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THE ACTIONS OF TUBOCURARINE AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The action of tubocurarine on voltage-clamped frog muscle end-plates has been re-examined by means of (*a*) equilibrium dose-ratio measurements, (*b*) current fluctuation measurements and (*c*) voltage-jump relaxation measurements.

2. The equilibrium measurements can be interpreted as implying that tubocurarine has (*a*) a competitive blocking action, with a dissociation constant of 0·34 μM, which is not dependent on membrane potential, and (*b*) an additional voltage-dependent blocking action.

3. In the presence of tubocurarine two kinetic components can be seen. The faster one is similar to, but rather faster than, the normal ion channel closing rate. The other is much slower (1–3 sec), and, in relaxation experiments it is in the opposite direction to the fast relaxation.

4. A number of alternative explanations for the results are discussed. The mechanism that fits them best appears to be a combination of competitive block (or block of shut channels), with a strongly voltage-dependent block of open ion channels by tubocurarine. Estimates of the rate constants for channel blocking (and their voltage dependence) are derived. From these estimates the dissociation constant for the binding of tubocurarine to open channels appears to be roughly 0·12 μM at -70 mV and 0·02 μM at -120 mV.

5. Several potential sources of error in the experiments, and in their interpretation, are discussed. The most serious of these are problems associated with diffusion in the small volume of the synaptic cleft, viz. (*a*) changes in cleft concentration consequent on changes in binding, and (*b*) ionophoretic flux of antagonist and agonist into the synaptic cleft.

INTRODUCTION

The action of tubocurarine on the post-synaptic membrane of the neuromuscular junction has been considered to be one of the classical examples of competitive antagonism. The classical work of Jenkinson (1960) strongly suggested that the effect of tubocurarine could be accounted for entirely by competition with acetylcholine for the acetylcholine receptors on the post-synaptic membrane. On the other hand, the action of tubocurarine on *Aplysia* neurones appears to be quite different; the

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drug appears to work primarily by blocking ion channels while they are open, rather than by competing with acetylcholine (Marty, Neild & Ascher, 1976; Ascher, Marty & Neild, 1978). Manalis (1977) observed that the effect of tubocurarine at the frog neuromuscular junction was dependent on membrane potential when the agonist was applied by ionophoresis, but not when it was applied by nerve stimulation, and he suggested that this effect might be the result of block of ion channels, of the sort seen in *Aplysia*.

We have investigated this problem in the frog, *Rana esculenta*. The methods that have been used were designed to allow us to assess, in particular, the following aspects of the action of tubocurarine. (*a*) The importance, and dependence on membrane potential, of the competitive action of tubocurarine, for which purpose application of known agonist concentrations is important so that dose ratios can be measured. Therefore agonist was applied in to the tissue bath (rather than by ionophoresis) in most experiments. (*b*) The rates, and their dependence on membrane potential, of the actions of tubocurarine. For this purpose it is necessary to use voltage-clamped end-plates, and kinetic measurements were made by both noise analysis, and by means of voltage-jump relaxations. Two kinetic components were clearly seen in the presence of agonist and tubocurarine by the voltage-jump method, but the slower component was too slow to be resolved by the noise method.

Some of the results in this paper have already been communicated to the Physiological Society (Colquhoun, Dreyer & Sheridan, 1978).

METHODS

Experiments were performed on the cutaneus pectoris muscle of the frog (*Rana esculenta* unless otherwise noted). The preparation was pinned to a thin layer of Sylgard (Dow-Corning) on the glass (microscope slide) base of a Perspex tissue bath. The muscle was continuously perfused with Ringer solution through a glass pipette (2 mm i.d. shank with a wide flattened end which was placed as close as possible to the impaled end-plate). This pipette was also used to apply drug solutions (cf. Cooke & Quastel, 1973). Unless otherwise stated, the Ringer solution contained (mM) NaCl 117, KCl 2·5, $CaCl_2$ 1·0, $MgCl_2$ 1·8, Na_3PO_4 buffer 2·0 and 100 nM-tetrodotoxin (Sigma Chemical Co.). The pH of this solution was 7·0–7·1. The inflowing solution was cooled by means of a jacketed tube through which cold water was circulated, and the tissue bath had a tube around its periphery through which cold water also flowed. The flow rate of solution was about 3 ml./min, and the temperature, which was routinely measured with a thermistor bead placed as close to the recording site as possible, was 8·5–9·5 °C unless otherwise stated. Carbachol was obtained from Aldrich Chemical Co. Inc. and (+)-tubocurarine, B.P., from Koch-Light Labs. or Sigma Chemical Company. Purified α-bungarotoxin was kindly given to us by Professor E. A. Barnard; a solution of 50 or 100 nM was perfused over the preparation until the desired degree of block was achieved, and then washed out with Ringer solution.

Recording of end-plate currents. End-plates were located visually by means of a Zeiss Nomarski differential interference contrast microscope with modified stage (Micro Instruments, Oxford). Normally a 40× water-immersion objective was used. The end-plate was penetrated with a voltage recording micro-electrode filled with 2·5–3 M-KCl (resistance 5–10 MΩ usually) and, close by, with a current recording electrode filled with 2 M-K citrate adjusted to pH 7·0 with citric acid (5–15 MΩ usually). The objective was then raised from the solution (mainly to aid temperature control) and the voltage clamp (similar to that of Dionne & Stevens, 1975) switched on. The size and rate of rise of miniature end-plate currents were used as an additional check on the location of the electrodes. The clamp gain, and the capacity compensation on the voltage follower, were adjusted by optimizing the response to a 5 mV rectangular command pulse.

Noise measurements were made as described by Colquhoun, Large & Rang (1977), except that the filtered signal was sometimes sampled on line by a PDP 11/40 computer. The computer also supplied command potentials for the voltage-jump experiments, each potential jump being followed by on-line sampling of the end-plate current, which was normally filtered above 2 or 3 kHz (Barr & Stroud EF3-02; 160 db/decade roll-off; damped mode). In most experiments the tissue bath was grounded directly via an Ag/AgCl wire electrode. In a few experiments the bath was clamped to ground potential by means of two Ag/AgCl (sintered) bath electrodes. One, used for voltage sensing, was connected to a voltage follower, so hardly any current flowed through it (thus avoiding polarization of the electrode), the follower output was inverted and fed back to the second bath electrode to maintain the bath at ground potential. Both methods gave similar results which indicates that electrode polarization was not a serious problem.

Analysis of current fluctuations (noise). The noise signal was edited visually and sections that contained miniature end-plate currents, or other interference, were removed. The usuable noise data, between faults, were divided into as many sections as possible of length $1/f_{res}$ seconds where f_{res} (Hz) was the desired resolution. From each section a power spectrum was calculated directly using a fast Fourier transform (uniform or 'boxcar' data window). The spectral densities calculated from the data were then averaged and the mean control spectrum (found from noise in the absence of agonist) was subtracted from the mean spectrum of noise observed in the presence of agonist. The net one-sided spectral density, $G(f)$, so found was fitted by Lorentzian components of the form, $G(f) = G(0)/[1+(f/f_c)^2]$, where f = frequency and f_c = half-power frequency. One such component, or the sum of several Lorentzians, was fitted by the method of weighted least-squares, using the Patternsearch method (see Colquhoun, 1971, 1978*b*). The points in the net spectrum were weighted in the following way. Say $G_d(f)$ and $G_c(f)$ are the mean spectral densities, at frequency f, in the presence and absence (control), respectively, of the drug. Thus the net spectral density is $G(f) = G_d(f) - G_c(f)$. The spectral estimates are distributed approximately as $\chi^2_2/2$ (see for example, Bendat & Piersol, 1971) so they have a constant coefficient of variation of $\sqrt{(2/n)}$, where n is the number of degrees of freedom of the spectral estimate. The spectrum is fitted to the $\log_{10} G(f)$ values, so if G_c and G_d are independent, the weight, $w(f)$, for each such value (i.e. the reciprocal of its variance) will be, approximately,

$$w(f) \cong \frac{2{\cdot}303^2 n_d}{2} \frac{(x-1)^2}{(x^2+y)},$$

where $x = G_d(f)/G_c(f)$, $y = n_d/n_c$, and n_d, n_c are the number of spectra averaged to get the mean spectrum with and without agonist present, respectively. Thus the weight will approach a maximum constant value, $2{\cdot}303^2 n_d/2$, when the control spectral density is far below that in the presence of agonist (x large), and will fall to zero as G_d approaches G_c. In practice weights were smoothed by averaging in groups (usually of eight frequency points) to avoid excessive fluctuation of the weights from point to point. The same weights were used to calculate approximate standard errors for the fitted parameters from the inverse Hessian matrix, as described by Colquhoun, Rang & Ritchie (1974).

The apparent single channel conductance, γ_{app}, was estimated as $\gamma_{app} = \text{var}\,(I)/m_I(V - V_{eq})$. In this expression var (I) 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, m_I is the mean drug-induced current, V is the membrane potential and V_{eq} the equilibrium potential (taken as zero: see Results). This value was corrected for loss of variance at frequencies below f_{min} and above f_{max}, the frequencies at which the observed noise was filtered. Suppose the spectrum is fitted by the sum of k Lorentzian components, the ith component being denoted $G_i(f)$ and having half-power frequency $f_c^{(i)}$. Then the fraction of the total variance between f_{min} and f_{max} will be

$$\frac{\sum_i \int_{f_{min}}^{f_{max}} G_i(f)\,df}{\sum_i \int_0^{\infty} G_i(f)\,df} = \frac{2\sum_i G_i(0) f_c^{(i)} [\tan^{-1}(f_{max}/f_c^{(i)}) - \tan^{-1}(f_{min}/f_c^{(i)})]}{\pi \sum_i G_i(0) f_c^{(i)}}$$

as long as the fitted spectrum describes the data accurately outside the observed range. The apparent conductance, γ_{app}, was divided by this factor to obtain an estimate of the mean single channel conductance, γ (see also Colquhoun, 1978*a*).

Analysis of relaxations. The sampling rate of the analogue to digital convertor was, when convenient, set to be faster for the earlier points following the voltage jump than for later points. This enabled both fast and slow relaxations to be resolved in a single jump. The membrane current obtained by performing the voltage jump in the absence of agonist (immediately before or after agonist application) was subtracted from that obtained by imposing the same jump in the presence of agonist, to obtain the net current flowing through channels opened by the agonist. All currents illustrated, or referred to, in this paper are net currents found in this way.

An exponential, or sum of exponentials, was fitted to the net current by equally weighted least squares. The asymptote was estimated along with the other parameters, rather than being guessed beforehand. When necessary (see Results) a sloping base line was fitted too. The early capacitative transient, and occasionally other undesired points, were assessed visually and omitted from the fitting process. Approximate standard errors were found as above, except that all weights were set to 1·0 and the diagonal elements of the inverted Hessian were multiplied by an estimate of experimental variance found from the scatter of points about the fitted line, viz. $S_{min}/(N-p)$, where S_{min} is the minimum sum of squared deviations, N is the number of points fitted and p is the number of parameters fitted. Some of the problems of fitting exponentials are discussed by Colquhoun (1978*b*).

When the reversal potential was estimated from instantaneous current–voltage plots (e.g. Fig. 6*A*) of jumps from a series of hyperpolarized membrane potentials, back to the holding potential, it was sometimes found that the estimate of the reversal potential was negatively correlated with the estimate of the time constant at the holding potential. This arose because the estimate of the time constant was often correlated negatively with the amplitude of the relaxation. Therefore those relaxations that gave a time constant that was too fast, also gave an estimate of instaneous current that was too negative. As a result of this phenomenon it was occasionally found desirable to estimate the instantaneous current, before fitting the relaxation, by taking the reversal potential as zero (see Results), and constraining the fit to go through this estimated instantaneous (zero time) current. The constraint was achieved by adding a penalty factor to the sum of squared deviations: this factor was a constant (usually 10 or 20) times the absolute value of the difference between the desired zero time current, and the calculated zero time current.

RESULTS

Experiments at equilibrium

Equilibrium responses and desensitization

Application of a moderate carbachol concentration (sufficient to produce an inward current of up to 40 or 50 nA), usually resulted in a stable plateau which was maintained for the duration of the application (not usually more than a minute or so). Such responses are illustrated in Fig. 1 (*A*1 at −70 mV and *B*1 at −120 mV). It is quite likely that some desensitization was present during the plateau because it was often observed that the amplitude of miniature end-plate currents was reduced. This was not investigated in detail. At higher agonist concentrations, a slow decline in the response was seen and this will be referred to as desensitization. Densitization was found to be a good deal more prominent with acetylcholine than with carbachol, so the latter agonist was used in most experiments.

In the presence of tubocurarine, with agonist concentration raised so as to keep the response similar to that before addition of tubocurarine, desensitization was rather faster than in its absence, but slower than the desensitization rate that the raised agonist concentration would have produced without tubocurarine (see Fig. 12).

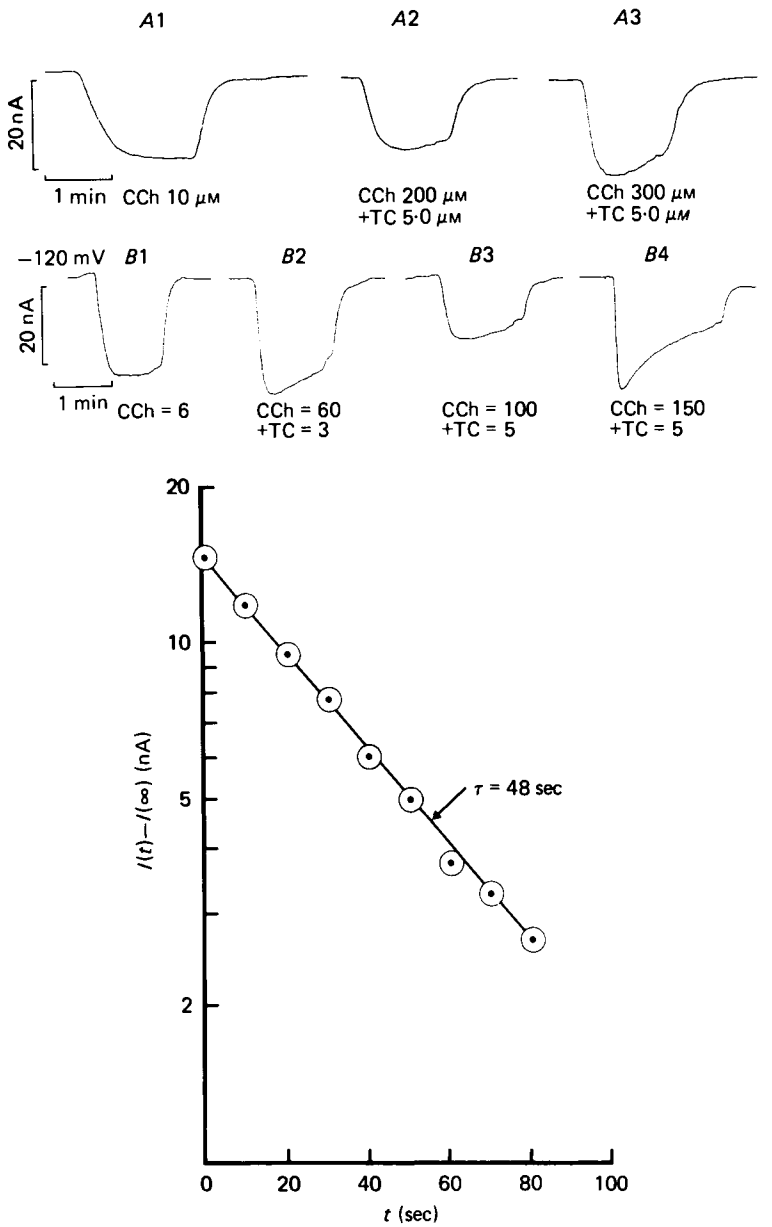


Fig. 1. Responses (net agonist-induced current) to application of carbachol (CCh) at (*A*) −70 mV, and (*B*) −120 mV. Inward current plotted downwards. *A*1, CCh, 10 μM; *A*2, CCh, 200 μM and tubocurarine, 5 μM; *A*3, CCh, 300 μM and TC, 5 μM. *B*1, CCh, 6 μM; *B*2, CCh, 60 μM and TC, 3 μM; *B*3, CCh, 100 μM and TC, 5 μM; *B*4, CCh, 150 μM and TC, 5 μM. *C*, semilogarithmic plot of the decay of the current in (*B*4).

The desensitization rate increased with agonist concentration (for a given tubocurarine concentration) as shown in Fig. 1 (*A*2 and *A*3 at −70 mV; *B*2–*B*4 at −120 mV). If the response was small enough there was no obvious desensitization even in the presence of tubocurarine. The decline is more or less linear in Fig. 1 (*A*1–*B*3) but if

the slope (0–0·13 nA sec^{-1}) is treated as the initial slope of an exponential curve, the observed amplitudes allow the statement that the time constant of the exponential must have been *at least* 40 sec for all of these responses. The response in Fig. 1

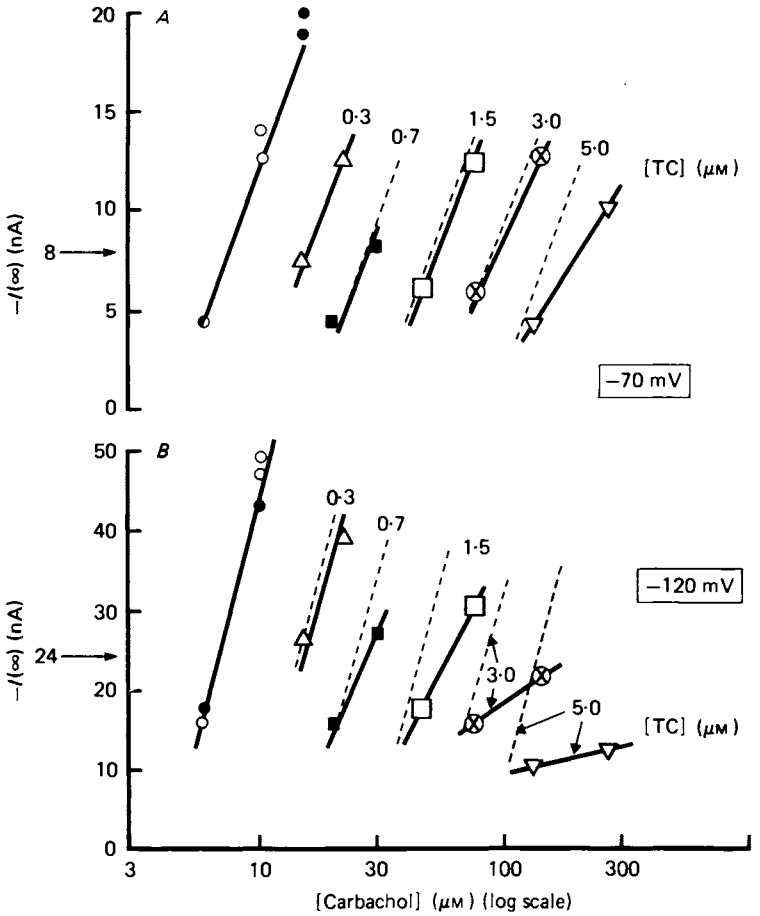


Fig. 2. Dose–response curves at equilibrium (or near equilibrium, see text). End-plate membrane clamped to (*A*) −70 mV, (*B*) −120 mV. Open circles show response to CCh before any application of TC, and filled circles show response after washing out TC. Other points determined in the presence of the indicated TC concentration (μM). Continuous lines show observed currents. The dashed lines show the responses predicted for a pure competitive antagonist with K_B = 0·27 μM (see Fig. 3 and text). Dose ratios for Fig. 3 were measured at (*A*) −8 nA and (*B*) −24 nA, as indicated.

(*B*4) was longer than usual; and the graph in Fig. 1*C* shows that it was close to a single exponential; the time constant was 48 sec, and the initial slope 0·36 nA sec^{-1}. This rate is comparable with those seen by Adams (1975) and, in electric eel, by Lester, Changeux & Sheridan (1975).

These 'desensitization' rates are quite slow compared with the reaction rates measured later. But the lack of a clear plateau under some circumstances did create problems for the measurement of equilibrium responses. The peak responses on curves such as that in Fig. 1 (*A*3) were used, although the system was clearly not in true equilibrium. Some possible consequences of this are mentioned later (p. 253).

Concentration–response curves

The classical method for investigation of antagonists (Arunlakshana & Schild, 1959; see also Thron, 1973 and Colquhoun, 1973) is to measure the shift of the equilibrium response *vs.* log agonist concentration curve that is produced by various concentrations of antagonist. In the case of a pure competitive antagonist, it is predicted, for a wide range of theories of agonist action, that the dose ratio, r (the factor by which agonist concentration must be increased to maintain a constant response in the presence of antagonist) will be given by the Schild equation

$$r = 1 + c_B. \tag{1}$$

The normalized concentration, c_B, of the antagonist (B) is defined as

$$c_B = x_B/K_B, \tag{2}$$

where x_B is the concentration of antagonist and K_B its equilibrium (dissociation) constant.

Fig. 2*A* shows an example of the dose–response curves determined under our conditions, with the end-plate clamped at -70 mV. The effect of tubocurarine was completely reversible, as shown by complete recovery of the control curve after removal of tubocurarine. The dashed lines in Fig. 2*A* show the parallel shifts predicted by eqn. (1) (taking $K_B = 0{\cdot}27$ μM, see below). The observations are quite close to these predictions (except for the response at the higher agonist concentration in 5 μM-tubocurarine; this was a consistent finding which is discussed below).

Fig. 2*B* shows dose–response curves determined in the same way, except that the end-plate was clamped at -120 mV. As at -70 mV, the effect of tubocurarine was completely reversible, and, as at -70 mV, the effect of the lowest tubocurarine concentration (0·3 μM) was close to that predicted for competitive antagonism (with $K_B = 0{\cdot}27$ μM as at -70 mV). However, above this tubocurarine concentration, increasingly large deviations from the competitive prediction were seen at -120 mV. The size of the deviations increases with agonist concentration (they are greater at the higher than at the lower concentration for any given tubocurarine concentration) as well as with tubocurarine concentration. And the deviations from competitive behaviour clearly increase as the end-plate is hyperpolarized.

The dose ratios (r) measured from Fig. 2 (at the arbitrarily chosen response levels specified therein) are plotted in Fig. 3, as $\log(r-1)$ *vs.* $\log x_B$. For a pure competitive antagonist the logarithmic form of eqns. (1) and (2),

$$\log(r-1) = \log x_B - \log K_B, \tag{3}$$

predicts that such a plot should be linear, with unit slope, and that the intercept on the abscissa at $r-1 = 1$ is an estimate of $\log K_B$. It can be seen that, at -70 mV, tubocurarine behaves like a competitive inhibitor. The point for 5 μM-tubocurarine lies above the line, but a crude correction for 'desensitization' by extrapolation of the response, such as that shown in Fig. 1 (*A*3), to zero time suggests that this deviation may be mainly the result of desensitization rather than non-competitive behaviour (see Discussion).

On the other hand, at -120 mV, a considerable deviation from simple competitive behaviour is seen in Fig. 3, as expected from Fig. 2*B*. In fact, the dose ratio for

5 μM-tubocurarine seemed to be infinite because the maximum response that could be obtained appeared to have been depressed below the level (24 nA) at which dose ratios were measured (though no attempt was made to measure the maximum response precisely). However, at low tubocurarine concentrations the two curves

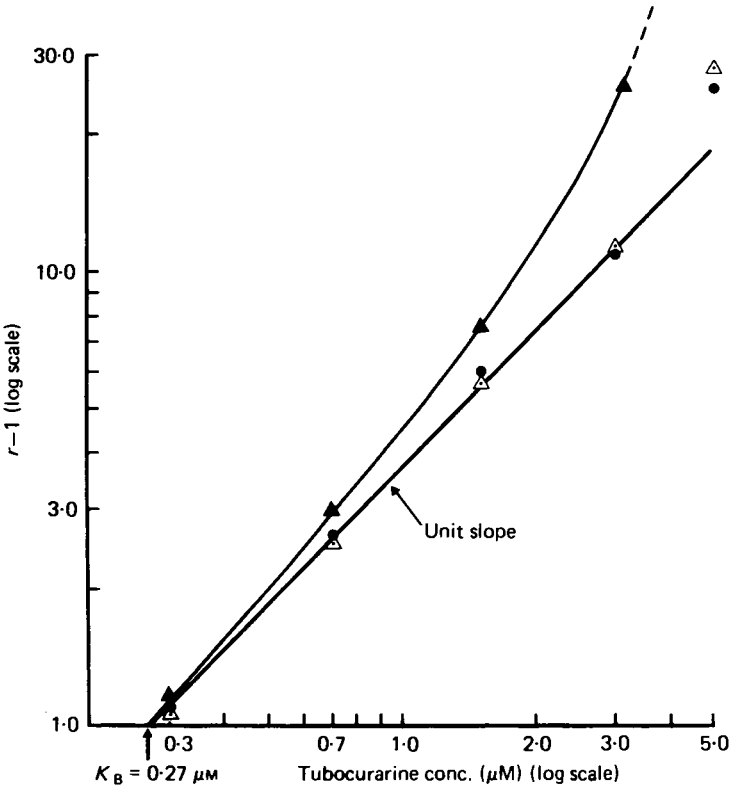


Fig. 3. Schild plot of dose ratio (r) against TC concentration (x_B) according to eqn. (3). ●, dose ratios from equilibrium current at −70 mV (from Fig. 2). ▲, dose ratios from equilibrium current at −120 mV (from Fig. 2). △, dose ratios from peak current at −120 mV (same experiment as Fig. 2; see text).

in Fig. 3 appear to converge, so the slope, even at −120 mV, approaches unity, and the estimates of K_B obtained from this graph are virtually identical, viz. 0·27 μM, at both −70 and at −120 mV.

Dose–response curves, such as those shown in Fig. 2, can also be plotted not with the equilibrium responses, but with the peak responses (i.e. the currents observed before the slow inverse relaxation has had any important effect, see Fig. 5 and p. 257). If dose ratios are measured from such curves, despite the fact that the responses are not at equilibrium, the antagonist behaves as though it were competitive even at hyperpolarized membrane potentials. For example in Fig. 3, the dose ratios measured from peak currents at −120 mV show virtually the same competitive behaviour as those measured at −70 mV, although the equilibrium dose ratios at −120 mV deviate considerably from simple competitive behaviour.

The obvious, but not the only, interpretation of the results shown in Figs. 2 and 3 is that tubocurarine is mainly a simple competitive antagonist at −70 mV, but, as the end-plate is hyperpolarized, a voltage-dependent, non-competitive form of

block appears in addition to the competitive action. According to this interpretation, Fig. 3 suggests that the affinity of tubocurarine for the agonist binding site is *not* voltage-dependent (K_B = 0·27 μM at both potentials). In eleven experiments (not all with such complete data as in Figs. 2 and 3), in which K_B estimates could be made, the values were (*a*) at −70 mV, $K_B = 0{\cdot}31 \pm 0{\cdot}02$ μM (6), (*b*) at −80 mV,

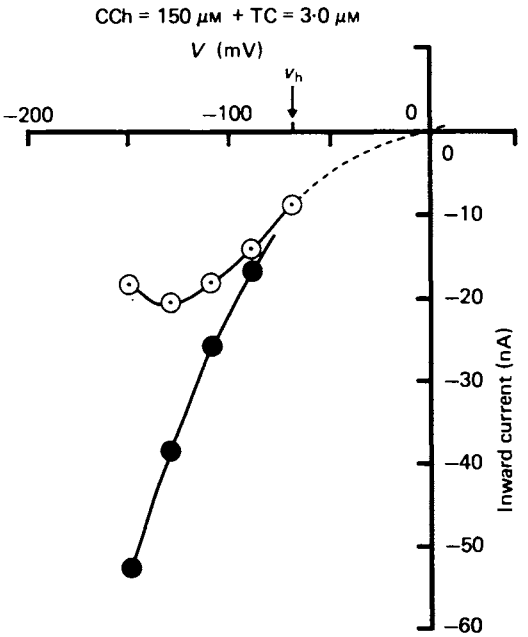


Fig. 4. Current–voltage relationships measured in the presence of CCh, 150 μM with TC, 3 μM. ○, equilibrium current. ●, peak current (see text).

$K_B = 0{\cdot}36 \pm 0{\cdot}04$ μM (4), (*c*) at −120 mV, $K_B = 0{\cdot}33 \pm 0{\cdot}03$ μM (6), and (*d*) at −140 mV, $K_B = 0{\cdot}41 \pm 0{\cdot}02$ μM (3). There is, therefore, no sign that K_B estimated in this way, is voltage-dependent. The over-all mean value is $0{\cdot}34 \pm 0{\cdot}02$ μM (19).

Voltage-dependent effect at equilibrium

Fig. 4 (open circles) shows more explicitly the voltage dependence of action of tubocurarine. At −70 mV, carbachol (150 μM) produced an inward current of about 9 nA in the presence of tubocurarine (3 μM). As the membrane is hyperpolarized, the driving potential increases and, normally, more channels open too, so the equilibrium current increases more than in proportion to the hyperpolarization, as shown later (see p. 258 and dashed line in Fig. 6*A*; the filled circles in Fig. 4 behave similarly and this will be discussed later). However, in the presence of tubocurarine it is seen that, although moderate hyperpolarization caused an increase in current, the curvature was in the opposite direction to usual, and eventually, further hyperpolarization actually caused a *decrease* in current, despite the continued increase in driving force. This observation is qualitatively similar to the anomalous voltage-dependence of inward current, in the presence of tubocurarine, observed by Manalis (1977) during prolonged application of acetylcholine by ionophoresis.

The single channel conductance

The mean single channel conductance, measured as described in Methods, was $\gamma = 20{\cdot}5 \pm 1{\cdot}5$ pS (6) at -70 or -80 mV in the absence of tubocurarine. This is slightly lower than the value of 25 pS reported by Colquhoun, Dionne, Steinbach & Stevens (1975), possibly because of the presence of magnesium in our Ringer (B. Sakmann & P. R. Adams, personal communication). The mean ratio, in nine experiments, of γ in the presence of tubocurarine (1–5 μM) to γ in its absence was $0{\cdot}95 \pm 0{\cdot}03$. At hyperpolarized potentials (-120 or -140 mV), at which the voltage-dependent block is more prominent, the ratio was $0{\cdot}92 \pm 0{\cdot}02$ (4); and near the resting potential (-70 or -80 mV) the ratio was $0{\cdot}98 \pm 0{\cdot}04$ (5). In eight experiments out of ten, some reduction of γ by tubocurarine was observed ($P = 0{\cdot}11$, sign test). Thus there is a hint of slight reduction of γ by tubocurarine at negative membrane potentials, but if real it is certainly small, and it cannot be regarded as well established.

Kinetic experiments

The changes in kinetic measurements produced by tubocurarine

The rates of the reactions underlying the equilibrium responses that have been described, were measured by two methods: (*a*) by following the relaxation of the membrane current that follows a step change in membrane potential, and (*b*) by measuring current fluctuations at equilibrium. With the former method, two exponential components could be clearly resolved in the presence of tubocurarine. The faster one is similar to the normal rate constant seen in its absence. Its time constant is known to give, under certain circumstances, a good estimate of the mean open lifetime of the ion channels that are opened by the agonist (see Anderson & Stevens, 1973; Neher & Sakmann, 1975; Colquhoun & Hawkes, 1977). The second component is very much slower. Noise analysis was used to measure the faster rate constant, but the slower one was much too slow to be resolved by this method.

The basic phenomenon that we have observed is illustrated in Fig. 5. It will be described briefly and qualitatively now; the details will be presented in subsequent sections.

Before zero time, in Fig. 5*A* the end-plate was held at -80 mV and the current through the channels opened by carbachol (10 μM) was about -20 nA. At time zero, the potential across the end-plate was stepped to -140 mV, there was an immediate ohmic jump in the current, to about -32 nA (the instantaneous current) as more current flowed through the channels that were already open, under the influence of the increased potential gradient. During the next 32 msec (which is shown on a greatly expanded time-scale), more channels open under the influence of the more hyperpolarized potential (Rang, 1973; Dionne & Stevens, 1975). Eventually, the equilibrium current, about -90 nA, appropriate to -140 mV, was reached. The time constant for this re-equilibration (relaxation) is denoted τ_f. It is about $6{\cdot}2 \pm 0{\cdot}3$ msec at -140 mV in Fig. 5*A*. Once the equilibrium current had been reached, it stayed more or less constant for the next 8 sec.

It will be noticed that there is a further increase in inward current between about 30 and 300 msec. This was frequently observed, and its time constant (in so far as it could be fitted

by an exponential) was usually between 30 and 300 msec, probably rather too slow for it to be attributed to the opening of extrajunctional channels. It is, no doubt, the same phenomenon as the 'sloping base line' observed by Neher & Sakmann (1975) and by Adams (1977*a*). They suggested that it might result from ionophoresis of agonist from the bulk solution into the synaptic cleft. This is one possibility, but the process seems rather slow for this mechanism. Another possibility is that it may result from slight movements caused by local contracture at the end-plate. The effect was usually reversible. The equilibrium current at −140 mV was taken as the asymptote of the first, major, exponential component.

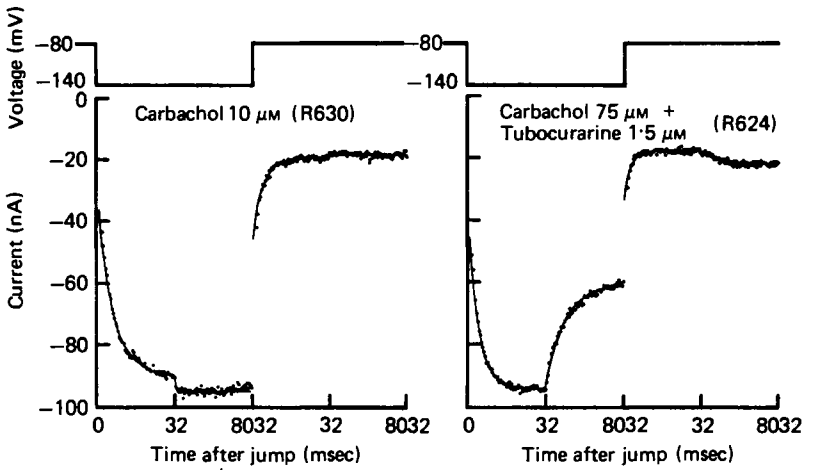


Fig. 5. Relaxation of agonist-induced current following the step changes in membrane potential indicated at the top of each curve. The current was recorded for 8032 msec after a step from −80 to −140 mV, and for a further 8032 msec after returning to −80 mV. Notice that the first 32 msec of each record is shown on a greatly expanded time scale so that the fast relaxations can be seen. Curves were fitted as described in Methods. *A*, CCh, 10 μM. *B*, CCh, 75 μM in the presence of TC, 1·5 μM.

After 8032 msec, the membrane potential was stepped back to −80 mV, and there was again an ohmic jump in current. Then channels shut during the first 32 msec (rather faster than they opened) until the equilibrium current at −80 mV (−20 nA, as it was before the voltage jump) was reached again. The current then remained constant until the agonist was removed.

The results of imposing the same voltage jump in the presence of tubocurarine (1·5 μM) are shown in Fig. 5*B*. The agonist concentration was increased so as to produce a similar (peak) current to that in the absence of tubocurarine. Before zero time the current at −80 mV was about −23 nA. The fast relaxations were very similar to those in the absence of tubocurarine, except that they are slightly faster (e.g. $\tau_f = 4{\cdot}3 \pm 0{\cdot}2$ msec at −140 mV in Fig. 5*B*) in the presence of tubocurarine. However, the *peak current* at −140 mV (about −95 nA) was not maintained, but waned slowly, until equilibrium at −140 mV was eventually reached at about −60 nA. The time constant for this slow inverse relaxation is denoted τ_s (it is about 2 sec in this example). When the potential was jumped back to −80 mV, after 8032 msec, the process reversed. After the rapid shutting of channels, the current reached a peak value of −18 nA, but then channels slowly opened again (with time constant denoted τ_s; about 1·3 sec in Fig. 5) until the equilibrium current at −80 mV (−23 nA, as it was before the jump) was eventually reached.

The reversal potential

The voltage-jump method allowed rapid, though not particularly accurate, estimation of the reversal potential for the ion channels opened by carbachol. Fig. 6*A* shows the instantaneous current–voltage relationship in the presence of carbachol (15 μM). The open symbols show the current flowing immediately *before* a jump, and the filled symbols show the ('instantaneous') current immediately *after* a step change to a new potential (found by extrapolation of the curve fitted to the ensuing relaxation back to the moment of the jump). The sort of experimental record from which this information was obtained is illustrated in Fig. 5. The points immediately before and after the jump (separated by such a short time that the number of open channels does not have time to change) are joined, and this line can be extrapolated linearly to zero current. Individual ion channels behave ohmically over the observed range of potentials in agreement with Magleby & Stevens (1972*b*) as shown by the linearity in Fig. 6. If they continue to behave ohmically at more depolarized potentials this procedure yields an estimate of the reversal potential, V_{rev}. In this example, V_{rev} was $3{\cdot}8 \pm 2{\cdot}7$ mV (5), and, although there was considerable scatter in the estimates, V_{rev} did not usually differ significantly from zero, so this value was used for all calculations.

After a step in the hyperpolarizing direction (upper curve in Fig. 6*A*), ion channels opened, with time constant τ_f, during the subsequent relaxation (see Fig. 5) until the current reached (or nearly reached, depending on the time allowed) a new equilibrium level at the hyperpolarized potential (open symbols on lower curves, Fig. 6). The dashed line drawn through the open symbols is, therefore, a close approximation to the *equilibrium* current–voltage relationship. The curvature of this relationship, seen in Fig. 6*A*, is now well known (Rang, 1973; Dionne & Stevens, 1975; Adams, 1976*a*; Mallart, Dreyer & Peper, 1976).

It is necessary, for the analysis of our results, to know whether the reversal potential is affected by the presence of tubocurarine or by long hyperpolarizing jumps. The experiment on which the Fig. 6*B* is based involved 8032 msec hyperpolarizing jumps from a holding potential of -70 mV, in the presence of carbachol (150 μM) and tubocurarine (3 μM). The set of instantaneous current–voltage curves look, in tubocurarine, more complicated than those without it in Fig. 6*A*, because of the large inverse relaxation (see Fig. 5*B* for example). The reversal potential, $-0{\cdot}95 \pm 5{\cdot}4$ mV (8) has not obviously changed from the value of zero assumed in the absence of tubocurarine, and was similar for jumps in the hyperpolarizing direction ($-0{\cdot}9 \pm 11$ mV (4)) and for jumps in the depolarizing direction after 8032 msec hyperpolarization ($-1{\cdot}0 \pm 3{\cdot}5$ mV (4)). Since 8032 msec is long enough for equilibrium to be closely approached the dashed line in Fig. 6*B* is near to the equilibrium

Fig. 6. Instantaneous current–voltage relationships. ○, agonist-induced current immediately before potential jump (close to equilibrium, so the dashed curve is close to the equilibrium current–voltage relationship). ●, instantaneous current immediately after the potential jump. *A*, in the presence of CCh, 15 μM. Measured by means of 64 msec jumps from -70 mV to various more hyperpolarized potentials, and back. *B*, in the presence of CCh, 150 μM with TC, 3 μM. Measured by means of 8032 msec jumps.

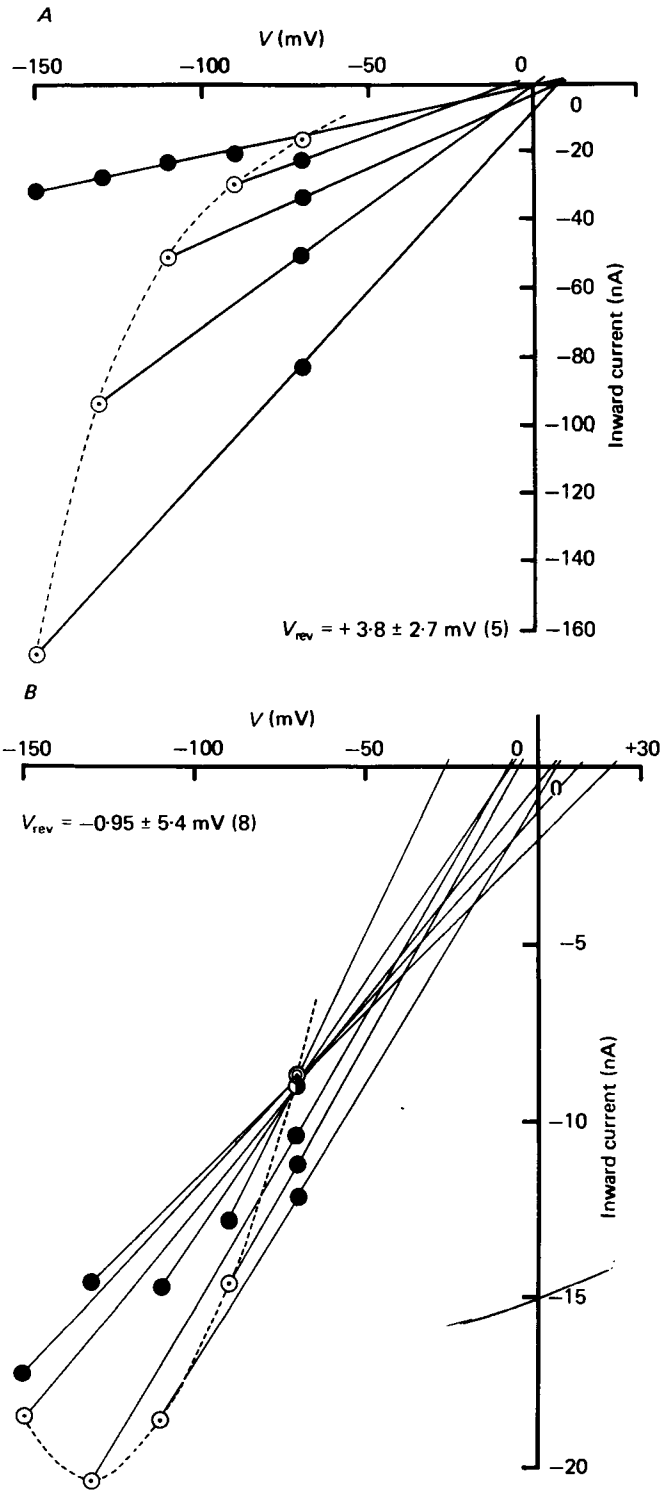


Fig. 6. For legend see opposite.

current–voltage relationship shown in Fig. 4. It is quite different from the analogous relationship in the absence of tubocurarine (Fig. 6*A*), because of its voltage-dependent blocking action. On the other hand, it is worth noting that plotting, against voltage, the peak (rather than equilibrium) current in the presence of tubocurarine (e.g. the most negative current in Fig. 5*B*) produces a curve (illustrated in Fig. 4) that is much more like the equilibrium curve in its *absence* (Fig. 6*A*) than that in its presence (Fig. 4 or Fig. 6*B*).

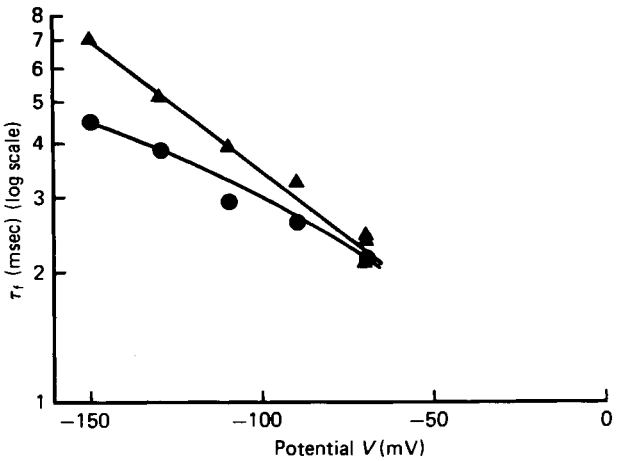


Fig. 7. Dependence of the time constant for the fast relaxation, τ_f, on membrane potential. Same experiment as Fig. 6. ▲, in the presence of CCh, 15 μM. Slope corresponds to H_f = 71 mV for an e-fold decrease in τ_f. ●, in the presence of CCh, 150 μM with TC, 3 μM. A straight line through the points would give H_f = 105 mV. The curve drawn through the points is from eqn. (5) with $k^*_{+B} = 2{\cdot}7 \times 10^7$ M^{-1} sec^{-1} at 150 mV and $H^*_{+B} = -59$ mV.

The voltage dependence of the fast kinetic component

If the control, by the clamp circuit, of the voltage across the end-plate membrane were seriously inadequate, one might expect that the voltage sensitivity of τ_f would be spuriously low (Magleby & Stevens, 1972*b*). In these experiments, in which the whole length of the end-plate was activated, it was therefore considered particularly important to check the voltage sensitivity of τ_f.

The time constants, τ_f, of the relaxation towards a new equilibrium current (see Fig. 5), for the experiment illustrated in Fig. 6*A*, are plotted against voltage in Fig. 7 (upper curve). The mean open lifetime of the channel opened by carbachol, as estimated by τ_f, was about 2·2 msec at $V = -70$ mV, and about 7 msec at $V = -150$ mV. The relationship is exponential, as was always found. If it is written

$$\tau_f(V)^{-1} = \tau_f(0)^{-1} \exp(V/H_f)$$

then H_f ,the potential change for an e-fold increase in rate, is 71 mV for the results in Fig. 7. Similar values were found in other experiments, whether τ_f was measured by the relaxation or by the noise method.

In twelve experiments, the average value of H_f was 78·7 ± 1·9 mV in the absence

of tubocurarine. The work of Anderson & Stevens (1973), who applied agonist to a restricted (and therefore more readily clamped) area of the end-plate by ionophoresis, suggests that H_f should be 70–80 mV at the temperature (8–9 °C) of our experiments; our results are compatible with these values.

An example of the voltage dependence of τ_f in the presence of tubocurarine (3 μM) is also shown in Fig. 7 (lower curve); it is from the same experiment that was shown in Fig. 6. The voltage dependence of τ_f is reduced by tubocurarine; a straight line through the lower curve yields an estimate of about 105 mV for H_f in the presence of tubocurarine. The line drawn through the lower curve in Fig. 7 was calculated from eqn. (5), taking $k^*_{+B} = 2{\cdot}7 \times 10^7$ M^{-1} sec^{-1} at -150 mV, and $H^*_{+B} = -59$ mV. Fig. 7 shows that, at any given membrane potential, τ_f gets faster when tubocurarine is present, especially at the more hyperpolarized potentials. This phenomenon is described in more detail below.

The effect of agonist concentration on the fast relaxation

Some experiments were done in which τ_f was measured in the presence of high carbachol concentrations, after treatment of the muscle with α-bungarotoxin (BuTX) to keep the current down to the normal level (less than 100 nA) at which adequate voltage clamp could be maintained. No clear change in τ_f was visible over the range 5–100 μM-carbachol, at any membrane potential (a sigmoid relationship between τ_f^{-1} and agonist concentration is expected; see, for example, Sakmann & Adams, 1976; Sheridan & Lester, 1977; Colquhoun, 1979*a*). At -70 mV a slight increase in rate (τ_f reduced by about 10 %) was visible with 200 μM-carbachol, a concentration greater than that used in most experiments. At -160 mV the increase in the rate with carbachol concentrations above 100 μM appeared to be more pronounced: τ_f was reduced by roughly 30 % with 200 μM-carbachol. These observations are consistent with the more extensive observations of B. Sakmann & P. R. Adams (personal communication) who found, at -80 mV, a 5 % decrease in τ_f with 100 μM-carbachol; a 17 % decrease in 200 μM-carbachol, and a 50 % decrease in 800–2000 μM-carbachol. The errors in inferences about tubocurarine action that might result from this effect of agonist concentration alone on τ_f are discussed later.

Effect of tubocurarine on the fast relaxation

It has usually been found, in the past, that tubocurarine has, at most, a small effect on acetylcholine channel lifetime, or on evoked end-plate current decay rate when cholinesterase is active (Beranek & Vyskočil, 1967; Katz & Miledi, 1972; Magleby & Stevens, 1972*a*). But Takeuchi & Takeuchi (1959) mention an acceleration of the evoked end-plate current by tubocurarine, and Mallart & Molgó (1978) recently observed a larger effect. Near the normal resting potential, the effect of tubocurarine on τ_f is indeed small (Figs. 7 and 11). But when the end-plate is hyperpolarized the effect becomes much more obvious. The voltage-jump experiment shown in Fig. 8 illustrates this phenomenon. In the presence of carbachol (6 μM) alone, τ_f was $6{\cdot}8 \pm 0{\cdot}1$ msec at -150 mV and $2{\cdot}3 \pm 0{\cdot}1$ msec at -70 mV (Fig. 8*A*). After equilibration with tubocurarine (5 μM) and a raised carbachol concentration (130 μM) to maintain a similar peak response, τ_f at -150 mV fell to $3{\cdot}1 \pm 0{\cdot}2$ msec,

46 % of its value is the absence of tubocurarine, but τ_f at -70 mV ($1{\cdot}8 \pm 0{\cdot}3$ msec) fell much less (Fig. 8*B*).

A similar acceleration of the fast kinetic component was seen in noise experiments. In Fig. 9, the net spectrum of current fluctuations produced by 6 μM-carbachol at

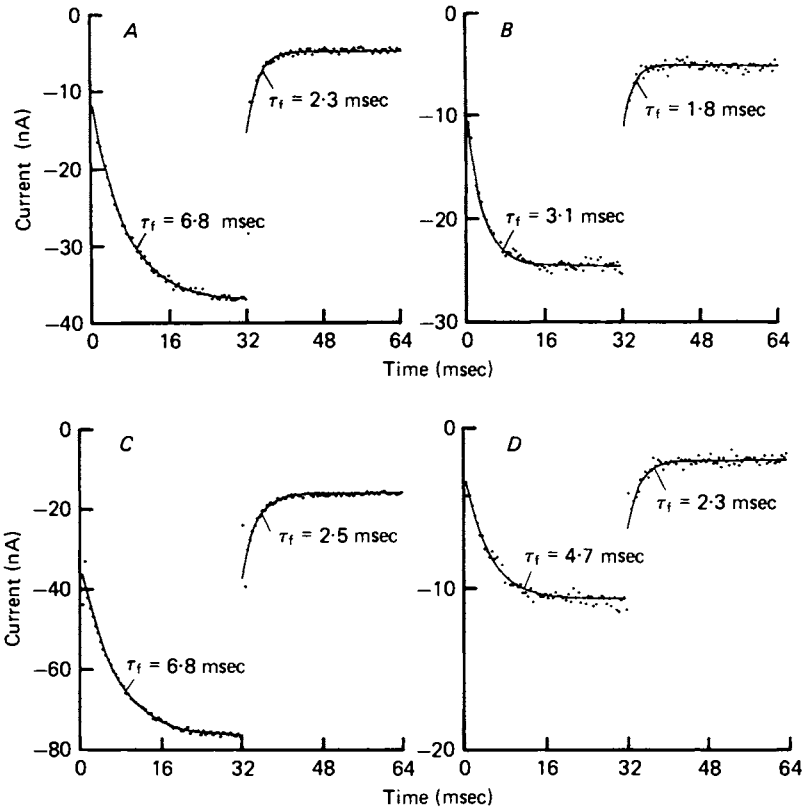


Fig. 8. Fast relaxations following 32 msec voltage jumps. Potential was stepped, at zero time, from -70 to -150 mV (*A*, *B*) or to -130 mV (*C*, *D*), and back (at $t = 32$ msec) to -70 mV. In *A* and *B* the response is maintained by increasing the agonist concentration in the presence of TC. In *C* and *D* the agonist concentration is kept constant. Curves were fitted as described in Methods. *A*, CCh, 6 μM. *B*, CCh, 130 μM with TC, 5 μM. *C*, CCh, 30 μM. *D*, CCh, 30 μM with TC, 1 μM.

a membrane potential of -120 mV is close to a single Lorentzian with a half-power frequency, f_c of $49{\cdot}4 \pm 1{\cdot}8$ Hz (i.e. $\tau_f = 1/2\pi f_c = 3{\cdot}2$ msec). However in the presence of 3 μM-tubocurarine (and a tenfold higher carbachol concentration to maintain the response) the spectrum (Fig. 9, open circles) had a higher half-power frequency, $f_c = 65{\cdot}6 \pm 2{\cdot}3$ Hz (i.e. $\tau_f = 2{\cdot}4$ msec), 33 % faster than in the absence of tubocurarine.

It might be objected that the changed rates reflected the increased agonist concentration rather than the presence of tubocurarine, but (qualitatively at least – see discussion) this is not so for the following reasons. (*a*) The decrease in τ_f observed when carbachol concentrations near 130 μM were tested, in the absence of tubocurarine (after BuTX treatment), were much smaller than the effect shown in Fig.

8*A* and *B* (see above). (*b*) The effect of tubocurarine on τ_f can be observed even when the agonist concentration is held constant, though not easily because any concentration of tubocurarine that is large enough to have a substantial effect on τ_f reduces the current so much that precise measurement is difficult. An example is shown in Fig. 8*C* and *D*. (*c*) The accelerating effect of tubocurarine can be seen on

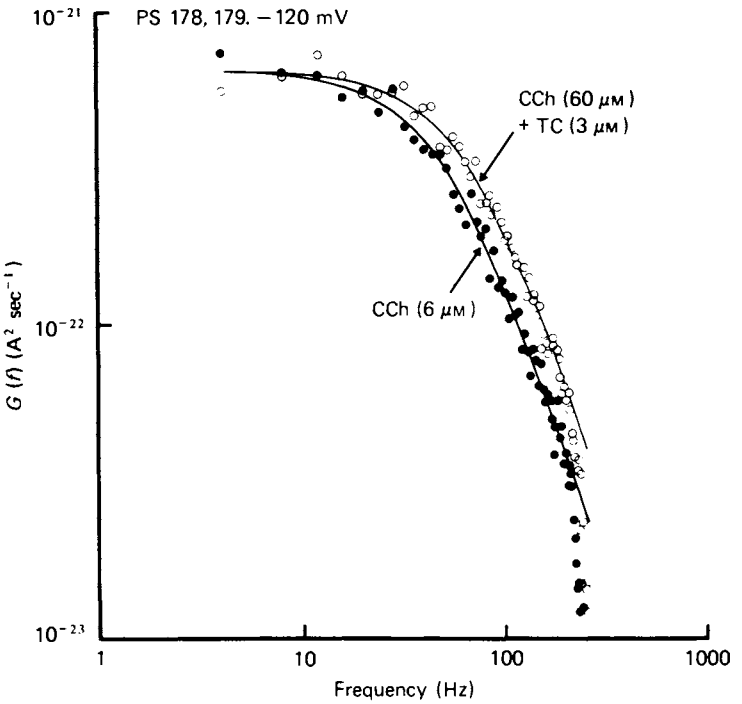


Fig. 9. Spectral density, $G(f)$, of current fluctuations measured at −120 mV. ●, CCh, 6 μM; fitted line is single Lorentzian with f_c = 49·4 Hz, τ_f = 3·2 msec. ○, CCh, 60 μM with TC, 3 μM; fitted line is single Lorentzian f_c = 65·6 Hz, τ_f = 2·4 msec; $G(f)$ multiplied by 1·41, so $G(0)$ is the same for both spectra.

the decay phase of the nerve-evoked end-plate current. The time constant of this decay is a good measure of τ_f (Katz & Miledi, 1972; Anderson & Stevens, 1973), and, because the agonist concentration is near zero during most of the decay phase, the effect of tubocurarine on τ_f cannot be attributed to high agonist concentration. An example of this effect is shown in Fig. 10; at −140 mV the time constant for the decay phase is 6·1 msec, but in 3 μM-tubocurarine it is reduced to 3·6 msec. Measurements of this sort have been exploited by Katz & Miledi (1978) in their recent study.

The effect of the tubocurarine concentration on the fast relaxation rate constant, τ_f^{-1}, in one experiment is plotted in Fig. 11. The lines are straight, within experimental error, and, as suggested by the results in Fig. 7, the slope (change in τ_f^{-1} per unit increment in tubocurarine concentration) increases as the end-plate is hyperpolarized. It will be shown later (p. 271) that the slope of the lines in Fig. 11 depends exponentially on voltage, and the average size of the effect will be discussed there.

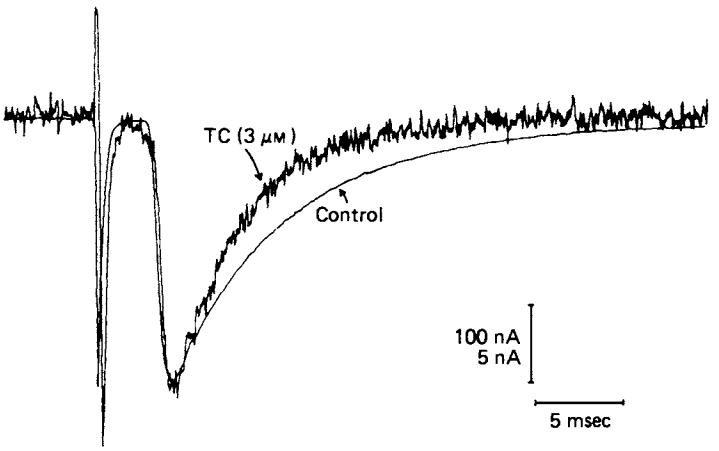


Fig. 10. Nerve evoked end-plate currents clamped at −140 mV. Currents with and without TC scaled to the same height, and superimposed. Temperature 11 °C. The control has an amplitude of 332 nA and a decay time constant of 6·1 msec. In the presence of TC, 3 μM, the amplitude is reduced to 17·7 nA (so the record looks noisier), and the time constant for decay is reduced to 3·6 msec.

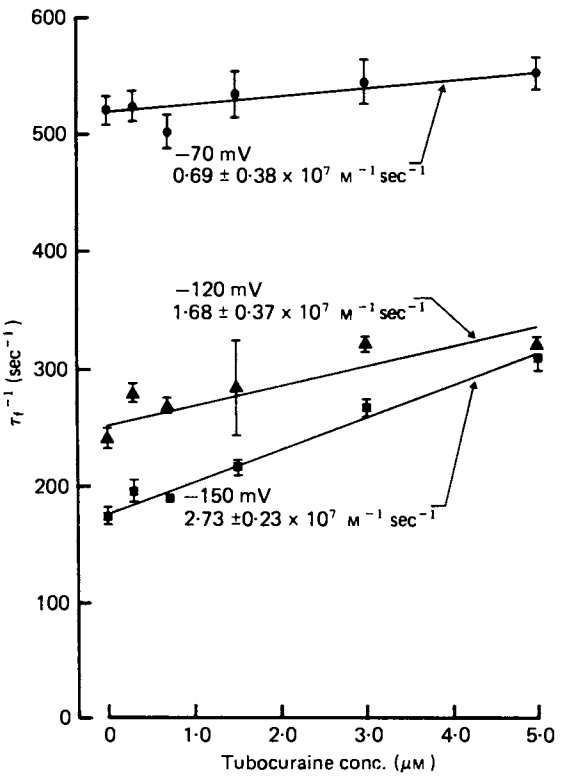


Fig. 11. The rate constant, τ_f^{-1}, for the fast relaxation (measured from voltage jumps) as a function of TC concentration. ●, at −70 mV. Slope of line $0{\cdot}69 \pm 0{\cdot}38 \times 10^7$ M^{-1}sec^{-1}. ▲, at −120 mV. Slope of line $1{\cdot}68 \pm 0{\cdot}37 \times 10^7$ M^{-1} sec^{-1}. ■, at −150 mV. Slope of line $2{\cdot}73 \pm 0{\cdot}23 \times 10^7$ M^{-1} sec^{-1}.

Effect of carbachol concentration on the slow relaxation

It is obvious that the decline in inward current as a result of desensitization (see p. 250) may interfere with the accurate measurement of the slow inverse relaxations shown in Fig. 5*B*). Examples of desensitization seen, during voltage jumps, with

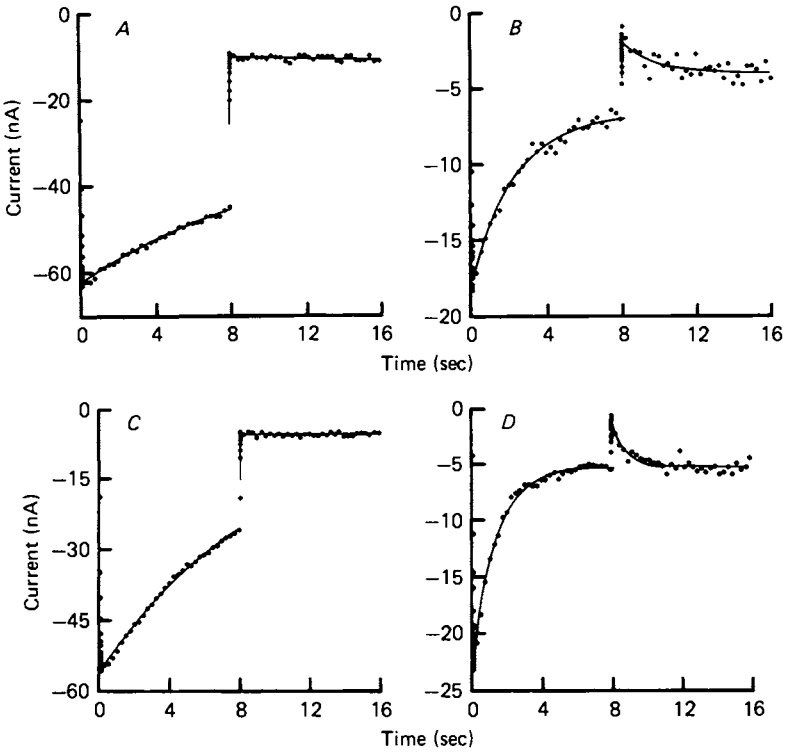


Fig. 12. Effect of high agonist concentration alone (*A*, *C*) or with a low TC concentration (0·4 μM in *B*, *D*) on slow relaxations. Muscle treated with α-bungarotoxin before the experiment, so that the high agonist concentrations used did not produce excessively large currents. Potential was changed, at zero time, from -70 to -120 mV, held there for 8 sec, and then stepped back to -70 mV at $t = 8$ sec. Curves were fitted as described in Methods. *A*, CCh, 100 μM. *B*, CCh, 100 μM with TC, 0·4 μM. *C*, CCh, 200 μM. *D*, CCh, 200 μM with TC, 0·4 μM.

higher concentrations of carbachol (100 and 200 μM after BuTX treatment) are shown in Figs. 12*A* and *C*. There is a decrease of inward current during the hyperpolarizing step, even in the absence of tubocurarine, and its amplitude increases with carbachol concentration. It is quite slow, $\tau_s = 11{\cdot}2 + 2{\cdot}45$ sec for 100 μM-carbachol (Fig. 12*A*) and $\tau_s = 9{\cdot}5 + 0{\cdot}5$ sec for 200 μM-carbachol (Fig. 12*C*). These rates are rather faster than the desensitization time constant of at least 40 sec described earlier (see Fig. 1, *A*1, *B*1), presumably because of the higher carbachol concentrations used in Figs. 12*A* and *C*. These, and similar measurements of slow relaxations in the absence of tubocurarine, are plotted in Fig. 14 (lower curve).

Effect of tubocurarine concentration on the slow relaxation

When a low concentration of tubocurarine (0·4 μM in Fig. 12) is added, without increasing the agonist concentration, clear inverse relaxations appeared (Fig. 12*B* and *D*), both at -120 and at -70 mV. At -120 mV, the time constants for these relaxations were $2{\cdot}5 \pm 0{\cdot}45$ sec (Fig. 12*B*) and $1{\cdot}25 \pm 0{\cdot}065$ sec (Fig. 12*D*). These are a good deal faster than in the absence of tubocurarine, so, even if it did not

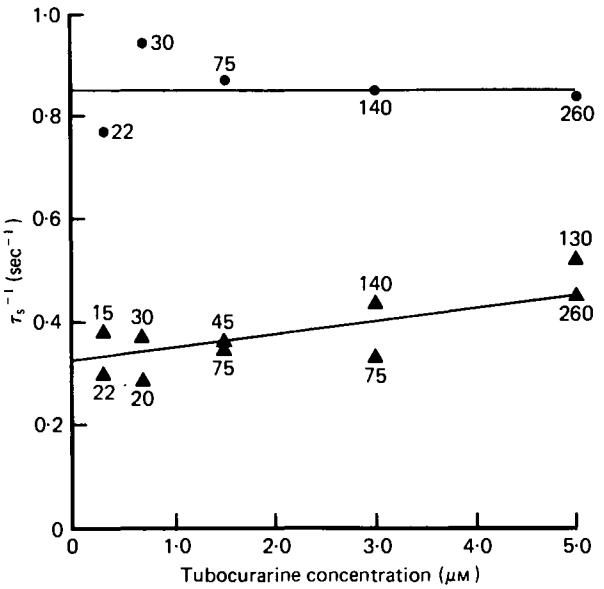


Fig. 13. The rate constant, τ_s^{-1}, for the slow relaxation as a function of TC concentration. The CCh concentration (marked, in μM, on each point) was increased along with the TC concentration, in such a way that the response size was kept in the easily measurable range. ●, at -70 mV. ▲, at -120 mV.

reduce the desensitization rate for a fixed agonist concentration, which it probably does (see Figs. 1 and 12), the measurement of τ_s should not be grossly distorted.

Measurements of τ_s at various tubocurarine and agonist concentrations are shown in Fig. 13, for two membrane potentials. In these experiments the muscle had not been treated with BuTX. At -70 mV, τ_s was around 1 sec, as was usually observed, and it did not noticeably depend (over the range shown) on either tubocurarine or carbachol concentration. At -120 mV, τ_s was slower (as can be seen in Figs. 5*B*, 12*B* and *D*), about 3 sec; and a slight increase in rate with both substances may be suspected from the results in Fig. 13. The dependence of τ_s on agonist concentration can be tested over a much wider range if the muscle is progressively treated with BuTX to maintain a reasonable response size. The results from several such experiments are shown in Fig. 14. The tubocurarine concentration was small, and constant throughout (approximately: see legend). The slow inverse relaxation rate, τ_s^{-1}, is seen to change little up to about 100 μM-carbachol, but further increases in agonist concentration caused a substantial increase in rate (see later, pp. 268, 274, for discussion of this observation). The rate is always a good deal faster than that observed in the absence of tubocurarine (lower curve). In a few experiments, voltage

jumps were not performed until the desensitization (as illustrated in Fig. 1*A*) had reached a plateau (open symbols in Fig. 14). This had no systematic effect on τ_s in the presence of tubocurarine, though the values in its absence usually appeared a bit faster.

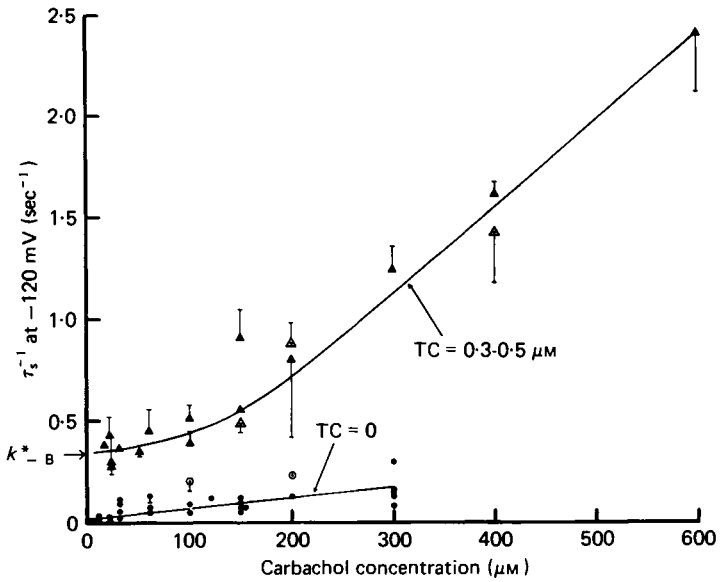


Fig. 14. The rate constant, τ_s^{-1}, for the slow relaxation at −120 mV as a function of agonist (CCh) concentration. The lower curve (circles) shows 'desensitization' rates measured in the absence of TC. The upper curve (triangles) is from experiments in the presence of 0·3–0·5 μM-TC. Muscles were progressively treated with α-bungarotoxin to keep response amplitudes in the measurable range. Filled symbols were derived from experiments in which the voltage jump was performed when the response to CCh (at the holding potential, usually −70 mV) was near its peak. Open symbols are from experiments in which the voltage jump was not performed until a plateau level of 'desensitizing action' had been reached (at the holding potential).

Interpretation of the observations

Only one interpretation of the observations in terms of mechanism has been attempted so far. It was suggested that affinity constants could be extracted from Schild plots of equilibrium observations (e.g. Fig. 3) even when, as at hyperpolarized potentials, they are curved. The hypothesis on which this is based is that a separate process, which is independent of competitive inhibition, supervenes at the higher tubocurarine concentrations and more negative membrane potentials, to cause the curvature. Our results are compatible with this hypothesis but do not prove it.

The interpretation of the equilibrium and kinetic observations will now be discussed. The most economical hypotheses are as follows.

(1) *Voltage-dependent competitive binding*

If binding of tubocurarine to the acetylcholine receptor increased sufficiently with hyperpolarization, the inverse relaxations would be expected, as would the shape of the equilibrium current–voltage relationship shown in Fig. 4. However,

this explanation seems unlikely for the following reasons. (*a*) Our interpretation of the equilibrium results suggest that the equilibrium constant for tubocurarine binding to acetylcholine receptors ($K_B = k_{-B}/k_{+B}$, see scheme (4) below) is not dependent on membrane potential. If it were, the Schild plots for different potentials would all have unit slope but would be shifted to the left by hyperpolarization, contrary to what is observed (Fig. 3). It is therefore, unlikely that either of the rate constants, k_{+B} or k_{-B} is strongly voltage dependent. (*b*) Such a mechanism cannot simply explain the increase in τ_f^{-1} produced by tubocurarine. (*c*) It cannot explain the steep increase of τ_g^{-1} with agonist concentation (see Fig. 14). (*d*) Voltage-dependent competitive block mechanisms predict that increase of the concentration of agonist and of antagonist should have opposite effects on the slow rate constant τ_g^{-1} (D. Colquhoun, in preparation). In fact both increase it (Figs. 13, 14 and 16). (*e*) Voltage-dependent competitive block cannot explain the fact that the normal slight voltage dependence of the *amplitude* of nerve-evoked end-plate currents is not much affected by tubocurarine (Takeuchi & Takeuchi, 1959; Manalis, 1977).

(2) *Effects of agonist concentration and desensitization*

The decrease in τ_f produced by tubocurarine might be a result of increased agonist concentration. Reasons for thinking that this is not correct, or at least not the only mechanism, have already been presented (p. 262). The slow inverse relaxation produced by tubocurarine might be a result of an enhancement of desensitization by tubocurarine. But desensitization in the presence of tubocurarine, as judged by the results in Fig. 1, is still quite slow, so it would be necessary to postulate the appearance of a second, much faster, component of desensitization in the presence of the drug. This is rather arbitrary, and also begs the question of the mechanism by which such a component might be produced.

(3) *Current carries tubocurarine into the synaptic cleft*

The current that flows into the synaptic cleft, to hold the end-plate at a fixed potential must, to some extent, be carried by tubocurarine, and by carbachol. This effect, and others that involve changes in the drug concentration in the cleft (see Discussion), could well be important because of the small volume of the cleft. The sort of numerical values assumed in this section, and in the Appendix, imply that when the tissue has equilibrated with 0·3 μM-tubocurarine, so that about half the binding sites are occupied by tubocurarine, there will be only one free molecule in the cleft for every 50 or more tubocurarine molecules that are bound to acetylcholine receptors. The work of Castillo & Katz (1957) suggests that tubocurarine diffuses slowly, with a time constant of the order of a second. When the end-plate is hyperpolarized, more inward current flows, and more tubocurarine will enter, thus causing channels to shut slowly; i.e. causing a slow inverse relaxation even if tubocurarine binding were purely competitive and not voltage-dependent. Furthermore, the rate and extent of this slow inverse relaxation would be expected to increase with agonist concentration (because of the larger inward current). And the rate should be increased by raising the tubocurarine concentration or by BuTX treatment, both of which should increase the diffusion rate of tubocurarine in the

cleft by reducing the number of binding sites. These predictions agree qualitatively with our observations. Suppose, to be on the safe side, that the tubocurarine has a similar mobility to all the other ions (e.g. Na), and so carries a fraction $x_B/0{\cdot}124$ of the current, where 0·124 is the molarity of ions in the Ringer solution. And suppose that only the net, drug-induced current flows into the synaptic cleft. Then a current I should produce a flow of $Ix_B/0{\cdot}124z\mathrm{F}$ mole s^{-1}, where z is the charge, taken as 2 at the pH of our experiments, and F is the Faraday. So in 5 μM-tubocurarine a current of -100 nA should cause a flux of the order of 10^7 cations of tubocurarine per second into the synaptic cleft. This is similar to the number of acetylcholine receptors in the cleft, so the effect is potentially large.

In the Appendix a method is proposed to make rough predictions about the extent and rate of the effects produced by ionophoretic flux of antagonist, on the assumption that the antagonist is purely competitive. Calculations have been made to simulate the effect of voltage jumps by numerical solution of eqn. (A 5). The value of agonist concentration, c_A^n, was chosen to give currents similar to those observed experimentally (-20 to -30 nA at -70 mV for example) and the time constant for diffusion in the *absence* of binding, τ_0, was taken, rather conservatively, as 10 msec for tubocurarine, which, in the absence of binding, would be expected to diffuse only slightly more slowly than acetylcholine. Other numerical values are specified in the Appendix. Very similar results were found, with far less trouble, by the use of eqns. (A 7) and (A 9). The expected characteristics, mentioned above, of the slow relaxation caused by ionophoresis of tubocurarine into the cleft were predicted by these calculations.

The predicted amplitude of the inverse relaxations was quite small; for $c_B = 1-20$ (e.g. 0·3–6 μM-tubocurarine) they were 0·5–1·5 nA at -70 mV, and 2–4 nA at -120 mV. This is considerably smaller than is actually observed. However, it is only just negligible, and it must be remembered that the method of calculation is rather simple-minded. The time constant predicted at -120 mV for $c_B = 1$ (e.g. 0·3 μM-tubocurarine) is about 280 msec, i.e. roughly 10 times faster than is actually observed. The predicted rate always increased slightly with hyperpolarization, quite contrary to the experimental observations. And, perhaps most striking, the time constant for the slow inverse relaxation is predicted to fall sharply with antagonist concentration, from 300 msec at $c_B = 1$ (e.g. 0·3 μM-tubocurarine), to 42 msec at $c_B = 5$ (e.g. 1·5 μM), and to 13 msec at $c_B = 20$ (e.g. 6 μM). This contrasts strongly with the very modest concentration dependence of the slow relaxation that is actually observed (see Fig. 13).

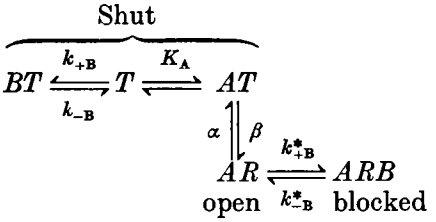
Similar arguments can be applied to the ionophoresis of agonist into the synaptic cleft. It would appear (e.g. from eqn. (A 2)) that the relative agonist concentrations, at equilibrium, in the cleft and in the bulk solution should be affected by ionophoretic flux of agonist to a similar extent to that predicted for tubocurarine. The fact that no evidence exists for the importance of this effect with agonist (e.g. Dionne & Stevens, 1975) is another indirect reason for thinking that it may not be a large effect with antagonists either. Furthermore, the effects of agonist and antagonist influx would be expected to cancel each other, though not exactly. It has been suggested (Adams, 1977*a*) that ionophoretic flux of agonist into the cleft might produce the 'sloping baseline' effect that we also see (see p. 256). However, eqn.

(A 3) suggests that an effect of this sort should reach equilibrium too quickly for this explanation to be very plausible, though the rate of equilibration could be slowed because ionophoretic influx of agonists will cause an increase in current and hence a greater influx.

(4) *Block of open ion channels plus competitive block*

In the light of other recent work on ion channel block (see Discussion), one obvious explanation for our results is that tubocurarine can block ion channels.

The simplest reaction scheme for the channel blocking mechanism is:



(4)

where A represent agonist, B represents blocking drug (tubocurarine in our case), T represents the closed conformation and R the open conformation of the ion channel. The equilibrium constant for agonist binding is K_A, and the other symbols are rate constants. This scheme is clearly too simple: for example (*a*) it ignores the well known cooperativity of the response to agonist, and (*b*) it ignores the possibility that the blocking agent may bind to the ion channel (as opposed to the agonist receptor) even when the channel is in its shut conformation (see para. 5, below). But the scheme is relatively simple, so it seems worth discussing the extent to which it can explain our observations before trying to take into account the complications of cooperativity and so on.

The predictions of scheme 4 will now be considered briefly. They are discussed in greater detail elsewhere (D. Colquhoun, in preparation). If the binding of agonist is assumed to be fast (this is not known for certain; e.g. Anderson & Stevens, 1973) then there are four kinetically distinguishable states in scheme (4), so there should be three kinetic components, the rate constants of which can be calculated as discussed, for example, by Colquhoun & Hawkes (1977). In fact we have never been able consistently to measure more than two rate constants, τ_f^{-1} and τ_s^{-1}. However, this is not entirely surprising: it can be shown (D. Colquhoun, in preparation) for that a pure competitive antagonist (state ARB omitted from scheme 4), although two kinetic components are predicted, one of them usually, under conditions like those in our experiments, has such a low amplitude that it would be unlikely to be detected in experiments (except, for example, when it has a rate close to α and would therefore be difficult to measure). Similar behaviour is predicted for scheme 4; under most conditions only two of the three components are likely to be detected. At sufficiently low concentrations of agonist we would expect that

$$\tau_f^{-1} \simeq \alpha + k^*_{+B} x_B, \tag{5}$$

where x_B is the concentration of the blocking drug, tubocurarine. And, when the slow component is very much slower than the fast one, as in the present case, we

would expect

$$\tau_s^{-1} \simeq k^*_{-B} \tag{6}$$

as long as the concentrations of agonist and antagonist are so small that τ_s^{-1} does not depend detectably on them.

In so far as these (rather drastic) approximations hold good, (*a*) the slope of the graph of τ_f^{-1} against x_B (e.g. Fig. 11) can be interpreted as an estimate of k^*_{+B}, and

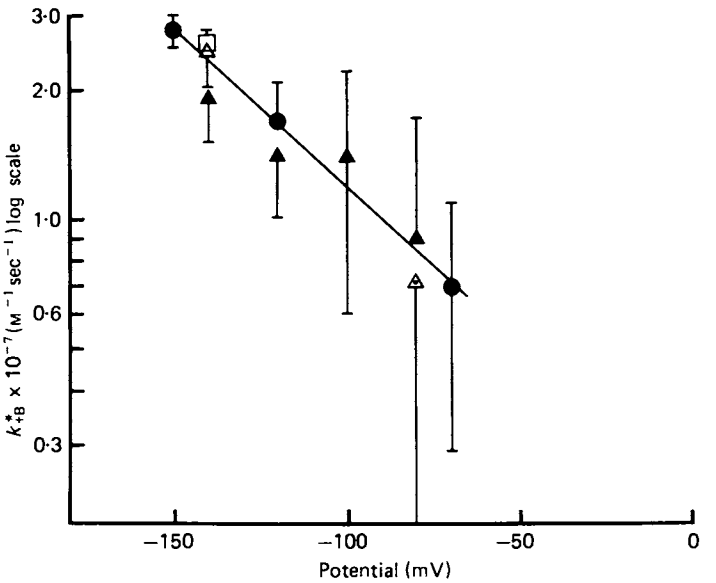


Fig. 15. The apparent channel blocking rate constant, k^*_{+B}, for TC as a function of membrane potential. Eqn. (5) was used to estimate k^*_{+B} from the accelerating effect of TC on τ_f. Open symbols are derived from experiments in which τ_f was measured by noise analysis. Filled symbols are from experiments in which τ_f was estimated by voltage-jump relaxation. Circles, triangles and squares represent three different experiments. The slope of the line corresponds with $H^*_{+B} = -59$ mV for an e-fold increase in k^*_{+B}.

(*b*) the observed slow relaxation rate can be interpreted as an estimate of k^*_{-B}. A number of estimates of the channel blocking rate constant k^*_{+B} made, from eqn. (5), in this way are plotted against membrane potential in Fig. 15. It can be seen that (*a*) very similar values were found, whether τ_f was estimated from voltage-jump relaxations, or from noise measurements, and (*b*) the channel blocking rate constant (unlike the inferred receptor block) increases quite sharply as the end-plate is hyperpolarized. The potential dependence appears to be exponential (Fig. 15) so it can be written

$$k^*_{+B}(V) = k^*_{+B}(0) \exp (V/H^*_{+B}), \tag{7}$$

where $k^*_{+B}(0) = 0{\cdot}24 \times 10^7$ M^{-1} sec^{-1}), and H_{B+}^*, the increment in membrane potential that produces an e-fold increase in k^*_{+B}, is -62 ± 8 mV (from pooled results). Thus, at -80 mV, k^*_{+B} is estimated to be about $0{\cdot}9 \times 10^7$ M^{-1} sec^{-1}, and at -150 mV k^*_{+B} is about $2{\cdot}7 \times 10^7$ M^{-1} sec^{-1}. Even if the theory were correct, there would be

several sources of error in these estimates; some of these are considered in the Discussion.

The rate constant for unblocking of channels, k^*_{-B} in scheme 4, also appears to be voltage dependent, though in the opposite direction to k^*_{+B} as might be expected. In Fig. 13, τ_s^{-1} is not strongly dependent on the concentrations of tubocurarine and carbachol, so eqn. (6) may be used as a rough approximation. This gives (see Fig. 13) estimates of $k^*_{-B} = 0{\cdot}86 \pm 0{\cdot}03$ sec^{-1} at -70 mV, and 0·32 sec^{-1} at -120 mV. However, there does appear to be some dependence on tubocurarine concentration in Fig. 13, and Fig. 14 shows a strong dependence of τ_s^{-1} on agonist concentration when the latter is increased sufficiently. Therefore an improvement on the approximation in eqn. (6) seems to be needed. When the rates are like those observed with tubocurarine, an improved approximation for the slow rate constant is (D. Colquhoun, in preparation)

$$\tau_s^{-1} \simeq \frac{k^*_{-B}}{s}\left[1+\frac{\beta''}{\alpha}(1+c^*_B)\right], \tag{8}$$

where s is the factor by which τ_f is speeded up by the addition of concentration x_B of the antagonist ($s \geqslant 1$);

$$c^*_B = x_B/K^*_B, \tag{9}$$

the blocker concentration normalized with respect to its equilibrium constant for binding to the open channel, $K^*_B = k^*_{-B}/k^*_{+B}$; and

$$\beta'' = \frac{\beta c_A}{1+c_A+c_B}, \tag{10}$$

where $c_A = x_A/K_A$ is the agonist concentration, x_A, normalized with respect to K_A, and $c_B = x_B/K_B$ is the blocker concentration normalized with respect to its equilibrium constant for binding to the agonist receptor ($K_B = k_{-B}/k_{+B}$). The second term in the square brackets in eqn. (8) shows that τ_s^{-1} is expected to increase with the concentration of agonist and with that of antagonist. For the analysis of experimental results, it is convenient to put eqn. (8) in a different form. If we define a new measure, $\lambda^\ddagger$ say, of the slow relaxation rate by multiplying the observed rate, τ_s^{-1}, by the observed speeding factor, s, and rearrange the eqn. (8) we get (D. Colquhoun, in preparation)

$$\lambda^\ddagger = s\tau_s^{-1} \simeq k^*_{-B} + \left(\frac{k^*_{+B}}{N\gamma}\right)\left(\frac{x_B I^\ddagger}{V-V_{rev}}\right). \tag{11}$$

In this equation, N is the total number of ion channels, γ is the conductance of a single open channel, V_{rev} is the reversal potential, and $I^\ddagger$ is the hypothetical current that *would have* been observed to flow at equilibrium (with concentrations x_A and x_B of agonist and antagonist) *if* the antagonist were a purely competitive blocker (no ion channel block). In those experiments with the most complete results it was possible to estimate all of the quantities needed to use eqn. (11). The value of $I^\ddagger$ can, for example, be read from the dashed lines in Fig. 2 (though in some cases this may involve rather dubious extrapolation of the observed concentration–response relationship). An example of this sort of analysis is shown in Fig. 16. The

value of $\lambda^{\ddagger}$ found from the observations, is plotted against the value of

$$x_B I^{\ddagger}/(V - V_{rev}),$$

similarly found (V_{rev} was taken as zero; see above). The slope of this graph should, from eqn. (11), be $k^*_{+B}/N\gamma$. The results at -120 mV have a slope, b say, of about

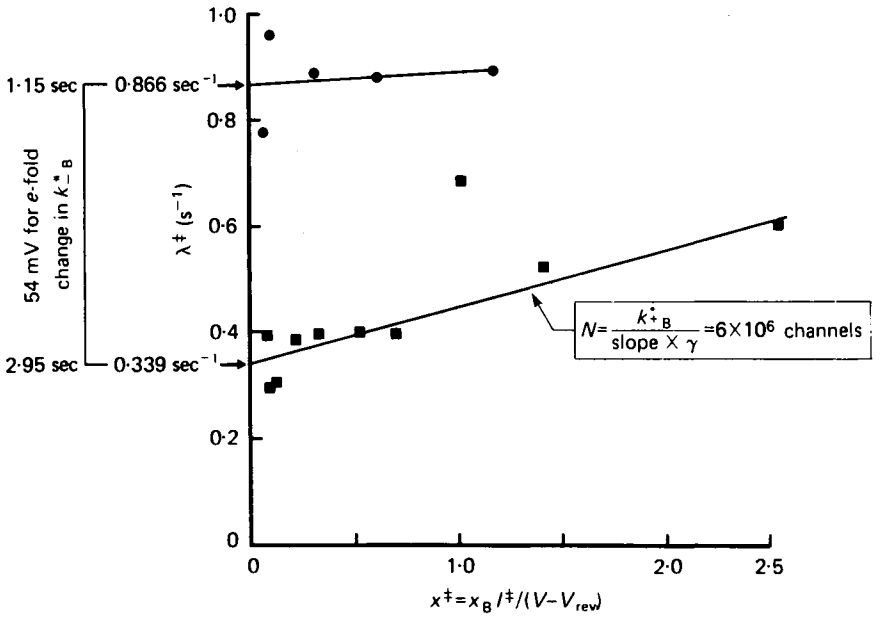


Fig. 16. Plot of the modified slow relaxation rate, $\lambda^{\ddagger}$, against the modified TC concentration, $x_B I^{\ddagger}/(V - V_{rev})$, according to eqn. (11). Lower curve (■) for results at -120 mV, Upper curve (●) for results at -70 mV. The slope of the line fitted to the results at -120 mV is 0·11 M^{-1} sec^{-1} pS^{-1}. The line drawn through the results at -70 mV was calculated from this as described in the text.

0·11 M^{-1} sec^{-1} pS^{-1} in Fig. 16. If we take $\gamma = 20$ pS (see p. 256), and

$$k^*_{+B} = 1{\cdot}65 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1},$$

as was estimated in this experiment, we can estimate the total number of channels to be about $N = k^*_{+B}/b\gamma = 0{\cdot}75 \times 10^7$. In two other experiments, estimates of N obtained in this way were $1{\cdot}0 \times 10^7$ and $0{\cdot}75 \times 10^7$, the mean being $0{\cdot}83 \pm 0{\cdot}1 \times 10^7$ (3). While this calculation can hardly be regarded as a precise method of estimating the number of ion channels at the end-plate, it is rather close to the value that would be expected from existing knowledge. Dreyer, Peper & Sterz (1978) estimate that carbachol can open, at most, about 8000 channels per μm of terminal; and Dionne, Steinbach & Stevens (1978) estimate that carbachol can open, at most, about half of all channels. Therefore a 500 μm terminal would have about $0{\cdot}8 \times 10^7$ channels (rather less than the number of BuTX binding sites which is $3\text{–}4 \times 10^7$ per end-plate; Barnard, Chiu, Jedrzejcyjk, Porter & Wiechowski, 1973; Matthews-Bellinger &

Salpeter, 1978). This agreement must be regarded as evidence in favour of a mechanism of the general type summarized in scheme (4).

The slope of the line at -70 mV in Fig. 16 should be lower in proportion to the value of k^*_{+B}; the slope drawn in Fig. 16 is that predicted from the observed values of k^*_{+B} at -70 mV and at -120 mV (together with the observed slope at -120 mV). The predicted slope at -70 mV is so shallow that it is not surprising that it cannot be estimated from the observations.

Only three experiments were sufficiently complete for the analysis shown in Fig. 16 to be done with reasonable precision. These experiments gave estimates of k^*_{-B} of 0·92 sec^{-1} at -70 mV, 1·0 sec^{-1} at -80 mV, 0·46 sec^{-1} at -100 mV and 0·34, 0·339 sec^{-1} (two experiments) at -120 mV. Many other experiments suggested values that were compatible with these estimates. If we suppose that the potential dependence of k^*_{-B} is again exponential,

$$k^*_{-B}(V) = k^*_{-B}(0) \exp (V/H^*_{-B}),$$

then the best estimates we can make are $k^*_{-B}(0) \simeq 3{\cdot}7$ sec^{-1} with H^*_{-B} about 50–70 mV. The value of about 0·34 sec^{-1} at -120 mV is confirmed by the fact that the curve in Fig. 14 appears to approach this value at low agonist concentration, as predicted by eqn. (8).

Eqn. (8) also predicts that the slow rate constant, τ_s^{-1}, should rise linearly with agonist concentration at first, and then flatten off. This is not the shape of the observed curve (Fig. 14). However, a shape of the general sort that is observed is actually expected, because of the cooperativity of the response, which has been neglected, for simplicity, in scheme 4. The reason for this expectation is roughly as follows (D. Colquhoun, in preparation). If the response is small (as it is) the fraction of channels that would be opened if tubocurarine were purely competitive, p' say, would be approximately β''/α. And if the concentration of blocker is high relative to its equilibrium constant for binding to open channels ($c^*_B \gg 1$) as is also the case in most experiments (see below), then eqn. (8) can be written, approximately, as

$$\tau_s^{-1} \simeq \frac{1}{s}[k^*_{-B} + p' k^*_{+B} x_B]. \tag{12}$$

Notice, that, apart from the factor s, this is simply the rate constant that would be expected for equilibration of binding to the open channel, viz. the sum of the dissociation rate, k^*_{-B}, and the association rate $p' k^*_{+B} x_B$. It would be expected that a factor such as p' would be involved because *open* channels are one of the reactants in the channel blocking reaction, and they make up a small fraction only of all channels. Now for non-cooperative reaction schemes, like scheme 4, p' will be hyperbolically related to agonist concentration. But in fact there *is* cooperativity and p' is actually related in a sigmoid manner to agonist concentration. The flat region at low agonist concentration, preceding the rise in τ_s^{-1} at higher agonist concentrations (Fig. 14) is, therefore, to be expected.

The equilibrium constant for binding to open channels. According to the interpretation of the observations in terms of scheme 4, it is found that the rate constant for ion channel blocking, k^*_{+B}, increases with hyperpolarization, and that for unblocking, k^*_{-B}, decreases to a similar (but less certain) extent. Therefore the equilibrium con-

stant for binding of tubocurarine to the open channel must, according to this interpretation, be strongly voltage dependent. It appears to decrease (i.e. affinity increases) e-fold for every 30 mV or so of hyperpolarization. At -120 mV, our best estimates of k^*_{+B} and k^*_{-B} give, for the equilibrium constant,

$$K^*_B = k^*_{-B}/k^*_{+B} = 0{\cdot}34/(1{\cdot}7 \times 10^7) = 20 \text{ nM}.$$

At -70 mV we similarly estimate that K^*_B is about $0{\cdot}12\ \mu$M. This is very similar to the value of K^*_B (at -80 mV) of $0{\cdot}25\ \mu$M found in *Aplysia* neurones by Ascher *et al.* (1978). It appears that the affinity is high. The reason that channel blocking is not more prominent, despite the high affinity, is that one of the reactants (open channels) is present in such low concentration.

(5) *Non-selective block of both open and shut channels*

We cannot rule out the possibility that tubocurarine can combine within channels even when they are shut, as well as when they open. Such a mechanism could produce results resembling those we have observed, at least if binding to the ion channel were voltage-dependent only when the channel was open. The main reason for attributing the non-voltage dependent part of the block to competitive block is the well known ability of both tubocurarine and acetylcholine to retard the binding of labelled bungarotoxin, acetylcholine or decamethonium. Fifteen such binding studies are being reviewed by D. Colquhoun (in preparation), and thirteen of them estimate the equilibrium constant for binding of tubocurarine to the acetylcholine (or bungarotoxin, or decamethonium) binding site to be in the range $0{\cdot}2$–$0{\cdot}6\ \mu$M, close to the value of $K_B = 0{\cdot}34\ \mu$M that we estimate.

DISCUSSION

The evidence in favour of various possible mechanisms for the actions of tubocurarine has been discussed in the Results section. There are, however, a number of potentially serious sources of error in both (*a*) our experimental methods and (*b*) our interpretation of observations even in the absence of technical errors. These will now be discussed.

Technical sources of error

Voltage clamp quality. The frog end-plate is so long that when it is all activated, as in the present experiments, the quality of voltage control at parts of the end-plate most distant from the electrodes must be dubious. Furthermore, the considerable currents that flow during jumps to hyperpolarized potentials must, to some extent, result in a potential drop across any resistance in series with the end-plate membrane (e.g. the narrow synaptic cleft) so that the imposed potential is not, as assumed, all dropped across the end-plate membrane.

The main evidence that poor clamp quality has not seriously affected our results is that the voltage dependence of τ_f with agonist alone (i.e. of the estimated mean open channel lifetime), agrees with that seen in experiments in which, because of localized ionophoretic agonist application, clamp quality is more certain (e.g.

Anderson & Stevens, 1973). The reversal potential of near zero is also in agreement with such experiments (Dionne & Stevens, 1975; Colquhoun *et al.* 1975; Mallart *et al.* 1976; Katz & Miledi, 1977). On the other hand, the time constant of about 2 msec that we observe for carbachol at -80 mV and about 8 °C is rather longer than the value of 0·9 msec reported by Dreyer, Walther & Peper (1976) for *R. esculenta* at -65 to -85 mV and 8–11 °C. If our value were genuninely longer, one reason could be imperfect voltage control.

The possibility of electrode polarization has already been mentioned in the Methods section.

Desensitization. It is clear that desensitization (as shown in Fig. 1) will affect, to some extent, estimates of (*a*) the size of equilibrium responses and (*b*) the rate of the slow relaxations. Evidence has been presented that suggests that the latter effect is not very large. But desensitization may well account for part of the non-parallelism of the dose–response curves (e.g. Fig. 2). In fact the ion channel block mechanism predicts a rather smaller degree of non-parallelism than that shown in Fig. 2, though exact predictions are difficult because of the lack of precise knowledge about the mechanism by which agonist causes channels to open (D. Colquhoun, in preparation).

Non-exponential control relaxations. In the presence of agonist, but no antagonist, we have found that relaxations following voltage jumps usually contain a second, relatively slow, component as well as the main exponential relaxation. During brief hyperpolarizing jumps this appears as a sloping base line and a similar phenomenon has been observed by other workers who have used this method in muscle (Neher & Sakmann, 1975; Adams, 1977*a*): the mechanism is unknown. In the presence of anything but the smallest tubocurarine concentrations the 'sloping baseline effect' is not usually obvious, but it may, nevertheless, cause some distortion in our results.

Errors consequent on the small volume of the synaptic cleft. At modest antagonist concentrations there must be at least fifty bound tubocurarine molecules for every molecule that is free in solution in the synaptic cleft (see Results). Molecules of tubocurarine, or of agonist, that are carried into the cleft by the clamp current may thus have a substantial effect on the drug concentration in the cleft. This problem has been considered in the Results and Appendix and the predicted effects of iono-phoretic tubocurarine influx do not agree well with our observations: however the method of prediction is over-simplified, and the size of the predicted effects is not obviously negligible, so it cannot be ruled out that effects of this sort distort our observations, and hence their interpretation.

The small cleft volume also means that any change in the number of bound tubocurarine molecules would be expected to produce an exaggerated change in the concentration in solution in the synaptic cleft. This perturbed cleft concentration would then have to re-equilibrate more or less slowly by diffusional exchange with external bulk tubocurarine solution: this process might distort the rates of changes in end-plate current that are observed. The ion channel block mechanism predicts only small changes in antagonist binding during the fast relaxations following voltage jumps, but substantial slow changes in tubocurarine binding are predicted. After a hyperpolarizing jump a considerable increase in the fraction of blocked channels (state ARB in scheme 4) is predicted. But if, as in scheme 4, there is also

voltage-independent competitive block, the fraction of receptor sites occupied by tubocurarine molecules will *decrease* following hyperpolarization, thus reducing the net change in antagonist binding that is predicted to follow a hyperpolarizing voltage step (D. Colquhoun, in preparation). Furthermore, at the tubocurarine concentration (1–5 μM) most commonly used for relaxation measurements, a considerable fraction of tubocurarine binding sites must be occupied, so it would be expected that the cleft concentration would re-equilibrate with the bulk concentration at a rate substantially faster than that of the observed slow relaxations. Nevertheless it is quite possible that this phenomenon may have caused distortion in our observations.

Mechanisms of action

The arguments presented in Results suggest that, of the various mechanisms considered there, the experimental results are described best by a combination of non-voltage-dependent competitive block with voltage-dependent block of open ion channels.

Block of open ion channels was first suggested (for ganglionic synapses) by Blackman (1959, 1970) and Blackman & Purves (1968). And evidence that quaternary local anaesthetics work in this way at the muscle end-plate was presented Steinbach (1968*a*, *b*). More recently it has been found that tubocurarine and hexamethonium, as well as procaine, seem to work primarily by block of open ion channels at *Aplysia* synapses (Marty *et al.* 1976; Ascher *et al.* 1978; Marty, 1978), and that a similar mechanism describes the action at the neuromuscular junction of (*a*) barbiturates (Adams, 1976*b*), (*b*) various local anaesthetics (Kordaš, 1970; Katz & Miledi, 1975; Beam, 1976; Adams, 1977*b*; Ruff, 1977; Neher & Steinbach, 1978), (*c*) the partial agonist, decamethonium (Adams & Sakmann, 1978) and, perhaps, other agonists (Colquhoun *et al.* 1975), (*d*) atropine (Katz & Miledi, 1973*b*; Adler & Albuquerque, 1976; Feltz *et al.* 1977; Adler, Albuquerque & Lebeda, 1978), (*e*) amantadine (Albuquerque, Eldefrawi, Eldefrawi, Mansour & Tsai, 1978), and (*f*) prednisolone (Dreyer, Peper, Sterz, Bradley & Müller, 1979).

Selective block of open ion channels alone will not adequately account for our observations with tubocurarine, but, as Manalis (1977) supposed in his work, a combination of this action with competitive block of the classical sort (Jenkinson, 1960) will describe most of our experimental results. We have recently found evidence for a similar, but faster (procaine-like) action of gallamine (Colquhoun & Sheridan, 1979). Our conclusions concerning both tubocurarine and gallamine are consistent with those of Katz & Miledi (1978), though they disagree (in the case of tubocurarine) with those of Albuquerque *et al.* (1978).

Problems of interpretation. In addition to the general sources of error already discussed, there are other problems of interpretation that are particular to competitive plus channel block mechanisms such as that shown in scheme 4. Even if a mechanism of this sort were supposed to be correct, it would still be necessary to consider two factors that have been omitted so far, viz. the effects of lack of knowledge about (*a*) the rate of competitive block and (*b*) the details of the cooperative mechanism of agonist action. More details will be given elsewhere (D. Colquhoun, in preparation).

It has already been mentioned that under a wide range of conditions it would be

expected that only two kinetic components would be large enough to be resolved experimentally. This will be true if the rate of equilibration of the antagonist with the acetylcholine binding site, considered in isolation, viz.

$$k_{+B}x_B + k_{-B} = k_{-B}(1 + c_B)$$

for scheme 4 (Hill, 1909), is either much faster, or much slower than the channel lifetime, α. If the equilibrium were much faster than α, the methods used to estimate channel blocking rates (k^*_{+B} and k^*_{-B}) should be fairly accurate. If the equilibration were much slower than α, then they would be less accurate; in particular k^*_{+B} would probably be over-estimated.

The observation that peak currents, which are attained with a rate constant of the order of α, behave in a way close to that expected for pure competitive block at equilibrium (see Fig. 3), suggests that the interaction of tubocurarine with the nicotinic receptor can reach equilibrium fairly rapidly (though this test is not a very critical one at lower tubocurarine concentrations). Numerical calculations show that the methods used to estimate the channel dissociation rate constant, k^*_{-B}, do not depend critically on the competitive rate constants. And, at the tubocurarine concentrations that are not too low, reasonable estimates of k^*_{+B} can be obtained if k_{-B} is at least 100 sec^{-1} (time constant for dissociation of tubocurarine from the acetylcholine binding site 10 msec or less). Since we estimate that $K_B = 0{\cdot}34\ \mu M$, this value implies that k_{+B} must be at least $3 \times 10^8\ M^{-1}\ sec^{-1}$. However, over some concentration ranges these calculations suggest that even a value of k_{-B} as slow as 10 sec^{-1} (time constant for TC dissociation of 100 msec, and $k_{+B} = 3 \times 10^7\ M^{-1}\ sec^{-1}$) might allow tolerable k^*_{+B} estimates. The experiments of Armstrong & Lester (1979) suggest that the dissociation time constant k^{-1}_{-B}, must be *at most* 100 msec, and other, much more indirect, estimates suggest that equilibration of tubocurarine with the acetylcholine receptor may be faster than this, with k^{-1}_{-B} = 1–40 msec (Ferry & Marshall, 1973; Blackman, Gauldie & Milne, 1975, and, in electric eel, Sheridan & Lester, 1977).

The arguments in the preceding paragraph all remain true for a variety of mechanisms which take into account the cooperativity of the agonist response (neglected in scheme 4). Further details will be given by D. Colquhoun (in preparation).

There is one prediction of scheme 4, and of related but cooperative mechanisms, that we have not observed. It is predicted that under certain conditions both the fast and slow relaxation that follow a depolarizing voltage jump may be opposite in direction to the relaxations that were usually observed (e.g. in the right-hand section of Fig. 5*B*). The predictions are as follows. (*a*) The fast relaxation should, at rather high antagonist concentrations, become inverted (i.e. represent a net opening of channels). We have observed this phenomenon under conditions similar to those for which it is predicted. (*b*) The slow relaxation should, at low agonist concentrations, reverse its normal direction (i.e. it should represent a net shutting of channels). We have never unambiguously observed this phenomenon, though at low agonist concentration virtually no slow relaxation can be seen. This may be because we have not been able to use sufficiently low agonist concentrations; in any case it does not seem to be a sufficiently large difference between prediction and experiment to rule out mechanisms of the type of scheme 4.

Can tubocurarine fit into an ion channel? The simplest interpretation of ion channel blockage is to suppose that tubocurarine can be inserted into, and hence plug, the ion channel. Crystallographic studies (Sobell, Sakore, Tavale, Canepa, Pauling & Petcher, 1972; Codding & James, 1973; Reynolds & Palmer, 1976) show that the molecule has a substantial degree of flexibility, and that the six rings do not all lie in the same plane. It can adopt a curved conformation with a convex surface that is largely hydrophilic and a concave surface that is almost entirely hydrophobic. Not enough is known about the structure of the ion channel for any judgment to be made, at the moment, about whether or not the charged group (or groups) of such a structure could be inserted into the mouth of the channel. But the mouth of the channel may be quite wide; some fairly large molecules can enter, and even penetrate, the channel (see, for example, Furukawa & Furakawa, 1959; Nastuk, 1959; Creese & Maclagan, 1970; Adams & Sakmann, 1978; and the references to local anaesthetic actions above). The voltage dependence of the 'channel block' equilibrium constant is inferred to be roughly 30 mV for an e-fold change. This parameter should be estimated by $RT/z\delta F$ (e.g. Woodhull, 1973) where δ is the fraction of the membrane field that acts on z charges. At 8 °C RT/F is about 24 mV, so to account for the observed voltage dependence, the tubocurarine molecule would have to sense about 80 % of of the membrane potential if only one charge were in the electric field, or about 40 % if both charges were in the field.

How important are the non-classical actions of tubocurarine? In view of the strong voltage dependence of the putative channel blocking component of the action of tubocurarine, it is most unlikely that it contributes appreciably to the block of single nerve-evoked end-plate potentials. However, the rate constants that apply to mammalian muscle at 37 °C are quite unknown, and it is possible that channel block, which is characteristically 'use-dependent', may contribute (along with decrease in transmitter release) to the deepening of paralysis that is often observed during high frequency stimulation in the presence of 'competitive' neuromuscular blocking agents (see, for example, Waud & Waud, 1971; Bowman & Webb, 1976).

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Appendix

Ionophoretic flux of drugs into the synaptic cleft

A simple, but rather crude, way to predict the result of the ionophoretic flux of a charged drug into the cleft, is to treat the cleft as a single well stirred compartment that contains binding sites. If diffusion into this compartment has rate constant k_0 in the absence of binding, and if the binding is supposed to reach equilibrium rapidly compared with the diffusion rate (which may not be true: see Discussion), then the rate of change of the normalized tubocurarine concentration inside the cleft, $c_1(t)$ say, should be

$$\frac{dc_1}{dt} = k_0(c_0 - c_1) - \frac{Ic_0}{0{\cdot}124zFV} - \frac{M}{KV}\frac{d(1-p_t)}{dt}. \qquad (A\ 1)$$

In this equation, I is the current (inward current taken as negative), c_0 is the normalized concentration of antagonist in the solution (i.e. molar concentration divided by K), K is the equilibrium dissociation constant for the antagonist, z is its charge (taken as 2 at the pH of our experiments), p_f is the fraction of free sites (those *not* occupied by antagonist), M is the number of moles of binding site in the cleft and V is the volume of the cleft. This is the same as eqn. (2) of Colquhoun & Ritchie (1972) except for the addition of a term for the ionophoretic flux. In the calculations, the current, I, is taken to be the net drug-induced current, i.e. it is assumed that all of this current, but none of the remainder of the clamp current, flows into the synaptic cleft. If we take $p_f = 1/(1+c_1)$, i.e. the effect of the agonist (which is present in low concentration) on antagonist binding is neglected, then, when the current, I, is *constant* the solution of this equation is exactly the same as given in Colquhoun & Ritchie (1972), as long as the appropriate equilibrium value for p_f is used. When the current, I, is constant this is

$$p_f(\infty) = \frac{1}{1+c_0(1+I/0{\cdot}124zFVk_0)}. \tag{A 2}$$

For small perturbations in p_f, the approach to equilibrium will be nearly exponential, with a time constant of about

$$\tau = \tau_0\left(1+\frac{M}{KV}\,p_f(\infty)^2\right), \tag{A 3}$$

where $\tau_0 = 1/k_0$ is the time constant for diffusion in the absence of binding. This result has also been derived by P. R. Adams (personal communication). For low ligand concentrations, τ approaches $\tau_0(1+M/KV)$. Although oversimplified, this approach may not be entirely misleading. For example if we suppose that there are 10^7 binding sites, that the volume of the cleft is about 450 μm^3, that $K = 0{\cdot}3$ μM for tubocurarine and $K \simeq 30$ μM for acetylcholine (Dreyer, Peper & Sterz, 1978 and, in electric eel, Sheridan & Lester, 1977), then $(1+M/KV)$ is about 124 for tubocurarine and 2·2 for acetylcholine. These are the factors by which binding is expected to slow down the diffusion rate (with low ligand concentration and rapid binding), and they are the sort of values that would be expected on the basis of present knowledge (Katz & Miledi, 1973*a*; Magleby & Terrar, 1975; Colquhoun *et al.* 1977; Sheridan & Lester, 1977; Armstrong & Lester, 1979).

However, in the case of tubocurarine, we have neglected the fact that the ionophoretic influx of tubocurarine into the cleft is expected to reduce the current that causes the influx. An approximate allowance for this can be made if we suppose that the current is given, at time t, by

$$I(t) \simeq N\gamma E c_A^n p_f(t)/K_c, \tag{A 4}$$

where N is the number of channels, γ is the single channel conductance, E is the membrane potential (reversal potential taken as zero, as before), c_A is the normalized agonist concentration (assumed constant), n is the low-concentration Hill coefficient and $K_c = \alpha/\beta$ is the equilibrium constant for opening of the ion channel. Eqn. (A 4) should apply, as an approximation, to a wide range of mechanisms for channel

opening. If eqn. (A 4) is substituted into eqn. (A 1) we get

$$\frac{dp_f(t)}{dt} = k_0\left[\frac{c_0 y p_f^3 - (c_0+1)\,p_f^2 + p_f}{1+\dfrac{M}{KV}\,p_f^2}\right], \tag{A 5}$$

where, for brevity, we have defined

$$y = \frac{N\gamma E c_A^n}{0{\cdot}124 zFVK_c k_0}. \tag{A 6}$$

At equilibrium, the fraction of free sites should be

$$p_f(\infty) = \frac{1}{(c_0+1)}\left[\frac{2}{1+\left(1-\dfrac{4c_0 y}{(c_0+1)^2}\right)^{\frac{1}{2}}}\right] \tag{A 7}$$

which, as expected, reduces to $1/(c_0+1)$ if there is no ionophoretic flux. Eqn. (A 5) has been solved, for a variety of conditions, by means of numerical integration. However, if the conditions are such that the denominator in eqn. (A 5) does not change much during the course of the reactions, then the equation can be integrated to give

$$t \simeq \frac{1}{2k'}\left\{\log\left[\left(\frac{p_f(t)}{p_f(0)}\right)^2\frac{X(0)}{X(t)}\right] + \frac{p_f(\infty)\,(c_0+1)}{2-p_f(\infty)\,(c_0+1)} \times \log\left[\frac{X(0)}{X(t)}\left(\frac{Y(t)}{Y(0)}\right)^2\right]\right\} \tag{A 8}$$

where, for brevity, we have defined

$$k' = \frac{k_0}{1+\dfrac{M}{KV}\,p_f(\infty)^2},$$

$$X(t) = 1-(c_0+1)\,p_f(t)+c_0 y p_f(t)^2,$$

and

$$Y(t) = -c_0 y p_f(t)+c_0+1+[(c_0+1)^2-4c_0 y]^{\frac{1}{2}}.$$

For small perturbations, this expression is well approximated by a simple exponential time dependence of $p_f(t)$, and hence, from our assumptions, of the membrane current. The time constant of this relaxation is approximately

$$\tau = \frac{1}{k'[2-p_f(\infty)\,(c_0+1)]} = \tau_0\left[\frac{\left(1+\dfrac{M}{KV}\,p_f(\infty)^2\right)}{2-p_f(\infty)\,(c_0+1)}\right]. \tag{A 9}$$

where $\tau_0 = 1/k_0$ is the time constant for diffusion in the absence of binding, and $p_f(\infty)$ is given by eqn. (A 7). Eqn. (A 9) reduces to eqn. (A 3) when there is no ionophoretic flux.

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