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THE EFFECT OF TUBOCURARINE COMPETITION ON THE KINETICS OF AGONIST ACTION ON THE NICOTINIC RECEPTOR

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1 The rates at which tubocurarine associates with, and dissociates from, the nicotinic receptor, while exerting its classical competitive effect, are still in doubt. We have investigated this problem by observing the effect of low concentrations of tubocurarine on the re-equilibration rate, following a step change in membrane potential, of the current produced by carbachol in voltage-clamped endplates of frog muscle.

2 It is expected, and observed, that in order to see the effects of *competition* (as opposed to ion channel block), sufficiently high agonist concentrations must be used so that the relaxation rate becomes faster than that seen at low agonist concentrations.

3 Small concentrations of tubocurarine were found to reduce this relaxation rate, towards a value appropriate to a lower agonist concentration.

4 The results suggest that tubocurarine equilibrates very rapidly with the nicotinic receptor.

5 Some of the possible technical problems of this sort of experiment are discussed. The results are similar to those already published for nicotinic receptors in eel electric tissue.

Introduction

One of the first quantitative pharmacological questions to be asked concerned the rate at which nicotine and curare combine with receptors in skeletal muscle (Hill, 1909). The answer is still uncertain.

Since Hill, other attempts have been made to measure the rate at which tubocurarine associates with, and dissociates from, nicotinic acetylcholine receptors. Some of these will be considered later (see Discussion). One way to approach the problem is to see how tubocurarine affects the rate at which the response to agonist re-equilibrates ('relaxes') after a perturbation, and a convenient way to perturb the equilibrium at the neuromuscular junction is to apply a step change in membrane potential ('a voltage jump') to the voltage-clamped endplate. This method was used (in *Electrophorus* electroplaques) by Sheridan & Lester (1977), whose results resemble ours (in skeletal muscle) in many ways. It can be shown that useful results are, in principle, obtainable only if the agonist concentration is high enough for the relaxation rate, in the absence of antagonist, to be substantially faster than is seen at low agonist concentrations (Sheridan & Lester, 1977). This is not easy to achieve in skeletal muscle, in which rapid desensitization complicates experiments with high agonist concentrations. We have attempted such experiments on endplates of frog skeletal muscle.

A preliminary account of these experiments has already been published (Colquhoun & Sheridan, 1980).

Methods

Experiments were performed on the *cutaneus pectoris* muscle of the frog, *Rana temporaria*. The preparation was pinned to a thin layer of Sylgard (Dow-Corning) on the glass 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 endplate in order to obtain a response with the fastest possible rise time (see Results). This pipette was also used to apply drug solutions (see Cooke & Quastel, 1973). Unless otherwise stated, the Ringer solution contained (mM): NaCl 117, KCl 2.5, $CaCl_2$ 1.0, $MgCl_2$ 1.8, sodium phosphate buffer 2.0 and 100 nM tetrodotoxin (Sigma Chemical Co.). 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

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3–6 ml/min, and the temperature, which was routinely measured with a thermistor bead placed close to the recording site, was 7.5 to 8.5°C. 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 the unbound toxin was washed out with Ringer solution.

Endplates 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 endplate was penetrated with a voltage recording microelectrode filled with 2.5 M KCl (resistance usually 3–5MΩ) and, close by, with a current injecting electrode filled with 2M potassium citrate adjusted to pH 7.0 with citric acid (4–10MΩ 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) was switched on. The size and rate of rise of miniature endplate 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 5mV rectangular command pulse.

A PDP 11/40 computer with a laboratory interface (Cambridge Electronic Design) was used to supply command potentials for the voltage-jump experiments, each potential jump being followed by on-line sampling of the endplate current, which was normally filtered above 1.5 or 2 kHz (Barr & Stroud EF3-02; 160 dB/decade roll-off; damped mode).

The membrane potential was held at −70mV throughout the experiment except during the voltage jumps. These usually consisted of a step change of potential to −150mV, followed by sampling of the membrane current at 2000 Hz for 64 ms (i.e. 128 points were collected), after which the membrane potential was stepped back to −70mV, and another 128 samples of membrane current were collected. In many experiments a series of jumps, separated by 5 s intervals, were imposed. This allowed the constancy of the relaxation time constant to be tested (see Results). In every case the currents observed in the absence of agonist were subtracted from those observed in the presence of agonist to obtain the net agonist induced current. A theoretical line was fitted to these currents by the method of least squares (equally weighted) (see also Colquhoun, 1979a, and Colquhoun, Dreyer & Sheridan, 1979). In almost all cases an adequate fit was obtained with the sum of one exponential and a straight line (see Results).

Results

In the experiments to be described we have measured the rate of re-equilibration ('relaxation') of the agonist-induced current through the muscle endplate, following a step change in membrane potential. The aim of these experiments was to measure such relaxations at much higher agonist concentrations than are commonly used (see also Sakmann & Adams, 1979), and in the presence and absence of tubocurarine. Only low concentrations of tubocurarine could be used, because it was necessary to avoid complications arising from the putative channel blocking action of tubocurarine (Katz & Miledi, 1978; Colquhoun *et al.*, 1979; see Discussion). The muscle was pre-treated with α-bungarotoxin as necessary, in order to keep the size of the response to high agonist concentrations within the range that could be adequately voltage-clamped.

Effects of response rise time and desensitization

The main problems that were encountered were the rapid desensitization that is produced by high agonist concentrations, and the necessity (see below) to obtain a response that rose as rapidly as possible following addition of the agonist. It is, of course, possible to obtain rapid responses by ionophoretic application of agonist, but in these experiments it was necessary to have a known, and uniform, agonist concentration at the endplate, so ionophoresis could not be used.

Examples of the inward current (plotted downwards) in response to various carbachol concentrations are shown in Figure 1. The response rose to a maximum only slowly with a low carbachol concentration (10 μM) in the cell illustrated in Figure 1a. There was little obvious desensitization. In the same cell, a high carbachol concentration (600 μM) caused a faster rise to a peak, followed by rapid desensitization as shown in Figure 1c. This rapid rise may have been partly a result of the more rapid diffusion of the agonist that would be expected as a result of both the α-bungarotoxin treatment (100 nM for 21 min) that preceded the response and the high agonist concentrations used (Colquhoun, Henderson & Ritchie, 1972; Katz & Miledi, 1973; Armstrong & Lester, 1979). But the main reason for the early peak in Figure 1c is, no doubt, that desensitization cuts short the rapid rise long before a plateau can be reached. When care was taken to position the inflow pipette as close as possible to the endplate and the maximum flow rate compatible with stability was used, much faster rise times could be achieved with low agonist concentrations. For example, the response to carbachol (10 μM) shown in Figure 1b rises to 90% of its equilibrium value in about 6 s. The rise time varied

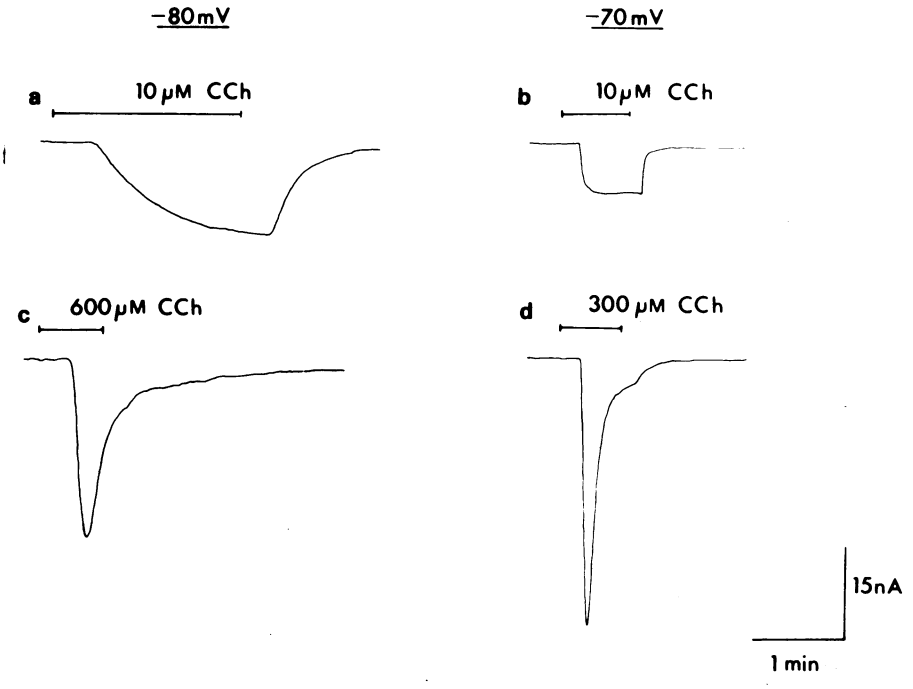


Figure 1 The response to low and high carbachol (CCh) concentrations at membrane potentials of −80(a and c) −70 mV (b and d). Inward currents are plotted downwards. The time lag between application of the agonist and the start of the response is due, almost entirely, to the time taken for the solution to flow through the cooling tube before it reached the tissue. (a) Shows the response to a low concentration (10 μM) in a cell with a rather slow response; (c) shows the response (after α-bungarotoxin treatment) of the same cells to a high carbachol concentration; (b) and (d) show responses to low (10 μM) and high (300 μM) carbachol concentrations in cells that responded much more rapidly.

substantially from cell to cell even when the flow rate, and pipette position were kept as constant as possible, doubtless partly as a result of anatomical differences (e.g. fast rise time could not be achieved when the endplate was on the underside of the muscle fibre). With our system, the fastest rise times that could be achieved with low agonist concentration was roughly 3 s, and such rapidly responding cells showed very fast responses, with high peaks, when high agonist concentrations were applied, as illustrated in Figure 1d.

The experimental procedure is shown in Figure 2. The sharp downward deflections on the current record show the position of responses to 64 ms hyperpolarizations to −150 mV (the responses are, of course, not faithfully reproduced by the pen recorder). A voltage jump was usually imposed near the peak of the response, and at 5 s intervals thereafter. The position of the jumps in the absence of the agonist, which were subtracted from those in its presence, are also shown in Figure 2b. In Figure 3a the rate constant found by fitting (see below) the relaxation that followed hyperpolarization, is plotted against the time (from the onset of the response) at which the voltage jump was imposed. The rise time of the response was relatively slow in this cell; even with 40 μM carbachol after moderate α-bungarotoxin treatment, it was about 10 s, and for the response to 200 μM carbachol shown in Figure 3, the rise time was about 6 s. It is clear from Figure 3 that the rate constant measured in the first jump, which was imposed at the peak of the response, is slower than the rate constants measured from the later jumps which were imposed while the response was decreasing because of desensitization. It therefore seems very probable that the agonist concentration at the endplate had not reached equilibrium at the time of the peak response, but was still rising (the position of the peak response being determined by both the increasing agonist concentration and the concomitantly increasing extent of desensitization). The rate constants measured from the 2nd, 3rd and 4th jumps were virtually constant, despite the fact that the response was desensitizing rapidly at this stage. The amplitude of the relaxation (the difference between the instantaneous current at zero time, to the asymp-

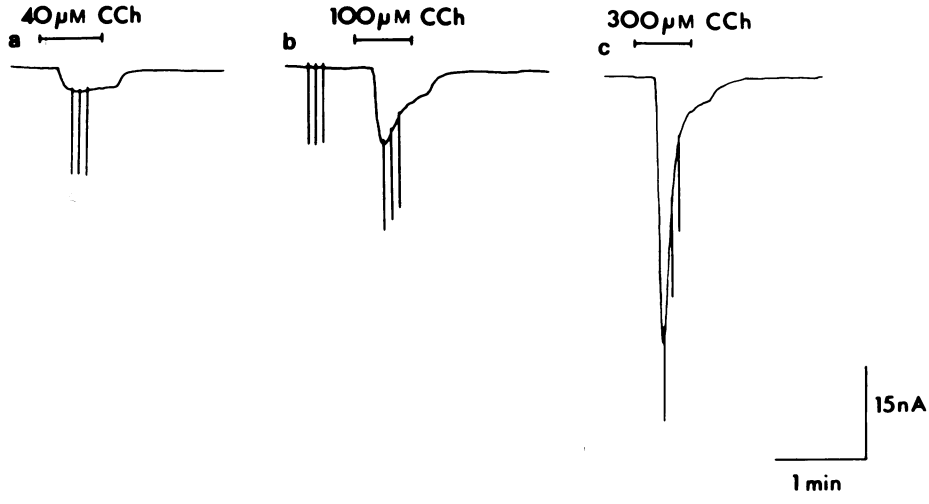


Figure 2 Response of one cell to three different carbachol (CCh) concentrations at −70 mV (a, 40 μM; b, 100 μM; c, 300 μM). This cell had been treated with α-bungarotoxin (100 nM for 17 min) before the responses shown here were elicited. The downward deflections show the positions of 64 ms voltage-jumps to −150 mV. In (b) the position of the control voltage jumps, imposed in the absence of agonist, are also shown.

tote inferred from the fitting procedure) is plotted on the same time scale in Figure 3b; this amplitude is a measure of the size of the response so the independence of the rate constant and the degree of desensitization, after about 10 s had elapsed from the start of the response, can be seen clearly. In cells with still slower responses to agonist, the rate constant appeared to increase continuously with time. It was therefore important to use only cells that responded rapidly to the application of agonist, and to test that the rate constant did not change with time. A rather better cell is illustrated in Figure 4. Even before α-bungarotoxin treatment, the rise time of the response to 15 μM carbachol was only 4 s in this cell. The response to 500 μM carbachol, shown in Figure 4, rose to a peak in about 1 s, and the first jump was imposed at 3 s after the onset of the response, when the current had already fallen to about half of its peak value. The rate constant (Figure 4a) was much the same for all four jumps in this case, although the degree of desensitization for the last three jumps was profound, as shown in Figure 4b.

Shape of the observed relaxations

Examples of the voltage-induced relaxations are shown in Figure 5. It was usually found that a single exponential curve was an inadequate fit to the observations, but the sum of an exponential curve and a straight line (i.e. a sloping baseline) provided an adequate fit in most cases. Even under optimum conditions it has been found that a sloping baseline is often needed (Neher & Sakmann, 1975; Adams, 1977; Colquhoun *et al.*, 1979), although the reason for this is not understood. In these experiments the conditions are far from optimum; a sloping baseline is to be expected because the relaxation is often superimposed on a more-or-less rapidly desensitizing response, and the responses were often small and therefore noisy.

Figure 5a shows the relaxation of the current induced by 40 μM carbachol, a fairly low concentration. The least squares estimate of the time constant, τ is 4.2 ms (i.e. the rate constant, $1/\tau = 238 s^{-1}$) and the estimate of the baseline slope is −0.034 nA/ms. This slope corresponds to a change in current of −1.1 nA in the first 32 ms of the relaxation (which is shown in Figure 5a), so the slope is not very steep compared with the signal that is being measured (though the slope of −34 nA/s is very steep on the time scale of the whole response). The relaxation of the current induced by 400 μM carbachol in the same cell is shown in Figure 5b; the estimated time constant, 2.21 ms ($1/\tau = 452 s^{-1}$) is shorter than with 40 μM carbachol and the estimated slope of the baseline is 0.045 nA/ms. The relaxation of the current induced by the same high carbachol concentration, 400 μM, measured after equilibration of the tissue with 0.4 μM tubocurarine, is shown in Figure 5c; the estimated time constant is longer in the presence of tubocurarine, 3.4 ms ($1/\tau = 292 s^{-1}$). The estimated slope of the baseline in this case was −0.013 nA/ms.

The effect of tubocurarine on relaxation rate

Whatever the concentration of agonist, one would

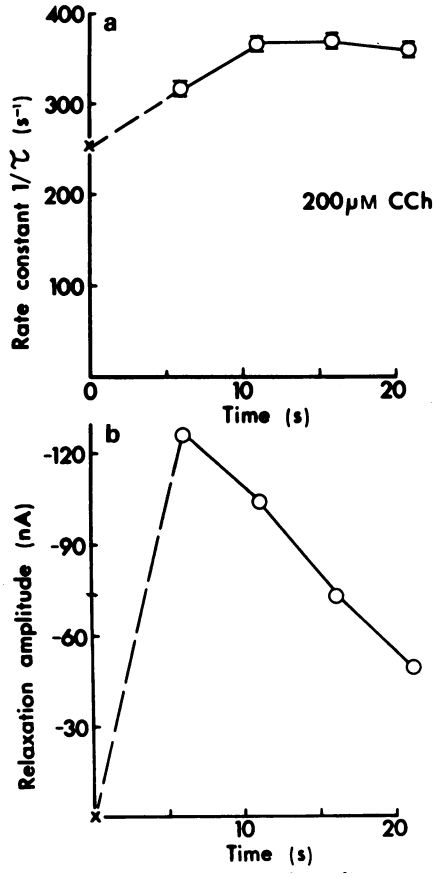


Figure 3 (a) The rate constant (reciprocal of the time constant) for the relaxation of the agonist-induced current following a voltage jump to −150 mV. The rate constant is plotted against the time at which the response to the agonist started (see Figures 1 and 2). The agonist was carbachol (200 μM). The cross indicates the rate constant ($\simeq\alpha$) for the relaxation with a low carbachol concentration. In this cell the onset of the response to a low carbachol concentration was quite slow. (b) The amplitude of the relaxation, the rate constant of which was plotted in (a). The amplitude declines with time as desensitization progresses (see Figures 1 and 2).

expect tubocurarine to speed up the relaxation rate, by virtue of its putative open-channel-blocking action. However, in this study the concentration of tubocurarine was kept low (0.4 μM or less) to avoid this effect; on the basis of the results of Colquhoun *et al.* (1979), it would be expected that 0.4 μM tubocurarine would increase the relaxation rate constant, at a membrane potential of −150 mV, by about 11 s^{-1} (see p. 82). This predicted effect is too small to be easily resolvable in the present experiments.

However, when the agonist concentration was sufficiently high to cause an increase in relaxation rate, the addition of a low concentration of tubocurarine always appeared to *decrease* the relaxation rate, as already illustrated in Figure 5b and c. This effect was seen qualitatively in many experiments, but its quantitative measurement was more difficult. This is because it was necessary to use only rapidly responding cells, to check that the relaxation rate had reached a plateau, and to obtain a substantial number of responses to high agonist concentration in the same cell with and without tubocurarine. The results of two of the more complete experiments are shown in Figures 6 and 7. The relaxation rate constant at a given agonist concentration is seen to be reduced by low tubocurarine concentrations. An alternative way of saying the same thing is to measure the dose-ratio: the carbachol concentration had to be increased by a factor of 1.6–1.8 in the presence of 0.4 μM tubocurarine (or by about 1.3 in 0.15 μM tubocurarine) in order to achieve the same relaxation rate as was seen in the absence of tubocurarine.

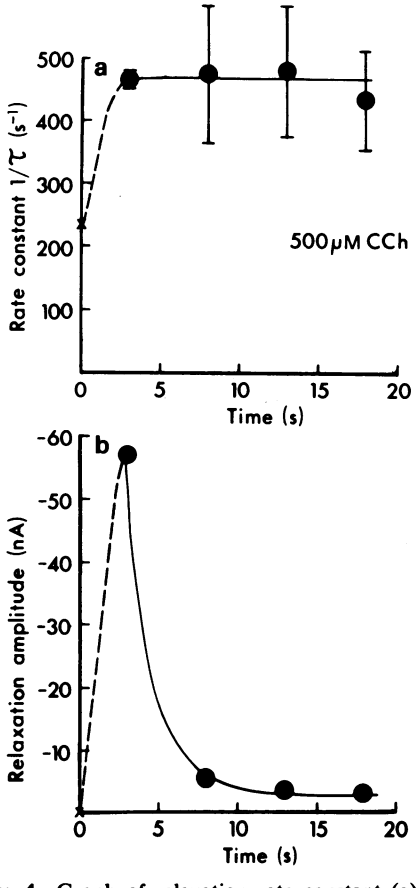


Figure 4 Graph of relaxation rate constant (a) and amplitude (b) against the time from onset of the response to agonist. These graphs are like those shown in Figure 3, except that this cell responded much more rapidly to application of a low agonist concentration.

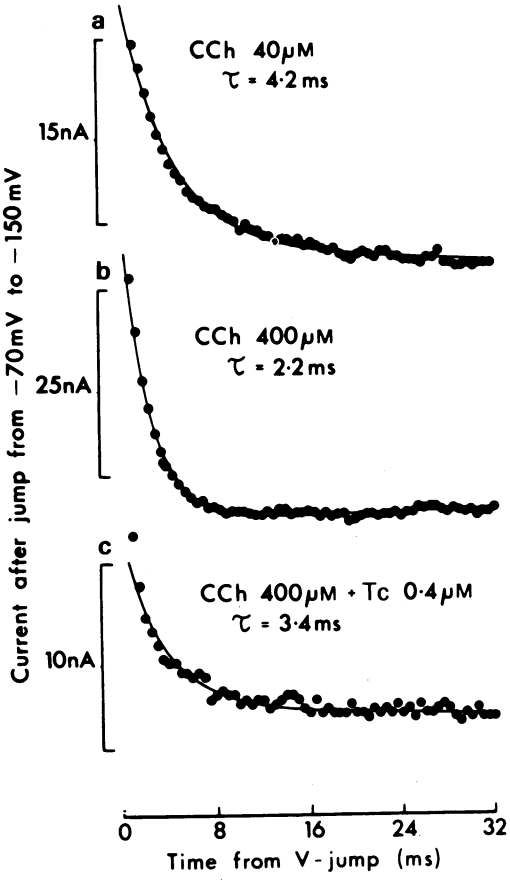


Figure 5 Examples of relaxations of the agonist induced current through the endplate membrane following a jump of membrane potential from −70 mV to −150 mV at $t=0$. (a) Carbachol (CCh) 40 μM; time constant 4.2 ms. (b) Carbachol, 400 μM, time constant 2.2 ms. (c) Carbachol, 400 μM, as in (b), but in the presence of tubocurarine (Tc) 0.4 μM; time constant 3.4 ms. See text for further details.

Interpretation of the tubocurarine effect

Three of the assumptions that will be made in the following discussions are as follows:

(i) The binding of agonist is sufficiently fast that the system can be represented, in the absence of antagonist, by the simple scheme

$$\text{shut} \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} \text{open} \qquad (1)$$

in which the effective opening rate constant, β', increases with agonist concentration. This assumption is discussed, for example, by Anderson & Stevens (1973), Colquhoun & Hawkes (1977, 1981) and Sakmann & Adams (1979). It may well not be strictly true (Colquhoun & Sakmann, 1981), but our inferences do not depend very critically on this assumption.

(ii) The putative channel blocking action of tubocurarine (Katz & Miledi, 1978; Colquhoun *et al.*, 1979) will not interfere seriously with the interpretation. The results of Colquhoun *et al.* (1979) suggest that the association rate constant for blocking of open ion channels should be about $2.7\times10^{7}\text{M}^{-1}\text{s}^{-1}$ at a membrane potential of −150 mV. The largest tubocurarine concentration used in these experiments was 0.4 μM; this concentration should make the control relaxation rate faster by about

$$(2.7\times10^{7})\times(0.4\times10^{-6}) = 11\,\text{s}^{-1} \qquad (2)$$

It will be assumed that the slow relaxation that is expected as a result of 'channel block' can be ignored when, as in these experiments, the voltage jump is brief (and a sloping base is fitted).

(iii) It will be assumed that the concentration of agonist and antagonist at the endplate surface remain constant throughout the relaxation. This assumption is necessary if the relatively simple analysis in terms

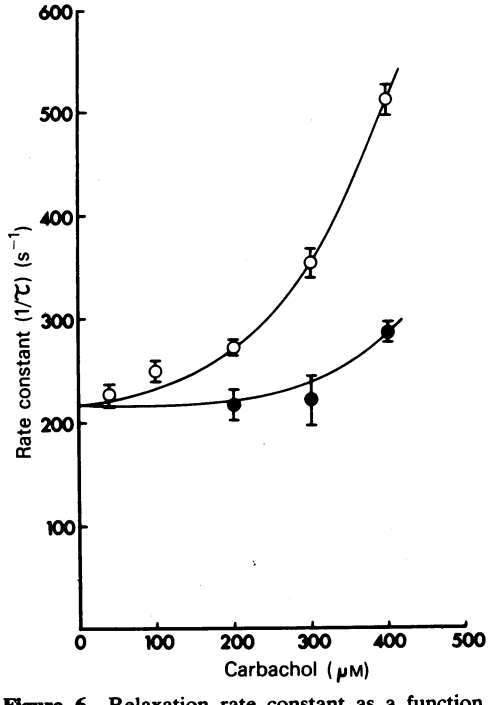


Figure 6 Relaxation rate constant as a function of agonist concentration. Determined in the absence of (○) of antagonist, and in the presence of 0.4 μM tubocurarine (●).

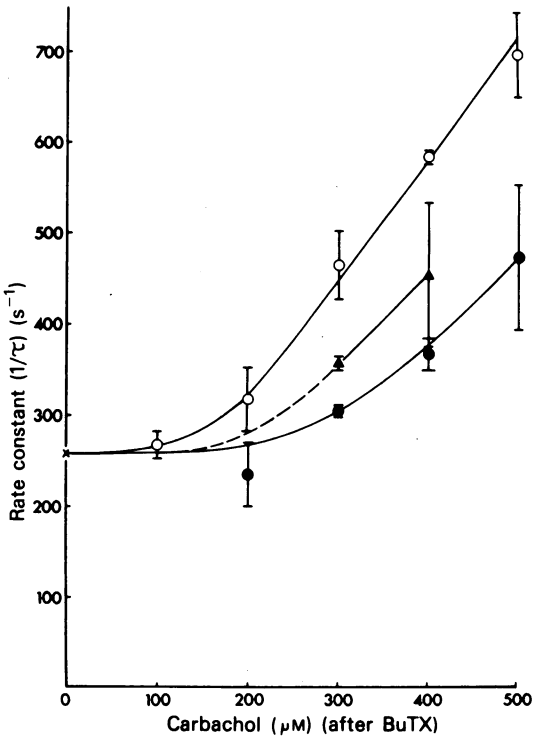


Figure 7 Relaxation rate constant as a function of agonist concentration. Determined in the absence (○) of antagonist, and in the presence of 0.15 μM (▲), or 0.4 μM (●) tubocurarine.

of exponentials (as described, for example, by Hill, 1909 and Colquhoun & Hawkes, 1977) is to be valid. This is probably the most dubious of all of the assumptions, but no results obtained so far have been sufficiently precise for it to be tested experimentally.

It is, of course, expected that (in the absence of antagonist) the time constant of the voltage-induced relaxations will decrease as the agonist concentration is increased (see Figures 6 and 7) because scheme (1) implies that the observed time constant will be

$$\tau = 1/(\alpha+\beta') = [1-p_1(\infty)]/\alpha \qquad (3)$$

where $p_1(\infty)$ is the fraction of ion channels that is open at equilibrium (see also Sheridan & Lester, 1977; Sakmann & Adams, 1979). The effect of *competitive antagonism* on the relationship between the relaxation time constant and agonist concentration can be predicted easily in two extreme cases, as follows:

(1) *Tubocurarine works slowly.* If the rate of equilibration of tubocurarine with the acetylcholine receptor were slow (on the time scale of the 64 ms voltage jumps) then the competitive antagonism would appear, on this time scale, to be irreversible. The number of receptors available for activation would be reduced, but the receptors that were not blocked would behave exactly as in absence of the antagonist. In this case, therefore, the time constant of the relaxation should be unchanged by tubocurarine. This prediction is clearly incompatible with the observations shown in Figures 5–7, but is apparently the case with antagonism by α-bungarotoxin.

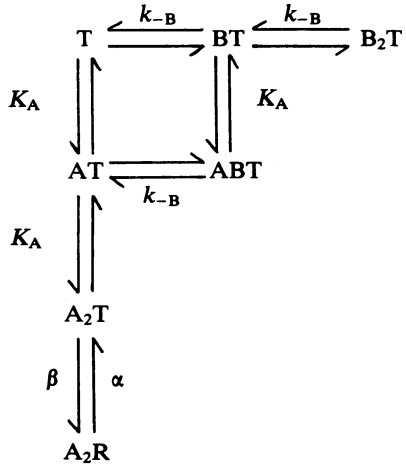
(2) *Tubocurarine works rapidly.* If the rate of equilibration of tubocurarine with the acetylcholine receptor were so rapid that equilibrium between tubocurarine, acetylcholine and receptor were maintained throughout the voltage jump, then tubocurarine should slow down the relaxation rate. The reason for this is as follows. It is observed (Jenkinson, 1960; Colquhoun *et al.*, 1979) that under conditions where antagonism by channel block is negligible at equilibrium (such as those employed here), the fraction of open channels at equilibrium obeys the Schild equation, i.e.

$$r = 1 + c_B$$

where r is the agonist dose ratio, $c_B = x_B/K_B$, x_B is the concentration of antagonist, and K_B is the equilibrium constant for competitive binding of tubocurarine to acetylcholine receptors. Now, under the assumption that tubocurarine equilibrates so fast that all shut states, blocked or not blocked, are at all times in equilibrium, the system can still be described by Scheme (1). Therefore the effective channel opening rate constant, β', must also obey the Schild equation, and hence the observed relaxation time constant, $\tau = 1/(\alpha+\beta')$, must do so as well. In other words, when a concentration x_B of competitive antagonist is present, the time constant at a given agonist concentration, x_A, should be increased (i.e. the rate constant $1/\tau$ should be reduced) to the value that corresponds to a lower effective agonist concentration $x_A/(1 + c_B)$. Analysis of the results in Figures 5 and 6 shows that a tubocurarine concentration of 0.15 μM produced a dose-ratio of around 1.3 (which implies $K_B \simeq 0.5$ μM), and 0.4 μM tubocurarine produced a dose ratio of 1.6–1.8. These values imply that K_B = 0.5–0.7 μM, close to the estimates of K_B, 0.43–0.73 μM, given by Jenkinson (1960) for *Rana temporaria* (the species used in these experiments). They are slightly larger than the estimate of K_B = 0.34 μM found in *Rana esculenta* by Colquhoun *et al.* (1979), though in view of the experimental errors (particularly in the present work), it is doubtful whether the difference is real. It is thus clear that, within experimental error, our observations are compatible with the hypothesis that competitive antagonism by tubocurarine equilibrates very rapidly. In order to see what this means in terms of rate con-

stants, it will be necessary to consider what would be expected if equilibration of tubocurarine with the acetylcholine receptor were neither very fast nor very slow.

(3) *Tubocurarine works at an intermediate rate.* In this case the prediction of the appearance of relaxation, in the presence of a competitive agonist and a high agonist concentration, is more complicated. The amplitude, and indeed the number, of kinetic components will depend on the mechanism of agonist action, as well as on the characteristics of the antagonist. This question will be considered in more detail elsewhere. Qualitatively it can be stated that there are substantial ranges of rate constants for the competitive binding of tubocurarine in which only one exponential component would be obvious (particularly in experiments of the sort presented here, in which slow processes would not be resolvable). If the competitive antagonist equilibrated rapidly, but not rapidly enough for the limiting case (2) to be attained, the component with the slowest rate constant would still be predominant but it would be rather slower than in the limiting case; thus curves such as those in Figures 5 and 6 should appear to be shifted by rather *more* than is predicted by the Schild equation. For a still slower competitive antagonist, more than one component should become visible, and eventually the fastest component would become predominant. At first this would be rather *faster* than the control value in the absence of tubocurarine, until the limiting case for a slow competitive antagonist is reached, in which the rate constant is the same in the presence and absence of antagonist (case (1) above). Thus, whatever the rate constants for the association and dissociation of the competitive antagonist, it is never predicted that a shift of *less* than that predicted by the Schild equation will be observed. Numerical calculations have been carried out on the basis of a simple mechanism in which two agonist molecules (denoted A) are assumed to bind sequentially before an ion channel can open. This is probably as close as we can get to the truth at present (e.g. Colquhoun, 1979b). In the presence of a competitive agent, B, the mechanism becomes



(4)

where T represents the shut conformation, and R the open conformation, of the receptor-ion channel. It was assumed (see start of this section) that agonist binding is fast, so this mechanism has four kinetically distinguishable states; the time course of voltage-jump relaxations should, therefore, be described by the sum of three exponential components (as long as the transition probabilities are independent of time, a condition which is not necessarily fulfilled; see discussion).

The time course of relaxation following a voltage step from −80 mV to −150 mV was calculated, for scheme (4), by the methods described by Colquhoun & Hawkes (1977). The time constant, and amplitudes for the three exponential components were computed over a range of (normalized) agonist concentrations, in the absence of the competitive blocker, and in the presence of blocker in a concentration (as in our experiments) close to its equilibrium constant (i.e. $c_B = 1$). The calculations were performed for a range of possible values for the microscopic dissociation rate constant, k_{-B}, of the blocker, viz 10, 100, 500, 1000 and $10^4\,s^{-1}$. In order to do these calculations it was assumed (1) that the microscopic equilibrium constant (K_A) for agonist binding was the same for the first and second molecules bound (the evidence for this is flimsy, but our conclusions are not critically dependent on this assumption), (2) K_A was independent of membrane potential, (3) that tubocurarine binding was independent of potential (Colquhoun *et al.*, 1979), (4) that β was $1000\,s^{-1}$ (see Sakmann & Adams, 1979) and independent of membrane potential, and (5) that $\alpha = 500\,s^{-1}$ at −80 mV, and $184\,s^{-1}$ at −150 mV. It was found that under most conditions (in particular, if the deviations from either of the two extreme cases discussed above were not too big) one of the three exponential components was predicted to have a much bigger amplitude than the other two. When the time constant for this dominant component was plotted against agonist concentration in the presence and absence of the blocker, in the same way as is used for the experimental observations in Figures 6 and 7, it was found that the results behaved quantitatively as discussed above, and that even with k_{-B} as large as $1000\,s^{-1}$ the shift of the curve by antagonist was still somewhat greater than that expected from the Schild equation. Our observations, on the other hand, show no evidence of the shift being greater than is predicted by the Schild equation. We therefore infer that our results imply that the rate constant for dissociation of tubocurarine from the acetylcholine receptor cannot be much less than $1000\,s^{-1}$ (i.e. tubocurarine cannot stay on the receptor for much longer than 1 ms, on average). It follows that if, as we have already assumed in mechanism (4), the binding of tubocurarine does not induce a conformation change then the association rate constant for its binding can be calculated from the ratio of k_{-B} to the equilibrium constant

(0.5–0.7 μM). This calculation would suggest an association rate constant greater than 10^9 M^{-1} s^{-1}.

Discussion

In addition to the method used by Sheridan & Lester (1977), and in this paper, two other approaches have been tried with the object of measuring the rate at which competitive antagonists associate with, and dissociate from, nicotinic receptors.

One method is to measure the rate of recovery of the response to an agonist following a brief ionophoretic application of tubocurarine (Castillo & Katz, 1957; Waud, 1967; Armstrong & Lester, 1979). However, it has been shown that rates measured in this way are likely to be limited by diffusion, even when the nerve terminal is lifted away from the endplate (Waud, 1967; Armstrong & Lester, 1979; R.D. Purves, personal communication). Therefore, the shortest time constant for recovery seen in such experiments, down to about 100 ms, provides only a minimum value (10 s^{-1}) for the dissociation rate constant of tubocurarine.

Another method that has been tried, is to measure the extent to which a very rapidly equilibrating antagonist (such as hexamethonium) can relieve the block of transient agonist application by a supposedly slower antagonist such as tubocurarine. This method has been used by Ferry & Marshall (1973) and Blackman, Gauldie & Milne (1975). The latter authors interpreted their results in terms of receptor interaction rates, according to the theory of Ginsborg & Stephenson (1974). They inferred that the half-time for dissociation of tubocurarine is around a millisecond in rat diaphragm endplates, and even briefer in amphibian endplates; that is, the dissociation rate constant is about 700 s^{-1} in rat, and faster in amphibia.

The voltage jump experiment on frog endplates in this paper, and those of Sheridan & Lester (1977) on *Electrophorus* electroplaques, lead to a similar conclusion to those of Blackman *et al.* (1975). As all of these authors point out, a dissociation rate constant of around 1000 s^{-1}, together with an equilibrium constant of about 0.5 μM, implies an association rate constant 2×10^9 M^{-1}s^{-1}. This value is very fast, faster than has been observed in most biochemical reactions, and faster than can reasonably be expected for a diffusion controlled reaction (Gutfreund, 1972). In the few cases where association rate constants as fast as this have been observed, it has been postulated that some special effect, such as lateral diffusion along the surface to the receptor, must exist (e.g. Richter & Eigen, 1974). It is possible (e.g. Colquhoun, 1973) that the binding of tubocurarine could be accompanied by a conformation change, in which case the above calculation of the association rate constant would be invalid. There is, at present, no strong evidence for such a conformation change.

For example, Katz & Miledi (1977) found no detectable effect of high tubocurarine concentrations on normal frog endplates (hyperpolarization of 0.004 ± 0.009 mV). In addition, most experiments have indicated that tubocurarine has no detectable tendency to favour the desensitized conformation of the nicotinic receptor (Rang & Ritter, 1969; Weber, David-Pfeuty & Changeux, 1975; Colquhoun & Rang, 1976; Weiland & Taylor, 1979), though a small effect cannot be excluded (Quast, Schimerlik, Lee, Witzemann, Blanchard & Raftery, 1978; Neubig & Cohen, 1979). On the other hand, there is no conclusive evidence *against* a conformation change either; further steps, following the initial binding of tubocurarine, would probably not be detected by present methods if they were not accompanied by changes in endplate conductance or desensitization.

The major problem with the interpretation of all of these experiments is, as usual, the problem of diffusion. It has been pointed out (D.R. Waud, personal communication; Colquhoun, 1975) that diffusion-limitation of observed rates may mimic the Ginsborg-Stephenson (1974) phenomenon, or at least render inaccurate the interpretation of that phenomenon in terms of receptor interaction rates. It is also quite possible that the diffusion problems could distort voltage jump relaxations of the sort described here, and by Sheridan & Lester (1977). During a hyperpolarizing jump, receptor occupancy by agonist should increase, and occupancy by antagonist should decrease, so there is a real danger that their concentrations in the synaptic cleft may change substantially during the relaxation (especially in the case of tubocurarine, for which the number of bound molecules on the endplate is a good deal bigger than the number of free molecules in the synaptic cleft). Such concentration transients are explicitly assumed not to occur in the conventional analysis (e.g. Colquhoun & Hawkes, 1977). It is, of course, possible to do elaborate calculations to try to predict the effect of such diffusion processes. We have done some simple calculations of this sort and the results do not suggest that such mechanisms are likely to account for our observations. However, much more detailed calculations would be necessary to put this conclusion on a firm basis, and the models used for such calculations contain a number of uncertainties. It therefore seems wiser, rather than to attempt to answer the diffusion problem theoretically, to develop new experimental approaches in order to discover whether the rate of interaction of tubocurarine with the nicotinic acetylcholine receptor is really fast as it appears to be.

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