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STUDIES ON THE MECHANISM OF ACTION OF ACETYLCHOLINE ANTAGONISTS ON RAT PARASYMPATHETIC GANGLION CELLS

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SUMMARY

1. The mode of action of ACh antagonists on the parasympathetic neurones of the submandibular ganglion of the rat was studied by means of a two-micro-electrode voltage-clamp technique. The currents produced by various agonists (carbachol, ACh, suberylcholine) were studied in steady state and after voltage steps, before and after perfusion of various antagonists.

2. For three antagonists (tubocurarine, hexamethonium, decamethonium) the blocking action increases with hyperpolarization. For three other antagonists (surugatoxin, trimetaphan, mecamylamine) the effects observed at low concentrations appear to be independent of membrane potential, although in some cases voltage dependence of the block was observed for mecamylamine.

3. The block produced by tubocurarine, hexamethonium and decamethonium increases with the agonist concentration, an observation which supports a 'sequential' scheme in which the antagonist blocks the 'open' channel–receptor complex. The block produced by trimetaphan and mecamylamine decreases slightly with increased agonist concentration, which in turn suggests that these two compounds are competitive antagonists, preventing binding of the agonists to the closed channel–receptor complex.

4. In the cases where the block is voltage dependent, voltage jumps trigger slow relaxations which are not present in control conditions. In the case of tubocurarine and hexamethonium, the relaxation following a hyperpolarizing voltage jump corresponds to a decrease in conductance. In the case of decamethonium, the slow relaxation is in the opposite direction.

5. The slow relaxations observed with tubocurarine and hexamethonium are speeded by an increase of the antagonist concentration; the slow relaxations observed with decamethonium are slowed by an increase of the decamethonium concentration.

6. The steady-state observations and the relaxations can be interpreted in terms of a scheme in which tubocurarine, hexamethonium and decamethonium act mainly by blocking the channels opened by the cholinergic agonists.

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7. The two types of slow relaxation are those predicted if tubocurarine and hexamethonium dissociate slowly from the channel, and decamethonium rapidly.

8. An additional effect of tubocurarine is described, which consists of a potentiation of the rising phase of the response to an ionophoretic pulse. Possible mechanisms of this effect are discussed.

INTRODUCTION

It has been assumed generally that non-depolarizing ganglion blocking drugs act by competing with cholinergic agonists for the receptors, though there is little evidence to support this. As early as 1959, Blackman suggested that hexamethonium might act on the sympathetic ganglion by blocking the ionic channels opened by acetylcholine (ACh) rather than by direct competition at the receptor. He later supported this suggestion by showing that the blocking action was increased markedly by hyperpolarization of the cells (Blackman, 1970). He argued that because hexamethonium is cationic an increase in the membrane potential would increase its affinity for a site lying within the transmembrane electric field. Though this evidence on its own does not rule out a competitive interaction, Blackman's theory has gained support from recent work on the actions of various blocking agents, including hexamethonium, decamethonium and tubocurarine on the ACh responses of *Aplysia* neurones (Marty, Neild & Ascher, 1976; Ascher, Marty & Neild, 1978*b*). In these studies the kinetic as well as the steady-state predictions of the channel-block theory were confirmed by analysis of ACh-induced noise and of the relaxation patterns occurring in voltage-clamped cells in response to an imposed voltage jump. Similar studies on the frog neuromuscular junction have shown that tubocurarine, gallamine and decamethonium, as well as interacting with the receptor, also have the ability to block the ionic channels (Manalis, 1977; Colquhoun, Dreyer & Sheridan, 1979; Katz & Miledi, 1978; Adams & Sakmann, 1978; Colquhoun & Sheridan, 1979). It had been shown earlier that a variety of compounds, including barbiturates (Adams, 1976), various local anaesthetics (Steinbach, 1968; Adams, 1977; Ruff, 1977; Beam, 1976*a*, *b*), atropine (Feltz, Large & Trautmann, 1977; Adler, Albuquerque & Lebeda, 1978) and quinacrine (Adams & Feltz, 1977) act probably by a channel-blocking mechanism, but it was surprising to find evidence for a similar mechanism among classical competitive antagonists at the neuromuscular junction.

In this study we have analysed the actions of a series of ganglion-blocking drugs on mammalian autonomic ganglion cells using the same basic approach of investigating the steady-state voltage dependence, and the transient relaxation patterns following a voltage step, as has been used for *Aplysia* neurones and the neuromuscular junction.

The preparation chosen was the rat submandibular ganglion recently described by Lichtman (1977). This preparation is well suited for the voltage-clamp technique with two independent micro-electrodes as the cells can easily be seen with Nomarski optics and lack large dendrites. High resistance micro-electrodes had to be used. This prevented us from resolving the response to voltage jumps in the first few milliseconds, so that we could only study relatively slow relaxations. For this reason our analysis is less complete than those of other systems. None the less the results strongly suggest that three of the drugs tested (hexamethonium, tubocurarine and

decamethonium) act mainly or entirely by channel block, whereas the others (mecamylamine, trimetaphan and surugatoxin) probably act mainly at the receptor.

Preliminary accounts of some of this work have been published (Ascher, Large & Rang, 1978, 1979).

METHODS

Dissection and mounting of ganglion

Rats were killed with chloroform. The chest was quickly opened, the inferior vena cava cut and Krebs solution (about 20 ml.) was slowly injected into the left ventricle in order to wash the blood out of the head and neck. The submandibular ganglia were exposed as described by Lichtman (1977). The ganglion cells lie in a sheet of connective tissue between the lingual nerve and the submandibular duct, and this sheet, with adjacent stretches of the nerve and duct, was excised and placed in oxygenated Krebs solution. The ganglion was pinned over a small pad of Sylgard resin in the recording chamber, and as much loose connective tissue as possible was removed. The base of the chamber consisted of a glass microscope slide and it was mounted on the stage of a Normarski microscope fitted with a water immersion (times 40) objective, which was electrically insulated from the body of the microscope by a Teflon insert.

The recording chamber was perfused continuously with oxygenated Krebs solution at 3–4 ml./min. Experiments were done at room temperature (20–23 °C).

Electrical recording

Micro-electrodes of resistance 50–150 MΩ were pulled from 1·5 mm glass containing an internal filament (Clark Electromedical) on a Narishige horizontal puller. The recording electrode was filled with 3 M-K acetate and the current-passing electrode with 0·5 M-K_2SO_4. The use of K_2SO_4 in the current-passing electrode seemed to give better stability and cell survival than other electrolytes. Many attempts at impalement with two electrodes were unsuccessful because of cell damage. In successful cells the membrane potential recorded immediately after the first impalement was usually 40–60 mV. Insertion of the second electrode often reduced the membrane potential to 20–40 mV and the input impedance was often low (less than 20 MΩ initially). The cell then gradually recovered until the membrane potential was about 50 mV and the input impedance was 50–200 MΩ. In most experiments the membrane potential was clamped at −50 mV except during brief command pulses, and the holding current was normally less than 0·5 nA at this potential. Once satisfactorily stabilized a cell could usually be held for an hour or more; occasionally cells were held for 3–4 h.

The voltage clamp circuit was that described by Dionne & Stevens (1975). The necessity for high resistance electrodes usually limited the gain that could be used because of the liability of the clamp to oscillate. With the gain optimally adjusted the response to a square command pulse settled in 5–10 msec, which was too slow to enable us to resolve fast components in the relaxation patterns. It is possible that the use of lower resistance bevelled micro-electrodes would improve the frequency of response. To reduce high frequency noise the current signal was passed through a low-pass filter (200–400 Hz cut-off; 80 db per decade).

The signals were recorded directly by means of a potentiometric recorder and were stored on FM tape for analysis. A transient recorder (Datalab 901) and X–Y plotter (Bryans) were used to produce permanent records.

Drugs were applied ionophoretically in the conventional way. Ejecting and retaining currents were generated by a current pump similar to that described by Dreyer & Peper (1974). With carbachol or suberylcholine, positioning of the ionophoretic pipette did not seem to be particularly critical for obtaining responses provided that the surface of the cell was not covered with connective tissue. When ACh was applied ionophoretically, and if no anticholinesterase had been added, responses could only be elicited from localized spots as described by Dennis, Harris & Kuffler (1971). With 100–200 MΩ pipettes containing 2 M-carbachol, ACh or suberylcholine a retaining current of about 1 nA and ejecting currents of 2–4 nA were normally used.

Noise analysis, which will be reported on more fully in a subsequent paper, was carried out on records obtained during bath application of depolarizing drugs. The current signal recorded for

60–120 sec during maintained responses was fed into a band-pass filter (0·3–200 Hz) and stored on magnetic tape. The signal was analysed with a PDP 11/40 computer, usually at a sampling frequency of 512 Hz, and the net power spectrum (after subtraction of the control spectrum recorded with no drug present) was calculated between 0·5 and 100 or 200 Hz. The net spectrum was fitted by least squares to a Lorentzian curve for determination of the channel conductance and cut-off frequency (Katz & Miledi, 1972; Anderson & Stevens, 1973).

Solutions and drugs

The Krebs solution used had the following composition (mM): NaCl, 119; KCl, 4·7; $CaCl_2$, 2·5; KH_2PO_4 1·2; $NaHCO_3$, 25; $MgSO_4$, 1·2; glucose, 11. The solution was bubbled with 95% O_2:5% CO_2 and the pH was 7·2. When it was necessary to inhibit cholinesterase for measurements with ACh, neostigmine (3 μM) or ecothiopate (10 μM) was added to the medium; in a few experiments the preparation was bathed in methanesulphonylfluoride (10 mM) for 1 hr before the start of the experiment.

Drugs used were acetylcholine bromide (Koch-Light) carbamylcholine chloride (Aldrich); hexamethonium bromide (Koch-Light); decamethonium iodide (Koch-Light); mecamylamine hydrochloride (Merck, Sharpe & Dohme); trimetaphan camsylate (Roche); (+)-tubocurarine chloride (Wellcome); neostigmine methylsulphate (Roche). Suberyldicholine was a gift from Dr A. Ungar; surugatoxin was a gift from Professor D. A. Brown; α-bungarotoxin was a gift from Professor E. A. Barnard.

RESULTS

Agonist-induced currents

Carbachol, ACh and suberylcholine applied by perfusion or ionophoretically caused an inward current which, as expected, increased in amplitude when the membrane was hyperpolarized, and the current–voltage relationship for the agonist-induced current was investigated in a number of cells.

Fig. 1 shows a perfusion experiment. Before application of carbachol the potential was manually changed in steps from the holding potential (−50 mV). The control current–voltage curve was linear over the range (−40 to −80 mV), with a slope of about 100 MΩ. Addition of carbachol (25 μM) with the potential held at −50 mV caused an inward current which reached a plateau within 2 min. Another sequence of voltage steps was then imposed and the carbachol-induced current measured at each potential as the difference between the currents measured before and during the application of carbachol. The relationship between carbachol-induced current and membrane potential is shown in Fig. 1*B*. The relationship is somewhat non-linear, the current tending to increase disproportionately as the membrane is hyperpolarized, and extrapolation suggests that the line would cross the abscissa at about −25 mV.

Similar results were obtained in thirty-nine experiments, for carbachol concentrations between 25 and 50 μM. The resting input resistance of the cells ranged between 40 and 200 MΩ. The mean value of the current produced by 25 μM-carbachol at −50 mV was 0·7 ± 0·1 nA (S.E. of mean).

In the ionophoretic experiments the agonists were applied in pulses of 2–10 sec at intervals of 30 sec or more. The duration of the pulse was chosen so that the agonist-induced current reached a plateau before the end of the pulse (Figs. 2 and 10). The intensity of the ionophoretic current was adjusted to produce a response similar in amplitude to those obtained in perfusing experiments (0·5–3 nA at −50 mV). As

the surface activated by an ionophoretic application is likely to be smaller than in a perfusion experiment, the mean concentration of agonist is likely to be larger. The intervals between the pulses were made long enough to ensure that desensitization did not lead to a progressive decrease of the responses. Once a stable response had

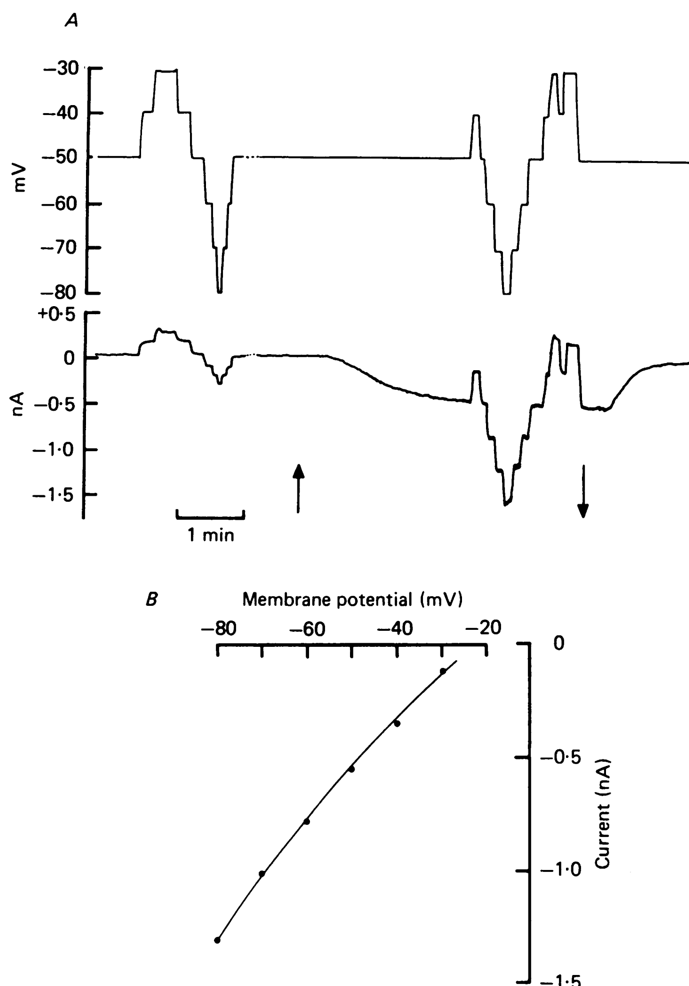


Fig. 1. *A*, effect of bath-applied carbachol (25 μM) on the current–voltage relationship of a voltage-clamped ganglion cell. Upper trace: membrane potential, which was changed manually in steps from the holding potential (−50 mV). Lower trace: membrane current (inward current downwards). The carbachol-induced current was measured as the difference, at a given membrane potential, between the current during the control step and during the step recorded in the presence of carbachol. The graph (*B*) shows the relationship between carbachol-induced current and membrane potential.

been obtained at a given potential, the potential was shifted and the height of the plateau current measured. When no antagonist was present the curves relating the agonist-induced current to membrane potential were very similar to those obtained in the perfusion experiments (Figs. 1*b*, 3 and 4).

In the experiment illustrated in Fig. 1, the voltage was changed 'manually' and

the change from one potential to the next took about 1 sec. In most experiments, however, the change of potential was triggered by a square command pulse. As illustrated in various Figures (Figs. 5–9) the agonist induced current, when measured in the absence of antagonists, appeared to reach its stead-state value immediately after the jump. It should be noted, however, that the first 5–10 msec following the jump could not be resolved because of the limited frequency response of the clamp. We therefore cannot exclude that a fast relaxation occurs in this initial period, as is the case in the other systems where the lifetime of the open channel is voltage-dependent (see below).

With suberylcholine as agonist a distinct slow relaxation was observed following a voltage jump, which superficially resembles the type of relaxation seen with agonists at the neuromuscular junction (Neher & Sakmann, 1975; Adams, 1975). This relaxation (time constant about 0·25 sec at -80 mV) is however much too slow to represent voltage-dependent channel opening (see section on noise analysis) and more probably results from an interaction of agonist and channel blocking activity as described for decamethonium at the neuromuscular junction (Adams & Sakmann, 1978).

Reversal potential of the agonist-induced current

From the relationship between drug-induced current and membrane potential it usually appeared that the current would reverse in direction at about -20 mV. The direct observation of a reversal of the agonist-induced current was difficult, however, because of a marked increase in membrane conductance (delayed rectification) which occurred when the membrane was depolarized beyond -40 mV. The large currents required to hold the membrane potential at more positive levels tended to block the high resistance micro-electrodes with which we were usually working. In a few experiments we used low resistance (40 MΩ) current electrodes which could support the large currents required, but these usually had a damaging effect on the cells. On these experiments we succeeded in approaching the reversal potential and in one case observed a clear reversal at -12 mV. This value is close to that obtained in other autonomic ganglion cells (Nishi & Koketsu, 1960; Blackman, Ginsborg & Ray, 1963; Dennis *et al.* 1971; Selyanko & Skok, 1979) and we will assume, therefore, that the reversal potential is about -10 mV.

Voltage dependence of the agonist-induced current

In most systems where ACh has excitatory effects, it has been found that the lifetime of the channels opened by ACh increases with hyperpolarization (e.g. Anderson & Stevens, 1973; Sheridan & Lester, 1975; Ascher, Marty & Neild, 1978*a*). However, there have been recent reports of cholinergic excitatory responses where this increase seems small (Dionne & Parsons, 1978; Kuba & Nishi, 1979) or even absent (MacDermott, Dionne & Parsons, 1978).

Where voltage dependence of the channel lifetime has been demonstrated this results in an increase in the mean fraction of open channels as the membrane is hyperpolarized; this in turn leads to a curvature of the steady-state current–voltage curve during hyperpolarization because the total current increase is the product of the increased number of open channels and the increased driving force (Dionne &

Stevens, 1975). As a result, linear extrapolation of a restricted number of points of the current–voltage relation at potentials negative to the reversal potential leads to an apparent reversal potential markedly different (more negative) from the true reversal potential (Mallart, Dreyer & Peper, 1976; Ascher, Marty & Neild, 1978*a*). Conversely a curvature of the current–voltage relation or a difference between extrapolated and directly measured values of the reversal potential (or both) may suggest that the lifetime of the channel opened by ACh is voltage-dependent, although there are other possible interpretations for such a curvature (see below).

As is evident from the data of Figs 1, 3 and 4, the current–voltage relation measured in the absence of antagonist was usually non-linear. To obtain a more precise evaluation of this curvature, we calculated the ratio of the steady-state currents produced by carbachol at −50 and −80 mV, $I(-80):I(-50)$.

Assuming that each channel has a linear current–voltage relationship (Anderson & Stevens, 1973; Dionne & Stevens, 1975) reversing at −10 mV, the ratio $I(-80):I(-50)$ would equal 1·75 if the number of channels open were not voltage-dependent. Instead we found the mean value of $I(-80):I(-50)$ to be $2{\cdot}25 \pm 0{\cdot}05$ in thirty-nine experiments with bath-applied carbachol. This result suggests that, as in other cells, the fraction of open channels is increased by hyperpolarization, because of an increase in mean channel lifetime. The magnitude of this effect may be similar in fact to the effect at the frog end-plate, where Magleby & Stevens (1972) found that the ratio of mean channel lifetimes at −80 and −50 mV is 1·26. For a reversal potential of −10 mV this would lead to a ratio of the steady-state currents of 2·20, close to the value we measured.

We have at present no evidence as to the linearity of the single channel current–voltage relationship in ganglion cells, and an appreciable non-linearity (cf. Lewis, 1979) would affect the tentative conclusion about the voltage dependence of the channel lifetime. Another mechanism by which a non-linear steady-state current–voltage curve could arise is by a secondary increase in K^+ conductance triggered by agonist-induced Ca^{2+} entry (Ascher, Marty & Neild, 1978*a*; Suzuki & Kusano, 1978).

Since for most of the analysis of the antagonist action, we do not need a precise value of the voltage dependence of the channel lifetime, we shall neglect it in a first approximation. Further studies (and in particular the study of the effect of membrane potential on the noise spectrum) will be required to obtain a more precise knowledge of this effect.

Comparison of the three agonists: carbachol, acetylcholine, suberylcholine

Most of the perfusion experiments were done with carbachol concentrations between 15 and 50 μM, which produced currents of 0·3–3 nA at the holding potential (−50 mV). In a few experiments we used ACh and suberylcholine. Suberylcholine was about five times as potent as carbachol. In the presence of an anticholinesterase, ACh was about five times as potent as carbachol, but with no cholinesterase inhibitor concentrations exceeding 500 μM were required to produce any response.

In the ionophoretic experiments carbachol was again found the most convenient agonist, because consistent responses did not require precise or close positioning of the ionophoretic pipette, and also because a plateau could usually be obtained after a relatively short application (a few seconds). The same was true if ACh was applied

after inactivation of cholinesterase, but in the absence of anticholinesterases very accurate localization of the pipette and close approximation to the cell were needed to produce consistent responses. Responses to ionophoretic application of suberylcholine were readily obtained, but they usually showed a slowly rising form which did not reach a plateau even with a 10 sec ionophoretic pulse. This is in contrast to the behaviour of ACh and carbachol, and the reason for the difference is not clear at present.

Noise analysis

An increase in the noise shown on the current record was always observed during the application of the agonist, as can be seen in most of the Figures.

Spectral analysis of steady responses to carbachol at -50 mV was performed in five cells. The power spectra were satisfactorily fitted by a single Lorentzian, with a mean cut-off frequency of 10·5 Hz. This corresponds to an average lifetime of $15{\cdot}2 \pm 1{\cdot}0$ msec (S.E. of mean; five cells). Similar analysis gave the mean channel lifetime at -50 mV for ACh as $30{\cdot}2 \pm 3{\cdot}8$ msec S.E. of mean, four cells), and for suberylcholine $35{\cdot}3 \pm 1{\cdot}9$ msec (S.E. of mean, six cells). These values are clearly higher than those obtained at the frog neuromuscular junction at room temperature (Katz & Miledi, 1972; Anderson & Stevens, 1973) and are closer to the values found in *Aplysia* neurones (Ascher, Marty & Neild, 1978*a*). Noise analysis on hyperpolarized cells met with technical difficulties due to the need to pass currents for prolonged periods through the high resistance micro-electrodes, and we do not have enough data so far to indicate with certainty whether the channel lifetime changes with membrane potential over the range -40 to -80 mV.

The mean channel conductance (assuming the reversal potential to be -10 mV) was calculated as: carbachol $19{\cdot}5 \pm 1{\cdot}9$ pS (five cells); ACh $21{\cdot}1 \pm 2{\cdot}4$ pS (five cells); suberylcholine $22{\cdot}4 \pm 1{\cdot}7$ pS. An average-sized response (1 nA at -50 mV) in our experiments thus corresponds to the opening of about 1000 channels.

Absence of muscarinic effects

In four experiments bethanechol was applied ionophoretically or by perfusion at concentrations of 0·5 and 1 mM. No response could be observed. We also found that the response to carbachol was completely abolished by hexamethonium (1 mM). It appears unlikely therefore that the cells of the submandibular ganglion bear muscarinic receptors similar to those described in *Necturus* heart ganglion (Hartzell, Kuffler, Stickgold & Yoshikami, 1977).

Absence of effect of α-bungarotoxin

α-Bungarotoxin (200 μM) applied for up to 90 min had no effect on the response to ionophoretic or bath-applied carbachol.

Effects of antagonists in the steady state

We have tested the effects of six antagonists. Hexamethonium, trimetaphan and mecamylamine are well-known ganglion blocking drugs; tubocurarine and decamethonium also produce a non-depolarizing type of ganglionic block; surugatoxin is a polypeptide shellfish toxin with specific ganglion blocking activity (Hayashi & Yamada, 1975; Brown, Garthwaite, Hayashi & Yamada, 1976).

The addition of ganglionic blocking drugs to the bathing solution reduced the amplitude of the response to carbachol. The effect was usually fully developed within 10 min of adding the blocking agent. With all drugs except mecamylamine, the block was reversible within about 30 min of washing out the drug. With mecamylamine, washing for 60 min or longer only partially restored the response.

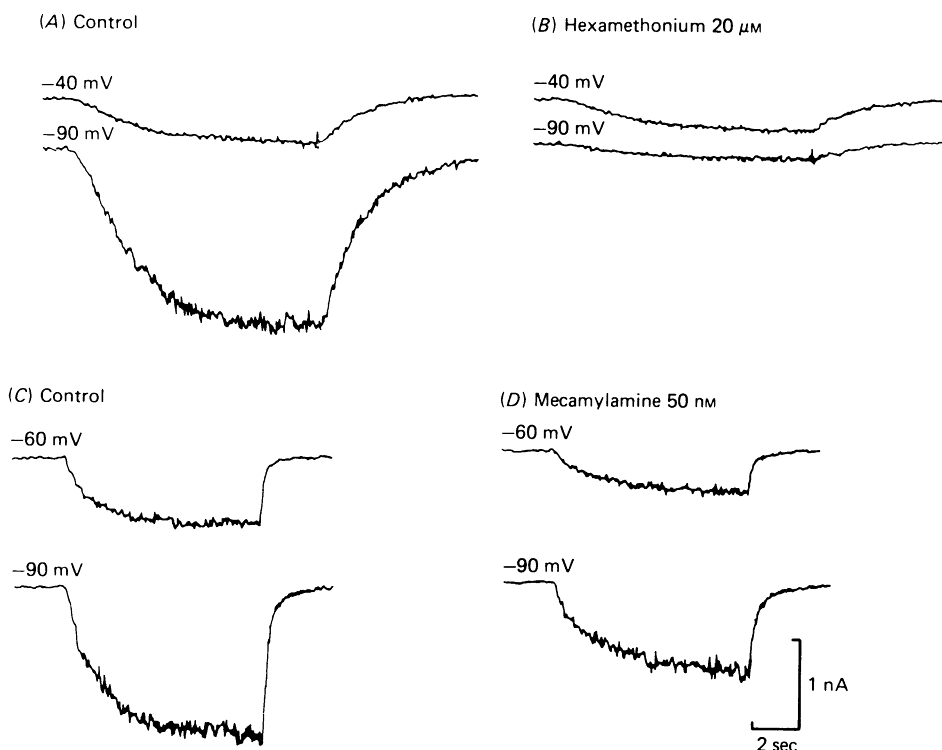


Fig. 2. Effect of antagonists on responses to ionophoretically applied carbachol. Records *A* and *C* show control responses in two cells to a pulse of carbachol lasting 10 sec, at two membrane potentials. Records *B* and *D* were obtained in the presence of hexamethonium (20 μM) and mecamylamine (50 nM) respectively. Hexamethonium inhibits the response at −90 mV much more than at −40 mV whereas mecamylamine shows no voltage dependence. The increased 'noise' during agonist application is clearly seen.

When the current–voltage relationship was investigated in the presence of antagonists two different patterns were observed.

(1) With hexamethonium, decamethonium or tubocurarine the blocking effect was clearly voltage-dependent, and increased markedly as the cell was hyperpolarized. This effect is seen in Fig. 2*A* and *B*) which shows responses to ionophoretically applied carbachol at −40 and −90 mV. Normally the current at −90 mV is much greater than that at −40 mV, but in the presence of 20 μM-hexamethonium, the response at −90 mV is considerably smaller than that at −40 mV, which is only slightly reduced compared to the control. This type of voltage-dependent block has the effect of distorting the current–voltage relationship for the agonist effect, so that hyperpolarization causes relatively less increase in current than expected from the

control curve (Fig. 3*A*, *B* and *C*). At higher antagonist concentrations the relationship was distorted so much that hyperpolarization beyond a certain point reduced rather than increased the agonist-induced current.

To express this effect quantitatively we have calculated Λ, the ratio of the current in the absence of antagonist to the current in the presence of antagonist. Λ = 1

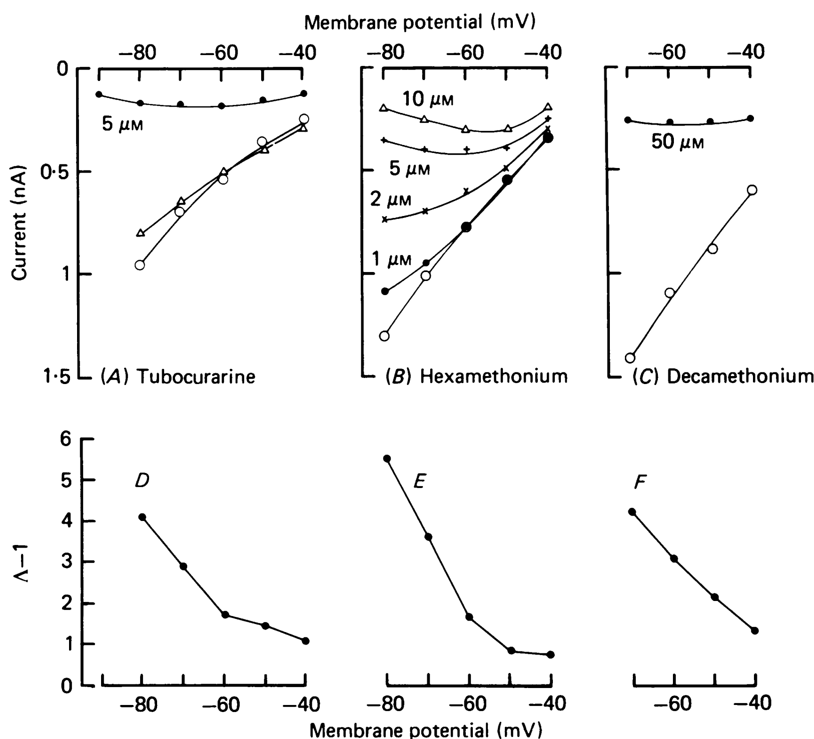


Fig. 3. Effect of voltage-dependent antagonists on the relationship between agonist-induced current and membrane potential. The agonist used was bath-applied carbachol (25 μM) in panels *A*, *C*, *D* and *F*, and ionophoretically applied carbachol in panels *B* and *E*. *A*, *B*, *C*: current–voltage curves obtained in the presence of different antagonists. Open circles show control curves, open triangles (*A*) show recovery after washing out tubocurarine. *D*, *E*, *F*: blocking effect, expressed as Λ − 1, as a function of membrane potential for the three antagonists. The line plotted in *E* is for 10 μM hexamethonium. In each case Λ − 1 increases markedly as the membrane is hyperpolarized.

corresponds to no inhibition, and Λ − 1 can be used as an index of the effect of the antagonist. With this first group of antagonists, as can be seen from Fig. 3*D*, *E* and *F*), Λ − 1 increased with hyperpolarization of the cell (i.e. the blocking action of the antagonist was markedly voltage-dependent). This voltage dependence was observed at all concentrations tested. In Fig. 3*B* an example is shown where 1 μM-hexamethonium had no blocking effect except when the membrane was hyperpolarized to beyond − 60 mV. Higher doses reduced the response at all membrane potentials, but the block always increased with hyperpolarization. In a few experiments, mecamylamine at concentrations exceeding 50 nM showed some degree of voltage dependence in its blocking action. This effect was, however, very variable and we are not clear at this stage what factors determine whether or not it occurs.

(2) Trimetaphan, surugatoxin and mecamylamine inhibited the agonist-induced current equally at all membrane potentials, and $\Lambda - 1$ showed no voltage-sensitivity. Fig. 2 (*C* and *D*) shows that mecamylamine (50 nM) reduced the response to carbachol equally at -60 and -90 mV and the plots in Fig. 4 show the lack of voltage dependence for other drugs in this group.

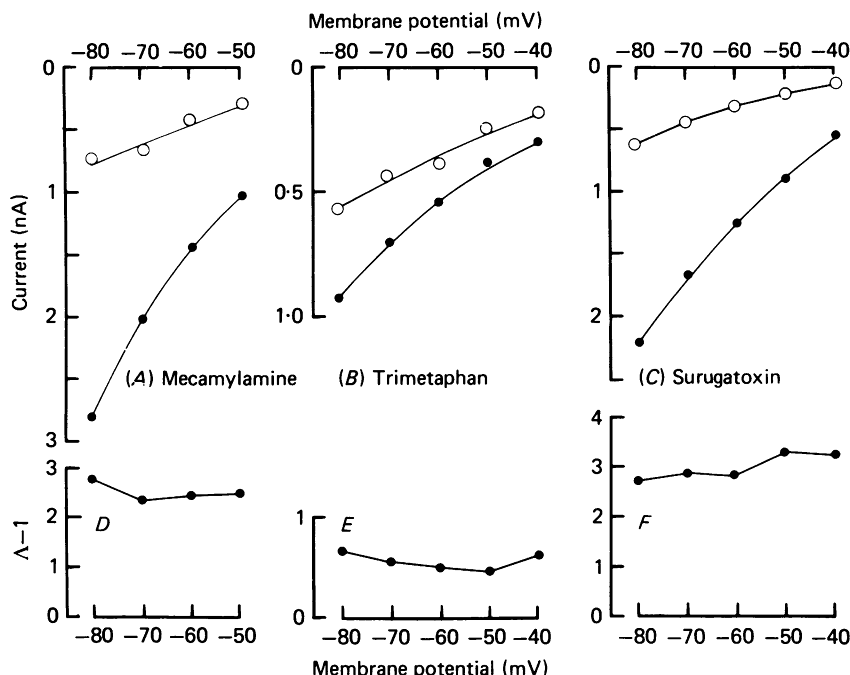


Fig. 4. Effect of non-voltage-dependent antagonists on the relationship between agonist-induced current and membrane potential. The agonist used was bath-applied carbachol (25 μM). *A*, *B*, *C*: current–voltage curves before (filled circles) and after (open circles) adding the antagonists. The antagonist concentrations used were: (*A*) mecamylamine 25 μM; (*B*) trimetaphan 2·5 μM; and (*C*) surugatoxin 0·2 μM. *D*, *E*, *F*: blocking effect, expressed as $\Lambda - 1$, as a function of membrane potential. With these antagonists, $\Lambda - 1$ is not appreciably voltage-dependent.

TABLE 1. Effect of membrane potential on blocking action of antagonists

Antagonist	$\frac{\Lambda(-80)-1}{\Lambda(-50)-1}$	Antagonist	$\frac{\Lambda(-80)-1}{\Lambda(-50)-1}$
Tubocurarine (5 μM)	3·13 ± 0·29 + (11)	Surugatoxin (0·1–0·2 μM)	0·86 ± 0·9 (3)
Hexamethonium (0·5–20 μM)	3·17 ± 0·22 (42)	Trimetaphan (2·5 μM)	1·18 ± 0·08 (12)
Decamethonium (50 μM)	2·87 ± 0·11 (7)	Mecamylamine (25–50 nM)	1·39 ± 0·15 (17)

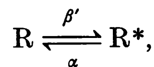
Λ is defined as the ratio of the carbachol responses measured in the absence and presence of the inhibitor, at the membrane potential indicated. Figures in parentheses are the number of measurements made with each drug.

The degree of voltage sensitivity shown by each antagonist is conveniently expressed as the ratio $\{\Lambda(-80)-1\}:\{\Lambda(-50)-1\}$ and values of this ratio for the six drugs tested are given in Table 1.

Dependence of the steady-state block on the agonist concentration

The experiments analysed above indicate that ganglionic blocking agents fall into two groups, differing in the sensitivity of the steady-state block to membrane potential. By analogy with other systems where a voltage-dependent block has been observed, it is reasonable to hypothesize that voltage-dependent block results from blocking of ionic channels at a site lying within the transmembrane electric field, and that the non-voltage-dependent block represents a competitive interaction at the receptor. However, these two interpretations are by no means the only possible ones and a more general distinction can be drawn between antagonists which block by preferential binding to the closed or open state of the channel–receptor complex.

If we represent the channel opening reaction by



$$R \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} R^*,$$

where R is the closed and R* the open state, and α and β' the closing and opening rate constants, then antagonists might act by binding to R (e.g. competitive antagonism) or R* (e.g. channel block) or both. In the above scheme β' is a function of agonist concentration. If the antagonist binds selectively to R, $\Lambda - 1$ will tend to decrease as the agonist concentration is increased; if the antagonist is selective for R*, $\Lambda - 1$ will increase as the agonist concentration is increased.

If we define the ratio $y_\Lambda = (\Lambda_1 - 1):(\Lambda_2 - 1)$, where Λ_1 and Λ_2 are measurements of the degree of block produced by the same concentration of antagonist in the presence of two different agonist concentrations, then in the case where the antagonist binds selectively to R,

$$y_\Lambda = \frac{\beta_2' + \alpha}{\beta_1' + \alpha}, \tag{1}$$

where β_1' and β_2' are values of β' for the two agonist concentrations. If the antagonist binds selectively to R^*

$$y_\Lambda = \frac{\beta_1'}{\beta_1' + \alpha} \div \frac{\beta_2'}{\beta_2' + \alpha}. \tag{2}$$

Thus y_Λ is equal to the ratio of the fraction of channels opened (i.e. to the ratio of the currents produced, $y_I = I_1 : I_2$) by the two agonist concentrations in the absence of any blocking agent.

If $\beta_2' < \beta_1'$, eqn. (1) predicts that y_Λ (which is a measure of the relative degree of block at the high, compared with the low, agonist concentration) should be less than one, whereas eqn. (2) predicts that y_Λ should exceed one.

Equation (1) makes two assumptions, namely that channel opening by the agonist shows no co-operativity, and that a receptor occupied by an agonist molecule has a high probability of opening. Both of these assumptions are open to question, but relaxing them does not alter the general conclusion that for an antagonist binding selectively to R, $y_\Lambda < 1$.

We have tested the effect of agonist concentration on Λ for various antagonists, the results being given in Table 2. In experiments with bath-applied carbachol in the presence of hexamethonium, two carbachol concentrations were tested (one double the other) which gave responses differing, on average, 2·9-fold. In the presence of hexamethonium (1–5 μM) the mean ratio y_Λ was 2·1.

Because of the difficulty of holding impalements for long enough, most experiments were done with ionophoretically applied agonists. Ionophoretic pulses lasting 5–10 sec were applied every 30–60 sec, with alternating large and small currents, these being adjusted so that the amplitudes of the responses produced differed 3–5-fold. The duration of the two pulses was the same. The relative degree of block, y_Λ, was then measured for various antagonists.

TABLE 2. Blocking effect of antagonists as a function of agonist concentration

Antagonist	Agonist	y_I*	y_Λ*	Number of tests
Hexamethonium (2 μM)	Carbachol (20–40 μM) (bath-applied)	2·94 ± 0·42	2·13 ± 0·32	4
Hexamethonium (2–5 μM)	Carbachol ionophoretic	3·75 ± 0·38	1·39 ± 0·10	6
Tubocurarine (5 μM)	Carbachol ionophoretic	3·06 ± 0·28	1·76 ± 0·31	3
Decamethonium (5–50 μM)	Carbachol ionophoretic	3·93 ± 0·28	1·73 ± 0·03	3
Trimetaphan (2·5 μM)	Carbachol ionophoretic	4·60 ± 0·67	0·89 ± 0·09	8
Mecamylamine (15–100 nM)	Carbachol ionophoretic	3·72 ± 0·34	0·77 ± 0·06	8

* $y_I = \frac{I_1}{I_2}$; $y_\Lambda = \frac{\Lambda_1 - 1}{\Lambda_2 - 1}$, where subscripts 1 and 2 represent different agonist concentrations (see text). Measurements were made at −80 mV holding potential.

During the response the concentration of agonist varies in time and in space in a complex way, but it can be assumed that at any point, at a given time after the beginning of the ionophoretic pulse, the concentration obtained with the large pulse is larger than that obtained with the small pulse. We can therefore assume that on each small area of membrane the fraction of open channels increases when the intensity of the ionophoretic pulse increases. It is, however, possible that saturation of the response or marked desensitization occurs in the area close to the pipette tip, which would lead to a error in our calculations.

In these experiments, the voltage-dependent antagonists all gave values of y_Λ greater than one, whereas with two non-voltage-dependent antagonists y_Λ was less than one. Surugatoxin could not be tested in these experiments because not enough was available. With the voltage-dependent drugs, y_Λ was in fact never as great as y_I, the ratio of control currents produced by the two agonist concentrations. With bath-applied carbachol, agreement was closer than with ionophoretic responses.

Despite this discrepancy, the results summarized in Table 2 strongly suggest a correlation between the effects of voltage and those of the agonist concentration. For tubocurarine, hexamethonium and decamethonium the block is increased by hyperpolarization and by increasing the fraction of open channels. For mecamylamine and trimetaphan, the block is insensitive to membrane potential and decreases slightly when the agonist concentration is increased.

The most likely interpretation of these results is that the non-voltage-dependent blocking action of mecamylamine, trimetaphan and surugatoxin results from binding to the closed state of the channel–receptor complex (possibly to the receptor itself) whereas the voltage-dependent effect of tubocurarine, hexamethonium and deca-

methonium results from binding to the open state of the channel–receptor complex (most probably to a site within the channel). The non-voltage-dependent site is assumed to lie on the external surface of the membrane while the channel blocking site lies within the membrane so that binding of cations to it would be favoured by hyperpolarizing the membrane. The voltage dependence could also be interpreted by assuming that the antagonist modifies the $I–V$ relation for a single channel. Bamberg & Laüger (1977) have studied such a case, in which the antagonist binds close to the mouth of the channel and leads to a curved $I–V$ relation. In this case, however, although hyperpolarization reduces the conductance it cannot reduce the current, but only makes it increase less than expected. This type of model, therefore, does not explain effects like those seen in Fig. 3*B*, where in the presence of hexamethonium, the current at -80 mV is smaller than at -40 mV.

TABLE 3. Estimates of equilibrium constants for non-voltage-dependent antagonists, from the relationship $K_B = X_B/(\Lambda - 1)$

Antagonist	Concentration range tested (μM)	K_B(μM) ± S.E. of mean	Number of tests
Trimetaphan	2·5–10	1·44 ± 0·12	10
Mecamylamine	0·025–0·1	0·05 ± 0·02	4
Surugatoxin	0·1–0·2	0·11, 0·07	2

Measurements were made at -80 mV holding potential.

Equilibrium constants for non-voltage-dependent antagonists

If the non-voltage-dependent antagonists act simply by blocking receptors competitively, or by combining at a site other than the receptor with the closed state of R of the channel–receptor complex, and if the interaction is bimolecular, the binding constant K_B can be calculated from

$$K_B \approx \frac{X_B}{\Lambda - 1}, \tag{3}$$

where X_B is the antagonist concentration. This approximation is acceptable so long as the fraction of open channels is small, and provided that the assumptions made in deriving eqn. (1) are fulfilled. Since, under our experimental conditions, a fourfold increase in the control agonist response had only a small effect on Λ (Table 2), the error introduced by neglecting the agonist concentration will be small, but an appreciable error may result from neglecting cooperativity. Table 3 shows the calculated values of K_B for the non-voltage-dependent antagonists.

Voltage-jump experiments

General pattern of relaxations

The voltage-jump technique was first introduced to analyse the kinetics of agonist action (Adams, 1975; Neher & Sakmann, 1975; Sheridan & Lester, 1975), and has recently been found useful in elucidating the mechanism of action of antagonists

(Marty *et al.* 1976; Adams, 1977*b*; Ascher, Marty & Neild, 1978*b*; Colquhoun *et al.* 1979; see also Adams & Sakmann, 1978). In most cases the relaxation observed after a voltage jump in the presence of an agonist follows a single exponential time course, representing the shift in the equilibrium between closed and open states ($R \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} R^*$). Even though this reaction involves both binding and channel opening reactions it appears that one of these reactions occurs at too high a rate to be resolved electrophysiologically, so that only the slower reaction is detected.

Voltage-jump relaxations recorded in the presence of certain antagonists have been shown to consist of two exponential components, respectively faster and slower than the relaxation seen with no antagonist present (e.g. Adams, 1977; Ascher, Marty & Neild, 1978*b*; Colquhoun *et al.* 1979). Concerning the slow relaxations, two different patterns have been recorded in response to a hyperpolarizing jump: (1) a slow exponential *decrease* of conductance (e.g. hexamethonium or tubocurarine in *Aplysia*; Ascher, Marty & Neild, 1978*b*); this slow decrease of conductance is referred to below as the 'outward' relaxation; it was termed the 'inverse' relaxation by Ascher, Marty & Neild (1978*b*); and (2) a slow exponential *increase* of conductance (e.g. procaine at the motor end-plate and in *Aplysia*; Adams, 1977; Marty, 1978) referred to below as the 'inward' relaxation. Because these various relaxation patterns have been shown to be consistent with a voltage-dependent channel block hypothesis (see later section), we were interested to see whether similar results could be obtained with ganglion cells.

The channel lifetimes estimated from noise analysis are relatively long compared with the response time of our voltage clamp and the indirect evidence from the curved current–voltage relationship for the agonist-induced currents which suggests that the channel lifetime may be voltage-dependent. We therefore expect to see inward relaxations in response to hyperpolarizing jumps when no antagonist is present. In fact we could not observe such relaxations (see Figs. 5–9); even on a faster time base these records showed no consistent relaxation pattern during the first 20–40 msec). At present we do not know the explanation for this discrepancy, and further studies on the agonist-induced noise are in progress to try to throw more light on the problem.

In submandibular ganglion cells in the presence of voltage-dependent antagonists both inward and outward slow relaxations were seen, depending on the antagonist used (Fig. 5). Fast relaxations also appeared to be present (see below) though their time course was too fast to be resolved. Therefore the relaxations seen in Figs. 5–8 are assumed to correspond with the slow relaxations seen by others.

It was found that tubocurarine and hexamethonium both caused slow outward (i.e. inverse) relaxations on hyperpolarization, whereas with decamethonium the slow relaxation was inward (i.e. normal). Sometimes pronounced outward relaxations were also seen with mecamylamine, though, as already mentioned, this drug gave inexplicably variable results.

As a first approximation, a channel blocking drug whose affinity for the open channel increases with hyperpolarization will produce a slow outward relaxation following a hyperpolarizing jump if the rate of the channel blocking reaction is slow compared with the channel opening reaction, because the open channels will slowly

become blocked after the jump. On the other hand, an inward relaxation will tend to occur if the blocking reaction is faster than the opening reaction, since the open channels at the beginning of the jump will be rapidly blocked, reducing the number of open channels, which will then be slowly replenished by re-equilibration of the

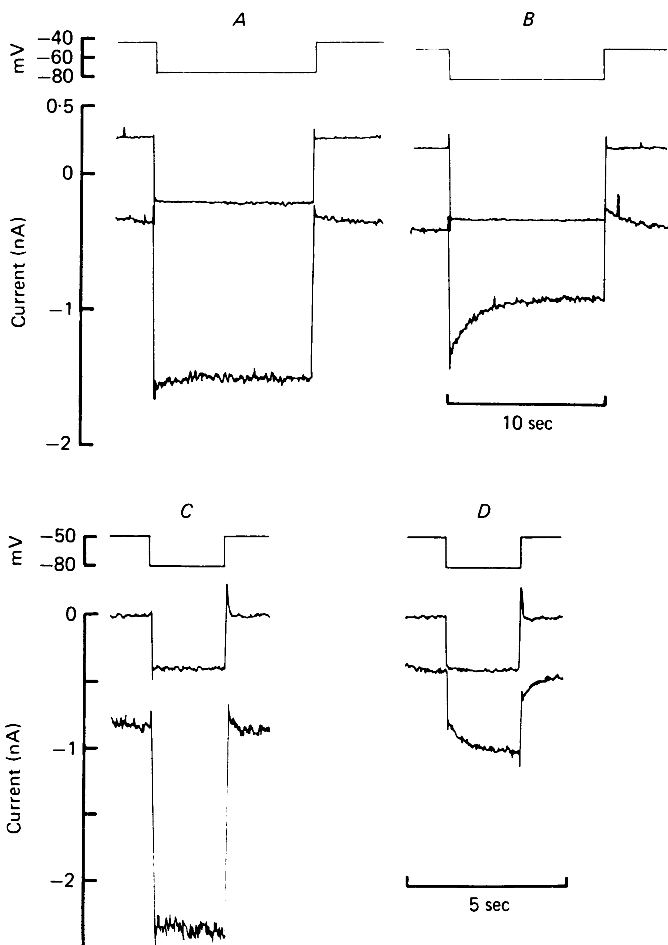


Fig. 5. Slow relaxations during hyperpolarizing voltage steps in the presence of tubocurarine and decamethonium. *A*, *C*: effect of 30 mV hyperpolarizing step on current in the absence (upper current record) and in the presence (lower current record) of carbachol. *B*, *D*: similar records obtained in the presence of tubocurarine 5 μM (*B*) and decamethonium 50 μM (*D*). Note the marked voltage dependence in both cases, the carbachol-induced current being similar at the two membrane potentials, and the appearance of slow relaxations (outward with tubocurarine and inward with decamethonium). In *A* and *B* bath-applied carbachol (25 μM) was used; in *C* and *D* carbachol was applied ionophoretically.

opening reaction. Though an over-simplification, this gives a picture of how the two types of relaxation can arise; a more detailed discussion is given later.

Examples of inward and outward slow relaxations are shown in Fig. 5. Fig. 5*A* and *C* shows the response of the cell to a voltage jump (-50 to -80 mV) lasting 10 sec. The upper current record in each panel shows the response when no agonist is

present. The current required to hyperpolarize the cell by 30 mV was about 0·5 nA. The lower current record was obtained in the presence of carbachol, which caused a steady inward current at -50 mV. After the hyperpolarizing jump, the inward current increased rapidly to a new value. Occasionally, as in Fig. 5*A*, a small outward relaxation occurred, but it was inconsistent and never large.

In Fig. 5*B*, the same experimental sequence was repeated in the presence of tubocurarine. The steady-state carbachol-induced current at the holding potential was only slightly reduced. When the jump was applied, the current increased immediately to a value quite close to that reached in control experiments, but then declined exponentially towards the steady-state level (outward relaxation).

The high resistance of the micro-electrodes prevented the analysis of the first few milliseconds following the jump. In the control conditions, the ratio $I(-80{:}I(-50)$ was 2·15, i.e. higher than the ratio predicted if the current increase was solely due to the increase in driving force. It is therefore probable that a better time resolution would allow the separation of the initial rise into an instantaneous increase of current followed by a fast (inward) relaxation.

In the presence of tubocurarine, the ratio of the initial current measured at -80 mV to the steady-state current at -50 mV was similar to that measured in control conditions. This suggests that, if a fast relaxation is present in control conditions, it also occurs in the presence of tubocurarine, and its amplitude is little modified (see Ascher, Marty & Neild, 1978*b*; Colquhoun *et al.* 1979).

Fig. 5*D* illustrates a similar experiment with decamethonium. As with tubocurarine, the voltage jump produced a slow relaxation when the antagonist was present, but in this case the slow relaxation was inward, i.e. the conductance slowly increased.

An additional marked difference between the two experiments is the low value of the current measured immediately after the jump to -80 mV in the presence of decamethonium. The ratio of this 'instantaneous' current to the steady-state current measured at -50 mV is 0·96 (Fig. 5 *D*), i.e. much less than the control value (Fig. 5*C*) of 2·39. This strongly suggests that the slow (inward) relaxation is preceded by a fast (outward) one, but this cannot be resolved at present.

The results presented so far are qualitatively compatible with a sequential scheme in which the antagonist acts by blocking the open state of the channel–receptor complex. The predictions of this hypothesis have been considered by many authors (see Adams, 1977; Ascher, Marty & Neild, 1978*b*). The scheme can be written

$$\mathrm{R} \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} \mathrm{R}^* \underset{k^*_{-\mathrm{B}}}{\overset{k^*_{+\mathrm{B}} X_{\mathrm{B}}}{\rightleftharpoons}} \mathrm{R}^*\mathrm{B}. \quad (4)$$

The rate constants $k^*_{+\mathrm{B}}$ and $k^*_{-\mathrm{B}}$ are the association and dissociation rates of the blocking agent (B) for the open channel; X_{B} is the concentration of B.

As discussed earlier β' is an unspecified function of agonist concentration. The blocking reaction $R^* \rightarrow \mathrm{R^*B}$ is assumed to be bimolecular, and the equilibrium constant is given by

$$K^*_{\mathrm{B}} = \frac{k^*_{-\mathrm{B}}}{k^*_{+\mathrm{B}}}. \quad (5)$$

The degree of block at equilibrium is

$$\Lambda - 1 = \frac{\beta'}{\alpha+\beta'} \cdot \frac{X_B}{K^*_B}. \quad (6)$$

A sudden perturbation of the equilibrium will lead to a relaxation represented by the sum of two exponential components. A voltage jump constitutes such a perturbation, provided that at least one of the parameters α, β', k^*_{-B} is voltage dependent.

The amplitude of the two components of the relaxations are given by quite complex relations (see Bernasconi, 1976, chapter 7; Ascher, Marty & Neild, 1978*b*, p. 227) which make it difficult to predict the direction of the relaxations in the general case. Such predictions are, however, much simpler in certain limiting cases, in particular if one of the two sequential reactions ($R \rightarrow R^*$, $R^*+B \rightarrow R^*B$) is much faster than the other (Ascher, Marty & Neild, 1978*b*).

If we assume that this condition is satisfied and, for simplicity, that only the second reaction is voltage-dependent there is no relaxation after a jump in the absence of antagonist. In the presence of an antagonist, two cases are possible. If the first reaction is much faster than the second, a hyperpolarizing voltage jump induces no immediate perturbation of the fast equilibrium $R \rightleftharpoons R^*$. Then, slowly, an increased block of the open channels develops, leading to a slow outward relaxation. If, on the other hand, the second reaction is fast compared to the first one, the initial effect of the jump will be a fast increase in the number of blocked channels (i.e. a fast outward relaxation). This will deplete the population of the R^* state, which will then be replenished slowly from R. This additional opening of channels will correspond to a slow inward relaxation.

If we keep the assumption that the two reactions have very different speeds, but now assume that both the first and the second are voltage-dependent, intuitive predictions concerning the direction of the fast relaxation become difficult. For the slow relaxation, however, one can still predict that with a fast dissociating antagonist a hyperpolarizing jump will produce a slow inward relaxation, while with a slowly dissociating antagonist a slow outward relaxation will occur.

It must be noted, however, that our experimental data, and the calculations described below indicate that the limiting conditions considered above are not strictly met. For the combination of carbachol 30 μM and tubocurarine 5 μM, for example, we have calculated $\alpha = 56$ sec^{-1} and $\beta' = 0{\cdot}85$ sec^{-1} *vs.* $k^*_B X_B = 25$ sec^{-1} and $k^*_{-B} = 0{\cdot}17$ sec^{-1}; these two sets of values are not sufficiently different to allow the use of the simplified equations calculated for the limiting cases (Ascher, Marty & Neild, 1978*b*).

In such conditions it could be misleading to attribute to a single factor the direction of the slow relaxations. Nevertheless, it appears reasonable to consider that the striking difference between the relaxations observed with tubocurarine and hexamethonium on one hand, and with decamethonium on the other, is mainly due to a difference in the value of k^*_{+B}, the rate at which the antagonist dissociates from the channel–receptor complex. A working hypothesis is therefore that tubocurarine and hexamethonium are slowly dissociating antagonists, while decamethonium is a rapidly dissociating one.

Effect of antagonist concentration on the slow relaxation rate

An interesting prediction of the sequential model concerns the effect of the antagonist concentration on the speed of the slow relaxation.

In the general case, the two time constants of the relaxations can be obtained by solving a quadratic equation (see e.g. Bernasconi, 1976, p. 27). The general expressions defining the two values simplify if the two roots are markedly different.

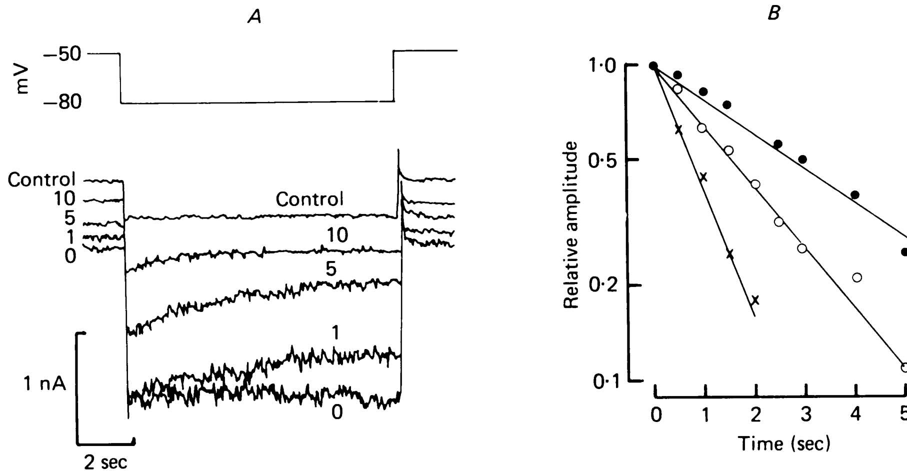


Fig. 6. Effect of tubocurarine concentration on the slow relaxation occurring after a hyperpolarizing step. *A*, responses to a 30 mV hyperpolarizing step. The upper current record shows the control response with no drug present. The other records show responses during the application of carbachol (25 μM). The numbers show the concentration (μM) of tubocurarine. *B*, semilogarithmic plots of the slow relaxation, showing that the relaxations are roughly exponential, and that the time constant, τ_s decreases with increasing tubocurarine concentration. ●, 1 μM-tubocurarine; ○, 5 μM; ×, 10 μM. The values of Λ and τ_s obtained from this experiment are shown in Table 4.

Therefore, as noted by Adams & Feltz (1977), the experimental observation that the two relaxations have markedly different time constants allows the fast and slow time constants to be calculated from the approximate solutions of the quadratic equations. For the slow relaxation τ_s, this leads to

$$\tau_s^{-1} = \frac{\beta' k^*_{+B} X_B + \alpha k^*_{-B} + \beta' k^*_{-B}}{\alpha + \beta' + k^*_{+B} X_B + k^*_{-B}}. \tag{7}$$

Two additional simplifying assumptions can be made: (1) $\beta' \ll \alpha$ (i.e. the fraction of channels opened by the agonist is small); and (2) $k^*_{+B} X_B \gg k^*_{-B}$, a necessary condition for appreciable equilibrium block to occur if $\beta' \ll \alpha$. Assumption (1) is justified by the fact that larger agonist concentrations could produce much larger currents than those that were normally used, and by the calculation from noise analysis that the number of open channels was normally about 1000; it seems safe to imagine that the cell possesses many more than 1000 receptors. With these simplifying approximations, eqn. (7) becomes:

$$\tau_s^{-1} \simeq \frac{\beta' k^*_{+B} X_B + \alpha k^*_{-B}}{k^*_{+B} X_B + \alpha}, \tag{8}$$

which can be written

$$\tau_s^{-1} = \beta' \left(\frac{k^*_{+B} X_B + \alpha k^*_{-B}/\beta'}{k^*_{+B} X_B + \alpha} \right). \tag{9}$$

From eqn. (9) it can be seen that an increase in the antagonist concentration X_B will *increase* the speed of the slow relaxation if $k^*_{-B}/\beta' < 1$, and *decrease* the speed of

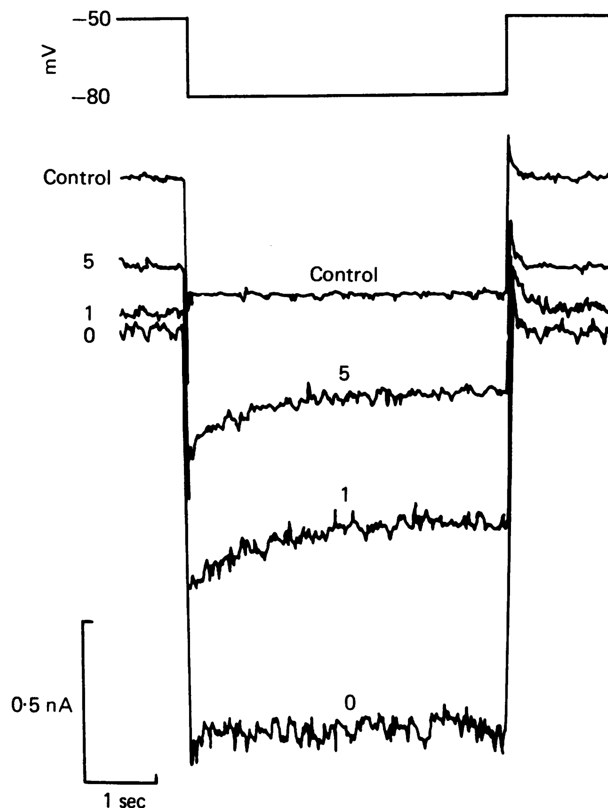


Fig. 7. Effect of hexamethonium concentration on the slow relaxation occurring after a hyperpolarizing step. The upper current record (control) was recorded during a 30 mV hyperpolarizing step with no drug present. The other records were recorded during ionophoretic application of carbachol in the presence of the hexamethonium concentration (μM) shown. Slow outward relaxations, whose rate increases at higher hexamethonium concentrations, are evident, similar to those seen with tubocurarine (Figs. 5 and 6). In this experiment the values of $\Lambda(-80)$ and τ_s were respectively 1·9 and 1·1 sec at 1 μM-hexamethonium, and 4·6 and 0·7 sec at 5 μM-hexamethonium (see Table 4).

the slow relaxation if $k^*_{-B}/\beta' < 1$. The first case will arise if the blocking agent dissociates slowly from the channel (k^*_{-B} small) and the second case if it dissociates rapidly (k^*_{-B} large).

We have investigated the dependence of τ_s^{-1} on antagonist concentration with hexamethonium, tubocurarine and decamethonium.

Fig. 6. illustrates an experiment using tubocurarine, similar to that of Fig. 5*B*, but with three different concentrations of tubocurarine. As illustrated in Fig. 6*B* the

speed of the outward relaxation increased as the concentration of tubocurarine was increased.

Fig. 7 illustrates a similar experiment for hexamethonium. The results resemble those obtained with tubocurarine. A significant difference, however, concerns the amplitude of the 'fast' relaxation. The existence of this relaxation is apparent from the fact that with hexamethonium the initial current measured after the jump falls considerably short of the value predicted by the increase in driving force and the

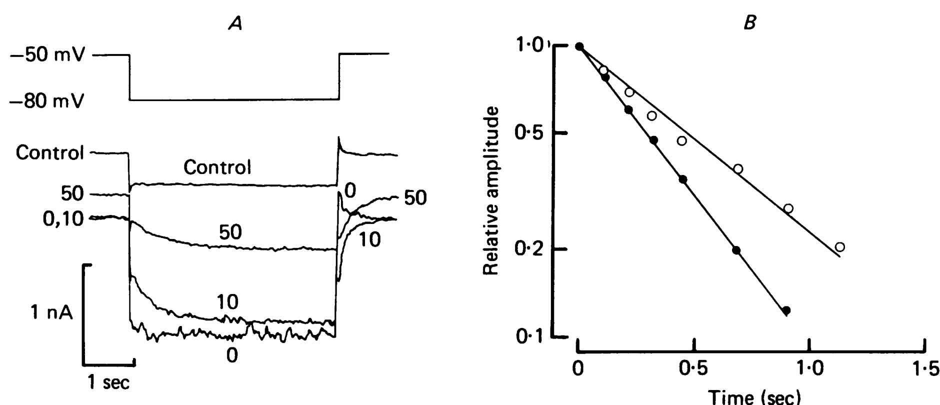


Fig. 8. Effect of decamethonium concentration on the slow relaxation occurring after a hyperpolarizing step. *A*, experimental records displayed as in Fig. 6*A*. The carbachol concentration was 30 μM. *B*, semilogarithmic plots of the slow relaxation, showing that τ_s increases with decamethonium concentration. ●, 10 μM-decamethonium; ○, 50 μM. In this experiment the values of $\Lambda(-80)$ and τ_s were respectively $\sim$ 1·1 and 0·42 sec at 10 μM-decamethonium and 2·4 and 0·68 sec at 50 μM-decamethonium.

current flowing immediately before the jump. As already discussed for the case of decamethonium in Fig. 5*D*, this suggests for hexamethonium that the slow outward relaxation is preceded by a *fast* outward one. With tubocurarine, on the other hand, the increase in current immediately after the jump (Figs. 5*A* and 6*A*) exceeds what would be expected from the increase in driving force alone, suggesting that the slow outward relaxation is preceded by a fast inward one. With tubocurarine, however, the unexpected potentiating effect (see later section) introduces a complication which is difficult to allow for, so conclusions drawn from the amplitude of the currents are necessarily tentative.

Fig. 8 illustrates an experiment in which two concentrations of decamethonium were used. With 10 μM-decamethonium, the carbachol-induced current at -50 mV is unchanged: when the jump is applied the initial current is much smaller than in the control, but then rises slowly (inward relaxation) towards a steady-state level which is not very different from that obtained in the control. At 50 μM, the same pattern is observed but the steady-state currents are now clearly reduced at -50 and -80 mV, and the time constant of the slow relaxation is larger than that measured with 10 μM-decamethonium.

The results obtained with 10 μM-decamethonium confirm an interesting prediction of the channel-block hypothesis in the case where k^*_{-B} is large, which is that at low

concentrations of antagonist the steady-state current may be unchanged by the antagonist, whose action can only be detected by kinetic measurements. Neher & Steinbach (1978) have pointed out that with low concentrations of the local anaesthetic QX222 and low concentrations of agonist, the *total* open time of the channel remains unchanged, but is distributed into a series of discontinuous events instead of

TABLE 4. Characteristics of slow relaxations following voltage jump to − 80 mV in presence of tubocurarine and hexamethonium

Tubocurarine				Hexamethonium			
Agonist	Tubocurarine concentration (μM)	Λ(− 80)	τ (sec)	Agonist	Hexamethonium concentration (μM)	Λ(− 80)	τ (sec)
Carbachol (50 μM)	5	2·56	1·44	Carbachol (25 μM)	1	2·32	0·94
Carbachol (ionophoretic)	5	2·40	2·21		5	5·00	0·75
ACh (ionophoretic)	5	2·17	1·52	Carbachol (25 μM)	1	1·91	1·08
ACh (iontophoretic)	5	2·42	2·77		5	4·88	0·69
Carbachol (30 μM)	1	1·39	4·15	Carbachol (50 μM)	1	1·93	1·47
	5	2·67	2·20	Carbachol (40 μM)	2	6·49	1·04
	10	5·33	1·20				

a single continuous open period. As long as the fraction of open channels remains low, such a phenomenon produces no change in steady-state current, although the effects of the blocking agent are detectable by various kinetic techniques. In the record of Fig. 8 it is noticeable that 10 μM-decamethonium reduces the apparent amplitude of the noise (presumably because it is shifted to higher frequencies) even though the current at − 80 mV is only marginally reduced. The characteristics of the slow relaxations recorded in the presence of tubucurarine and hexamethonium are summarized in Table 4.

Non-voltage-dependent antagonists (such as trimetaphan, Fig. 9) did not give rise to any discernible slow relaxations. Mecamylamine, as already mentioned, varied unpredictably in the degree of voltage dependence that it showed; in those instances where the steady-state block showed marked voltage dependence we usually observed slow outward relaxations following a hyperpolarizing jump with the time constants of about 1–3 sec, but these were not analysed in detail. The presence of a tertiary nitrogen in mecamylamine raises the possibility of intracellular accumulation of the drug which may have been a major complicating factor in these experiments and may account for the very slow reversibility of this compound.

The effect of agonist concentration on the slow relaxation

Eqn. (9) predicts for the channel-block hypothesis that increasing the agonist concentration (i.e. increasing β') ought to speed up the slow relaxation. We did not test this systematically, but in one experiment with decamethonium (50 μM) we found that increasing the ionophoretic pulse of carbachol so as to increase the current at -50 mV fourfold increased the rate constant of the slow relaxation roughly twofold, which is qualitatively consistent with the channel-block hypothesis.

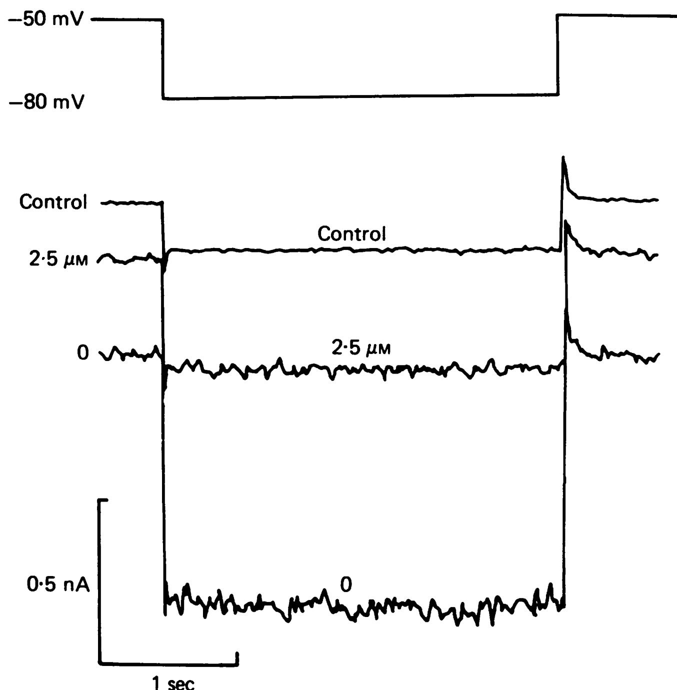


Fig. 9. Response to hyperpolarizing step recorded in the presence of trimetaphan 2·5 μM. The records are arranged as in Fig. 6*A*. Carbachol (25 μM) was applied in the bathing solution. As expected for a non-voltage-dependent antagonist, no slow relaxation occurred in the presence of trimetaphan.

An unexpected effect of tubocurarine

While analysing the effects of tubocurarine on the responses to *ionophoretically* applied agonists, we noticed that during the rising phase of the response the current was actually greater in the presence of tubocurarine than in the control, though the plateau level was less. This effect was observed at all membrane potentials tested. In Fig. 10 the effect of tubocurarine on responses to ionophoretically applied carbachol are shown at two membrane potentials. The equilibrium blocking effect of tubocurarine at -40 mV is very small, so the plateau current is very little reduced. The early part of the response is, however, considerably enhanced. At -90 mV, when the equilibrium blocking effect is substantial, the two curves cross as the early enhancement gives way to the later inhibition.

The ratio between the early potentiation and the equilibrium blocking effect can also be altered by changing the agonist concentration. In the experiment in Fig. 11 carbachol was applied with alternating large and small ionophoretic currents. When tubocurarine was added, its only effect on the small response was an increased rate of

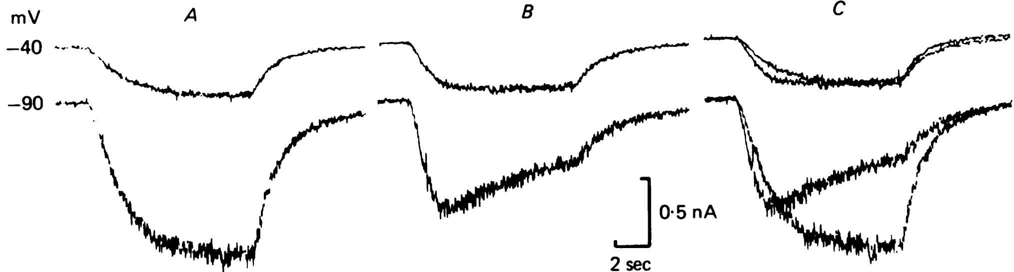


Fig. 10. Early potentiation of the response to ionophoretically applied carbachol by tubocurarine. *A*: control responses to 8 sec carbachol pulses at −40 and −90 mV. *B*: responses recorded in the presence of 5 μM tubocurarine. *C*: *A* and *B* superimposed to show early enhancement of response (similar at both membrane potentials) with later inhibition (more pronounced at −90 mV than at −40 mV).

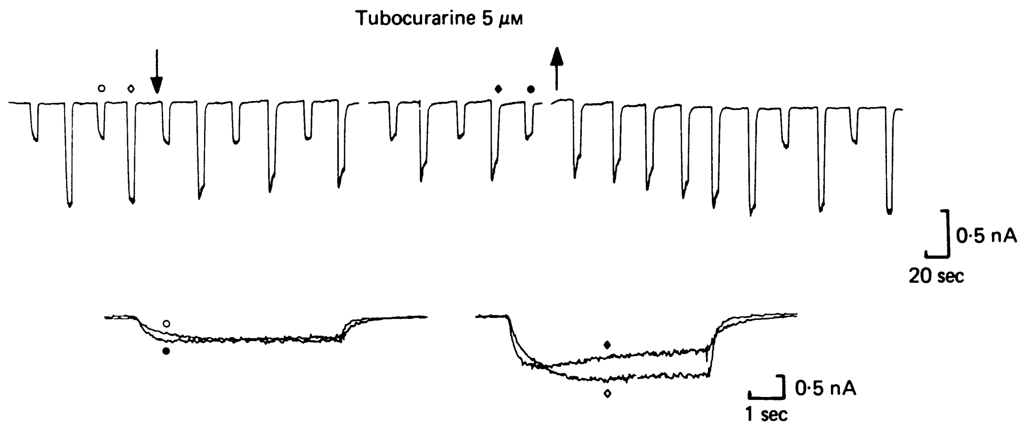


Fig. 11. Effect of tubocurarine on the response to large and small ionophoretic pulses of carbachol. The upper record is a chart recording of the current produced by alternate large and small carbachol pulses of 5 sec duration applied every 30 sec. Tubocurarine was added to the bathing solution at the first arrow and washed out at the second arrow. The break in the trace represents a gap of about 5 min during which the responses remained constant. The lower records show superimposed responses on an expanded time scale. The early potentiation is about the same for the large and small pulses, but the later inhibition is more marked with the large pulse. The effect of increasing the carbachol concentration is thus similar to that of hyperpolarizing the membrane (Fig. 10).

rise; the steady-state level was not changed. On the other hand, the large response showed an early increase and a later decrease below the control level. The differences produced by increasing the agonist mimicked closely the effects of hyperpolarization illustrated in Fig. 10. Since, as already discussed, the steady-state blocking effect of tubocurarine increases with both hyperpolarization and increasing agonist con-

centration, this result implies that the early potentiating effect shows less dependence on these two variables.

A consequence of the effect just described, and of the slow development of the tubocurarine block, is that tubocurarine can actually lead to a marked *potentiation* of the response to a short pulse of carbachol or ACh. This is illustrated in Fig. 12. The response to a brief carbachol pulse was potentiated to the same extent at all membrane potentials tested by addition of tubocurarine at 5 μM. With a higher

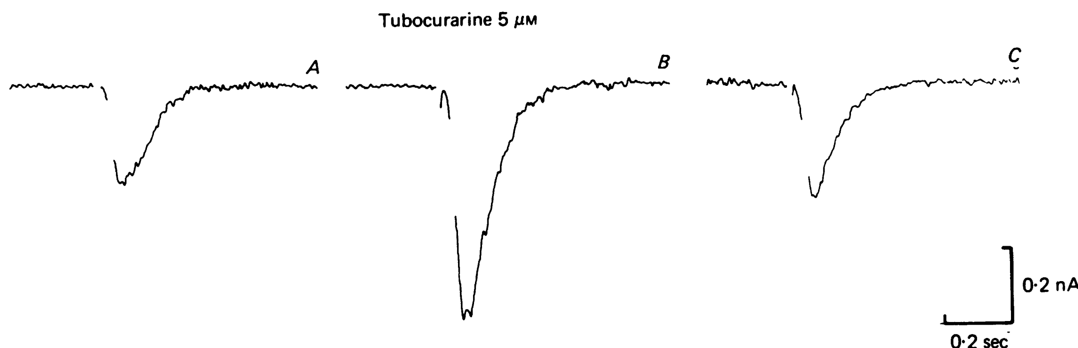


Fig. 12. Potentiating effect of 5 μM-tubocurarine on brief ionophoretic responses to carbachol. *A*, initial control. *B*, in presence of tubocurarine. *C*, after recovery. The artefacts occurring at the beginning and end of the ionophoretic pulse have been blanked out.

tubocurarine concentration (10 μM) the typical voltage-dependent blocking effect was seen, even with short carbachol pulses, though it was presumably offset by the potentiating effect. The two effects seemed to appear at different rates, since addition of 10 μM-tubocurarine increased the response for the first 2–3 min, and later decreased it.

The potentiating effect observed with tubocurarine was not observed with the other antagonists tested. With trimetaphan and mecamylamine the current during the rising phase was reduced in equal proportion to the equilibrium current; thus the value of Λ measured early in the response, e.g. after 0·5 sec, was not significantly different from the value measured in steady state. With hexamethonium and decamethonium, a similar result was obtained in most experiments, but sometimes Λ was larger at the beginning of the response, and then decreased, which is the opposite of what was seen with tubocurarine. This effect was small, irregular, and was not further investigated.

One possible explanation of the potentiating effect of tubocurarine is that the drug blocks sites of loss which normally bind agonist molecules and slow down the rate of rise of agonist concentration at the receptors. Such sites of loss would retard the rising phase of the response but would not affect the steady-state agonist concentration. It is known that sympathetic ganglia contain α-bungarotoxin binding sites (Greene, 1976; Fumagalli, de Renzis & Miani, 1976) which appear to be unrelated to ACh receptors (Brown & Fumagalli, 1977; Carbonetto, Fambrough & Muller, 1978), and it seemed possible that these sites might be acting as sites of loss

for agonists. However, application of α-bungarotoxin (200 nM for 90 min) produced no potentiation of the response to carbachol, nor did it affect the potentiation produced by 5 μM-tubocurarine tested subsequently. There thus appears to be no relation between the bungarotoxin binding sites and the observed potentiation by tubocurarine. It is however well known (see Chagas, 1959) that tubocurarine binds non-specifically to numerous tissues, and it is possible that competition with car-carbachol at such sites is responsible for the potentiation. In calculating the degree of block at equilibrium for tubocurarine we have ignored this potentiating effect, on the assumption that it occurs only transiently, affecting the rate of rise of the response rather than its equilibrium level, but so far there is no direct evidence to support this assumption.

One aim of the experiments with ionophoretically applied carbachol was to see whether a 'concentration jump' could produce the same type of relaxation as a voltage jump in the presence of tubocurarine, and to compare the rate constant for the slow relaxation in each type of experiment. Though the records shown in Figs. 10 and 11 agree qualitatively with the expected results, the presence of the potentiating effect makes the interpretation difficult, since the relaxation could result partly or entirely from the decline of the potentiation. It will be necessary to clarify the mechanism of the potentiation, and if possible find a way of eliminating it, in order to determine whether or not the relaxation seen in Figs. 10 and 11 is associated with channel block.

DISCUSSION

Our analysis of the steady-state currents in the presence of various ganglionic blocking agents leads to a clear distinction between two types of antagonists. In the first type the effect of the antagonist is increased both by hyperpolarization and by an increase of the agonist concentration; in the second type neither of these two effects occurs. These two groups are likely to correspond to the situation in which the antagonist combines preferentially with (*a*) the open state of the channel–receptor complex, R* and (*b*) the closed state of the complex, R.

The predictions of a sequential model in which the antagonist binds to the open state of the channel–receptor complex (e.g. to the channels) are in many respects simpler to evaluate than the predictions of a scheme in which the antagonist binds to R. In particular, in the case of a scheme where the antagonist enters the open channel and blocks it, it is reasonable to postulate that the reaction between R* and B is bimolecular; and no hypothesis is required about the mechanism by which the agonist opens the channel, or on the form of the relationship between the rate of channel opening, β', and the agonist concentration. On the other hand, the effect of an antagonist combining selectively with R can be produced by a number of different mechanisms, and our results do not allow us to distinguish between competition for the ACh binding site and binding to a separate site on the receptor or channel. The conventional view of the action of non-depolarizing ganglion blocking drugs is that they compete directly with ACh for the receptors but the evidence for this is by no means conclusive (van Rossum, 1962). The main part of this discussion will be concerned with the analysis of the open-state block.

Estimation of rate constants for the channel-block model

Our kinetic analysis is too incomplete for a full analysis of the rate constants in scheme (4) for the different antagonists. In particular, experimental determinations of the fast relaxation kinetics are missing. We can nevertheless put approximate limits on some of the rate constants, assuming that tubocurarine, hexamethonium and decamethonium bind exclusively to R*.

If the fraction of channels opened by the agonist is small (i.e. $\beta' \ll \alpha$), eqn. (6) can be rearranged to give

$$\Lambda = \frac{\beta'}{\alpha}\frac{X_B}{K_B^*}+1. \tag{10}$$

Combining this with eqn. (8) gives

$$\tau_s^{-1} = k_{-B}^*.\Lambda.\frac{\alpha}{k_{+B}^* X_B+\alpha}. \tag{11}$$

Thus, if $k_{+B}^* X_B$ is small with respect to α, we can calculate k_{-B}^* from Λ and τ_s^{-1} which are both measured experimentally. From the values obtained in the experiments shown in Figs. 5 and 6 for 1 μM-antagonist at -80 mV membrane potential, we obtain $k_{-B}^* = 0{\cdot}17$ sec^{-1} for tubocurarine, and 0·48 sec^{-1} for hexamethonium. If $k_{+B}^* X_B$ is not much smaller than α this calculation will underestimate k_{-B}^*, so these values must be regarded as lower limits. The test of whether the approximation is realistic rests in measuring whether or not the estimate of $k_{-B}^* \simeq \tau_s^{-1}/\Lambda$ varies with antagonist concentration, X_B. In the experiment with tubocurarine in Table 4 τ_s^{-1}/Λ did not change with tubocurarine concentration in the range 1–10 μM, so the estimate of k_{-B}^* is probably accurate.

With hexamethonium (Table 4) τ_s^{-1}/Λ appeared to be somewhat smaller at the higher antagonist concentration, so the estimate of k_{-B}^* is probably too small, but more experiments will be needed to determine it accurately.

For decamethonium, the inverse dependence of τ_s^{-1} on antagonist concentration (Fig. 8) implies (from equation (9)) that $\beta'/k_{-B}^* < 1$. Eqns. (9) and (10) can be combined to give

$$\tau_s^{-1} = \frac{\Lambda\beta'}{\Lambda-1+\dfrac{\beta'}{k_{-B}^*}}. \tag{12}$$

If we assume that β'/k_{-B}^* is negligible, we can estimate β' from

$$\beta' \simeq \tau_s^{-1}\frac{(\Lambda-1)}{\Lambda}. \tag{13}$$

For the experiment of Fig. 8 at 30 μM-carbachol this gives $\beta' \simeq 0{\cdot}85$ sec^{-1}. Because of the approximation used, this is a lower limit.

Noise analysis showed the channel lifetime for carbachol at -50 mV to be 15 msec, corresponding to a rate constant for channel closing, α, of 66 sec^{-1}. If the voltage dependence of α is the same as at the end-plate (Magleby & Stevens, 1972), the value of α at -80 mV would be 52 sec^{-1}. Thus for 30 μM-carbachol at -80 mV the fraction of channels opened $\beta'/(\beta'+\alpha)$ is about 0·02.

As mentioned in the results section the average response in our experiments corresponded to the opening of about 1000 channels. Thus, the total number of channels per cell is likely to be of the order of 50,000.

From the estimates of β' and α and the experimental measurements of Λ we can use eqn. (10) to obtain approximate values for the equilibrium constant K_B^* for binding to the open channel. From the experiments shown in Figs. 6–8 we obtain the values given in Table 5.

TABLE 5. Approximate values for the parameters describing association of antagonists with the open state of the channel–receptor complex, calculated from the experiments shown in Figs. 6–8, for −80 mV membrane potential

Antagonist	K^*_B (M)	k^*_{+B} (M^{-1} sec^{-1})	k^*_{-B} (sec^{-1})
Tubocurarine	$3{\cdot}4 \times 10^{-8}$	5×10^{6}	0·17
Hexamethonium	$2{\cdot}2 \times 10^{-8}$	$2{\cdot}2 \times 10^{7}$	0·48
Decamethonium	6×10^{-7}	—	—

Comparison with other systems

In the case of tubocurarine, the calculated value of k^*_{-B} is in the same range as the values calculated by Ascher, Marty & Neild (1978*b*) in the case of *Aplysia* neurones (0·1 sec^{-1} at −80 mV and 12 °C) and Colquhoun, Dreyer & Sheridan (1979) for the frog neuromuscular junction (1·0 sec^{-1} at −80 mV and 8 °C).

The value for k^*_{-B} for hexamethonium also agrees with that calculated for *Aplysia* neurones (0·5 sec^{-1}) at −80 mV and 12 °C.

The equilibrium constants for blocking of the open channel correspond to affinities 3–6 times greater than the values calculated for tubocurarine and hexamethonium on *Aplysia* neruones, and our estimate for tubocurarine corresponds to an affinity roughly three times that for channel block by tubocurarine at the frog end-plate (Colquhoun *et al.* 1978), though the measurements were made at somewhat different membrane potentials.

In the case of decamethonium, the slow inward relaxation observed after a hyperpolarizing jump in rat ganglion (Figs. 5 and 8) resembles the relaxation described in frog muscle by Adams & Sakmann (1978) but contrasts with the slow outward relaxation reported in *Aplysia* by Ascher, Marty & Neild (1978*b*). One possibility to account for this difference is that k^*_{-B} is comparatively smaller in *Aplysia* than in the rat. Another possibility is that k^*_{-B} has similar values in the two systems, but that in the *Aplysia* experiments β' was higher. As discussed by Marty (1978) for a fast dissociating antagonist, an increase in the agonist concentration (and therefore of β') can lead from a slow inward relaxation to a slow outward one.

Combined block of open and closed channels

The effect on Λ of increasing the agonist concentration (Table 2) showed that there was a discrepancy between the effect of increased agonist concentration on the blocking effect, expressed as y_Λ and on the control current, expressed as y_I. For a drug that acts only on open channels these two quantities should be identical.

With the ionophoretic experiments, the spatial and temporal non-uniformity of the agonist concentration may be responsible for the discrepancy between y_Λ and

y_I, but the existence of a similar discrepancy in the results with bath-applied carbachol suggests that it may be significant. An obvious possibility is that, like tubocurarine at the neuromuscular junction (Colquhoun *et al.* 1979), hexamethonium acts on both open and closed channels. For such a combined effect, provided that the fraction of channels opened during the response is small, the effect of agonist concentration on the degree of block can, with the same assumptions as in eqns. (1) and (3), be expressed as

$$y_\Lambda = \frac{1 + y_I \dfrac{\beta_2' K_B}{\alpha K_B^*}}{1 + \dfrac{\beta_2' K_B}{\alpha K_B^*}}, \tag{14}$$

where K_B and K_B^* are the equilibrium constants for binding of the blocking agent to R and R*, respectively. Thus

$$\frac{K_B}{K_B^*} = \frac{\alpha(y_\Lambda - 1)}{\beta_2'(y_I - y_\Lambda)}. \tag{15}$$

From results on the slow relaxation in the presence of decamethonium we calculate that $\alpha/\beta_2' \approx 50$. Applying eqn. (15) to the results for hexamethonium given in Table 2 gives $K_B/K_B^* \approx 70$ at -80 mV. The affinity of hexamethonium for the open channel, R*, is thus much greater than its affinity for the closed state, R. A relaxation of the assumptions stated for eqn. (1) will clearly affect these numerical estimates, but would not be expected to change the general conclusion.

The mean value of $\Lambda - 1$ for 2 μM-hexamethonium and 25 μM-carbachol at -80 mV was 2·0. For a mixed block

$$\Lambda - 1 = \frac{X_B}{K_B}\left[1 + \left(\frac{\beta' K_B}{\alpha K^*_B}\right)\right]. \tag{16}$$

The values of K_B and K^*_B for hexamethonium at -80 mV calculated from eqns. (15) and (16) are 4·9 and 0·07 μM, respectively. These can only be regarded as rough estimates because of the considerable scatter in the results of Table 2. If the affinity for the closed channel is ignored (K_B assumed to be very large), the estimated K^*_B value for hexamethonium calculated from eqn. (16) becomes 0·04 μM. Thus ignoring the closed channel block (as in the analysis of Table 5) probably leads to some underestimate of K^*_B. Without more accurate estimates of the relative affinities of the different blocking agents for R and R* however, it seems unjustified to correct for this error.

Voltage dependence of the blocking effect

For the three voltage-dependent antagonists the ratio $\Lambda(-80) - 1 : \Lambda(-50) - 1$ was close to 3 (Table 1). This implies a similar degree of voltage dependence to that calculated for channel blocking drugs in *Aplysia* neurones and on the end-plate. It is interesting that the difference between tubocurarine and hexamethonium found in *Aplysia* was not present in our studies. This may be related to the fact that at the higher pH of artificial sea water the tubocurarine molecule bears a net positive charge of less than two, whereas it will bear two positive charges in Krebs solution.

For tubocurarine and decamethonium we have only measured the agonist con-

centration dependence of Λ by the ionophoretic method, which appears to be quantitatively unreliable. Furthermore, with tubocurarine, calculation of Λ may be made complicated by the potentiating effect. Thus for these drugs estimation of the relative importance of open- and closed-state block is uncertain. Colquhoun *et al.* (1979) showed that tubocurarine has roughly equal affinity for R^* and R at -70 mV. This resulted in voltage-dependent block and a deviation from conventional competitive antagonism only at fairly high degrees of block (i.e. at dose ratios exceeding 10). In our experiments these effects appeared as soon as any block was produced; we conclude that with tubocurarine block of R^* compared with R is much more pronounced at the ganglion than at the end-plate, but we cannot exclude a small degree of competitive block at the ganglion.

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