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Effects of Hexamethonium and Decamethonium on End-Plate Current Parameters

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SUMMARY

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The effects of neuromuscular blocking concentrations of hexamethonium and decamethonium on end-plate current parameters have been studied in frog sartorius nerve-muscle preparations. In the presence of either drug, hyperpolarization of the cell membrane reduces the peak amplitude of the end-plate current despite the increase in driving force. The effect is larger for hexamethonium than for decamethonium. In the presence of decamethonium, hyperpolarization reduces the time to peak of the end-plate current and splits the decay phase into two components, one faster and the other slower than in controls. Similar but less dramatic effects on parameters of end-plate current shape occur in the presence of hexamethonium. For both drugs, the decay of the end-plate current can be fitted by the sum of two exponential functions. Hyperpolarization speeds the initial fast component and slows the subsequent slow component. These results are consistent with the hypothesis that both drugs act in part by direct block of open ion channels.

INTRODUCTION

Hexamethonium, a short-chain symmetrical bisquaternary ammonium compound, blocks synaptic transmission in autonomic ganglia and (in higher concentrations) at the skeletal neuromuscular junction by a nondepolarizing postsynaptic action. In contrast, decamethonium, which differs from hexamethonium structurally only by the insertion of two methylene groups, has little effect on ganglia, but initially depolarizes the skeletal neuromuscular junction and eventually blocks transmission by a "desensitizing" mechanism ("phase II block"). Because of their structural resemblance to acetylcholine, both drugs have been generally assumed to exert their blocking effects at the binding site for acetylcholine, and there is indeed some evidence that hexamethonium is a weak competitive inhibitor of cholinergic agonists (1–4). However, as early as 1959 Blackman (5), on the basis of structure-activity studies, suggested that a range of ganglion-blocking compounds might block transmission by competing with cations for entry into the ion channels which mediate the synaptic current (see also ref. 6). Subsequently, he supported this hypothesis by demonstrating that hyperpolarization enhances block of postsynaptic potentials by hexamethonium in sympathetic ganglia, as might be expected if the drug were to bind to sites deep within the membrane, which would be strongly influenced by the transmembrane electric field (7) (see also ref. 8). Similar effects on postsynaptic potentials have been observed with both hexamethonium and decamethonium using the toad (*Bufo marinus*) sartorius nerve-muscle preparation (8, 9). These observations, while suggesting a common site of action for the drugs, do not rule out the possibility of voltage-sensitive binding to the acetylcholine receptor.

These competing theories can be tested by examining in detail the end-plate current and its voltage dependence. Under appropriate conditions, drugs which block open subsynaptic ion channels might be expected to shorten the time to peak of the end-plate current and to endow it with a biphasic decay. Drugs which simply occlude a proportion of acetylcholine receptors should exhibit neither of these effects.

The former effect is readily understood: as channels open under the influence of the transmitter, a proportion of them becomes blocked and the peak of the end-plate current occurs earlier because the effective rate of channel closure is greater. Furthermore, if the affinity of the drug for the ion channel is enhanced by hyperpolarization, as might be expected for cationic drugs, then the time to peak will be shortened by hyperpolarization. The biphasic decay (10) reflects rapid block of open channels

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by the drug as well as delayed closure of channels after the drug molecules have vacated them either by re-entering the synaptic cleft or by entering the cytoplasm (11). The two components of the decay phase might be expected to depend differently on membrane potential, inasmuch as the efficiency of the channel-block process will depend on the mean channel lifetime, which at the neuromuscular junction is voltage-sensitive (12). Specifically, membrane hyperpolarization should speed the early component by facilitating channel block by cations, and slow the late component by reducing the dissociation rate of drug molecules from ion channels. A range of neuromuscular blocking drugs has been shown to exhibit some or most of these effects (see Discussion).

Therefore, we report a detailed parametric analysis of the end-plate current under conditions of neuromuscular block by hexamethonium, decamethonium, and magnesium as a control. The results indicate that, at the concentrations required to block transmission, both drugs block open ion channels directly and this mechanism contributes to neuromuscular block.

A preliminary report of these results has been presented previously (13).

METHODS

The preparation used in these experiments was the isolated sartorius muscle from the summer North American grass frog *Rana pipiens*, supplied by Mogul-Ed (Oshkosh, Wisc.). The salt solution, which was maintained at approximately 13° with a Peltier cooling device, had the following ionic composition (millimolar): Na^+, 122; K^+, 2.5; Ca^{2+}, 1.8; Cl^-, 123; HPO_4^-, 2.2; $H_2PO_4^-$, 0.9. The pH of the solution was adjusted to 7.4 before use. Drugs added directly to the bathing solution were hexamethonium dibromide (0.75–1.5 mM; Sigma Chemical Company, St. Louis, Mo.) or decamethonium dibromide (0.05–0.10 mM; K. & K. Laboratories Inc., New York, N. Y.).

Experiments were conducted in preparations in which end-plate potentials were reduced to just subthreshold levels with one of the drugs or by addition of $MgCl_2$ (total Mg^{2+} concentration 7.5–10.0 mM). An end-plate region was located by insertion of a microelectrode in a region where the motor nerve gave off fine branches. Because end-plates could not be directly visualized, satisfactory electrode placement was obtained by searching for a position where the time to peak of the end-plate potential (at approximately 13°) was less than 4 msec. A second current-passing electrode was then inserted close to the first, and the membrane potential was clamped at a holding potential of either −60 or −80 mV. The gain of the voltage clamp amplifier was adjusted for maximal frequency response and maximal reduction of the voltage change occurring during the end-plate current. Details of the voltage clamp apparatus and current-monitoring circuit have been described previously (14). Beveled glass capillary electrodes (resistance 3–5 Mohm) filled with 2 M potassium citrate were used for recording and for current injection.

The end-plate current produced by stimulation of the nerve with a suction electrode at 0.5–2 Hz was sampled at 10 KHz (500 samples) and averaged on-line using a PDP-11/34 computer. Digitized averages were stored on a magnetic disc for subsequent analysis. The membrane potential was shifted sequentially to different levels in increments of 20 mV, and averages of 5–15 end-plate currents were recorded at each level after waiting at least 10 sec for stabilization of the end-plate current. End-plate currents occurring within this 10-sec interval were not analyzed in detail. In many experiments the entire sequence was repeated and the responses at given levels of membrane potential were averaged again. Finally, measurements were taken at the original clamped level of membrane potential. The recording electrode was then withdrawn from the cell, and results were accepted if the potential returned to the preimpalement baseline level. For the analysis, the baseline was set at the value obtained immediately prior to the stimulus artifact or just prior to the start of the end-plate current. The time to peak of the response was defined as the time elapsed between the initial deflection and peak value. The decay phase of the end-plate currents was analyzed off-line by using a simultaneous two-exponential nonlinear least-squares curve-fitting procedure. The starting point for the curve fit was set arbitrarily at 10% after the peak of the end-plate current, and zero time as the initial downward deflection of the end-plate current.

RESULTS

End-plate current amplitude. Hyperpolarization altered the amplitude and the decay phase of the end-plate current in a manner which depended upon the blocking drug used. Figure 1 shows the voltage dependence of the end-plate current in preparations in which neuromuscular transmission had been blocked presynaptically with Mg^{2+} or postsynaptically with decamethonium or hexamethonium. In the control situation (Mg^{2+} block), the end-plate current increased in amplitude and broadened as the membrane was hyperpolarized (Fig. 1A). In contrast, when transmission had been blocked with either decamethonium or hexamethonium, hyperpolarizing the membrane beyond −100 mV reduced the peak amplitude of the end-plate current. In addition, the decay portion of the end-plate current became clearly biphasic with decamethonium (Fig. 1C), and this effect, although smaller, was also evident with hexamethonium (Fig. 1B).

Figure 2A shows the voltage dependence of the peak amplitude of the end-plate current. In the control situation (Mg^{2+}) the peak amplitude increased almost linearly with membrane potential over the range −60 to −100 mV, and exhibited slight curvature outside this range. These results are similar to those reported for preparations in which neuromuscular transmission was blocked with tubocurarine or in which excitation-contraction coupling was blocked (15, 16).

In contrast, when transmission was blocked with either hexamethonium or decamethonium, the end-plate current first increased in amplitude then decreased dramatically as the membrane potential was shifted in the hyperpolarizing direction. These results are in the direction expected if the drugs bind to a site in the membrane which is very sensitive to the transmembrane electric field.

Time to peak. Measurements of the time to peak of

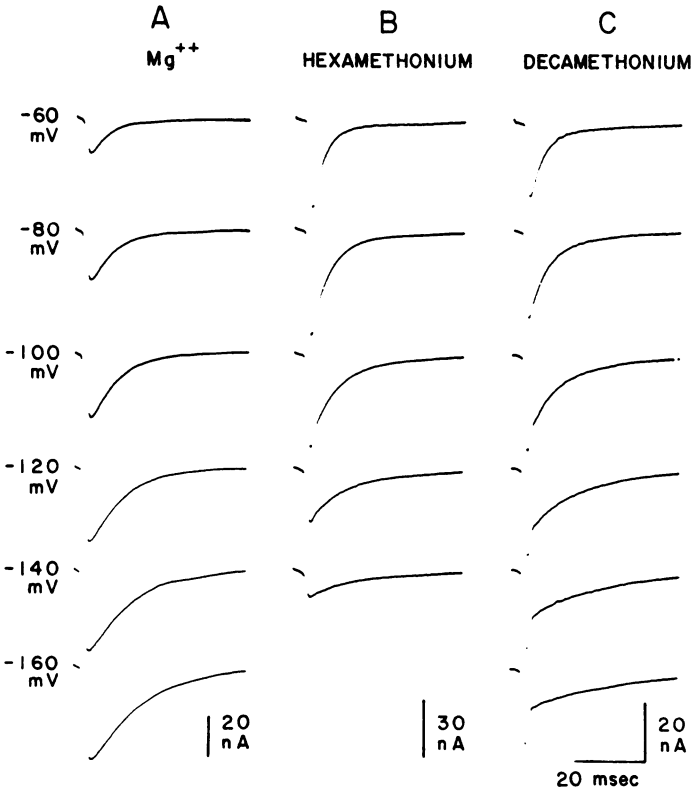

FIG. 1. *The effects of membrane hyperpolarization on averaged end-plate currents in the presence of neuromuscular blocking concentrations of magnesium (7.5 mM), hexamethonium (1 mM), and decamethonium (0.05 mM)*

As the membrane is hyperpolarized, the amplitude of the end-plate currents increases in the presence of Mg^{2+} but decreases in the presence of hexamethonium and decamethonium. Note that hyperpolarization leads to a marked biphasic decay of the end-plate current in the presence of decamethonium.

the end-plate current support the hypothesis that both hexamethonium and decamethonium reduce the peak amplitude of the end-plate current by blocking open ion channels. Figure 2B shows that the time to peak of the end-plate current was smaller for the quaternary ammonium compounds than for controls. The differences were statistically significant (one-sided pooled *t*-test, $p < 0.05$) over the range −80 to −140 mV for decamethonium, and −120 to −140 mV for hexamethonium. The time to peak also increased with hyperpolarization in controls but decreased with hyperpolarization at drug-treated junctions. The slopes of the linear regression lines for the drugs differed significantly from each other and from the regression line for control experiments ($p < 0.05$). These results would not be expected if hyperpolarization merely increased the affinity of the drugs for the acetylcholine receptor.

Decay phase. Drugs which act by blocking open ion channels typically split the decay phase of the end-plate current into two components, one faster than the normal channel closure rate and one slower (see Introduction). Furthermore, hyperpolarization should enhance this effect. At membrane potentials beyond −100 mV it was clear that decamethonium did indeed split the end-plate current decay (Fig. 1C). The effect was much smaller for hexamethonium (Fig. 1B). We therefore analyzed the results further by fitting the end-plate current decay phase at each level of membrane potential with a two-exponential curve (see Methods).

Figure 3 shows the voltage dependence of the fast- and slow-rate constants of the end-plate current decay phase for both drugs as well as for control experiments performed with Mg^{2+}. Over the range of membrane potential examined, the early component of the decay phase was much faster than that in controls, and the late component was similar to that of controls, or slower, for both drugs. The disparity between the fast- and slow-decay rate constants was greatly enhanced by hyperpolarization, and this effect was particularly pronounced for decamethonium.[3] In addition to the time constants, the coefficients of the fast and slow exponential functions were also obtained by the curve-fitting procedure. The ratios of the "fast" and "slow" coefficients were similar for both drugs (approximately 1.75 at −80 mV) and were enhanced by hyperpolarization (see also ref. 11). These data, however, cannot sustain detailed quantitative analysis, since the estimates of the "fast" coefficient were poorly resolved by the technique owing to the fact that small errors in the fast-time constant estimate lead to large errors in the estimate of the coefficient of the exponential with the fast-time constant.

Although these results graphically illustrate major differences between drug-treated junctions and controls, at hyperpolarized levels of membrane potential they also show that the differences are much smaller near the resting potential (−60 to −90 mV). Comparison of the slow rate constants showed that the drug-treated junctions differed significantly from controls at membrane potentials beyond −100 mV for hexamethonium and −80 mV for decamethonium (one-sided pooled *t*-test; $p < 0.05$). It should be noted that Mg^{2+}-blocked preparations may not provide an ideal control in these experiments, because divalent cations may reduce the end-plate current decay rate constant by a "screening" effect on the postsynaptic membrane (ref. 19; however, see refs. 20 and 21). The differences between the slow-rate constants may therefore be even greater than our results indicate. The effect will be to make less negative the value of membrane potential beyond which the curves differ significantly.

DISCUSSION

Our results show that block of the end-plate current by hexamethonium and desensitizing (phase II) block by decamethonium are strongly enhanced by hyperpolarizing the cell membrane. These results extend those obtained on the end-plate potential in the Fijian cane toad

[3] The curve-fitting procedure identified an early "fast" component of end-plate current decay in approximately half of the Mg^{2+} controls. It is possible that Mg^{2+} itself may have a channel-blocking action (see ref. 17) which would induce a biphasic decay. However, in our experiments the fast component did not have any systematic properties, in contrast to the fast components in drug-treated preparations. It seems likely, therefore, that the fast component in controls is artifactual, possibly arising from a bias in the curve-fitting procedure or incomplete spatial clamping of the end-plate (see ref. 18).

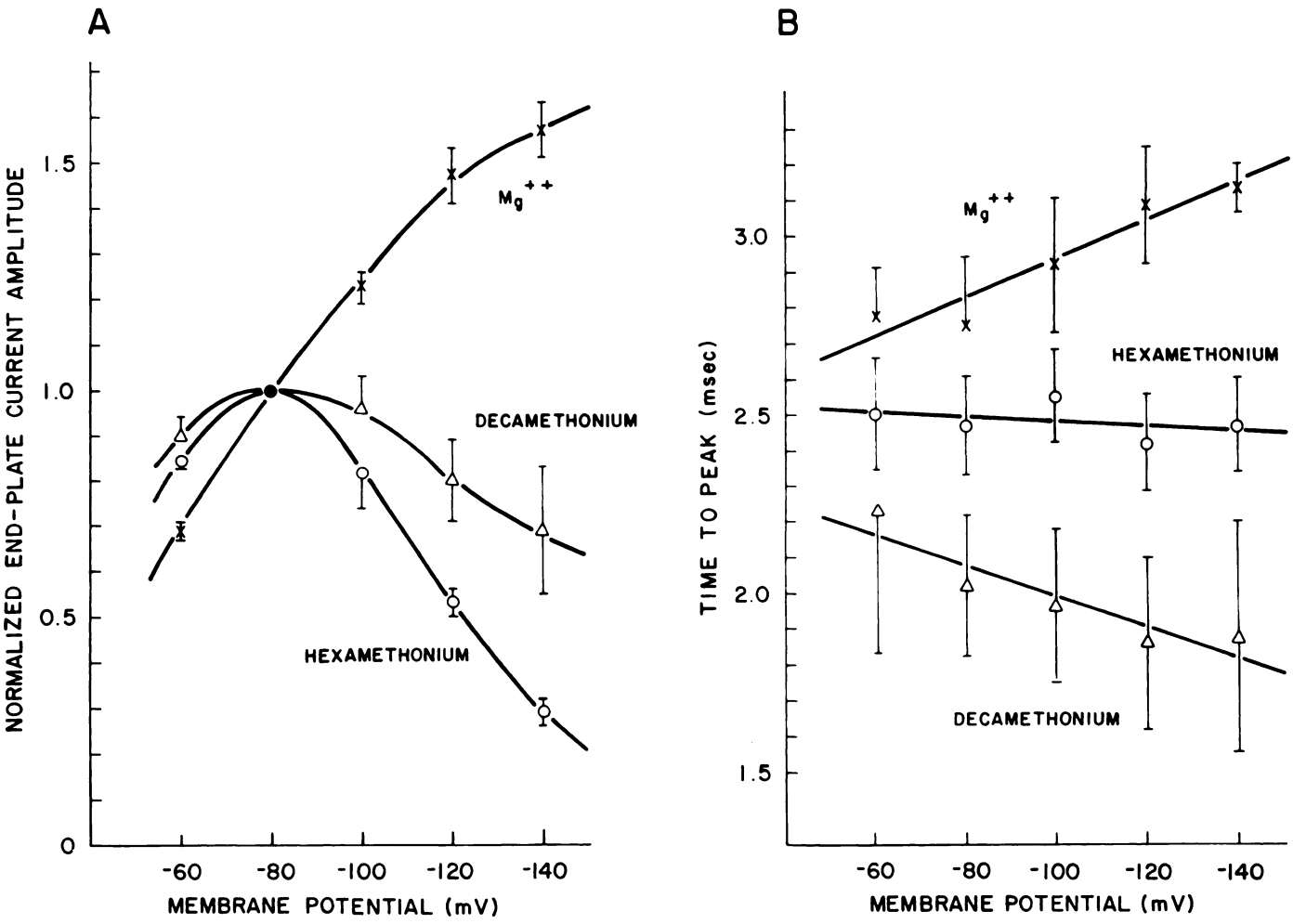

FIG. 2. *The effects of membrane hyperpolarization on the amplitude (A) and time to peak (B) of the end-plate current, in the presence of neuromuscular blocking concentrations of Mg^{2+}, hexamethonium, and decamethonium*

Each drug was tested in 5–7 different cells. Data points represent means ± standard error of the mean of measurements taken in different cells, and each measurement represents the average of 5–50 end-plate currents. Curves in A were fitted by eye whereas straight lines in B were fitted by linear regression analysis. Data in A are normalized to the response amplitude at −80 mV.

B. marinus (8, 9) and suggest a common site of action for the two functionally dissimilar, structurally homologous, drugs. The observed modifications of parameters of end-plate current shape (time to peak and decay rate), as well as their voltage-sensitivity, are consistent with the hypothesis that, at the concentrations required to block neuromuscular transmission, both drugs directly occlude ion channels opened under the influence of the transmitter.

Recent studies by Adams and Sakmann (11) have shown that decamethonium splits the decay phase of miniature end-plate currents into two components with opposite voltage sensitivities, an observation equivalent to ours (see also ref. 22). These authors concluded on the basis of voltage jump and fluctuation analysis that decamethonium blocks open ion channels (11). Our results for decamethonium therefore provide independent confirmation of their conclusion.

Hexamethonium appears to be a less effective channel-blocking compound than decamethonium, since the parameters of end-plate current shape (Figs. 2B and 3) were intermediate between those of decamethonium and controls. This might be expected, since its smaller molecular size and relatively rigid conformation (23) would allow it to pass through open channels even more readily than decamethonium (24) and therefore block them less effectively. The relative potencies of the two drugs (decamethonium is roughly 20 times more potent than hexamethonium) may result in part from their relative efficacies as channel blockers; however, this conclusion must be tempered by the possibility that both drugs have additional actions at sites other than the ion channel. In particular decamethonium, like acetylcholine (25), may "desensitize" in part by a mechanism other than channel block.

Our results can be used to estimate rate constants. If a simple sequential model of the channel-block process is assumed (see refs. 10, 11, 26), the decay phase of the end-plate current is described by a second-order differential equation whose coefficients are explicit functions of the rate constants and drug concentration (it is assumed that the channel opening rate is negligible over the range of measurements of end-plate current decay). If the roots of the corresponding secular equation are

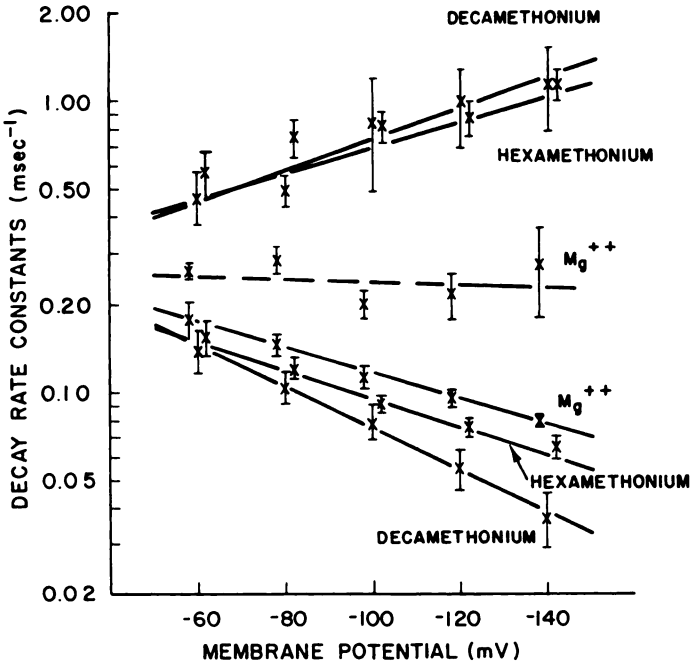

FIG. 3. *The effects of membrane hyperpolarization on the "fast" and "slow" rate constants of the decay phase of averaged end-plate currents, determined from a two-exponential nonlinear curve-fitting procedure*

Straight lines were fitted by log-linear regression analysis. - - -, an early "fast" component of end-plate current decay observed in the control experiments (see footnote 3). Data points are means ± standard error of the mean. At hyperpolarized levels the rate constant for the slow decay of the end-plate current in hexamethonium and decamethonium is less than the rate constant of the Mg^{2+} controls.

defined as r_1 and r_2, then

$$r_1 + r_2 = k_2 + k_3B + k_4$$

and

$$r_1r_2 = k_2k_4$$

where k_3 and k_4 are the forward and backward rates for binding to the open-ion channel, k_2 is the channel closure rate, and B is the concentration of antagonist. Solving for k_3 and k_4 and substituting measured parameters (k_2 is obtained from control results) yields values for decamethonium of 0.60×10^7 M^{-1} sec^{-1} and 0.51 $msec^{-1}$ for the forward and backward rate constants, respectively, at −130 mV. These estimated values agree satisfactorily with those obtained independently by voltage jump and fluctuation techniques (1.7×10^7 M^{-1} sec^1 and 1.0 $msec^{-1}$; ref. 11). The corresponding values for hexamethonium at −130 mV are 1.8×10^5 M^{-1} sec^{-1} and 0.80 $msec^{-1}$. Therefore the estimated dissociation constants of decamethonium and hexamethonium for binding to the ion channels are 86 μM and 4.5 mM, respectively (13°, −130 mV). For both drugs, the dissociation constants are higher at −80 mV, implying that the channel-block process is less effective at or near the resting potential.

Although our observations are consistent with the hypothesis that both hexamethonium and decamethonium block open-ion channels, it does not necessarily follow that this mechanism is primarily responsible for reducing the peak amplitude of the end-plate current. An alternative explanation is that the drugs reduce the peak amplitude by blocking and/or desensitizing receptors, and that their effects on open-ion channels, like those of tubocurarine (27), are incidental. However, it appears that tubocurarine does not appreciably change the time to peak of the end-plate current (fig. 10 in ref. 27). In contrast, both hexamethonium and decamethonium reduce the time to peak (see Results), which implies that a significant proportion of open channels becomes blocked before the natural peak of the end-plate current is reached. Furthermore, the voltage sensitivity of the time to peak in the presence of both drugs implies that the process which reduces the end-plate current amplitude is enhanced by hyperpolarization. We conclude, therefore, that block of open ion channels contributes appreciably to the observed reduction of the peak amplitude of the end-plate current. A corollary would be that the dramatic voltage dependence of end-plate current amplitude observed with both drugs reflects the voltage sensitivity of the channel-block mechanism.

A problem with this interpretation is that the voltage sensitivity of the end-plate current amplitude is greater for hexamethonium than for decamethonium, even though parameters of end-plate current shape indicate that decamethonium blocks open ion channels more effectively. A related problem emerges from quantitative considerations. On the basis of our data, hexamethonium's dissociation constant for binding to ion channels is approximately 4.5 mM (at −130 mV), which implies that its blocking action at open-ion channels is exceedingly weak, even when the membrane is hyperpolarized. In contrast, the equilibrium dissociation constant of hexamethonium in amphibian muscle has been variously estimated by equilibrium dose-ratio methods at 30–50 μM (1–4). Therefore we suggest that hexamethonium may act at some other site within the transmembrane electric field. The acetylcholine recognition site is one candidate, although this possibility seems less attractive in view of the voltage insensitivity of tubocurarine's action at this site (27). Alternatively, hexamethonium may bind to the closed form of the ion channel, as originally proposed for procaine (28) and more recently for piperocaine (29) and tetraethylammonium (30). The present experiments cannot decide between these possibilities.

The observations reported in this paper may be compared with the effects of a range of putative channel-blocking compounds on parameters of end-plate current. Procaine (31), tetraethylammonium (30), atropine (32), and scopolamine (33) exhibit a nonlinear relationship between end-plate current amplitude and membrane potential. The latter three drugs, as well as quinacrine (34, 35), also shorten the time to peak. All of these compounds, as well as histrionicotoxin (36) and some barbiturates (10), have been shown to shorten the end-plate current decay phase and to reduce its voltage sensitivity. Indeed, it is interesting that hexamethonium and decamethonium at the concentrations used in this study do not simply reduce but actually reverse the voltage sensitivity of the early phase of end-plate current decay, as has also been shown for higher concentrations of piperocaine. Finally, procaine (37), lidocaine (38), scopolamine

(33), and methyprylone (10) exhibit the second, slower phase of end-plate current decay which has been ascribed to delayed closure of previously blocked channels (11, 39; see also ref. 26). Hexamethonium, decamethonium, and also tubocurarine appear to block open ion channels in mammalian parasympathetic ganglion cells (40) and in neurons of *Aplysia* (41). However, the relative importance of receptor and channel sites for the drugs' effects on nicotinic cholinergic transmission have not been ascertained in these preparations.

To conclude, our data are consistent with a model in which both hexamethonium and decamethonium block neuromuscular transmission at least in part by occluding open ion channels. More detailed quantitative studies of each drug, along with specific biophysical models of their effects on the end-plate current, are needed to ascertain the relative contributions of receptor and channel binding sites to neuromuscular block.

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