MOLECULAR PHARMACOLOGY, 26:293-303

Interactions of Bupivacaine with Ionic Channels of the Nicotinic Receptor

Electrophysiological and Biochemical Studies

S. R. IKEDA,[1] R. S. ARONSTAM,[2] J. W. DALY,[3] Y. ARACAVA,[4] AND E. X. ALBUQUERQUE

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201

Received January 25, 1984; Accepted June 29, 1984

SUMMARY

The actions of the tertiary local anesthetic bupivacaine were studied on the nicotinic receptor-ionic channel complex (AChR) using electrophysiological and biochemical methods. Voltage clamp studies of the frog sartorius and cutaneous pectoris neuromuscular junction revealed a concentration-dependent depression of the decay time constant of the end-plate (τ_{EPC}) and spontaneous miniature end-plate (τ_{MEPC}) currents. The relationship of the reciprocal of either τ_{EPC} or τ_{MEPC} and bupivacaine concentration up to 100 μM was linear. Voltage dependence of EPC over the range +60 to −150 mV was reduced, whereas both EPC and MEPC decays were adequately described by a single exponential function at all concentrations tested. Peak MEPC and EPC amplitudes were also depressed in a concentration-dependent manner such that 100 μM bupivacaine reduced peak amplitude by about 50%. The current-voltage relationship remained linear under all conditions tested. Nerve-evoked responses were difficult to study at concentrations greater than 100 μM because of apparent blockade of nerve conduction. Extracellular recording of the MEPC afforded results similar to those obtained with EPCs. The τ_{MEPC} could be reduced to less than 300 μsec at a bupivacaine concentration of 400 μM. Fluctuation analysis showed that bupivacaine at concentrations of 10 and 25 μM did not change channel conductance but decreased single-channel lifetime to 76% and 39% of control values, respectively. Biochemical studies were performed on *Torpedo californica* membrane fragments using [^{3}H]phencyclidine ([^{3}H]PCP) and [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX) as channel probes. Bupivacaine inhibited the binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX with inhibition constants (K_i) of 32 and 25 μM, respectively. The corresponding inhibition constants for bupivacaine methiodide were 1.8 and 3.2 μM. The preincubation of the membranes with carbamylcholine increased the affinity of bupivacaine for the ionic channel sites 5- to 8-fold and the affinity of bupivacaine methiodide 3- to 4-fold. Bupivacaine, however, had no affinity for the agonist recognition site as determined by [^{3}H]ACh and [^{125}I]α-bungarotoxin bindings. The electrophysiological and biochemical studies indicate that bupivacaine reacts primarily with the ionic channel of the nicotinic AChR. The results are consistent with a sequential model in which the drug interacts with the sites at the ionic channel of AChR in its open conformation, producing species with little or no conductance. From the present studies there is no evidence for an interaction of bupivacaine with the agonist binding site or closed states of AChR.

INTRODUCTION

Certain local anesthetics react with the AChR[5] located in the postsynaptic membrane of the vertebrate neuromuscular junction, thereby producing accelerated single and biphasic EPC decays. These effects have been successfully analyzed by postulating a sequential series of reactions in which the drug molecule reversibly binds to

This research was supported by United States Army Research and Development Command Contract DAMD-17-81-C-1279, by Army Research Office Grant DAAG-29-81-K-0161, and by United States Public Health Service Grant NS-12063.

[1] Present address, National Institute on Alcohol Abuse and Alcoholism, Rockville, Md. 20852.

[2] Department of Pharmacology, Medical College of Georgia, Augusta, Ga. 30912.

[3] Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, and Kidney and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20205.

[4] Recipient of a fellowship from FAPESP, Brazil. On leave of absence from Department of Pharmacology, ICB, University of Sao Paulo, 05508 Sao Paulo, Brazil.

0026-895X/84/050293-11$02.00/0

the conducting species to form a species with little or no conductance (1–5). The sequential model can be represented by:

$$\text{nACh} + \text{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ACh}_n\text{R} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{ACh}_n\text{R}^* + D \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{ACh}_n\text{R}^*D$$

where ACh is the transmitter, R is the receptor-channel complex ACh_nR^* is the conducting species, and ACh_nR^*D is the blocked species with its rate constants as indicated.

Bupivacaine (1-*n*-butyl-DL-piperidine-2-carboxylic acid 2,6-dimethyl anilide hydrochloride, Marcaine) is a tertiary amine local anesthetic containing an aromatic nucleus linked to a piperidine moiety via an amide group (see Fig. 1, *inset*). It has a pK_a of 8.1 and in physiological solution at pH 7.1 this compound is 90% in the protonated form. Bupivacaine is similar in structure to mepivacaine and lidocaine and is used clinically to induce prolonged caudal, epidural, and peripheral nerve block (6). In addition to blocking nerve and muscle action potential generation, bupivacaine has been found to have a myotoxic action which results in muscle degeneration followed by regeneration (7, 8). Furthermore, experiments in this laboratory have shown bupivacaine to be a potent agent for producing terminal sprouting in rats (9). During the course of these experiments, it became apparent that bupivacaine produced a profound acceleration of the MEPP decay phase at the rat neuromuscular junction. Subsequent studies of voltage-clamped MEPCs confirmed these findings and revealed that these effects occurred without marked attenuation of peak amplitude.

In light of these findings and the previous studies with chemically similar agents, we decided to use electrophysiological and biochemical techniques to investigate the effects of bupivacaine on the nicotinic AChR in order to understand the molecular target of this agent at the junctional regions of the neuromuscular synapse. A short communication of this work has been presented previously (10).

MATERIALS AND METHODS

Electrophysiological Techniques

EPC experiments were performed on *Rana pipiens* sciatic nerve-sartorius muscle preparations treated with 600 mM glycerol to abolish excitation-contraction coupling. MEPC and noise analysis experiments were performed on *R. pipiens* cutaneous pectoris muscles. The muscles were carefully dissected free of connective tissue and pinned to a Sylgard plate with slight longitudinal and lateral spread. All experiments were carried out at room temperature (22°) unless otherwise noted.

The physiological solution used in these experiments had the following composition (millimolar): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7. The solution was bubbled with 100% O_2 and the pH was 6.9–7.1. When necessary, membrane excitability was blocked with 0.3 μM tetrodotoxin. Bupivacaine, 10–400 μM, was applied to all preparations by superfusion into the bathing medium, and the recordings were started 30 min after addition of the drug.

EPC recording. The recording details of EPCs using glass microelectrode (filled with 3 M KCl, 1–3 Mohm) are described elsewhere (11). The d.c. current and voltage traces (with adequate amplification) were sent on-line to a digital computer for later analysis.

MEPC recording. The studies on MEPCs were performed using both conventional voltage clamp and extracellular recording techniques. Voltage clamp recordings were made in a manner similar to EPCs except that a different voltage clamp circuit was employed. The clamp was similar to that described by Dionne and Parsons (12). Current was monitored by a virtual ground circuit employing an AD515 (Analog Devices, Norwood, Mass.) operational amplifier. MEPCs were recorded on FM tape (d.c.-10 KHz), as was a high gain a.c.-coupled potential trace. MEPCs were later "trapped" on a digital oscilloscope (Gould OS4000) and sent to the computer via an analogue output coupled with a trigger pulse. The signal was filtered before analysis to increase the signal-to-noise ratio (0.2–2500 Hz, fourth-order Butterworth response). MEPCs were considered adequate for analysis if there was no deformation of the peak ("spiking") or large deflection in the voltage trace, both of which are indications of inadequate clamping (13).

MEPCs were extracellularly recorded using specially constructed electrodes. Glass electrodes were pulled from hematocrit capillaries in a manner similar to that described for patch electrodes (14, 15). Tip diameter was estimated to be 1–3 μm, and electrode resistance, when filled with normal physiological solution, about 0.5–2 Mohm. End-plates were identified by carefully probing with the electrode until the spontaneous currents could be seen on the oscilloscope. This search was greatly facilitated by increasing the MEPP frequency by addition of 25 mM sucrose to the bathing medium. MEPCs were usually recorded at resting membrane potential (−80 to −95 mV). Intracellular potential was sometimes determined directly by inserting a conventional microelectrode into the same fiber. Occasionally, when MEPCs were recorded at different membrane potentials, conventional microelectrodes were inserted on each side of the extracellular electrode (usually within 100 μm), and the membrane potential was altered by application of a constant current through one of the microelectrodes. Extracellular MEPCs were recorded and analyzed similarly to voltage-clamped MEPCs except that no additional filtering was necessary.

ACh noise analysis. Noise analysis experiments were done using the same voltage clamp as that used for EPCs. ACh-induced currents were produced by iontophoresis of ACh from a low-resistance electrode containing 3 M ACh positioned about 100 μm above the surface of the muscle. Details of this technique are described elsewhere (16). All noise experiments were carried out at 10°. Temperature in the bath was monitored with a thermistor positioned near the preparation.

Data analysis of the EPCs and MEPCs. EPCs were digitized at 100-μsec intervals by a 12-bit A/D converter and subsequently analyzed by a computer program which scans the digitized current and potential traces and determines holding potential, peak amplitude, rise time, half-decay time, time constant (τ_{EPC}) of decay, and peak clamp error. The time constant of decay is determined by linear regression on the logarithms of the decay (20–80%). MEPCs are digitized at 56- or 28-μsec intervals and analyzed by a similar program. Each MEPC is analyzed for peak amplitude, rise time (0–100% of the rising phase) and τ_{MEPC}. In addition, a signal averaged MEPC is generated by aligning all MEPCs according to position of the peak current. τ_{MEPC} values are determined from the signal average MEPC rather than from the grand mean of the individual currents.

The method for noise analysis has been described previously (16, 17).

Biochemical Techniques and Tissue Preparation

Electric organs of *Torpedo californica*, purchased from Pacific Biomarine (Venice, Calif.) and stored for up to 3 months at −70°, were used for the biochemical studies. The tissue was chopped into small pieces and homogenized with a blender in 5 volumes of 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonic acid to inhibit proteolysis. The homogenate was filtered through three layers of

[5] The abbreviations used are: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor-ionic channel complex; EPC, end-plate current; MEPP, miniature end-plate potential; MEPC, miniature end-plate current; BGT, α-bungarotoxin; PCP, phencyclidine; H_{12}-HTX, perhydrohistrionicotoxin; τ_{EPC}, decay time constant of EPC.

cheesecloth to remove collagenous and other disrupted material. The filtrate was centrifuged at 30,000 × *g* for 20 min, and the resulting pellets were resuspended in buffer at a concentration of 1–2 mg of protein per milliliter and kept at 0° for up to 2 days before use. Protein content was determined by a modification of the method of Lowry *et al.* (18), using bovine serum albumin as the standard.

Receptor and ion channel binding measurements: Nicotinic receptor binding was measured using [^{125}I]BGT or [^{3}H]ACh. [^{125}I]BGT binding was assayed by filtration. *Torpedo* membranes (10–15 μg of protein) were incubated with the appropriate ligands (e.g., bupivacaine or carbamylcholine) for 20 min in 50 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$ (incubation volume = 1 ml) before addition of 0.5 nM [^{125}I]BGT (New England Nuclear Corporation, Boston, Mass.). After 20 min, the reaction was quenched by addition of 0.5 ml of methylated bovine serum albumin (10 mg/ml) (Sigma Chemical Company, St. Louis, Mo.). The suspensions were then filtered through Whatman GF/B filters which had been soaked in the albumin solution. The radioactivity content of the filters was determined by gamma scintillation counting.

[^{3}H]ACh ([*N-methyl*-^{3}H]ACh iodide, 80 Ci/mmol; New England Nuclear Corporation) binding was assayed by equilibrium dialysis. An aliquot of suspended *Torpedo* membranes was placed inside cellulose tubing (Spectra/Por 2, Fisher Scientific Company), and the tube was sealed at both ends and placed in a 20-ml bath containing 0.5 nM ([^{3}H]ACh, 1 mM $CaCl_2$, and 100 mM Tris-HCl (pH 7.4). After incubating for 4 hr at room temperature on a rotary shaker, aliquots were taken from the bath and sealed tubes and analyzed for radioactivity content by liquid scintillation counting. The tissue was pretreated with 100 μM diisopropylfluorophosphate for 30 min at room temperature to inhibit acetylcholinesterase. Nonspecific binding was determined by including 100 μM nicotine in a parallel set of incubations.

Ion channel binding of bupivacaine and bupivacaine methiodide (a quaternary derivative) was measured using [^{3}H]PCP ([*piperidyl*-3,4-^{3}H(*N*)]PCP, 48 Ci/mmol, New England Nuclear Corporation) and [^{3}H]H_{12}-HTX (about 54.5 Ci/mmol, prepared by reduction of octahydrohistrionicotoxin with tritium gas; New England Nuclear Corporation) as probes. An aliquot of the membrane suspension (40–60 μg of protein) was added to an incubation medium containing 3 nM [^{3}H]PCP or 2 nM [^{3}H]H_{12}-HTX and Tris buffer in a final volume of 1 ml. Parallel experiments were run in the presence or absence of 1 μM carbamylcholine. Nonspecific binding was determined in the presence of 100 μM amantadine, a potent channel blocker (19). After incubation for 60 min, the suspensions were filtered through Whatman GF/B glass-fiber filters that had been wetted with a 1% organosilane preparation (Prosil-28; PCR Research Chemicals, Gainesville, Fla.) to reduce nonspecific binding to the filters. The filters were washed twice with 5 ml of Tris buffer and removed for determination of their radioactivity content by liquid scintillation counting.

Inhibition constants (K_i) were calculated from the IC_{50} concentrations for inhibition of 3 nM [^{3}H]PCP and 2 nM [^{3}H]H_{12}-HTX binding according to the relationship:

$$K_i = \frac{IC_{50}}{1 + L/K}$$

where L and K are the concentration and dissociation constant of the labeled probe, respectively. [^{3}H]PCP and [^{3}H]H_{12}-HTX dissociation constants were 0.54 and 0.14 μM, respectively, in the absence of carbamylcholine, and 0.097 and 0.089 μM in the presence of 1 μM carbamylcholine.

Drug Solutions

ACh chloride (crystalline salt, Sigma Chemical Company), bupivacaine hydrochloride (Sterling-Winthrop, Lot XBO-058), and bupivacaine methiodide were used to prepare the stock solutions. Bupivacaine methiodide was prepared by treatment of bupivacaine free base with excess methyl iodide in acetonitrile at room temperature for 24 hr. After evaporation of solvent *in vacuo*, the material was recrystallized from methanol-diethyl ether. Bupivacaine methiodide was analytically and chromatographically pure (no detectable tertiary amine).

Statistical Analysis

The analysis of data was performed using Student's unpaired *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of bupivacaine on nerve-evoked EPCs. The effects of bupivacaine on the peak amplitude and decay time constant of EPCs (τ_{EPC}) recorded from surface fibers of the glycerol-treated frog sartorius muscle at room temperature (22°) are shown in Fig. 1. In normal physiological solution, the peak amplitude of the EPC was linearly related to membrane potential between +60 and −150 mV. τ_{EPC} was exponentially dependent on holding potential and could be adequately fit to the following equation (20):

$$1/\tau = B \exp(AV)$$

where V is voltage, and A and B are constants. The values for A and B as determined from linear regression of the logarithms of τ_{EPC} versus membrane potential were 0.0069/mV and 1.00/msec, respectively. These results are similar to previously published values (20–22) for control EPCs.

Bupivacaine (25, 50, and 100 μM) produced a concentration-dependent decrease in both the peak EPC amplitude and τ_{EPC}. These changes were usually evident within 5 min subsequent to superfusion of the drug and reached a steady state within 30 min. The decrease in peak EPC amplitude was significant at bupivacaine concentrations of 50 and 100 μM at all membrane potentials tested (except near the reversal potential, where small amplitudes render measurements less reliable). In addition, the relative magnitude of this depression was similar throughout the range of membrane potentials tested; thus, in the presence of the drug, the current-voltage (I-V) relationship remained linear (Fig. 1A).

Bupivacaine significantly decreased the τ_{EPC} decay at all concentrations and membrane potentials tested (Fig. 1B). The degree of acceleration of EPC decay was not uniform with voltage. Greater effects were observed at the more hyperpolarized membrane potentials. At a holding potential of +40 mV, 25, 50, and 100 μM bupivacaine decreased τ_{EPC} to 86, 75, and 40% of control values; at −140 mV the depression was to 51, 33, and 19% of control values, respectively. Thus, bupivacaine reduced the voltage dependence of τ_{EPC} in a concentration-dependent fashion. Under all experimental conditions, the decay of the EPC remained a single exponential function of time. Assuming that the actions of bupivacaine on the decay of the EPC can be described by a subset of the sequential model in which the rate constant for drug dissociation (k_{-3}) is considered negligible, then τ should be described by the expression:

$$1/\tau = k_{-2} + [D]k_3$$

where k_{-2} is the rate constant of decay in the absence of drug, $[D]$ is the drug concentration, and k_3 is the second-order forward rate constant for drug binding. The linearity of the $1/\tau_{EPC}$ versus drug concentration relationship

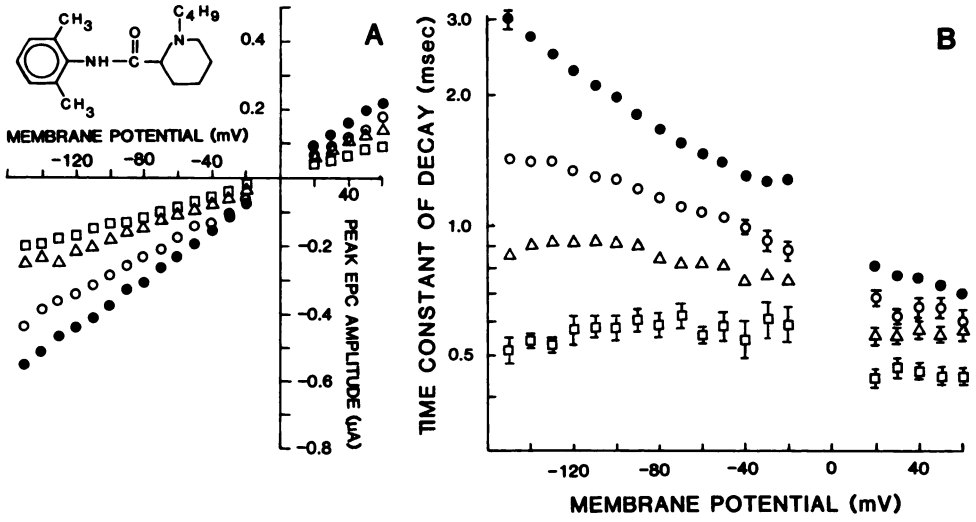

FIG. 1. *Concentration-dependent decrease of the peak amplitude and voltage sensitivity of the τ_{EPC}s produced by bupivacaine*

A. Relationship between the peak amplitude of EPC and membrane potential under control conditions (●) and after 30–60 min exposure to bupivacaine [25 (○), 50 (△), or 100 μM (□). Each symbol represents the mean of 8–24 fibers from two to three muscles. B. Relationship between the logarithm of τ_{EPC} and membrane potential under control conditions and in the presence of different concentrations of bupivacaine (symbols same as in A). Each point represents the mean ± standard error of the mean of 8–24 fibers from two to three muscles. *Inset*, molecular structure of bupivacaine.

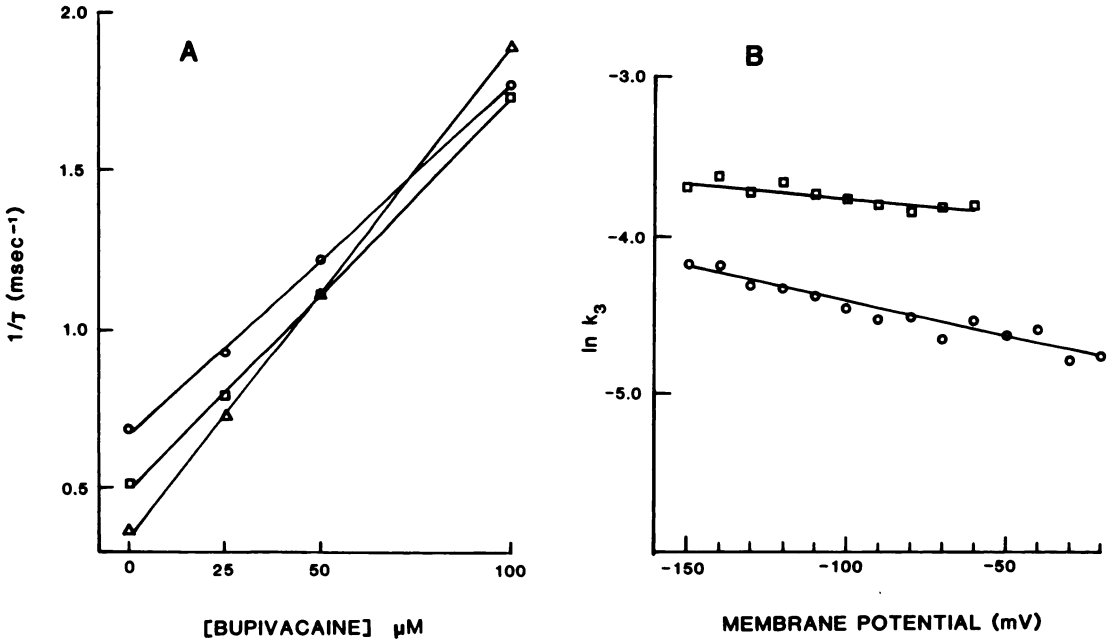

FIG. 2. *Relationship between the reciprocal of τ_{EPC} and bupivacaine concentration (A) and voltage sensitivity of the forward rate constant for bupivacaine blockade of the ionic channel (B)*

A. Membrane potentials were −50 mV (○), −100 mV (□), and −140 mV (△). The *solid lines* represent the best fits obtained from linear regression of the data. B. Forward rate constants (μM^{-1} $msec^{-1}$) were determined from the plots of the slope of the reciprocal of τ versus bupivacaine concentration (A) for EPCs (○). For MEPCs, k_3 (□) was determined in a similar way. The *solid lines* represent the best fits obtained from linear regression. The slope indicates that bupivacaine senses 11% and 4.5% of the membrane electric field for EPCs and MEPCs, respectively.

(Fig. 2A) is consistent with this notion and allows estimation of k_3 for different membrane potentials. Previous investigators (1, 5, 23, 24) have shown that the experimental dependence of k_3 on voltage for different cationic drugs can be described by a Boltzmann distribution as follows:

$$k_3(V) = k_3(0) \exp(-neV\delta/kT)$$

where $k_3(0)$ is the rate constant at 0 mV, ne is the charge of drug, k is Boltzmann's constant, T is absolute temperature, k_3 is the rate constant for drug binding at a given voltage V, and δ is the fraction of the membrane potential that influences k_3. The plot of ln k_3 versus membrane potential (Fig. 2B) reveals good agreement with the prediction and allows estimation of δ from the slope. For bupivacaine the slope was significantly different from 0, and δ was determined to be 0.11.

Effects of bupivacaine on MEPCs. MEPCs arise from discrete regions of the nerve terminal; thus the ability of the two-electrode voltage clamp to provide adequate control of membrane potential is improved. To take advantage of this fact, MEPCs arising in the region of adequate clamp control must be discriminated from those arising from more distant regions. In this series of experiments, MEPCs recorded from surface fibers of the frog cutaneous pectoris muscle were selected for analysis based on clamp error (i.e., transient deflections in the potential trace) and deformation of the peak of the MEPC (13). MEPCs exhibiting inadequate clamping were not included in the analysis.

In physiological solution (containing 0.3 μM tetrodotoxin), peak amplitude and decay time constant of MEPCs (τ_{MEPC}) were voltage-sensitive in a manner similar to that of EPCs. The constants A and B, describing the voltage dependence of τ_{MEPC} were 0.0098/mV and 2.18/msec, respectively. Superfusion of bupivacaine in the bathing solution produced a decrease in both peak MEPC amplitude and τ_{MEPC} (Fig. 3). The depression of peak MEPC amplitude was less than that observed for peak EPC amplitudes. For example, at −140 mV holding potential, 25, 50, and 100 μM bupivacaine decreased peak EPC amplitudes to 70, 43, and 35% of control values, whereas peak MEPC amplitudes (mean of 10–40 MEPCs from 3–19 fibers) were reduced to 81, 71, and 66% of control values, respectively. This result suggests that part of the depression in EPC peak amplitude produced by bupivacaine could result from actions on targets prior to the postsynaptic AChR. Alternatively, the discrepancy could be attributed to differences in the release function for EPCs and MEPCs. The rising phase of the MEPC is briefer than that of the EPC (25), presumably reflecting asynchrony of quantal release in the latter (26). An agent which accelerates EPC and MEPC decays could be expected to cause the closing of a larger proportion of activated ionic channels prior to the peak of the current, thus leading to a decrease in peak amplitude—an effect which would have greater expression in the EPC. In contrast to the effect of bupivacaine on peak amplitude of the EPC, the magnitude of τ_{MEPC} depression was similar to that of EPCs. At −140 mV holding potential, 25, 50, and 100 μM bupivacaine decreased τ_{MEPC} to 58, 37, and 17% of control values, respectively. As in the EPCs, MEPC decays were adequately described by a single exponential function of time under all experimental conditions. Determination of the influence of voltage on the calculated k_3 for MEPCs reveals that k_3 (Fig. 2B) is exponentially dependent on voltage. The slope of ln k_3 versus membrane potential is significantly different from 0, and δ was determined to be 0.045. Although the slopes of the plot of ln k_3 versus membrane potential are significantly different by t-test for MEPCs and EPCs, the data (especially the dependent variable) have undergone so many transformations that it would be overly optimistic to assume that this is a valid test (27).

Effect of bupivacaine on MEPCs recorded with extracellular electrodes. The sequential model presented thus far predicts a linear relationship between the reciprocal of the time constant of decay and drug concentration. Results presented for EPCs and voltage-clamped MEPCs have been consistent with this prediction. However, the range of concentrations tested has been limited in both instances. MEPCs recorded with extracellular electrodes have better signal-to-noise ratios and frequency response than those recorded with conventional two-electrode voltage clamp (28, 29), thus allowing higher concentrations of drug to be tested. In addition, extracellular electrodes record MEPCs only from the immediate vicinity of the extracellular electrode (Fig. 4) and not from the entire end-plate, thereby obviating the uncertainties associated with two-electrode voltage clamping.

As fewer studies have been done with MEPCs recorded

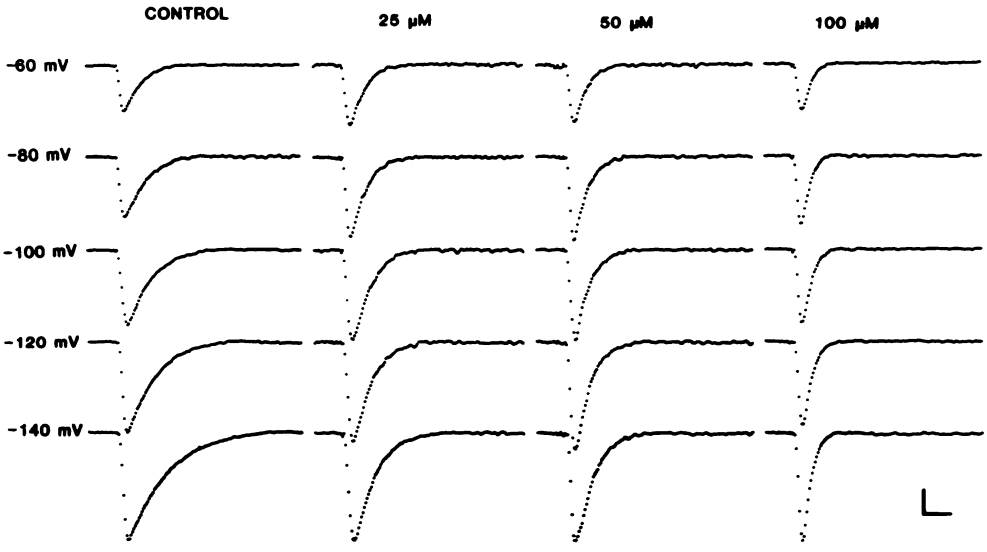

FIG. 3. *Effect of bupivacaine on spontaneous MEPCs*

Families of digitized MEPCs recorded from the frog cutaneous pectoris muscle. Each trace represents the signal average of 16–45 MEPCs from a single end-plate. Horizontal calibration was 1 msec and vertical calibrations were 1.58, 0.91, 0.95, and 0.73 namp for control and bupivacaine at concentrations of 25, 50, and 100 μM, respectively.

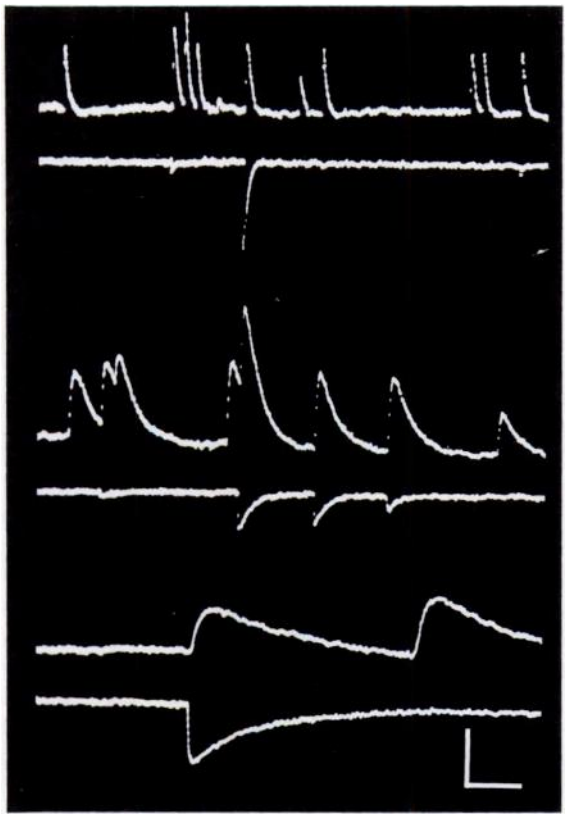

FIG. 4. *Simultaneously recorded intra- and extracellular MEPPS*
MEPPs recorded with an intracellular electrode (upward deflections) and extracellular electrode (downward deflections) in the frog cutaneous pectoris muscle. Membrane potential was held at −150 mV by a third electrode inserted into the same fiber. Vertical calibration were 1.1 mV for the intracellular traces and 2.5 mV for the extracellular traces. Horizontal calibrations were 50, 10, and 2 msec (*top* to *bottom*). Note that the extracellular electrode records only a fraction of the MEPPs detected by the intracellular electrode.

with extracellular electrodes, it was important to determine whether the characteristics of these currents were similar to those obtained with the voltage clamp. Figure 5A shows results obtained from surface fibers of the frog cutaneous pectoris muscle at 22°. In this set of experiments, the voltage control of the postsynaptic membrane was established by inserting conventional intracellular electrodes on either side of the extracellular electrode. One electrode was used to pass current from a d.c. source and the other to monitor membrane potential. MEPC decay was adequately described by a single exponential function of time at all membrane potentials (Fig. 5B), and the time constant of decay was exponentially dependent on membrane potential. The constants A and B, describing the voltage dependence, were determined to be 0.0101/mV and 1.63/msec, respectively. These results compare favorably with the voltage-clamped MEPC results in this study and the results obtained by Adams and Sakmann (28) for frog MEPCs recorded with extracellular electrodes.

In addition to the decay phase, the rising phase of the MEPC could be studied reliably with extracellular recordings. The results obtained for five fibers at various holding potentials (−140 to +60 mV) are similar to previously published values (13, 29). In the cells in which MEPCs could be recorded at positive holding potentials, it appears that, in contrast to the decay phase, depolarization produces a lengthening of the rising phase (30).

The effect of bupivacaine on MEPCs recorded with extracellular electrodes at resting membrane potential (−80 to −95 mV) is shown in Table 1 and Fig. 6A. Bupivacaine at concentrations of 25–400 μM produced a concentration-dependent decrease in τ_{MEPC}. MEPC decays could be adequately described by a single exponential function of time at all bupivacaine concentrations tested (Fig. 6B). The plot of $1/\tau$ versus bupivacaine concentration remained linear over the concentrations tested (Fig. 7). Bupivacaine at 400 μM significantly depressed the rise time to 76% of control values (Table 1, Fig. 6A).

Effect of bupivacaine on ACh-induced EPC fluctuations. The effects of bupivacaine on single-channel lifetime and conductance were determined from fluctuation analysis on voltage-clamped surface fibers from frog cutaneous pectoris muscle. Bupivacaine (10 μM) significantly ($p < 0.01$) decreased the channel lifetime from 4.09 ± 0.22 msec in control conditions to 3.09 ± 0.14 msec ($n = 5$ spectra obtained from five different junctional regions). At 25 μM, the drug further decreased channel lifetime from 6.69 ± 0.20 msec to 2.58 ± 0.10 ($n = 8$). A sample of spectra obtained in the presence of these two concentrations of bupivacaine is shown in Fig. 8. Single-channel conductance at these concentrations was not significantly altered. All experiments were performed at 10° and at a membrane potential of −80 mV.

Bupivacaine inhibition of ligand binding to receptor and ion channel sites. Bupivacaine inhibited the binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX to the AChR complex in membranes isolated from *Torpedo californica* electric organ with K_i values of 32 and 25 μM, respectively (Figs. 9 and 10; Table 2). The quaternary derivative, bupivacaine methiodide, showed a more potent inhibition of the binding of these ionic channel probes (Fig. 10; Table 2). Inhibition of both [^{3}H]PCP and [^{3}H]H_{12}-HTX binding by 100 μM bupivacaine was reversed 80% by washing the membranes twice by dilution and centrifugation. The affinity of bupivacaine and bupivacaine methiodide for the ionic channel sites was increased 5- to 8-fold and 3- to 4-fold, respectively, in the presence of 1 μM carbamylcholine. The affinity of most ionic channel ligands is increased when receptor binding sites are occupied (31).

In contrast, bupivacaine had little affinity for the cholinergic receptor (i.e., the ACh binding site) (Fig. 11). Bupivacaine did not inhibit [^{125}I]BGT or [^{3}H]ACh binding to the receptor by more than 10% when present at a concentration of 1 mM.

DISCUSSION

The present electrophysiological and biochemical results suggest that bupivacaine interacts with the activated conducting ("open") species (ACh_nR^*) of the nicotinic AChR to produce a blocked species (ACh_nR^*D) with little or no conductance in a manner kinetically consistent with the sequential model presented earlier. Moreover, this mechanism of action is believed to be

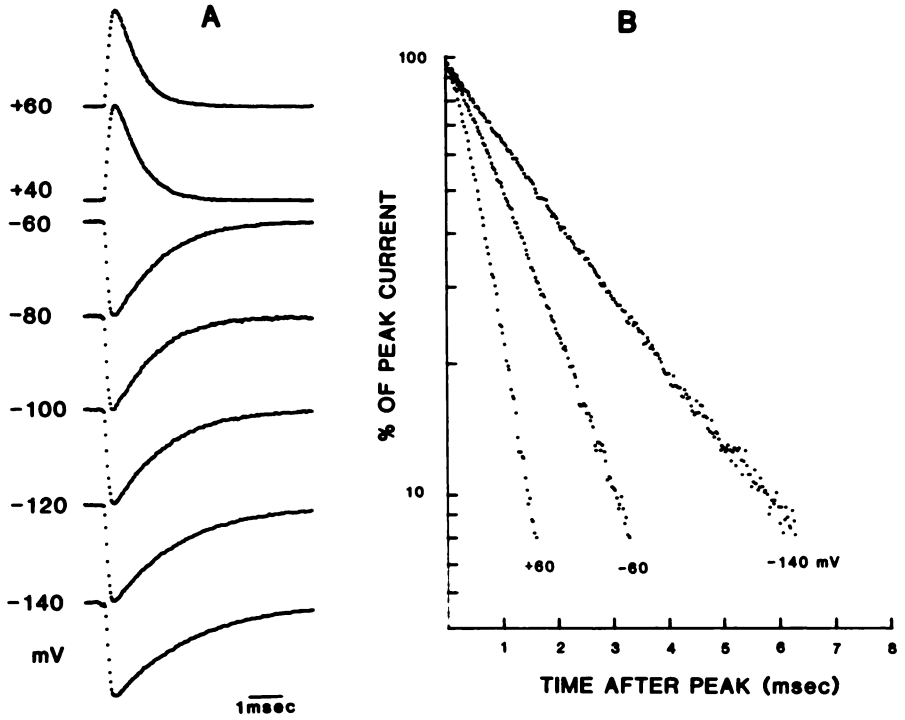

FIG. 5. *Extracellularly recorded MEPCs under control conditions*

A. Digitized MEPCs recorded from the frog cutaneous pectoris muscle under control conditions at various membrane potentials. Membrane potential was controlled by insertion of a current-passing electrode into the same muscle fiber. Each trace represents the signal average of 30–85 MEPCs and is normalized to the same amplitude. B. Decays are plotted from signal-averaged MEPCs from the frog cutaneous pectoris muscle under control conditions. The decays are adequately described by a single exponential function of time at all membrane potentials.

TABLE 1

Effect of bupivacaine on rise time and τ in extracellularly recorded MEPCs from the frog cutaneous pectoris muscle at resting membrane potential (−80 to −95 mV)

N represents the total number of fibers used to determine the mean. Rise time means (SEM) were determined from individual MEPCs in each fiber. The means were then pooled to give the grand mean presented here. τ values were determined in a similar fashion, although the mean from each fiber was determined from a signal-averaged waveform. Temperature = 22°.

Drug concentration	*N*	Rise time	τ
μM		*msec*	*msec*
0	11	0.291 ± 0.011	1.17 ± 0.10
25	2	0.357 ± 0.029[a]	1.15 ± 0.11
50	4	0.304 ± 0.019	0.92 ± 0.06
100	3	0.277 ± 0.014	0.61 ± 0.02[a]
200	5	0.251 ± 0.008	0.43 ± 0.02[b]
400	9	0.220 ± 0.009[b]	0.29 ± 0.04[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

sufficient to explain the majority of the results observed. Thus, bupivacaine represents a very specific "open channel" blocker and is devoid of actions on other targets comprising the AChR macromolecule.

This suggestion is based on several observations. First, bupivacaine alters neither the single exponential nature of EPC and MEPC decays nor the single Lorentzian function disclosed by fluctuation analysis. This strongly indicates that the rate constant for drug dissociation (k_{-3}) is negligible (3, 4, 5, 32). Second, the reciprocal of τ_{EPC} and τ_{MEPC} versus drug concentration (25–400 μM) relationship is linear, as predicted by the sequential model. Although this model predicts a linear relationship for infinite drug concentrations, it is clear that at some point other processes, such as the decay of ACh concentration in the synaptic cleft, would become rate-limiting (33). Since bupivacaine shortened τ_{MEPC} to less than 300 μsec without apparent deviation from the linearity, it appears that little or no contamination from other rate-limiting reactions is present even at such a degree of shortening. Furthermore, the inherently greater frequency responses of extracellular recordings versus conventional two-electrode voltage clamp recordings (29), strengthens this argument. Third, channel conductances in the presence of 10 and 25 μM bupivacaine, as determined from ACh-induced fluctuation analysis, were not significantly different from those obtained in physiological solution alone. Moreover, patch clamp studies have revealed that single-channel amplitudes are unaltered in the presence of bupivacaine (15, 34). Thus, channels which open do so with normal conductance, and those channels which are blocked have negligible conductance. Fourth, bupivacaine binds to sites associated with the ionic channel of the nicotinic AChR as determined from the displacement of radiolabeled channel ligands. The presence of the agonist carbamylcholine increases the affinity of bupivacaine to an activated species. Furthermore, bupivacaine is devoid of action on the agonist-binding site at concentrations used in this study.

Although the sequential model (in the form presented here) describes only the effect of drugs on decay phase

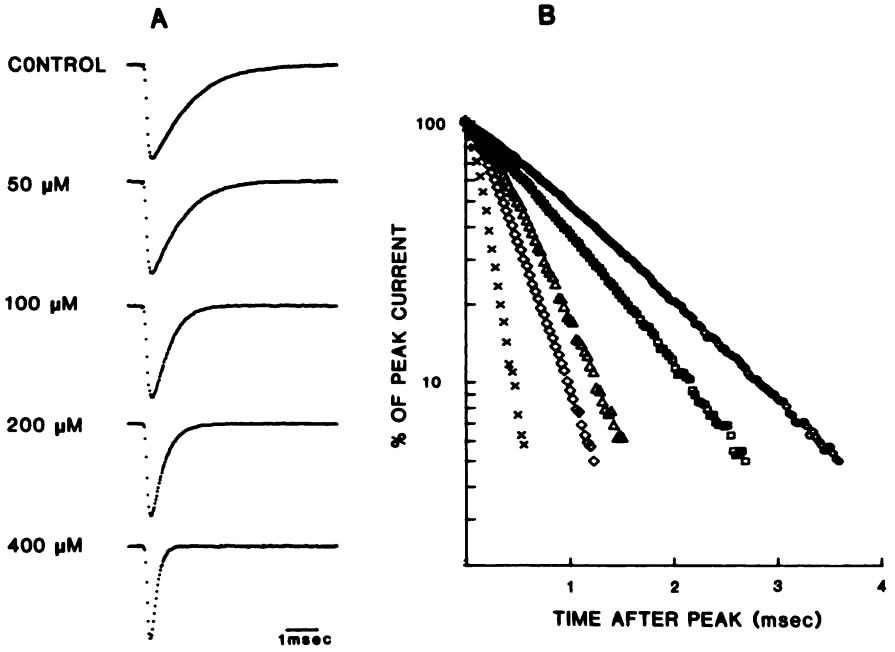

FIG. 6. *Effect of bupivacaine on peak amplitude and decay phase of the extracellularly recorded MEPCs*

A. Digitized MEPCs were recorded in the absence and presence of various concentrations of bupivacaine at resting membrane potential (−80 mV). Each trace represents the signal average of 72–104 MEPCs. MEPCs are normalized to the same amplitude. Horizontal calibration was 1 msec. B. Control (○) and bupivacaine 50 (□), 100 (△), 200 (◇) and 400 μM (×). Decays remained single-exponential at all drug concentrations.

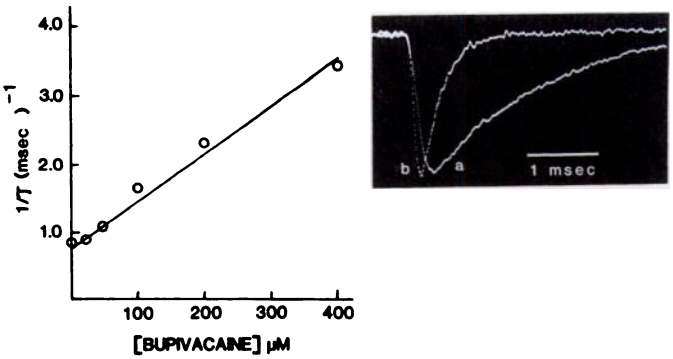

FIG. 7. *Relationship between the reciprocal of the extracellularly recorded MEPC decay time constant and bupivacaine concentration*

Each point represents the value obtained from 6–195 signal averaged MEPCs from 2–11 fibers. Recordings were done at resting membrane potential. The *solid line* represents the best fit obtained from linear regression. Recordings (*inset*) are representative of extracellularly recorded MEPCs in the absence (*a*) and presence (*b*) of 400 μM bupivacaine. Traces are superimposed and the amplitude normalized.

kinetics, bupivacaine also decreased the peak amplitude of EPC and MEPC in a concentration-dependent manner. It could be argued that this decrease in amplitude was secondary to a decrease in channel conductance or inhibition of ACh binding to the agonist recognition site. Both possibilities are unlikely in light of the electrophysiological and biochemical results present. Another possibility is that bupivacaine interacts with a site (other than the agonist binding site) prior to channel opening. This mechanism has been proposed for piperocaine (35), phencyclidine (36), tricyclic antidepressants (22), histrionicotoxins (33), and meproadifen (16). All of these drugs, however, produce concavity in the current-voltage relationship and time- and voltage-dependent blockade of the peak EPC amplitude. Bupivacaine, on the other hand, does not produce these effects and probably does not share a common mode of action. The question then arises as to whether this decrease in amplitude can be attributed solely to blockade of the open channel. Unfortunately, this question cannot be answered without accurately modeling the entire EPC or MEPC time course (4, 37). A possible explanation for the decrease in amplitude is that an abbreviation of channel lifetime caused a greater proportion of channels to close before the peak current was attained than would occur normally.

Analysis of the influence of membrane potential on the forward rate constant for drug binding (k_3) reveals that bupivacaine "senses" between 4.5% and 11% of the membrane potential at its rate-limiting energy barrier. This determination assumes that the rate constants are exponentially dependent on voltage. As there was deviation from this relationship if the depolarized membrane potentials were used (38), only negative potentials were considered in this determination. This estimate is similar to that determined for phencyclidine methiodide and piperocaine methiodide (23) and for quinuclidinyl benzilate (24). On the other hand, under equilibrium conditions, procaine (1, 38) and QX-222 (5) have been estimated to sense between 30–50% and 78% of the membrane electric field, respectively. In light of the structural similarities between QX-222 and bupivacaine, it is difficult to reconcile the great disparity in this picture. If a comparison is allowed to be made, the terminal 1-*n*-butyl piperidine group of bupivacaine could provide a greater steric obstacle to the channel lumen (a proposed binding site) than the terminal trimethyl group of QX-222. This raises a question concerning the location of the binding site for bupivacaine on the ionic channel. Injection of

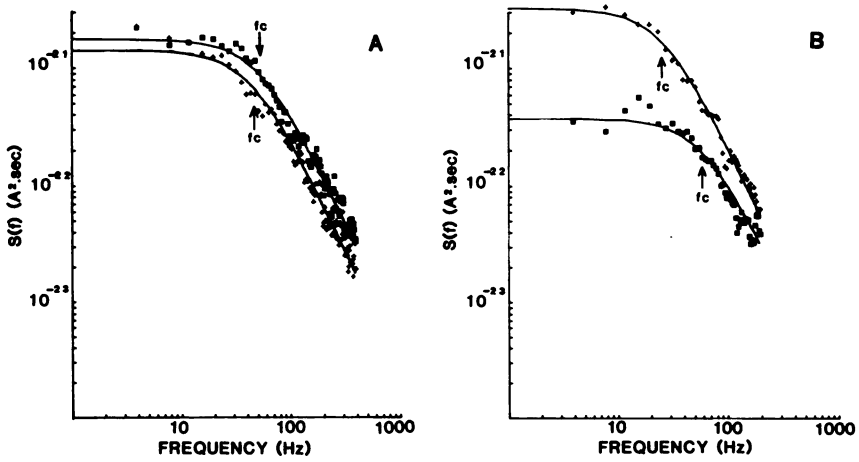

FIG. 8. *Shortening of channel lifetime produced by bupivacaine as revealed by ACh-induced EPC fluctuations in the frog cutaneous pectoris muscle*

A. Power density spectra of ACh-induced EPC fluctuations in the absence (+) and presence (□) of 10 μM bupivacaine. The *solid lines* represent the least-squares fits to a single Lorentzian function. The half-power frequencies, represented by the *arrows*, are 43.7 Hz for control and 51.5 Hz for drug. Single-channel conductance and lifetime were 23.0 pS and 3.64 msec for control and 20.2 pS and 3.09 msec for drug, respectively. The holding potential was −80 mV and temperature 10°. B. Power density spectra in the absence (+) and presence (□) of 25 μM bupivacaine. Conditions are the same as in A. The half-power frequencies are 25.8 Hz for control and 59.2 Hz for drug. Single-channel conductance and lifetime were 23.7 pS and 6.17 msec for control and 21.7 pS and 2.69 msec for drug, respectively.

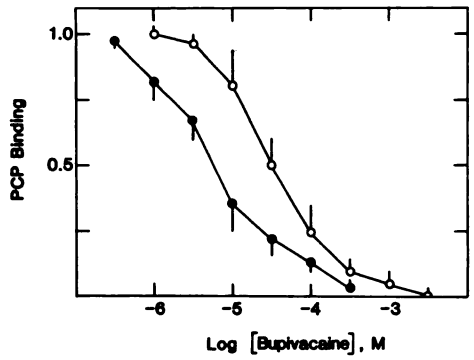

FIG. 9. *Bupivacaine inhibition of [³H]PCP binding to ion channel binding sites in membranes from Torpedo electroplax*

The binding of 2 nM [³H]PCP was measured in the presence of the indicated concentrations of bupivacaine in the absence (○) or presence (●) of 1 μM carbamylcholine. Binding is expressed as fraction of specific (i.e., amantadine-sensitive) binding measured in the absence of carbamylcholine. The points and bars indicate the mean and standard deviations from four determinations.

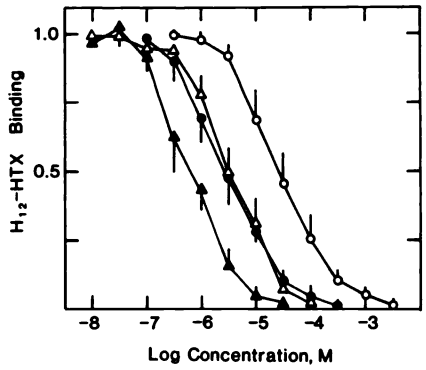

FIG. 10. *Bupivacaine and bupivacaine methiodide inhibition of [³H] H_{12}-HTX binding to ion channel sites in membranes from Torpedo electric organ*

The binding of 1 nM [³H]H_{12}-HTX was measured in the presence of the indicated concentrations of bupivacaine (○, ●) and bupivacaine methiodide (△, ▲) in the absence (○, △) and presence (●, ▲) of 1 μM carbamylcholine. Binding is expressed as fraction of specific (i.e., amantadine-sensitive) binding measured in the absence of competing ligands. The points and bars indicate the mean and standard deviation from four determinations.

QX-222 into the interior of muscle cells produces no effect on end-plate kinetics (39); thus the drug appears to approach its binding site exclusively from the exterior of the membrane. Conversely, the internal application of piperocaine methiodide and phencyclidine methiodide (23) and procaine (38) produced marked alterations in end-plate kinetics, suggesting alternate binding sites (i.e., internal and external) and access routes (i.e., penetration through the lipid phase). No specific experiments with bupivacaine were undertaken to address this question. However, the quaternary derivative, bupivacaine methiodide, produced results similar to the parent compound when tested on EPCs.[6] Bupivacaine methiodide, like bupivacaine, inhibited binding of channel probes such as [³H]PCP and [³H]H_{12}HTX to the nicotinic receptor channel complex in *Torpedo* membranes (Table 2). In patch clamp experiments, bupivacaine methiodide had no effect on the intracellular components of the AChR (15). Thus, it is probable that the charged form of bupivacaine is responsible for its action, and that the site of action is located on the extracellular portion of the AChR complex.

In addition to the above-mentioned effects on decay kinetics, bupivacaine also seems to alter the rising phase of the MEPC, although the results are not nearly so dramatic. It appears that the extreme shortening of MEPC decay by bupivacaine may accelerate the rising phase.

In summary, these results suggest that bupivacaine

[6] S. R. Ikeda, Y. Aracava, and E. X. Albuquerque, unpublished observation.

TABLE 2

Bupivacaine and bupivacaine methiodide inhibition of [^{3}H]H_{12}-HTX and [^{3}H]PCP binding to ion channel binding sites associated with the nicotinic acetylcholine receptors from Torpedo californica electric organ

K_i is the inhibition constant associated with bupivacaine inhibition of [^{3}H]PCP and [^{3}H]H_{12}-HTX binding to ion channel sites in *Torpedo* electroplax. Binding was measured in the absence (control) or presence of 1 μM carbamylcholine (Carb). The means and standard deviations from four to five determinations are listed.

Drug	K_i			
	[^{3}H]H_{12}-HTX		[^{3}H]PCP	
	Control	Carb	Control	Carb
	μM			
Bupivacaine	25 ± 5	3 ± 1	32 ± 3	6 ± 1
Bupivacaine methiodide	3.2 ± 0.4	0.75 ± 0.08	1.8 ± 0.4	0.56 ± 0.06

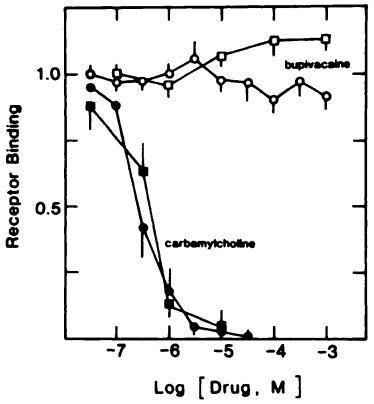

FIG. 11. *Influence of bupivacaine on [^{125}I]BGT and [^{3}H]ACh binding to receptor sites in membranes from Torpedo electroplax*

The binding of 5 nM [^{125}I]BGT (○, ●) and 0.5 nM [^{3}H]ACh (□, ■) was measured in the presence of the indicated concentrations of bupivacaine (○, □) or carbamylcholine (●, ■). Binding is expressed as the fraction of total specific binding measured in the absence of competing ligands. Bupivacaine had little influence on either [^{125}I]BGT or [^{3}H]ACh binding, whereas carbamylcholine inhibited the binding of both probes with an IC_{50} below 1 μM. The points and lines indicate the mean and standard deviations from three determinations.

reacts with the ionic channel of the nicotinic receptor in the open conformation to produce a species with no conductance. There is no evidence for interaction with the agonist binding site or closed channel species.

ACKNOWLEDGMENTS

We would like to thank Mrs. Lauren Aguayo and Ms. Mabel Zelle for computer and technical assistance, and Dr. Vincent E. Dionne for kindly supplying the voltage clamp schematic.

REFERENCES

1. Adams, P. R. Voltage jump analysis of procaine action at frog end-plate. *J. Physiol. (Lond.)* **268**:291–318 (1977).
2. Ruff, R. L. Local anesthetic alteration of miniature endplate currents and end-plate current fluctuations. *Biophys. J.* **16**:433–439 (1976).
3. Ruff, R. L. A quantitative analysis of local anesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol. (Lond.)* **264**:89–124 (1977).
4. Adler, M., E. X. Albuquerque, and F. J. Lebeda. Kinetic analysis of end plate currents altered by atropine and scopolamine. *Mol. Pharmacol.* **14**:514–529 (1978).
5. Neher, E., and J. H. Steinbach. Local anesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol. (Lond.)* **277**:153–176 (1978).
6. Ritchie, J. M., and P. J. Cohen. Cocaine, procaine and other synthetic local anesthetics, in *The Pharmacological Basis of Therapeutics* (L.S. Goodman and A. Gilman, eds.). MacMillan, New York, 379–403 (1975).
7. Sokoll, M. D., B. Sonesson, and S. Thesleff. Denervation changes produced in an innervated skeletal muscle by long-continued treatment with a local anesthetic. *Eur. J. Pharmacol.* **4**:179–187 (1968).
8. Benoit, P. W., and W. D. Belt. Destruction and regeneration of skeletal muscle after treatment with a local anesthetic bupivacaine (Marcaine®). *J. Anat.* **107**:547–556 (1970).
9. Deshpande, S. S., E. C. B. Hall-Craggs, and E. X. Albuquerque. Electrophysiological and morphological investigation of bupivacaine-induced myopathy and terminal sprouting in the rat. *Exp. Neurol.* **78**:740–764 (1982).
10. Ikeda, S. R., R. S. Aronstam, and E. X. Albuquerque. Interactions of bupivacaine with ionic channel of nicotinic receptors. *Neurosci. Abstr.* **8**:499 (1982).
11. Kuba, L., E. X. Albuquerque, J. Daly, and E. A. Barnard. A study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.* **189**:499–512 (1974).
12. Dionne, V. E., and R. L. Parsons. Characteristics of the acetylcholine-operated channel at twitch and slow fibre neuromuscular junctions of the garter snake. *J. Physiol. (Lond.)* **310**:145–158 (1981).
13. Gage, P. W., and R. N. McBurney. Effects of membrane potential, temperature and neostigmine on the conductance change caused by a quantum of acetylcholine at the toad neuromuscular junction. *J. Physiol. (Lond.)* **244**:385–407 (1975).
14. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
15. Aracava, Y., S. R. Ikeda, J. W. Daly, N. Brookes, and E. X. Albuquerque. Interactions of bupivacaine with ionic channels of the nicotinic receptors: analysis of single-channel currents. Mol. Pharmacol. **26**:304–313 (1984).
16. Maleque, M. A., C. Souccar, J. B. Cohen, and E. X. Albuquerque. Meproadifen reaction with ionic channel of the acetylcholine receptor: potentiation of agonist-induced desensitization at the frog neuromuscular junction. *Mol. Pharmacol.* **22**:636–647 (1982).
17. Anderson, C. R., and C. F. Stevens. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.)* **235**:655–691 (1973).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. Tsai, M.-C., N. A. Mansour, A. T. Eldefrawi, M. E. Eldefrawi and E. X. Albuquerque. Mechanism of action of amantadine on neuromuscular transmission. *Mol. Pharmacol.* **14**:787–803 (1978).
20. Magleby, K. L., and C F. Stevens. The effect of voltage on the time course of end-plate currents. *J. Physiol. (Lond.)* **233**:151–171 (1972).
21. Magleby, K. L., and C. F. Stevens. A quantitative description of end-plate currents. *J. Physiol. (Lond.)* **233**:173–197 (1972).
22. Schofield, G. G., B. Witkop, J. E. Warnick, and E. X. Albuquerque. Differentiation of the open and closed states of the ionic channels of the nicotinic acetylcholine receptors by tricyclic antidepressants. *Proc. Natl. Acad. Sci. U. S. A.* **78**:5240–5244 (1981).
23. Aguayo, L. G., B. Pazhenchevsky, J. W. Daly, and E. X. Albuquerque. The ionic channel of the acetylcholine receptor: regulation by sites outside and inside the cell membrane which are sensitive to quaternary ligands. *Mol. Pharmacol.* **20**:345–355 (1981).
24. Schofield, G. G., J. E. Warnick, and E. X. Albuquerque. Elucidation of the mechanism and site of action of quinuclidinyl benzilate (QNB) on the electrical excitability and chemosensitivity of the frog sartorius muscle. *Cell. Mol. Neurobiol.* **1**:209–230 (1981).
25. Gage, P. W., and R. N. McBurney. Miniature end-plate currents and potentials generated by quanta of acetylcholine in glycerol-treated toad sartorius fibres. *J. Physiol. (Lond.)* **226**:79–94 (1972).
26. Katz, B., and R. Miledi. The measurements of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. Roy. Soc. Lond. B. Biol. Sci.* **161**:483–495 (1965).
27. Colquhoun, D. Fitting curves: the relationship between two variables, in *Lectures on Biostatistics*. Oxford University Press, London; Clarendon Press, Oxford, 214–278 (1971).
28. Adams, P. R., and B. Sakmann. Agonist-triggered end-plate channel opening. *Biophys. J.* **21**:53a (1978).
29. Dwyer, T. M. The rising phase of the miniature endplate current at the frog neuromuscular junction. *Biochim. Biophys. Acta* **646**:51–60 (1981).
30. Gage, P. W., R. N. McBurney, and D. Van Helden. Octanol reduces end-plate channel lifetime. *J. Physiol. (Lond.)* **274**:279–298 (1978).
31. Aronstam, R. S., A. T. Eldefrawi, I. N. Pessah, J. W. Daly, E. X. Albuquerque, and M. E. Eldefrawi. Regulation of [^{3}H]perhydrohistrionicotoxin binding of *Torpedo ocellata* electroplax by effectors of the acetylcholine receptors. *J. Biol. Chem.* **256**:2843–2850 (1981).
32. Adams, P. R. Drug blockade of open end-plate channels. *J. Physiol. (Lond.)* **260**:531–552 (1976).
33. Spivak, C. E., M. A. Maleque, A. C. Oliveira, L. M. Masukawa, T. Tokuyama, J. W. Daly, and E. X. Albuquerque. Actions of the histrionicotoxins at the

ion channel of the nicotinic acetylcholine receptor and at the voltage-sensitive ion channels of muscle membranes. *Mol. Pharmacol.* **21**:351–361 (1982).
34. Aracava, Y., S. R. Ikeda, N. Brookes, and E. X. Albuquerque. Blockade of acetylcholine (ACh)-induced channels by bupivacaine. *Fed. Proc.* **42**:991 (1983).
35. Tiedt, T. N., E. X. Albuquerque, N. M. Bakry, M. E. Eldefrawi, and A. T. Eldefrawi. Voltage- and time-dependent actions of piperocaine on the ion channel of the acetylcholine receptor. *Mol. Pharmacol.* **16**:909–921 (1979).
36. Albuquerque, E. X., M.-C. Tsai, R. S. Aronstam, A. T. Eldefrawi, and M. E. Eldefrawi. Site of action of phencyclidine. II. Interaction with the ionic channel of the nicotinic receptor. *Mol. Pharmacol.* **18**:167–178 (1980).
37. Wathey, J. C., M. M. Nass, and H. A. Lester. Numerical reconstruction of the quantal event at nicotinic synapses. *J. Biophys.* **27**:145–164 (1979).
38. Gage, P. W., O. P. Hamill, and R. E. Wachtel. Sites of action of procaine at the motor end-plate. *J. Physiol (Lond.)* **335**:123–137 (1983).
39. Horn, R., M. S. Brodwick, and W. D. Dickey. Asymmetry of the acetylcholine channel revealed by quaternary anesthetics. *Science (Wash. D. C.)* **210**:205–207 (1980).

Send reprint requests to: Dr. E. X. Albuquerque, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, MD 21201.