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Neuromuscular blocking profile of the vecuronium analogue, Org-9487, in the rat isolated hemidiaphragm preparation

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1 The neuromuscular effects of the short-acting aminosteroid muscle relaxant Org-9487 have been studied in the *in vitro* rat phrenic nerve/hemidiaphragm preparation by use of twitch tension and electrophysiological recording techniques.

2 Org-9487 (5-100 μM) produced a concentration-dependent decrease in the amplitude of twitches (0.1 Hz) and tetanic contractions (50 Hz) evoked by motor nerve stimulation. The compound produced fade of force during both 50 Hz stimulation and train-of-four stimulation at 2 Hz, indicating a prejunctional component of action.

3 Anticholinesterases only partially reversed the effect of Org-9487 on twitch responses. This was possibly because, at the concentrations required to block twitches in the rat, Org-9487 itself was found to possess significant anticholinesterase activity.

4 Org-9487 (3 μM) increased the rundown of endplate current amplitudes during a 2 s train of 50 Hz nerve stimulation. This was because Org-9487 increased the quantal content of the first endplate current in the train without affecting acetylcholine release towards the latter part of the train.

5 Org-9487 (10 μM) produced a voltage-dependent decrease in the time constant of decay of endplate currents at 32°C and 0.5 Hz, indicative of a block of endplate ion channels. The blocking rate constant increased with membrane hyperpolarization.

Keywords: Org-9487; neuromuscular transmission; endplate currents; rat phrenic nerve/hemidiaphragm; anticholinesterase

Introduction

Org-9487, the 16N-allyl, 17β-propionate analogue of vecuronium, is a non-depolarizing aminosteroid muscle relaxant currently undergoing clinical trial (Wierda *et al.*, 1994). These studies show that it has one-quarter the potency of the recently introduced muscle relaxant rocuronium (Org-9426) and is approximately 25-30 fold less potent than vecuronium (Wierda *et al.*, 1994). The clinical attraction of Org-9487 is its rapid onset and short duration of action. It has been suggested (see e.g. Bowman *et al.*, 1988; Wierda *et al.*, 1993) that the rapid onset associated with compounds such as Org-9487 and rocuronium is partly a consequence of the large quantity of the compounds that have to be administered given their relatively low potency. However, low potency can lead to an increased risk of adverse side-effects. Thus, Org-7617, one of the least potent muscle relaxants ever tested in man, has an onset time as fast as that of suxamethonium but has uncertain clinical value because of its unwanted side-effects (van den Broek *et al.*, 1994).

In this study, we have used twitch tension and electrophysiological techniques to examine, in detail, the neuromuscular effects of Org-9487 in the rat isolated hemidiaphragm nerve/muscle preparation. We have focused on two separate aspects of the neuromuscular activity of Org-9487. Firstly, we have examined the relative pre- and postjunctional neuromuscular blocking actions of the compound by assessing the effects of Org-9487 on responses to high and low frequency nerve stimulation respectively. Secondly, we have studied the extent to which Org-9487 exhibits neuromuscular effects in addition to its competitive nicotinic acetylcholine (ACh) receptor (AChR) blocking activity. We show that, in the rat, Org-9487 is a low potency muscle relaxant with a similar profile to vecuronium and rocuronium. In addition, it has several other neuromuscular effects including: (*a*) an enhancement of ACh release at low frequencies of nerve stimulation; (*b*) inhibition of acetylcholinesterase (AChE) and (*c*) a blocking effect on the endplate ion channel. The marked evidence of these effects in the rat is most likely a consequence of the unusually low nicotinicAChR blocking potency that the compound exhibits in this species. However, the clean neuromuscular blocking profile seen with Org-9487 in initial clinical evaluations of the compound in man (Wierda *et al.*, 1994) suggests that the additional actions described here do not pose a problem in a species where the nicotinicAChR blocking potency of the compound is relatively high.

Methods

Rat hemidiaphragm preparation

Each hemidiaphragm muscle, along with 15-20 mm of its associated phrenic nerve, was isolated from male Sprague-Dawley rats (150-250 g) killed by CO_2 anaesthesia followed by immediate exsanguination. Preparations were mounted in Krebs-Henseleit solution (see below). The phrenic nerve was stimulated via a pair of silver wires by a Grass S88 stimulator and a Grass SIU5 stimulus isolation unit with pulses of 50-100 μs duration and voltage greater than that required to produce maximal responses.

Tension recording

All tension experiments were performed at 32°C with resting tension of the muscle preparation set to give the maximum developed force when the motor nerve was stimulated. Responses were monitored by a force displacement transducer (Grass, FT03C) linked to a flat-bed recorder (Grass, 79D). Two experimental protocols were adopted. In the first, the motor nerve was stimulated continuously at 0.1 Hz, except

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every 10 min it was alternately subjected to either a train-of-four at 2 Hz or a high frequency tetanus (50 Hz for 2 s). Once reproducible responses to all frequencies were obtained, each preparation was exposed to a single concentration of Org-9487 (5, 10, 20 or 40 μM). The drug was added 5 min before a tetanus and left in contact with the muscle for 80 min. In the second protocol, Org-9487 was added cumulatively to indirectly stimulated (0.1 Hz) preparations until twitch force was reduced by 70–80%. Subsequently, either neostigmine (0.5 μM), edrophonium (50 μM) or 3,4-diaminopyridine (10 μM) was added to determine the reversibility of the effect of Org-9487. For comparison, the effects of the anticholinsterases (anti-AChEs) were also tested against a similar degree of twitch block produced by vecuronium. In control preparations, there was no change in indirectly elicited twitch force following 150 min of stimulation at 0.1 Hz; twitch force at 150 min was 97.7±5.4% ($n=6$) of initial value. To show that any effect of Org-9487 on twitches was not due to a direct effect on muscle contractility, the effects of 80 μM of the compound on directly elicited twitch force (at 0.1 Hz) were determined in preparations in which neuromuscular transmission was blocked with 0.3 μM α-bungarotoxin.

Tension data analysis

In each muscle, twitch and tetanic force in the presence of Org-9487 were expressed as a percentage of their respective control values. Train-of-four fade, defined as the loss of twitch force by the fourth stimuli at 2 Hz, was expressed as a percentage of the first twitch in the group. Tetanic fade, defined as the loss of force at the end of the tetanus, was expressed as a percentage of the maximum tetanic force. In control preparations no tetanic or train-of-four fade was measurable. In the reversal studies, percentage reversal of neuromuscular block was calculated using the following equation:

$$\text{Reversal} = 100 \times \frac{T_{rev} - T_{blk}}{T_{con} - T_{blk}}$$

where T_{con} is the control twitch force, T_{blk} is the twitch force at maximal block, before addition of the reversal agent and T_{rev} is the twitch force at maximal reversal. For the reversal studies, maximum twitch block before addition of the reversal agent was expressed as a percentage as follows:

$$\text{Block} = 100 \times \frac{T_{con} - T_{blk}}{T_{con}}$$

Electrophysiological recordings

All recordings were made in rat hemidiaphragm muscle/phrenic nerve preparations (Barstad & Lilleheil, 1968) using the cut muscle preparation as described elsewhere (for review see Prior *et al.*, 1993). Following isolation, preparations were dissected free of connective tissue and mounted on the Sylgard (Dow Corning) base of a 5 ml bath perfused (5–15 ml min^{-1}) with Krebs-Henseleit solution (see below) at either room temperature (18–22°C) or at 32°C. Miniature endplate currents (m.e.p.cs) and endplate currents (e.p.cs) were recorded from motor endplates by a conventional two-microelectrode voltage-clamp technique. Signals were monitored by either a purpose built system modified from that of Dionne & Stevens (1975) linked to the output of a model 701-A electrometer (World Precision Instruments) on the voltage side or alternatively an Axoclamp 2A (Axon Instruments). Microelectrodes were filled with either 3 M KCl (voltage recording, 5–10 MΩ) or 0.6 M K_2SO_4 (current passing, 2–5 MΩ). Voltage-clamp feedback gain and bandwidth were adjusted so that the voltage escape during the e.p.c. did not exceed 1% of the holding potential.

To determine the relative pre- and postjunctional effects of Org-9487, in each fibre, m.e.p.cs and e.p.cs (elicited at 50 Hz for 2 s) were recorded at room temperature (18–22°C) and at a single holding potential of −50 mV in the presence and absence of a single concentration of Org-9487 (3 μM). Room temperature was chosen for these experiments to allow comparison with previously obtained data from our laboratory for other clinically used muscle relaxants (Gibb & Marshall, 1984; 1987; Tian *et al.*, 1992). The concentration of 3 μM was chosen to produce an approximate 30% reduction in the amplitude of m.e.p.cs. The simultaneous recording of m.e.p.cs and e.p.cs in each fibre allowed an analysis of quantal ACh release to be performed. To assess the endplate ion channel blocking activity of Org-9487, in each fibre studied, between 10 and 30 e.p.cs (elicited at 0.5 Hz) were recorded at 32°C at a range of holding potentials (−30 to −70 mV) in the absence and presence of 10 μM Org-9487. The ion channel blocking experiments were performed at 32°C to minimise the effects of repetitive binding of ACh to its postjunctional receptors on the decay phase of e.p.cs. For all the electrophysiological protocols, Org-9487 was applied to the tissue by superfusion (5–15 ml min^{-1}) for 5 min. No evidence for a depolarizing mechanism of action of Org-9487 was observed.

Electrophysiological data acquisition and analysis

Current signals were recorded on either FM-tape (Racal Store 4DS, bandwidth: d.c.–5 kHz) or video-tape (Panasonic AG6200 VCR with a modified Sony PCM-701 digital audio processor, bandwidth: d.c.–20 kHz) for subsequent off-line analysis. Taped signals were low-pass filtered at 5 kHz and digitised at 25 kHz with a standard laboratory interface (Data Translations DT2801A or National Instruments Lab-PC) and stored on computer by a suite of signal acquisition and analysis programs (Dempster, 1993) running on a laboratory microcomputer (Vanilla 386SX).

Analysis of quantal ACh release For each m.e.p.c. record, 13–59 individual m.e.p.cs were sequentially averaged to create a single averaged signal. Averaged m.e.p.cs were analysed for peak amplitude and time constant of decay (τ_{mepc}) as described elsewhere (Prior *et al.*, 1993). The peak amplitude of the averaged m.e.p.c. was used as m.e.p.c.$_{amp}$ in the quantal analysis. In each train of e.p.cs, all individual e.p.cs were analysed for peak amplitude. The amplitudes of the 81st–100th e.p.cs were used to calculate the mean e.p.c. amplitude (e.p.c.$_{amp}$) and the variance of e.p.c. amplitudes (e.p.c.$_{var}$). Transmitter release parameters were calculated by use of a simplified version of the binomial method described by Miyamoto (1975) and Glavinovic (1979):

$$m = \text{e.p.c.}_{amp}/\text{m.e.p.c.}_{amp}$$

$$n_q = (\text{e.p.c.}_{amp})^2/[(\text{e.p.c.}_{amp} \times \text{m.e.p.c.}_{amp}) - \text{e.p.c.}_{var}]$$

$$p = \text{e.p.c.}_{amp}/(n_q \times \text{m.e.p.c.}_{amp})$$

where m is the number of quanta of ACh released by each impulse (i.e. the quantal content), n_q is the size of the pool of quanta available for immediate release and p is the probability of release of each individual quantum. No correction was made within the calculations for either m.e.p.c. amplitude variance or the contribution of background noise variance to the total recorded e.p.c. amplitude variance (for explanation see Tian *et al.*, 1994). Full binomial analysis of quantal ACh release was performed, in both the presence and absence of 3 μM Org-9487 in each of 6 different muscle fibres, each from a separate hemidiaphragm preparation.

Analysis of endplate ion channel block At each holding potential, 10–30 individual e.p.cs were sequentially averaged to give a single averaged signal. Averaged e.p.cs were analysed for peak amplitude and time constant of decay (τ_{epc}) as described elsewhere (Prior *et al.*, 1993). Exponential fits to the decay phase of the averaged e.p.c., starting at 80% of the peak amplitude were

used to determine τ_{epc}. Peak amplitude and τ_{epc} were measured at five membrane potentials (−30 to −70 mV in 10 mV steps) in the presence and absence of 10 μM Org-9487. All current signals could be fitted by single exponentials. In each fibre, the ion channel blocking rate constant (G) was calculated from τ_{epc} values according to the sequential model of ion channel block (see e.g. Adams, 1976; 1977; Ruff, 1977; 1982):

$$G.\,[Org9487] = \frac{1}{\tau_{drug}} - \frac{1}{\tau_{control}}$$

where $\tau_{control}$ is τ_{epc} measured in the absence of Org-9487 and τ_{drug} is τ_{epc} measured in the presence of 10 μM Org-9487. Results determined from 5 different muscle fibres, each from a different hemidiaphragm muscle preparation, were averaged to give the data presented in the figures.

Acetylcholinesterase assay

The ability of Org-9487 to inhibit the AChE activity of rat brain homogenates was determined at room temperature, by use of a modification of the method described by Ellman *et al.* (1961). For each determination, the inhibitory activity of 5–6 different concentrations of Org-9487 (10–200 μM) was measured and, using non-linear least squares regression analysis, the best fit Hill equation was used to determine the IC_{50} and the slope coefficient (*SC*); this latter measure being analogous to the Hill coefficient determined for binding data:

$$\text{Activity (as \% control)} = 100/(1 + ([Org9487]/IC_{50})^{SC})$$

The presented data for the IC_{50} and slope coefficient are the mean and s.e.mean of values derived from 3–4 separate determinations.

Drugs, solutions and statistics

All tension and electrophysiological experiments were performed in a standard Krebs-Henseleit solution (mM): NaCl 118, KCl 5, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1, glucose 11, gassed with 5% CO_2 in 95% O_2 to a pH of 7.2–7.4. Org-9487 (1-[2β, 3α, 5α, 16β, 17β)-3-(acetyloxy)-17-(1-oxopropoxy)-2-(1-piperidinyl)androstan-16-yl]-1-(2-propenyl)piperidinium bromide) and vecuronium, as the bromide salts, were gifts from the Organon Scientific Development Group (Newhouse, U.K.). For the electrophysiological and tension studies, stock solutions of the Org-9487 (1–5 mM) and vecuronium (200 μM) were made in buffer containing (mM): citric acid 4.3, Na_2HPO_4 4.6 and mannitol 13.4. These stock solutions were kept at 4°C and appropriate dilutions were made, on the day of use, in physiological buffer. The maximum citrate concentration that preparations were exposed to was 0.04 mM; this concentration did not affect neuromuscular transmission. However, citrate ions did affect the sensitivity of the AChE assay. Therefore, for these studies, solutions of Org-9487 were made in buffer directly from the bromide salt immediately before use. Other drugs used were neostigmine methylsulphate, edrophonium bromide and 3,4-diaminopyridine (supplied by Sigma Chemical Co, Poole, Dorset).

All data are presented as mean and s.e.mean of values from 3–7 individual experiments. Differences between sets of data were tested for by a two-tailed paired Student's *t* test with the level of significance set at $P<0.05$.

Results

Effects of Org-9487 on twitch tension

Org-9487 produced a concentration-dependent decrease in the amplitudes of nerve evoked twitches and tetanic contractions (Figure 1a). In addition, it produced concentration-dependent degrees of fade of nerve evoked trains-of-four and tetani (Figure 1b). However, 80 μM Org-9487 had no effect on directly elicited twitches (0.1 Hz) in α-bungarotoxin-treated preparations; directly elicited twitch force following 80 min exposure to 80 μM Org-9487 was 94.7±5.0% ($n=4$) of the initial control.

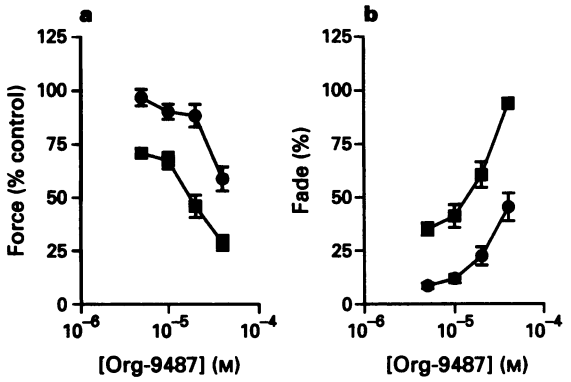

Figure 1 (a) Concentration-effect curves for the effect of Org-9487 on the peak height of single twitches at 0.1 Hz (●) and the peak height of tetanic (50 Hz) contractions (■). The force of contraction is expressed as a percentage of peak control force. (b) Concentration-effect curves for the effect of Org-9487 on train-of-four fade at 2 Hz (●) and the fade of 50 Hz tetani (■). Train-of-four fade and tetanic fade were calculated as described in the Methods. All contractile responses were nerve evoked. All experiments were performed at 32°C and in all cases preparations were exposed to Org-9487 for a minimum of 80 min before any readings were taken. Each data point is the mean and s.e.mean of between 5 and 7 individually measured responses.

Neither edrophonium (50 μM) nor neostigmine (0.5 μM) fully reversed the effect of Org-9487 on twitch force. Rather, both produced a partial restoration of the twitch responses (Table 1). Neostigmine (at 46%) was significantly more effective ($P<0.05$, two sample Student's *t* test) in reversing the effects of Org-9487 than edrophonium (at 25%). The reversal of Org-9487-induced twitch block was only transient; with both anti-AChEs, following the initial increase, twitch height gradually fell towards zero. In contrast, both agents produced an almost complete (around 75%) and sustained reversal of the effects of vecuronium (Table 1). For both anti-AChEs, the reversal of the effects of vecuronium-induced twitch block was significantly greater than that seen with Org-9487 ($P<0.05$, two sample Student's *t* test) and, unlike with Org-9487, there was no difference in the efficacy of the two anti-AChEs against

Table 1 Effect of three 'reversal agents' on the block of twitch force produced by Org-9487 and vecuronium

Blocking agent	n	*Concn.* (μM)	*Maximum block* (%)	*Reversal agent*	*Maximum reversal* (%)
Org-9487	5	81.0±10.2	79.0±2.2	NEO	46.4±4.2
Org-9487	6	75.0±5.6	81.0±2.5	EDR	25.3±7.5[b]
Org-9487	5	74.0±7.7	73.7±2.1	DAP	112.5±4.6
Vecuronium	6	1.35±0.07	81.4±2.0	NEO	71.5±7.6[a]
Vecuronium	6	1.22±0.04	75.0±2.7	EDR	76.6±2.2[a]

All data are mean and s.e.mean values from the number of individual experimental recordings indicated in *n*. Concn. indicates the concentration of blocking agent required to produce the percentage block shown in the table. Maximum block and maximum reversal were calculated as described in the Methods. Reversal agents: neostigmine 0.5 μM (NEO), edrophonium 50 μM (EDR) or 3,4-diaminopyridine 10 μM (DAP). [a]$P<0.05$ versus reversal of Org-9487-induced twitch block by same compound (two-tailed, two sample Student's *t* test). [b]$P<0.05$ versus reversal of Org-9487 with neostigmine (two-tailed, two sample Student's *t* test).

vecuronium-induced twitch block. The effects of Org-9487 on twitch force were completely reversed by 3,4-diaminopyridine 10 μM (Table 1).

Org-9487 inhibited rat brain AChE in a concentration-dependent manner in the same range of concentrations as that used to block twitch responses in the hemidiaphragm muscle (Figure 2). The mean IC_{50} for AChE inhibition by Org-9487 was 48.2±1.7 μM ($n=4$) and the mean slope coefficient for the fitted sigmoid inhibition curves was 0.906±0.057 ($n=4$) which is not significantly different from unity ($P>0.05$, one sample Student's t test).

Effects of Org-9487 on quantal transmitter release

Org-9487 (3 μM) reduced m.e.p.c. amplitude by approximately 25% (Figure 3, Table 2) with no effect on the frequency of occurrence of m.e.p.cs: control, 0.48±0.09 Hz; Org-9487, 0.52±0.28 Hz ($n=6$). However, 3 μM Org-9487 had no effect on the amplitude of the first e.p.c. in a 50 Hz train of stimuli (Figure 3, Table 3). Thus, Org-9487 increased the quantal content of the first e.p.c. (Table 3). Org-9487 (3 μM) reduced the mean amplitude of the 81st to 100th e.p.cs in the 50 Hz train of stimuli by approximately 30% (Figure 3, Table 3); a similar reduction to that seen in the m.e.p.c. Thus, towards the latter part of the train, quantal content was unaffected by 3 μM Org-9487 (Table 3). Analysis of the amplitudes of the 81st to 100th e.p.cs in the presence and absence of Org-9487 indicated that Org-9487 had no effect on either the size of the pool of quanta available for immediate release or the probability of release of a single quantum (Table 3). As a consequence of its enhancing effect on the quantal content of the first e.p.c. in the train, 3 μM Org-9487 significantly increased ($P<0.05$, paired Student's t test) the rundown of e.p.c. amplitudes from 52.3±6.1% (control, $n=6$) to 63.3±4.9% (Org-9487, $n=6$).

Ion channel blocking effects of Org-9487

Org-9487 (3 μM) reduced τ_{mepc} recorded at room temperature and −50 mV from 1.32±0.15 ms (control, $n=6$) to 1.01±0.07 ms (3 μM Org-9487, $n=6$, $P<0.05$ paired Student's t test). There was no evidence for Org-9487 producing m.e.p.c.

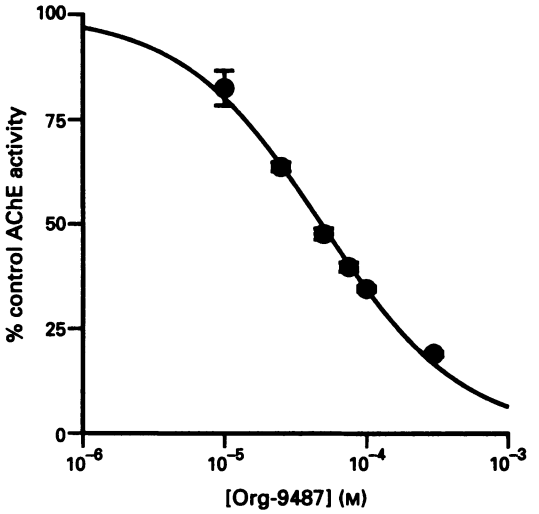

Figure 2 Plot of inhibition of rat brain acetylcholinesterase (AChE) activity versus concentration for Org-9487. Each point is the mean and s.e.mean of data from 3–4 separate determinations of the inhibitory activity of Org-9487. Curve is the best fit sigmoid, calculated using a single mean data value for each Org-9487 concentration.

Table 2 Effect of Org-9487 3 μM on the peak amplitude of m.e.p.cs and e.p.cs recorded from the rat hemidiaphragm at room temperature and voltage-clamped at −50 mV

	Control (nA)	*Org-9487* (3 μM) (nA)	(%)
m.e.p.c.	2.08±0.13	1.57±0.09*	75.7±1.2
$e.p.s._1$	94.1±13.4	83.7±8.4	92.6±6.5
$e.p.c._{81-100}$	43.7±7.8	30.4±4.7*	70.8±4.8

All values are mean and s.e.mean of data from the same six preparations. $E.p.c._1$ denotes the first e.p.c. in a 2 s train of 50 Hz stimuli. $E.p.c._{81-100}$ denotes the average of the 81st to 100th e.p.cs in a 2 s train of 50 Hz stimuli. *$P<0.05$ versus control, paired Student's t test.

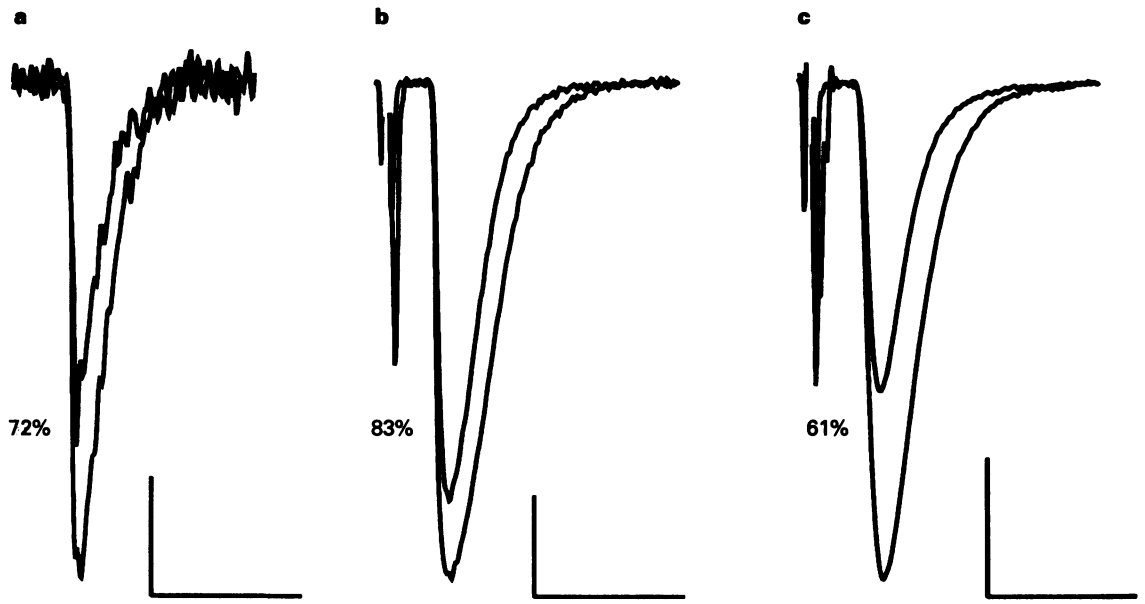

Figure 3 Representative examples of miniature endplate currents (m.e.p.cs) (a) and endplate currents (e.p.cs) (b, c) recorded at −50 mV and room temperature from a single fibre of a rat hemidiaphragm muscle preparation in the absence (larger signal of each pair) and presence (smaller signal of each pair) of 3 μM Org-9487. M.e.p.c. signals are the sequential average of 31 and 48 individually recorded m.e.p.cs. The e.p.cs shown in (b) are the first of a 2 s train of e.p.cs at 50 Hz while those in (c) are the sequential averages for the 81–100th e.p.cs from the same trains of stimuli. All control signals have been scaled to appear the same size on the Figure to emphasize the effects of Org-9487 on the current amplitudes. Numbers on the figure indicate the sizes of the currents in the presence of Org-9487 3 μM expressed as a percentage of the control response. Averaged percentage data for all fibres studied are shown in Table 3. Calibration bars: horizontal, 5 ms for all panels; vertical, 0.5 nA in (a), 25 nA in (b) and 20 nA in (c).

Table 3 Effect of Org-9487 3 μM on the binomial parameters of ACh release in the rat hemidiaphragm at room temperature

	Control (nA)	*Org-9487* (3 μM) (nA)	(%)
m_1	44.9±5.0	53.3±4.4*	122.2±8.3
m_{81-100}	21.0±3.4	19.4±2.8	93.8±6.8
n_q	24.9±4.0	25.0±3.7	102.5±11.1
p	0.84±0.03	0.79±0.04	93.0±5.0

Binomial parameters of acetylcholine (ACh) release were calculated from e.p.c. and m.e.p.c. amplitudes as described in the Methods. All values are mean and s.e.mean of data from the same six preparations. m_1 denotes the quantal content of the first e.p.c. in a 2 s train of 50 Hz stimuli and m_{81-100} denotes the average quantal content of the 81st to 100th e.p.cs in a 2 s train of 50 Hz stimuli. n_q and p denote the size of the pool of quanta available for release and the probability of release of a quantum respectively. Both these latter measures were calculated over the 81st to 100th e.p.cs from the 50 Hz train of stimuli. *$P<0.05$ versus control, paired Student's t test.

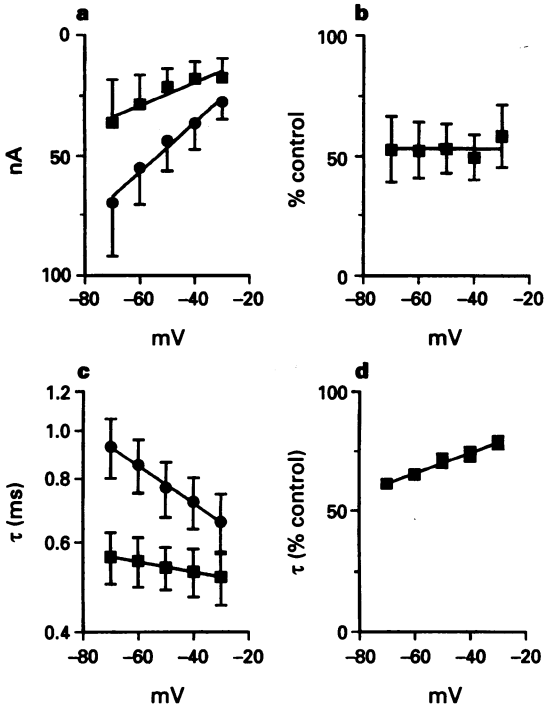

Figure 4 Plots of end plate current (e.p.c.), amplitude (a and b) and τ_{epc} (c and d) versus holding membrane potential for e.p.cs recorded, at 32°C, in the absence (●) and presence (■) of Org-9487 (10 μM). E.p.c. measures are expressed as absolute values (a and c) and, for Org-9487, as a percentage of the control value at the same holding membrane potential (b and d). All data are mean and s.e.mean of values from the same 5 fibres. Lines in (a), (c) and (d) are best-fit to the data. Line in (b) is the mean of the data over all holding membrane potentials. Note the strong voltage-dependence of the effects of Org-9487 on τ_{epc} as indicated in (d).

decays composed of more than one exponential component. This suggests that Org-9487 possesses an endplate ion channel blocking action. This was further investigated by examining the effect of a higher concentration of Org-9487 (10 μM) on τ_{epc} over a range of holding membrane potentials at 32°C. Both peak e.p.c. amplitude (Figure 4a) and τ_{epc} (Figure 4c) were decreased at all potentials. The decrease in amplitude was approximately 50% at all potentials studied (Figure 4b). However, the decrease in τ_{epc} was voltage-dependent, being greater at more hyperpolarized potentials (Figure 4d). Thus there was a change in the slope of the relationship between membrane potential and the logarithm of τ_{epc}. The slope was

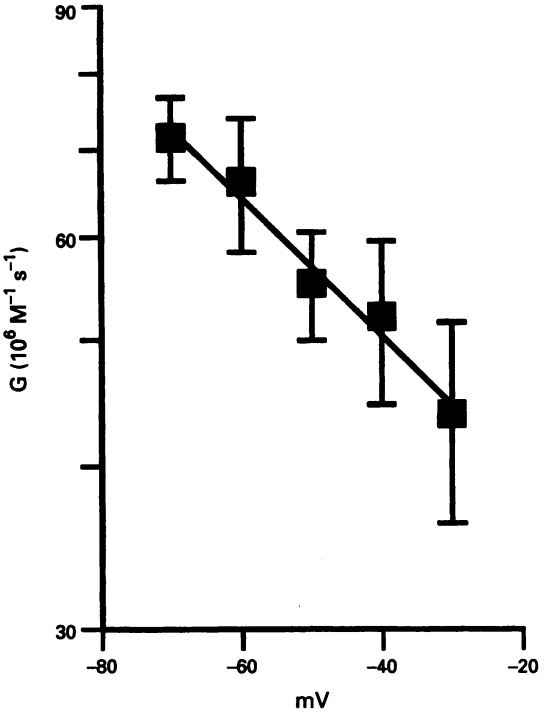

Figure 5 Plot of the ion channel blocking rate constant (G) versus holding membrane potential for Org-9487. Values were derived from the τ_{epc} data shown in Figure 4. Data are mean and s.e.mean of values from the same 5 fibres. Note the tendency for G to increase with membrane hyperpolarization. Value of G at −70 mV is significantly greater than value at −30 mV ($P<0.05$, paired Student's t test). Vertical scale is logarithmic.

reduced from 3.78±0.21 V^{-1} (control, $n=5$) to 1.17±0.21 V^{-1} (10 μM Org-9487, $n=5$, $P<0.05$, paired Student's t test). As expected for a positively charged quaternary amine, the ion channel blocking rate constant (G) for Org-9487 showed significant voltage-dependence, being greater at more hyperpolarized potentials (Figure 5). The slope of the relationship between the logarithm of G and membrane potential was 5.9±1.7 V^{-1} ($n=5$, $P<0.05$ versus zero, one sample Student's t test).

Discussion

Mechanism of twitch block produced by Org-9487

The effects of Org-9487 on directly and indirectly evoked tension responses and the lack of an effect on nerve action potentials (Prior, unpublished observation) confirm that the compound inhibits neuromuscular transmission. The electrophysiological observations eliminate either a suxamethonium-like depolarizing mechanism of action or a magnesium-like block of prejunctional calcium channels. At the concentration used (10 μM), 3,4-diaminopyridine enhances ACh release through a blocking action on nerve terminal potassium channels with little effect on muscle membrane potassium channels (Harvey & Marshall, 1977). Therefore, the rapid and complete reversal of Org-9487-induced twitch block by 10 μM 3,4-diaminopyridine suggests that the main action of Org-9487 is a competitive antagonism of postjunctional nicotinic AChR.

Anticholinesterase activity of Org-9487

The inability of anti-AChEs to reverse the effects of Org-9487 on twitches suggests that Org-9487 has another neuromuscular effect in addition to blocking the nicotinic AChR. That Org-9487 inhibits AChE in the concentrations used in the twitch study was confirmed biochemically. Based on the unitary value of the slope coefficient of the inhibition curve, the anti-AChE

activity of Org-9487 follows simple competitive kinetics. The anti-AChE activity of Org-9487 in species other than the rat is unknown. However, unlike in the rat isolated hemidiaphragm, the neuromuscular blocking effects of Org-9487 in the cat *in vivo* and in man can be easily reversed by neostigmine (A.W. Muir, unpublished observations; Wierda *et al.*, 1994). Thus, even if Org-9487 is active against human AChE, the selectivity of the compound for the nicotinic AChR over AChE must be greater in man than in rats. Vecuronium also has anti-AChE activity in man, with an IC_{50} of 66 μM (Marshall *et al.*, 1980), and this is not generally regarded as a problem with this compound.

Neostigmine (Fiekers, 1985) and edrophonium (Yost & Maestrone, 1994) block endplate ion channels. Therefore, with Org-9487, the decline in twitch force following the initial partial reversal by these anti-AChEs might be due to the development of a significant degree of endplate ion channel block through the combined activities of the two compounds. That neostigmine was more effective than edrophonium in temporarily reversing the twitch block produced by Org-9487 could be due to edrophonium having a greater propensity for ion channel block than neostigmine or, alternatively, could indicate an additional prejunctional effect of neostigmine to increase ACh release, such as suggested by Braga *et al.* (1993). Finally, the anti-AChE effect of Org-9487 would result in an underestimation of the true nicotinic AChR blocking activity of the compound from *in vivo* and *in vitro* tension studies.

Prejunctional effects of Org-9487

In the rat, in spite of its anti-AChE activity, Org-9487 produced a similar profile of activity on twitch force at both high and low frequencies of nerve stimulation to previously examined muscle relaxants (Gibb & Marshall, 1986; Blount *et al.*, 1992; Tian *et al.*, 1992). The ability of Org-9487 to produce tetanic and train-of-four fade suggests that the compound produces an enhanced fall-off in ACh release during the course of high frequency stimulation. This supposition was confirmed by electrophysiological analysis of the amplitudes of e.p.c. evoked at 50 Hz. Thus Org-9487, in common with other clinically used muscle relaxants such as tubocurarine (Gibb & Marshall, 1984), vecuronium (Gibb & Marshall, 1987) and rocuronium (Tian *et al.*, 1992), enhanced the rundown of e.p.c. amplitudes recorded at 50 Hz from rat diaphragm muscles. With Org-9487, the enhanced rundown of e.p.c. amplitudes during high frequency stimulation was a result of raised quantal content of the initial e.p.c. with no effect of the compound on quantal content during the terminal part of the train. Such an effect has previously been shown for tubocurarine (Wilson, 1982) and for the aminopyridines (Thomsen & Wilson, 1983). These results were attributed to an effect of tubocurarine on a prejunctional negative-feedback nicotinic AChR and the effect of the aminopyridines on prejunctional potassium channels respectively. With regard to a possible action of Org-9487 on motor nerve terminal potassium channels, it has been shown that the compound does block the delayed rectifier in rabbit isolated heart muscle (J. Leboeuf, personal communication). However, if nerve terminal potassium channel block were to account for the augmentation seen with Org-9487 then it is unlikely that the compound is a potent potassium channel blocking agent. Thus, although Thomsen & Wilson (1983) showed that the aminopyridines increase endplate potential (e.p.p.) amplitude rundown, they also saw an aminopyridine-induced increase in quantal content during the latter part of the high frequency train; an effect that we did not observe with Org-9487. In addition, in our tension studies we found that the effects of high concentrations of Org-9487 could be reversed by 3,4-diaminopyridine.

The effects of Org-9487 on quantal content contrasts markedly with those of tubocurarine and vecuronium previously determined in our laboratory (Tian *et al.*, 1994). Both tubocurarine and vecuronium decreased quantal content during high frequency stimulation whilst tubocurarine, but not vecuronium, increased quantal content during continuous low frequency stimulation. The decrease in quantal content associated with both agents at high frequencies was attributed to an action of the compounds on a putative positive-feedback prejunctional nicotinicAChR responsible for enhancing ACh mobilization during periods of sustained ACh release (Bowman, 1980; Bowman *et al.*, 1990). It has been postulated (Tian *et al.*, 1994) that this positive-feedback nicotinicAChR is similar to the muscle-type receptor found at the motor endplate. So why does Org-9487 not decrease quantal content at high frequencies of stimulation? Perhaps, despite its action at the postjunctional nicotinic AChR, Org-9487 is devoid of activity at the putative prejunctional positive-feedback nicotinicAChR. Alternatively, the enhanced ACh release, seen at the beginning of the train of stimuli, may persist throughout the train and thus masks any effect of the compound to block the positive-feedback nicotinic AChR. Whether the prejunctional effect of Org-9487 is mediated via an action at prejunctional positive-feedback and/or negative-feedback nicotinic AChRs, or even through a partial block of prejunctional potassium channels cannot be determined from the present data. However, any combination of these three mechanisms could account for the observed effects of Org-9487 on trains-of-four and tetani.

Org-9487 and endplate ion channel block

Vecuronium, rocuronium (Org-9426) and Org-9991 all reduce τ_{epc} recorded from rat hemidiaphragm muscle at room temperature (Gibb & Marshall, 1987; Muir *et al.*, 1991; Tian *et al.*, 1992). However, because the effects of these compounds on τ_{epc} were not voltage-dependent, they were attributed to a decrease in the repetitive binding of ACh to its receptors (Magleby & Terrar, 1975) rather than to ion channel block. The relatively high degree of repetitive ACh binding in control was attributed, in turn, to low AChE activity at room temperature. For a number of reasons, it is unlikely that a similar mechanism underlies the effects of Org-9487 on τ_{epc}. Firstly, the effects of Org-9487 on τ_{epc} were observed at 32°C. At this higher temperature, increased AChE activity would decrease the synaptic lifetime of released ACh compared to at room temperature and consequently repetitive ACh binding would not be so prevalent. Secondly, a similar effect of Org-9487 was seen on τ_{mepc}, where repetitive ACh binding is less likely. Similar values for G were obtained, at -50 mV, for both e.p.cs at 32°C ($55.3 \pm 5.3 \times 10^6$ M^{-1} s^{-1}, $n=5$) and m.e.p.cs at room temperature ($71 \pm 22 \times 10^6$ M^{-1} s^{-1}, $n=6$) further suggesting that a change in ACh repetitive binding was not the basis of the effects of Org-9487 on τ_{epc}. Finally, G for Org-9487 was voltage-dependent. An increase in G with hyperpolarization is characteristic of the ion channel blocking properties of a positively charged compound such as a quaternary amine (Colquhoun *et al.*, 1979). It is unlikely that the ion channel blocking activity of Org-9487 contributes significantly to its neuromuscular block in the rat since, if this were the case, the twitch block would not be reversed by 3,4-diaminopyridine.

In conclusion, at high concentrations, Org-9487 blocks neuromuscular transmission in the rat hemidiaphragm muscle preparation. It is likely that the principal mechanism of action of the compound is a competitive antagonism of the postjunctional nicotinic AChR. The compound also possesses a number of other neuromuscular effects which could potentially enhance or depress its neuromuscular blocking activity. However, based on the initial clinical evaluation of the compound (Wierda *et al.*, 1994), it is likely that the additional neuromuscular effects of Org-9487, seen in rat tissue, would not complicate or compromise its use as a muscle relaxant in species, including man, where it has a relatively higher nicotinic AChR blocking potency.

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