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DIFFERENT TYPES OF BLOCKADE OF CRUSTACEAN ACETYLCHOLINE-INDUCED CURRENTS

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SUMMARY

1. The voltage dependence, concentration dependence, and agonist dependence of blocking and unblocking produced by anticholinergic agents on the ionophoretically activated cholinergic currents of the lobster gastric mill 1 (g.m.1) muscle were examined. Although the ionophoretic technique provides only qualitative information as to blocking mechanisms it is useful in revealing slow components of the blocking action of some drugs. At least two qualitatively different types of voltage-dependent block of the crustacean cholinergic currents were observed.

2. For pempidine, mecamylamine and decamethonium (also chlorisondamine: Lingle, 1983), a slowly developing voltage-dependent block was produced that led to the formation of a stable–blocked state. Recovery from this stable–blocked state is largely dependent on subsequent application of agonist.

3. In contrast, recovery from the voltage-dependent block produced by QX-222, atropine, procaine and curare either proceeds independently of agonist application or occurs too rapidly to be observed by the present methods.

4. Blockade by hexamethonium reveals anomalous voltage dependence, being enhanced over some voltages and relieved with additional hyperpolarization.

5. Blockade by trimetaphan is largely independent of membrane potential except at higher concentrations.

INTRODUCTION

Certain muscles of the decapod foregut receive a cholinergic excitatory motor innervation from identifiable cholinergic motoneurones (Marder, 1976). The acetylcholine-activated potentials found on one of these muscles, the gastric mill 1 (g.m.1), are sensitive to a variety of ganglionic nicotinic blockers and, in terms of sensitivity to agonists and insensitivity to α-bungarotoxin appear similar to ganglionic nicotinic responses (Marder & Paupardin-Tritsch, 1980). In a recent paper (Lingle, 1983), the mechanism by which one such ganglionic blocker, chlorisondamine, reduces cholinergic currents on the lobster g.m.1 muscle was examined. Evidence was presented indicating that chlorisondamine interacts primarily with the opened ACh channel, reduces cholinergic currents following a diffusionally controlled binding

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reaction and, subsequently, results in the formation of an apparent closed–blocked state that requires re-exposure to agonist for unblocking to occur. The following scheme that accounts for both the kinetic determinations and the qualitative steady-state observations was proposed:

$$\underset{\text{closed}}{\text{AR}} \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} \underset{\text{open}}{\text{AR}^*} \underset{b^*}{\overset{f^*C}{\rightleftharpoons}} \underset{\text{open–blocked}}{\text{AR}^*\text{C}} \underset{k_o'}{\overset{k_c}{\rightleftharpoons}} \underset{\text{closed–blocked}}{\text{ARC}} \qquad (1)$$

where α and β' have the usual meaning, f^*C and b^* correspond to the drug-binding reactions, and k_c and k_o' correspond to the closing and opening respectively of the open–blocked channel.

The formation of the closed–blocked state in the presence of chlorisondamine is somewhat novel among blocking reactions of transmitter-gated channels examined to date. Thus, one aim of the present study was to examine qualitatively the mechanisms of action of other cholinergic blockers on the lobster g.m.1 preparation in order to determine whether the occurrence of the closed–blocked state is characteristic of only some or all blockers. In addition, since previous studies had indicated similarities between the crustacean cholinergic response and the nicotonic ganglionic response of vertebrates, this study also sought to provide an additional comparison between these preparations.

Intracellular pharmacological studies of nicotinic ganglionic receptors have been minimal (Ascher, Large & Rang, 1979; Rang, 1982; Selyanko, Derkach & Skok, 1981). To a large extent the mechanisms of action of ganglionic blockers are unexamined. For example, three compounds, chlorisondamine, mecamylamine and pempidine, produce antihypertensive effects through nicotinic ganglionic blockade characterized by unusually long durations of action (Volle & Koelle, 1975). Of these three, only mecamylamine has been examined by intracellular methods on a ganglionic preparation and found to produce a relatively voltage-independent block of ACh-activated currents that is only slowly reversible (Ascher *et al.* 1979). The cause of this lack of reversibility is unknown.

The results presented here indicate that of a series of cholinergic blockers, mecamylamine, pempidine, and to some extent decamethonium, share with chlorisondamine the somewhat unique ability to produce the blocked–closed state of the ACh channel. In contrast, other agents including curare, QX-222, procaine, and trimetaphan produced a block that can be accounted for by other mechanisms. The properties of blockade by hexamethonium may represent a limiting case of the first group of blockers.

METHODS

The materials and procedures used in this paper are similar to those used previously (Lingle, 1983). Briefly, spiny lobsters, *Panulirus interruptus*, were obtained from Pacific Biomarine, Venice, CA and maintained in 12–14 °C Instant Ocean Aquaria. The g.m.1 muscle was isolated from the animal by dissection through the dorsal carapace. The muscle and attached nerve were placed in a 1–3 ml Sylgard-lined chamber and continuously superfused with cooled (10–14 °C) physiological *Panulirus* saline. The saline composition was (mM): NaCl, 479; KCl, 12·7; $CaCl_2$, 13·7; $MgSO_4$, 10; Na_2SO_4, 3·9; maleic acid, 3·6; Tris base, 8·3. The pH was 7·3–7·5.

Tubocurarine chloride, decamethonium chloride, hexamethonium bromide, mecamylamine, procaine, atropine, acetylcholine and carbachol were obtained from Sigma. Chlorisondamine was the gift of Ciba-Geigy, trimetaphan camsylat was the gift of Hoffman-LaRoche, and pempidine was provided by May & Baker. Drugs were applied to the preparation through the superfusion system (2–10 ml/min).

Muscle fibres were voltage-clamped with a Dagan two-electrode clamping system with 5–15 MΩ K acetate/KCl electrodes. Agonists were applied by current pulses through an ionophoretic electrode. Even for responses of 200 nA, more than 95% of the currents were clamped. To maintain steady ionophoretic responses, relatively slow rise time responses of several hundred milliseconds were frequently used, although, even for small responses, rise times were at least 100–200 ms. In order to minimize problems that might be associated with slow changes in ionophoretic electrode position or desensitization, the standard protocol was to obtain agonist-induced responses at a given holding potential both before and after a series of responses at a test potential. Thus, if necessary, responses could be normalized to the responses obtained at the holding potential.

RESULTS

The experiments described here qualitatively examine the voltage- and time-dependence of drug-induced blockade of ionophoretically activated cholinergic currents on the g.m.1 muscle of the spiny lobster, *Panulirus interruptus*. In addition, the requirement for receptor activation in the blocking and unblocking process is examined for each blocking molecule. Two principal groups of voltage-dependent blocking agents are distinguishable by the above parameters. One group, which includes QX-222, curare, atropine and procaine, produces a voltage-dependent block from which unblocking occurs in the absence of agonist application. The second group, which includes chlorisondamine (Lingle, 1983), mecamylamine, pempidine and decamethonium, manifests a slowly developing voltage-dependent blockade that requires agonist application for unblocking to occur. The voltage-dependent blocking action of hexamethonium is not clearly similar to either group. The blocking action of trimetaphan is largely independent of voltage, except at higher concentrations and very hyperpolarized potentials.

A more detailed description of the properties of the cholinergic response of the g.m.1 preparation has been presented elsewhere (Lingle, 1983; Lingle & Auerbach, 1983). Little information is available concerning the density and distribution of the cholinergic receptors. However, ionophoretic responses can be elicited at any position on the muscle fibres. The properties of the ionophoretic responses suggest that the actual density of cholinergic receptors is quite low and that measureable current flow results from the activation of receptors over a substantial area of membrane. Thus, the magnitude of the cholinergic responses activated by ionophoresis may reflect the size of the area of membrane affected by agonist rather than a specific concentration of agonist.

Compounds for which agonist application is not required for unblocking QX-222, atropine and procaine

The effect of QX-222 on carbachol-induced currents on the g.m.1. muscle is shown in Fig. 1*A*. From a holding potential of −60 mV, the membrane potential was stepped to more hyperpolarized levels and sequential pulses of carbachol applied. QX-222 produces a block of the carbachol currents that is more pronounced at

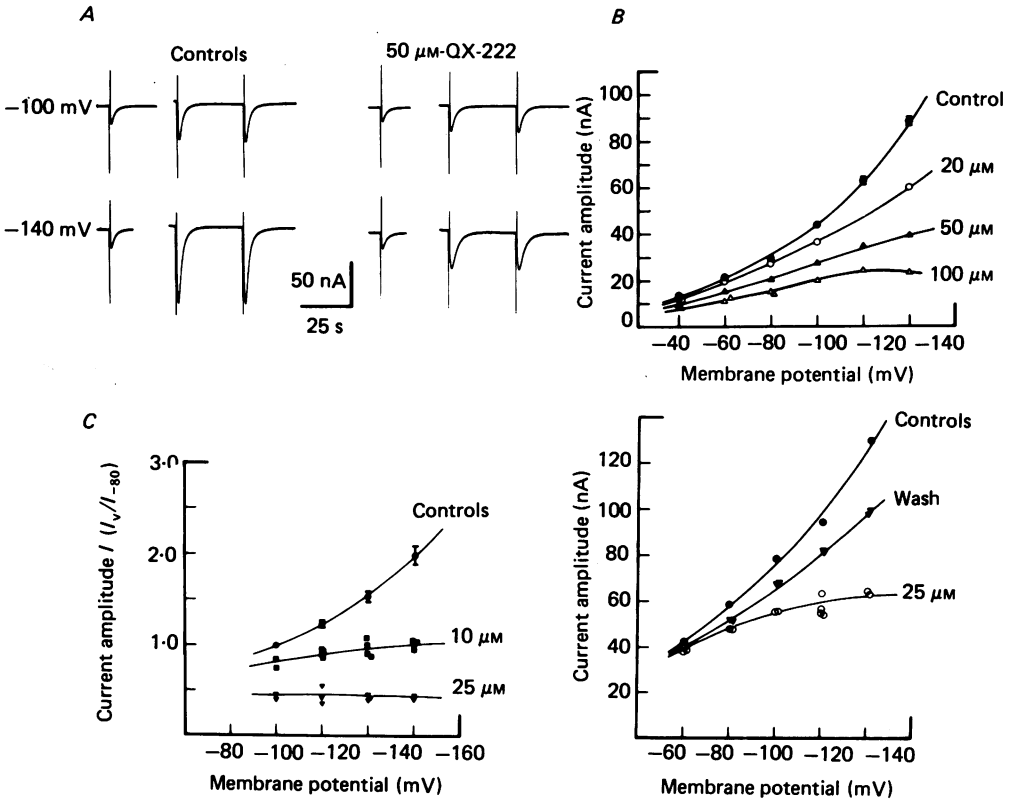

Fig. 1. *A*, the blocking action of QX-222 on cholinergic currents of the g.m.1 muscle. Ionophoretic pulses of carbachol were applied to a voltage-clamped region of the g.m.1 muscle. On the left in normal saline (with 20 mM-$MnCl_2$) control responses at a holding potential of −60 mV were followed by a hyperpolarizing voltage step to the indicated membrane potential. On the right, the effect of 50 μM-QX-222 on the carbachol-induced currents is shown. Horizontal calibration: 25 s. Vertical calibration: 50 nA. Temperature: 9·8 °C. *B*, the voltage dependence of blockade by QX-222. From an experiment similar to that in *A*, responses to carbachol application over a range of membrane potentials at three concentrations of QX-222 are shown. The filled circles are control and wash responses with S.E.M. of mean either shown by the error bars or less than the size of the point. Open circles are 20 μM-QX-222, filled triangles are 50 μM-QX-222, and open triangles are 100 μM-QX-222. Vertical axis is carbachol-induced current in nA and the horizontal axis is membrane potential. *C*, effect of atropine and procaine on ACh-activated currents. On the left, amplitudes of ACh-activated currents are plotted as a function of membrane potential for two concentrations of atropine. Amplitudes of cholinergic currents were normalized to responses obtained at −80 mV just prior to and after a membrane potential step. Circles indicate control responses with +/− S.D.; squares were obtained in 10 μM-atropine; triangles were obtained in 25 μM-atropine. Two applications of both drug concentrations were used to generate this Figure. Temperature: 12·5 °C. On the right, amplitudes of carbachol-activated currents are plotted as a function of membrane potential in the presence and absence of 25 μM-procaine. Filled circles indicate responses in normal saline (with 20 mM-$MnCl_2$); open circles, in procaine; and triangles, after 15 min of wash-out of procaine. Responses following recovery from blockade by procaine consistently failed to reach pre-procaine values. Temperature: 12·5 °C.

hyperpolarized levels. No indication of interaction between sequential pulses is seen, even at pulse separations close to 1 s. Fig. 1*B* summarizes the effects of a range of QX-222 concentrations and indicates that, at −40 mV, 50% blockade of the carbachol response occurs at a concentration greater than 100 μM while at −140 mV a concentration of less than 50 μM is sufficient for 50% blockade.

The lack of change in amplitude of the carbachol-induced currents during a sequence of responses at a new potential indicates that the blocking reactions and opening and closing processes of the cholinergic channels are essentially complete before or during the time course of these slow ionophoretic responses. This is further supported by the observation that following wash-out of QX-222 without application of agonist, the initial response following wash-out shows complete recovery. Similarly, following introduction of QX-222 to the preparation without agonist application, block of the cholinergic currents is fully developed with the first agonist response.

Atropine also produced a voltage-dependent blockage of cholinergic ionophoretic currents on the g.m.1 muscle. During trains of pulses, blockade was fully developed during the first agonist pulse. Wash-out of atropine without agonist application produced full recovery with the first subsequent pulse of agonist. The voltage- and concentration-dependence of the action of atropine is illustrated in Fig. 1*C*. 50% block at −140 mV occurred at about 10^{-5} M-atropine and, at −80 mV, about 2×10^{-5} M.

The action of procaine, although similar to that of the above compounds, displays some complicating aspects. At concentrations above 1 μM, procaine produces a substantial increase in amplitude of responses to ACh. The absence of such an enhancement of carbachol-induced responses suggests that procaine is a potent inhibitor of the crustacean acetylcholinesterase. With carbachol as agonist, procaine produces a voltage-dependent reduction in the amplitude of ionophoretically activated currents. This reduction is complete during the first response in a train of agonist pulses after a voltage step or upon initial introduction of procaine into the muscle bath. Following wash-out of procaine the initial response to agonist is identical in amplitude to subsequent responses. However, in contrast to the above blockers, these responses remain reduced relative to the amplitude of control responses that occurred prior to procaine application (Fig. 1*C*). Agonist application or membrane potential steps do not facilitate recovery from the residual blockade that persists following procaine wash-out.

Trimetaphan

Blockade by trimetaphan was not very voltage-dependent (Fig. 2). Only at higher concentrations at membrane potentials between −100 and −140 mV did a trend toward increases in block with hyperpolarization become apparent. As illustrated in Fig. 2*A*, blockade by trimetaphan was essentially complete prior to or during the time course of the first response at a given membrane potential. In the presence of trimetaphan, pairs of ionophoretic responses showed no interaction at pulse separations down to about 5 s. Following wash-out of trimetaphan in the absence of agonist application, initial agonist responses exhibited full recovery. A distinguishing feature of the action of trimetaphan is that of the agents for which unblocking was independent of agonist application, trimetaphan was the most potent, with 50%

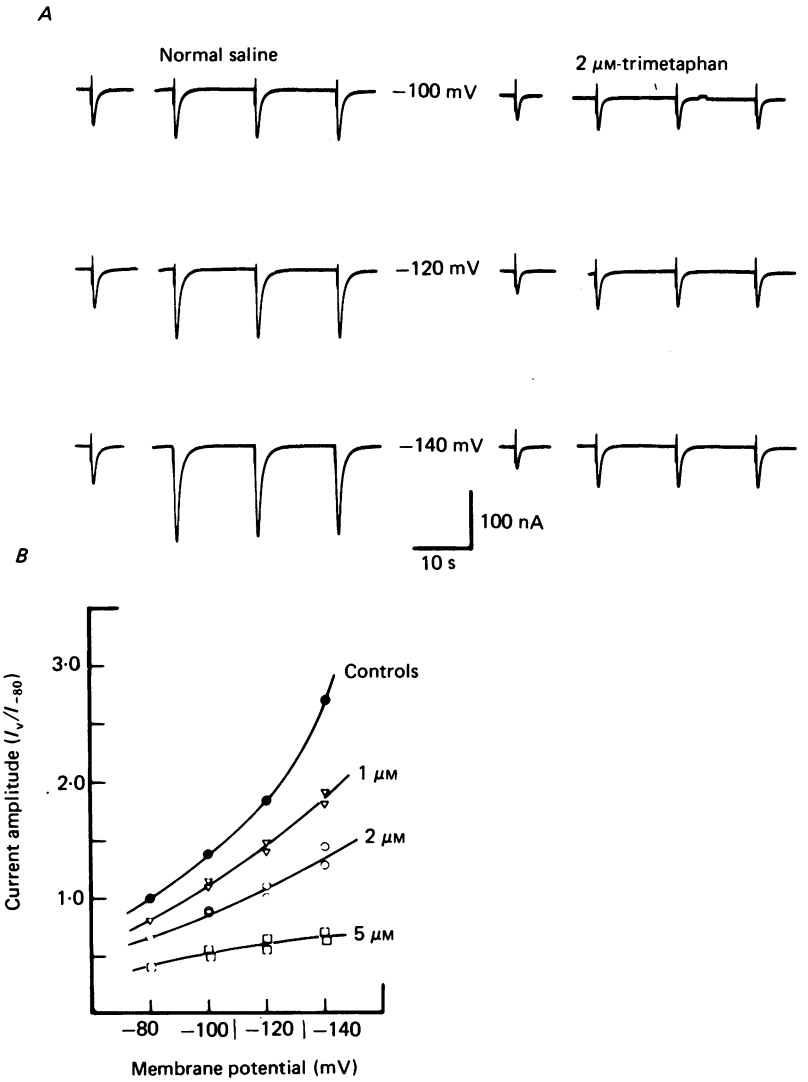

Fig. 2. *A*, blockade of cholinergic currents by trimetaphan. Ionophoretically activated ACh currents were elicited at a voltage-clamped region of a g.m.1 muscle. On the left, control responses at a holding potential of −80 mV with subsequent hyperpolarizing steps to −100, −120 and −140 mV, from top to bottom, are shown. On the right similar responses in the presence of 2 μM-trimetaphan are displayed. Horizontal calibration: 10 s; vertical calibration: 100 nA. Temp: 14·3 °C. *B*, voltage and concentration dependence of trimetaphan blockade. The amplitude of ionophoretically activated cholinergic currents is plotted as a function of membrane potential for three concentrations of trimetaphan. The horizontal axis plots membrane potential and the vertical axis shows the current amplitude normalized to the amplitude at −80 mV just prior to voltage step to a new potential. The filled circles were obtained in normal saline, the open triangles in 1 μM-trimetaphan, the open circles in 2 μM-trimetaphan, and the open squares in 5 μM-trimetaphan. Experiment was in saline containing 20 mM-$MnCl_2$.

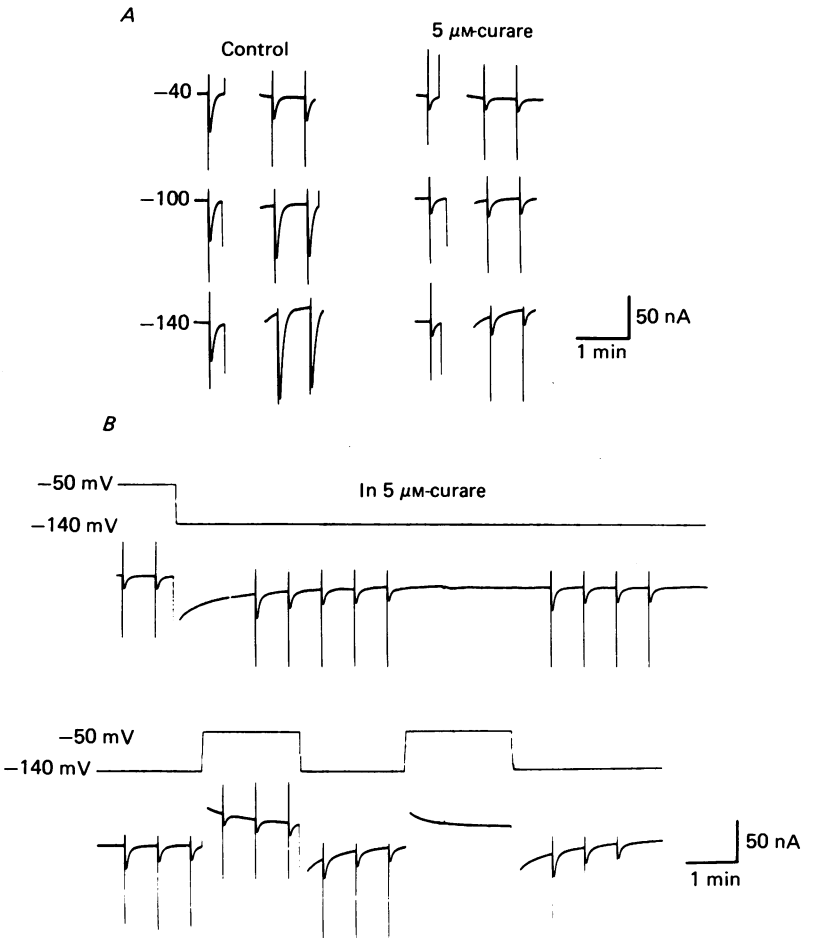

Fig. 3. *A*, effect of curare on ionophoretically activated cholinergic currents. Traces show ACh-activated currents obtained at a holding potential of −80 mV and the first currents following a voltage step to the indicated potential. Test potentials were from top to bottom, −40 mV, −100 mV and −140 mV. The left traces were obtained in normal saline and on the right with 5 μM-tubocurare. Vertical calibration: 50 nA; horizontal calibration: 1 min. Temp: 12·5 °C. *B*, use-dependent blockade by curare. In each pair of traces, the top trace displays membrane potential steps between −50 mV and −140 mV while the bottom trace monitors membrane currents. Acetylcholine was applied ionophoretically during the continual presence of 5 μM-curare. Following a hyperpolarizing voltage step, a slowly developing increase in block is observed. The top trace shows that recovery from that increase in block occurs with cessation of ACh pulses. The bottom trace shows that recovery from block occurs at −50 mV with or without agonist application. Vertical calibration: 50 nA; horizontal calibration: 1 min.

blockade of cholinergic ionophoretic responses at about 2 μM at −140 mV and about 3 μM at −80 mV.

Curare

The block of cholinergic currents produced by curare is illustrated in Fig. 3*A* and is markedly voltage-dependent. In contrast to the above agents, during a train of

agonist responses following a step in membrane potential in curare, a slowly developing component of blockade was observed (Fig. 3*B*). The onset of the slowly developing block did not occur until a sequence of agonist pulses was initiated, and the depth of the block that occurred during a train of pulses was dependent on the frequency of application of agonist pulses. Thus, relief from this slow component of blockade can be produced solely by a period of cessation of agonist application. Similarly, if curare is washed from the preparation, the first response to agonist once curare is removed shows full recovery.

The slow component of the action of curare is qualitatively similar to the slow open channel blocking action of curare on an *Aplysia* excitatory ACh response (Ascher, Marty & Neild, 1978) and the action of curare and quinacrine at the frog neuromuscular junction (Colquhoun, Dreyer & Sheridan, 1979; Adams & Feltz, 1980*a*, *b*). On the g.m.1 muscle in the presence of curare, if the percentage recovery between pairs of ionophoretic responses is measured, an approximately single exponential recovery process is observed. With 10^{-6} M-curare at -120 mV and 12 °C a time constant of about 7 s is obtained. This time constant slows with increases in curare concentration and, when plotted as a function of curare concentration, yields a y-intercept of about 0·2/s. Evaluated according to a simple blocking scheme, this intercept would correspond to b^*, the unbinding rate of drug from its blocking site. This value is similar to unblocking rates observed for curare on *Aplysia* and the frog (Ascher *et al.* 1978; Colquhoun *et al.* 1979). Although the present data are certainly not sufficient to establish a mechanism of curare action on the g.m.1 preparation, the results show that a component of the blocking action of curare requires ACh receptor activation and a fairly slow unbinding of curare from its blocking site, consistent with an action on the opened ACh channel.

Compounds for which recovery from block requires agonist application

In contrast to the action of the agents described above, several blockers were identified for which a portion of the blockade involves the formation of a stable-blocked pool of receptor channels that requires agonist application for unblocking to occur. This is similar to the action of chlorisondamine on this same preparation for which some quantitative information is available that addresses possible mechanisms of blockade (Lingle, 1983).

Pempidine and mecamylamine

Pempidine and mecamylamine share with chlorisondamine a long lasting antihypertensive action (Volle & Koelle, 1975). On the g.m.1 muscle, both pempidine and mecamylamine produce a slowly developing voltage-dependent blockade of the cholinergic response (Fig. 4*A* and *B*). Similar to chlorisondamine, the slowly developing portion of the blockade does not begin until the sequence of agonist pulses is initiated. The rate of development of the blockade is a function of the number of agonist pulses in a blocking sequence and not on the interval between pulses. Following the development of block in either pempidine or mecamylamine during a series of agonist pulses at a given membrane potential, no unblocking occurs at periods over 1 min prior to the next agonist pulse (Fig. 5). However, relief from blockade can be produced by depolarizing voltage steps but only if agonist is applied during the

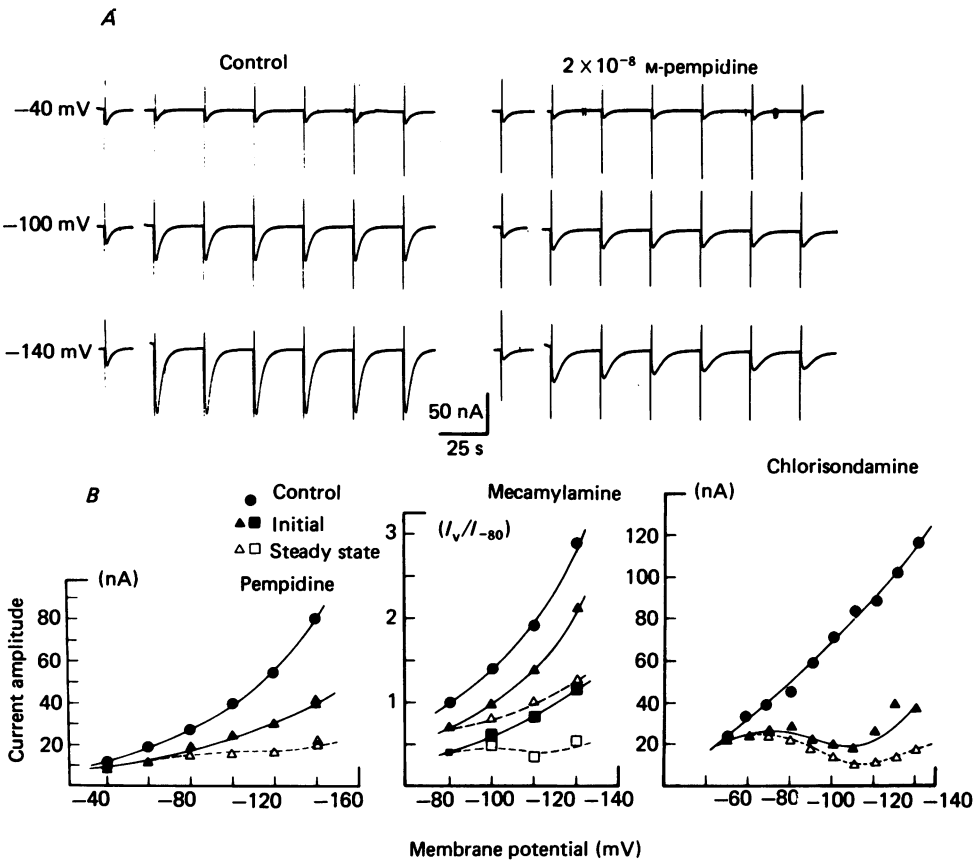

Fig. 4. *A*, effect of pempidine on cholinergic currents. Carbachol-induced currents over a range of membrane potentials in the presence and absence of 20 nM pempidine are illustrated. The membrane potential was held at −60 mV and stepped to the indicated test potentials (from top to bottom, −40 mV, −100 mV and −140 mV). Responses at −60 mV were in all cases continuous with the subsequent responses at the test potential. On the left, responses in normal saline are shown and, on the right, in the presence of pempidine. Vertical calibration: 50 nA; horizontal calibration: 25 s. Temp: 9·5 °C. *B*, voltage dependence of blockade by pempidine, mecamylamine and chlorisondamine. Amplitudes of responses to cholinergic agonist are plotted as a function of membrane potential. Because of the slow blocking action of these agents, both initial and steady-state levels of block following a step-change in membrane potential are shown. On the left: 50 nM-pempidine, carbachol as agonist; in the middle, 20 nM- and 100 nM-mecamylamine, with ACh as agonist and response amplitudes normalized to responses at −80 mV; on the right, 2 μM-chlorisondamine, with carbachol as agonist.

voltage step. Fig. 6 dramatically illustrates the agonist dependence of the unblocking from pempidine. In the case illustrated, the relief from blockade that is observed at the first response after wash-out of pempidine largely reflects the fact that the last response in the presence of pempidine occurred at −80 mV. Thus, at least the predominant portion of the steady-state blockade produced by pempidine results from the formation of a stable–blocked pool of receptor channels that requires agonist application in order for unblocking to occur. Essentially identical results were obtained with mecamylamine.

The present experiments do not allow one to assess directly what portion of the blockade by pempidine or mecamylamine involves exclusively the agonist-dependent formation of a pool of stable–blocked receptor channels. Arguments in support of the idea that most of the blockade results from an agonist-dependent action are presented in the Discussion. This uncertainty does not detract from the primary observation that at least a substantial component of the blockade requires activation of channels and that this portion results in a stable–blocked pool of channels.

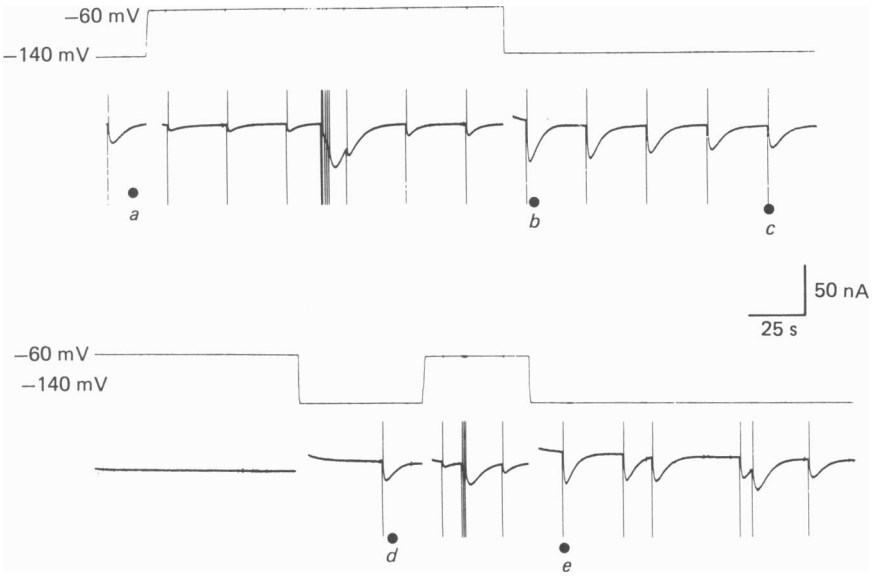

Fig. 5. Role of agonist application and membrane potential in recovery from slowly developing portion of pempidine block. Each pair of traces shows, on the top, membrane potential stepped between −140 mV and −60 mV, and, on the bottom, membrane current. Carbachol-induced currents were obtained in the continual presence of 20 nM-pempidine. At *a*, a steady-state blocked level is present. During the subsequent step to −60 mV, unblocking is observed and, following a burst of ionophoretic pulses, recovery is somewhat enhanced. From *b* to *c*, slow blockade at −140 mV is again allowed to develop. A subsequent step to −60 mV in the absence of agonist application fails to produce any recovery (*d*) from the slowly developing blockade. At *e*, recovery has again been produced by a series of agonist pulses applied at −60 mV. Vertical calibration: 50 nA; horizontal calibration: 25 s.

The voltage dependence of the steady-state blockade produced by pempidine and mecamylamine are presented along with similar results for chlorisondamine in Fig. 4*B*. An interesting feature of these plots, which can be seen most clearly in the case of chlorisondamine, is the tendency towards a relief from the voltage-dependent block at very hyperpolarized potentials. This was also observed for decamethonium. The voltage dependence of blockade by QX-222, procaine, curare, trimetaphan and atropine never demonstrated this trend.

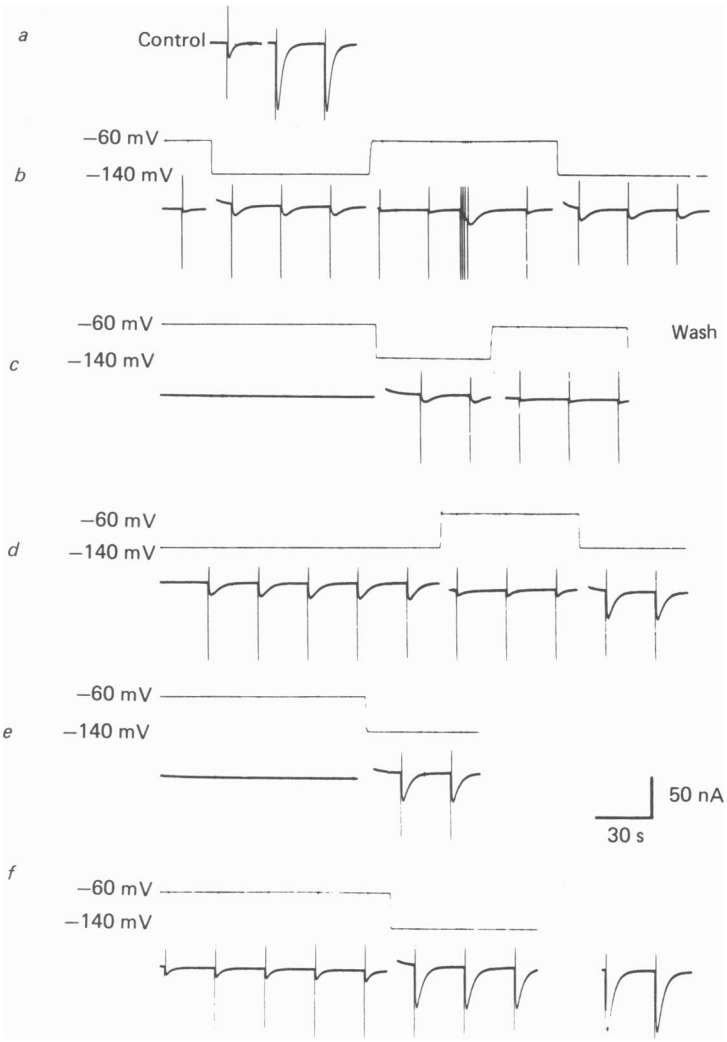

Fig. 6. Effect of drug wash-out and agonist application on recovery from pempidine blockade. Each pair of traces, from *b* to *f*, show, on the top, membrane potential steps between −60 mV to −140 mV and, on the bottom, membrane currents. Records in *b–f* are continuous except for a gap without agonist application between *c* and *d*. In *a*, responses in normal saline at −60 mV and −140 mV are shown. Traces in *b* and *c* are in the presence of 100 nM-pempidine. As in Fig. 5, the effect of agonist application on relief from block can be seen. At end of trace *c*, pempidine was washed from the preparation for 15 min and ionophoretic agonist pulses were suspended, while the membrane potential was held at −140 mV. Beginning at *d*, agonist pulses were again initiated. A gradual recovery in amplitude can be observed in *c–e* which is facilitated by depolarization to −60 mV but only if agonist is applied during the step to −60 mV. The last record in *f* indicates responses following about 30 min wash with agonist application at various membrane potentials. Vertical calibration: 50 nA; horizontal calibration: 30 s.

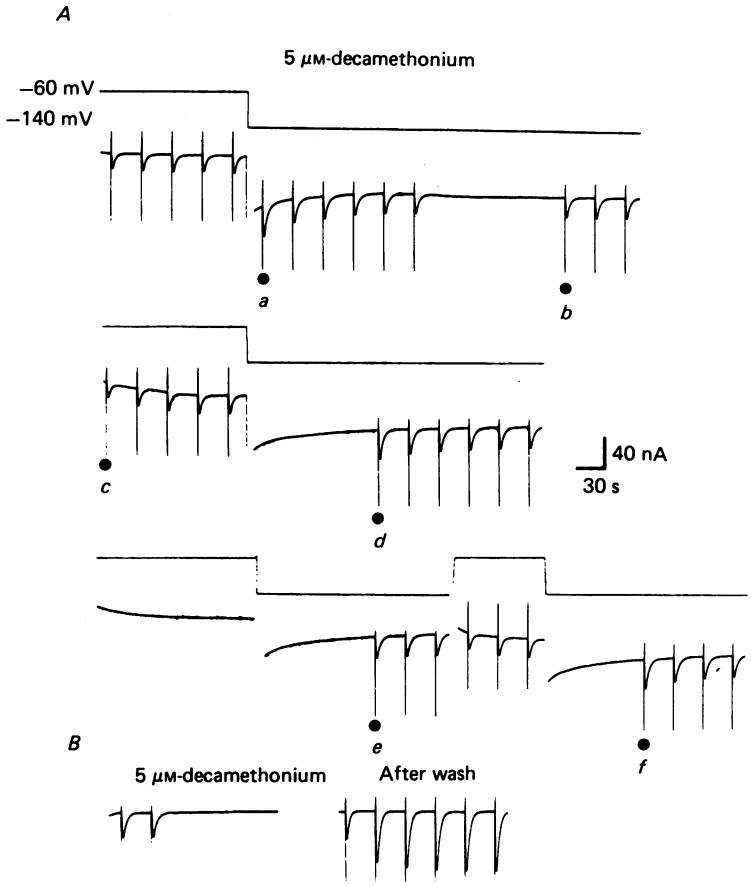

Fig. 7. *A*, evidence for a stable-blocked state induced by decamethonium. Each pair of traces shows membrane potential steps between −60 mV and −140 mV on the top and membrane current on the bottom. Traces are continuous and are in the presence of 5 μM-decamethonium. At *a*, following a voltage step to −140 mV, a slowly developing block develops. As seen at *b*, no relief from that block occurs in response to cessation of agonist pulses. At *d*, following a depolarizing step to −60 mV (*c*) during agonist application, relief from block has occurred (a comparison of responses at *a* and *d* also indicates that onset of block does not begin until agonist application is initiated). At *e*, following a voltage step to −60 mV in the absence of agonist application, no relief from the slowly developing block has occurred, while, at *f*, depolarization with agonist application produces relief from blockade. Vertical calibrations: 40 nA; horizontal calibrations: 30 s. *B*, effect of agonist applications on recovery from blockade following wash-out of decamethonium. Membrane currents at −140 mV are shown. On the left, response to ACh in 5 μM-decamethonium is shown and, on the right, response to ACh following 10 min wash in normal saline. No agonist pulses were applied between the left- and right-hand traces. Vertical calibration: 40 nA. Horizontal calibration: 30 s.

Decamethonium

Decamethonium also produces a voltage-dependent reduction in the amplitude of cholinergic responses on the g.m.1 muscle. As in the case of pempidine, chlorisondamine and mecamylamine, a portion of the blockade produced by decamethonium involves

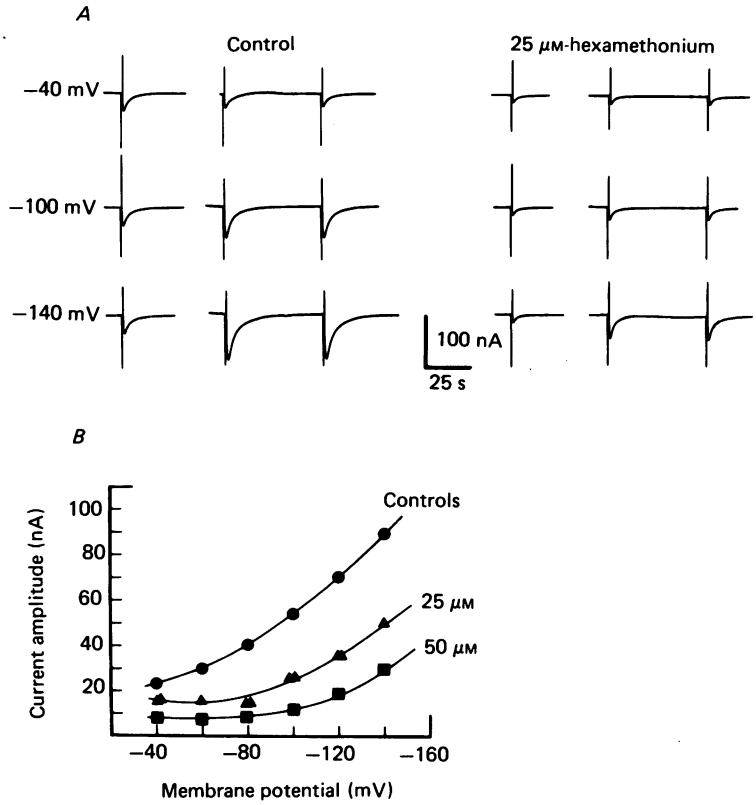

Fig. 8. *A*, effect of hexamethonium on cholinergic currents. As in previous Figures, responses to carbachol first at a holding potential of −60 mV and subsequently at test potentials are shown. Responses on the left were in normal saline and on the right in 25 μM-hexamethonium. Test potentials were from top to bottom, −40 mV, −100 mV and −140 mV. Vertical calibration: 100 nA; horizontal calibration: 25 s. *B*, voltage dependence of blockade by hexamethonium. Amplitudes of responses to carbachol are plotted as a function of membrane potential for normal saline and two concentrations of hexamethonium. Circles represent responses in normal saline, triangles in 25 μM-hexamethonium, and squares in 50 μM-hexamethonium.

the formation of a stable–blocked state (Fig. 7*A*). The development of the agonist-dependent blockade by decamethonium tends to occur with fewer agonist pulses than for the other related blockers. Similarly, the agonist-dependent recovery from block by decamethonium requires fewer agonist pulses for full recovery (Fig. 7*B*). Although the first response after decamethonium wash-out is larger than the last response in decamethonium, this probably is indicative that some unblocking occurs during the rising phase of the first ionophoretic response. Again, it is possible that an agonist-independent action of decamethonium contributes to its blocking action. However, the results clearly demonstrate that a portion of the blockade is entirely dependent on agonist application and leads to a stable–blocked state.

The properties of blockade by hexamethonium

Hexamethonium also reduces cholinergic currents on the g.m.1 muscle (Fig. 8). Similar to the action of the first group of blockers, the blockade produced by hexamethonium does not show any slowly developing component nor evidence of a stable–blocked state. On wash-out of hexamethonium, the first response to agonist application shows full recovery. However, the blockade produced by hexamethonium is voltage-dependent over only a limited range of membrane potentials (Fig. 8*B*). With sufficient hyperpolarization, a remarkable abolition of the voltage dependence of blockade occurs.

This relief from blockade produced by hyperpolarization is similar to the trends seen with chlorisondamine and, to some extent, with other agents that produce a stable–blocked state of the cholinergic response. Since 50 % blockade of the cholinergic currents at −80 mV occurs at about 10–20 μM-hexamethonium, it is possible that hexamethonium may actually result in the formation of the blocked–closed state but that upon channel activation unblocking occurs so rapidly that it is complete during the rising phase of the ionophoretic response. However, even with responses with rise times as fast as 200 ms, no evidence for the formation of a stable–blocked state was observed.

DISCUSSION

Two types of voltage-dependent blockade

In this investigation, the qualitative features of the blocking action of a variety of anti-cholinergic agents on a nicotinic cholinergic response on the lobster g.m.1 muscle were examined. All of the agents, except trimetaphan, produced a steady-state blockade of ionophoretically activated cholinergic currents that was voltage-dependent. Although the qualitative nature of these experiments precludes detailed mechanistic interpretations, the types of voltage-dependent blockade could be separated into at least two groups based primarily on the role of agonist application in the unblocking process. The principal observation that distinguishes the two groups is that for some compounds, when blocking drug is removed during the cessation of agonist application, recovery from blockade is complete on the first response to agonist after wash-out. For the other compounds, unblocking does not proceed until agonist application is initiated.

Compounds with agonist-dependent actions

Of primary interest are the blockers that produce a stable–blocked state of the cholinergic response that requires agonist application for unblocking to occur. The simplest physical interpretation of this state is that it represents a closed state of the channel with the blocking molecule still trapped in its blocking position. The combined implications of both the kinetic and pseudo-steady-state information available for chlorisondamine has allowed the proposal of a kinetic mechanism that could account for this type of blocking action (Lingle, 1983). The model suggests that the portion of the agonist-dependent blockade that leads to the formation of a stable–blocked state results from the binding of blocking drug to the opened ACh

channel and that some fraction of channels bound by blocking drug can close with the drug trapped in a blocking site (eqn. (1)). Qualitatively, at least a substantial portion of the actions of mecamylamine, pempidine and decamethonium are consistent with this type of mechanism. In short, the effect of these blockers can be most simply viewed as an action on the activated form of the ACh channel. Following interaction with the activated channel, some transition to a stable–blocked state occurs. The irreversible slow blocking reaction that proceeds to a steady-state level cannot be accounted for simply by a one-step blocking scheme, but requires that the blocking reaction be composed of at least two sequential steps.

A limitation of the present experiments is that the slow time course of the ionophoretic responses to cholinergic agonists precludes a determination of the true instantaneous block produced prior to agonist application. Thus, although a portion of the blockade produced by mecamylamine, pempidine, decamethonium and chlorisondamine is clearly agonist-dependent, the reduction in the amplitude in the first response in a train of agonist pulses following a hyperpolarizing step relative to a response in the absence of blocker may involve an action on the closed state of the receptor channel. Yet, since the slow ionophoretic responses involve the repeated openings and closings of many channels prior to peak amplitude, the initial response in a blocking sequence will always underestimate the portion of the block that strictly requires agonist application. Additionally, a more compelling argument against the idea that a substantial closed channel block is occurring derives from the observation that following wash-out of drug virtually no unblocking occurs until agonist application is initiated. If these drugs can produce a blockade of cholinergic currents by an agonist-independent action on the closed receptor channel, one would expect that molecules producing such a block should also be able to leave their blocking sites without the requirement for activation of the channel by agonist. If drug could not leave such agonist-independent blocking sites, with sufficient time a complete blockade of cholinergic sensitivity would occur prior to any application of agonist. It would, therefore, seem rather difficult to argue that the steady-state block at low concentrations of chlorisondamine, pempidine, mecamylamine and decamethonium involves both open and closed channel actions, since one would be forced to say that both the closed and open channel blockade can be relieved only by activation of the channel.

The compounds that produce the stable–blocked state include not only diquaternary amines, but also a tertiary amine, mecamylamine, and a secondary amine, pempidine. The pK values of both pempidine (11·25; Merck Index, 1981) and mecamylamine (11·2; Goldstein, Aronow & Kalman, 1974) are extraordinarily high for a secondary and tertiary amine, suggesting that the protonated species may be active. In this study no tests have been made in this regard. It might be tempting to attribute the particularly slow, agonist-dependent blocking and unblocking from pempidine and mecamylamine to a reservoir of molecules partitioned into hydrophobic domains with access to blocking sites. However, since agonist application readily promotes unblocking during depolarizing voltage steps and is responsible for the major portion of the unblocking process, it seems unlikely that the slow depletion of a pool of blocking molecules in a hydrophobic reservoir can account for these results. In fact, one might expect that, if such a reservoir existed, following an unblocking sequence

of agonist pulses at a depolarized potential, a slow reblocking might occur from this reservoir even in the absence of blocker in the bath. Since this is not observed and diquaternary blockers seem to exert qualitatively the same action, it does not seem necessary to postulate a route through hydrophobic pathways.

From the above considerations, the most simple view of the action of these blockers is that the blockade results primarily from an action on the activated form of the receptor channel that leads to the formation of a stable–blocked state probably corresponding to a closed–blocked channel.

Compounds with agonist-independent unblocking

A question arises as to whether the present experiments exclude the possibility that the actions of QX-222, trimetaphan, curare, procaine and atropine result in the formation of a closed–blocked state. For rapidly equilibrating blockers, blocking and unblocking would essentially occur 'instantaneously' with the first response in a blocking or unblocking sequence, and a closed–blocked state could not be discerned by the present ionophoretic procedures.

At least in the case of curare, an argument can be made against this possibility. Since blockade by curare involves a slowly developing component which probably reflects the slow unbinding of curare from a blocking site on the agonist-activated channel, if the channel were to close with curare trapped in this blocking site one would not expect the agonist-independent recovery observed for curare. For the other compounds, if the failure to observe the stable–blocked state reflected rapid kinetics, one might expect that blocking efficacy of such compounds, assuming similar association kinetics, would always be somewhat less than for decamethonium, the least potent of the blockers leading to the stable–blocked state. In these experiments, at -140 mV, 50% blockade produced by atropine, procaine and QX-222 occurred at concentrations at least 5- to 10-fold greater than that required for decamethonium (about 2 μM) to produce 50% blockade. Consequently, given the limitations of the present techniques, the formation of a blocked–closed state with QX-222, procaine and atropine might be obscured by rapid reaction rates.

Hexamethonium

The action of hexamethonium appears to represent an additional facet of possible blocking actions. No stable–blocked state can be detected by the present procedures. Of primary interest, the voltage dependence of block by hexamethonium is unusual. Indications of similar relief from blockade with hyperpolarization for drugs leading to a stable–blocked state raise the possibility that hexamethonium may act in a similar fashion. One explanation might be that with sufficient hyperpolarization some blockers can permeate the ACh-opened ion channel at a reasonable rate. In the absence of additional data, this can only be viewed as a suggestion. However, at the vertebrate neuromuscular junction there is evidence that decamethonium permeates the cholinergic channel quite readily (Creese & MacLagan, 1970). The results of Adams & Sakmann (1978) clearly demonstrate an open-channel blocking action of decamethonium. The results, however, cannot be considered to rule out the possibility that some portion of open-blocked channels can close, since any value of $k_c \ll b^*$ (see eqn. (1)) will yield kinetic results virtually indistinguishable from the open-channel blocking model.

Comparison to blockade of other cholinergic responses

The observation of a stable–blocked state of the cholinergic receptor channel that requires exposure to agonist to become unblocked has not previously been observed by electrophysiological means on any cholinergic system. The fact that chlorisondamine, mecamylamine and pempidine are all known as long lasting anti-hypertensive agents raises the possibility that a similar stable–blocked state may occur with some vertebrate nicotinic responses.

On rat parasympathetic ganglion neurones, mecamylamine has in fact been observed to produce a very slowly reversible blockade of cholinergic currents (Ascher, Large & Rang, 1979). The factors influencing the recovery from this blockade have not been determined. A principle argument in support of open-channel block on parasympathetic ganglion cholinergic responses is that with increased doses of agonist the percentage blockade produced by a given concentration of blocker is increased (Ascher *et al.* 1979). For mecamylamine, a non-voltage-dependent blocker, no increase in block is produced by increases in agonist dose, leading to the suggestion that mecamylmine is a closed channel blocker. However, the proposed two-step kinetic scheme (eqn. (1)) also predicts little effect of changes in agonist concentration on the depth of drug blockade. Consequently, the lack of effect of agonist concentration on the depth of block produced by mecamylamine on ganglionic neurones cannot be taken as evidence against an action on the opened ACh channel. Rather, the result only argues against a simple, open-channel blocking action.

More recently, the effect of curare, trimetaphan and hexamethonium on parasympathetic ganglionic synaptic currents has been reported (Rang, 1982). The results are complicated by apparently two kinetically distinct populations of ACh-activated channels. However, the nature of the results agrees quite well with the present observations. Curare exhibits a voltage-dependent action consistent with an action on the opened ACh channel. The reduction in synaptic currents by trimetaphan is largely voltage-independent with no effect on synaptic current decays, consistent with an action on the closed form of the channel. For hexamethonium, a voltage-dependent decrease in synaptic current amplitude and a shortening of synaptic current decays is observed. However, the reduction in current amplitude exceeds that predicted solely by a simple blocking scheme. Although part of the reduction may reflect the asynchronous activation of cholinergic currents (Rang, 1982), some portion of the voltage-dependent block produced by hexamethonium remains unexplained.

On the excitatory ACh response of *Aplysia* neurones (Ascher *et al.* 1978) the effects of hexamethonium, decamethonium and curare were all consistent with an action on the ACh-opened ion channel. Similar to the present observations, curare exhibited slow unblocking kinetics. The blocking effects of hexamethonium and, less dramatically, decamethonium over the range of -80 mV to -120 mV, exhibited systematic trends towards a new increase in conductance. These results are similar to the present observations. The effects of blockers on the *Aplysia* excitatory ACh channel, based on voltage-jump-induced relaxations, are not sufficient to exclude the possibility that some portion of open-blocked channels can close.

At the vertebrate neuromuscular junction ACh receptor, no stable–blocked state has been observed in response to any drug. Although unblocking from procaine is

retarded relative to its removal from the muscle bath, there is no indication that such unblocking requires or is facilitated by agonist application (Adams, 1977). This, in fact, is similar to the present observations. The discrepancies between the steady-state blockade by procaine and the kinetic rates of procaine block imply an action of procaine on both the closed and open forms of the cholinergic channel (Adams, 1977). Although a cyclical model has been proposed to account for procaine action, transitions from the open–blocked to closed–blocked state appear to be negligible and are thus clearly different from the formation of a stable–blocked state observed in this study.

Overview

At first glance, this paper might seem to over-emphasize a phenomenon that may represent a kinetically small portion of the total blocking action of a unique group of drugs. However, the phenomenon appears clearly different from the action exerted by slow open-channel blockers such as quinacrine (Adams & Feltz, 1980*a*, *b*) and curare (Colquhoun *et al.* 1979) and possibly is indicative of a different site of channel blocking action. The possibility that channels can close while occupied by blocking molecules would create some constraints on the shape and structure of the pathway for ion permeation and on the structural changes the channel must undergo during the gating process. As a consequence, the fact that such a phenomenon might occur at all raises some critical issues.

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