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THE MODE OF ACTION OF ANTAGONISTS OF THE EXCITATORY RESPONSE TO ACETYLCHOLINE IN *APLYSIA* NEURONES

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SUMMARY

1. The mode of action of various antagonists of acetylcholine (ACh) excitatory effects on *Aplysia* neurones was studied under voltage clamp. ACh was applied by iontophoresis whereas antagonists were applied in the bath. Tubocurarine and hexamethonium were the most thoroughly studied compounds.

2. The 'elementary current', calculated as the ratio of the variance of the ACh noise to the mean ACh induced current, was not modified by any of the antagonists tested.

3. The evolution of the ACh induced current after a voltage jump, which is normally described by a single exponential, was modified by all the antagonists tested. A common feature of the modified relaxations was the appearance, over a certain concentration range of the antagonist, of two successive and opposite exponential components.

4. The characteristics of the composite relaxations depend on the antagonist. For a given antagonist they vary with membrane potential, ACh concentration, and antagonist concentration.

5. The noise power spectra of the ACh induced current showed changes consistent with those of the relaxations.

6. In the absence of antagonists, the current induced by a steady application of ACh increases linearly with hyperpolarization. In the presence of antagonists, the $I-V$ curve shows a marked curvature, indicating a proportionally larger reduction of the ACh response at more negative membrane potentials.

7. The voltage sensitivity of the blocking action of hexamethonium and decamethonium is noticeably stronger than that of monovalent antagonists.

8. A model is proposed which accounts for the observed effects. It assumes that the antagonists studied bind preferentially to the 'activated' ACh-receptor complex, and convert it to a non-conducting state. Kinetic constants can be calculated for this reaction; e.g. for curare, at 12 °C and -80 mV, the dissociation and association constants were estimated at 0·1 sec^{-1} and 4×10^5 $M^{-1} sec^{-1}$.

9. Partial replacement of the extracellular Na by Tris modifies the relaxations observed in the presence of hexamethonium. Hexamethonium appears less effective in the presence of Tris, which supports the hypothesis that the binding site of the antagonists is linked to the ionic channel.

INTRODUCTION

In the preceding paper (Ascher, Marty & Neild, 1978) we have analysed the excitatory effects of ACh on some *Aplysia* neurones. From the study of ACh noise and of relaxations following voltage jumps, we have determined the average size of the current, i_{el}, flowing through the individual channels opened by ACh, as well as the average duration of opening of one channel, τ. In the present paper we report on similar experiments done in the presence of various ACh antagonists.

From data obtained on other cholinergic synapses it was expected that some antagonists, but not others, would modify the ACh noise and the relaxations of the ACh induced current. At the frog neuromuscular junction, for example, curare alters neither the elementary current nor the noise power spectrum (Katz & Miledi, 1972). On the other hand a number of compounds, known to alter the end-plate current time course, also alter the noise power spectra and the relaxation (Steinbach, 1968; Katz & Miledi, 1975; Adams, 1977; Feltz, Large & Trautmann, 1977; see Steinbach & Stevens, 1976). In most cases the description of the system in the presence of these antagonists requires the use of two time constants, one faster and one slower than the single time constant sufficient to characterize the response in the control situation. These changes have been interpreted in the frame of a variety of models (cf. Gage, 1976; Adams, 1977) which have in common the assumption of a non-competitive antagonism.

Our initial interest in applying this kind of approach to the excitatory ACh response of *Aplysia* neurones was our knowledge that the pharmacology of this response differs from that of the most studied nicotinic receptors of the motor end-plate and of the electroplaque. Since the initial observation by Tauc & Gerschenfeld (1962) that hexamethonium was a selective antagonist of the excitatory ACh response in *Aplysia*, a number of indications have accumulated to suggest that the ACh receptor mediating ACh excitation in *Aplysia* neurones resembles more that of the vertebrate autonomic ganglia than those of the frog muscle or electroplaque (Kehoe, 1972; cf. Ascher & Kehoe, 1975). It was therefore interesting to see whether noise and relaxation analysis in *Aplysia* neurones would, again, separate two classes of compounds: those altering the noise spectra and the relaxations, and those without effect. In fact, all the antagonists tested, and in particular curare and hexamethonium, showed time and voltage dependent effects, and provoked striking changes in the pattern of relaxations observed after voltage steps. As will be seen, the study of these changes, combined with noise analysis, supports the hypothesis that the antagonists tested bind preferentially to the 'activated' (conducting) receptor-channel complex, converting it to a non-conducting state. Some of the results support a more specific hypothesis, first proposed by Blackman (1959) and recently developed by Adams (1975, 1976) according to which antagonists could act as 'pore-blockers'.

METHODS

The neurones and the basic experimental procedures used have been described in the preceding paper (Ascher *et al.* 1978). Records of the currents were first taken in control sea water, then in a solution containing the antagonist. In experiments done at 12 °C, measurements started only 5 min following changes in the bathing solution, to insure temperature equilibration. In the absence of ACh, none of the antagonists had any effect on the clamp current.

Most experiments were done in sea water buffered with Tris (10 m-mole/l., pH 7·8) at 12 and 22 °C. Some experiments were done in sea water buffered with $NaHCO_3$ (5 m-mole/l., pH 7·8, e.g. Fig. 2). Some were done in low Na, Tris substituted sea water (e.g. Fig. 12) (see Table 1 in Ascher *et al.* 1978).

Variance measurements. The frequency range for variance measurements was chosen according to the shape of the corresponding power spectra. In general, the cut-off frequency of the low-pass filter was set at around 10 times the value of the cut-off frequency of the fast component of the spectrum. The corresponding error on i_{el} is then less than 10 %. The cut-off frequency of the high pass filter (first order) was either 1·5 or 0·1 Hz. Some experiments were done with DC recording. In the experiments using AC recording, the value of i_{el} obtained from the variance reading was corrected by assuming that the power spectrum was flat between 0 and 2 Hz. This correction was always less than 20 %. It is possible that with certain antagonists, and in certain concentration ranges, the corrected value of i_{el} will lead to an underestimate of i_{el} as the power spectra can be expected to present a slow component in the lowest frequency range (Katz & Miledi, 1975; Ruff, 1977).

Noise power spectra and relaxations induced by voltage jumps. In principle the time constants calculated from the cut-off frequencies of the components of the spectrum are identical to the time constants of the components of the relaxation. The relative amplitudes of the various components are not the same with the two methods, however. In practice, we found the study of the relaxation more convenient than that of the spectra. The main reason was that it lent itself better to the study of the slowest components, which were often barely detectable in the spectra but prominent in the relaxation. An additional reason for preferring relaxation was that the power spectrum analyser was not on line, whereas relaxations could be observed while the experiment was in progress.

In the voltage jump experiments the holding potential was usually −40 mV, and hyperpolarizing steps were applied when the response to a steady application of ACh had reached its plateau value. Because a Ca induced increase in K permeability often obscured the interpretation of the relaxation following the return to the holding potential (see the preceding paper) emphasis was put on the relaxation following the onset of hyperpolarizing pulses.

Effect of the ACh concentration and anticholinesterase effects. In most of the experiments (for noise or relaxation studies) the iontophoretic current of ACh was kept at the same value before and after the application of the antagonist. In some experiments, however, its value was increased after application of the antagonist until the response reached the same amplitude as in the control. Under such conditions, quantitative differences in the relaxation characteristics were observed, particularly with low antagonist concentration.

Such observations indicating that the ACh concentration affects the relaxation have interesting implications for the mode of action of the antagonists; but they also raise a particular experimental problem since many of the antagonists tested have anticholinesterase effects. This is particularly marked for decamethonium, less for hexamethonium. The effect was revealed by the fact that the application of the antagonist at low concentration increased the ACh response, but not the response to carbachol or the response to ACh in the presence of neostigmine (5×10^{-5} M). The practical implication of this anticholinesterase effect was that we could not assume that, for the same iontophoretic current, the ACh concentration was the same before and after application of the antagonist. As a result, experiments involving a quantitative evaluation of the spectra or relaxations were usually made in the presence of neostigmine 5×10^{-5} M, applied at least one hour before the beginning of the recordings. Neostigmine by itself had no effect on the noise and relaxation characteristics (Ascher *et al.* 1978).

RESULTS

Invariance of i_{el} in the presence of antagonists

The elementary current, i_{el}, was measured as described in the preceding paper, i.e. as the ratio of the noise variance to the total ACh induced current. None of the antagonists tested (curare, hexamethonium, atropine, procaine) modified the value of i_{el} at the membrane potentials between −40 and −120 mV. This is illustrated in Fig. 1.

Constancy of i_{el} is what is expected from competitive antagonists (Katz & Miledi, 1972) but it is also expected if non-competitive antagonists block the open channels in all-or-none fashion. It may be shown quite generally that if each possible state of the channel, however many those are, has either a conductance of zero, or a conductance corresponding to an elementary current i_{el}, then $i_{el} = (\text{var}(I)/\langle I \rangle)$ on the two conditions that the number of channels having an elementary current i_{el} is small compared with the number of non conducting channels (in the present case, either closed or blocked by an antagonist) and that each channel behaves independently of the others (see Colquhoun & Hawkes, 1977). In the more general case

$$i_{el} = (\text{var}(I)/\langle I \rangle k),$$

where k is the fraction of channels with zero conductance. In most of the experiments of Fig. 1, the iontophoretic current was kept constant for the entire experiment (before

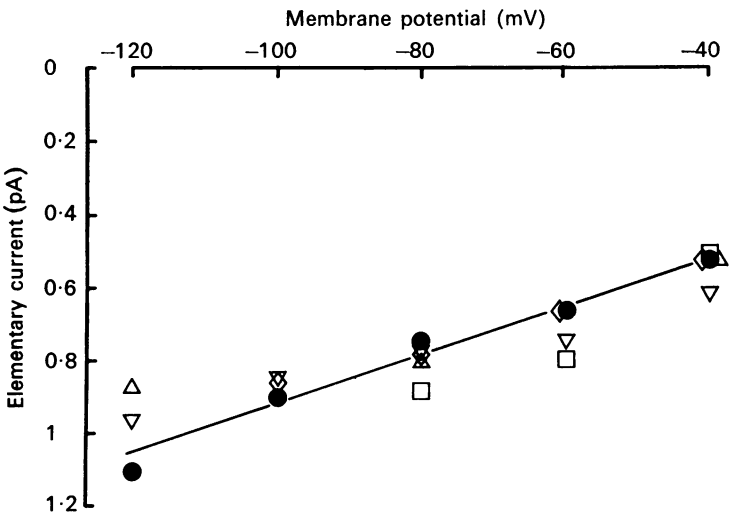

Fig. 1. Measurement of i_{el} in the presence of antagonists. ◇, tubocurarine (5×10^{-5} M); □, hexamethonium (4 or 5×10^{-5} M); △, atropine (5×10^{-5} M and 10^{-4} M); ▽, procaine (2×10^{-4} and 4×10^{-4} M); ●, control values taken from Fig. 2 of the preceding paper. Each point is the mean of a number of experiments ranging from 1 to 4. S.E. never exceeded 20 % of the mean value. Temp. 12 °C. The band width of the variance to DC converter was adapted to the shape of the noise power spectra. The low-pass filter was at 500 Hz in the case of atropine and procaine, where the cut-off frequency of the fast component of the spectrum was of the order of 50 Hz; it was at 100 Hz in the case of hexamethonium and curare. The high-pass filter was at 0·1 or 1·5 Hz. In this last case, a correction was introduced as indicated in the Methods section.

and after the addition of the antagonist) and we can assume that the 'low concentration limit' was satisfied (see Ascher *et al.* 1978), so that $k \simeq 1$. But even in some experiments where the iontophoretic current was increased in the presence of antagonists we did not observe a decrease of $\text{var}(I)/\langle I \rangle$.

We will now consider the data indicating that the antagonists studied do not behave as competitive antagonists. We will first consider in detail the case of tubocurarine (curare) which was the prototype of the compounds used.

The effects of curare

The effect of curare depends on the dose of ACh. A first indication that curare does not act as a competitive inhibitor of ACh in the neurones studied can be found by comparing the effects of a given dose of curare on the responses to two ACh applications differing in intensity. The predictions of competitive inhibition are that the fractional inhibition of the ACh response should be independent of the ACh concentration (for low ACh concentrations) or should decrease when the ACh concentration increases. In

Fig. 2. Effects of curare on the responses to two doses of ACh. Responses to a 20 sec application of ACh (arrows) in normal sea water (*A*, *C*) and after addition of curare (*B*, *D*). *A*, *B*: low ACh dose (calibration: 0·5 nA, 4 sec). *C*, *D*: high ACh dose (calibration: 5 nA, 4 sec). Curare reduces the maximal response by a factor of 1·9 in *A*, *B*, a factor 3·3 in *C*, *D*. Membrane potential −70 mV. Temp. 12 °C. $NaHCO_3$ buffer. Neostigmine 5×10^{-5} M.

fact, it was found that the response to higher doses of ACh was proportionally more reduced than was the response to the smaller doses. This is illustrated in Fig. 2. In this experiment, the ACh pipette was placed quite far (about 100 μm) from the cell. The control responses are shown at different amplifications but on the same time scale. For the low dose of ACh, the response had not reached its maximum at the end of the ACh pulse; for the higher dose, the plateau was reached after 20 sec. After perfusion with tubocurarine 10^{-4} M, the response to the low intensity ACh pulse (measured at the end of the pulse) was reduced by a factor of 1·9 (from 1·05 to 0·55 nA) whereas the response to the high intensity pulse was reduced by a factor of 3·3 (from 11·5 to 3·5 nA).

Thus the ratio of the control response to the response in the presence of curare, which we call Λ, increases with ACh concentration.

Time dependence of the effect of curare. Fig. 3 again illustrates the effects of curare on the response to a long ACh pulse, but in this experiment the ACh pipette had been brought closer to the neurone: under these conditions, the control response has a faster rise time; it also shows a progressive, slow decline after reaching its peak. In the presence of curare, the rising phase is nearly identical, but the decline following the peak is faster and more pronounced than in the control. As a result, the ratio of the control response to the response in the presence of curare, Λ, varies with time: it is smaller at the beginning of the ACh application than at the end.

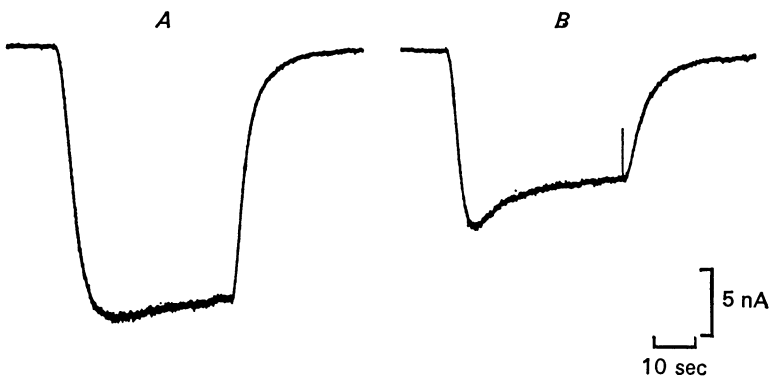

Fig. 3. Time-dependent effects of curare. A, control. B, curare 2×10^{-5} M. In this experiment the ACh pipette was placed closer to the cell surface than in the experiment of Fig. 2, to obtain a faster rise. After reaching a maximum, the current showed a slow decrease. In the presence of curare the initial part of the response was little affected, but declined more rapidly (time constant 11 sec) to a lower plateau value. The peak was reached 14 sec after the beginning of the injection in the control and after only 7 sec in the presence of curare. Calibration 10 sec, 5 nA. Membrane potential −90 mV. Temp. 12 °C. Tris buffer. Neostigmine 5×10^{-5} M.

It is not possible in the experiment illustrated to consider the ACh application as instantaneous. However, this experiment suggests that if ACh could be applied instantaneously, the initial peak might not be reduced at all by curare. In other words, most of the effect of curare occurs after the onset of the ACh application.

Voltage dependence of the effects of curare. The effects of curare are proportionally larger when the membrane is hyperpolarized. In the experiment of Fig. 4, ACh was applied at −60 and at −100 mV, before and after perfusion with curare (2×10^{-5} M). The plateau response at −60 mV was reduced by a factor of 1·3 (from 6 to 4·7 nA) whereas the response at −100 mV was reduced by a factor 2·0 (from 15 to 7·5 nA). As expected from the time dependence of the effects of curare, the difference was less marked on the initial peak of the response.

A more detailed analysis of the kind of experiments described in Figs. 2 to 4 is limited by various factors. The size and the time course of the ACh response are subject to slow variations with time, possibly linked with variations of the ACh concentration gradient inside the iontophoretic pipette, and of the level of desensitization of the receptors. In addition, changes in solutions often lead to changes in the

relative positions of the pipette and the cell. To circumvent some of these difficulties, we turned to voltage step experiments, taking advantage of the fact that the voltage dependence of the effects of curare is reflected in changes in current relaxation patterns.

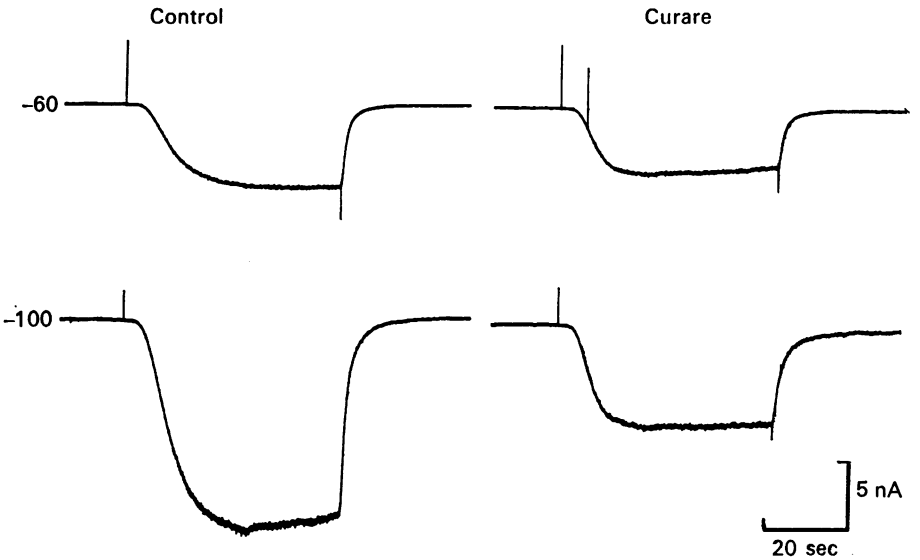

Fig. 4. Voltage-dependent effects of curare. The responses to long identical applications are shown at -60 mV (upper traces) and at -100 mV (lower traces) in normal sea water (left) and in the presence of 2×10^{-5} M curare (right). The ratio of the control response over that observed in curare is 1·3 at -60 mV, and 2·0 at -100 mV. As a result the ratio of the maximum currents measured at -100 and -60 mV goes from 2·5 (control) to 1·6 (curare). Calibrations: 20 sec, 5 nA. Temp. 12 °C. Neostigmine 5×10^{-5} M.

Voltage jumps in the presence of curare. The two-component relaxations. Fig. 5 illustrates how the effects of a voltage jump on the ACh-induced current are modified in the presence of curare. The membrane potential was stepped from -40 to -80 mV and back; two pulse lengths were used (300 msec and 7 sec). Before application of curare, the relaxation was as described in the preceding paper: the hyperpolarizing step triggered an immediate jump of the ACh current to a value noted I_{in} (resulting from the 'instantaneous' increase of the driving force) followed by an exponential increase towards a steady value (I_{ss}). The same experiment was then repeated in the presence of 5×10^{-5} M curare, first using the same dose of ACh as in the control, then using a slightly higher dose adjusted to give the same steady current at -40 mV as in the control. In both cases, the hyperpolarizing pulse triggered again an instantaneous jump followed by an exponential increase of the current, but then the current decreased slowly before reaching a steady value. As expected from the experiment of Fig. 4, we found that the steady current was much more decreased by curare at -80 than at -40 mV, i.e. that the effects of curare are increased by hyperpolarization.

Because the two components of the relaxation observed in the presence of curare have very different speeds, it is possible to study them separately. Thus, the time constant of the fast component can readily be obtained by taking for its asymptote a straight line extrapolated from the slow component. This time constant is the same

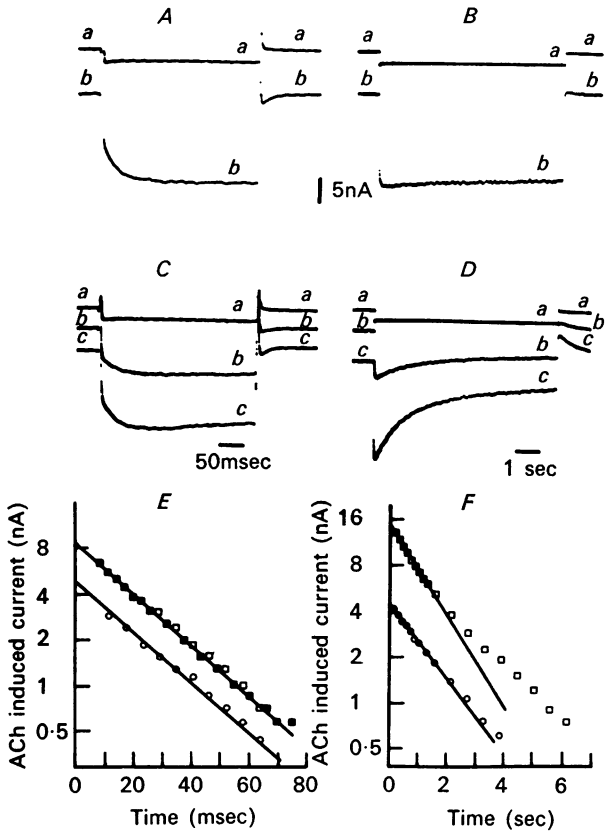

Fig. 5. A relaxation experiment in the presence of curare. *A*, this control relaxation was obtained in the same condition as that illustrated in Fig. 5 *A* of the preceding paper (Ascher *et al.* 1978). A hyperpolarizing pulse bringing the membrane potential from −40 to −80 mV was first applied in the absence of ACh, then during an iontophoretic application of ACh. The voltage trace is not illustrated. The difference between the current recorded in the absence of ACh (*a*) and during the ACh application (*b*) represents the ACh induced current. The hyperpolarizing pulse lasted 300 msec. Calibration as in *C*: 50 msec. *B*, same experiment as in *A*, but the sweep speed was slow (horizontal calibration as in *D*: 1 sec) and the hyperpolarizing pulse lasted 7 sec. *C*, same experiment as in *A* (fast sweep speed) but after addition of tubocurarine (5×10^{-5} M). The upper current trace (*a*) shows the current in the absence of ACh; the middle current trace (*b*) the current in the presence of the same dose of ACh as in *A* (*b*); the lower trace (*c*), the membrane current recorded after the iontophoretic current had been increased to give, at −40 mV, a steady-state current matching that recorded at −40 mV in *A*. *D*, same experiment as in *C* (tubocurarine 5×10^{-5} M) but the sweep speed was slow, as in *B*, and the hyperpolarizing pulse lasted 7 sec. *E*, *F*, semilogarithmic plot of the relaxations. *E* corresponds to the fast 'direct' relaxations shown in *C*; *F* corresponds to the slow, 'inverse' relaxations shown in *D*. Only the 'on-relaxation' (those following the hyperpolarization to −80 mV) have been plotted. The base line for the fast component was obtained by extrapolation of the slow component. ■, values taken from *A*; ○, values taken from the middle trace, *b*, of *C* and *D*; □, values taken from the lower trace, *c*, of *C* and *D*. The time constant of the fast relaxation (27 msec) is the same for all records. The slow relaxation is not exponential. It is clear, however, that it is accelerated by increasing the ACh dose. Temp. 12 °C. Neostigmine 5×10^{-5} M.

as in the control for the two ACh doses (Fig. 5). Furthermore the relative amplitude of the relaxation is as in the control: this may be seen from the fact that at the high ACh dose (which elicited the same current at -40 mV as in the control), the amplitude of the exponential is the same as in the control (Fig. 5). Finally, it is possible to calculate the instantaneous current $I_{in}(-80)$ by back extrapolation of the first component, and to calculate the reversal potential E_{rev} by comparison with $I_{ss}(-40)$ (Ascher *et al.* 1978). The value found is also as in the control (about $+20$ mV). On the whole, it appears that the relaxation starts 'normally' in the presence of curare, and is then followed by what we will call the inverse component of the relaxation (or simply the inverse relaxation).

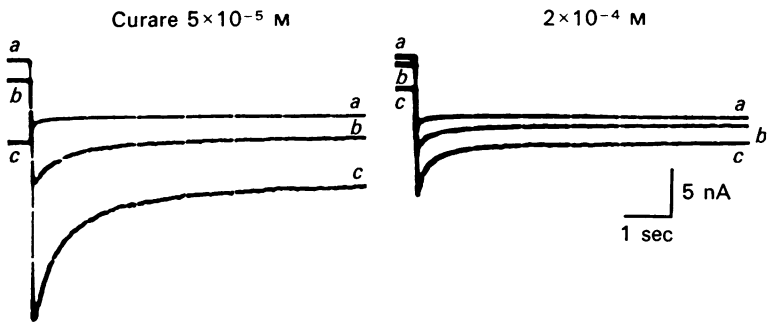

Fig. 6. Effects of the curare concentration on the relaxation. This experiment used voltage steps from -40 to -80 mV, in the presence of two concentrations of curare: 5×10^{-5} M (left) and 2×10^{-4} M (right). The upper trace, *a*, corresponds to the current recorded in the absence of ACh; the middle and lower trace (*b*, *c*) to the currents recorded during the application of two different doses of ACh. The time constants of the inverse relaxation are approximately 850 msec for the middle trace of the left, 670 msec for the lower left trace, and 380 msec for the lowest trace on the right, which corresponds to the same ACh dose as the lowest trace on the left. Thus both an increase in the dose of ACh and an increase in the dose of curare speed up the inverse relaxation. Calibrations: 1 sec, 5 nA. Temp. 12 °C.

The inverse component was not purely exponential in the experiment of Fig. 5, a frequent finding with high ACh concentrations. A clue to this phenomenon is given by comparing the time course of the inverse component for the two different ACh doses in Fig. 5. Their initial parts can be fitted with exponentials having time constants of 1·7 sec for the low ACh dose and 1·4 sec for the high ACh dose. It therefore appears that the inverse component is faster for a higher ACh concentration. As the ACh concentration is certainly not homogeneous in our conditions of iontophoretic application, it is not surprising that the total response is not a pure exponential.

Increasing the ACh concentration has three effects on the relaxation: it decreases $I_{ss}(-80)/I_{ss}(-40)$, it speeds up the inverse component, and it increases its relative amplitude. These effects can be seen in the experiment of Fig. 5. They are also apparent in Fig. 6, which shows the result of a relaxation experiment done at two ACh doses and at two curare concentrations (5×10^{-5} and 2×10^{-4} M).

Increasing the curare concentration accelerates the inverse relaxation and decreases $I_{ss}(-80)/I_{ss}(-40)$. An example of the effect of curare concentration (between 10^{-5} M and 5×10^{-5} M) on the steady-state value of the ACh response is illustrated in Fig. 7. The ratio of the control response to the response in the presence of curare increases

approximately linearly as a function of the curare concentration. The relationship holds both at -40 and -80 mV, the difference between the slopes of the two lines reflecting the voltage dependence of the effect of curare.

An example of the effect of the curare concentration on the time constant of the inverse relaxation, τ_2, is illustrated in the experiment of Fig. 6, where the time constant of the initial part of the inverse relaxation observed with the higher ACh dose was

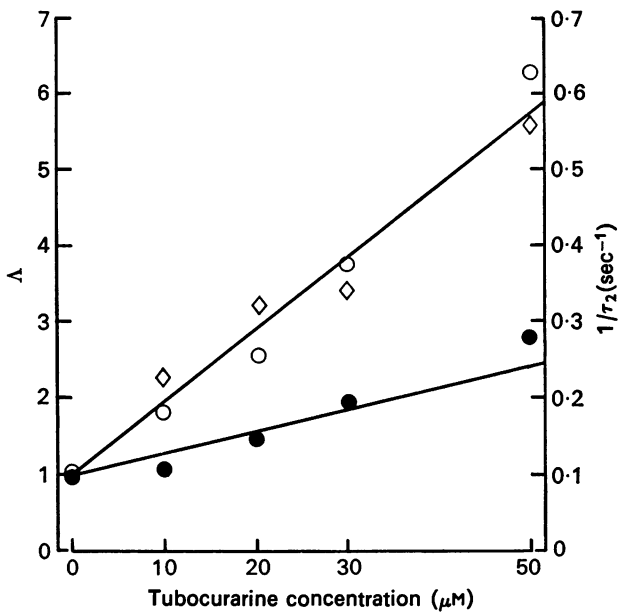

Fig. 7. Effects of the concentration of curare on the reduction of the stationary current and on the time constants of the inverse relaxation. Relaxation experiments were performed in the presence of curare, using long pulses (11 sec) which allowed the measure of the stationary current at the test potential as well as the measure of the time constant of the inverse relaxation. The ratio Λ of the plateau current before the application of curare to the plateau current in the presence of curare is plotted on the left vertical scale. These values were obtained both at -40 (●) and at -80 mV (○). Right vertical scale: inverse of the time constant τ_2 of the slow relaxation. Because of the presence of a Ca-induced K permeability (see text), τ_2 was only determined at -80 mV (◇). The concentration of curare, indicated in the abscissa, varied between 0 and 5×10^{-5} M. Within this range both Λ and $1/\tau_2$ are approximately linearly related to the curare concentration. The slope is 10^{-6} M^{-1} sec^{-1} for $1/\tau_2$ (-80), 10^5 M^{-1} for Λ (-80 and $2{\cdot}9 \times 10^4$ M^{-1}) for Λ (-40). At -80 mV, both the line relating $1/\tau_2$ to the curare concentration [B] and Λ to [B] cross the abscissa at the same point (about -10^{-5} M). The intersect is at $-3{\cdot}4 \times 10^{-5}$ M for the relationship between Λ (-40) and [B]. Temp. 12 °C. Neostigmine 5×10^{-5} M.

670 msec in 5×10^{-5} M curare, and 380 msec in 2×10^{-4} M curare. Thus, increasing the curare concentration at a given ACh dose decreases τ_2. This effect has been plotted in Fig. 7, where it is shown that $1/\tau_2$ is a linear function of the curare concentration in the range between 10^{-5} M and 5×10^{-5} M. With an appropriate scaling factor, the $1/\tau_2(V)$ and $\Lambda(V)$ relationships may be approximated by the same line, which suggests that in this range of concentration $1/\tau_2$ and Λ are proportional. It will be seen in a later section how this proportionality between the two parameters can be used

in the frame of a quantitative analysis of the effects of curare. Table 1 shows other examples of experiments where τ_2 and Λ were simultaneously measured in the presence of 5×10^{-5} M curare.

An additional effect of curare concentration (not shown) was a reduction of the relative amplitude of the 'direct' component of the relaxation, which eventually became indistinguishable at high concentrations (around 2×10^{-4} M). The time constant τ_2 of this component became simultaneously slightly smaller than in control conditions. A similar effect will be described in more detail for hexamethonium (see below) and for procaine (Marty, 1978).

TABLE 1. Values of τ_2 and Λ in the presence of curare (5×10^{-5} M). The temperature was 12 °C for cells 1–4, 22 °C for cell 5. Neostigmine (5×10^{-5} M) was only present in the experiments of cells 4 and 5

Cell	Membrane potential (mV)	τ_2 (sec)	Λ	$\frac{1}{\Lambda . \tau_2}$ (sec^{-1})
1	−80	1·6	3·5	0·18
2	−40	1·4	1·5	0·47
	−80	2·0	3·6	0·14
3	−40	1·0	3·7	0·27
	−80	1·0	6·0	0·17
4	−40	—	2·8	—
	−80	1·8	6·2	0·089
5	−40	—	2·9	—
	−80	0·63	4·4	0·36

I–V curves in the presence of curare. Two types of $I-V$ curves were obtained from relaxation experiments, one for the instantaneous current changes induced by the voltage steps, the other for the steady-state values.

The evaluation of the instantaneous currents involves some approximations inasmuch as it necessitates the extrapolation to $t = 0$ of the complex relaxations observed under curare. Nevertheless, the results indicate that the voltage dependence of the instantaneous $I-V$ curve is not modified by curare as already mentioned for the experiment of Fig. 5. This confirms the observation that the elementary current i_{el}, calculated from the ratio of the noise variance to the mean ACh induced current, is identical in normal sea-water and in the presence of curare up to concentrations of 10^{-3} M. These results suggest that curare does not modify the amplitude of the elementary current, but only alters the probabilities of opening and closing of the ACh sensitive channels.

The curve relating the steady state value of I_{ACh} to membrane potential is profoundly altered by curare, as expected from the fact that the reduction of the ACh response, Λ, is larger at −80 mV than at −40 mV. Fig. 8 illustrates how the $I_{ss}(V)$ relationship is changed in the presence of curare.

The amount of deformation of the $I-V$ curve increased progressively with the concentrations of curare, but not indefinitely. When the concentration of both ACh and curare were increased, the changes in shape became smaller, so that for high concentrations of both ACh and curare the curve appeared to reach a 'limiting shape' (see below, p. 225 and Fig. 14).

Noise power spectrum in the presence of curare. The noise power spectrum in the presence of curare was identical to that obtained in control conditions: it was described by a single Lorentzian, and the cut-off frequency was the same as in the absence of curare. This might appear surprising inasmuch as one can expect the two components of the relaxations to be reflected in the power spectrum, whatever model is adopted for the interpretation of the effects of curare. However, the time constant of the slow relaxation is of the order of 1 sec or more (e.g. Fig. 5) so that the corresponding value

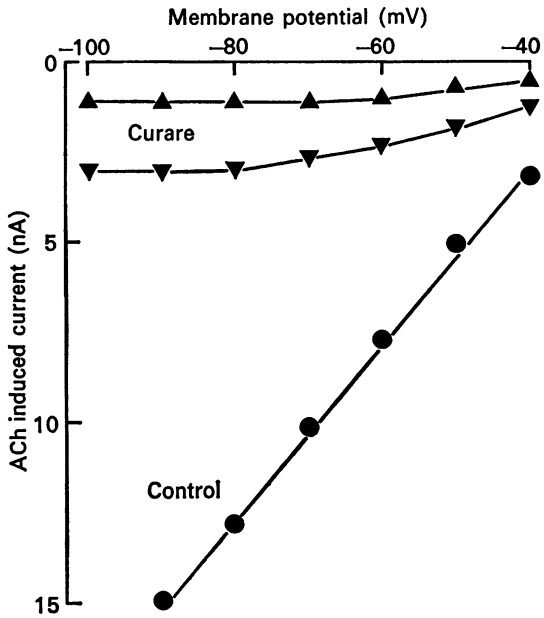

Fig. 8. Voltage dependence of the steady-state current in the presence of curare. The steady-state current produced by a constant application of ACh was measured at various membrane potentials in the absence (●) then in the presence of curare at two concentrations: 5×10^{-5} M (▼) and $1{\cdot}67 \times 10^{-4}$ M (▲). Notice the increasing curvature of the $I-V$ relationship. Temp. 22 °C. $NaHCO_3$ buffered sea water. Neostigmine 5×10^{-5} M.

of the cut-off frequency would be 0·2 Hz or less. As this frequency is below the range of our noise analysis, it is not surprising that it was not detected. The identity of the fast component in the presence and in the absence of curare is predicted from the constancy of the corresponding time constant in the relaxation experiments (Fig. 5).

This analysis of the effects of curare suggests that curare is not a competitive antagonist, and that its blocking action develops only after ACh has started opening channels. This blocking action of curare is a slow process, favoured by hyperpolarizing the cell, and speeded by increasing either the ACh concentration or the curare concentration.

When we analysed other antagonists of the excitatory ACh responses of *Aplysia* neurones, we found that they acted like curare. In particular we observed inverse relaxations with hexamethonium, decamethonium, atropine and procaine. Quantitatively, however, the ratio of the direct and inverse relaxations, their amplitudes, and their speeds, were different for each antagonist. We shall describe briefly below the

results obtained with hexamethonium, and then examine general features observed for the blocking action of all the drugs examined. We shall leave for the following paper (Marty, 1978) the peculiar relaxations observed in the presence of procaine.

The effects of hexamethonium and of other antagonists

Effects of hexamethonium. Hexamethonium does not change i_{el}; its blocking effect is increased by hyperpolarization; in its presence, voltage jumps trigger biphasic relaxations, in which the inverse component is accelerated by increasing either the ACh concentration or the hexamethonium concentration. In these respects, hexamethonium

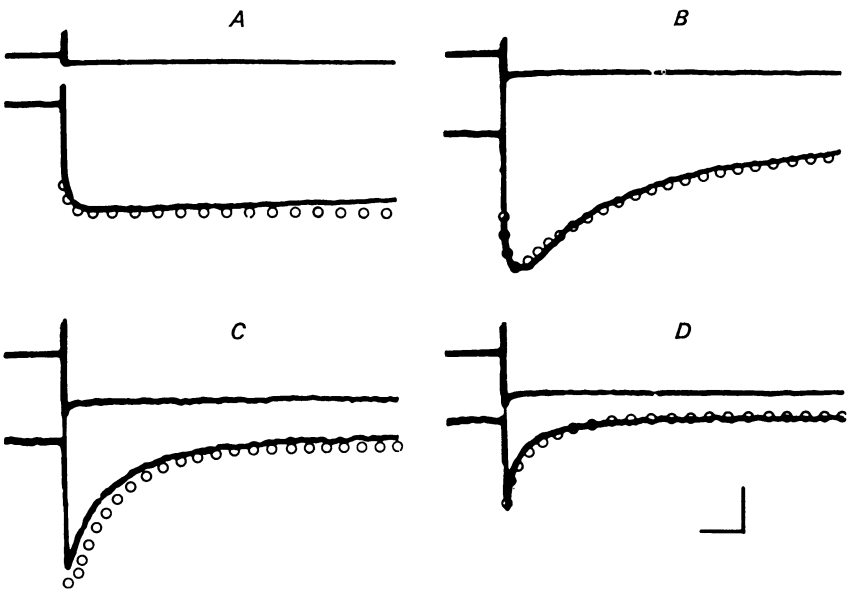

Fig. 9. Effects of the hexamethonium concentration on the relaxation. Voltage steps from -40 to -80 mV were applied in control conditions (A) and in the presence of increasing hexamethonium concentrations: 10^{-5} M (B), 5×10^{-5} M (C) and 10^{-4} M (D). The iontophoretic current was the same for all records. In B the relaxation is biphasic; in C and D only the inverse component is visible. The time constant of the inverse relaxation is 260 msec in B, 140 msec in C, 62 msec in D (the fit by an exponential, satisfactory for B and C, is only an approximation in D). Calibration: 10 nA in A, 5 nA in B, 2 nA in C and D; 100 msec. Temp. 12 °C. Neostigmine 5×10^{-5} M. Dotted traces show theoretical curves obtained with a HP 9825 calculator for the sequential model. The parameters used are $q_{-80} = 40\,\text{sec}^{-1}$, $q_{-40} = 80\,\text{sec}^{-1}$, $p = 10\,\text{sec}^{-1}$, $\rho_{-80} = 7{\cdot}5 \times 10^{-5}\,\text{M}^{-1}\,\text{sec}^{-1}$, $s_{-80} = 0{\cdot}5\,\text{sec}^{-1}$, $K_B(-40) = 6K_B(-80)$.

acts like curare. Fig. 9 shows the results of a relaxation experiment performed in the presence of increasing concentrations of hexamethonium. The response at -80 mV (the test potential) shows a two component relaxation at a moderate hexamethonium concentration (10^{-5} M). However, the initial relaxation is faster and of much smaller amplitude than in the control – an effect not observed with curare – and the time constant of the inverse relaxation (250 msec) is definitely smaller than in the presence of curare (even at high doses of curare and ACh). At higher hexamethonium concentrations no initial component of the relaxation is apparent and the inverse component has an even shorter time constant (Fig. 9C and D). Another difference with curare

appears by studying the steady state currents I_{ss}. The ratio $I_{ss}(-80)/I_{ss}(-40)$ falls to 0·4 and 0·35 in 5×10^{-5} and 10^{-4} M hexamethonium respectively (Fig. 10) whereas this ratio in curare relaxation experiments never falls below 0·7, even when both ACh and curare concentrations have been increased until the $I-V$ curve has reached its 'limiting

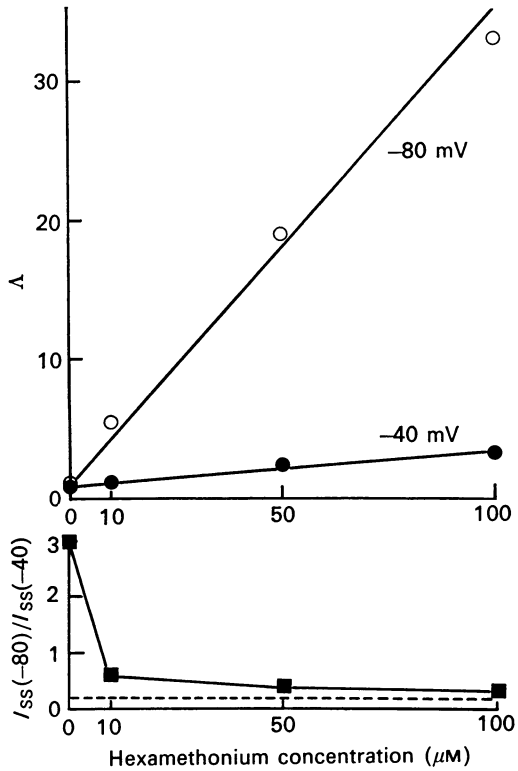

Fig. 10. Effects of the hexamethonium concentration on the steady state currents.

Upper graph. The ratio of the control steady-state current to the steady current in the presence of hexamethonium, Λ, was calculated from the experiment illustrated in Fig. 9, both at −80 (○) and −40 mV (●). Λ depends linearly on the concentration of hexamethonium, in the range studied. The slope is $3{\cdot}4 \times 10^5$ M^{-1} at −80 mV and $2{\cdot}8 \times 10^4$ M^{-1} at −40 mV. The ratio of the two slopes is 12, to be compared with a value of 3·4 in the case of curare (Fig. 7).

Lower graph. The ratio of the steady-state current measured at −80 mV to that measured at −40 mV (for the same ACh application) has been calculated again from the experiment of Fig. 9. When the hexamethonium concentration increases, this ratio decreases sharply at first, and then more slowly. The limiting value of 0·2 (dashed lines) is from Fig. 14.

shape' (see preceding section). If Λ is plotted as a function of hexamethonium concentration, a linear relationship is obtained both at −40 and at −80 mV (Fig. 10) and the ratio of the slopes is roughly 12, to be compared with 3·4 in the case of the experiment of Fig. 7, in the presence of curare.

Qualitatively the effects of hexamethonium resemble those of curare. Quantitatively, in the case of hexamethonium, the inverse relaxation is faster and the voltage sensitivity of Λ is more pronounced.

The higher speed of the inverse relaxation has an important consequence on the shape of the noise power spectrum obtained in the presence of hexamethonium. Contrary to what was observed for curare, the noise power spectrum in hexamethonium differed from that of the control. In the presence of 4 or 5×10^{-5} M hexamethonium, it could be fitted by the sum of two Lorentzians, although it was not possible to obtain a very good estimate of the cut-off of the low frequency component. As a rule the high frequency component was more conspicuous than the low frequency component in the power spectra, whereas the reverse was observed for current relaxations in the same conditions (Fig. 11).

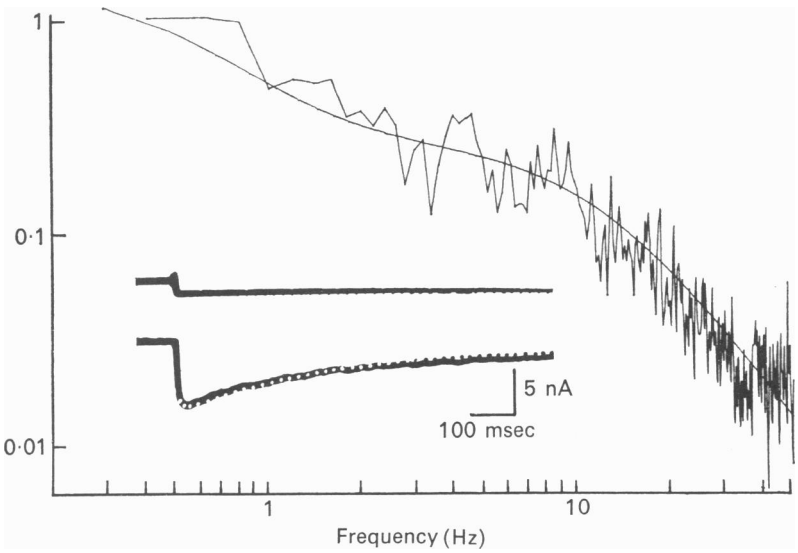

Fig. 11. Two component power spectrum obtained in the presence of hexamethonium. The spectrum was drawn between 0·3 and 50 Hz. from a current induced by ACh in the presence of hexamethonium 4×10^{-5} M. Double logarithmic co-ordinates. Membrane potential -60 mV. Temp. 12 °C. The continuous line is the theoretical curve obtained with the following parameters: $p = 20\,\text{sec}^{-1}$; $q = 50\,\text{sec}^{-1}$; $r = 10\,\text{sec}^{-1}$; $s = 1\,\text{sec}^{-1}$. Insert: current relaxation induced by a voltage step from -40 to -60 mV, on the same cell, for the same iontophoretic current of ACh. Calibration: 5 nA; 100 msec. The dots correspond to the curve obtained by using the same parameters as above at -60 mV and $q(-40) = 75\,\text{sec}^{-1}$; $s/r(-40) = 2{\cdot}7s/r(-60)$. These values were obtained from Fig. 4 of the preceding paper (Ascher *et al.* 1978) and from Fig. 15 of this paper.

Effects of Tris on the relaxations observed in the presence of hexamethonium. We have shown in the preceding paper that in the absence of antagonists, addition of Tris does not modify the time constants of the relaxations following a voltage jump, but greatly reduces i_{el} .We have suggested that Tris does not interfere with the activation of the receptor by ACh, but competes with permeant ions at sites located inside the ionic channel. A possible explanation of the effects of the antagonists studied in the present paper is that they also bind inside the ionic channel (see Discussion). Thus it was of interest to see how Tris would affect the relaxations observed in the presence of antagonists.

Table 2 shows the results of experiments done with hexamethonium (10^{-4} M) either in normal sea water or after partial replacement of the extracellular NaCl by TrisCl

(both in the presence and in the absence of neostigmine). It appears from the data of Table 2 that hexamethonium causes a smaller reduction in a response obtained in Tris substituted sea water than in a response obtained in normal sea water. Thus the reductions due to Tris and hexamethonium are not independent, which suggests that the two compounds compete for the same blocking site.

TABLE 2. Antagonism of Tris and hexamethonium. The value of Λ (ratio of the control response to the response in the presence of hexamethonium) was measured in 'normal sea-water' (containing 10 mM-Tris) and in low Na sea water (containing 350 mM-Tris) at two membrane potentials (-40 and -80 mV). The data of the last column were all obtained on the same cell. The concentration of hexamethonium was 10^{-4} M in a and c, 2×10^{-4} M in b.

The values of Λ obtained in Na sea water were smaller than in other experiments where neostigmine (5×10^{-5} M) was added (e.g. Figs. 9, 10). This difference probably indicates that hexamethonium has some anticholinesterase effect

Tris concentration (mM)	Membrane potential (mV)	*a* 10^{-4} M 12 °C	*b* 2×10^{-4} M 12 °C	*c* 10^{-4} M 22 °C
10	−40	1·6	6·7	2·5
10	−80	6·1	25	16
350	−40	1·0	1·7	2·0
350	−80	2·6	4	2·5

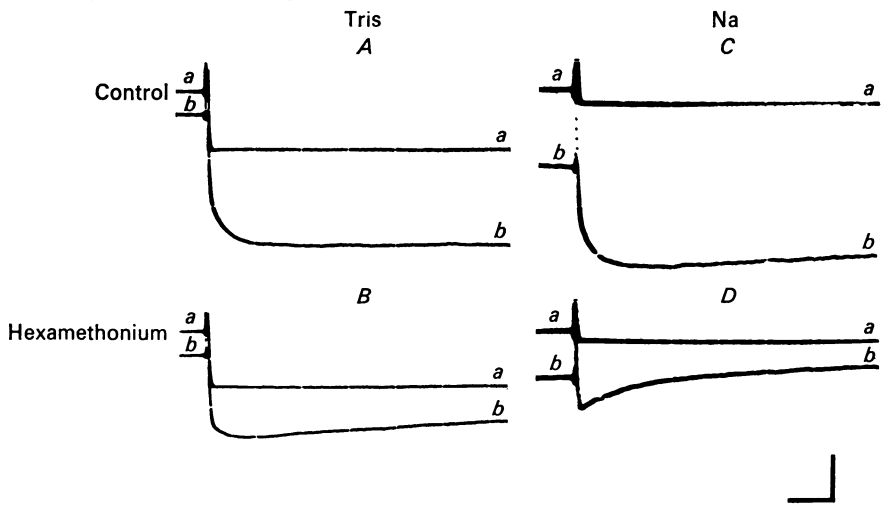

Fig. 12. Effects of Tris on the relaxations observed in the presence of hexamethonium. The four sets of records correspond to the currents obtained in the absence of ACh (upper traces, *a*) and during the application of ACh (lower traces, *b*) in response to a hyperpolarizing pulse (-40 to -80 mV). The iontophoretic current of ACh was the same for the four records. *A*, *B*, Tris substituted sea water (Na 192 mM; Tris 350 mM); *C*, *D*, Na sea water (Na 480 mM; Tris 10 mM). *A*: no hexamethonium present. *B*: hexamethonium 10^{-4} M. *C*: hexamethonium was washed for 25 min while Na sea water replaced Tris sea water. *D*: hexamethonium 10^{-4} M. Calibrations 100 msec; 2 nA (*A*, *B*); 5 nA (*C*, *D*). No neostigmine present. *A*, the time constant of the direct relaxation is 32 msec. *B*, the relaxation is biphasic. The steady-state current is not reduced at -40 mV, but reduced by a factor 2·8 at -80 mV. *C*, the time constant of the direct relaxation is 36 msec. There is a slight inverse component, which may indicate an incomplete elimination of hexamethonium. Notice that the vertical scale is different, and that the currents are larger than in *A* and *B*. *D*, from *C* to *D* the steady-state current is reduced by a factor of 1·9 at -40 mV, and a factor of 7·5 at -80 mV.

Fig. 12 illustrates one of the experiments summarized in Table 2 and shows the effects of Tris on the relaxations. In the control sea water (with 10 mM-TrisCl) the inverse relaxation dominates, as it always does in the presence of a 10^{-4} M concentration of hexamethonium; in the Tris sea water (TrisCl 350 mM) the relaxation is biphasic, and resembles the relaxation which is normally observed for a lower concentration of hexamethonium (e.g. 10^{-5} M, see Fig. 10).

We will return to these observations in the Discussion. At this point, they may be summarized by saying that the main effect of Tris on the ACh current relaxations appears to be equivalent to that of a reduction in the hexamethonium concentration.

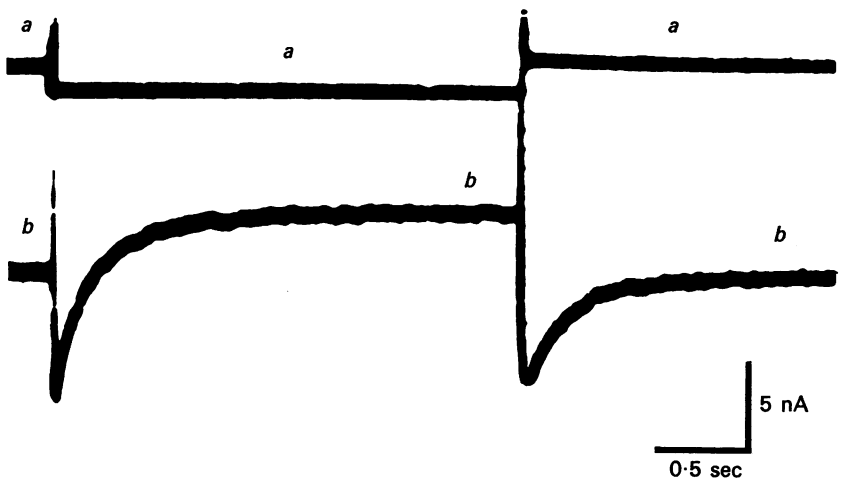

Fig. 13. Effects of decamethonium on the relaxations of the ACh induced currents. Decamethonium was applied in the perfusion medium at 10^{-4} M. Voltage jumps from -40 to -80 mV were applied in the absence of ACh (upper trace, *a*), then during the (electrophoretic) application of ACh (lower trace, *b*). The relaxations observed when the membrane potential was brought from -40 to -80 mV consisted of an initial relaxation (too fast to be visible at this sweep speed) followed by an inverse, slow component (time constant 280 msec). When the membrane was repolarized one observed a large inward peak, relaxing with a time constant of 250 msec.

Effects of other antagonists. Decamethonium does not depolarize the *Aplysia* neurones excited by ACh, but acts as an antagonist of ACh (see Ascher & Kehoe, 1975). The concentrations needed for a given degree of block were higher than for curare and hexamethonium. A striking feature of the relaxation patterns was that an inverse relaxation was observed both when the cell was hyperpolarized and when it was depolarized (Fig. 13). This phenomenon has not been studied in detail. The voltage dependence of the effects of decamethonium on steady-state currents is illustrated in Fig. 14: it is remarkably similar to that of hexamethonium for membrane potentials between -40 and -80 mV.

Atropine is a selective antagonist of the excitatory effects of ACh on *Aplysia* neurones (Kehoe, 1972). It did not alter the elementary current, i_{el}, calculated from the measurement of the noise variance. Its effects on the steady-state ACh induced currents varied with membrane potentials in a way very similar to that obtained with curare (Fig. 14). The same was true for TEA (Fig. 14).

Procaine is an antagonist of ACh in the *Aplysia* neurones studied. Its effects on the relaxations will be described in detail in the following paper (Marty, 1978). However the values of i_{el} obtained in the presence of procaine have been incorporated in Fig. 1, and the voltage dependence of the blockade of the steady-state currents is illustrated in Fig. 14.

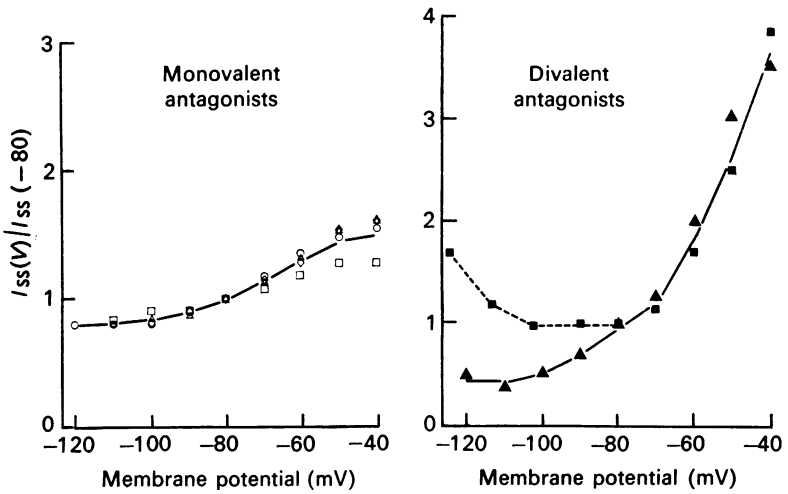

Fig. 14. Normalized I–V curves at high antagonist concentrations. Steady-state ACh induced currents (I_{ss}) were measured during a long ACh application, in the presence of various antagonists at high concentration. ACh was applied while the membrane potential was held at −40 mV. When the ACh induced current had reached a steady level, a hyperpolarizing pulse brought the membrane potential to a value V, and kept it there until the current had reached a new stationary level, $I_{ss}(V)$. The curves are normalized curves obtained by plotting the ratio $I_{ss}(V)/I_{ss}(-80)$ as a function of membrane potential. The antagonists were applied at high concentrations, at which the normalized I–V curves had reached their 'limiting shape' (see text). Left: data obtained for 'monovalent' antagonists: □, tubocurarine 4×10^{-4} M; ○, procaine 10^{-3} M; ◇, TEA 10^{-3} M; △, atropine 2×10^{-4} M. Right: data for divalent antagonists: ■, hexamethonium 4×10^{-4} M; ▲, decamethonium 10^{-3} M. Lines drawn by eye. For hexamethonium there was a systematic trend towards a new increase of conductance at potentials more negative than −80 mV (dashed line). Temp. 12 °C.

Responses to fast ACh applications

The sequential model for blocking action which is developed below predicts that the response to a fast and brief application of ACh should be less affected than the steady-state response to a prolonged application. This difference should be particularly marked for antagonists displaying slow kinetics, that is curare and to a lesser degree hexamethonium. In one experiment at 15 °C, we observed that the ratio of the peak currents $I_p(-80)/I_p(-40)$ for an ACh pulse (the time to peak was about 800 msec) was 2·6 in the control, 2·3 in 2×10^{-5} M-curare, and 2·0 in 5×10^{-5} M-curare. For the same iontophoretic current, the corresponding ratios for the steady-state responses obtained with a long ACh application were about 1·0 in 2×10^{-5} M curare and 0·8 in 5×10^{-5} M curare. Thus, this experiment suggests that the shape of I–V curves is much less affected by curare in the case of short applications of ACh. The reduction of the response and the distortion of the I–V curve should be even smaller for synaptic

currents, which have a fast rise time, than for the responses to short ACh pulses. This could not be tested on the cells used in this study, for which no presynaptic neurones are known.

Voltage sensitivity of the plateau current at high concentrations of antagonists

For all compounds tested, the ACh induced current at very negative membrane potentials was proportionally more reduced than at less negative membrane potentials. This effect, leading to a curvature of the steady-state $I-V$ curve, was more marked when either the ACh concentration or the antagonist concentration were increased (see for example Fig. 6). However, as mentioned above, the process has a limit and for high concentrations of the antagonist a 'limiting' shape of the $I-V$ curve could be reached. In the frame of the sequential model of the antagonists action (see below) one can calculate from such curves the dissociation constant K_B of the reaction of the antagonist with the 'activated' channel receptor complex. This constant is voltage dependent, and its variation with membrane potential has been plotted in Fig. 15. The experimental conditions of Fig. 14 were empirically determined so that a further increase in antagonist concentration would not significantly modify the $I_{ss}(V)$ curve. The iontophoretic dose was larger than the minimal values used in the preceding paper (Ascher *et al.* 1978). However, it is probable that the condition $p \ll q$, which is necessary to derive K_B from Fig. 14 (see below), was still valid in the experimental conditions of Fig. 14. This is indicated *a posteriori* by the good agreement between the results of Fig. 15 and the voltage dependence of K_B as obtained from experiments of the type illustrated in Figs. 7 and 10. In practice, the $I_{ss}(V)$ curves of Fig. 14 were directly obtained in the presence of antagonists, without the relevant control for the same ACh dose (which would have led to currents too large to be recorded by our voltage clamp system); however, the iontophoretic current was kept at a low value so that typical values of $I_{ss}(-40)$ were only 2 and 5 nA in Fig. 14 *A* and *B* respectively. Much larger currents could be obtained by further increasing the ACh dose, which again suggests that the condition $p \ll q$ was still valid under these experimental conditions.

The results of Fig. 14 appear to fall into two groups:

(1) for curare, procaine, atropine and TEA all limiting $I_{ss}(V)$ curves are virtually identical. The resulting $K_B(V)$ curve is a straight line in semilogarithmic co-ordinates (at least between -100 and -40 mV) with a slope corresponding to an e-fold change in 49 mV. In other words, K_B can be written in this potential range as

$$K_B(V) = K_B(-80)\exp(-80+V/49)$$

if V is in mV;

(2) for hexamethonium and decamethonium the limiting $I_{ss}(V)$ curve is much steeper. Again, it appears strikingly similar for the two compounds. The resulting $K_B(V)$ can now be fitted in the -100 to -40 mV potential range by the equation

$$K_B(V) = K_B(-80)\exp(-80+V/23).$$

It is interesting that antagonists bearing one positive charge all fall in the first class, whereas hexamethonium and decamethonium, which are both divalent, fall in the second class. It is also interesting that tubocurarine behaves as a 'monovalent' antagonist. This could be due to the fact that, at physiological pH, tubocurarine

possesses only one net charge, that of the quaternary nitrogen group; the charge contributed by the tertiary nitrogen group (which is close to its pK) is balanced by the charge of a hydroxyl group which is also close to its pK (Everett, Lowe & Wilkinson, 1970; Maclagan, 1976). One cannot exclude, however, that even at a pH where the net charge of the tubocurarine molecule would be different, only the quaternary nitrogen would be involved in the voltage dependent interaction with the membrane.

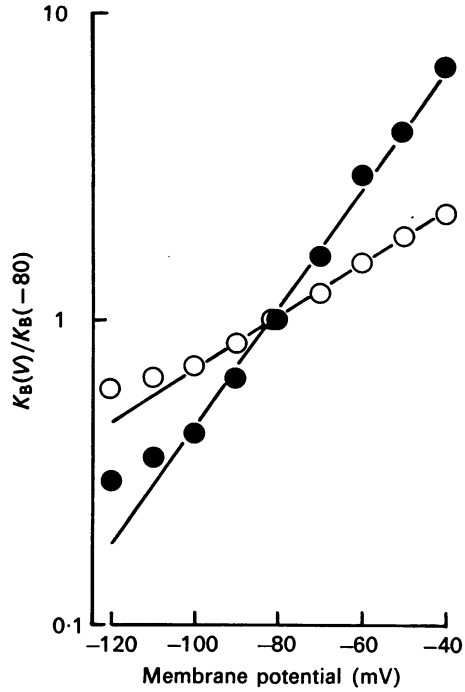

Fig. 15. Voltage dependence of the antagonists' dissociation constant, K_B. K_B was calculating using eqn (10) from the continuous lines drawn in Fig. 14. The values plotted are the ratio $K_B(V)/K_B(-80)$. The ordinate is logarithmic. ○, monovalent antagonists; ●, divalent antagonists. The slopes of the lines correspond to an e-fold change of K_B for a membrane potential change of 49 mV (○) or 23 mV (●).

Whether the distinction between the two classes of antagonists is due to the monovalent divalent distinction, or to that between monoquaternary and biquaternary compounds, it is striking that the slope of the log $K_B(V)$ curve for class 2 is nearly twice the corresponding slope for class 1. Finally, it may be noted that the voltage dependence for the monovalent antagonists resembles that of $1/\tau$ (except that the relationship is somewhat steeper for K_B) in the control sea water as described in the preceding paper.

A model allowing the simulation of the relaxation data

Although, as will be shown in the Discussion, our results can be interpreted by more than one model, we will present here the one we consider the most adequate. The model allows the prediction of changes in the shape of the relaxation with ACh or antagonist concentration.

We assume that (1) in the presence of low concentrations of ACh, and in the absence

of antagonist, the receptor-channel complex can take two possible states, R (closed) and R_A (conducting)

$$R \underset{q}{\overset{p}{\rightleftharpoons}} R_A,$$

where p is the probability of transition from R to R_A, q the probability of the reverse transition. Only p depends on the ACh concentration (see Ascher *et al.* 1978); (2) the blocking action of the particular class of antagonists that we have studied is supposed to result from the transformation of the conducting complex R_A in a non-conducting (blocked) complex R_{AB}

$$R_A \underset{s}{\overset{r}{\rightleftharpoons}} R_{AB},$$

where r and s are again probabilities of transition. r depends on the concentration of antagonist, [B]; s is independent of [B]; (3) [B] cannot bind to R, which implies in particular that R_{AB} cannot return to R without going through R_A.

The model is a variant of the sequential model (Steinbach, 1968; Adams, 1975, 1977; Ruff, 1977). The reasons for choosing it will be considered in the Discussion. We will examine here some of its predictions, and consider how they apply to our observations.

Variance. In the presence of an antagonist, the current through each channel will alternate between 0 and a finite value i_{el} which is the same as in control conditions. Provided that the 'low concentration limit' is not exceeded, the ratio of the variance to the mean of the ACh induced current will then be equal to i_{el} both in the absence and in the presence of antagonists (see above, p. 210). This is what we observed (Fig. 1).

Relaxations and noise power spectra. The two reactions postulated lead to

$$\frac{dR_A}{dt} = pR - (q+r)R_A + sR_{AB}, \tag{1}$$

$$\frac{dR_{AB}}{dt} = -sR_{AB} + rR_A. \tag{2}$$

If the system is moved away from equilibrium the concentration R_A will relax towards its new equilibrium value as a linear combination of two exponential functions of time

$$R_A = L_1 \exp(-\lambda_1 t) + L_2 \exp(-\lambda_2 t) + Cte. \tag{3}$$

The values of λ_1, λ_2 are

$$\lambda_1, \lambda_2 = \tfrac{1}{2}(p+q+r+s) \pm \tfrac{1}{2}\sqrt{\Delta}$$

with $\Delta = (p+q+r+s)^2 - 4(pr+ps+qs)$. We will also use the notation $\tau_1 = 1/\lambda_1$ (fast component) and $\tau_2 = 1/\lambda_2$ (slow component).

The values of L_1 and L_2 are

$$L_1 = \frac{R_T}{\lambda_1 - \lambda_2}\left[\left(\frac{1}{1+q/p+r/s} - \frac{1}{1+q_0/p_0+r_0/s_0}\right)(p+q+r+\lambda_2) + (s-p)\left(\frac{1}{1+s_0/p_0(1+q_0/p_0)} - \frac{1}{1+s/r(1+q/p)}\right)\right]$$

$$L_2 = \frac{R}{1+q_0/p_0+r_0/s_0} - \frac{R_T}{1+q/p+r/s} - L_1,$$

R_T being the total number of receptors. The values of the parameters before the voltage step are noted with 0 subscripts

The autocorrelation function ψ follows the same differential equation as R_A (except for the constant term). The noise power spectrum will therefore be the sum of two Lorentzian components, with cut off frequencies $2\pi\lambda_1$ and $2\pi\lambda_2$.

Simplified expressions for λ can be obtained if

$$pr+ps+qs \ll p+q+r+s.$$

Two limiting cases in which this condition is satisfied are the following:

(1) $$q \gg p \sim r \gg s.$$

Then
$$\lambda_1 \simeq q \quad \text{(fast)}, \tag{4}$$
$$\lambda_2 \simeq s+\frac{pr}{q} \quad \text{(slow)}. \tag{5}$$

One of the time constants measured in the presence of the antagonist will be identical to that measured in the control; the other will be much slower.

(2) $$r \gg s \sim q \gg p.$$

Then
$$\lambda_1 \simeq r \quad \text{(fast)}, \tag{6}$$
$$\lambda_2 \simeq \frac{qs}{r}+p \quad \text{(slow)}. \tag{7}$$

Case 1 corresponds to a situation where the $R \rightleftharpoons R_A$ reaction is fast compared to the $R_A \rightleftharpoons R_{AB}$ reaction, and case 2 to the inverse situation. Note that in case 1 the slow relaxation will get faster if the antagonist concentration, and therefore r, is increased, but it will get slower in case 2.

Steady-state current. The ACh induced steady-state current is supposed to be proportional to R_A:

$$R_A = \frac{R_T}{1+\frac{r}{s}+\frac{q}{p}}. \tag{8}$$

Assuming that $q \gg p$ (low ACh concentration), the response in the presence of an antagonist is divided by a factor

$$\Lambda = 1+\frac{pr}{qs}. \tag{9}$$

At a given antagonist concentration, the reduction will be larger for higher ACh concentrations (p larger). This is consistent with our observation that the blocking effect of the antagonist increases when the ACh concentration increases (see for example Fig. 2).

Bimolecular character of the antagonist action. In our experiments we could not evaluate the concentration of ACh, but we knew that of the antagonist, [B]. We could therefore attempt to check the prediction that the effect of [B] on R_A results from a simple bimolecular reaction. In this hypothesis, r is proportional to the antagonist concentration [B]. This implies (eqn. (9)) that Λ should also be a linear function of [B], and in the limiting cases 1 and 2 that one of the rate constants of the relaxation should be a linear function of [B] (eqns. (5) and (6)).

As we will see, experiments performed in low curare concentrations appear to fall into the category of case 1. Since we have shown that in the case of curare (Fig. 7) Λ and λ_2 depend linearly on [B], we conclude that the effect of [B] on R_A is indeed due to

a bimolecular reaction. A similar conclusion may be reached by considering the results obtained with procaine, which fall in case 2 at high procaine concentration (Marty, 1978). Then λ_1 is found to be proportional to [B], as expected from eqn. (6) if r is proportional to [B].

As shown below the results of voltage jump experiments and of noise analysis are in agreement with the predictions outlined above. For each compound, over a given concentration range, the relaxations can be described as a sum of two exponentials; the time constants of these relaxations follow the qualitative predictions of the model when the ACh concentration or the antagonist concentration is increased; and the steady-state current depends linearly on the antagonist concentration (Figs. 7 and 10).

The model allows, in addition, some quantitative evaluations of the rate constants characterizing the antagonist binding. s is the backward rate constant. We shall call ρ the forward rate constant, which is related to r by $r = \rho[\mathrm{B}]$, and note $K_\mathrm{B} = s/\rho$.

The limiting $I_{ss}(V)$ curve is obtained by making $\Lambda \gg 1$ in eqn. (9). Then $r/s \gg q/p$ and eqn. (8) yields

$$R_\mathrm{A} \simeq R_\mathrm{T}\frac{s}{r} = \frac{R_\mathrm{T}K_\mathrm{B}}{[\mathrm{B}]}.$$

Since

$$\frac{I_{ss}(V)}{I_{ss}(-80)} = \frac{R_\mathrm{A}(V)}{R_\mathrm{A}(-80)} \times \frac{i_{el}(V)}{i_{el}(-80)},$$

one obtains

$$\frac{I_{ss}(V)}{I_{ss}(-80)} = \frac{K_\mathrm{B}(V)}{K_\mathrm{B}(-80)} \times \frac{i_{el}(V)}{i_{el}(-80)}. \tag{10}$$

This is the equation used to calculate $K_\mathrm{B}(V)$ in Fig. 15.

$K_\mathrm{B}(-40)/K_\mathrm{B}(-80)$ may also be obtained from experiments of the type illustrated in Figs. 7 and 10. Comparing the slopes of the Λ(B) relationships with their theoretical values (eqn. (9)) yields $K_\mathrm{B}(-40)/K_\mathrm{B}(-80) = 2{\cdot}0$ and $8{\cdot}2$ for curare and hexamethonium respectively (taking $q(-40)/q(-80) = 1{\cdot}7$ from Ascher *et al.* (1978)) which are in good agreement with the values $2{\cdot}2$ and $6{\cdot}7$ of Fig. 15*A* and *B*.

Tubocurarine. The slowness of the inverse relaxation requires that $s \ll q$. The fact that the direct relaxation has, at least at low curare concentration, the same time constant as in the control, indicates that $r \ll q$. Thus we are in the limiting case 1 and combining eqns. (5) and (9) yields

$$s = \frac{\lambda_2}{\Lambda},$$

where λ_2 is the rate constant of the inverse relaxation, and Λ the reduction factor for the steady-state current.

The data of Table 1 allow the evaluation of $s \simeq 0{\cdot}1\,\mathrm{sec}^{-1}$ (at -80 mV, and 12 °C). To calculate the forward rate constant, we can take advantage of an interesting prediction of the model, namely that for a certain concentration of antagonist, the voltage jump induced relaxation will have an initial slope of 0. This was observed with concentrations of curare of about 2×10^{-4} M (the same phenomenon is observed for hexamethonium 6×10^{-5} M and procaine 6×10^{-5} M). In terms of the model it corresponds to

$$\left(\frac{dR_\mathrm{A}}{dt}\right)_{t=0} = 0,$$

which bringing in eqn. (1), and arranging, gives

$$r(-80) = \frac{q(-40) - q(-80)}{1 - \frac{K_B(-80)}{K_B(-40)}}. \tag{10}$$

The ratio $K_B(-80)/K_B(-40)$ is about $\frac{1}{2}$ from the data of Fig. 15. Putting the experimental values of $q(-40)$ and $q(-80)$ into eqn. (11) (80 and 40 sec^{-1} respectively, from Ascher *et al.* 1978) then yields $\rho(-80) \simeq 4 \times 10^5$ $M^{-1} sec^{-1}$ and $K_B(-80) \simeq 2{\cdot}5 \times 10^{-7}$ M. The last step is to calculate p. This can be done by measuring the slopes of the straight lines in Fig. 7 and comparing them with their theoretical value p/qK_B. The result is $p = 0{\cdot}8\ sec^{-1}$ and $p/q = 0{\cdot}02$ at -80 mV. Since p/q is the fraction of activated receptors

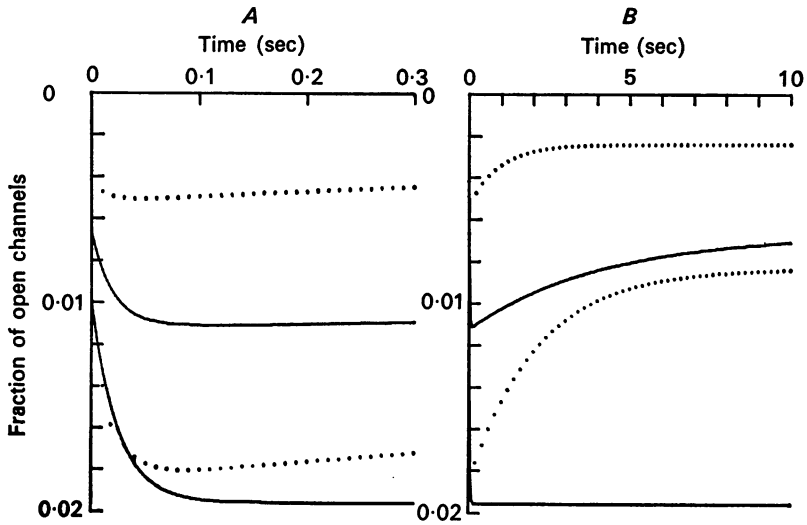

Fig. 16. Simulation of the relaxations observed in the presence of curare. The relaxations following a voltage step from -40 to -80 mV were simulated by using eqn. (3) for various concentrations of curare. The left and right Figures differ only by the time scale (100 msec in *A*, 1 sec in *B*). The vertical scale is the same in *A* and *B*, and represents the fraction of open channels. This fraction, which is proportional to the ACh induced current, has been plotted downwards to facilitate the comparison with the experimental records. The continuous lines and the dotted lines correspond to two different concentrations of ACh (higher for the dotted line). Lower continuous line: control relaxation. Upper continuous line and lower dotted line: curare 5×10^{-5} M. Upper dotted line: curare 10^{-4} M. The values of the parameters ρ and s, obtained as described in the text, were, at -80 mV $\rho = 4 \times 10^5$ $M^{-1} sec^{-1}$ and $s = 0{\cdot}1\ sec^{-1}$. We took $K_B(-40) = 2 \times K_B(-80)$ from Fig. 15. The values of q ($q(-80) = 40\ sec^{-1}$; $q(-40) = 80\ sec^{-1}$) were taken from Fig. 4 of the preceding paper. p was chosen as $0{\cdot}8\ sec^{-1}$ (continuous lines) and $3{\cdot}2\ sec^{-1}$ (dots) which leads to a fraction of open channels of 0·02 and 0·08 in the absence of curare. The various curves mimic well the currents recorded in Figs. 5 and 6, and changes of these currents when curare and ACh concentrations were modified.

in the control, this last result shows that our analysis is consistent with the initial assumption of low concentration limit. All the parameters involved in the model being now calculated for curare, it is possible to perform a simulation of the relaxation data. Fig. 16*A* shows the result of such a simulation for -40 to -80 mV steps in the presence of various concentrations of curare. The agreement with experimental data

(compare with Figs. 5 and 6) is good. In Fig. 16*B*, it can be seen that increasing p (the 'ACh concentration') accelerates the reverse relaxation, in agreement with experimental data. The major source of differences between simulated and experimental data presumably originates from ACh concentration heterogeneities over the cell surface during iontophoretic application. The total response must then be considered as the sum of heterogeneous responses with kinetics differing according to the local ACh concentration. It is therefore not surprising that the reverse relaxation could not always be fitted with a single exponential, especially at high ACh doses.

Hexamethonium. For hexamethonium the values of λ_1 and λ_2 did not allow the simplification to a limiting case such as 1 or 2. The values of the constant were approximated by empirical fitting of the relaxation as indicated in Fig. 9. The values found for Fig. 9 (at -80 mV and 12 °C) are listed in Table 3.

TABLE 3. Kinetic parameters for various antagonists at 12 °C and -80 mV

	Curare	Hexamethonium	Procaine†	Decamethonium QX222†
$s(\text{sec}^{-1})$	0·1	0·5	10	> 10
$\rho(\text{M}^{-1}\,\text{sec}^{-1})$	$4{\cdot}4 \times 10^5$	$7{\cdot}5 \times 10^5$	$1{\cdot}3 \times 10^6$	—
$K_B(\text{M})$	$2{\cdot}3 \times 10^{-7}$	$6{\cdot}7 \times 10^{-7}$	$7{\cdot}7 \times 10^{-6}$	—

† Data from Marty (1978).

DISCUSSION

We have shown that we can account for the effects of the various antagonists tested, in particular curare and hexamethonium, by assuming that they bind to the conducting receptor channel, complex and convert it into a non-conducting complex. This is a sequential model, which has been used by a number of authors in other systems (e.g. Steinbach, 1968; Armstrong, 1969; see Gage, 1976; Adams, 1976; Armstrong, 1975). Qualitatively, a variety of other models could account for non-competitive effects of ACh antagonists. We will leave for the following paper (Marty, 1978) the discussion of the 'parallel models', and will only consider here whether we can exclude, for curare and hexamethonium, the models in which these compounds bind both to the closed and to the open receptor-channel complex.

The binding of the antagonist on the 'closed' complex could assume various forms. For example, the binding site of B on R can be supposed to be the same as that of ACh on R, and different from the binding site of B on R_A. Alternatively, B can bind at the same site on R and R_A. In the second case the transition $RB \rightleftharpoons R_A B$ is possible and although it will not lead to a permeability change, it will modify the kinetics of the system. Both cases are described by a cyclic model,

$$\begin{array}{ccc} R & \overset{p}{\underset{q}{\rightleftharpoons}} & R_A \\ + & & + \\ B & & B \\ K'_B \,\Vert\!\downarrow & & \uparrow\!\Vert\, K_B \\ RB & \overset{p'}{\underset{q'}{\rightleftharpoons}} & R_A B \end{array}$$

considered by a number of authors (e.g. Rang & Ritter, 1970; Adams, 1977). In the first case, the transition rates p' and q' are negligible. The cyclic model predicts that

$$\Lambda = 1 + \frac{[B]}{K'_B} + \frac{[B]p}{K_B q}.$$

In this equation, p is the only parameter sensitive to the ACh concentration. The relation is only valid if $p \ll q$, i.e. for low ACh concentration. The fact that Λ increased when the ACh concentration increased (e.g. Fig. 2) implies that the third term of eqn. (9) is not small, and therefore

$$K_B \ll K'_B,$$

i.e. the affinity of the antagonist for the closed receptor-channel complex is much smaller than its affinity for the open complex. A second argument against an important contribution of a binding of B on R comes from the study of the $I_{ss}(V)$ relationship. According to the cyclic model, I_{ss} should be proportional to

$$\frac{i_{el}}{\frac{q}{p}\left(1 + \frac{[B]}{K'_B}\right) + \frac{[B]}{K_B}}.$$

Unless one postulates an *ad hoc* voltage sensitivity of K'_B (which would have to be different from that of K_B since q also depends on voltage) the existence of a limiting I–V curve (Fig. 14) independent of [B] and [ACh] implies that the term in K'_B must be negligible in the expression of I_{ss}.

These arguments do not allow one to exclude completely a contribution of a direct binding of B onto the R form. However, they show that such a contribution, if present, has to be small, and they validate the use of the sequential model to account for our data.

Values of ρ and s. Table 3 shows the values of the kinetic constants ρ and s obtained in the frame of the sequential model for curare, hexamethonium and procaine. We have also indicated in Table 3 that calculations done on the limited data obtained with decamethonium (Fig. 13) and QX 222 (Marty, 1978) indicate that these compounds must have s values larger than $10\,sec^{-1}$.

The main difference between the compounds appears in the values of the dissociation constants, s, which vary by a factor 100 from tubocurarine to procaine. On the other hand the values of the association rate constants, ρ, vary in a much more restricted range – less than four between curare and procaine.

The values of ρ listed in Table 3 may be compared to the rate ρ_{Na} at which Na ions enter the channel. An estimate of ρ_{Na} can be obtained by transposing an argument of Armstrong (1966). If we assume that the elementary current measured at $-80\,mV$ corresponds mainly to an influx of Na, and that the rate limiting step of Na permeation is the entry into the channel, then i_{el} is related to the extracellular Na concentration, Na_0, by $i_{el} = \rho_{Na} Na_0 e_0$, where e_0 is the charge of the electron. With $i_{el} = 0{\cdot}8 \times 10^{-12}\,A$ (Ascher *et al.* 1978) and $Na_0 = 480\,mM$, we find $\rho_{Na} = 10^7\,M^{-1}\,sec^{-1}$. Thus the rate of association calculated for Na is only one order of magnitude larger than the rate calculated for the antagonists.

Both the rates of access for Na and for the antagonists are low if compared to theoretical values for a diffusion limited process. The discrepancy is even larger if one takes into account the probable presence of a negative surface potential, which

should increase the apparent rates of entry by increasing the cation concentrations at the membrane-external solution interface. The low value of ρ may be attributed to diffusion hindrance and (or) to repulsive forces of electrostatic origin (Parsegian, 1969) in the channel (see below).

Site of action of the antagonists. The voltage sensitivity of the effects of the antagonists can be explained by assuming that their binding site is located at some depth in the membrane, in a region sensitive to an applied electric field (Kordaš, 1970). The fact that all monovalent antagonists have the same voltage sensitivity suggests that they all bind to the same site. Assuming that the conditions for applying the Boltzmann equation are satisfied (see e.g. Armstrong, 1969) one can calculate that the site should be located at a depth of about one half of the total thickness of the membrane.

The higher voltage sensitivity found for the divalent antagonists (hexamethonium and decamethonium) remains to be explained. It is tempting to suggest that their positive ends bind simultaneously to two sites (cf. Barlow, 1964). The short distance between the two ionized groups (10 and 14 Å for hexamethonium and decamethonium respectively) makes it unlikely that the two sites are on different receptor-channel complexes if these complexes are organized in a lattice structure similar to that found in *Torpedo*, with a centre to centre distance of 90 Å (e.g. Cartaud, Benedetti, Cohen, Meunier & Changeux, 1973).

The assumption of a 'pore-block' by the antagonist is a specific version of the above interpretation, in which the deep binding site of the antagonist is situated in the very structure crossed by the permeating ions. This hypothesis was already proposed by Blackman in 1959 for synaptic channels, and developed after the experiments of Armstrong on the axon K channels (Armstrong, 1971, 1975); it has since been extensively used, in particular for the axon Na channels (e.g. Strichartz, 1973; Woodhull, 1973; Yeh & Narahashi, 1977) and for synaptic channels (Adams, 1975, 1976; Barker & Gainer, 1973). We think that it receives additional support from our observation of a competition between Tris and hexamethonium, inasmuch as Tris itself appears to act as a channel blocker, an hypothesis discussed in the preceding paper.

Effects of curare and hexamethonium in other systems. How general are the actions of curare and hexamethonium that we have described? Three types of transmitter mediated conductance increases are obvious candidates for a generalization.

The pharmacological similarity between the ACh excitatory effects on autonomic ganglion cells and on *Aplysia* neurones (Tauc & Gerschenfeld, 1962; Kehoe, 1972; cf. Ascher & Kehoe, 1975) may suggest that the voltage and time dependent blocks described in *Aplysia* will also be observed in ganglion cells. Indeed Blackman (1970) has reported that the effects of hexamethonium in sympathetic ganglion were increased by hyperpolarization.

We have stressed in the preceding paper (Ascher *et al.* 1978) the similarity between the ACh induced channels of the frog endplate and those of *Aplysia* neurones. If curare and hexamethonium can block the channels, some voltage dependent effects of curare may be expected in the frog, and in fact have been recently observed (Manalis, 1977).

Finally it would be interesting to know whether similar effects of curare and hexamethonium could be observed, in *Aplysia*, on non-cholinergic systems. Curare

blocks the excitatory effects of a number of putative transmitters in *Aplysia* neurones (e.g. dopamine, serotonin), and it is therefore tempting to consider these effects as a channel block (Carpenter, Swann & Yarowsky, 1977). However, curare does not block all excitatory responses in *Aplysia*; hexamethonium does not block all the responses blocked by curare; and curare blocks some inhibitory responses (cf. Ascher & Kehoe, 1975). Thus the 'channel-block' hypothesis will certainly have to be refined to account for the specificity of the effects which have been observed.

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