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PRE- AND POST-JUNCTIONAL EFFECTS OF TUBOCURARINE AND OTHER NICOTINIC ANTAGONISTS DURING REPETITIVE STIMULATION IN THE RAT

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SUMMARY

1. The effects of tubocurarine and trimetaphan have been examined at voltage-clamped rat diaphragm neuromuscular junctions during (a) single and repetitive stimulation of the phrenic nerve in cut muscles and (b) repetitive ionophoretic application of acetylcholine (ACh).

2. Tubocurarine ($2{\cdot}5 \times 10^{-7}$–10^{-6} M) produced a concentration-dependent reduction in the amplitude of neurally evoked end-plate currents (e.p.c.s). It also reduced their time constant of decay ($\tau_{e.p.c.}$) in a maner that was independent of membrane potential, and not markedly dependent on the tubocurarine concentration. Likewise the snake α-neurotoxin, erabutoxin b, reduced the e.p.c. amplitude and produced a voltage-independent shortening of $\tau_{e.p.c.}$

3. Estimates of mean channel lifetime (τ_{noise}) from ACh-induced e.p.c. fluctuations revealed that (a) τ_{noise} was $46{\cdot}4 \pm 3{\cdot}7$ % shorter than $\tau_{e.p.c.}$ measured at the same end-plate. At these same end-plates in the presence of tubocurarine ($2{\cdot}5 \times 10^{-7}$ M) $\tau_{e.p.c.}$ was $32{\cdot}6 \pm 1{\cdot}0$ % shorter than the control $\tau_{e.p.c.}$ but tubocurarine did not change τ_{noise}, (b) trimetaphan ($2{\cdot}5 \times 10^{-5}$–2×10^{-4} M) produced a concentration-dependent and voltage-dependent reduction of $\tau_{e.p.c.}$, and a concentration-dependent reduction of peak e.p.c. amplitude. Trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) produced a 50 % reduction of τ_{noise}.

4. (a) Both tubocurarine and trimetaphan produced concentration-dependent increases in the run-down of trains of neurally evoked e.p.c.s (50 Hz, 0·4 s). This effect did not vary with membrane potential in tubocurarine, but was voltage dependent when induced by trimetaphan. (b) Erabutoxin b reduced the e.p.c. amplitude but did not produce any increase in the run-down of trains of neurally evoked e.p.c.s.

5. During 50 Hz repetitive ionophoretic application of ACh, tubocurarine ($2{\cdot}5 \times 10^{-7}$ M) reduced the amplitude of each current in the train without inducing any run-down of the current amplitudes. This effect was not dependent on the membrane potential. In contrast trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) induced a voltage-dependent run-down of trains of ionophoretically evoked e.p.c.s.

6. We conclude that tubocurarine and erabutoxin b reduce the e.p.c. amplitude

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by blocking the post-junctional ACh receptor. Tubocurarine produces tetanic run-down of e.p.c.s by a prejunctional mechanism, whereas the effects of trimetaphan during single and repetitive stimulation are at least partly due to block of the open ion channel associated with the ACh receptor.

INTRODUCTION

A rapidly waning tension ('tetanic fade') and the tetanic run-down of end-plate potentials (e.p.p.s) and end-plate currents (e.p.c.s) in a train, are characteristic of partial neuromuscular block produced by tubocurarine and related nicotinic antagonists (Paton & Zaimis, 1952; Liley & North, 1953; Glavinovic, 1979*b*).

There is evidence indicating that tubocurarine may have actions both pre- and post-junctionally in addition to a simple block of the post-junctional acetylcholine (ACh) receptor recognition sites (see Bowman, 1980, for review), and these actions may play a part in tetanic run-down. Controversy has arisen as to whether the action of tubocurarine which leads to run-down of trains of e.p.p.s or e.p.c.s during repetitive stimulation may be prejunctional in origin (Blaber, 1970, 1973; Hubbard & Wilson, 1973; Glavinovic, 1979*b*; Magleby, Pallotta & Terrar, 1981), or whether it represents a use-dependent block of the receptor-associated ion channel (Dreyer, 1982), of the type first suggested for tubocurarine by Mánalis (1977) and Katz & Miledi (1978) and later comprehensively studied by Colquhoun, Dreyer & Sheridan (1979).

In this study we have attempted to differentiate between these two mechanisms by comparing the effects of tubocurarine on trains of e.p.c.s produced, in the voltage-clamped cut hemidiaphragm of the rat, either by nerve stimulation or by repetitive ionophoretic application of ACh. In addition, we have compared the actions of tubocurarine with those of trimetaphan and the snake α-neurotoxin, erabutoxin b, as these two drugs have contrasting effects on tetanic tension. During partial neuromuscular block, trimetaphan produces a rapid waning of tetanic tension, resembling in appearance that produced by tubocurarine, whereas erabutoxin b produces no waning of tetanic tension (Gibb, Marshall & Bowman, 1982). We have found that the main action of trimetaphan can be ascribed to ion channel block, whereas erabutoxin b appears to block the ACh receptor selectively. Preliminary reports of some of these results have appeared in abstract form (Gibb & Marshall, 1982, 1983).

METHODS

All experiments were performed on phrenic nerve hemidiaphragm preparations from male Sprague Dawley rats (200–300 g in weight). The preparations were continuously perfused at room temperature (20–25 °C) with Krebs solution of composition (mM): NaCl, 118; KCl, 5; $CaCl_2$, 2·5; $NaHCO_3$, 30; KH_2PO_4, 1; $MgSO_4$, 1; glucose, 11; and pH of 7·4 when bubbled with a mixture of 95% O_2 and 5% CO_2 (v/v). A similar solution containing only 2 mM-KCl was used while cutting the muscle fibres to prevent nerve conduction block (Randic & Straughan, 1964; Glavinovic, 1979*a*).

Cut muscles were prepared essentially as described by Glavinovic (1979*a*), except that the muscle fibres were cut approximately 2 mm dorsal to, and 6 mm ventral to the main intramuscular nerve branch under a continuous stream of low-K Krebs solution (3–4 ml min^{-1}). Cutting in this manner left the main regions of surface end-plates lying in a band across the centre of the preparation. Occasionally fibres deep in the muscle continued to twitch in response to nerve stimulation. When

this occurred the mid-section of the fibres on the abdominal side of the muscle was removed. In addition to preventing movement in response to nerve stimulation this increased the resolution possible when the preparation was viewed under the microscope. Each preparation was gently stretched and pinned to a thin layer of Sylgard resin (Dow Corning) coating the base of a 4 ml Perspex tissue bath. After perfusing the cut muscle for a further 10–15 min with low-K Krebs solution, the solution was changed to one containing 5 mM-KCl, and the experiment begun in a further 5–10 min.

A Watson binocular compound microscope fitted with a Leitz ×20 long-range working distance objective giving a total magnification of ×300 was used to view the preparation. End-plates were located by following the finest nerve branches along the surface of the muscle to their ending, where the end-plate region could often be distinguished as an area of lighter colouring than the remainder of the muscle fibre. Microscope and micromanipulators were mounted on an air-damped flotation table (Ealing Vibration Isolation System).

The two-micro-electrode voltage-clamp system used was similar to that described by Dionne & Stevens (1975), in that membrane voltage was measured as the potential difference between the tip of the voltage recording electrode and the bathing solution which was held at virtual ground by means of a Ag/AgCl pellet placed in the bath. Clamp current was measured as the voltage drop across a 1 MΩ resistor placed in series with the current-passing electrode. The clamping circuit was arranged so that the clamp gain varied inversely with frequency by inserting a capacitor in series with the resistor in the feed-back loop of the clamp amplifier. The exact relationship of gain to frequency could be varied by independently changing both the resistance and the capacitance in the feed-back loop. The voltage follower, clamp amplifier and current monitor circuits had a band width of d.c.–10 kHz. The voltage recording electrodes were filled with 3 M-KCl and had resistances of 8–12 MΩ. The current-passing electrodes were filled either with 3 M-KCl or 0·6 M-K_2SO_4 (Dionne & Parsons, 1981) and had resistances of 4–8 MΩ. The amplitude and rate of rise of the miniature end-plate currents (m.e.p.c.s) was used to indicate if the end-plate region was satisfactorily clamped. M.e.p.c.s with a rise time (base line to peak) of less than 0·5 ms were regarded as satisfactory. The clamp gain was adjusted so that the membrane potential changed by less than 1% of the driving potential during an e.p.c.

E.p.c.s were elicited by stimulating the phrenic nerve with rectangular pulses of 0·05 ms duration. During single shock stimulation the nerve was stimulated at 0·5 Hz and during tetanic stimulation at 50 Hz for 0·4 or 1·9 s. In addition e.p.c.s were elicited by ionophoretic application of ACh. ACh was applied to the end-plate via ionophoretic electrodes of resistance 30–60 MΩ when filled with 2 M-ACh chloride. The electrodes were rejected if, when their tip touched the end-plate, they required a backing voltage of more than 0·8 V (backing current > 30 nA) to prevent any increase in holding current due to ACh leakage. The electrode was connected via an isolation device (W-P Instruments breakaway box model L BB-1) to a Grass S88 square-wave stimulator which supplied the command pulses to eject ACh from the electrode tip. Pulses of 10 μs duration and between 10 and 150 V in amplitude were used to apply ACh to the end-plate. Ionophoretically evoked e.p.c.s of short duration were recorded from whole muscles, which were prevented from contracting by adding tetrodotoxin (10^{-7} M) to the solution perfusing the muscle. The ionophoretic electrode was carefully manipulated around the end-plate until a 'hot spot' region was located (Kuffler & Yoshikami, 1975) where currents of less than 20 ms total duration could be elicited. These currents had rise times of less than 3 ms and decayed exponentially between 85 and 15% of their peak amplitude with time constants in the range of 3–6 ms.

Neurally or ionophoretically evoked e.p.c.s were initially recorded on FM tape (Racal 4DS, band width d.c.–5 kHz). Responses were subsequently analysed either manually after averaging with a Digitimer Neurolog NL750 signal averager and filming on 35 mm film for later enlargement, or they were digitized and analysed off-line using a PDP 11/23-based minicomputer. The computer interface (Cambridge Electronic Design Ltd. 502) was set to digitize at 25 kHz when sampling neurally evoked e.p.c.s and at 10 kHz when sampling trains of neurally evoked e.p.c.s or trains of ionophoretically evoked e.p.c.s. Computer analysis then consisted of calculating the e.p.c. amplitude from the difference between the 1 ms of base line before the e.p.c. and the point of largest amplitude during the e.p.c. This avoided contamination of the e.p.c. with its stimulus artifact which typically falls 3–4 ms before the neurally evoked e.p.c. A single exponential was fitted to the decay of the e.p.c. between 85 and 15% of its peak amplitude. With ionophoretically evoked e.p.c.s, the stimulus artifact falls immediately before the e.p.c. and may intrude upon the rising phase of the e.p.c.

Therefore, in some experiments, ionophoretically evoked e.p.c.s were measured manually from photographic records.

The experimental protocol for recording e.p.c.s was to voltage clamp a satisfactory end-plate at −60 mV and record e.p.c.s (0·5 Hz) and trains of e.p.c.s (50 Hz). The degree of run-down of these trains was quantified by calculating the mean amplitude of the eleventh to the twentieth e.p.c. in the train and expressing the difference between this and the amplitude of the first e.p.c. in the train as a percentage of the first e.p.c. in the train. The same recording procedure was used at −90 mV and at −35 mV and in addition e.p.c.s (0·5 Hz) were recorded at −20, −50, −70 and −80 mV. With some cells it was also possible to record e.p.c.s and trains at −120 mV. Cells were rejected at this stage if the peak current–voltage relation exhibited any obvious non-linearity, or if the e.p.c.s at any holding potential appeared to decay in a non-exponential manner. The membrane potential was then clamped at or near the resting potential, and the perfusing solution changed to one containing the drug concentration to be studied before repeating the above measurements in the presence of the drug.

ACh-induced e.p.c. fluctuations were elicited either from cut muscles or from muscles treated with tetrodotoxin (10^{-7} M) by backing the ionophoretic electrode off the end-plate region by 20–50 μm and then applying 5–10 s bursts of ACh to the end-plate. The ACh-induced e.p.c. fluctuations were recorded on one FM tape channel as a low-gain d.c. record (d.c.–5 kHz), and on a separate channel as a high-gain a.c. record (2 Hz–5 kHz). The amplitude of the drug-induced current was then measured from the low-gain record, while the high-gain signal was used for later analysis on the PDP 11/23 minicomputer. Data aquisition for analysis consisted of collecting separate 250 ms segments of data which had been digitized at 2 kHz after filtering (high-pass 2 Hz and low-pass 800 Hz by a Cawkell 1471 variable filter of 34 dB octave^{-1} roll-off) to avoid aliasing errors (Katz & Miledi, 1972; Anderson & Stevens, 1973).

These segments of data were then edited visually, and any segments containing m.e.p.c.s or obvious artifacts were deleted before analysis. Between four and thirty-two segments of control noise and sixteen to sixty-four segments of ACh-induced noise were collected in this way from each instance where ACh was applied to the end-plate. The data points in each segment were then reduced to differences from their mean and their variance calculated. A 0·1 cosine taper data window was applied and spectral density as a function of frequency was determined by fast Fourier transform. Power spectral density was then averaged around thirty-six frequencies, exponentially distributed between 0 and 999·2 Hz. A base-line spectrum was then calculated by averaging the spectra obtained from the control data segments and this was subtracted from the average of the ACh-induced spectra to yield the final spectrum. A single-sided Lorentzian function of the form

$$S(f) = \frac{S(0)}{1+(2\pi f\tau_n)^2},$$

where $S(f)$ is the power spectral density at frequency f, $S(0)$ is the d.c. power spectral density and $\tau_n = 1/2\pi f_c$ where f_c is the frequency at which the power spectral density falls to one-half of $S(0)$. Channel opening was assumed to follow the sequential scheme proposed by Del Castillo & Katz (1957):

$$A + T \underset{k-1}{\overset{k+1}{\rightleftharpoons}} AT \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR, \qquad (1)$$

where A represents the agonist, acetylcholine, T the receptor, AT the agonist–receptor complex and AR the conducting form of the agonist–receptor complex. The rate constants $k+1$ and $k-1$ refer to the association and dissociation of agonist with receptor and β and α represent the opening and closing rates of the ion channel. If it is assumed that $k+1$ and $k-1$ are much faster than α and β then τ_{noise} will be

$$\frac{1}{\alpha+\beta[AT]/([T]+[AT])}.$$

In the limiting situation where agonist concentration is low (relative to $k-1/k+1$) then τ_{noise} will tend to the mean lifetime of the channels, $1/\alpha$. Thus, τ_{noise} will reflect the mean channel lifetime when only a small fraction of the total end-plate channels are activated by ACh (Anderson & Stevens, 1973; Colquhoun, 1981). In these experiments the mean drug-induced current was approximately 30 nA in the absence of any blocking drug and single channel conductance was around 37 pS. This corresponds to an average of 15000 ion channels open which is 0·15 % of a possible

total of 10^7 channels at an end-plate (Fambrough & Hartzell, 1972; Colquhoun *et al.* 1979). In the presence of a drug able to block the ion channel, the channel can be closed by the normal route or due to drug binding to state AR, thus

$$T \rightleftharpoons AT \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR + D \underset{G}{\overset{F}{\rightleftharpoons}} ARD \tag{2}$$

(Adams, 1976, 1977; Ruff, 1977, 1982; Neher & Steinbach, 1978), where F and G are the blocking and unblocking rate constants respectively. In this situation

$$\frac{1}{\alpha + F[D]} \tag{3}$$

will equal the mean lifetime of the channels in the presence of the channel blocker (Colquhoun & Sheridan, 1981). The time constants for noise or relaxation will approximate to this if it is assumed that $F \gg G$ and $F[D]+\alpha > G$. This situation was assumed to arise in the presence of trimetaphan as e.p.c.s and m.e.p.c.s in its presence decayed as single exponentials of more rapid decay than in its absence. Therefore, although scheme (2) predicts that the macroscopic fluctuations from a three state system will have two kinetically distinguishable states, and hence produce spectra which are the sum of two Lorentzian components of different half-power frequencies, only single Lorentzians were fitted to the spectra in the presence of trimetaphan over the frequency range 7·8–800 Hz. These spectra rolled off with a limiting slope not significantly different from $1/f^2$ indicating that any second Lorentzian component was of low power in this frequency range. The resolution of the spectra in the lower frequency range did not allow the measurement of a second Lorentzian component in the presence of trimetaphan.

Single channel conductance γ was calculated from the relationship

$$\gamma(\text{spec}) = \frac{S(0)}{4\mu(V_m - E_0)\tau_n},$$

and from the variance of the noise induced by the drug by the relationship

$$\gamma(\text{var}) = \frac{S^2}{\mu(V_m - E_0)},$$

where S^2 is the variance of the drug-induced current calculated as the difference between the directly calculated variances of the edited noise in the presence and absence of the agonist, μ is the mean drug-induced current, V_m, the membrane potential, and E_0 the reversal potential. γ(var) was corrected to allow for high frequency loss due to filtering using the formula

$$\gamma(\text{var corr}) = \gamma(\text{var}) 2/\pi \tan^{-1}(2\pi f_f \tau_n),$$

where f_f is the filter cut-off frequency used during the data aquisition and τ_n is the mean channel lifetime calculated from the spectrum (Colquhoun, Large & Rang, 1977).

Drugs and statistics

The drugs used were ACh chloride, tetrodotoxin and (+)-tubocurarine chloride (all Sigma), trimetaphan camphosulphonate (Roche) and erabutoxin b, a neurotoxin from the venom of *Laticauda semifasciata* supplied by Dr A. L. Harvey. Drugs were diluted from concentrated stock solution to the desired concentration with the Krebs solution that was superfusing the preparation during the experiment. Each drug concentration was examined in four to seven different preparations. Generally, only one cell was used from each preparation and only occasionally was it possible to record the effects of more than one concentration of a drug on any individual cell. Where results were obtained from more than one cell in any one preparation, the preparation was washed for between 35 and 50 min with drug-free Krebs solution before control responses from the second cell were recorded. Statistical differences between mean values were tested by Student's t test ($P < 0.05$ being regarded as significant).

RESULTS

End-plate responses to single shock stimulation in the cut rat hemidiaphragm

In the cut hemidiaphragm the peak amplitude of the e.p.c. increased progressively with hyperpolarization of the post-junctional membrane. In amphibian muscle fibres hyperpolarization produces a smaller increase in the e.p.c. amplitude than would be predicted from a linear current–voltage relationship (Dionne & Stevens, 1975). However, in the rat, a linear current–voltage relationship was found in most fibres (Fig. 1*A*) and in those fibres not showing a linear relationship, hyperpolarization increased the e.p.c. amplitude more than would have been predicted. The null potential was estimated in some experiments as the potential at which no current deflexion was seen to occur in response to nerve stimulation and was $-6{\cdot}9 \pm 0{\cdot}6$ mV ($n = 23$ fibres). The e.p.c.s decayed as single exponentials (85–15 % of peak) according to the relationship $I(t) = I(0) \exp(-t/\tau)$, where $I(t)$ is the e.p.c. amplitude at time t after its peak, $I(0)$ is the amplitude at $t = 0$ of the exponential fitted to the e.p.c. decay phase and τ is the time constant of decay of the e.p.c. $\tau_{e.p.c.}$ increased exponentially with hyperpolarization according to the relationship: $\tau\ (V_m) = \tau\ (0) \exp(-V_m/H)$ where $\tau(V_m)$ is the decay time constant at membrane potential V_m, $\tau(0)$ is the decay time constant at zero membrane potential and H is the change in membrane potential required to produce an e-fold change in τ. Thus, semi-logarithmic plots of $\tau_{e.p.c.}$ *versus* membrane potential resulted in straight-line plots (Fig. 1*C*). The control e.p.c. quantal content, as assessed by the direct method of e.p.c. amplitude/m.e.p.c amplitude, was 57 ± 5 quanta ($n = 27$ fibres). The decay characteristics of m.e.p.c.s and e.p.c.s recorded from cut muscle preparations were not significantly different from those of m.e.p.c.s recorded from tetrodotoxin-treated preparations. For example, at -90 mV $\tau_{m.e.p.c.} = 1{\cdot}46 \pm 0{\cdot}07$ ms and $\tau_{e.p.c.} = 1{\cdot}47 \pm 0{\cdot}07$ ms ($n = 26$) in cut muscles whereas $\tau_{m.e.p.c.} = 1{\cdot}56 \pm 0{\cdot}08$ ms ($n = 14$) in tetrodotoxin-treated preparations. The voltage dependence of $\tau_{e.p.c.}$ in cut muscles ($H = -136 \pm 10$ mV, $n = 26$) was slightly less than the voltage dependence of $\tau_{m.e.p.c.}$ in tetrodotoxin-treated preparations ($H = -90 \pm 8$ mV, $n = 14$).

Effects of tubocurarine

Tubocurarine ($2{\cdot}5 \times 10^{-7}$–10^{-6} M) produced a concentration-dependent reduction in peak e.p.c. amplitude that reached a steady level for each concentration in 5–7 min. In the presence of tubocurarine, the relationship between membrane potential and peak e.p.c. amplitude remained linear (Fig. 1*A*). In all concentrations studied, tubocurarine decreased $\tau_{e.p.c.}$, the e.p.c.s decaying as single exponential functions in its presence (Fig. 1*B*). Tubocurarine ($2{\cdot}5 \times 10^{-7}$ M) did not significantly change the voltage-dependence of $\tau_{e.p.c.}$ and $5{\cdot}0 \times 10^{-7}$ M and $7{\cdot}5 \times 10^{-7}$ M produced a slight increase in the voltage sensitivity of $\tau_{e.p.c.}$ (Fig. 1*C*). Thus the voltage changes producing an e-fold change in $\tau_{e.p.c.}$ (H value) were -159 ± 63 mV, -111 ± 18 mV and $-87 \pm 13{\cdot}3$ mV in 2·5, 5·0 and $7{\cdot}5 \times 10^{-7}$ M-tubocurarine respectively, compared to a control H value of -146 ± 13 mV. Control values shown in Fig. 1*C* are the mean of all control values from each of the three groups of experiments. As control values varied between groups of experiments, the positions of the lines for the three tubocurarine concentrations, relative to the control line, do not reflect their position

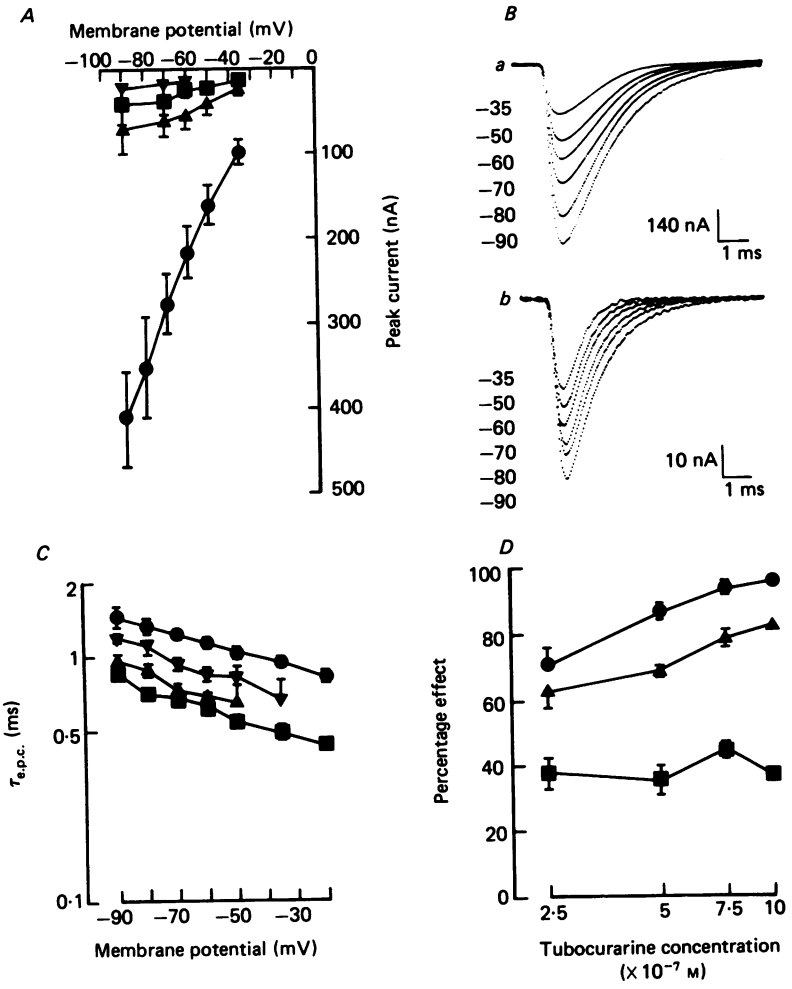

Fig. 1. Effects of tubocurarine on e.p.c.s neurally evoked at 0·5 Hz from a cut rat hemidiaphragm. *A*, relationship between holding potential and the peak amplitude of the e.p.c. in the absence of tubocurarine (●) and in the presence of $2{\cdot}5 \times 10^{-7}$ M (▲), $5{\cdot}0 \times 10^{-7}$ M (■) and $7{\cdot}5 \times 10^{-7}$ M (▼) tubocurarine. *B a*, digitized e.p.c.s from a single end-plate recorded between −35 and −90 mV at the holding potentials shown by the symbols in *A* and superimposed to allow comparison of their amplitude and time course. *B b*, e.p.c.s from the same end-plate and at the same holding potentials as *a* recorded in the presence of $7{\cdot}5 \times 10^{-7}$ M-tubocurarine. *C*, semilogarithmic plots of the effect of membrane potential on the time constant of decay ($\tau_{e.p.c.}$) of the e.p.c. recorded in the absence (●) of tubocurarine, and in the presence of $2{\cdot}5 \times 10^{-7}$ M (▲), $5{\cdot}0 \times 10^{-7}$ M (■) and $7{\cdot}5 \times 10^{-7}$ M (▼) tubocurarine. *D*, concentration dependence of the percentage reduction of the peak e.p.c. amplitude (●), percentage run-down of trains of e.p.c.s neurally evoked at 50 Hz (▲) and percentage reduction of $\tau_{e.p.c.}$ (■) produced by tubocurarine.

relative to their own control line. Thus, as shown in Fig. 1*D* each concentration of tubocurarine produced essentially the same percentage reduction in $\tau_{e.p.c.}$ The mean channel lifetime measured at −60 mV in cut muscle preparations by noise analysis was 46·4 ± 3·7 % smaller than control $\tau_{e.p.c.}$ ($n = 4$) and was not significantly changed by tubocurarine ($2{\cdot}5 \times 10^{-7}$ M), whereas tubocurarine reduced $\tau_{e.p.c.}$ by 32·6 ± 1·0 % (Fig. 2).

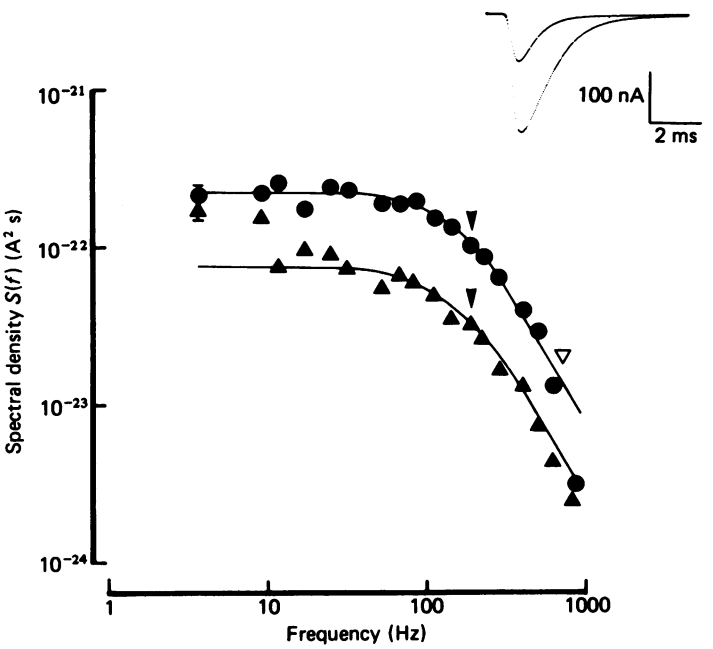

Fig. 2. Representative log–log plots of power spectral density, $S(f)$, *versus* frequency, of ACh-induced e.p.c. fluctuations recorded from a single end-plate of a cut rat hemidiaphragm in the absence (●) and in the presence (▲) of $2{\cdot}5 \times 10^{-7}$ M-tubocurarine. Membrane potential −60 mV, 22 °C. Each spectrum contained thirty-six points which were averaged in pairs for ease of plotting to produce the spectra shown. Each continuous line is the best-fit single-sided Lorentzian function calculated as described in the Methods. The error bars at 5·8 Hz indicate the standard error of the spectra which was produced by averaging the spectra from thirty-two 250 ms stretches of ACh-induced noise. The symbol ▽ indicates the filter cut-off frequency used during data acquisition (800 Hz) and the arrows indicate the half-power frequency (f_c) of the spectra: in the absence of tubocurarine $f_c = 188$ Hz indicating a mean channel lifetime, $\tau_{noise} = 0{\cdot}85$ ms with single channel conductance $\gamma = 21{\cdot}3$ pS. In the presence of tubocurarine $f_c = 192$ Hz, $\tau_{noise} = 0{\cdot}83$ ms, $\gamma = 21{\cdot}7$ pS. The inset represents superimposed, digitized e.p.c.s recorded at −60 mV from the same end-plate in the absence ($\tau_{e.p.c.} = 1{\cdot}29$ ms, amplitude = 239 nA) and in the presence of tubocurarine ($\tau_{e.p.c.} = 0{\cdot}85$ ms, amplitude = 93 nA). In four experiments $\tau_{e.p.c.} = 1{\cdot}11 \pm 0{\cdot}08$ ms, $\tau_{noise} = 0{\cdot}65 \pm 0{\cdot}05$ ms and $\gamma = 31{\cdot}1 \pm 2{\cdot}92$ pS in the absence of tubocurarine and $\tau_{e.p.c.} = 0{\cdot}77 \pm 0{\cdot}05$ ms, $\tau_{noise} = 0{\cdot}71 \pm 0{\cdot}06$ ms and $\gamma = 42{\cdot}7 \pm 10{\cdot}6$ pS in the presence of tubocurarine.

Effects of erabutoxin b

The irreversible post-junctionally active snake toxin erabutoxin b was more difficult to study than tubocurarine, as its effects on the e.p.c. did not reach a steady state but gradually increased until the e.p.c.s were no longer measurable. This made

the study of a range of concentrations of little significance, and hence a concentration of 1 μg ml^{-1} (mol. wt. = 6800) was selected. In this concentration complete block of the e.p.c. responses occurred in 4–25 min, and e.p.c.s and trains of e.p.c.s were recordedwhen the e.p.c. had been reduced to approximately 50 % of control amplitude. Like tubocurarine, erabutoxin b produced around a 30 % reduction of $\tau_{e.p.c.}$ at all membrane potentials studied (Fig. 3), the e.p.c.s decaying as single exponentials. The voltage dependence of $\tau_{e.p.c.}$ in erabutoxin b corresponded to an H value of $-94{\cdot}8 \pm 12{\cdot}2$ mV compared to a control value of $-124{\cdot}5 \pm 6{\cdot}8$ mV ($n = 7$).

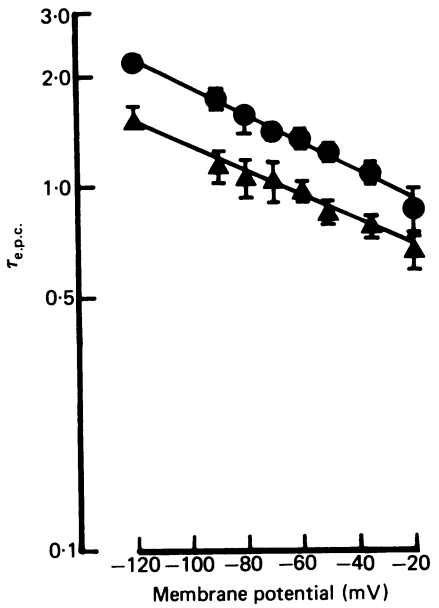

Fig. 3. Relationship between $\tau_{e.p.c.}$ and holding potential in control (●) and after approximately 50 % reduction of the e.p.c. amplitude by 1 μg ml^{-1} erabutoxin b (▲), $n = 7$ preparations.

Effects of trimetaphan

Trimetaphan ($2{\cdot}5 \times 10^{-5}$–2×10^{-4} M) produced a concentration-dependent decrease in the amplitude of intracellularly recorded e.p.c.s and m.e.p.c.s and of extracellularly recorded miniature end-plate potentials (m.e.p.p.s). Current–voltage plots of the e.p.c. amplitude in the presence of trimetaphan remained linear (Fig. 4*A*). Trimetaphan also produced a concentration-dependent shortening of the decay of e.p.c.s, m.e.p.c.s and extracellularly recorded m.e.p.p.s which decayed as single exponentials in its presence (Fig. 4*D*). The reduction in $\tau_{e.p.c.}$ was greatest at the more negative membrane potentials studied (Fig. 4*B*). Thus, $2{\cdot}5 \times 10^{-5}$ M-trimetaphan reduced the voltage dependence of $\tau_{e.p.c.}$, and 10^{-4} M and 2×10^{-4} M produced a reversal of the voltage dependence of $\tau_{e.p.c.}$ ($H = -313 \pm 55$ mV, $n = 7$; $+206 \pm 238$ mV, $n = 5$; and

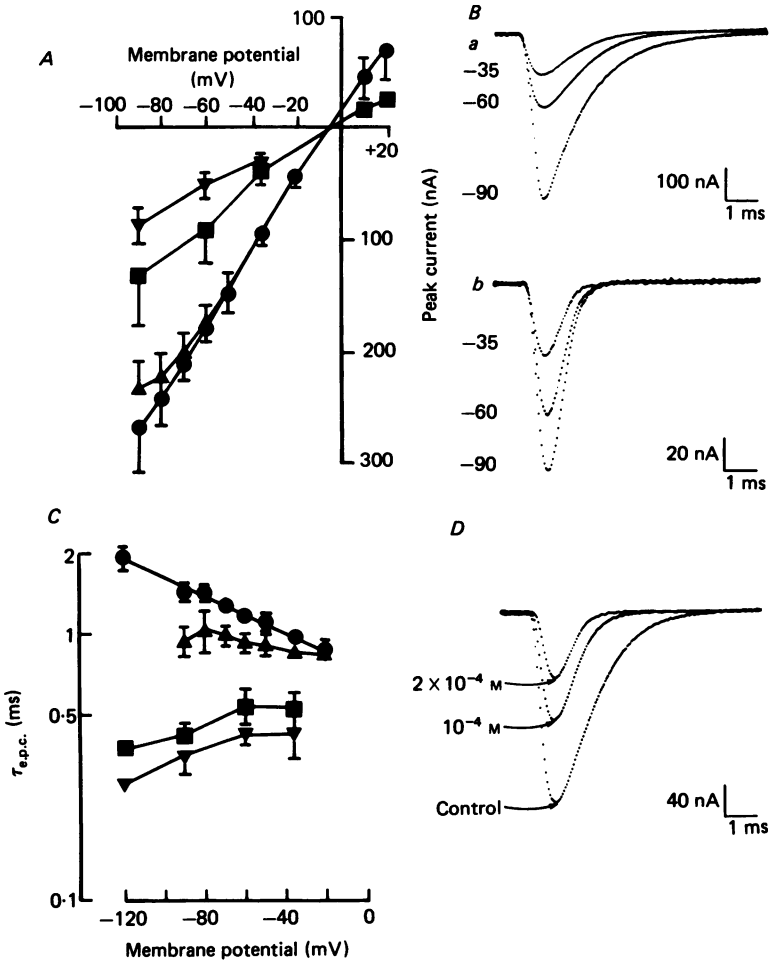

Fig. 4. *A*, current–voltage plot in the absence (●) and in the presence of trimetaphan $2{\cdot}5 \times 10^{-5}$ M (▲), 10^{-4} M (■) and $2{\cdot}0 \times 10^{-4}$ M (▼). *B*, digitized e.p.c.s recorded from a single end-plate in control (*a*) and in the presence of trimetaphan $2{\cdot}0 \times 10^{-4}$ M (*b*) at −35, −60 and −90 mV holding potential. *C*, relation between $\tau_{e.p.c.}$ and holding potential in the absence of trimetaphan (●) and in the presence of $2{\cdot}5 \times 10^{-5}$ M (▲), 10^{-4} M (■) and $2{\cdot}0 \times 10^{-4}$ M (▼) trimetaphan. *D*, digitized e.p.c.s from a single end-plate showing the concentration dependence of the effects of trimetaphan (10^{-4} M and $2{\cdot}0 \times 10^{-4}$ M) at a holding potential of −60 mV on both the amplitude and time course of the e.p.c.

$+190 \pm 116$ mV, $n = 4$ respectively compared to a control H value of $-139 \pm 10{\cdot}4$ mV). The effects of trimetaphan were similar on both cut muscles and tetrodotoxin-treated preparations. Trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) also reduced the mean channel lifetime estimated by noise analysis at −90 mV in muscles paralysed by tetrodotoxin (10^{-7} M) (Fig. 5).

Effects of tubocurarine, trimetaphan and erabutoxin b on trains of neurally evoked e.p.c.s.

Both tubocurarine and trimetaphan produce a fade of tension responses evoked by 50 Hz stimulation of the phrenic nerve (Gibb & Marshall, 1982, 1983), and both compounds produced a concentration-dependent run-down of trains of neurally evoked e.p.c.s elicited at 50 Hz (Figs. 6 and 7). In the absence of any drug trains of e.p.c.s run down by 55 ± 4 % as estimated from the ratio of the mean amplitude of

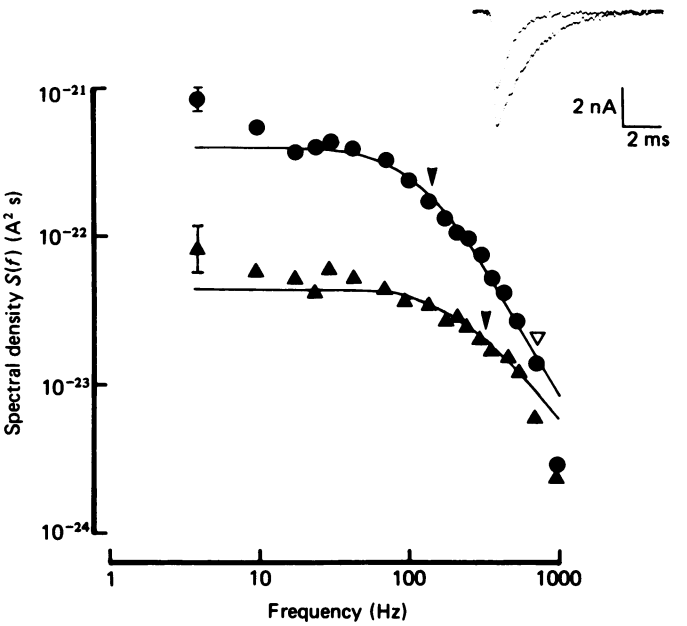

Fig. 5. Representative log–log plots of power spectral density, $S(f)$, *versus* frequency of e.p.c. fluctuations recorded from a rat hemidiaphragm paralysed with tetrodotoxin (10^{-7} M). The spectra were constructed as described for Fig. 2. Membrane potential −90 mV, 22 °C. The half-power frequency of the spectra $f_c = 138$ Hz corresponding to $\tau_{noise} = 1{\cdot}15$ ms and $\gamma = 41$ pS in the absence (●) of trimetaphan and $f_c = 317$ Hz, $\tau_{noise} = 0{\cdot}502$ ms, $\gamma = 43$ pS in the presence (▲) of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M). Inset are shown superimposed m.e.p.c.s recorded from the same end-plate in the absence of trimetaphan: $\tau_{m.e.p.c.} = 1{\cdot}5$ ms, amplitude = 5·6 nA and in the presence of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) $\tau_{m.e.p.c.} = 0{\cdot}65$ ms, amplitude = 3·6 nA. In four experiments $\tau_{m.e.p.c.} = 1{\cdot}43 \pm 0{\cdot}12$ ms, $\tau_{noise} = 1{\cdot}23 \pm 0{\cdot}14$ ms and $\gamma = 43{\cdot}4 \pm 11{\cdot}5$ pS in the absence of trimetaphan and $\tau_{m.e.p.c.} = 0{\cdot}65 \pm 0{\cdot}05$ ms, $\tau_{noise} = 0{\cdot}50 \pm 0{\cdot}02$ ms and $\gamma = 55{\cdot}5 \pm 3{\cdot}7$ pS in the presence of trimetaphan.

the eleventh to the twentieth e.p.c. in each train divided by the amplitude of the first e.p.c. in the train and this run-down did not vary with membrane potential (Figs. 6 and 7). The control quantal content of the first e.p.c. in the train calculated by the direct method was $65 \pm 5{\cdot}4$ ($n = 19$, $E_m = -90$ mV) and the quantal content in the plateau of the train was $26 \pm 2{\cdot}2$. Thus the percentage fall-off in quantal content during these control trains was $59 \pm 3{\cdot}1$ %. It was not possible to calculate quantal content by the direct method in the presence of a blocking drug. Therefore, the fall-off in e.p.c. amplitude during trains has been expressed throughout as a percentage

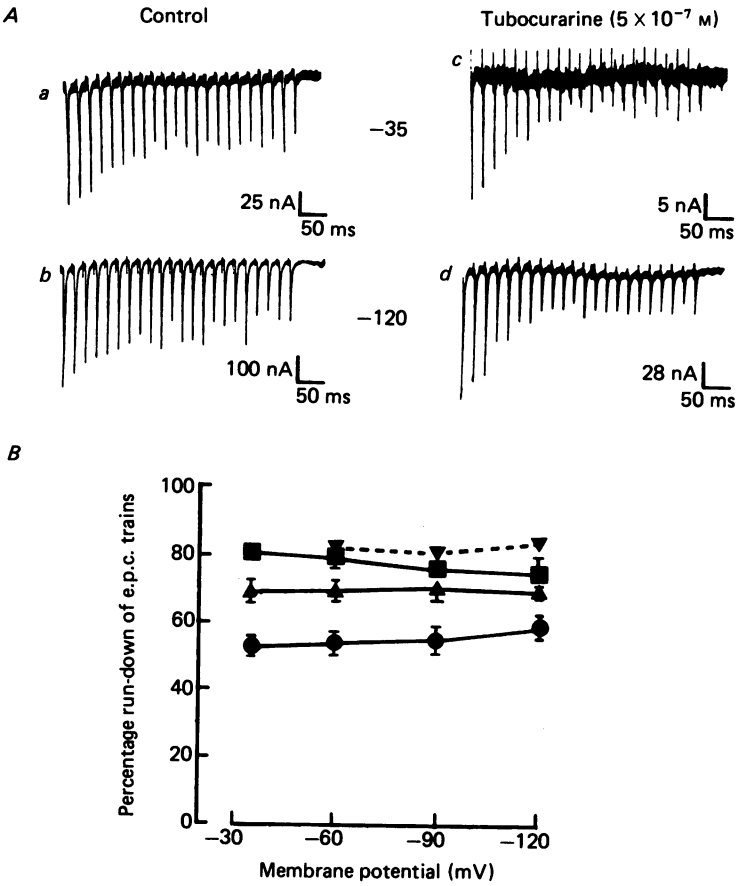

Fig. 6. Concentration dependence, but lack of voltage dependence of the effects of tubocurarine on strains of e.p.c.s neurally evoked at 50 Hz. *A*, control trains at −35 mV (*a*) and −120 mV (*b*). Trains at −35 mV (*c*) and −120 mV (*d*) from the same end-plate as *a* and *b* in the presence of tubocurarine (5×10^{-7} M). *B*, percentage run-down of e.p.c. trains plotted against membrane potential in the absence (●) and in the presence of 5×10^{-7} M (▲, $n = 6$), $7{\cdot}5 \times 10^{-7}$ M (■, $n = 6$) and 10^{-6} M (▼, $n = 1$) tubocurarine. (See text for details of calculations.)

run-down of the train. As previously reported by Magleby *et al.* (1981), the increase in e.p.c. train run-down produced by tubocurarine was independent of membrane potential (Fig. 6). In contrast, the run-down produced by trimetaphan was voltage dependent, being greater at more negative potentials (Fig. 7). In relation to the degree of e.p.c. run-down, trimetaphan produced a much smaller reduction of the amplitude of the first e.p.c. in the train than did tubocurarine (Figs. 6 and 7). Erabutoxin b (1 μg ml^{-1}) produced no significant change in the run-down of trains of e.p.c.s, run-down in control being $57{\cdot}5 \pm 4{\cdot}01$ % and after erabutoxin b $61{\cdot}2 \pm 3{\cdot}4$ %.

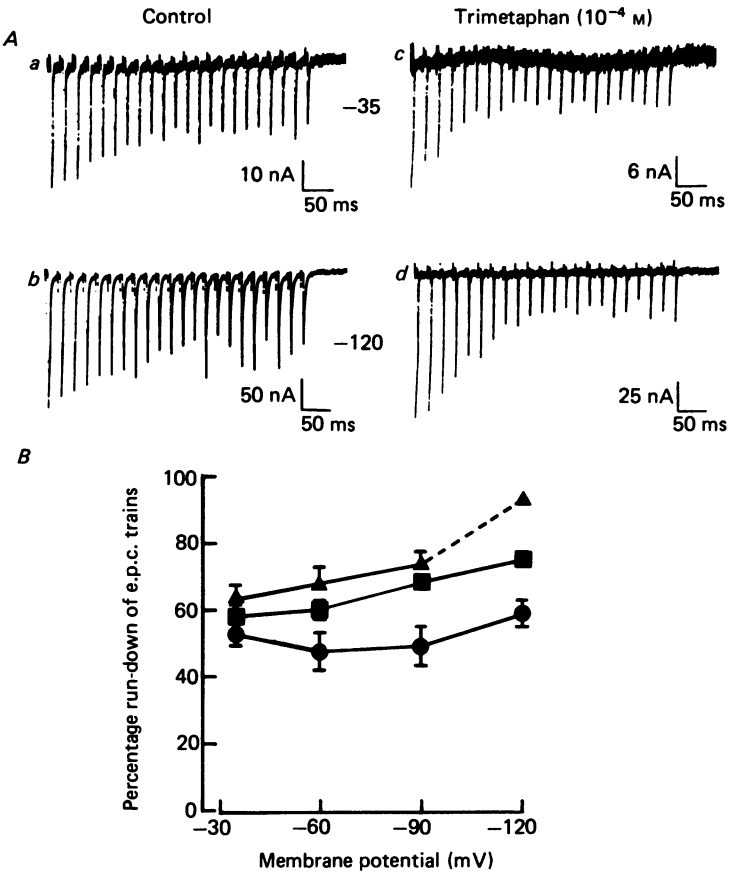

Fig. 7. Concentration and voltage dependence of the effects of trimetaphan on trains of e.p.c.s neurally evoked at 50 Hz. *A*, trains at −35 mV (*a*) and −120 mV (*b*) in the absence of trimetaphan and at −35 mV (*c*) and −120 mV (*d*) from the same end-plate as *a* and *b* in the presence of trimetaphan (10^{-4} M). *B*, percentage run-down of e.p.c. trains plotted against membrane potential in the absence (●) of trimetaphan and in the presence of trimetaphan 10^{-4} M (■, $n = 5$) and 2×10^{-4} M (▲, $n = 4$). The point joined by the dashed line is the result of a single experiment. (See text for details of calculations.)

Comparison of the effects of tubocurarine and trimetaphan on ionophoretically elicited trains of e.p.c.s

Because of the lack of voltage dependence of the e.p.c. train run-down in tubocurarine, Magleby *et al.* (1981) concluded that the effect was not a consequence of channel block and must therefore be the result of a prejunctional action. To test this hypothesis, the effects of tubocurarine and trimetaphan were examined on trains of e.p.c.s evoked by repetitive (50 Hz) ionophoretic application of short pulses of ACh to the end-plate, and these effects were compared to their effects on trains of neurally evoked e.p.c.s. Both tubocurarine ($2{\cdot}5 \times 10^{-7}$ M) and trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) reduced the amplitude of the ionophoretically evoked e.p.c.s. Tubocurarine produced a similar percentage reduction of the amplitudes of the e.p.c.s in

both ionophoretically evoked and neurally evoked trains (Fig. 8). The concentration of trimetaphan used ($2{\cdot}5 \times 10^{-5}$ M), produced a much greater effect on the initial ionophoretically evoked e.p.c. than on the initial neurally evoked e.p.c.: it produced a 60% reduction of the ionophoretically evoked e.p.c. amplitude, but no significant effect on the neurally evoked e.p.c. amplitude (Fig. 9). Provided that the ionophoretically evoked e.p.c.s were of less than 20 ms duration, it was possible to elicit trains of such e.p.c.s at 50 Hz without summation of the currents. In the

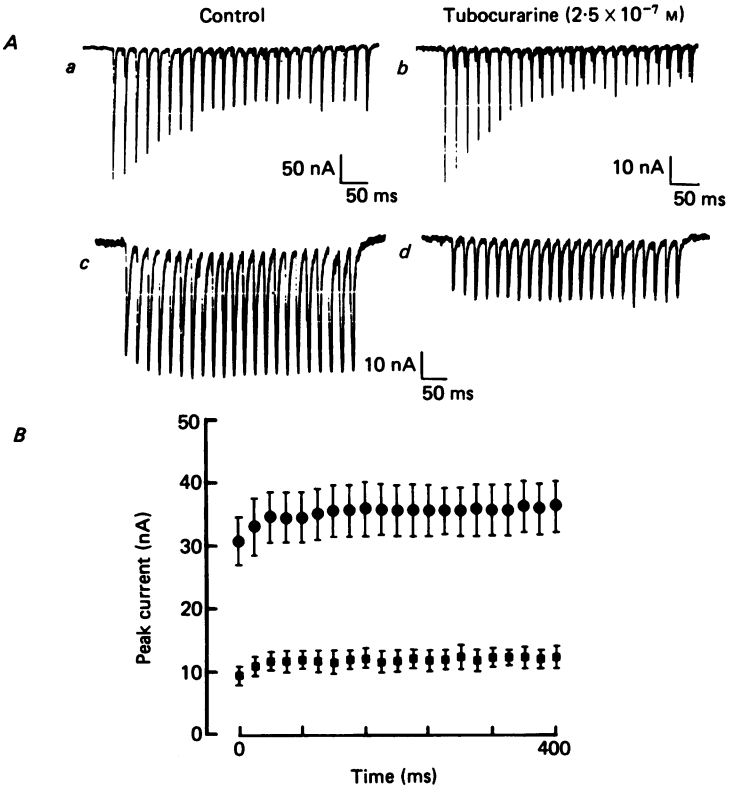

Fig. 8. Effect of tubocurarine ($2{\cdot}5 \times 10^{-7}$ M) on trains of ionophoretically evoked e.p.c.s. *A a* and *b* show for comparison, the run-down of trains of e.p.c.s neurally evoked at 50 Hz in the absence (*a*) and in the presence (*b*) of tubocurarine ($2{\cdot}5 \times 10^{-7}$ M). *c* and *d* show trains of e.p.c.s ionophoretically evoked at 50 Hz in the absence (*c*) and in the presence (*d*) of tubocurarine ($2{\cdot}5 \times 10^{-7}$ M). *B*, plot of the mean ±S.E. of mean amplitude ($n = 6$) of each ionophoretically evoked e.p.c. in the train in the absence (●) and in the presence (■) of tubocurarine ($2{\cdot}5 \times 10^{-7}$ M). Membrane potential −90 mV, 22 °C.

absence of either drug trains of ionophoretically evoked e.p.c.s increased in amplitude over the first three responses of the train and then remained constant in amplitude during the remainder of the train.

Tubocurarine ($2{\cdot}5 \times 10^{-7}$ M), in contrast to its effect on neurally evoked e.p.c.s, produced no run-down of trains of ionophoretically evoked e.p.c.s, but simply reduced the amplitude of each e.p.c. in the train by $55{\cdot}6 \pm 9{\cdot}8\%$ ($n = 6$, $E_m = -90$ mV) compared to the corresponding e.p.c. in the control train (Fig. 8).

There was no change in the profile of these trains evoked in the presence of tubocurarine over the range of membrane potentials studied (−60 to −140 mV). In contrast to tubocurarine, trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) produced essentially similar effects on the run-down of both neurally and ionophoretically evoked e.p.c. trains (Fig. 9). The run-down of ionophoretically evoked trains in trimetaphan was significantly greater at more negative membrane potentials ($41{\cdot}1 \pm 7{\cdot}5$ % at −90 mV and $52{\cdot}7 \pm 6{\cdot}1$ % at −120 mV, $P < 0{\cdot}05$, paired t test, Fig. 9).

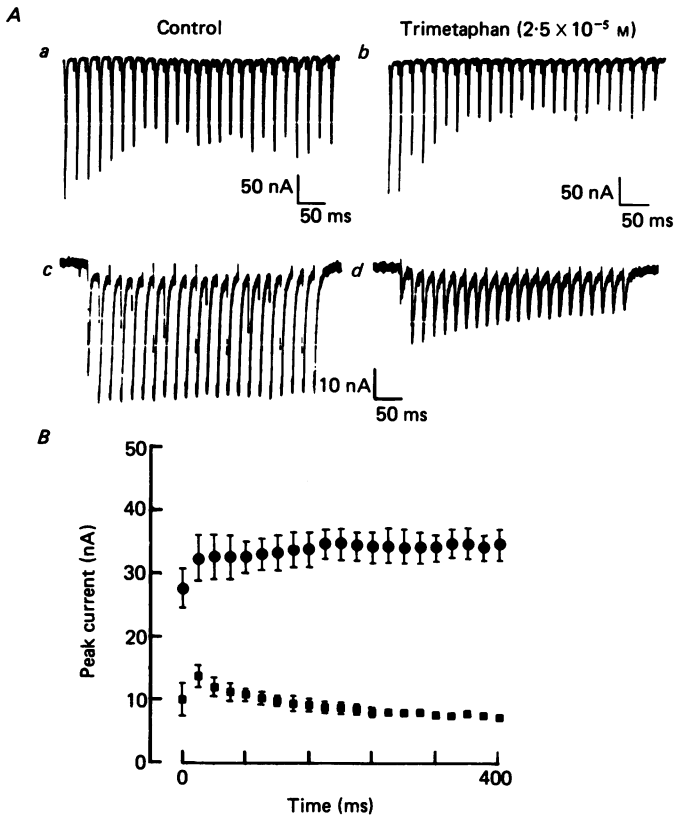

Fig. 9. Effect of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) on trains of ionophoretically evoked e.p.c.s. *A a* and *b* show for comparison the run-down of trains of e.p.c.s neurally evoked at 50 Hz in the absence (*a*) and in the presence (*b*) of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M). *c* and *d* show trains of e.p.c.s ionophoretically evoked at 50 Hz in the absence (*c*) and in the presence (*d*) of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M). *B*, plot of the mean ±S.E. of mean amplitude ($n = 4$) of each ionophoretically evoked e.p.c. in the train in the absence (●) and in the presence (■) of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M). Membrane potential = −90 mV, 22 °C.

DISCUSSION

The results presented demonstrate that the increased run-down of trains of e.p.c.s produced by tubocurarine, is independent of its post-junctional effects. Thus, addition of low concentrations of tubocurarine to the solution bathing the isolated rat diaphragm leads to a reduction in the amplitude of e.p.c.s elicited by either

stimulation of the phrenic nerve or by ionophoresis of ACh onto the end-plate region. However, tubocurarine produces an increase in the run-down of the amplitude of 50 Hz trains of e.p.c.s only when they are elicited by nerve stimulation and not when they are evoked ionophoretically. This result demonstrates that the ability of tubocurarine to produce e.p.c. train run-down is mediated prejunctionally.

Liley & North (1953) demonstrated that in the rat hemidiaphragm paralysed with tubocurarine, repetitive stimulation of the phrenic nerve produces a run-down of the e.p.p. amplitudes. These workers assumed that this was a consequence of a fall-off in transmitter output which was a physiological response to high-frequency stimulation and was merely unmasked, rather than caused by tubocurarine. Otsuko, Endo & Nonomura (1962) reached a similar conclusion. The development of the cut hemidiaphragm preparation (Barstad, 1962; Randic & Straughan, 1964; Lilleheil, 1965; Barstad & Lilleheil, 1968) allowed the electrophysiological measurement of normal levels of transmitter release in the absence of blocking drugs. Subsequent experiments in cut rat diaphragms (Hubbard, Wilson & Miyamoto, 1969; Hubbard & Wilson, 1973) and in cut cat tenuissimus muscles (Blaber, 1970, 1973) showed that tubocurarine increases the run-down of e.p.p.s in a high-frequency train compared to the run-down in the absence of any drug. This effect is accompanied by an increase in the coefficient of variance of the e.p.p. amplitudes in the plateau region of the train, compared to control, in both cat and rat, and was interpreted in terms of an inhibition of transmitter mobilization by tubocurarine, leading to a fall-off in quantal release during rapid stimulation, and hence to a reduced quantal content of the e.p.p.s in the plateau of the train.

Voltage-clamp techniques allow the recording of end-plate responses without the non-linear summation associated with potential recording. Our results agree well with those of Glavinovic (1979*b*) and Magleby *et al.* (1981) who demonstrated tubocurarine-induced e.p.c. run-down under voltage-clamp conditions, thereby proving that tubocurarine-induced run-down was not merely due to the unmasking of an already present run-down undetectable in non-clamped cut muscle due to the effects of non-linear summation. Although most of these workers concluded that tubocurarine-induced run-down was mediated prejunctionally, e.p.c. run-down might also be produced by any use-dependent post-junctional action of a drug. Blockade of the ACh-activated ion channel is one such mechanism and has been demonstrated for a wide range of compounds exemplified by the barbiturates (Adams, 1976, 1981) and local anaesthetics (Beam, 1976*a*, *b*; Ruff, 1976, 1977, 1982). Tubocurarine possesses such an action in the frog (Manalis, 1977; Katz & Miledi, 1978; Colquhoun *et al.* 1979; Lambert, Volle & Henderson, 1980; Shaker, Eldefrawi, Aguayo, Warnick, Albuquerque & Eldefrawi, 1982) and this action has been postulated as the mechanism by which tubocurarine produces e.p.c. train run-down at the neuromuscular junction (Dreyer, 1982). Magleby *et al.* (1981) tested this hypothesis by examining if tubocurarine-induced run-down increased with membrane hyperpolarization. The rationale behind this approach lies in the fact that the channel blocking action of tubocurarine is strongly voltage dependent in the frog. Thus, there is an approximately e-fold increase in the rate at which tubocurarine associates with the ion channel and an e-fold decrease in the rate at which tubocurarine dissociates from the channel for every 50 mV hyperpolarization of the post-junctional membrane

(Colquhoun *et al.* 1979). For any drug with a slow dissociation rate there would be an accumulation of blocked channels during a train of e.p.c.s, resulting in less channels being available for activation during each successive e.p.c. and hence the amplitude of the e.p.c.s would fall off. In view of the strong voltage dependence of the channel blocking action of tubocurarine in the frog (Colquhoun *et al.* 1979) the degree of e.p.c. train run-down might be expected to increase markedly as the post-junctional membrane is hyperpolarized. In fact Magleby *et al.* (1981) found no change in the degree of e.p.c. train run-down on hyperpolarization from −60 to −120 mV in rat, mouse and frog. Our results with tubocurarine confirm and extend those of Magleby *et al.* (1981). Thus we have found no voltage dependence in the effects of tubocurarine on either neurally or ionophoretically evoked e.p.c. trains. We have found no evidence for a channel blocking action of tubocurarine of sufficient magnitude to account for the effects of tubocurarine in this preparation, in a concentration that produced both e.p.c. train run-down and fade of tetanic tension *in vitro* (Gibb & Marshall, 1983).

The resolution of the spectra obtained by noise analysis is such that a small change in the mean channel lifetime due to this low concentration of tubocurarine would not have been detected. If it is assumed that tubocurarine does however possess a similar channel blocking potency in this preparation to that measured in the frog, i.e. association rate constant of around $6{\cdot}5 \times 10^6$ M^{-1} s^{-1}, dissociation rate constant of around 1·36 s^{-1} at −60 mV (Colquhoun *et al.* 1979), then in a concentration of $2{\cdot}5 \times 10^{-7}$ M one would expect $1/\tau_n$ to increase by only about 1·6 s^{-1}. In the presence of this concentration of tubocurarine the amplitude of single e.p.c.s is reduced by about 65% and the increased run-down of a train of e.p.c.s results in a steady state of perhaps 25000 channels opening in the plateau of the train (the peak conductance of single e.p.c.s was 4·8 μS which, assuming a single channel conductance of 35 pS, indicates 138000 channels open at the peak of a single e.p.c. or 2400 channels opened per quantum). The mean quantal content of the first e.p.c. of trains was 65, corresponding to 156000 open channels which, allowing for 55% run-down of the train, indicates that the plateau e.p.c. amplitude in control trains corresponds to 70000 channels opened per e.p.c. Allowing for 65% receptor block in the presence of tubocurarine leaves 25000 channels opened per e.p.c. in the plateau of the train. Using the above rate constants, 0·0016 of these channels will be blocked per millisecond or 40 per e.p.c. With an unblocking rate constant of 0·00136 ms^{-1} this indicates that a steady-state level of block will be obtained with a time constant of $1/(0{\cdot}00136 + 0{\cdot}00008) = 694$ ms. The plateau level of the steady state will be 0·944 of the plateau amplitude of the e.p.c.s assuming that only around 25000 channels are available for activation during each e.p.c. Such a small effect would be smaller still if a larger population of channels is available as discussed below in relation to the effects of trimetaphan. Thus the effects of ion channel blockade, even during prolonged nerve stimulation, are unlikely to be significant with this concentration of tubocurarine. In fact, e.p.c. train run-down occurs quickly over a period of 150–200 ms (Fig. 6) and thereafter the amplitude of the e.p.c.s in the train remains essentially constant both in the absence and in the presence of tubocurarine. A similar effect of tubocurarine has been shown in the frog by Spivak, Maleque, Oliveira, Masukawa, Tokuyama, Daly & Albuquerque (1982; fig. 5) who demonstrated clearly the difference in the profile of trains recorded in the presence of tubocurarine or perhydrohistrionicotoxin which, like trimetaphan, produces a voltage-dependent run-down of e.p.c. trains (Albuquerque, Kuba & Daly, 1974).

Although we found that tubocurarine shortens the decay of the e.p.c., this effect was neither concentration dependent nor voltage dependent. Tubocurarine in the concentration used does not alter τ_{noise}, the value of which is consistently 30–40% shorter than the e.p.c. decay time constant. Thus it is clear that the time constant of decay of the e.p.c. does not reflect the lifetime of the ACh-activated ion channels in this preparation. Therefore, it is likely that the e.p.c. in these experiments was

prolonged by repetitive binding of the transmitter as it diffuses from the junctional cleft. Similar findings have been described for m.e.p.c.s in the rat and mouse diaphragms (Colquhoun, Large & Rang, 1977; Pennefather & Quastel, 1981; Head, 1983) and for e.p.c.s in the frog (Katz & Miledi, 1973, 1978; Magleby & Terrar, 1975; Mallart & Molgo, 1978).

It is of interest to note that the snake toxin, erabutoxin b, reduces the e.p.c. amplitude and shortens the decay time constant of the e.p.c. independently of the membrane potential in a manner similar to the effects seen with tubocurarine in this study and with α-bungarotoxin (Katz & Miledi, 1973, 1978; Pennefather & Quastel, 1981). However, unlike tubocurarine, erabutoxin b produces no change in the run-down of the e.p.c. train.

The effects of the ganglion blocking drug trimetaphan contrast markedly with those of tubocurarine and erabutoxin b in that the effects of trimetaphan on $\tau_{e.p.c.}$ and e.p.c. train run-down are voltage dependent. As the lowest concentration of trimetaphan used in these experiments also reduces the mean channel lifetime estimated by noise analysis, the results indicate that the main effect of trimetaphan at the neuromuscular junction is to block the ACh-activated ion channel. Previously, trimetaphan has been shown to shorten the excitatory current at the rat submandibular ganglion at a concentration of $2{\cdot}5 \times 10^{-5}$ M (Rang, 1982). This is the lowest concentration used in the present study; it produced a 20–30% voltage-dependent shortening of $\tau_{e.p.c.}$ and $\tau_{m.e.p.c.}$, and a 5–10% voltage-dependent increase in e.p.c. train run-down. This concentration of trimetaphan did not significantly reduce the amplitude of the e.p.c. or m.e.p.c., which contrasts with the effects of trimetaphan at the submandibular ganglion (Rang, 1982) in which $2{\cdot}5 \times 10^{-5}$ M-trimetaphan produced a marked reduction in the amplitude of the excitatory post-synaptic current, emphasizing the differences between motor end-plate and ganglionic nicotinic receptors. The reverse occurs with tubocurarine, which has a high receptor blocking potency in the diaphragm but is less potent and mainly blocks the ion channel at the ganglion (Rang, 1982). The results may be an indication that while the nicotinic receptors at the ganglion and neuromuscular junction have different recognition sites, their associated ion channels are not dissimilar.

In order to distinguish between pre- and post-junctionally mediated effects we have compared the actions of tubocurarine and trimetaphan on both neurally and ionophoretically evoked trains of e.p.c.s. In the presence of trimetaphan the profile of the ionophoretically evoked e.p.c. train changed from being flat to showing a run-down similar to that seen with neurally evoked e.p.c. trains. The increase in run-down was voltage dependent. This is consistent with the voltage-dependent effects of trimetaphan on e.p.c.s, m.e.p.c.s and trains of e.p.c.s. The effects of trimetaphan contrast with those of tubocurarine, which reduces the amplitude of ionophoretically evoked e.p.c.s but did not produce any run-down of ionophoretically evoked trains. Therefore, we conclude that tubocurarine-induced run-down of trains of neurally evoked e.p.c.s is mediated prejunctionally.

Despite the evidence for a prejunctional action of tubocurarine, drugs that block the end-plate ion channel can also produce a use-dependent block, as has been shown with the barbiturates (Adams, 1976) and with prednisolone (Dreyer, Peper, Sterz, Bradley & Muller, 1979). A voltage-

dependent run-down of e.p.c.s has also been shown in the frog with perhydrohistrionicotoxin (Albuquerque *et al.* 1974). The results found for trimetaphan in this study demonstrate that this is also the case in the rat diaphragm. The use dependence of the effect of any channel blocking drug during trains of e.p.c.s will be dependent, primarily, upon the dissociation rate of the drugs from the ion channel, although the association rate must also be fast enough to allow significant blockade of the ion channels before they close normally. The slower the dissociation rate, the greater will be the degree of use dependence of the block. If the sequential model for channel activation and blockade (scheme 2) is assumed to hold for the action of trimetaphan then the association rate of trimetaphan with the ion channel can be calculated from the results of the noise analysis from the relationship given in (3). This was $4{\cdot}75 \times 10^7$ M^{-1} s^{-1} at -90 mV.

It is not possible to estimate the dissociation rate, G, for trimetaphan from the time course of the e.p.c.s in the presence of trimetaphan as no second exponential component of the e.p.c. decay was detectable. Nor was a second Lorentzian component distinguishable in the noise spectra. However, it is possible to estimate the dissociation rate, G, from the rate of run-down of the trains of ionophoretically evoked e.p.c.s recorded at -90 mV in the presence of trimetaphan using the same principle as that applied by Adams (1976) and Dreyer *et al.* (1979) when applying pairs of ionophoretically produced pulses of ACh.

If the rate that the ionophoretically evoked e.p.c. amplitudes fall due to open channel block during each e.p.c. is taken as B and the rate that trimetaphan dissociates from the channel is G, then the time constant of the run-down of the ionophoretically evoked e.p.c. amplitudes, when expressed as a fraction of the control e.p.c. amplitudes, will be $1/(B+G)$. The run-down of the ionophoretically evoked e.p.c.s shown in Fig. 9 was fitted by least-squares linear regression from response 2–21 to an exponential of time constant 99 ms and amplitude 0·238 of the control e.p.c.s after subtracting a steady-state amplitude of 0·216 of the control e.p.c.s. Assuming that the fall in amplitude from response 2 to response 3 is due to block of the ion channels uncontaminated by significant unblocking, indicates that the rate of reduction of the e.p.c. amplitudes in the absence of unblocking would be 0·79 % of the control e.p.c. amplitudes per millisecond. Thus $B = 0{\cdot}0079$ ms^{-1} and $1/(B+G) = 99$ ms implying that G, the unblocking rate is 0·018 ms^{-1}, a time constant of 56 ms. Thus trimetaphan has an apparent affinity for the ion channel at -90 mV of around $1{\cdot}8 \times 10^{-6}$ M.

In order to determine if the blocking and unblocking rates calculated above can account for the observed effects of trimetaphan on neurally evoked e.p.c. trains, it is necessary to calculate the degree of block that would be predicted in the plateau of the neurally evoked e.p.c. train. As $2{\cdot}5 \times 10^{-5}$ M-trimetaphan did not significantly reduce the amplitude of single e.p.c.s evoked at 0·5 Hz it is assumed that 156000 channels open during the first e.p.c. during trains of e.p.c.s in the presence of trimetaphan and that the increase in run-down is due to an accumulation of blocked ion channels. Assuming a blocking rate of $4{\cdot}75 \times 10^7$ M^{-1} s^{-1} and assuming the channels are open for an average of $\tau_{noise} = 1{\cdot}11$ ms at -90 mV then, in the presence of $2{\cdot}5 \times 10^{-5}$ M-trimetaphan, all of the channels opened during each e.p.c. will be blocked by trimetaphan. Run-down in the absence of trimetaphan fell to a level approximately 45 % of the first e.p.c. during 50 Hz stimulation over the first 7–10 e.p.c.s to a plateau level in the train equivalent to around 70000 open ion channels per e.p.c. Therefore, assuming no unblocking during this period and a total of 10^7 end-plate channels available for activation (Fambrough & Hartzell, 1972; Colquhoun *et al.* 1979) indicates that around 7 % of the end-plate channels will be blocked after ten e.p.c.s. This cannot account for the 12 % reduction of the plateau amplitude of the e.p.c.s recorded in the presence of this concentration of trimetaphan ($2{\cdot}5 \times 10^{-5}$ M) at -90 mV. However, unblocking must occur during this period as the e.p.c. train reaches a steady state after 7–10 e.p.c.s. If no unblocking occurs or unblocking is very slow, the e.p.c. amplitude will fall continually during the e.p.c. train (cf. effect of perhydrohistrionicotoxin, Spivak *et al.* 1982). Thus, considerably less than 10^7 end-plate channels must be available for activation during each e.p.c. if open channel block is to account for the effects of trimetaphan on e.p.c. trains.

The reduction in the plateau e.p.c. amplitude can be related to the fraction of the total channels, N, available for activation when a proportion of these (B) are blocked by the relationship

$$\frac{N-B}{N} \times 70000 = 51500 \qquad (4)$$

$$\Rightarrow \quad B = 0{\cdot}2643\, N.$$

In the steady state 51000 channels will be blocked during each e.p.c. and the same number must become unblocked during the 20 ms interval between each e.p.c. Thus

$$B - 51500 = B \exp(-G20 \text{ ms})$$
$$\Rightarrow \quad B = 170350.$$

Therefore, there will be on average, 170350 end-plate channels blocked during the plateau of the e.p.c. train. Thus from eqn. (4) above, N, the total number of channels available for activation in the plateau of a train is predicted to be 645000. Using the previously calculated blocking and unblocking rates, N was estimated from the effects of 10^{-4} M and 2×10^{-4} M-trimetaphan at -90 mV on e.p.c. trains to be 364000 and 268000 respectively and by calculating G from the run-down of ionophoretically evoked e.p.c. trains recorded at -120 mV in the presence of $2{\cdot}5 \times 10^{-5}$ M-trimetaphan and F from the e.p.c. data in Fig. 4*C*, N was estimated to be 330000. Thus if the effects of trimetaphan on e.p.c. train run-down are to be ascribed to ion channel blockade, it appears that only a small fraction (perhaps around 400000) of the total end-plate receptors can be reached by the normal release of ACh during a train of nerve stimulations. It seems plausible that a small quantal release will have access to only a small fraction of the total end-plate area. If the true total is greater than this, then ion channel block cannot completely account for the effects of trimetaphan on neurally evoked e.p.c. trains. However, the voltage dependence of the effects of trimetaphan on e.p.c. train run-down suggest that ion channel block at least plays a part in this effect although they do not preclude an additional prejunctional effect. The blocking and unblocking rates, G and F, indicate that in the presence of $2{\cdot}5 \times 10^{-5}$ M-trimetaphan the e.p.c. amplitudes should approach a steady state with a time constant of 22 ms which is compatible with the fact that the run-down in the presence of trimetaphan was of similar time course to that in control. Therefore we conclude that the ability of trimetaphan to produce e.p.c. train run-down is at least partly mediated post-junctionally by an open channel blocking mechanism of action.

The effects of tubocurarine are different from those of trimetaphan and cannot be explained in terms of ion channel blockade. As discussed peviously, we conclude that tubocurarine-induced e.p.c. train run-down is mediated prejunctionally. Although the present experiments were performed at room temperature, tubocurarine also produces e.p.c. train run-down at 37 °C (Magleby *et al.* 1981; A. J. Gibb, unpublished observation) indicating that the mechanism that tubocurarine affects to produce e.p.c. train run-down also operates at body temperature. The nature of this mechanism is uncertain, but as tetanic fade and run-down are produced by agents that act as antagonists at nicotinic receptors this may indicate the existence of a prejunctional nicotinic receptor. Recent evidence indicates that α-bungarotoxin does not bind to nerve terminals (Jones & Salpeter, 1983), a finding consistent with our observation that the similarly acting erabutoxin b does not appear to possess a prejunctional action like that of tubocurarine. Thus, if a prejunctional nicotinic receptor exists, it appears to have a different binding specificity from that of the postjunctional nicotinic recognition site.

The exact mechanism involved in the prejunctional effect of tubocurarine remains unknown. Bowman & Webb (1976) and Bowman (1980) have suggested that tubocurarine blocks a prejunctional nicotinic cholinoceptor that is normally stimulated by the transmitter and functions to facilitate transmitter mobilization during repetitive stimulation. Conversely, Wilson (1982) concluded that tubocurarine acts prejunctionally to block a negative feed-back mechanism, the result of the block being an initial increase in quantal content and a subsequent run-down that is a consequence of the initial elevated release. Wilson's conclusion supports those of Miledi, Molenaar & Polak (1978, 1983) who, on the basis of direct measurements of ACh release after inhibition of cholinesterase, also concluded that the transmitter activates a negative

feed-back mechanism. The results on which these suggestions are based are capable of different interpretations. Our results show that tubocurarine does exert a prejunctional action when producing run-down of trains of neurally evoked e.p.c.s.

We conclude that both post-junctional ion channel block and a prejunctional effect can produce e.p.c. train run-down. Although post-junctional mechanisms do not appear to be involved in tubocurarine-induced e.p.c. train run-down, ion channel block does contribute to trimetaphan-induced e.p.c. train run-down. An additional prejunctional effect of trimetaphan cannot, however, be ruled out by these results.

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