Endplate Channel Block by Guanidine Derivatives

JERRY M. FARLEY, JAY Z. YEH, SHIGENORI WATANABE, and TOSHIO NARAHASHI

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611. J. M. Farley's present address is the Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi 39216. S. Watanabe's present address is the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan.

ABSTRACT The effects of the *n*-alkyl derivatives of guanidine on the frog neuromuscular junction were studied using the two-microelectrode voltage clamp and other electrophysiological techniques. Methyl-, ethyl-, and propylguanidine stimulated the nerve-evoked release of transmitter. However, amyl- and octylguanidine had no apparent presynaptic action. All of the derivatives blocked the postsynaptic response to acetylcholine, the potency sequence being octyl- > amyl- > propyl-, methyl- > ethylguanidine. Methyl- and octylguanidine did not protect the receptor from α-bungarotoxin block, suggesting that these compounds do not bind to the receptor but probably block the ionic channel. Methyl-, ethyl-, and propylguanidine shortened inward endplate currents but prolonged outward currents. Amylguanidine prolonged both inward and outward endplate currents, and the currents became biphasic at negative membrane potentials. Octylguanidine increased the rate of decay of endplate currents at all potentials. All of the derivatives blocked inward endplate currents more markedly than outward currents, resulting in a highly nonlinear current-voltage relation. Methyl-, ethyl-, and propylguanidine reversed the voltage dependence of endplate current decay, while amyl- and octylguanidine reduced the voltage dependence of endplate current decay. Octylguanidine appears to block the ionic channel in both the open and the closed state. The block of the open channel follows pseudo-first-order kinetics with a forward rate constant of 4–6 $\times 10^7$ M^{-1} s^{-1}.

INTRODUCTION

A variety of compounds have been proposed to interact with ionic channels of endplate membranes. Neher and Steinbach (1978) have recorded single-channel currents from endplate membranes and concluded that two quaternary derivatives of lidocaine, QX-222 and QX-314, block the ionic channels when they are open. It has been proposed that decamethonium and amantadine also interact with the open channel (Adams and Sakmann, 1978; Tsai et al., 1978). Procaine, barbiturates, histrionicotoxin (HTX), and tetraethylammonium (TEA) appear to interact with the closed as well as the open channel (Adams, 1976, 1977; Masukawa and Albuquerque, 1978; Adler et al., 1979).

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Gage (1978) has suggested that another group of compounds, the long-chain alcohols, modify the kinetics of channel closing by affecting the fluidity of the membrane.

The *n*-alkyl derivatives of guanidine have recently been found to block the action of acetylcholine (ACh) postsynaptically (Watanabe et al., 1978; Farley et al., 1979 *a* and 1979 *b*). Guanidine compounds are of particular interest for several reasons. First, it has been suggested that guanidine is an important group of tetrodotoxin (TTX) and saxitoxin (Hille, 1975; Ritchie and Rogart, 1977) in their highly specific blocking action on the sodium channel in nerve membranes (Narahashi et al., 1964). Thus, these simple guanidine compounds are expected to provide insight into the molecular structure and topography of ionic channels. Second, the *n*-alkyl derivatives form a simple structure-activity series, the only differences between these derivatives being the length of the alkyl side chain. Third, at least one of the compounds, methylguanidine, is clinically significant. During renal failure many neuropathological symptoms occur which may be correlated with the accumulation of methylguanidine (Giovannetti et al., 1969).

The present study is concerned with the mechanism by which *n*-alkyl guanidine derivatives block the postsynaptic action of ACh. We have found that the ionic channels, but not the ACh receptors of the endplate membrane, are blocked by the *n*-alkylguanidine compounds. The potency and characteristics of the blocking action are a function of the length of the alkyl side chain. Both the open and the closed channel are affected by these compounds.

METHODS

The sartorius and cutaneous pectoris muscle preparations from the frog *Rana pipiens* were used. Southern frogs were obtained from Texas and maintained at room temperature in a tank with cold running water. The muscles were pinned out in a Plexiglas chamber. The nerves were isolated and stimulated via a suction electrode. The bath was continuously perfused with Ringer's solution. Muscle contraction was eliminated by treating the preparation with formamide, a technique developed by del Castillo and de Motta (1977). The muscle was treated for 25–35 min with a Ringer's solution containing 2 M formamide and 100–500 nM TTX. The TTX reduced large contractions that occurred when the muscle was exposed to the hypertonic formamide Ringer's solution. The muscle was then washed out with normal Ringer's solution. At least 1 h was allowed for recovery. With this method the resting potential of ~ −90 mV was regularly recorded after treatment with formamide. Neuromuscular transmission was intact and apparently unaltered.

The Ringer's solution used contained (mM); 115 NaCl, 5.4 KCl, 1.8 $CaCl_2$, and 2 *N*-2-hydroxethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES). The pH was adjusted to 7.3 with NaOH. In some experiments *d*-tubocurarine or TTX was added to the bathing solution. Methylguanidine as the hydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. Ethyl-, propyl-, and octylguanidine as the sulfate salt were synthesized for us by Dr. John Dutcher of the National Cancer Institute, Bethesda, Md. Amylguanidine was available from our own laboratory. Acetylcholine chloride was obtained from Sigma.

The endplate was voltage clamped using the two-microelectrode technique origi-

nally developed by Takeuchi and Takeuchi (1959). A 5- to 10-MΩ micropipette filled with 3 M KCl was used to measure membrane potential. The current-injection micropipette was filled with either 3 M KCl or 2 M potassium citrate and had resistance ranging from 2 to 5 MΩ. The location of the endplate was determined by the occurrence of miniature endplate potentials (MEPPs) with ~0.5 ms rise time. The voltage-measuring and current-injection electrodes were inserted in the endplate region within 20–30 μm of each other. Both electrodes were shielded with metal to decrease interelectrode capacitative coupling. The endplate currents (EPCs) were recorded through a virtual ground circuit connected to the bath. The rise time of a voltage command pulse applied to a cell during voltage clamp was typically <200 μs, and the capacitative current transient was longer, generally lasting <300 μs.

In some experiments, acetylcholine was applied iontophoretically to the endplate. A short constant-current pulse (0.5–2 ms) was injected into a micropipette filled with 1 M ACh and of 40–100 MΩ resistance. A small braking current was applied to stop diffusion of ACh from the micropipette tip.

All experiments were performed at 20°–22°C.

RESULTS

Endplate Potential

Whenever EPPs and EPCs are used as a measure of the postjunctional blocking action, it must be determined whether the compound in question has any effects on the presynaptic elements. It is well known that guanidine itself increases transmitter release at the neuromuscular and synaptic junctions of the frog, rat, and squid (Otsuka and Endo, 1960; Teravainen and Larsen, 1975; Kusano, 1970). The shorter chain *n*-alkyl derivatives of guanidine, methyl-, ethyl-, and propylguanidine have also been found to exert this action (Farley et al., 1979 *a*). Methylguanidine at a concentration of 5 mM greatly increased the endplate potential amplitude, as shown in Fig. 1. The increase in EPP amplitude took 30 min to reach a steady state. When the methylguanidine was washed out, there was initially a further increase in the amplitude of the EPP (Fig. 1 *C*). This suggests that two processes are occurring, one that increases EPP amplitude (presynaptic) and one that decreases EPP amplitude (postsynaptic). Extended washing decreased the amplitude of the EPP to the control level (Fig. 1 *D*). Complete reversal of the increase in EPP amplitude required 60–90 min of washing. Methyl-, ethyl-, and propylguanidine all affected the EPP in a similar manner. All of these compounds decreased the amplitude of MEPPs and abolished them completely at higher concentrations. This observation is also compatible with their postsynaptic blocking action.

The longer-chain derivatives of guanidine, amyl- and octylguanidine, have no apparent presynaptic action (Farley et al., 1979 *a*). That the shorter-chain alkyl derivatives affect transmitter release will be considered when the effects of these compounds on the time-course of the EPC are discussed.

The guanidine derivatives had no effect on the membrane potential of the muscle, with the exception of octylguanidine, which consistently caused the membrane to hyperpolarize by a few millivolts.

Dose-Response Relation

The effectivenss of *n*-alkylguanidines to block the postsynaptic action of ACh was measured by determining the dose-response relationship for inhibition of the iontophoretically induced ACh potential. These responses were obtained at resting potentials of −85 to −95 mV and typically had rise times between 10 and 30 ms. The amplitude of the responses varied from 10 to 15 mV. The dose-response curve for the effect of octylguanidine on the amplitude of the ACh potential is shown in Fig. 2 *A*. The effective dose 50 (ED_{50}) values for endplate block were estimated to be 4 mM, 0.4 mM, 65 μM, and 6 μM for ethyl-, propyl-, amyl-, and octylguanidine, respectively. These data are shown graphically in Fig. 2 *B*, in which the logarithm of the ED_{50} values are plotted

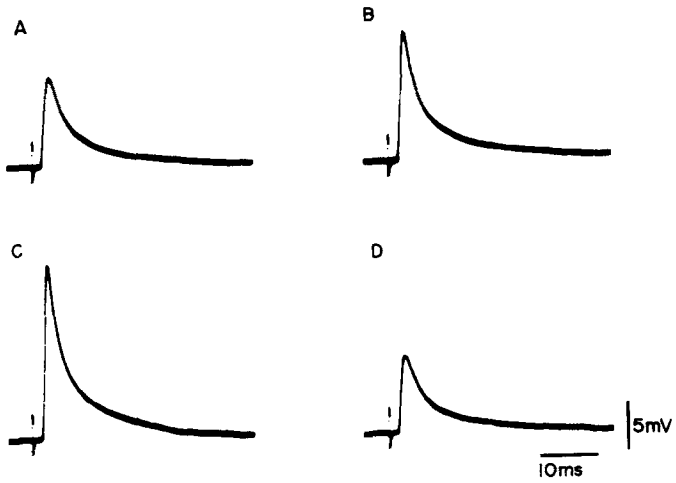


FIGURE 1. The effects of 0.5 mM methylguanidine on endplate potential. (*A*) Control. (*B*) After a 10-min exposure to solution containing 5×10^{-3} M methylguanidine. (*C*) After wash-out of methylguanidine for 3 min. Note a further increase in amplitude. (*D*) Reversal of methylguanidine's effects after 60 min of washing.

against the number of carbons in the alkyl side chain. Clearly, there is an increase in potency as the side chain is lengthened. This increase may be attributed to either molecular size or hydrophobicity. This will be discussed below.

The block of the endplate could occur either at the channel or at the receptor. To distinguish between these two possibilities, a "protection" experiment was performed.

Protection Experiments

If a compound binds to the ACh receptor, a high concentration of that compound should protect the receptor from binding by a highly specific, irreversible antagonist such as α-bungarotoxin (α-BuTX) (Chang and Lee,

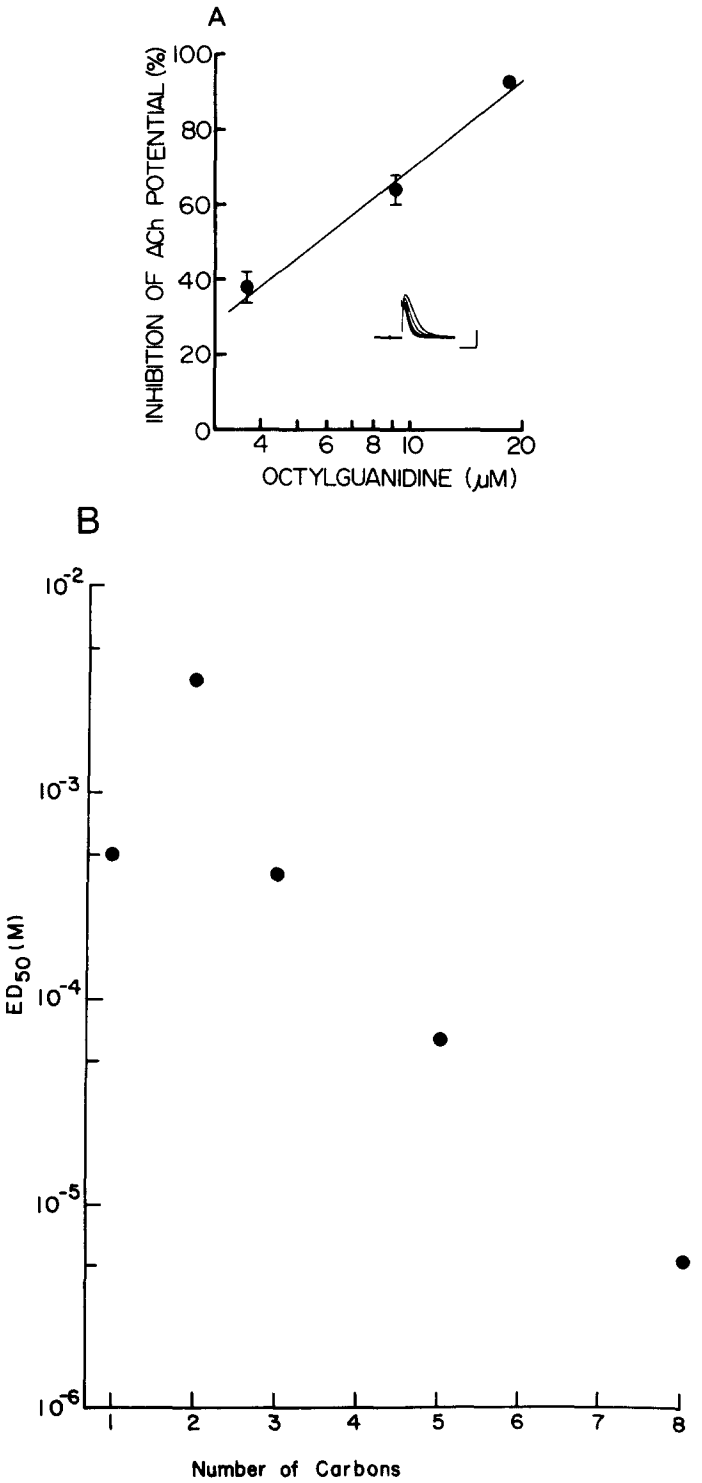
1963). Such a protection of the receptor from α-BuTX block demonstrates that the compound binds to the receptor. The following protocol was used to determine whether methyl- or octylguanidine could protect the receptor from α-BuTX binding. The muscle was incubated in 20 mM methylguanidine or 180 μM octylguanidine for 30 min before and during a 20-min exposure of the muscle to 0.05–0.2 μg/ml α-BuTX. Higher concentrations of octylguanidine caused an irreversible damage to the muscles. After washout of all the drugs, nerve stimulation failed to induce action potentials or EPPs in the surface fibers. The blocking action of 20 mM methylguanidine or 180 μM octylguanidine applied alone for the same length of time was reversible upon washing. In similar experiments, 100 μM *d*-tubocurarine protected the receptor from block by α-BuTX, because after washing large EPPs and/or action potentials could be observed.

These findings suggest that the *n*-alkylguanidine derivatives do not bind to the receptor but block the channel. Further evidence in favor of a channel-blocking mechanism has been obtained by characterizing the EPC blocking action. The characteristics of the EPC block are not consistent with receptor block.

Decay of Endplate Current

The guanidine derivatives had a marked effect on the EPC time-course. Four families of EPCs are shown in Fig. 3. The EPC family on the left was obtained in normal Ringer's solution at membrane potentials ranging from − 100 to + 40 mV in 20-mV steps. The EPC was elicited 10 s after the membrane potential was stepped to a new potential from −50 mV. The amplitude of the control EPCs was approximately a linear function of the membrane potential. The decay of the EPCs could be described by a single exponential function, and the rate of decay was dependent on the membrane potential, becoming faster as the membrane was depolarized. In the presence of the guanidine derivatives, these characteristics were markedly altered.

All the derivatives used inhibited inward endplate currents more than outward currents. The short chain derivatives methyl-, ethyl-, and propylguanidine caused the inward currents to decay faster, while the outward currents were prolonged. The time-course of decay remained approximately exponential. However, a slight nonlinearity of the decay, especially that of outward EPCs, was observed when they were plotted on semilogarithmic coordinates. The nonexponential decay may arise from the effect of propylguanidine on transmitter release. An example of EPCs obtained in the presence of 1.7 mM propylguanidine from the same endplate as in Fig. 3 *A* is shown in Fig. 3 *B*. Note that the outward currents in Fig. 3 *B* are larger than those in the control. This is caused by the presynaptic action of this compound to stimulate transmitter release. The effects of 0.26 mM amylguanidine on the EPC decay shown in Fig. 3 *C* are quite different from those of the shorter-chain derivatives. Amylguanidine prolonged the inward EPCs and caused them to decay in a biphasic manner. In contrast, the outward currents were prolonged but decayed as a single exponential. These currents were obtained


A
INHIBITION OF ACh POTENTIAL (%)
100
80
60
40
20
0
4
6
8
10
20
OCTYLGUANIDINE (μM)
B
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
ED_{50} (M)
1
2
3
4
5
6
7
8
Number of Carbons

at membrane potentials ranging from −100 to +50 mV. In the presence of 18 μM octylguanidine (Fig. 3 *D*), the EPCs decayed faster and with a single exponential time-course at all potentials. The membrane potential ranged from −90 to +70 mV in 20-mV steps for the currents shown.

The time constant of decay (τ) of the normal EPC changes with the membrane potential, depolarization decreasing the time constant. The voltage dependence of the rate of decay is described by the equation $\tau = \tau\ (0)\ \exp(-0.011 \times E_m)$, where E_m denotes the membrane potential. This voltage dependence is similar to that observed by Magleby and Stevens (1972) and by Ruff (1977). In the presence of 2.6 mM propylguanidine, the dependence of τ on voltage was reversed such that as the membrane was depolarized, the currents were prolonged (Fig. 4 *A*). A similar effect was also observed in the

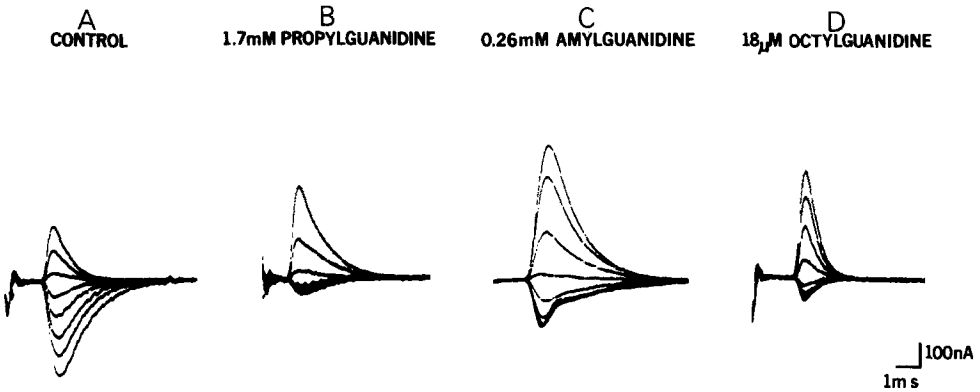


FIGURE 3. Families of EPC recorded at various membrane potentials. The family on the left (*A*) was obtained in control solution and the other families (*B*, *C*, and *D*) in the presence of the compounds shown. Records *A* and *B* were obtained at the same endplate. Each EPC was elicited 10 s after a step change of the membrane potential.

presence of methyl- and ethylguanidine. Because of the effect on transmitter release, it is difficult to quantify the purely postsynaptic action of short-chain guanidine derivatives on the EPC decay.

The decay of the inward EPCs in the presence of amylguanidine was biphasic (Fig. 3 *C*). Amylguanidine had no apparent presynaptic action. The

FIGURE 2. (*A*) Dose-response curve for the inhibition of the iontophoretically induced ACh potential by octylguanidine obtained at membrane potentials of −85 to −95 mV. The data represent the mean ± SEM of at least three experiments. The *inset* is typical iontophoretic acetylcholine responses before and during the wash-in of 9.1 μM octylguanidine. The scales are 40 ms and 5 mV. (*B*) The ED_{50} values for the inhibition of iontophoretically induced acetylcholine potentials by the *n*-alkylguanidine compounds plotted against the number of carbons in the alkyl side chain. The points are means of at least three experiments.

decay of the inward EPCs in the presence of amylguanidine could be separated into the sum of two exponential components by curve stripping. In Fig. 4 *B*, the voltage dependence of the time constant of the slow (squares) and fast (triangles) components of decay in the presence of 0.13 mM amylguanidine is illustrated together with the control (circles). In the presence of amylguanidine the EPCs decay more slowly than in the control solution at all potentials, as is shown by the slow component. However, the slope for the voltage dependence of the slow phase of the EPC decay in 0.13 mM amylguanidine is less than the control. The voltage dependence of the slow phase of EPC decay can be described by the equation $\tau_{slow} = \tau(0)_{slow} \exp(-0.0047 \times E_m)$. Amylguanidine prolonged the currents more at a concentration of 0.26 than at 0.13 mM. The voltage dependence of decay remains similar, however, and is described by the equation $\tau_{slow} = \tau(0)_{slow} \exp(-0.0045 \times E_m)$. The fast component of the EPC decay in 0.13 and 0.26 mM amylguanidine consistently had a voltage dependence reversed from that of the slow decay. However, there is a considerable error involved in obtaining the time constant of the fast component.

Octylguanidine caused the EPCs to decay faster at all potentials (Fig. 3 *D*). The rate of decay was increased as the concentration of octylguanidine was raised as is shown in Fig. 5 *A* for 9.1 and 27.3 μM. The voltage dependence of the decay was reduced by octylguanidine, although it can still be described by a single exponential function. The decrease in the voltage dependence of the decay was dependent on the concentration of octylguanidine. In 9.1 and 27.3 μM octylguanidine, the decay is described by the equations, $\tau = \tau(0) \exp(-0.0051) \times E_m)$ and $\tau = \tau(0) \exp(-0.0028 \times E_m)$, respectively. Octylguanidine at a concentration of 1.8 μM only slightly affected the EPC decay and did not alter the voltage dependence of τ appreciably. However, it caused a 33% reduction of the peak EPC amplitude at −90 mV.

If the channel blocking action of octylguanidine is a pseudo-first-order process, then a plot of the decay rate of the EPC against the concentration of octylguanidine should yield a straight line, the intercept being equal to the normal EPC decay rate and the slope to the rate at which octylguanidine blocks the channel (Adams, 1976). Such plots are shown in Fig. 5 *B* for the decay of EPCs at −90, −50, and +50 mV. The lines through the points are drawn by least square fits. The correlation coefficients for the fits are 0.971, 0.965, and 0.929, respectively. The linearity of this plot suggests that the kinetics of the block of the endplate channels by octylguanidine are consistent with a pseudo-first-order process and that the dissociation (unblocking) rate is negligibly low. The slopes of the lines are 5.0×10^7 M^{-1} s^{-1} at −90 mV, 5.4×10^7 M^{-1} s^{-1} at −50 mV, and 4.6×10^7 M^{-1} s^{-1} at +50 mV. The rate of block is not significantly voltage dependent.

Current-Voltage Relationship

It has been shown that the magnitude of the channel block caused by a number of compounds such as procaine, QX-222, histrionicotoxin, amantadine, and decamethonium is dependent on the membrane potential (Kordas, 1970; Deguchi and Narahashi, 1971; Ruff, 1977; Masukawa and Albuquer-

que, 1978; Tsai et al., 1978; Adams and Sakmann, 1978). We have also observed that the magnitude of the EPC block caused by the guanidine derivatives is dependent on the membrane potential.

All the *n*-alkylguanidine derivatives used in this study caused the EPC-

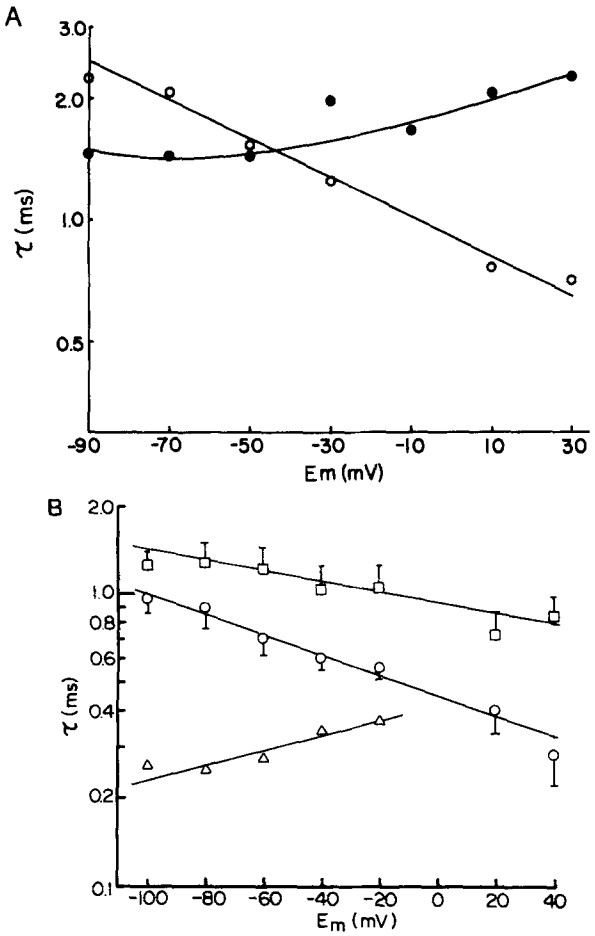


FIGURE 4. Logarithm of the time constant of EPC decay (τ) plotted as a function of membrane potential in control solution (○) and in the presence of 2.6 mM propylguanidine (●). Note that the voltage dependence of the EPC decay is reversed by propylguanidine. These data were obtained at a single endplate. (*B*) The effect of 0.13 mM amylguanidine. The logarithm of the time constant of decay (τ) is plotted against membrane potential. Open circles represent measurements in control solution, and the triangles and squares are the fast and slow components in 0.13 mM amylguanidine, respectively. The measurements are the means ± SEM of six experiments.

voltage (*I-V*) relationship to become extremely nonlinear under certain conditions (Fig. 3). It was observed, however, that the shape of the *I-V* curve obtained in the presence of octylguanidine differed, depending on the protocol used. This is illustrated in Fig. 6 *A*. The open circles represent the control *I-V*

relationship obtained 5 ms after each 2.2-s potential step from the holding potential of −50 mV. The control *I-V* curve is approximately linear, regardless of the time at which it is obtained after the beginning of voltage step. However, in the presence of octylguanidine, the curve was different, depending

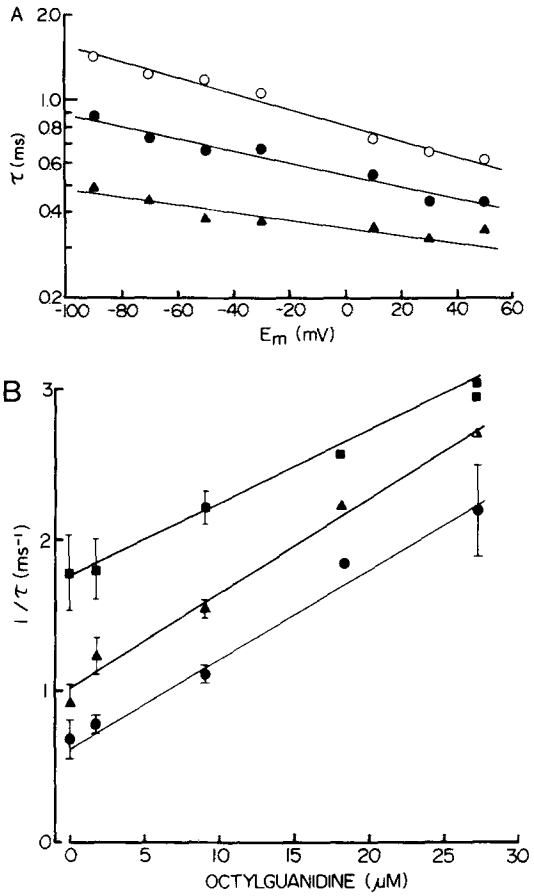


FIGURE 5. (*A*) The effect of octylguanidine on the voltage dependence of EPC decay time constant (τ). The open circles are the measurements in control solution. The filled circles and triangles represent the effect of 9.1 and 27.3 μM octylguanidine, respectively. The data were obtained at a single endplate. (*B*) The rates of EPC decay ($1/\tau$) as a function of the concentration of octylguanidine. The data were obtained at membrane potentials of −90 (●), −50 (▲), and +50 mV (■). The lines are least square fits to the data. The data represent the means ± SEM of at least three experiments.

on when an EPC is elicited after the beginning of each potential step. The *I-V* curve obtained 5 ms after each potential step is linear (closed circles), whereas the *I-V* curve obtained 2 s after each step is highly nonlinear (triangles). We envisage that the block of peak EPC measured 5 ms after the

potential step represents the sum of open-channel block associated with each potential step and the closed-channel block at −50 mV. The block measured at 2 s represents the sum of open- and closed-channel block at each potential step. That the *I-V* curve obtained 5 ms after each potential step is linear suggests the open channel block is voltage independent. It is interesting to note that the EPCs obtained 5 ms after the potential step exhibit less inhibition than those at 2 s at membrane potentials more negative than −50 mV. At positive membrane potentials the opposite relationship holds. The results strongly suggest that the closed-channel block is highly voltage dependent.

Block Development and Recovery

The time-course of block development was determined by eliciting an EPC at various times after the beginning of a 2-s voltage step. The membrane was held at −50 mV for 8 s between these episodes. This time interval was determined to be adequate for full recovery at −50 mV. When the membrane was hyperpolarized from −50 mV, there was a time-dependent increase in the blocking action of 10 μM octylguanidine, as shown in Fig. 6 *B* for membrane potentials of −70 and −90 mV.

The half-time of block development ranged from 200 to 500 ms with no clear voltage dependence. However, the steady-state level of block was highly voltage dependent. For example, at membrane potentials of −110, −90, and −70 mV, the ratios of steady-state (i.e., at 2 s) to initial (i.e., at 5 ms) EPC amplitude were 0.2, 0.4, and 0.7, respectively. Such voltage dependence is reflected in the *I-V* relationship as the difference obtained at 5 ms and 2 s after the voltage step.

When the membrane was depolarized from −50 to +30 mV in 10 μM octylguanidine, a time-dependent removal of block was observed. This is illustrated in Fig. 6 *C*. The half-time of recovery at +30 mV is 500–600 ms, and the ratio of steady-state to initial EPC amplitude ranges from 1.5 to 2. This removal of block is reflected in the *I-V* curves (obtained at 2 or 10 s) at membrane potentials positive to −50 mV (Fig. 6 *A*).

Amylguanidine (200 μM) also induced a time-dependent block, which developed with a half-time of ~300 ms at −100 and −90 mV. Thus, both amyl- and octylguanidine block the channel in a highly voltage-dependent manner independent of channel opening.

DISCUSSION

The potency and characteristics of the blocking action of the *n*-alkyl derivatives of guanidine vary with the length of the side chain. Methyl-, ethyl-, and propylguanidine cause inward EPCs to decay faster but prolong outward EPCs, similar to the action of amantadine (Tsai et al., 1978). Amylguanidine prolongs the inward currents and causes them to decay biphasically. Outward currents are also prolonged, but the decay can still be described by a single exponential function. These effects are similar to those of QX-222 and procaine (Ruff, 1977; Kordas, 1970; Deguchi and Narahashi, 1971; Adams, 1977). Octylguanidine causes both inward and outward EPCs to decay faster,

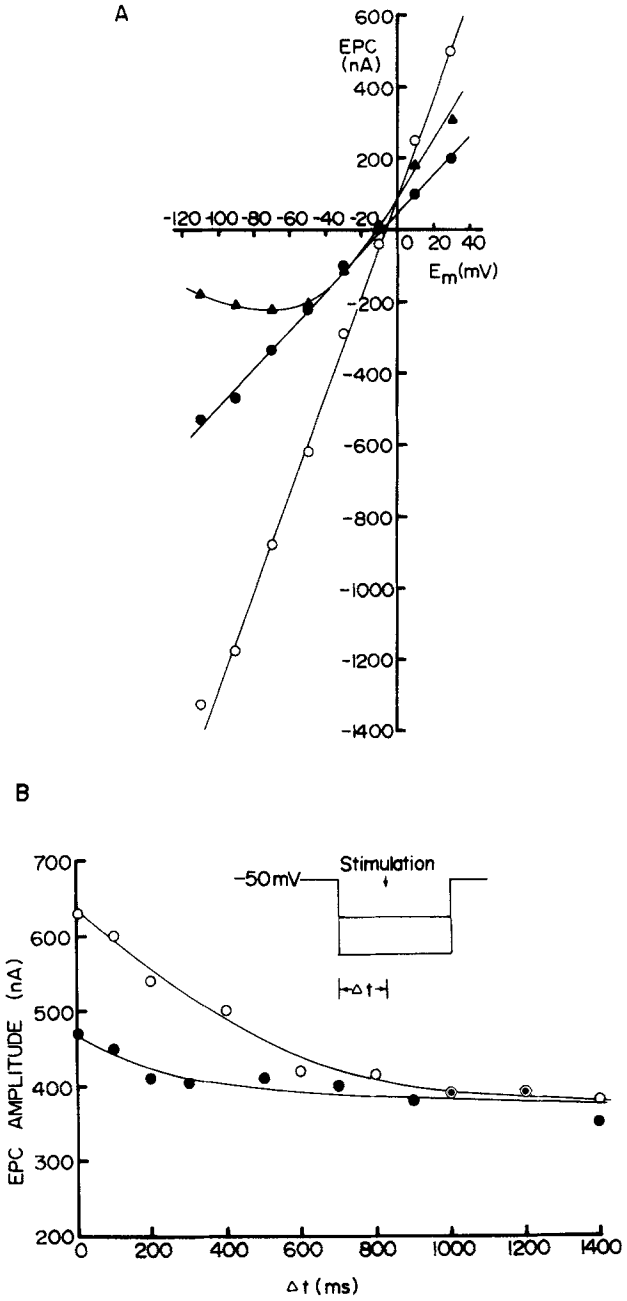


FIGURE 6. (*A*) Current-voltage relationships obtained from the control end-plate (○) and in the presence of 10 μM octylguanidine (● and ▲). The measurements represented by the closed and open circles were obtained by eliciting a single EPC 5 ms after the beginning of a 2.2-s voltage step, and the closed triangles by eliciting an EPC 2 s after the voltage step. (*B*) The time-course of the development of octylguanidine block. Peak EPC amplitude is

and the decay time-course remains single exponential. This is similar to the action of QX-314. All of the guanidine derivatives cause a marked nonlinearity of the current-voltage relationship, indicating that the block is voltage dependent. A nonlinear current-voltage relationship is also observed in the presence of local anesthetics, amantadine and histrionicotoxin (Deguchi and Narahashi, 1971; Tsai et al., 1978; Masukawa and Albuquerque, 1978), although in general the nonlinearity of the current-voltage relationship is not as great as that observed for the *n*-alkylguanidines.

The *n*-alkylguanidine derivatives present an excellent structure-activity relationship from which information about channel blocking action can be obtained. The obvious structural difference between these compounds is the

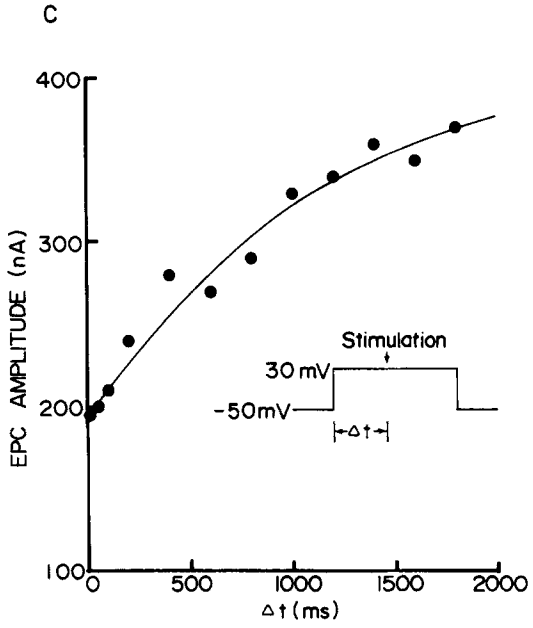


FIGURE 6

length of the alkyl side chain. It is also clear that the alkyl side chain affects the potency and kinetics of drug action. The blocking potency is enhanced by lengthening the side chain.

The relationship is similar to those observed for the block of sodium and potassium channels by quaternary ammonium derivatives (Rojas and Rudy, 1976; Shoukimas and French, 1979). These authors concluded that the observed increase in potency was related to the increase in side-chain hydrophobicity. This conclusion also seems reasonable as an explanation of the

plotted as a function of the time (Δt) after the beginning of a 2.2-s voltage step from −50 to −70 mV (●) or to −90 mV (○). Only one EPC is elicited during each step. (*C*) The time-course of the removal of octylguanidine block. The protocol is similar to *B* except the potential step is from −50 to +30 mV.

increase in potency with side-chain length in the alkylguanidine series. In support of this idea, the standard free-energy change of the blocking reaction for each methylene added can be calculated from the data on the effect of chain length on potency of the derivatives. One-to-one binding was assumed, and the ED_{50} values were used as estimates of the dissociation constant. The change in standard free energy is estimated to be 560 cal/mol of methylene. The theoretical free-energy change per methylene transferred from an aqueous to a hydrophobic environment is ~1,000 cal (Kauzmann, 1959). It is noteworthy that free-energy change per methylene for the interaction of *n*-alkylguanidine with the sodium channels of *Electrophorus electricus* (Reed and Trzos, 1979) and that of squid axons (Kirch et al., 1980) were found to be 590 and 650 cal, respectively. Rojas and Rudy (1976) also determined a similar free-energy change per methylene for interaction of tetraethylammonium derivatives with the sodium channels of squid.

The length of the alkyl side chain also alters dramatically the effects of these compounds on EPC amplitude and kinetics. These changes may arise from differences in their kinetics of interaction with the channels. It has been proposed that compounds such as QX-222 and QX-314 can bind to the channel only when it is open (Ruff, 1977; Neher and Steinbach, 1978). This is schematically represented by

$$\text{CLOSED} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{OPEN} \underset{F}{\overset{G}{\rightleftharpoons}} \text{BLOCKED}, \qquad \text{(Scheme 1)}$$

where CLOSED is the ACh bound receptor, channel nonconducting, OPEN the ACh bound receptor, channel conducting, BLOCKED the open but blocked state of channel, and G, F, α, and β the rate constants for the various reaction steps. The actions of the *n*-alkylguanidines will be compared with the predictions of this model.

One prediction of this model is that the amplitude of the EPC will only be reduced if there are alterations in the EPC kinetics. Both amyl- and octylguanidine decrease EPC amplitude before they measurably alter EPC decay. For example, at 1.8 μM, octylguanidine reduces EPC amplitude by 33% (at −100 mV) but has no effect on EPC kinetics. Preliminary results from noise analysis suggest that amylguanidine decreases the amplitude of the iontophoretically induced ACh current before it alters channel kinetics.[1]

A second prediction of this model is that if the rate of dissociation of the blocking compound from the channel is slow (as suggested for octylguanidine), iontophoretically induced ACh responses will be inhibited to a greater extent than will the EPC. However, octylguanidine reduces the EPC and iontophoretic ACh response to a similar degree. This suggests a process that affects the amplitude of the postjunctional response independent of the kinetics of that response.

Another feature of the sequential model is that the channel must be opened before it can be blocked. However, it is clear that amyl- and octylguanidine

[1] Farley, J. M., and C. H. Wu. Unpublished observation.

can block the channel in the absence of channel activation. This block is highly voltage dependent and develops or is relieved with a half-time of several hundred milliseconds after a hyperpolarizing or depolarizing voltage step. Therefore, the sequential blocking mechanism does not adequately describe the action of these compounds.

To further substantiate this idea, we have simulated EPCs using the model of Magleby and Stevens (1972) with the added feature that EPC kinetics can be modified by the action of channel blockers. To do this we incorporated the simple sequential blocking mechanism into their model.

Fig. 7 *A* is a family of computer-generated normal EPCs. These currents were generated by assuming that the driving function used by Magleby and Stevens (1972) had exponential rising and falling phases with 0.1- and 0.2-ms time constants, respectively, and that the reversal potential for the EPC was −5 mV. The rate of decay of the EPC was calculated from the equation $1/\tau = 1.6 \times 10^3 \exp(0.0083 \times E_m)\ s^{-1}$. Fig. 7 *B* shows a family of EPCs obtained from a voltage-clamped endplate. The model simulates the observed EPCs quite satisfactorily. The EPC in QX-222 and QX-314 were also adequately simulated by this model as well as the voltage dependence of their blocking action. The rate constants for the block and unblock of channels by QX-222 and QX-314 were taken from Neher and Steinbach (1978).

Octylguanidine was found to block EPCs by a pseudo-first-order reaction. The EPCs decay faster than in the control at all potentials in the presence of octylguanidine. The forward rate constant (G) for the block of EPCs by octylguanidine is estimated to be in the range of $4–6 \times 10^7\ M^{-1}\ s^{-1}$, which is slightly larger than that for QX-222 and QX-314 (Neher and Steinbach, 1978) but is of the same order of magnitude. The forward rate constant of block for octylguanidine is not voltage dependent, which is different from the effects of QX-222 (Neher and Steinbach, 1978; Ruff, 1977). To simulate the effect of octylguanidine on EPCs, a forward rate constant of $5 \times 10^7\ M^{-1}\ s^{-1}$ was used, and the reverse rate constant (F) was assumed to be much smaller than G, in this case $0.01\ s^{-1}$ for octylguanidine. This assumption seems reasonable, because the blocking action of octylguanidine follows pseudo-first-order kinetics. The currents predicted from these kinetic data are shown in Fig. 8 *A* for 9.1 μM octylguanidine. A family of EPCs experimentally obtained in the presence of 9.1 μM octylguanidine is shown in Fig. 8 *B*. The time-course of the currents in the presence of octylguanidine is simulated well, the decay rate being increased at all membrane potentials. This model also simulates the linear current-voltage relation for peak EPC measured 5 ms after each potential change (see Fig. 6 *A*), which probably represents the sum of the open-channel block at various potentials and the closed-channel block at −50 mV. However, the magnitude and voltage dependence of the steady-state block of peak currents measured at 2–10 s (Fig. 6 *A*) are obviously not well simulated by the model. The blocking potency and voltage dependence of the blocking action are greatly underestimated. For example, at −100 mV, 9.1 μM octylguanidine reduces the EPC amplitude by ~75%, whereas the model predicts only an ~10% reduction in amplitude. This vast difference is probably attributable to the closed-channel block shown in Fig. 6 *A*.

Amylguanidine causes inward currents to decay in two exponential functions. Such alterations in EPC time-course can be predicted by the sequential model (Adams, 1976; Ruff, 1977; Neher and Steinbach, 1978). Therefore, as a first approximation, we assume that the alterations in EPC decay arise

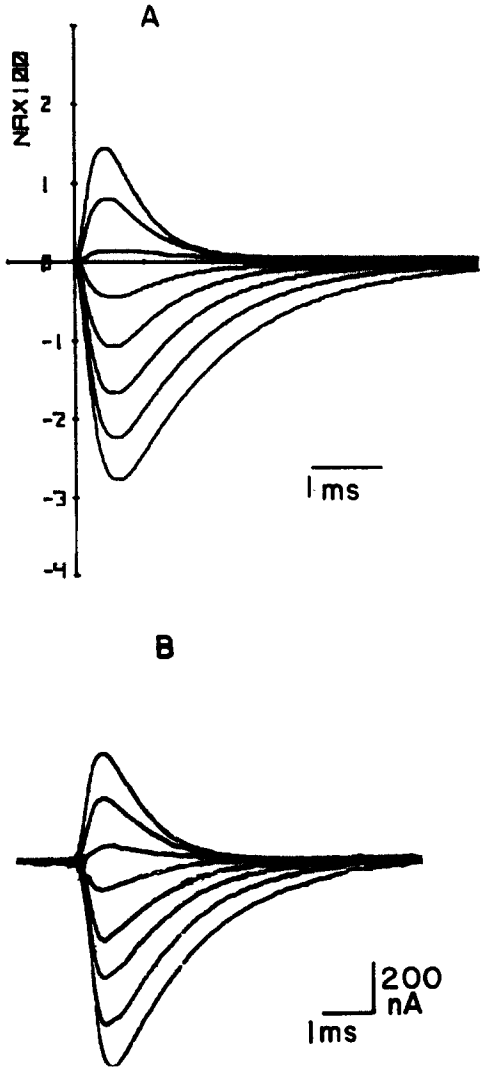


FIGURE 7. (*A*) Computer simulation of an EPC family using the model of Magleby and Stevens (1972). (*B*) An EPC family recorded at a voltage-clamped endplate (control). The membrane potential ranged from −100 to +40 mV in 20-mV steps for each family. See the text for further explanation.

mainly from open-channel block. If this is true then the forward and reverse rate constants can be estimated from the fast and slow rates of decay of the inward EPC by the relationships derived by Adams (1976), Beams (1976), and Ruff (1977). In the case of amylguanidine, the forward and reverse rate

constants of block are estimated to be $\sim 3 \times 10^6$ M^{-1} s^{-1} and 10^3 s^{-1}, respectively, at −100 mV. The forward rate constant for octylguanidine block is estimated to be on the order of $4\text{–}6 \times 10^7$ M^{-1} s^{-1}. The reverse rate constant, however, has to be much smaller in this case to obtain a single exponential decay and is assumed to be on the order of 0.01 s^{-1}. Thus, the length of the alkyl side chain apparently affects rate constants of the blocking reaction. The reverse rate constant appears to be greatly decreased as the side chain lengthens and the forward rate constant increases somewhat. Thus, the longer side-chain derivative comes off the channel much more slowly than does the shorter side-chain derivative.

However, it should be noted that the rate constants determined for the blocking action of amylguanidine will not even qualitatively predict the time-

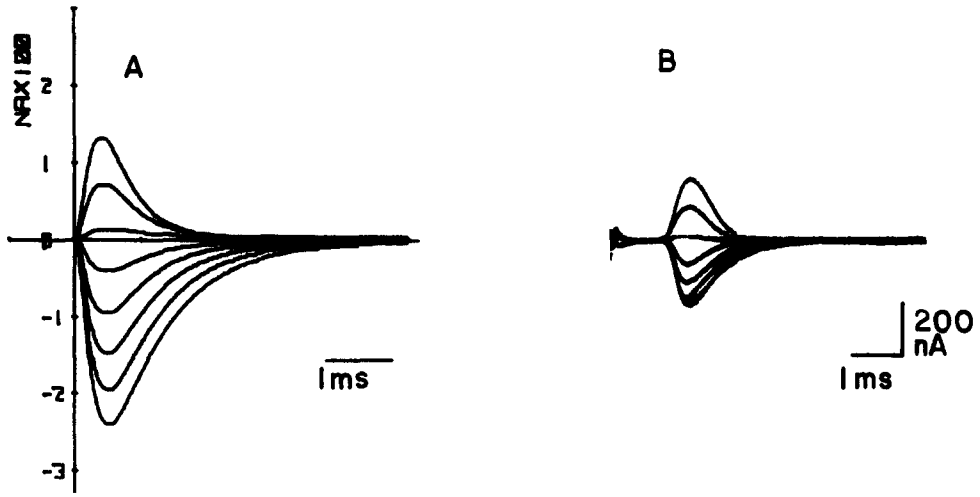


FIGURE 8. (*A*) Computer simulation of an EPC family in the presence of 9.1 μM octylguanidine assuming that it only interacts with the open channel. (*B*) An EPC family recorded at a voltage-clamped endplate in the presence of 9.1 μM octylguanidine. The membrane potential ranged from −100 to +40 mV in 20-mV steps. The current traces at −100 and −80 mV overlap.

course of the inward EPC altered by amylguanidine. To generate two exponential decay phases it is necessary to have a forward rate constant of $\sim 10^7$ M^{-1} s^{-1}. The reverse rate constant should be $< 10^4$ s^{-1}. Rate constants similar to these were estimated for the action of QX-222, which is known to cause the EPC to decay as the sum of two exponential functions (Ruff, 1977; Neher and Steinbach, 1978). An underestimate of the forward and reverse rate constants in the presence of amylguanidine could arise from two sources. First, the fast decay phase of the EPC is small, and thus the rate of decay is difficult to extract. Second, the transmitter release function may alter the fast rate constant of decay.

The outward currents in amylguanidine cannot be simulated adequately. To describe the fast and slow components of EPC decay, we made calculations

using our computer simulations and the equations derived by other investigators (Adams, 1976; Beams, 1976; Ruff, 1977). We presume that this slow decay of outward EPC arises from the same reaction scheme as that producing the slow inward EPC decay and that the initial fast component of EPC is indiscernible because of the fast dissociation constant, F. Thus, the outward EPC exhibits a single exponential decay. The sequential model will predict a prolongation of the EPC up to 2.5 times that of the control.

The time-course of EPC could be affected in other ways. For example, a drug could alter transmitter release, affect acetylcholinesterase activity (Goldner and Narahashi, 1974; Magleby and Terrar, 1975), or change the rate constant of channel closure, α (Gage et al., 1978). However, amylguanidine has no effect on acetylcholinesterase activity[2] and on transmitter release (Farley et al., 1979 *a*). It is not known whether the rate of channel closing is affected by amylguanidine.

Thus, it would seem that the sequential model, in its simplest form, cannot adequately predict the time-course of EPC altered by amylguanidine. As with octylguanidine, it is difficult to simulate the magnitude and voltage dependence of the block.

These findings suggest that the mechanisms by which amyl- and octylguanidine block the endplate channels cannot be totally described by the simple sequential model shown in Scheme 1 for the action of local anesthetics. Another mechanism of block must also be considered to explain the large voltage dependence and magnitude of the block. As suggested previously, it is possible that the alkylguanidines may also affect the closed channel such that it does not open or, if opened, that its conductance is greatly decreased. This is similar to "resting block" in the sodium channels in nerve. The closed-channel block must also be voltage dependent to explain the voltage dependence of the block that we have observed.

Adams (1977) has proposed that procaine blocks the resting state of the endplate ionic channel through a cyclic mechanism. The voltage dependence of this block was not determined, although it was suggested that it is less than that for block of the open channel. Masukawa and Albuquerque (1978) have shown that the block of the endplate channel by histrionicotoxin is voltage dependent and that part of the blocking action can occur in the absence of receptor activation. Thus, histrionicotoxin also acts by a mechanism that could be termed resting or closed-channel block. This type of block is intensified as the membrane is hyperpolarized. Tetraethylammonium has also been shown to have a similar blocking action on the closed channel (Adler et al., 1979).

The 10-fold difference in potency between amyl- and octylguanidine may be caused in part by differences in their ability to block the closed channel. It is unclear whether or not the shorter chain derivatives can block the closed channel, because transmitter release is increased at the same concentrations at which the EPC is blocked (Farley et al., 1979 *a*). It would be interesting to

[2] Phillips, T., J. M. Farley, and A. M. Boyne. Unpublished observation.

determine whether the amount of closed channel block is a function of the alkyl side-chain length.

The lack of voltage dependence of the forward rate and of the magnitude of the proposed open-channel block by octylguanidine suggests that it does not penetrate far into the potential field of the membrane when blocking the channel. On the other hand, the large voltage dependence of closed channel block (Fig. 6 *A*) suggests that a large fraction of the membrane field must be crossed to reach the blocking site. This implies that two separate blocking sites exist, one near the mouth of the channel and the other well into the membrane field. However, we cannot rule out the possibility that octylguanidine binds to a single site because the voltage profile across the channel could differ for the closed and the open configurations.

Adams (1977) has proposed that procaine blocks the ACh channel by a cyclic mechanism in which procaine could reach the same blocking site in both the closed and the open channel by separate pathways. Hille (1977) has proposed that two pathways exist for the block of the open and the closed sodium channel of nerve membranes by local anesthetics. Thus, it is also plausible that two separate pathways exist for the channel block by alkylguanidines.

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REFERENCES

ADAMS, P. R. 1976. Drug blockade of open end-plate channels. *J. Physiol. (Lond.)* **260:**531–552.

ADAMS, P. R. 1977. Voltage jump analysis of procaine action at frog end-plate. *J. Physiol. (Lond.)* **268:**291–318.

ADAMS, P. R., and B. SAKMANN. 1978. Decamethonium both opens and blocks end-plate channels. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2994–2998.

ADLER, M., A. C. OLIVERIA, M. E. ELDEFRAWI, A. T. ELDEFRAWI, and E. X. ALBUQUERQUE. 1979. Tetraethylammonium; voltage dependent action on end-plate conductance and inhibition of ligand binding to postsynaptic proteins. *Proc. Natl. Acad. Si. U.S.A.* **76:**531–535.

BEAMS, K. G. 1976. A quantitative description of end-plate currents in the presence of two lidocaine derivatives. *J. Physiol. (Lond.)* **258:**301–322.

CHANG, C. C., and C. Y. LEE. 1963. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch. Int. Pharmacodyn. Ther.* **144:**316–332.

DEGUCHI, T., and T. NARAHASHI. 1971. Effects of procaine on ionic conductances of end-plate membranes. *J. Pharmacol. Exp. Ther.* **176:**423–433.

DEL CASTILLO, J., and G. E. DE MOTTA. 1977. Influence of succinic anhydride on the decay of end plate currents evoked by high frequency stimulation. *Soc. Neurosci. Abstr.* **3:**371.

FARLEY, J., M. GLAVINOVIC, S. WATANABE, and T. NARAHASHI. 1979 *a*. Stimulation of transmitter release by guanidine derivatives. *Neuroscience.* **4:**1511–1519.

FARLEY, J., S. WATANABE, J. YEH, and T. NARAHASHI. 1979 *b*. Mechanism of end-plate channel block by n-alkyl guanidine derivatives. *Biophys. J.* **25**(2, Pt. 2):16 *a*. (Abstr.).

GAGE, P. W., R. N. MCBURNEY, and D. VAN HELDEN. 1978. Octanol reduces end-plate channel lifetime. *J. Physiol. (Lond.)*. **274**:279–298.

GIOVANNETTI, S., M. BIAGINI, P. L. BALESTRI, R. NAVALESI, P. GIAGNONI, A. DE MATTEIO, P. FERRO-MILONE, and C. CORFETTI. 1969. Uremialike syndrome in dogs chronically intoxicated with methylguanidine and creatinine. *Clin. Sci. (Oxf.)*. **36**:445–452.

GOLDNER, M. M., and T. NARAHASHI. 1974. Effects of edrophonium on end-plate currents in frog skeletal muscle. *Eur. J. Pharmacol.* **25**:362–371.

HILLE, B. 1975. The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. *Biophys. J.* **15**:615–619.

HILLE, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* **69**:497–515.

KAUZMANN, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14**:1–63.

KIRSCH, G. E., J. Z. YEH, J. M. FARLEY, and T. NARAHASHI. 1980. Interaction of *n*-alkylguanidines with sodium channels of squid axon membrane. *J. Gen. Physiol.* **76**:315–335.

KORDAS, M. 1970. The effect of procaine on neuromuscular transmission. *J. Physiol. (Lond.)*. **209**: 689–699.

KUSANO, K. 1970. Effect of guanidine on the squid giant synapse. *J. Neurobiol.* **1**:459–469.

MAGLEBY, K. L., and C. F. STEVENS. 1972. A quantitative descritpion of end-plate currents. *J. Physiol. (Lond.)*. **223**:173–197.

MAGLEBY, K. L., and D. A. TERRAR. 1975. Factors affecting the time course of decay of end-plate currents. A possible cooperative action of acetylcholine on receptors at the frog neuromuscular junction. *J. Physiol. (Lond.)*. **244**:467–495.

MASUKAWA, L. A., and E. X. ALBUQUERQUE. 1978. Voltage- and time-dependent action of histrionicotoxin on the endplate current of the frog muscle. *J. Gen. Physiol.* **72**:351–367.

NARAHASHI, T., J. W. MOORE, and W. R. SCOTT. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* **47**:965–974.

NEHER, E., and J. H. STEINBACH. 1978. Local anesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol. (Lond.)*. **277**:153–176.

OTSUKA, M., and M. ENDO. 1960. The effects of guanidine on neuromuscular transmission. *J. Pharmacol. Exp. Ther.* **128**:273–282.

REED, J. K., and W. TRZOS. 1979. Interactions of substituted guanidines with the tetrodotoxin-binding component in *Electrophorus electricus*. *Arch. Biochem. Biophys.* **2**:414–422.

RITCHIE, J. M., and R. B. ROGART. 1977. The binding of saxitoxin and tetrodotoxin to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.* **79**:1–50.

ROJAS, E., and B. RUDY. 1976. Destruction of the sodium conductance inactivation by a specific protease in perfused nerve fibres from *Loligo*. *J. Physiol. (Lond.)*. **262**:501–531.

RUFF, R. L. 1977. A quantitative analysis of local anaesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol. (Lond.)*. **264**:89–124.

SHOUKIMAS, J. J., and R. J. FRENCH. 1979. The effects of symmetrical quaternary ammonium ions of varying size on K-channel conductance. *Biophys. J.* **25**(2, Pt. 2):307 *a*. (Abstr.).

TAKEUCHI, A., and N. TAKEUCHI. 1959. Active phase of frog's end-plate potential. *J. Neurophysiol.* **22**:395–411.

TERAVAINEN, H., and A. LARSEN. 1975. Effects of guanidine on quantal release of acetylcholine in mammalian myoneural junction. *Exp. Neurol.* **48**:601–609.

Tsai, M.-C., N. A. MANSOUR, A. T. ELDEFRAWI, M. E. ELDEFRAWI, and E. X. ALBUQUERQUE. 1978. Mechanism of action of amantadine on neuromuscular transmission. *Mol. Pharmacol.* **14**:787–803.

WATANABE, S., and T. NARAHASHI. 1979. Cation selectivity of acetylcholine-activated ionic channel of frog endplate. *J. Gen. Physiol.* **74**:615–628.

WATANABE, S., J. FARLEY, and T. NARAHASHI. 1978. End-plate block by guanidine derivatives. *Soc. Neurosci. Abstr.* **4**:375.