neuromuscular junction. Assuming block of the postjunctional acetylcholine receptor produces tension depression, a second or third site must be involved in producing tetanic fade.

7 The possibility that tetanic fade results from block of the ion channel associated with the postjunctional acetycholine receptor or from the block of a prejunctional nicotinic receptor is discussed.

Introduction

Nicotinic antagonists, such as tubocurarine, produce both a reduction in peak tetanic tension evoked by repetitive nerve stimulation and a waning of tetanic tension during the period of stimulation ('tetanic fade') (Paton & Zaimis, 1952). In the past, fade and the increase in potency seen with increasing rates of stimulation, have generally been ascribed to a naturally-occurring fall-off in transmitter output that is unmasked once the safety margin of transmission is removed as a consequence of postjunctional receptor block (e.g. Hutter, 1952; Paton & Waud, 1967).

However, if tetanic fade is due to the same action as that producing twitch block and depression of the peak tetanic tension, then a given degree of tension depression should be associated with a given degree of tetanic fade regardless of the drug in use. There have been a few indications that this is not the case. For example, the neuromuscular blocking substances toxiferine VI (Paton & Perry, 1951) and α-bungarotoxin (Lee *et al.*, 1977) exhibit little propensity to produce tetanic fade in anaesthetized cats. Tubocurarine and pancuronium produce different amounts of tetanic fade in cats (Hunter, 1970; Bowman & Webb, 1976) and similar differences have been observed between several neuromuscular blocking agents in man (Williams *et al.*, 1980).

Different abilities of drugs to produce tetanic fade sugests that the two effects, tension depression and tetanic fade, may be mediated at different receptor sites at the neuromuscular junction (See Bowman, 1980 for review). In this study, the effects of a range of nicotinic antagonists have been tested on rat isolated phrenic nerve-hemidiaphragms. In isolated preparations problems of interpretation which could arise due to cumulative effects, redistribution and elimination of drugs *in vivo* can be avoided. In addition, *in vitro* preparations can be exposed to known drug concentrations and steady-state conditions generally obtained before measurement of the effect of a drug.

Method

Isolated phrenic nerve-hemidiaphragm preparations

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(Bülbring, 1946) were used from male Sprague-Dawley or Wistar rats (200–300 g). Preparations were bathed in Krebs-Henseleit solution of composition (mM): NaCl 118, KCl 5, $CaCl_2$ 2.5, $NaHCO_3$ 30, KH_2PO_4 1, $MgCl_2$ 1 and glucose 11, maintained at 32 ± 0.8 C and pH 7.4 when aspirated with a mixture of 95% O_2 and 5% CO_2 (v/v).

The phrenic nerve was stimulated with rectangular pulses of 0.2 ms duration and of a voltage greater than twice that required to elicit a maximal twitch (3–5 V). Single twitches were elicited at 0.1 Hz, trains of four twitches were elicited at 2 Hz and tetani at 50 Hz for 1.9 s. Twitch tension and peak tetanic tension were measured as a percentage of control. Train of four fade was calculated as $1 - T_4/T_1$, expressed as a percentage, where T_4 and T_1 are the first and fourth twitch tensions respectively. Tetanic fade was calculated as $1 - T_e/T_p$, expressed as a percentage, where T_e and T_p are the end and peak tetanic tensions respectively.

Preparations were suspended in 10 ml tissue baths under a resting tension of 2.5 g from Grass FT30C or FT10C semi-isometric force displacement transducers.

Drugs were dissolved in deionized water to form stock solutions such that volumes between 0.1 and 0.5 ml added to the tissue bath gave the desired final bath concentration. Atracurium was dissolved in deionized water acidified to pH 4 with acetic acid for stability reasons (J. B. Stenlake, personal communication).

Each preparation was exposed to only one drug concentration and each drug concentration was tested in 6 separate preparations unless otherwise stated. The effects of each drug were allowed to reach a steady state before measurement of tension parameters. This protocol avoided possible complications which could arise if fade and tension depression develop with different time courses with different drugs (Sugai *et al.*, 1976).

Statistical methods were as described by Colquhoun (1971). Results are expressed as mean ± s.e.mean throughout unless otherwise indicated.

Results

Effects on tension and tetanic fade

In all experiments, in the absence of any drugs, tetanic tension was well maintained during repetitive stimulation. In the absence of drugs twitch tension was 16.2 ± 1.1 g and tetanic tension was 92.1 ± 4.3 g.

The potency of the drugs studied was in the order tubocurarine>pancuronium>atracurium>trimetaphan>hexamethonium. The potency of the irreversible snake α-neurotoxin, erabutoxin b, could not be easily assessed as its effects did not reach a steady state, the twitches and tetani continuing to decline throughout the experiment.

Concentration-effect relationships for twitch block, block of peak tetanic tension, train of four fade and tetanic fade are shown in Figure 1. EC_{50} values calculated by linear regression of concentration-effect relationships for twitch block and tetanic fade, and their ratio, are shown in Table 1. The effects of atracurium were difficult to quantify as atracurium breaks down at pH 7.4 (Merritt *et al.*, 1983) and hence its effects never reached a steady state. Therefore, the effects of atracurium were measured after 30 min

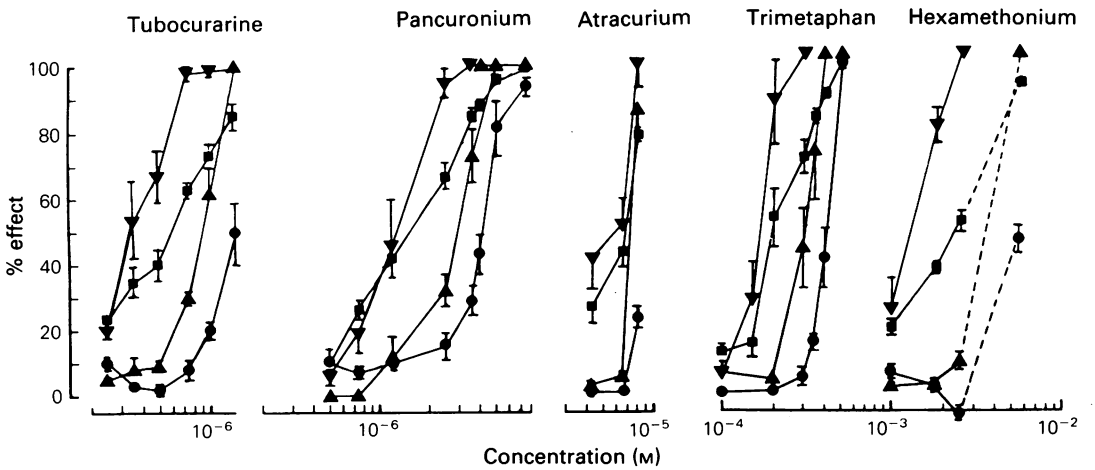

Figure 1 Concentration-effect relationships for nicotinic antagonists producing twitch block (●), train of four fade (▲), block of peak tetanic tension (■) and tetanic fade (▼). Points joined by lines are the mean of 4 experiments.

Table 1 EC_{50} values for twitch block and tetanic fade

	Tetanic fade	*Twitch block*	*EC_{50} twitch block/ EC_5 tetanic fade*
Tubocurarine	0.36 μM	1.4 μM	3.9
Pancuronium	1.1 μM	4.2 μM	3.8
Trimetaphan	0.17 mM	0.41 mM	2.5
Hexamethonium	1.2 mM	5.4mM	4.5

exposure which was approximately the time of maximum effect. In general, the ability of atracurium to produce tetanic fade was similar to that of tubocurarine.

The effects of erabutoxin b on tetanic fade were also difficult to quantify because its effects do not reach a steady state but continue to increase until muscle responses are completely blocked (Figure 2). Erabutoxin b was tested in concentrations of 0.25 μg ml^{-1}, 0.32 μg ml^{-1} and 0.42 μg ml^{-1} (mol. wt. = 6800). With increasing concentration, erabutoxin b produced neuromuscular block more rapidly, but the depression of twitches and of tetanic tension was not associated with the production of tetanic fade.

Figure 3 shows tetanic fade plotted against peak tetanic tension depression observed with the main drugs of this study. Clearly, with the drugs studied, tension depression was associated with different amounts of tetanic fade.

Effects of 3, 4-diaminopyridine on tetanic fade

In a separate group of experiments the effect on tetanic fade of the facilitatory drug 3, 4-diaminopyridine (5×10^{-5} M), which increases quantal content, was tested during partial neuromuscular block produced by the nicotinic antagonists used. Concentrations of nicotinic antagonists were chosen which would produce approximately 25% tetanic fade in order that any increase in fade would be on the linear part of the concentration-effect relationship for tetanic fade. 3, 4-Diaminopyridine (3, 4-DAP) produced a significant increase in tetanic fade with each drug studied except for erabutoxin b. The results of these experiments are summarized in Table 2. The magnitude of the increase

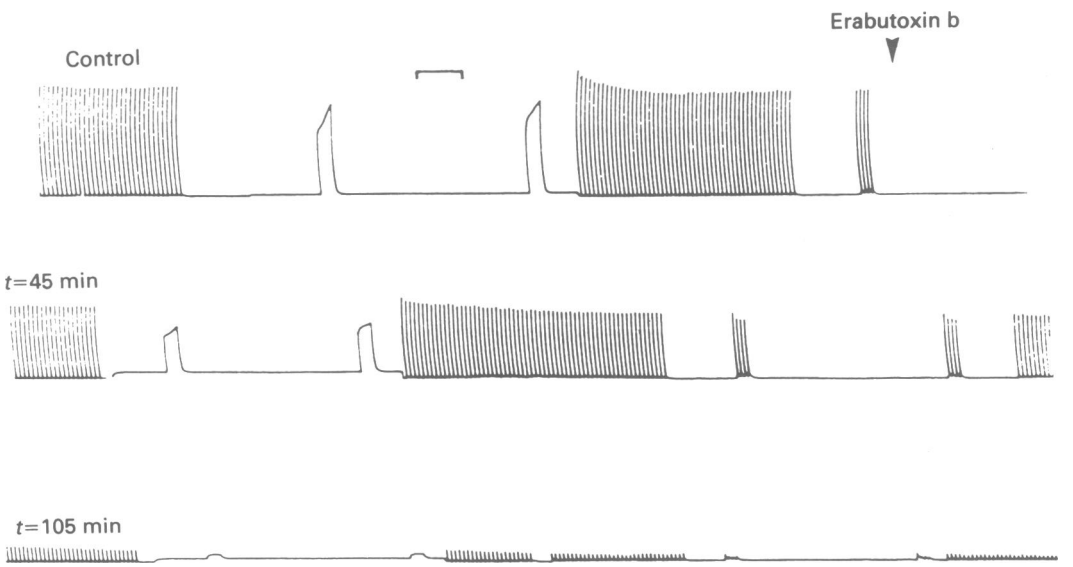

Figure 2 Time course of the effects of erabutoxin b on twitches, trains of four twitches and tetani recorded from a rat isolated diaphragm. The scale bar represents 4 s when recording tetani and trains of 4 and 2 min when recording twitches. The gain was reduced by a factor of 5 when recording tetani. Note that even when twitches were almost completely blocked there was no train of four or tetanic fade.

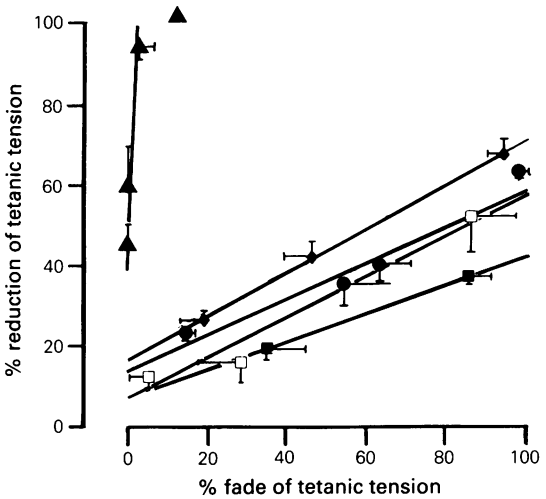

Figure 3 Relationship between tetanic fade and block of peak tetanic tension for erabutoxin b (▲), pancuronium (◆), tubocurarine (●), trimetaphan (□) and hexamethonium (■). For each drug points were used from where the concentration-effect relationships for tetanic fade and block of the peak tetanic tension overlap on the concentration axis.

in fade produced by 3,4-DAP varied with different drugs and followed the same order as that for the ability of the drugs to produce tetanic fade. In the continued presence of 3, 4-DAP and the nicotinic antagonists which produced fade, the anticholinesterase drug neostigmine (3.3×10^{-7}M) produced a partial reversal of tetanic fade in each case (Table 2).

Discussion

The ability of different nicotinic antagonists to produce tetanic fade, relative to block of the peak tetanic tension, was found to vary between different antagonists. Thus erabutoxin b produced no tetanic fade even when twitches and tetani were almost completely blocked, whereas hexamethonium produced marked tetanic fade at concentrations that produced no twitch block. These results make unlikely the assumption that all the drugs used in this study act at only one site at the neuromuscular junction.

The lack of correlation between the degree of tetanic fade and the degree of tension depression produced by different drugs indicates that at least two receptive sites are involved in the effects of nicotinic antagonists at the neuromuscular junction. With the techniques employed in this study it is not possible to determine the physical nature or position of these receptive sites. However, as α-neurotoxins such as erabutoxin b which produce neuromuscular block but not tetanic fade, are regarded as specific ligands for the postjunctional acetylcholine receptor, it may be assumed that tension depression is produced by block of the postjunctional receptor. The exact site involved in tetanic fade remains less certain.

Tetanic fade is presumably due to rundown of the amplitude of successive endplate potentials during a train of nerve stimuli. Nicotinic antagonists such as tubocurarine (Liley & North, 1953; Hubbard & Wilson, 1973) and pancuronium (Blaber, 1973) cause such a rundown and in the case of tubocurarine, this rundown has since been shown to be mediated prejunctionally (Magleby *et al.*, 1981; Gibb & Marshall, 1984). In contrast, erabutoxin b does not produce rundown (Gibb & Marshall, 1984) and trimetaphan, produces rundown by a use-dependent block of the receptor activated ion channel (Gibb & Marshall, 1984). Of the drugs used in this study, tubocurarine, pancuronium and hexamethonium have all been shown to be capable of blocking the receptor-activated ion channel at the frog neuromuscular junction, although in the cases of tubocurarine and pancuronium, only at concentrations around 10 times higher than were used in this study (Lambert *et al.*, 1983 for review). In addition, Rang & Rylett (1984) have observed that hexamethonium blocks the acetylcholine-receptor (AChR)-activated ion channel in the rat omohyoid muscle at concentrations greater than

Table 2 Effects of 3, 4-diaminopyridine (DAP) on tetanic fade and reversal by neostigmine.

	Before DAP	*After DAP*	*Mean change*	*After neostigmine*	*Mean change*
Tubo: ($n = 8$)	22 ± 8	49 ± 8	26 ± 7*	26 ± 11 ($n = 4$)	15 ± 4*
Hex: ($n = 6$)	25 ± 9	62 ± 6	38 ± 7*	55 ± 4 ($n = 4$)	12 ± 2*
Atra: ($n = 6$)	20 ± 5	53 ± 4**		23 ± 4 ($n = 6$)**	
Ebtx: ($n = 5$)	5 ± 5	15 ± 6		18 ± 2 ($n = 5$)	1.3 ± 5

Tubo = tubocurarine, Hexo = hexamethonium, Atra = atracurium, Ebtx = erabutoxin b. *Significant change, paired *t* test; **significant change, unpaired *t* test. Results are expressed as percentages.

0.4 mM. Thus, this mechanism could account for tetanic fade produced by the millimolar concentrations of hexamethonium used in this study. Rang & Rylett (1984) also demonstrated that hexamethonium produced AChR block and inhibition of acetylcholinesterase. Cholinesterase inhibition would be expected to increase fade produced by a use-dependent mechanism such as ion channel block, yet fade due to hexamethonium was partially reversed by neostigmine. It may be that the action of hexamethonium embodies a combination of ion channel block, anticholinesterase action and a prejunctional effect which is similar to that produced by tubocurarine.

Wilson (1982) has postulated that tubocurarine-induced endplate potential (e.p.p.) train rundown is due to blockade of a presynaptic nicotinic receptor that normally mediates a negative feedback on transmitter release. Tubocurarine-induced blockade of this receptor is assumed to increase release for the first e.p.p. in the train and hence lead to increased rundown during subsequent e.p.ps. This, combined with a reduction in the margin of safety due to postjunctional AChR block would be expected to produce tetanic fade. 3, 4-Diaminopyridine has been shown to increase release by increasing the quantal content of the first e.p.p. in the train without producing significant postjunctional block (Thompson & Wilson, 1983). However, in this study 3, 4-DAP was shown to increase tetanic fade only when used in combination with drugs that already produce tetanic fade. 3, 4-Diaminopyridine did not produce significant tetanic fade even when transmission was almost completely blocked by erabutoxin b. Therefore, it seems from these results that a mechanism such as that suggested by Wilson (1982), that is a combination of facilitation and postjunctional block, cannot account for nicotinic antagonist-induced tetanic fade.

Like Wilson, Blaber (1973) also found that tubocurarine could increase release, as well as cause increased e.p.p. train rundown in cut cat tenuissimus muscles. However, Blaber found that pancuronium and dimethyltubocurarine, which also increase train rundown, did not increase release. An additional effect of tubocurarine in increasing release could explain why in this study tubocurarine had a slightly higher propensity to produce tetanic fade than, for example, did pancuronium.

In conclusion, the results of the present experiments confirm those of Bowman & Webb (1976), although less marked differences between different nicotinic antagonists were observed in the present experiments than in the *in vivo* experiments of Bowman & Webb. These authors proposed that tetanic fade is mediated by block of a prejunctional nicotinic receptor which normally mediates a positive feedback on transmitter mobilisation. In view of the observations that neostigmine reverses tetanic fade and 3, 4-diaminopyridine does not increase it in the presence of erabutoxin b, the hypothesis of Bowman & Webb seems to fit best the results presented in this paper.

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