MOLECULAR PHARMACOLOGY, 15, 559-580

Voltage-Dependent and Voltage-Independent Blockade of Acetylcholine Receptors by Local Anesthetics in *Electrophorus* Electroplaques

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(Received June 19, 1978)
(Accepted November 21, 1978)

SUMMARY

KOBLIN, DONALD D. & LESTER, HENRY A. (1979) Voltage-dependent and voltage-independent blockade of acetylcholine receptors by local anesthetics in *Electrophorus* electroplaques. *Mol. Pharmacol.*, **15**, 559-580.

This study employs voltage-clamp techniques to survey the action of several local anesthetics on the nicotinic acetylcholine receptor of *Electrophorus* electroplaques. These drugs partially block the steady-state activation of receptors during bath application of agonist. Certain anesthetics alter the kinetics of voltage-jump relaxations during such application and alter the waveform of neurally-evoked postsynaptic currents. Procaine, tetracaine, and QX-222 act similarly at concentrations of 25-50 μM. When the agonist is carbamylcholine, acetylcholine, or suberyldicholine, the steady-state blockade depends on voltage, with a greater fractional inhibition of agonist-induced currents at more negative membrane potential. Alterations of voltage-jump relaxations are most noticeable with suberyldicholine. Under some conditions, at least two exponential relaxation components are seen; one component is much faster and another much slower than the single component seen in the absence of procaine. In contrast, the percentage blockade by dibucaine and chlorpromazine is about the same at all membrane potentials, and these compounds have little influence on suberyldicholine relaxation kinetics. The uncharged local anesthetic, benzocaine, at a concentration ten times that of the other local anesthetics employed (250 μM), only slightly decreases suberyldicholine-induced currents and has little effect on the relaxation rates. Thus, voltage affects the relative potencies of local anesthetics in blocking agonist-induced currents. At low negative membrane potentials, the potency of the anesthetics parallels their lipid solubility. At high negative membrane potentials this correlation disappears. The results suggest a dual mode of action for the local anesthetics: an indirect interaction with the lipids surrounding the receptor, and a direct, voltage-dependent interaction with the receptor-channel complex.

INTRODUCTION

Recent studies provide new insights on the gating of ion channels by nicotinic ACh[2] receptors. Channel opening and closing rates are revealed by electrophysiological studies on spontaneous and evoked postsynaptic currents (1-5), by fluctuation analysis (6-9), by voltage-jump relaxations (10-14), by recordings of single channel events (15), and by agonist concentration jumps (16). Such studies yield results in essential agreement.

Further information concerning the ki-

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[2] The abbreviations used are: ACh, acetylcholine; Carb, carbachol; Sub, suberyldicholine; MSF, methanesulfonyl fluoride; PSC, postsynaptic current.

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netics of ACh receptor function is obtained by altering the time course of postsynaptic currents with anesthetic agents (for reviews, see refs. 17 and 18). Furukawa (19) first observed that procaine induced a fast initial component and a slow late component of the endplate potential. Steinbach (20) later showed that the actions of several lidocaine derivatives on the time course and amplitude of the endplate potentials were unrelated to their ability to block conduction in nerve. More recent investigations have led to detailed molecular models for local anesthetic action on ACh receptors (21–27).

The present investigation employs *Electrophorus* electroplaques and examines the action of several local anesthetics a) on neurally evoked postsynaptic currents, and b) on voltage-jump relaxations during steady application of cholinergic agonists (12, 13, 28). We find that certain local anesthetics (procaine, tetracaine, QX-222) but not others (benzocaine, dibucaine, chlorpromazine) alter relaxation kinetics in a manner strongly dependent on membrane potential. In addition, at low negative potentials there is a correlation between the lipid solubility of the anesthetics and their ability to block acetylcholine sensitivity. In contrast, at large negative membrane potentials, the blockade is due primarily to a charge-sensitive mechanism.

METHODS

Isolated electroplaques from the organ of Sachs of *Electrophorus electricus* were employed in these experiments as described previously (13). The cell was mounted to expose the innervated face to one pool of Ringer's solution (pool A); the noninnervated face was exposed to another pool (B). The Ringer's solution in pool B contained 6 mM glucose; that in pool A contained 3 mM Ba^{++} to maintain the anomalous rectifier in a linear, low-conductance state (29). For the voltage-jump experiments with bath-applied agonists, pool A also contained 5×10^{-7} M tetrodotoxin to eliminate Na^+ activation.

Chemicals. Procaine, tetracaine, ACh, Carb[2] and TTX were products of Sigma. Dibucaine, chlorpromazine, and QX-222 were generous gifts from CIBA, Smith Kline and French, and Astra Pharmaceuticals, respectively. The tertiary amine local anesthetics were in the form of hydrochlorides and QX-222 was the chloride salt. Benzocaine and MSF were obtained from Eastman. Sub[2] was kindly provided by J. Heesemann.

Bath application experiments. Drugs were applied in pool A (0.5 ml) with a push-pull syringe system; 10 ml of the desired solution was transferred through the pool in approximately a 10 second period. Measurements were taken when the agonist-induced currents reached a plateau value; this required from 30 sec to 5 min depending upon the cell and the agonist examined. Immediately after the voltage-jump measurements were taken the cell was washed with Ringer's solution. Upon washing, the agonist-induced currents disappeared and the membrane potential was allowed to recover before the next bath application. The concentrations of agonists used in these experiments (50 μM or 60 μM carbamylcholine, 2 μM suberyldicholine, 10 μM ACh) were chosen because they produce an easily measurable response but cause little desensitization in the time period examined.

For experiments involving bath application of ACh, acetylcholinesterase was inactivated by exposing the innervated face of the electroplaque to 10 mM MSF[2] for 1 hr at 15°, followed by at least four washes with Ringer's solution. In addition, several cells tested with Sub were pretreated with MSF. Although the response to Sub varied from cell to cell, the potency of this agonist seemed to be greatest in those cells treated with MSF, suggesting that suberyldicholine may be a substrate for the esterase. Qualitatively, however, the results obtained with Sub did not depend upon the functional state of the esterase.

For experiments involving local anesthetics, the procedure was as follows: First, the agonist was applied in pool A and the voltage-jump relaxations were measured. The agonist was washed out with Ringer's solution and the preparation was preincubated for 10 min with the desired local anesthetic. None of the anesthetics significantly altered the membrane potential or

the resting conductance. Next, the agonist was applied with the anesthetic, the relaxations measured, and the preparation washed. Finally the agonist alone was reapplied to test for reversibility. Analyses were performed only on those cells in which the resting potentials showed little or no decline during the course of the experiment. In all, over 100 cells were examined. Experiments were carried out near 15°.

Measurement of agonist-induced currents. In a voltage-jump relaxation experiment, agonist is applied to the solution bathing the electroplaque and the membrane voltage is stepped from one value to another under feedback control. Following this voltage step, the agonist-induced conductance changes on a millisecond time scale. In most of the experiments passive and capacitative currents were subtracted with the "single-trial" method, which is particularly useful for measurements of small agonist-induced currents. This method has been described in detail in an earlier paper (13). The single-trial method is a slight modification of the procedure employed to search for gating currents in squid axons (30). We have found currents with similar properties in electroplaques. They largely precede Na-channel ionic currents, have voltage- and temperature-dependent kinetics, and are abolished by sustained depolarization. In the present experiments, these gating currents rendered it especially difficult to analyze relaxation kinetics for the small, rapid agonist-induced currents during exposure to some of the drugs.

Multiple exposure of a cell to agonist. During these experiments, we noted that the responses to the first application of agonist differed consistently from the later ones. Therefore, we devoted several experiments to examining the reproducibility of kinetic measurements during repeated exposure of a cell to a given concentration of agonist.

Figure 1 shows the depolarizing action of 50 μM Carb applied four times in succession to a single cell. During each agonist application, the cell depolarizes approximately 40 mV after a 4 min period. Upon washing with Ringer's solution, the membrane potential recovers almost completely to its initial value. These results resemble those described in the earlier literature (31).

Despite the rather constant depolarizing action of 50 μM Carb, the agonist-induced currents and voltage-jump relaxation time constants change markedly with each bath application (Fig. 2). The steady-state agonist-induced currents progressively decrease and the reciprocal relaxation time constants $1/\tau$ progressively increase. All four trials are summarized in Table 1.

A decrease in steady-state currents was also found with repetitive bath application of the agonists ACh and Sub (Table 2). For all three agonists, the agonist-induced currents during the second exposure are approximately 25% smaller than those measured during the first exposure to agonist. Although repetitive Carb applications usually result in increased reciprocal time constants, no significant changes in time constants are found with repetitive Sub or ACh bath applications.

The data of Fig. 2 exemplify some of the most dramatic changes seen in steady-state currents and in relaxation kinetics with repetitive agonist application. It should be emphasized that with many cells responses are essentially constant beginning with the second or third exposure. Nevertheless, in experiments which involve several bath applications of agonist, the effects of multiple exposure to a cell must be considered and the responses are smaller than for a fresh cell.

RESULTS

1. *Voltage-Dependent Blockade with Large Kinetic Changes*

Procaine: action on postsynaptic currents. In control cells, neurally-evoked PSCs[2] (Fig. 3) generally conformed to an earlier description (13). The PSC decays have approximately exponential time courses; the rate constants for decay, α, decrease as the voltage becomes more negative. The PSC decay rates were estimated by a least-squares fit to the slopes of the semilogarithmic plots shown in the bottom of Fig. 3. At the most hyperpolarized potentials, the waveform sometimes deviated

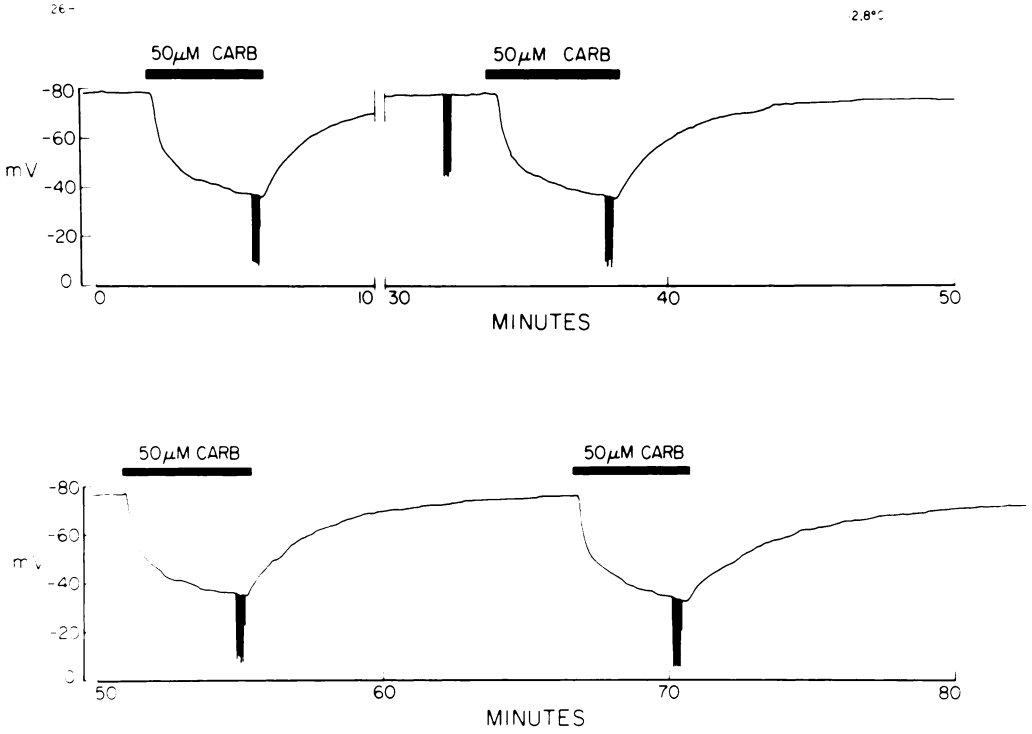


FIG. 1. *Membrane depolarization during successive bath applications of 50 μM Carb*
Carb was present during periods indicated by the black horizontal bars. Voltage-clamp episodes are indicated by the groups of rapid deflections.

from a single exponential decay. A similar effect was noted for frog endplate currents (1).

In procaine, the PSC decays more rapidly (Fig. 3). The decays seem to remain exponential in the presence of procaine. A very slow terminal current phase, such as that found at the frog endplate, either is not present or accounts for less than 10% of the PSC. The decay phase becomes progressively faster with increasing procaine concentration (Fig. 4).The fractional increase in rate constant is greatest at high negative potentials, and in the presence of 100 μM procaine, the decay rates are approximately four-fold greater than in the control. The action of procaine is mostly reversed upon washing for 30 min.

Voltage-jump relaxations. Procaine markedly alters the response to Sub (Fig. 5A). There is a marked decrease in equilibrium agonist-induced currents and voltage-jump relaxations have a rapid component. These effects of procaine are partially reversible upon washing.

The Sub relaxations in the presence of procaine were further analyzed on a faster time scale (Fig. 5B). For some voltages, at least two relaxation components are seen. One component is very slow and appears as a sloping baseline in a few of the traces. This slow component distorted the I-V plot (Fig. 5c) for Sub in the presence of procaine, because in several cases the currents do not quite reach a steady-state value on the time scale of Fig. 5B. However, we have performed the same experiments over a time scale long enough to attain complete equilibria, and the I-V plot has essentially the same shape as the one shown in Fig. 5C. It is clear from such data that procaine causes a large decrease in Sub-induced currents and that the fractional inhibition is greatest at high negative potentials (11, 28).

For all Sub relaxations in the presence of procaine, there is an initial fast conduct-

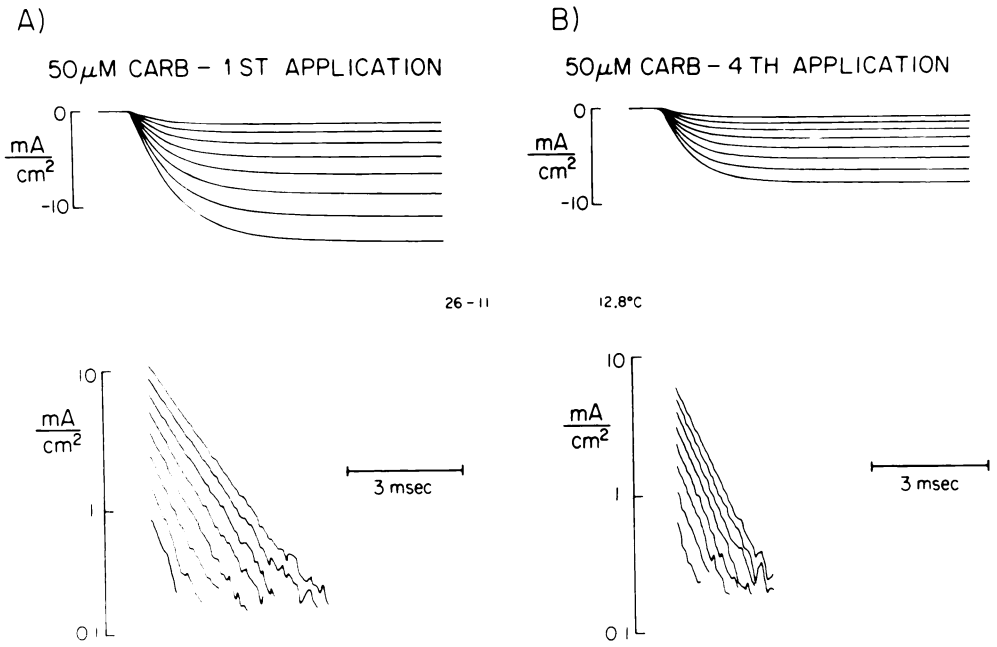


FIG. 2. *Agonist-induced currents measured during (A) the first application, and (B) the fourth application of 50 μM Carb in the experiment of Fig. 1*

The top traces are the relaxations for voltage jumps from +50 mV to −70, −85, −100, −115, −130, −145, −160, and −175 mV. The bottom traces show semilogarithmic plots of the approach to steady state. Slopes define the reciprocal time constant $1/\tau$. Same time axis applies to upper and lower panels.

TABLE 1

Alterations in equilibrium currents and relaxation rates upon repetitive application of 50 μM carbamylcholine

mV[a]	−I (mA/cm²)[b]				1/τ (msec⁻¹)[c]			
	1st[d]	2nd	3rd	4th	1st	2nd	3rd	4th
−85	2.9	2.4	2.4	1.9	0.86	0.85	0.80	0.86
−115	5.7	4.6	4.4	3.6	0.57	0.61	0.80	0.80
−145	9.7	7.7	6.9	5.7	0.50	0.60	0.73	0.76
−175	14.8	11.5	9.9	8.2	0.56	0.56	0.70	0.72

[a] Potential jump was from +50 mV to indicated voltage.
[b] Magnitude of equilibrium agonist-induced currents.
[c] Relaxation rate constants; standard errors are less than 8% of any given value.
[d] Number of exposures to carbamylcholine. Eel 26–11; temp. = 12.8°.

ance increase. This component's rate constant was calculated (Fig. 5C) by using the slow relaxation component as a sloped baseline. The values given in Fig. 5C are the best fits to a single exponential and are only approximate. The rate constant for this component is roughly an order of magnitude faster, and its amplitude several times smaller, than for the relaxation seen in the absence of procaine.

Procaine also markedly alters the response to ACh relaxations and reduces the equilibrium ACh-induced currents (Fig. 6). As with Sub, at some potentials the drug induces at least two exponential voltage-jump relaxations. Again, the rate constant for one component is much faster, and for another much slower, than for the relaxation in the absence of procaine. The increase in rates and the decrease in ACh-

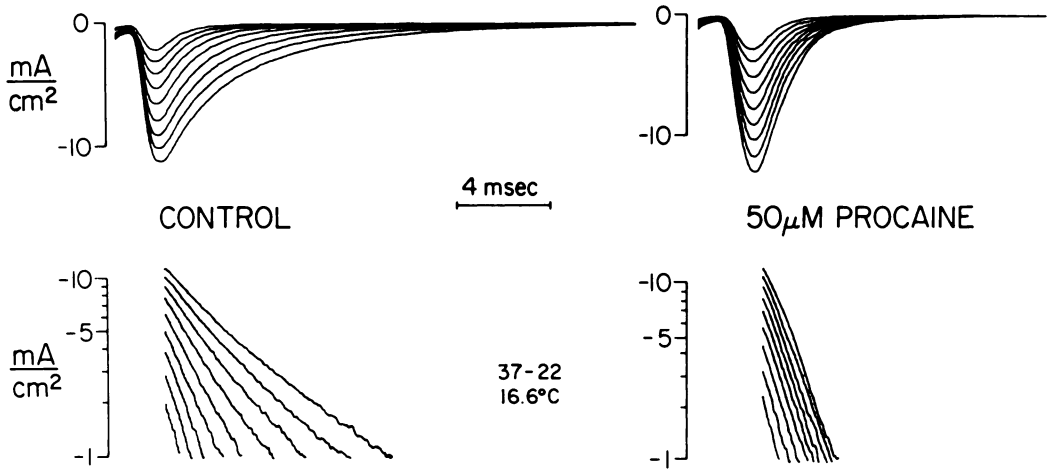


FIG. 3. *Postsynaptic currents in the absence (left) and in the presence (right) of 50 μM procaine*
The top traces are superimposed PSCs at membrane potentials from −25 to −160 mV, in −15 mV increments. Passive currents have been subtracted. The bottom traces show semilogarithmic plots of the decay. Same time axis applies to the upper and lower panels.

TABLE 2

Percentage decrease in equilibrium agonist-induced currents with repetitive bath application

Agonist	$\frac{I_1 - I_2}{I_1}^{a} \times 100$	n[b]
Carb (50 μM)	24 ± 9.4[c]	12
Sub (2 μM)	19.6 ± 9.8	13
ACh (10 μM)	29 ± 9.2	3

[a] I_1 is the equilibrium agonist-induced current measured at −175 mV during the first bath application of agonist to a cell. I_2 is the current measured at −175 mV during the second bath application.
[b] n = number of cells.
[c] ±SD.

induced currents is greatest at high negative potentials. All these effects are partially reversible upon washing.

Procaine affects Carb relaxations less than Sub or ACh relaxations (11). At a concentration of 25 μM, procaine decreases the Carb-induced currents by 60% at −100 mV and by slightly more at high negative potentials. The Carb relaxations seem to remain exponential in the presence of procaine, but the reciprocal relaxation times increase. This increase is more marked with increasingly negative potentials, the reciprocal relaxation times never exceeding twice the control values. The decrease in currents and the increase in rate constants are partially reversible upon washing. The

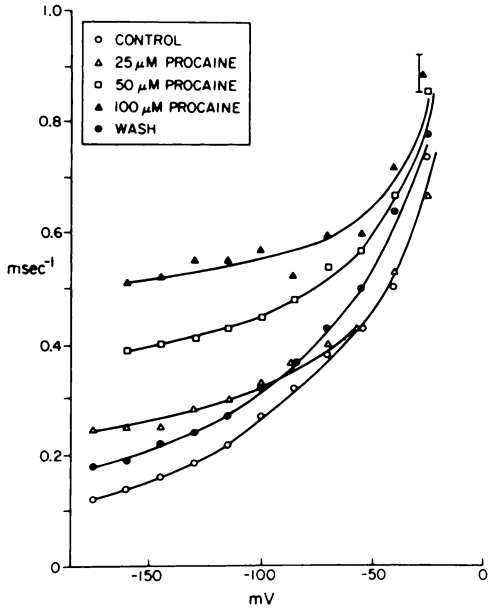


FIG. 4. *Plots of PSC decay rates versus potential from an experiment to test the effect of various procaine concentrations*

effect of procaine increases with concentration; however, at high procaine concentrations (>100 μM), the Carb-induced currents are too small for an accurate measurement of the rate constants.

Several sources of uncertainty made it impractical to characterize completely the

two or more components of voltage-jump relaxations in the presence of procaine. Firstly, the time course and magnitude of the multicomponent relaxations varied considerably from cell to cell and even from one trial to another on the same cell. Secondly, even without procaine there were often changes in relaxation kinetics and in steady-state currents from one agonist application to the next (see METHODS). Finally, small currents and fast relaxations were often distorted by the presence of gating currents. Nonetheless, these observations on procaine qualitatively agree with Adams' data at frog endplates. At concentrations of 10–100 μM, procaine decreases steady-state currents. Voltage-jump relaxations have two components, one faster and the other slower than seen in the absence of procaine, and the effect of procaine increases at more negative voltages.

Although the conductance change of a voltage-jump relaxation depends on both the initial and final voltages, the rate constants depend only on the final voltage (13). In the study by Adams (11), the hyperpolarizing voltage-jump started from −60 to −90 mV; thus some channels were already open and the fast relaxation involved a conductance decrease. In the present study, the jump started with very few open channels since the first voltage was +50 mV. Therefore, the fast relaxation involved a conductance increase.

The following experiments examine the action of other local anesthetics, possessing different physical-chemical properties, on the kinetics of ACh-receptor function. Suberyldicholine was employed in most of the bath application studies, since this agonist showed the most dramatic changes with procaine.

Tetracaine. Tetracaine greatly decreases the magnitude of the Sub-induced currents and produces a fast component of the relaxation (Fig. 7). A complete I-V plot shows that tetracaine exerts a voltage-dependent blockade; the fractional inhibition is largest at high negative potentials (Fig. 7B). Other experiments show that 25 μM tetracaine has qualitatively the same effect as 25 μM procaine in increasing the rates and decreasing the steady-state currents for Carb relaxations. Thus, tetracaine affects agonist-induced currents like procaine and at approximately the same concentration.

PSCs are not observed at a tetracaine concentration of 25 μM, presumably because the drug blocks impulse propagation in the motor nerve terminals. At a lower concentration (10 μM), tetracaine slightly increases the rate of the PSC decays.

QX-222. QX-222 (Fig. 8) is a derivative of lidocaine containing a permanently charged quaternary amine. At a concentration of 25 μM, QX-222 greatly decreases the Sub-induced currents and there are at least two exponential components in the Sub relaxation (Fig. 8, middle). One component has a fast time course, similar to that observed with Sub relaxations in the presence of procaine or tetracaine, with a rate constant on the order of one to two $msec^{-1}$. Another component has a very slow time course and appears as a downward sloping baseline on the trace at −85 mV, and as an upward sloping baseline at the most negative potentials (−175 mV). The Sub relaxation seen at −175 mV in the presence of QX-222 actually intersects the relaxations seen at the less negative membrane potentials. Such "inverse" relaxations have also been observed at frog endplates treated with quinacrine (32) and at *Aplysia* neurons in the presence of procaine (27). An accurate calculation of these components is vitiated by the difficulties given above (section on procaine). Upon washing, the action of QX-222 is largely reversible (Fig. 8, bottom).

QX-222 (50 μM) also markedly alters the PSC decays. At voltages more positive than −55 mV, the PSC decays seem to remain exponential and there is little change in PSC decay rates compared to the control values. At voltages near −100 mV, at least two components are present in the PSC decays (Fig. 9). The rate constant for one component is faster, and another slower, than in the control. At high negative potentials (<−130 mV), the prolonged component has an extremely small amplitude and becomes difficult to measure.

II. Voltage-Independent Blockade with Smaller Kinetic Effects

Dibucaine. Dibucaine (25 μM) strongly

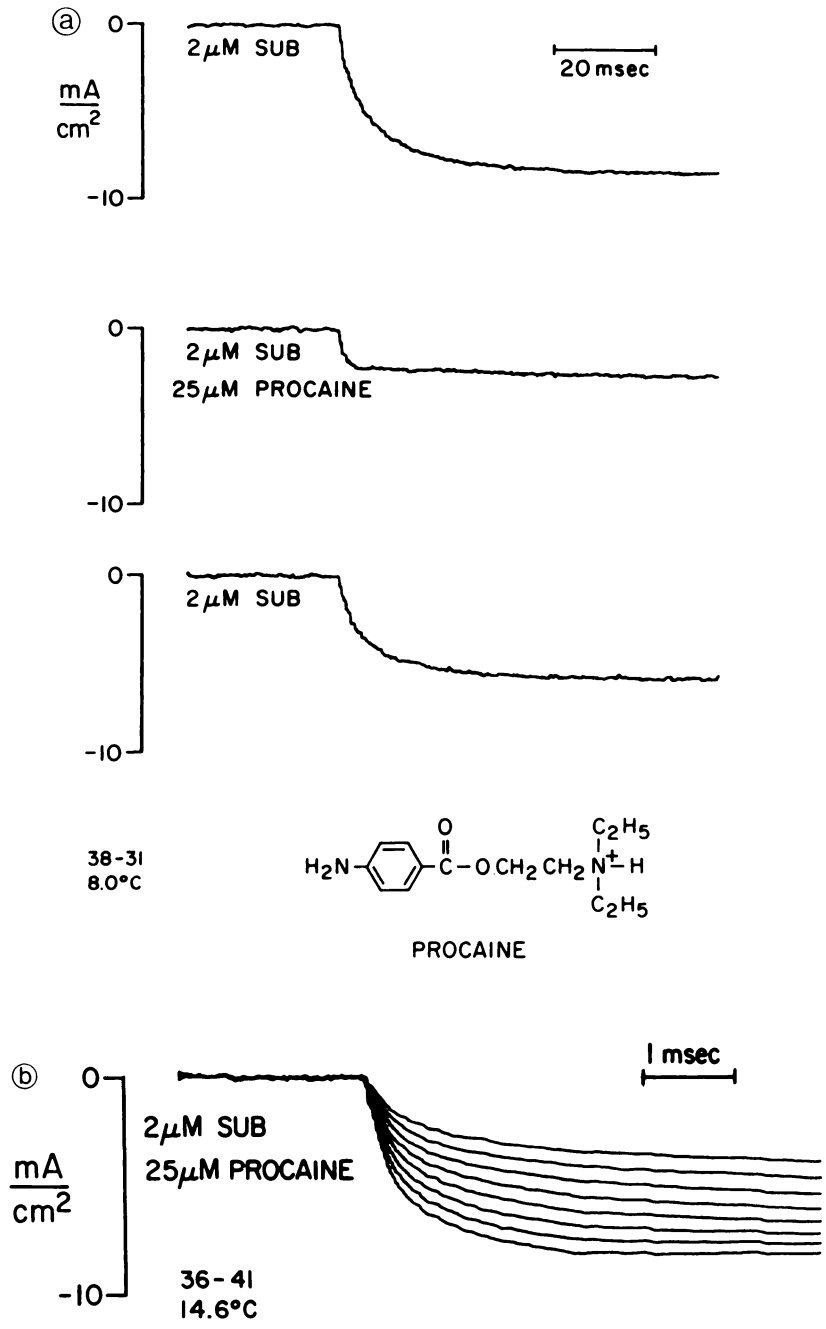
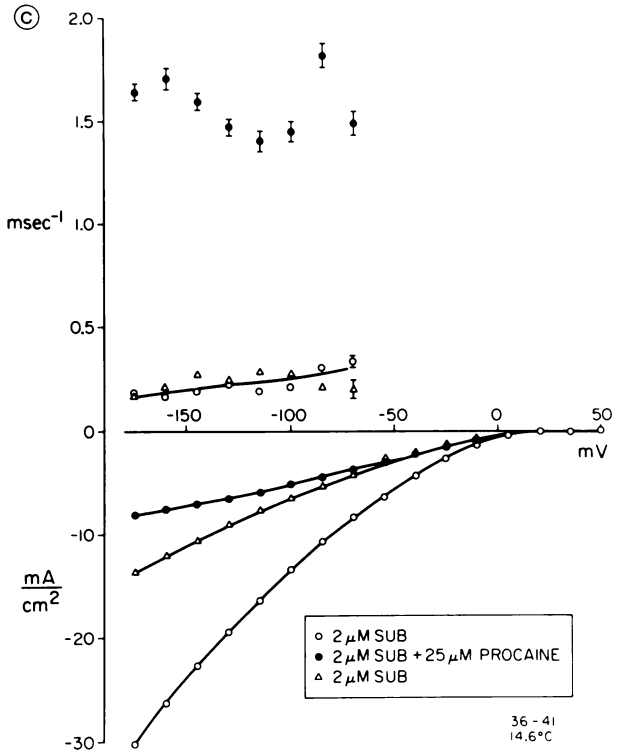


FIG. 5. *Action of procaine on voltage-jump relaxations in the presence of Sub*

(A) Voltage-jump relaxations with 2 μM Sub before (top), during (middle), and after (bottom) exposure to 25 μM procaine. Voltage was jumped from +50 mV to −175 mV. MSF treated cell. (B) Relaxations in the presence of 2 μM Sub + 25 μM procaine on another cell, over a range of final membrane potentials (−70 mV to −175 mV; −15 mV intervals). Note the much briefer time scale than in (A). (C) Steady-state Sub-induced currents (bottom) and reciprocal time constant of relaxations (top) as a function of membrane potential. Cell of panel (B).



suppresses the equilibrium Sub-induced currents (Fig. 10). This local anesthetic has a variable effect on the Sub-relaxation kinetics: the reciprocal relaxation times increased by one- to three-fold, depending on the membrane potential and the cell examined. For the experiment shown in Fig. 10, 25 μM dibucaine increased the reciprocal relaxation time at −175 mV by approximately a factor of two. At this concentration of dibucaine, the Sub-induced currents are so small that it becomes difficult to analyze the relaxation rates except at the most hyperpolarized potentials. Lower concentrations of dibucaine (10 μM) result in larger agonist-induced currents, but have little effect on the relaxation rates. Thus, dibucaine decreases agonist-induced currents in a manner similar to procaine and tetracaine, yet has less effect than these anesthetics on the kinetics of voltage-jump relaxations.

Dibucaine abolished PSCs at a concentration of 10 μM, probably by blocking impulse propagation in the motor nerve terminals. Lower concentrations of dibucaine had essentially no effect on the time course of the PSC decays.

Chlorpromazine. Figure 11A shows the action of 25 μM chlorpromazine on the Sub-induced currents for a voltage-jump from +50 mV to −175 mV. Chlorpromazine decreases the equilibrium agonist-induced currents but with little or no effect on the relaxation kinetics (Fig. 11). The fractional decrease in currents by chlorpromazine is approximately the same at all membrane potentials. For the four cells examined, the blockade of steady-state currents by chlorpromazine was essentially irreversible.

Benzocaine. Benzocaine (Fig. 12) is a derivative of procaine lacking the tertiary amine moiety. At 25 μM, benzocaine had little effect on the kinetics or equilibria; even at 250 μM (Fig. 12), this drug produced relatively small increases in reciprocal relaxation times and small decreases in currents. Unlike procaine, benzocaine did not promote a greater fractional decrease in currents and increase in rates at high neg-

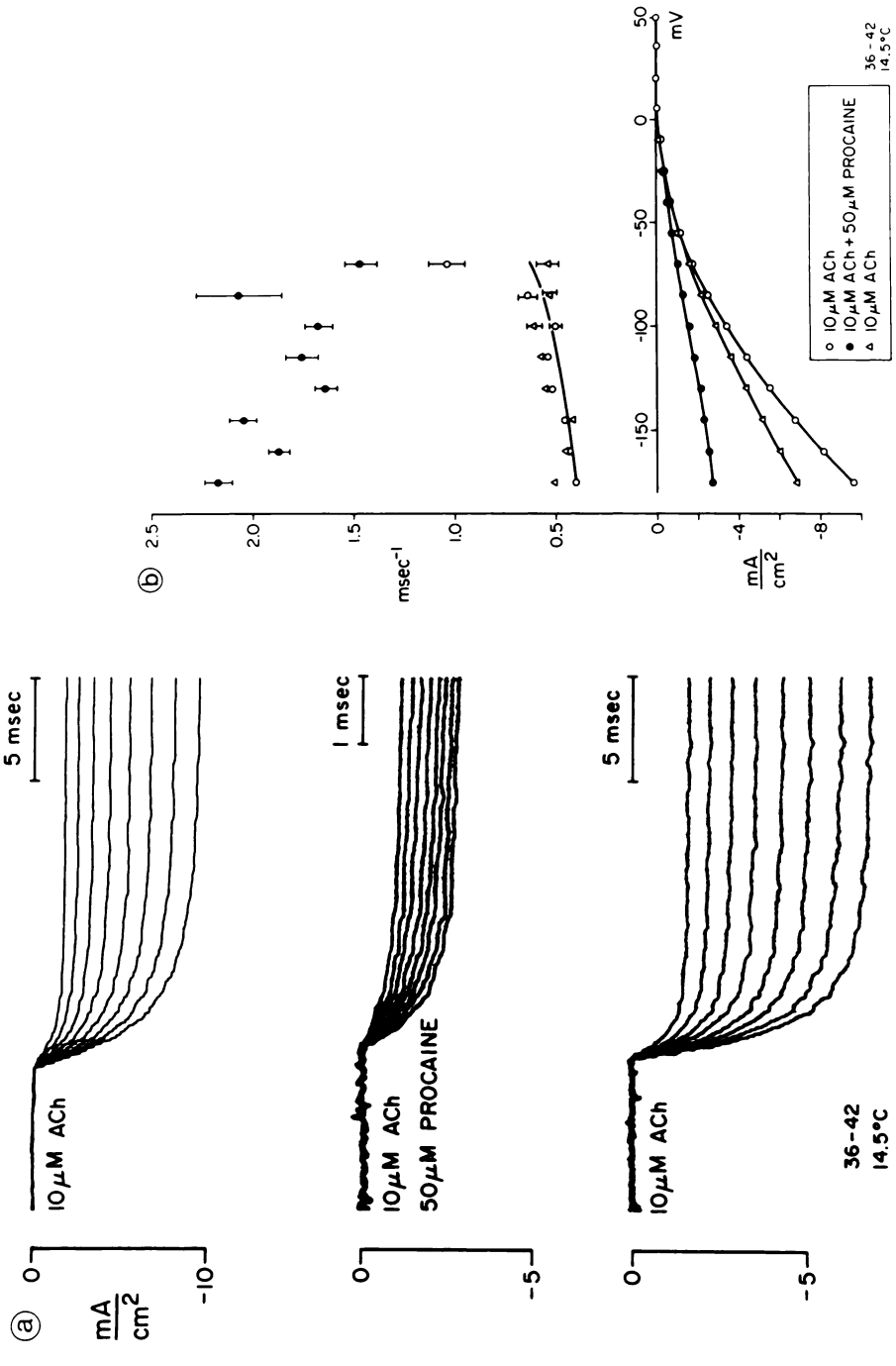


FIG. 6. *The action of procaine on ACh responses*

(A) Voltage-jump relaxations with 10 μM ACh before (top), during (middle), and after (bottom) exposure to 25 μM procaine. Final voltages ranged from −70 to −175 mV, with −15 mV increments. Note changes in the current and time scales. (B) Equilibrium ACh-induced currents and the reciprocal time constant of relaxations versus membrane potential.

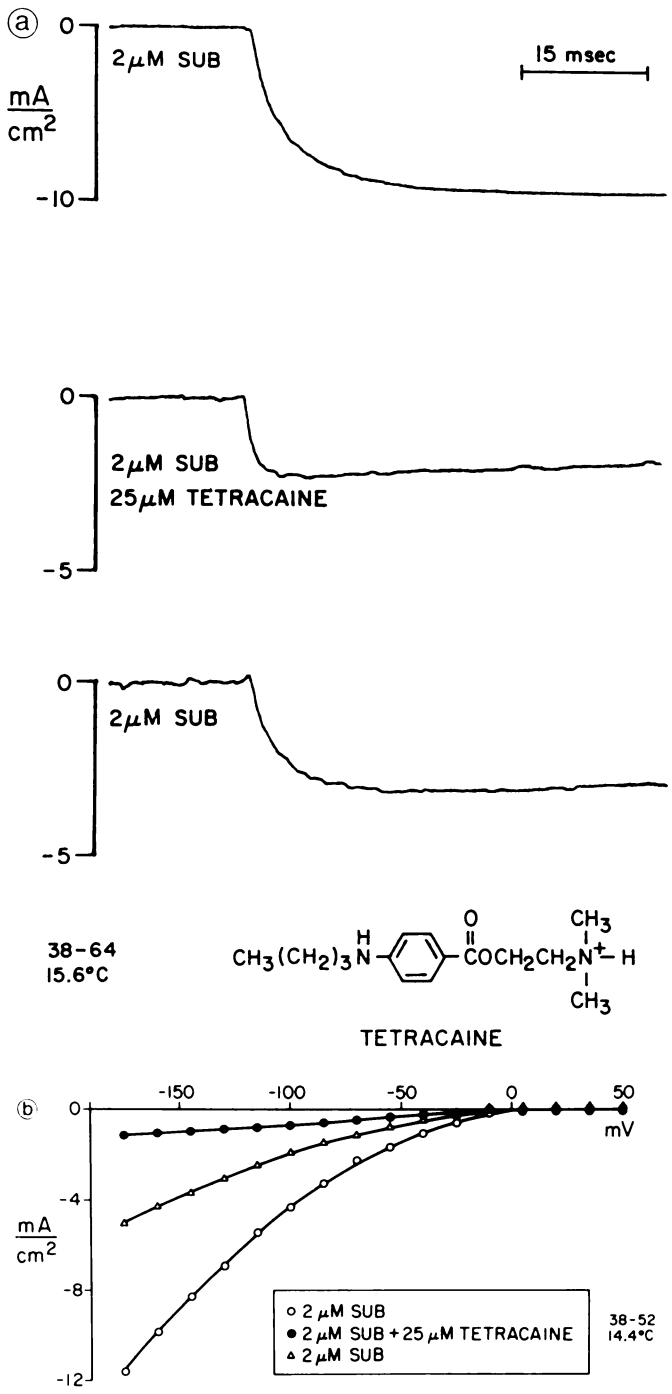


FIG. 7. *Action of tetracaine on responses to 2 μM Sub*

(A) Voltage-jump relaxations from +50 mV to −175 mV before (top), during (middle), and after (bottom) exposure to 25 μM tetracaine. The current scale of the middle and bottom traces is one half that of the top trace. (B) Equilibrium agonist-induced currents versus voltage for another cell.

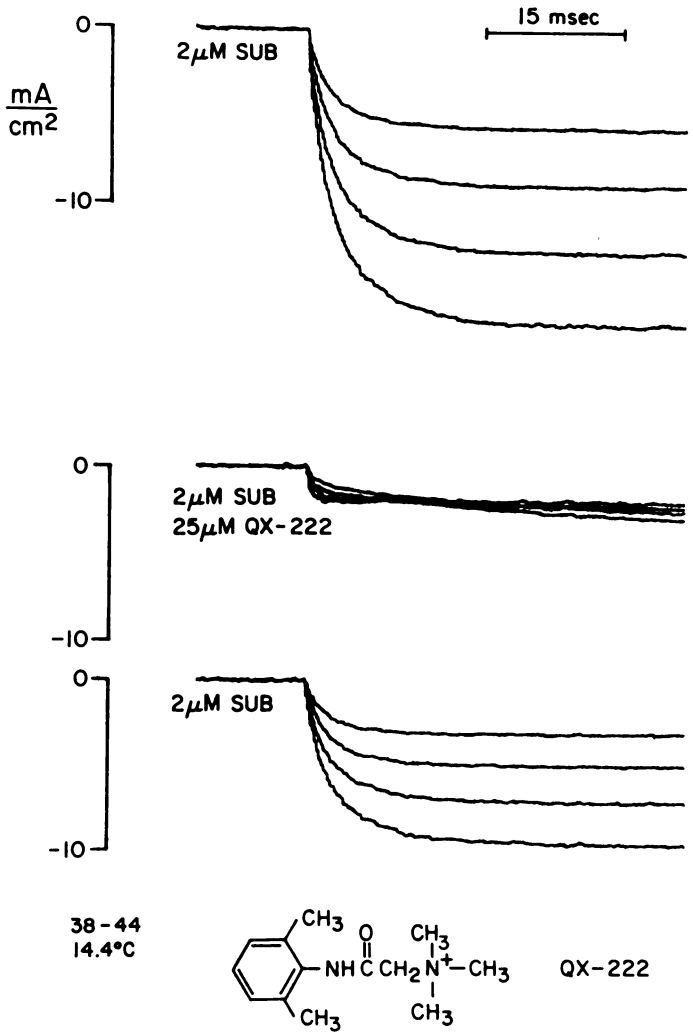


FIG. 8. *Action of QX-222 on responses to 2 μM Sub*

Voltage-jump relaxations from +50 mV to −85, −115, −145, and −175 mV, before (top), during (middle) and after (bottom) exposure to 25 μM QX-222.

ative membrane potentials. In fact, benzocaine may exert a slightly smaller blockade at high negative membrane potentials (Fig. 12).

Neurally evoked postsynaptic currents are not affected by 25 μM benzocaine. A 250 μM benzocaine concentration slightly increases the PSC decay rates; the increase in rate is approximately the same at all membrane potentials.

DISCUSSION

These experiments have examined the effects of local anesthetics on rates and equilibria at nicotinic acetylcholine receptors. The major findings are that: 1) procaine, tetracaine, and QX-222 modify kinetic and equilibrium aspects of the cholinergic response; 2) these drugs exert a blockade which is fractionally greater at high negative potentials; 3) other drugs block the steady-state currents with much smaller effects on kinetics and with little voltage dependence.

Multiple exposure of the electroplaque to agonist. In these experiments a major technical limitation was the progressive change in voltage-jump relaxations with re-

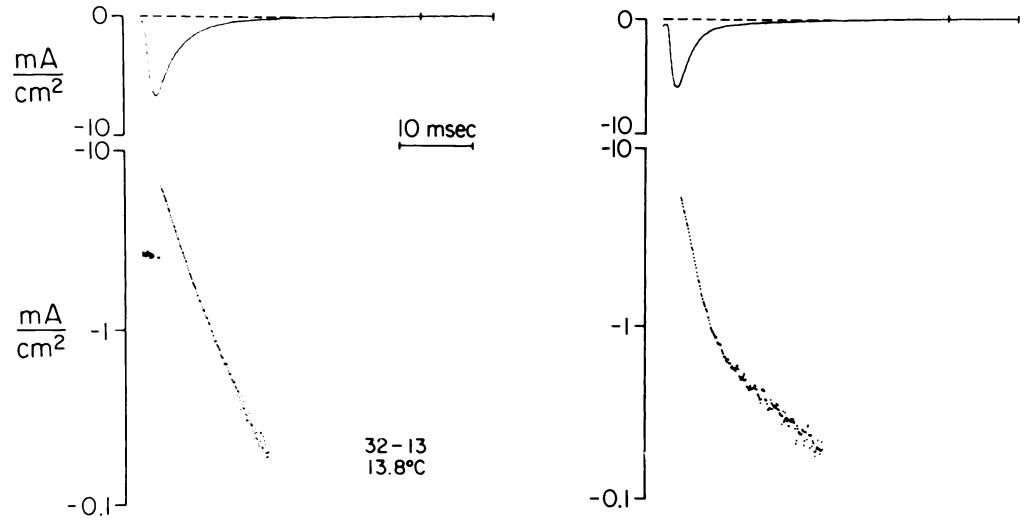


FIG. 9. *Neurally evoked postsynaptic currents in the absence (left) and the presence (right) of 50 μM QX-222* Top traces are PSCs at −100 mV. The dashed lines represent the zero current levels calculated as the average current betweens the short vertical lines at the end of each trace. The bottom traces are semilogarithmic plots of the decay. In the bottom left trace, the decay is fit to a single exponential to give a rate constant of 0.32 $msec^{-1}$. In the bottom right trace, the decay is fit to a sum of two exponentials, giving rate constants of 0.43 $msec^{-1}$ and 0.095 $msec^{-1}$.

peated exposure to agonist. These changes are largest between the first and second bath applications, becoming less noticeable with each application of agonist. The changes in steady-state currents are most predictable, always decreasing between the first and second bath application. In some cells only a 5% decrease in steady-state currents is seen, whereas in other cells reductions of over 35% have been recorded.

We do not yet know the cause for this effect. The cell's ionic composition changes during prolonged exposure to agonist (33, 34). However, the progressive changes still occur when there is a complete recovery of the membrane potential after each bath application (Fig. 1), signaling a complete recovery of the K ion gradient. Nonetheless, we did not monitor Na and Ca gradients in these experiments and they may have undergone permanent changes. Another possibility is that some receptors become permanently desensitized or otherwise inactivated. In several experiments we looked for changes in synaptic function by examining PSC decay rates before and after bath application of Carb to the cell. No significant differences in PSC decay rates were observed, even after several exposures to agonist. Therefore, the sensitive population could well be extrasynaptic receptors.

In the experiments presented above, the steady-state currents often recovered incompletely after removal of the anesthetic. This result can be explained, at least in part, by the effects of repetitive bath application.

Receptor blockade parallels lipid solubility at small potentials but not at large ones. Skou (35) first showed that the potency of tertiary amine local anesthetics in blocking nerve conduction correlated with their lipid solubility. He found the following minimum blocking concentrations for local anesthetics in frog nerve: 4.6 mM procaine, 10 μM tetracaine, and 5 μM dibucaine. At these concentrations, each of the anesthetics caused the same expansion of a nerve-lipid monolayer (35). Several later studies correlated the blocking potency of local anesthetics with their binding to phospholipid model membranes (36–39) and it is often thought that membrane lipids play a major role in the binding and action of local anesthetics.

Table 3 shows that receptor blockade

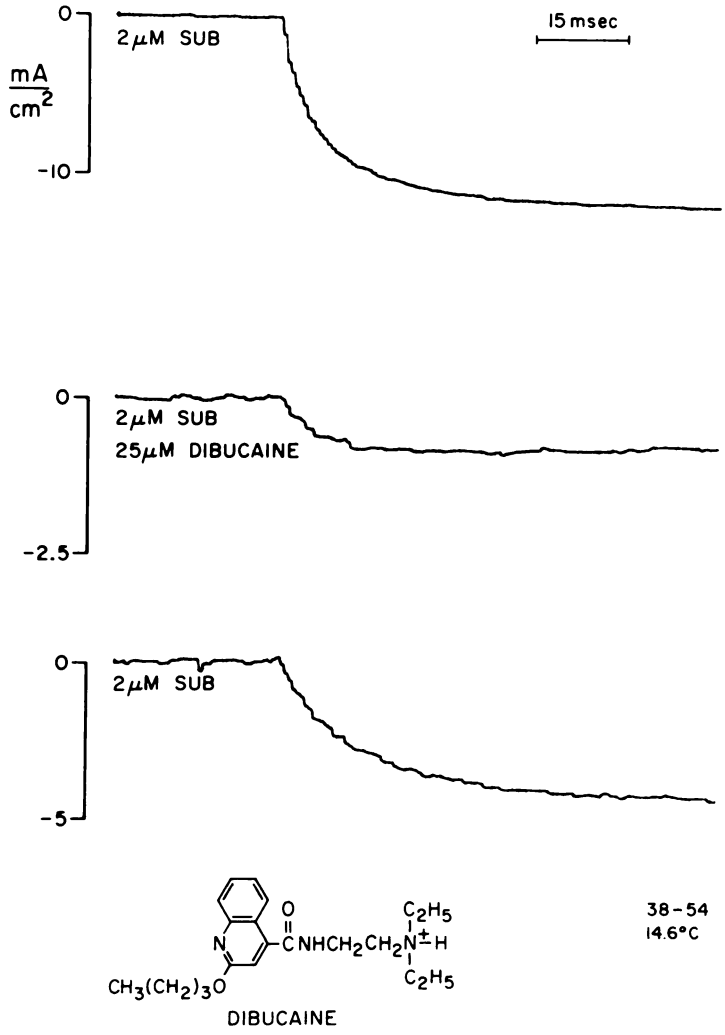


FIG. 10. *Action of dibucaine on responses to 2 μM Sub*

Voltage-jump relaxations from +50 mV to −175 mV before (top), during (middle), and after (bottom) exposure to 25 μM dibucaine. The current scale of the middle trace is one-fourth, and that of the bottom trace one-half of the current scale for the top trace.

correlates well with lipid solubility at low negative voltages but not at large negative voltages. At −40 mV, 25 μM procaine effects little blockade of Sub-induced currents, whereas tetracaine and dibucaine inhibit Sub-induced currents by about 50% and 90%, respectively (Table 3). These are rough values due to the difficulties in accurately measuring small agonist-induced currents. Nevertheless, it seems clear that these anesthetics block equilibrium Sub-induced currents at low negative potentials in the order dibucaine > tetracaine > procaine; this sequence follows the lipid solubility of the anesthetics. In contrast, at a high negative potential (−175 mV), steady-state blockade does not parallel the lipid solubility of these compounds and equimolar concentrations of these anesthetics decrease Sub-induced currents to approximately the same extent. These data emphasize again that the drugs we studied fall into two groups with regard to their voltage sensitivity: one whose blockade depends on

voltage and another with voltage-independent action.

Mechanisms of Voltage-Dependent Blockade

Procaine, tetracaine, and QX-222 have similar potencies. As at the nerve-muscle synapse (11, 22–26), receptor blockade by certain local anesthetics on the electroplaque depends on the membrane potential. Earlier studies demonstrated that procaine inhibits decamethonium-induced currents most effectively at high negative potentials (28). The present investigation extends this result to other local anesthetics and to other agonists. Like procaine, tetracaine and QX-222 cause a greater percentage inhibition of Sub-induced currents at high negative potentials and markedly affect the Sub relaxation rates.

For these experiments, the agonist suberyldicholine was employed because of its long channel lifetime compared to other agonists (7, 13–15) and because the anesthetics caused the most marked changes in the relaxations with this agonist. At concentrations of 25 μM, procaine (Fig. 5A), tetracaine (Fig. 7A), and QX-222 (Fig. 8), and at a potential of −175 mV, the relaxations acquire a rapid component roughly ten times faster than normal. In addition, each of these local anesthetics decreases the equilibrium Sub-induced currents at −175 mV to approximately the same extent (Table 3). As mentioned previously, the values for steady-state currents are not exact because in some records obtained with a local anesthetic, the relaxations have a small component with a long time course (e.g., Figs. 5 and 8; see ref. 11). Nevertheless, it seems that *equimolar* concentrations of procaine, tetracaine, and QX-222 have a qualitatively similar effect on the Sub-relaxations. Thus, although tetracaine blocks nerve conduction (35, 39, 40) and expands lipid monolayers (35, 41) at concentrations one to two orders of magnitude lower than does procaine, the voltage-jump relaxations in the electroplaque are modified equally by equal concentrations of these local anesthetics.

These findings show that some local anesthetics modify receptor gating to an extent that correlates neither with their membrane (lipid) solubility nor with their ability to block conduction in nerve. Such drugs may well act on the receptor-channel complex rather than by altering the physical state of the lipids surrounding the receptor (11, 24–27).

Local anesthetics are active in their positively charged form. Voltage-dependent action of a drug suggests that charged groups play a role in its action. The tertiary amine local anesthetics employed all have pK_a's in the region of 8–9 and therefore bear positive charges at experimental pH. In addition, the permanently charged quaternary amine QX-222 is quite active in altering the Sub relaxations, whereas benzocaine, an uncharged anesthetic, has little or no effect. These observations indicate that the anesthetics are active in their positively charged form, in agreement with earlier studies (20).

Quaternary amine anesthetics, such as QX-222, are relatively ineffective when applied outside nerve fibers but are active when applied inside (42–44). However, external application of QX-222 affects ACh receptors of the electroplaque (this study) and of nerve-muscle synapses (20, 22, 24, 26). Therefore, either 1) the site of action of the positively charged anesthetics is accessible only from the external surface, as found with acetylcholine (45); or 2) the postsynaptic membranes of the electroplaque and the frog neuromuscular junction, unlike axonal membranes, are permeable to quaternary amine anesthetics, possibly by movement through the channel, and exert their effect from the cytoplasmic side of the cell; or 3) both explanations are valid, and the anesthetics can act from either side of the membrane.

As previously noted, the agonist-induced currents never return completely to their original value after a local anesthetic has been washed out of the experimental chamber. A portion of this irreversibility is due to the effect of repetitive bath application but there may also be incomplete removal of the anesthetic. This idea is supported by the finding that in cells treated with pro-

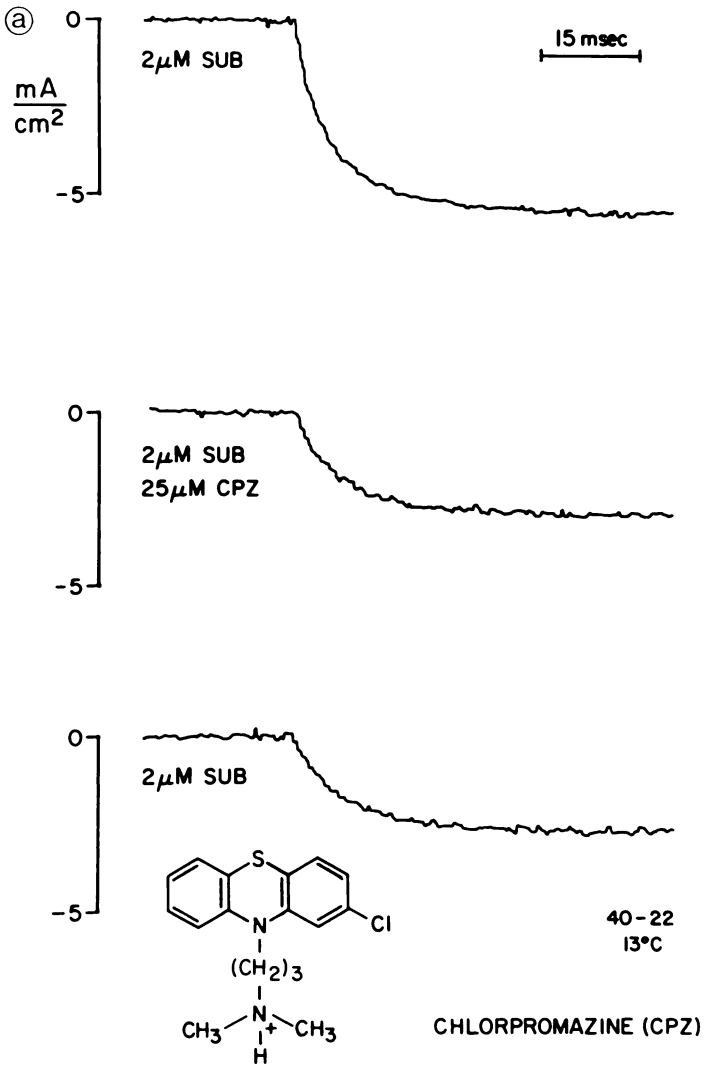
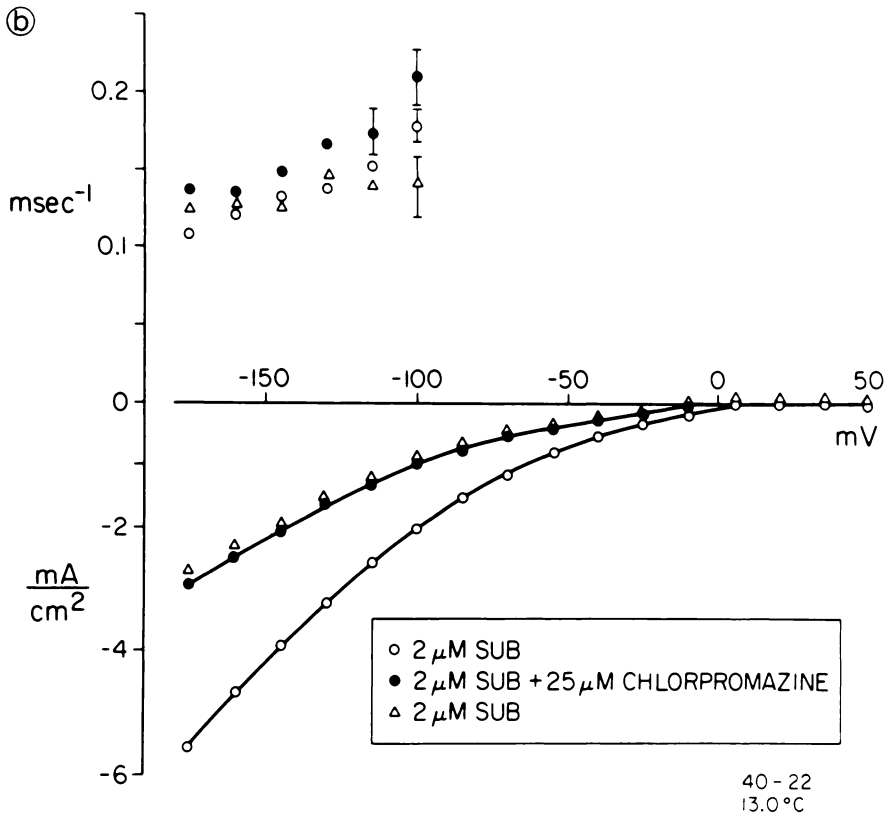


FIG. 11. *Action of chlorpromazine on responses to 2 μM Sub*

(A) Voltage-jump relaxations from +50 mV to −175 mV before (top), during (middle), and after (bottom) exposure to 25 μM chlorpromazine. (B) Equilibrium Sub-induced currents and reciprocal time constants.

caine, PSC decay rates do not return to their control values even after a thorough washing (Fig. 4). If the local anesthetics are able to penetrate the membrane and exist in a positively charged form on the cytoplasmic surfaces, it would be difficult to remove the anesthetic completely by an exhaustive washing of the external surface (46).

Specific molecular mechanism. The present results are in qualitative agreement with earlier studies on some of these drugs at nerve-muscle and nerve-electroplaque synapses. For procaine and QX-222, in particular, the data agree with the notion that the receptor channel is terminated prematurely (20). A specific such model, derived from the single-channel studies of Neher and Steinbach (26), states that QX-222 molecules (Q) bind and unbind rapidly from the open state (R*) producing a flickering conductance:



$$R \underset{\alpha}{\overset{\delta}{\rightleftharpoons}} R^* \underset{F}{\overset{G}{\rightleftharpoons}} R^*Q \qquad (1)$$

Note that in this formulation the blocked channel (R*Q) cannot close.

This model predicts PSC decays with two exponential components. Their rate constants $\lambda_\pm$ are related to the parameters of the model by:

$$\lambda_\pm = \frac{(\alpha + G[Q] + F) \pm [(\alpha + G[Q] + F)^2 - 4F\alpha]^{1/2}}{2} \qquad (2)$$

$$F = \lambda_+\lambda_-/\alpha \qquad (3)$$

$$G[Q] = (\lambda_+ + \lambda_-) - \alpha - \lambda_+\lambda_-/\alpha \qquad (4)$$

The results in Fig. 9 give $\alpha = 0.32$ msec^{-1}, $\lambda_+ = 0.43$ msec^{-1}, $\lambda_- = 0.095$ msec^{-1}, for QX-222 (50 μM, 13.8°, −100 mV). Therefore, $G[Q] = 0.08$ msec^{-1} and $F = 0.13$ msec^{-1}. These rate constants are about an order of magnitude smaller than those found at the frog endplate (26), but yield an equilibrium dissociation constant F/G of about 80 μM, close to that (54 μM) calculated from expressions given by Neher and Steinbach (26).

As shown by Adams (11), equation (1) can explain slow components in a voltage-jump relaxation. With QX-222 (Fig. 8), the slow component changes from a conductance increase to a conductance decrease as the voltage becomes more negative. This fits the suggestion (26) that G increases and F decreases at more negative voltages.

The procaine PSCs in the electroplaque lack a slow phase. This situation could arise because F is small, as suggested for QX-314 at the nerve-muscle synapse (26). If this interpretation is correct, F is at most 0.05 msec^{-1} (larger values would give a measurable slow phase) and the PSC decay rate is simply $\alpha + G[Q]$. From the results of Fig. 4, on PSCs in the presence of procaine at 16.6°, it may be estimated that G is the order of 4×10^6 M^{-1} sec^{-1} at −100 mV and

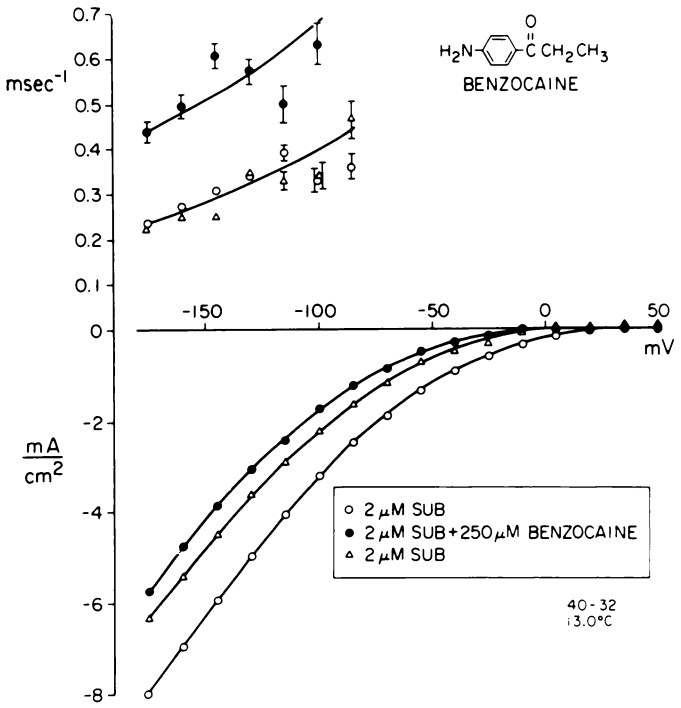


FIG. 12. *Effect of benzocaine on responses to 2 μM Sub*

TABLE 3

Decreases in sub-induced equilibrium currents by local anesthetics[a]

Anesthetic	Concentration	% Decrease = $(I_s - I_a) \times 100/I_s$[b]	
		−40 mV	−175 mV
Procaine	25 μM	2.7 ± 21 (7)[c]	73 ± 10 (8)
QX-222	25 μM	23 ± 30 (3)	90 ± 2 (3)
Tetracaine	25 μM	48 ± 34 (3)	80 ± 15 (3)
Dibucaine	10 μM	45 (1)	52 (1)
	25 μM	87 (2)	88 (2)
Chlorpromazine	25 μM	35 (2)	49 ± 15 (3)
Benzocaine	100 μM	40 (1)	14 (1)
	250 μM	43 (1)	29 (1)

[a] Concentration of suberyldicholine = 2 μM.

[b] I_s = equilibrium currents seen after first addition of 2 μM Sub;
I_a = equilibrium currents seen after addition of 2 μM Sub plus anesthetic.

[c] Number in parentheses indicates number of experiments. Values are mean ± SD.

decreases with depolarization. These rates are several-fold lower than those reported for procaine on the nerve-muscle synapse (11), and are comparable to the association and dissociation rate constants found with procaine on *Aplysia* neurons (27).

Thus, equation (1) seems to explain much of the data for procaine and QX-222 on the electroplaque. The association and dissociation rate constants for the blocking process are within one order of magnitude of those obtained in frog muscle membrane and in *Aplysia* neurons. It is difficult to present a more complete analysis of the data since 1) the effects of the local anesthetics were only partially reversible, 2) the agonist-induced currents in the presence of local anesthetics were of low magnitude and hard to measure, and 3) the relaxations gave only approximate fits to exponential components. Further information may come from experiments using light-activated blocking drugs (47).

Mechanisms of Voltage-Independent Blockade

The relatively hydrophobic local anesthetics dibucaine (Fig. 10) and chlorpromazine (Fig. 11) exert a roughly equal

blockade at all voltages and have little effect on the relaxation kinetics. At high negative potentials, dibucaine (25 μM) decreases the currents to approximately the same extent as procaine, QX-222, and tetracaine (Table 3), but has a smaller effect on the time course of the Sub relaxations (Fig. 10). Chlorpromazine (25 μM) has a lesser effect on the Sub-induced currents (Table 3) and essentially no effect on the Sub relaxation rates (Fig. 11). Chlorpromazine blocks nerve conduction at a concentration nearly 500 times lower than procaine and has a 500 fold greater membrane/buffer partition coefficient (48) but the relative potencies are reversed for effects on the ACh receptor.

Dibucaine and chlorpromazine mimic the action of α-bungarotoxin, which blocks agonist-induced currents without altering the gating kinetics (13, 49, 50). However, since displacement of agonist from isolated ACh receptors occurs only at concentrations much higher than those employed in the present experiments (51), it seems unlikely that dibucaine and chlorpromazine act by blocking the access of agonists to the receptor site. Instead, the results could be explained by assuming that channel gating depends on the physical state of the lipids surrounding the receptor-channel complex, and that dibucaine and chlorpromazine act by altering the properties of membrane lipids.

Effects on membrane lipids are also suggested by the observation that dibucaine, at concentrations of the same order as that used in the present experiments (25 μM), decreases the phase transition temperature of pure and mixed phospholipid systems (52, 53), alters the surface charge of phospholipid membranes (54, 55), and expands lipid monolayers (35, 41). (In contrast, procaine has essentially no effect on phospholipid membranes at concentrations of the order 25 μM). It would be purely speculative to suggest which, if any, of these anesthetic actions on lipids results in decreased agonist-induced currents. Nevertheless, it is apparent that low concentrations of dibucaine can alter the physical state of membrane lipids, and that such an alteration in the lipids surrounding the ACh receptor may influence the flow of ions through the channel.

Doubtless, some anesthetics act by a combination of the charge-sensitive, voltage-dependent mechanism and the hydrophobic, voltage-independent mechanism. For instance, tetracaine seems to interact specifically with the receptor at high negative potentials and significantly alters the kinetics of Sub relaxations. However, since it also has high lipid solubility, lipid interactions might also underlie part of tetracaine's very potent blockade of Sub-induced currents.

Blockade of Agonist-Induced Depolarization

Vertebrate skeletal muscle fibers and electroplaques are depolarized by bath application of nicotinic agonists. Such depolarizations are often interpreted in terms of receptor activation, on the assumption that the depolarization arises because the agonist-induced conductance short-circuits E_K. For *Electrophorus* electroplaques the situation is more complex. With bath-applied agonist, the sustained permeability increase leads to a depletion of ionic gradients. Therefore E_K moves toward zero. The depolarization primarily reflects the changed E_K and depends mostly on the history of the agonist-induced conductance, not on its present value (28, 33, 34).

Nonetheless agonist-induced depolarizations can be interpreted qualitatively in terms of events at the receptor if two points are kept in mind. (1) During the usual bath-application series, depolarizations are measured at a standard time after agonist is added (56). Assuming that drugs have no additional slow effects on ion conductances or on ion pumping, the depolarization thus reflects some average of drug-receptor interactions during this time. (2) The membrane voltage varies between −85 mV and zero during this time, and usually agonist-induced currents bring the membrane potential to the range −20 to −60 mV. Therefore in Table 3 the more relevant voltage-clamp measurements are those at −40 mV. We think the blockade at −40 mV reflects

primarily the "voltage-independent" blocking action.

These considerations explain most available data on the blockade of agonist-induced depolarizations. For instance, in agreement with the data of Table 3, Weber and Changeux (51) found that local anesthetics block membrane depolarizations with the relative potencies dibucaine > tetracaine > procaine; dibucaine was nearly 100 times more potent than procaine. In our experiments we observed the same pattern. Furthermore, in the present experiments, the relatively hydrophobic local anesthetics dibucaine, chlorpromazine, and tetracaine (at 25 μM) were the only anesthetics which significantly blocked depolarization during bath application of agonist; the other anesthetics tested had little or no effect. Because agonist-induced depolarizations are measured at membrane potentials near zero, local anesthetics affect these measurements primarily through a voltage-independent mechanism which correlates with their lipid solubility and nerve blocking potency.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant NS-11756; by a Grant-in-Aid from the Muscular Dystrophy Association, Inc.; by a Muscular Dystrophy Postdoctoral Fellowship to D.D.K.; and by an Alfred P. Sloan Fellowship and N.I.H. Career Development Award NS272 to H.A.L.

REFERENCES

1. Magleby, K. L. & Stevens, C. F. (1972) The effect of voltage on the time course of end-plate currents. *J. Physiol. (Lond.)* **223,** 151–171.
2. Magleby, K. L. & Stevens, C. F. (1972) A quantitative description of end-plate currents. *J. Physiol. (Lond.)* **223,** 173–197.
3. Kordas, M. (1972) An attempt at an analysis of the factors determining the time course of the end-plate current. I. The effects of prostigmine and of the ratio of Mg^{+2} to Ca^{+2}. *J. Physiol. (Lond.)* **224,** 317–332.
4. Kordas, M. (1972) An attempt at an analysis of the factors determining the time course of end-plate current. II. Temperature. *J. Physiol. (Lond.)* **224,** 333–348.
5. Dionne, V. E. & Stevens, C. F. (1975) Voltage dependence of agonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *J. Physiol. (Lond.)* **251,** 245–270.
6. Katz, B. & Miledi, R. (1972) The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol. (Lond.)* **224,** 665–699.
7. Katz, B. & Miledi, R. (1973) The characteristics of 'end-plate noise' produced by different depolarizing drugs. *J. Physiol. (Lond.)* **230,** 707–717.
8. Anderson, C. R. & Stevens, C. F. (1973) Voltage clamp analysis of acetylcholine induced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.)* **235,** 655–691.
9. Colquhoun, D., Dionne, V. E., Steinbach, J. H. & Stevens, C. F. (1975) Conductance of channels opened by acetylcholine-like drugs in muscle end-plate. *Nature* **253,** 204–206.
10. Adams, P. R. (1974) Kinetics of agonist conductance changes during hyperpolarization at frog end-plates. *Br. J. Pharmacol.* **53,** 308–310.
11. Adams, P. R. (1977) Voltage jump analysis of procaine action at frog end-plate. *J. Physiol. (Lond.)* **268,** 291–318.
12. Sheridan, R. E. & Lester, H. A. (1975) Relaxation measurements on the acetylcholine receptor. *Proc. Nat. Acad. Sci. USA* **72,** 3496–3500.
13. Sheridan, R. E. & Lester, H. A. (1977) Rates and equilibria at the acetylcholine receptor of *Electrophorus* electroplaques: A study of neurally evoked postsynaptic currents and of voltage-jump relaxations. *J. Gen. Physiol.* **70,** 187–219.
14. Neher, E. & Sakmann, R. (1975) Voltage-dependence of drug-induced conductance in frog neuromuscular junction. *Proc. Nat. Acad. Sci. USA* **72,** 2140–2144.
15. Neher, E. & Sakmann, B. (1976) Single channel currents recorded from the membrane of denervated frog muscle fibres. *Nature* **260,** 799–802.
16. Lester, H. A. & Chang, H. W. (1977) Response of acetylcholine receptors to rapid, photochemically produced increases in agonist concentration. *Nature* **276,** 373–374.
17. Gage, P. W. (1976) Generation of end-plate potentials. *Physiol. Rev.* **56,** 177–247.
18. Magazanik, L. G. (1976) Functional properties of postjunctional membrane. *Ann. Rev. Pharmacol. Toxicol.* **16,** 161–175.
19. Furukawa, T. (1957) Properties of the procaine end-plate potential. *Jap. J. Physiol.* **7,** 199–212.
20. Steinbach, A. B. (1968) Alteration by xylocaine (lidocaine) and its derivatives of the time course of the end-plate potential. *J. Gen. Physiol.* **52,** 144–161.
21. Katz, B. & Miledi, R. (1975) The effect of procaine on the action of acetylcholine at the neuromuscular junction. *J. Physiol. (Lond.)* **249,** 269–284.
22. Beam, K. G. (1976) A voltage clamp study of the effect of two lidocaine derivatives on the time course of end-plate currents. *J. Physiol. (Lond.)* **258,** 279–300.
23. Beam, K. G. (1976) A quantitative description of

end-plate currents in the presence of two lidocaine derivatives. *J. Physiol. (Lond.)* **258,** 301–322.

24. Ruff, R. L. (1976) Local anesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *Biophys. J.* **16,** 433–439.
25. Ruff, R. L. (1977) A quantitative analysis of local anesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol. (Lond.)* **264,** 89–124.
26. Neher, E. & Steinbach, J. H. (1978) Local anesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.* **277,** 153–176.
27. Marty, A. (1978) Noise and relaxation studies of acetylcholine induced currents in the presence of procaine. *J. Physiol.* **278,** 237–250.
28. Lester, H. A., Changeux, J. P. & Sheridan, R. E. (1975) Conductance increases produced by bath application of cholinergic agonists to *Electrophorus* electroplaques. *J. Gen. Physiol.* **65,** 797–816.
29. Ruiz-Manresa, F., Ruarte, A. C., Schwartz, T. L. & Grundfest, H. (1970) Potassium inactivation and impedence changes during spike electrogenesis in eel electroplaques. *J. Gen. Physiol.* **55,** 33–47.
30. Armstrong, C. M. (1975) Ionic pores, gates, and gating currents. *Quart. Rev. Biophys.* **7,** 179–210.
31. Higman, H. B., Podleski, T. R. & Bartels, E. (1963) Apparent dissociation constants between carbamylcholine, d-tubocurarine and the receptor. *Biochim. Biophys. Acta* **75,** 187–193.
32. Adams, P. R. & Feltz, A. (1977) Interaction of a fluorescent probe with acetylcholine-activated synaptic membrane. *Nature* **269,** 609–611.
33. Karlin, A. (1967) Permeability and internal concentration of ions during depolarization of the electroplax. *Proc. Nat. Acad. Sci. USA* **58,** 1162–1167.
34. Lester, H. A. (1978) Analysis of sodium and potassium redistribution during sustained permeability increases at the innervated face of Electrophorus electroplaques. *J. Gen. Physiol.* **72,** 847–862.
35. Skou, J. C. (1954) Local anesthetics VI. Relation between blocking potency and penetration of a monomolecular layer of lipoids from nerves. *Acta Pharmacol. Toxicol.* **10,** 325–337.
36. Feinstein, M. B. (1964) Reaction of local anesthetics with phospholipids: a possible chemical basis for anesthesia. *J. Gen. Physiol.* **48,** 357–374.
37. Papahadjopoulos, D. (1972) Studies on the mechanism of action of local anesthetics with phospholipid model membranes. *Biochim. Biophys. Acta* **265,** 169–186.
38. Giotta, G. J., Chan, D. S. & Wang, H. H. (1974) Binding of spin-labeled local anesthetics to phosphatidylcholine and phosphatidylserine liposomes. *Arch. Biochem. Biophys.* **163,** 453–458.
39. Blaustein, M. P. & Goldman, D. E. (1966) Action of anionic and cationic nerve-blocking agents: experiment and interpretation. *Science* **153,** 429–432.
40. Courtney, K. R., Kendig, J. J. & Cohen, E. N. (1978) Frequency-dependent conduction block: The role of nerve impulse pattern in local anesthetic potency. *Anesthesiol.* **48,** 111–117.
41. Hendrickson, H. S. (1976) The penetration of local anesthetics into phosphatidylcholine monolayers. *J. Lipid Res.* **17,** 393–398.
42. Narahashi, T., Frazier, D. T. & Moore, J. W. (1972) Comparison of tertiary and quaternary amine local anesthetics in their ability to depress membrane ionic conductances. *J. Neurobiol.* **3,** 267–276.
43. Strichartz, G. (1976) Molecular mechanisms of nerve block by local anesthetics. *Anesthesiol.* **45,** 421–441.
44. Hille, B., Courtney, K. & Dum, R. (1975) Rate and site of action of local anesthetics in myelinated nerve fibers. *Molecular Mechanisms of Anesthesia* **1,** 13–20.
45. del Castillo, J. & Katz, B. (1955) On the localization of acetylcholine receptors. *J. Physiol.* **128,** 157–181.
46. Narahashi, T. & Frazier, D. T. (1975) Site of action and active form of procaine in squid giant axons. *J. Pharmacol. Exp. Ther.* **194,** 506–513.
47. Lester, H. A., Krouse, M. E., Nass, M. M., Erlanger, B. F. & Wassermann, N. H. (1978) Light-activated blocker of acetylcholine receptor channels. Sixth International Biophysics Congress Abstracts p. 294.
48. Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24,** 583–655.
49. Katz, B. & Miledi, R. (1973) The effect of α-bungarotoxin on acetylcholine receptors. *Br. J. Pharmacol.* **49,** 138–139.
50. Adams, P. R. (1977) Relaxation experiments using bath-applied suberyldicholine. *J. Physiol.* **268,** 271–289.
51. Weber, M. & Changeux, J. P. (1974) Binding of *Naja nigricollis* [^{3}H] α-toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs. III. Effects of local anesthetics on the binding of the tritiated-neurotoxin. *Molecular Pharm.* **10,** 35–40.
52. Papahadjopoulos, D., Jacobson, K., Poste, G. & Shepherd, G. (1975) Effects of local anesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. *Biochim. Biophys.*

Acta **394,** 504–519.

53. Lee, A. G. (1976) Interactions between anesthetics and lipid mixtures. Amines. *Biochim. Biophys. Acta* **448,** 34–44.
54. Singer, M. A. (1974) Effects of amiloride and dibucaine on a model phospholipid membrane-structure-activity relationships. *Biochem. Pharmacol.* **23,** 2939–2950.
55. McLaughlin, S. (1975) Local anesthetics and the electrical properties of phospholipid bilayer membranes. *Progress in Anesthesiology* **1,** 193–220.
56. Changeux, J. P. & Podleski, T. R. (1968) On the excitability and cooperativity of the electroplax membrane. *Proc. Nat. Acad. Sci. USA* **59,** 944–950.